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W. Hiddemann T. Büchner B. Wörmann
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Acute Leukemias VII

Experimental Approaches and Novel Therapies

With 334 Figures and 331 Tables

Springer

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Preface

In the last few years a dramatic increase in the knowledge about the biology of acute leukemias and especially the underlying genetic and molecular events has been experienced. These processes lead to the deregulation of central transcription factors and genes regulating cell prliferation and differentiation and ultimately determine the response to chemotherapy and the longterm prognosis of patients suffering from these disorders. The increasing insights into the biology of acute leukemias also provide the basis for pathogenesis oriented new therapeutic strategies which aim at interfering with pathologically activated gene products or augmenting the antileukemic immune response. They give also new information about the mechanisms of action of conventional treatments and cytostatic agents in particular and thus allow to optimize drug regimens accordingly.

The seventh volume "Acute Leukemia VII – Experimental Approaches and Novel Therapies" provides new updates on these topics from leading basic scientists and clinicians around the world. The high quality of contributions makes this book an overview over most recent achievements which translate into new treatment strategies and hence an improved outlook for patients suffering from acute leukemias.

The editors wish to thank Beate Kosel for an excellent contribution as a coordinator of the editorial work.

September, 1997

W. Hiddemann · T. Büchner · B. Wörmann
J. Ritter · U. Creutzig · M. J. Keating · W. Plunkett

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Leukemia Cell Biology

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Alterations in DNA Repair: Implications for Leukemia Cell Biology

J. THOMALE[1], M. R. MÜLLER[2], C. BUSCHFORT[1], S. SEEBER[2], M. F. RAJEWSKY[1]

Abstract. The sensitivity of human tumor cells to chemotherapeutic agents has recently been correlated to their ability to eliminate drug-induced DNA damage. However, the exact mechanisms by which specific DNA adducts cause cell death are still not fully understood nor are the relative efficacies of distinct pathways within the complex DNA repair network of mammalian cells. To elucidate the link between repair and drug resistance in primary cancer cells we have measured the functional DNA repair capacity of normal and leukemic human lymphocytes ex vivo. Two sensitive assays (the immunocytological assay, ICA, detecting specific DNA adducts, and the comet assay, measuring the kinetics of DNA strand break processing) were used to determine the repair capacity at the single cell level.

The individual kinetics for the removal of primary or secondary lesions from nuclear DNA varied significantly (by factors > 10) between cell specimens derived from both healthy individuals and CLL patients. An inverse correlation was observed between the DNA repair capacity of CLL lymphocytes and their in vitro sensitivity to various mono- and bifunctional alkylating agents.

The application of modifiers which block distinct steps of DNA repair pathways provided information about their relative contribution to damage processing in individual cell samples or in cell subpopulations. Furthermore, this strategy revealed a loss of stringent control of specific repair functions in leukemic cells in comparison to their normal counterparts.

Keywords: drug resistance, inter-individual variation; DNA repair pathways, repair modifiers, CLL

Introduction

Many widely used chemotherapeutic anticancer drugs form complex patterns of different reaction products with nuclear DNA, monoadducts as well as intra- and interstrand crosslinks [1]. The exact mechanisms by which these adducts cause cell death and the relative cytotoxic potential of specific lesions are still not known.

Enzymatic removal of DNA damage is a key feature of cells to protect their genomic integrety. Our knowledge about the DNA repair machinery of mammalian cell has dramatically increased during recent years and a type of "repair netwok" is emerging with various main routes and a number of subpathways [2,3] (see Fig. 1). The loss of specific functions within this structure has successfully been attributed to the risk of cancer, both in experimental systems and in human epidemiology [4].

On the other hand, considerable effort has been made to correlate the (over-)expression of individual repair genes in cell lines and in tumor specimens to chemosensitivity

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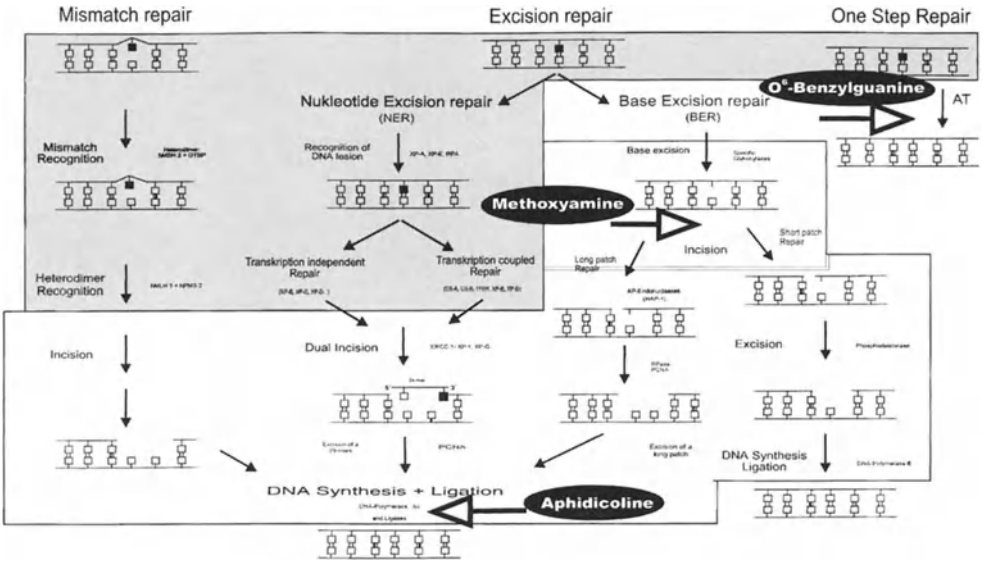

Fig. 1. Multiple mechanisms of DNA repair in mammalian cells. Primary DNA lesions are detectable by adduct-specific antibodies and can be measured by quantitative immuno-fluorescence analysis (ICA; *shaded area*); repair intermediates can be recognized by the comet assay (*boxed area*). The inhibition of distinct steps by three repair modifiers is indicated (O^6-benzylguanine inactivates the alkyltransferase AT; methoxyamine prevents DNA incision by AP-endonuclease(s); aphidicoline inhibits gap filling by DNA polymerases)

profiles or to the therapeutic response. Due to the unexspected complexity of the mammalian repair machinery many attempts to explain biological findings by single factor analyses were misleading, especially in transformed cells. The functional measurement of repair efficiency in primary tumor cells was hampered until now by a lack of sufficiently sensitive methods. Therefore, the clinical significance of DNA repair as a major determinant of therapy resistance remains still controversal.

Chronic lymphocytic leukemia (CLL) represents a model system which is particularly suited to study the development of cellular and clinical drug resistance. At first presentation CLL patients are usually responsive to oral chemotherapy with alkylators. In many cases the disease is becoming resistant to this regimen later on. Homogeneous populations of tumor cells are available from the peripheral blood and they can be analyzed for their chemosensitivity profiles as well as for their repair characteristics after DNA damage in vitro.

In this study we have used two functional assays covering different sections of the repair network to measure time courses of DNA damage processing in CLL lymphocytes at the single cell level. Additional information about the activity of specific pathways and their relevance for chemosensitivity was collected by the application of repair inhibitors.

Material and Methods

Chemicals. N-ethyl-N-nitrosourea (EtNU; Serva, Heidelberg, Germany) was recrystallized twice, and stored in DMSO as a stock solution (100 mg/ml) at –20 °C. Before use, the EtNU concentration was measured by UV spectroscopy in MES buffer (60 mM NaCl, 1mM 2-morpholino-ethan-sulfonacid, 0.5 mM EDTA; pH 6). The decomposition rate of EtNU at 37 °C in PBS (phosphate-buffered saline supplemented with 25 mM HEPES at pH 7.25) was determined spectrophotometrically.

Methoxyamine and aphidicoline (Sigma, Deisenhofen, Germany) were prepared as

stock solutions in sodium phosphate buffer (100 mM, pH 7.25) and stored at –20 °C. O^6-Benzylguanine was synthesized at our institute (courtesy of W. Drosdziok, IFZ Essen) and stored as a stock solution in DMSO at –20 °C. Agaroses (Seakem LE and NuSieve GTG for the comet assay, were purchased from Biozyme, Hameln, Germany.)

Mouse anti-(O^6-alkyl-2′-deoxyguanosine) monoclonal antibody (Mab) EM-2-3 [5] was purified from cell culture supernatant by FPLC-separation using Mono Q and Mono S columns (Pharmacia, Uppsala, Sweden). Secondary (anti-mouse) antibodies (IgG F[ab]2-fragments labeled with rhodamine isothiocyanate [TRITC]) were obtained from Dianova, Hamburg, Germany.

Preparation of Lymphocytes. Lymphocytes were isolated from peripheral blood obtained from healthy donors or from patients with chronic lymphatic leukemia prior to chemotherapy and analyzed on the same day.

Briefly, heparinized blood (10 ml) was layered onto 10 ml Ficoll-Hypaque and centrifuged for 25 min at 200 g at room temperature. Cells from the interface were removed, washed twice with PBS, and resuspended in pre-warmed RPMI medium supplemented with 10% FCS. The resulting cell suspensions contained >90% lymphocytes, as confirmed by light microscopy, and more than 95% of all cells were in G1/G0 (FACS analysis). All clinical specimens were isolated from patients with B-CLL, as determined by immuno-phenotyping carried out at first presentation. Lymphocytes were kept in RPMI medium at 37 °C in a humified atmosphere containing 5% CO_2.

Exposure to EtNU. Cells were washed once with pre-warmed PBS and resuspended in PBS, supplemented with Ca^{2+}, Mg^{2+}, and HEPES (25 mM; pH 7.25). EtNU was added from the stock solution at various concentrations and the cells were incubated at 37 °C for 20 min. Cells were then washed twice with PBS and resuspended in pre-warmed RPMI supplemented with 10% FCS. Cell aliquots were taken immediately and at several times after EtNU treatment. Where appropriate, cells were pre-incubated with RPMI medium containing methoxyamine (5 mM) or O^6-benzylguanine (50 µM) 1 h prior to EtNU exposure and throughout the experiment.

Comet Assay (Single Cell Gel Electrophoresis, SCGE). The comet assay was performed essentially as described (5). Briefly, after exposure to EtNU, cells were washed twice with icecold PBS and adjusted to 5x103 cells/µl. Aliquots of 10µl were suspended in low melting point (LMP) agarose (0.5%). Cells were immediately spread onto fully frosted microscope slides pre-coated with a thin layer of 0.7% LE agarose by placing a coverslip on top of the drop. A top-layer of 0.5% LMP agarose was used as a cover. Cells were lysed in situ by placing the slides into lysis buffer (2.5 M NaCl, 100mM EDTA, 10 mM Tris, 10% DMSO, 1% TritonX100 and 1% sodium sarcosinate; pH 10) at 4 °C overnight. Slides were washed twice and kept in an alkaline solution (300 mM NaOH, 1 mM EDTA; pH 12) for 20 min at 4 °C to denature the DNA. Slides were then subjected to alkaline electrophoresis (solution as above; 20 min at 4 °C and 4 V/cm), neutralized 3x10 min in Tris HCI (0.4 M; pH 7.5), and stained with ethidium bromide (2 µg/ ml).

Immunofluorescence Staining. lmmunofluorescence staining of O^6-EtGua in the nuclear DNA of single cells was performed essentially as described [6]. Briefly, cells were spread on microscope slides, air dried, fixed in methanol for 15 min at room temperature, rehydrated in 2X standard saline citrate (SSC) buffer (300 mM NaCI, 30 mM sodium citrate), and treated with RNases (RNase A; 200 µg/ml; RNase T; 50 units/ml) for 1 h at 37 °C. Cells were then washed with 0.14 M NaCI, and the DNA was partly denatured by alkali treatment (NaOH, 70 mM in 0.14 M NaCl, 5 min, 0 °C). After two rinses with PBS and pre-incubation with PBS containing 20% BSA for 20 min at room temperature, cells were incubated with (anti-O^6-alkylguanine) Mab EM-2-3 at a concentration of 0.2 µg/ml in PBS and 1% BSA for 16 h at 4 °C. Slides were then washed twice with PBS and stained with goat anti-mouse antibodies (Ig F[ab]2 fragments conjugated with TRITC; 2 µg/ ml PBS/BSA) for 3h at room

temperature. The nuclear DNA was counterstained with the fluorescent dye DAPI (3 x 10-7 M in PBS; 10 min). To reduce fading of fluorescence, slides were then covered with a solution containing 50 mM Tris-HCI, 33 mM 1,4-dithioerythritol (DTE), 30% glycerol, and 10% Elvanol in PBS (pH 8.2).

Quantification of Fluorescence Signals in the Nuclear DNA of Individual Cells (ICA). A Zeiss fluorescence photo-microscope (Axioplan) equipped with an HBO 100 W mercury arc lamp, and Zeiss standard filter combinations 2 (for DAPI), 14 (for TRITC) and 15 (for ethidium bromide) were used for the visualization of fluorescence of the individual cells. Fluorescence signals were amplified and recorded by a dual mode CCD camera (Photonics, Hamamatsu City, Japan), and fed into a four parameter image analysis program (ACAS; Ahrens, Bargteheide, Germany). This program performs integration of fluorescence images with low signal to noise ratios. Thresholds were set to discriminate background from DNA staining signals and to determine the image points to be included in the evaluation. Average fluorescence values were computed from the fluorescence intensities of 100–150 individual nuclei.

Detection of Abasic Sites and Single Strand Breaks. The integral amount of DNA damage in single cells was determined with the comet assay by measuring the total area of nuclear DNA and the fluorescence intensity by image analysis. Thresholds were defined to give homogeneously positive integrated areas throughout individual comets. Comet related DNA damage is expressed as the relative increase in the area of nuclear DNA in EtNU-exposed cells compared to the area of untreated cells from the same donor in the same experiment.

Measurement of O^6-Ethylguanine (O^6-EtGua). The amount of O^6-EtGua residues in the nuclear DNA of single cells was determined after intensifying DNA-DAPI and antibody-TRITC fluorescence signals. In particular, antibody and DNA derived fluorescence from the same cell can be processed separately or in parallel. The selected pixels were expressed as the integrated values (average signal x number of selected pixels) for each nucleus, and antibody-derived signals were corrected for nuclear DNA content.

Chemosensitivity Profiles of CLL Cells. The cytotoxicity of various DNA alkylators with or without the addition of repair modifiers was determined in isolated CLL lymphocytes by the MTT assay as described earlier [7,8]

Results

Functional measurement of DNA Repair

Normal and leukemic lymphocytes were analyzed for their DNA repair capacity after short time exposure to the standard alkylator N-ethylnitrosourea (EtNU) in vitro. The use of EtNU is advantageous for this purpose because it is a direct acting compound with a short half-life of < 10 min enabeling the separate analysis of adduct formation on repair. Furthermore, all reaction products with nuclear DNA are well characterized and sensitive immunanalytical methods for their detection are available [9]. At various time points during and after the alkylation period cell aliquots were taken and measured at the single cell level for DNA adduct concentrations (immunocytological assay, ICA) and for the amount of DNA strand breaks (comet assay, SCGE).

In the latter case, the relative electrophoretic mobility of the DNA of individual cell nuclei was recorded in comparison to untreated control cells from the same donor. As EtNU is a direct alkylating agent, the initial levels of DNA alkylation damage were homogeneous among all cells of a given sample and between individual cell specimens. Repair kinetics for primary alkylation products, e.g., O^6-etyhlguanine (O^6-EtGua [6, 7]) and for secondary lesions (abasic sites and single strand breaks), induced by DNA repair processes, were considerably different for individual cell samples (Fig. 2). As measures for the cellular repair capacity we have calculated the required time periods after pulse exposure to EtNU to eliminate 50% of the DNA alkylation product (t50 ICA) or to

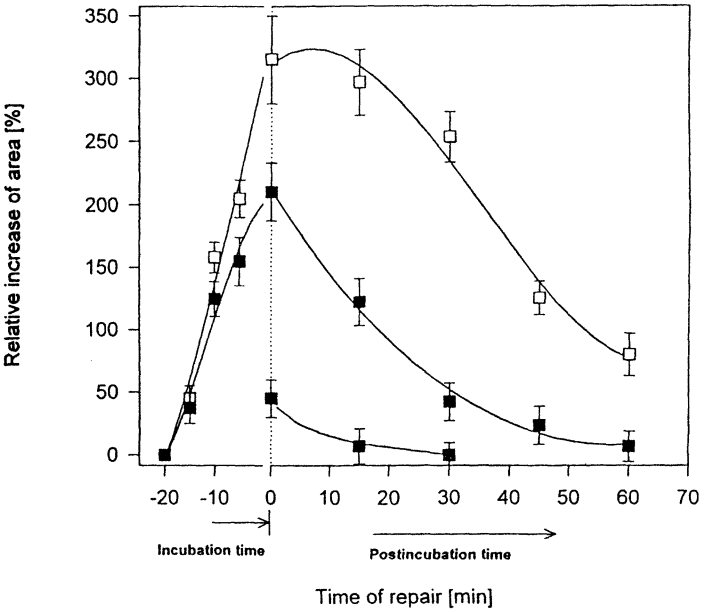

Fig. 2. Kinetics of strand break formation and persistence in the nuclear DNA of three individual samples of CLL lymphocytes (as measured at the single-cell level by the comet assay during and after exposure to EtNU in vitro)

reduce the "initial" comet area by 50% (t50 SCGE).

Interindividual Differences

Analyses of individual samples of normal or leukemic lymphocytes and of AML blasts exhibited broad spectra of repair times, both, for adduct removal and for strand break processing [5, 8] (Fig. 3). Time intervals for processing of 50% of both types of damage differed among cell samples by factors of > 20 (0.4 to 8.5 h for adduct elimination; 0.2 to 4.1 h for intermediate strand breaks) indicating an extensive regulation of rate limiting repair steps. The stability of individual „repair phenotypes" was proven by

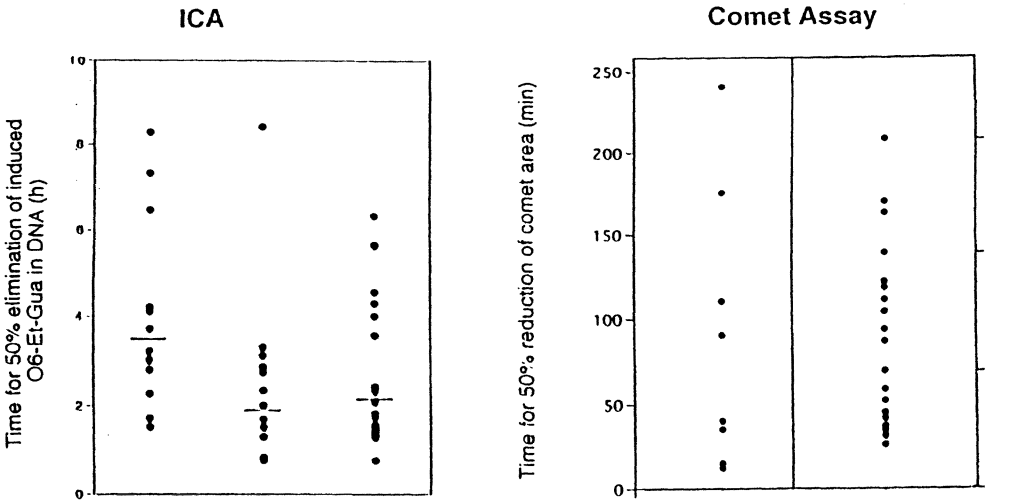

Fig. 3. Interindividual variations of cellular capacity for the elimination of DNA alkyl adducts (O^6-EtGua; ICA analysis) and of strand break processing (comet assay) in specimens of normal (NL) and CLL lymphocytes and in leukemic blast cells (AML) from different patients. t50% values were calculated from repair kinetics after pulse exposure of cells to EtNU (see Fig. 2)

repeated analyses of normal lymphocytes isolated from blood samples of the same donor after various time intervals (data not shown).

DNA Repair Capacity in Relation to Chemosensitivity in Vitro and to Clinical Status

Cellular DNA repair capacities of CLL lymphocytes (as determined by ICA and comet assay) were correlated with their respective chemosensitivity to various alkylating agents including chlorambucil (CLB). We have found significant ranking correlations between t50% repair times and the ID50 values for mono- and bifunctional alkylators which form different types and patterns of DNA adducts [7, 8]. The coincidence of fast / slow repair with cross-resistance / cross-sensitivity of CLL cells to various DNA reactive drugs suggests that these two features are causally connected.

This notion got additional support when the samples were grouped according to the clinical status (Fig. 4). Cells from untreated and from treated sensitive CLL patients showed wide scatters of DNA repair capacities. Specimens from patients refractory to treatment with alkylating agents, however, all displayed very fast damage processing through early and late steps without accumulation of intermediate strand breaks.

Repair Modifier

The relative usage of different repair pathways in individual samples of normal and leukemic lymphocytes was analyzed by the pretreatment of cells prior to alkylation with repair modifiers (RMs), small molecules interfering with early or late constituents of these multistep processes.

Modulation of Comet Formation by O^6-Benzylguanine, Methoxyamine, and Aphidicoline

Three RMs, O^6-benzylguanine (O6-BG), methoxyamine (MX), and aphidicoline (Aph), have been used to interrupt damage processing in EtNU treated lymphocytes at different key positions of the DNA repair network (see Fig. 1):

(i) MX, reacting with abasic sites in DNA, blocks the early incision step along the BER pathway;

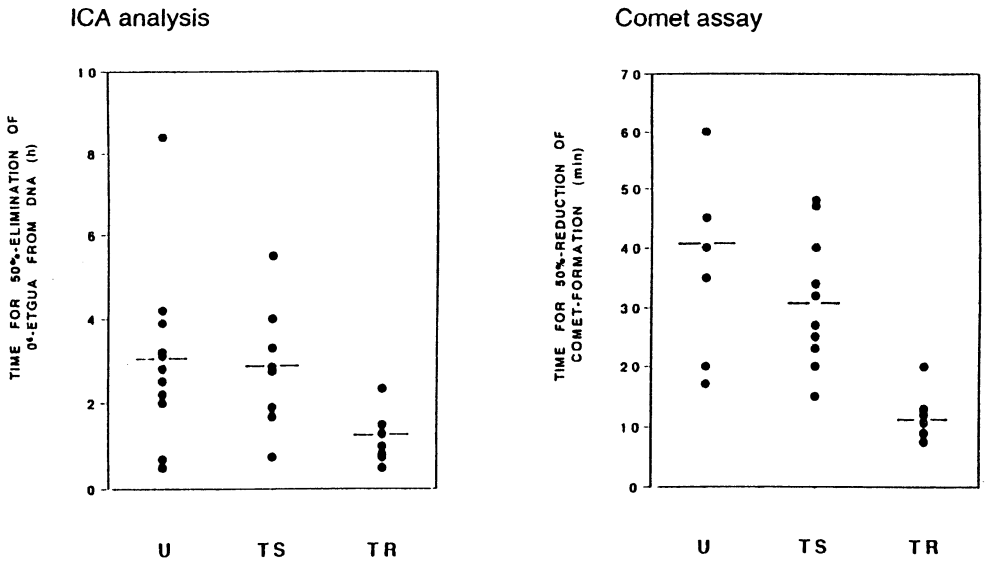

Fig. 4. Cellular DNA repair capacities of B-CLL lymphocytes for primary alkylation products (ICA) and for secondary lesions (comet assay) in correlation to the clinical status. Specimens were obtained from CLL patients either untreated (U), or sensitive (TS), or resistant (TR) to treatment with alkylating agents including chlorambucil

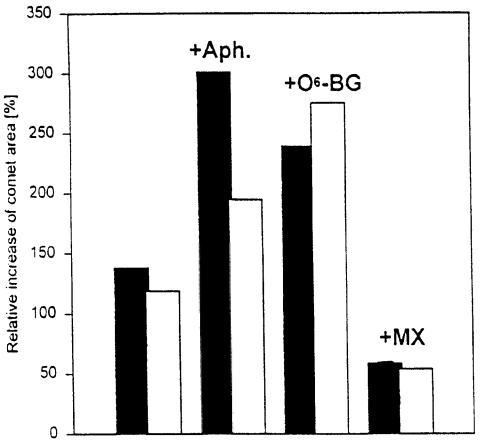

Fig. 5. Modulation of DNA strand break formation in EtNU exposed lymphocytes after pretreatment with repair modifiers. Two samples of normal lymphocytes (black and white bars) were puls treated with EtNU (100 µg/ml) alone or in combination with aphidicoline (*Aph*), O^6-benzylguanine (*O^6-BG*) or methoxyamine (*MX*) and subjected to comet analysis

(ii) Aph prevents the downstream filling of single strand gaps by DNA polymerases after excision repair;

(iii) O^6-BG inhibits the direct, one step removal of O^6-alkylation at guanines by inactivating the AT protein.

The observed effects of these modifiers on strand break processing in EtNU exposed lymphocytes (Fig. 5) were as exspected for MX (inhibition of an early repair step led to reduced frequencies of incisions) and for Aph (prevented resealing of repair patches resulted in accumulation of strand breaks). AT inhibition, however, was not "comet neutral" as assumed, but resulted in significantly increased DNA fragmentation which is most likely due to DNA incision at persisting O^6-EtGua residues by an alternative repair mechanism. Rodent cells have been shown to eliminate this adduct with high efficiency in a gene-specific manner from transcriptionally active sequences via an excision pathway [10].

The relative contribution of different DNA repair pathways to comet formation was evaluated by pre-incubating lymphocytes with MX prior to EtNU exposure. MX reacts with abasic sites in DNA, protecting them from enzymatic or alkali induced conversion into single strand breaks [5]. Comet areas were significantly reduced by MX in all samples of normal lymphocytes compared to cells exposed to EtNU only (Fig. 6). The significant diminution of comets by co-application of MX indicates that the majority of strand breaks (60-80%) were initiated

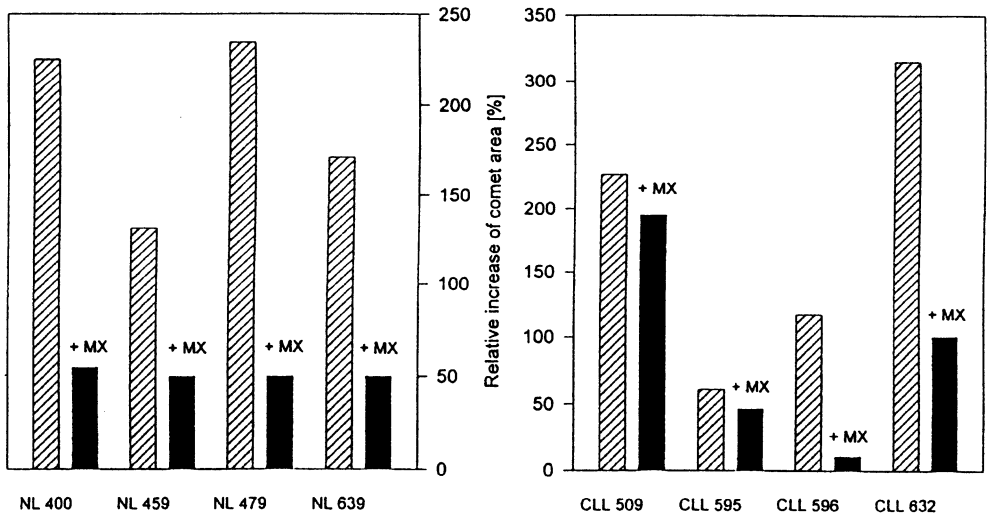

Fig. 6. Reduction of repair-induced DNA strand breaks in lymphocytes by methoxyamine (MX). Individual samples of normal (*NL*) or B-CLL lymphocytes (*CLL*) were alkylated by EtNU for 20 min with (*black bars*) or without (*hatched bars*) pre-exposure to MX (5 mM) and analyzed for DNA strand breaks by the comet assay [5]

by DNA glycosylases, either via the "classical" BER pathway or as a result of mismatch recognition. In almost all samples of normal lymphocytes the remaining levels of strand breaks were similar. These lesions were most likely induced by glycosylase-independent DNA repair (such as nucleotide excision repair [NER] or mismatch recognition and incision{MMR}).

CLL lymphocytes also showed reduced comet areas when pretreated with MX prior to EtNU (Fig. 6). In contrast to normal B cells, however, the rate of reduction (ranging from 20 to 90%) and, especially, the remaining amount of DNA strand-breaks varied greatly between individual cell specimens. This finding may be indicative for a loss of stringent (co-) regulation of various repair components or pathways in CLL compared to normal B cells.

Sensitization of CLL Lymphocytes to Alkylating Anticancer Drugs by RMs

To investigate the relative contribution of a defined DNA alkylation product to cell killing we have determined the cytotoxicity of a monofunctional alkylating agent (dacarbazine; DTIC) with and without blocking of the repair protein AT by the specific inhibitor O^6-BG (Fig. 7).

As non-repaired O^6-methylguanines are believed to be the major cytotoxic DNA lesions formed by DTIC, it is surprising that only 40% (11 out of 28) of the CLL cell specimens were sensitized by inhibition of the relevant AT repair pathway [11] (see Fig. 1). In this group of patients sensitization ratios up to five were observed for individual cell samples and the effect was predominantly pronounced in cells exhibiting high ID_{50} values for DTIC. Recent observations with MMR defective cell lines and MMR knock out mice suggest an important role of this pathway for the toxification of persisting O^6-alkylguanines (and other drug induced adducts) in DNA by futile repair cycles [12–14]. We are currently analyzing the CLL cell specimens which could not be sensitized by O^6-BG for a possible loss of specific MMR functions.

Discussion

For a mammalian cell it is most likely that unsuccessful attempts to replicate and / or to repair damaged DNA in time cause persisting single strand gaps or double strand breaks. So both, anticancer drug induced adducts and secondary lesions initiated by mechanisms of DNA metabolism can lead to cell death or genetic instability.

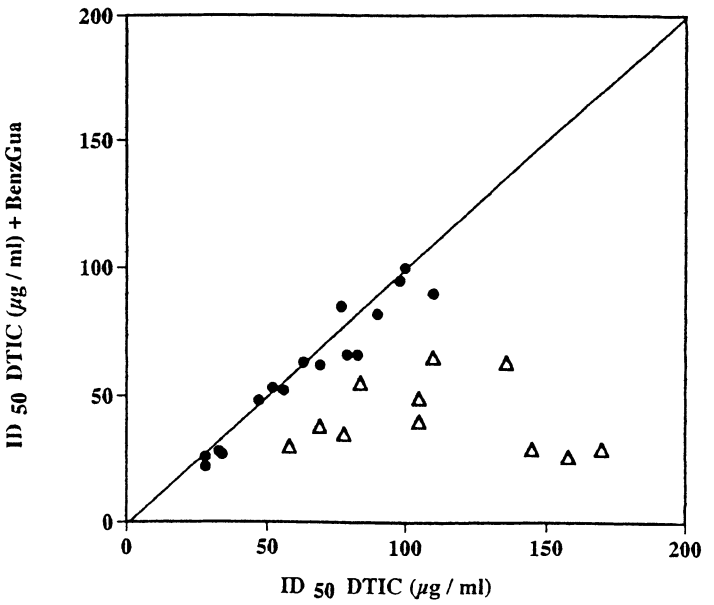

Fig. 7. Modulation of DTIC cytotoxicity in individual CLL specimens by a repair inhibitor: ratios of ID_{50} values for DTIC allone (x-axis) versus DTIC in the presence of O^6-BG (50 µM; y-axis). *Triangles in the lower field* represent cell samples sensitized by AT inhibition

It has been shown that treatment of lung cancer patients with chlorambucil did not lead to a (reactively) increased chemoresistance of normal lymphocytes from these patients [15]. In all specimens of B-CLL lymphocytes derived from patients who have developped resistance to chemotherapy with alkylating drugs (including CLB) we have measured high DNA repair capacities. This finding may hint to a clonal selection of tumor cells with this repair phenotype. This conclusion is further supported by a limited number of logitudinal studies showing acceleration of both, adduct elimination and strand break processing, with the number of therapeutic cycles (our own unpublished data). In addition, in CLL cells from refractory patients we have observed cross resistance to a variety of different mono- and bifunctional alkylators (e.g., EtNU, DTIC, BCNU, MAF or CLB;) [7, 8]. Together, our results argue for a causative role of DNA repair functions in the development of cellular and clinical resistance in CLL. For therapeutic intervention it will be crucial to identify rate limiting steps and possible regulatory elements of DNA repair pathways in leukemic cells.

There is increasing evidence that defects in mismatch repair (MMR) systems may be associated with the development of acute [16, 17] and chronic leukemias [18]. The contribution of MMR functions (loss or overexpression) for cellular and clinical drug resistance remains still to be clarified.

One possible lesson from the quickly accumulating knowledge in this field is to design more effective strategies for overcoming the repair component of drug resistance, whether intrinsic or acquired during therapy. Therefore, there is an urgent need for the development of more selective inhibitors for distinct repair steps / components and for a targeted delivery of these RMs to cancer cells.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Functional Characterization of Leukemic Stem Cells in Acute Myeloid Leukemia

B. Wörmann, K. Vehmeyer, M. Feuring-Buske, D. Grove, S. Könemann, W. Hiddemann

Introduction

The classification of acute leukemias is based on the morphological and immunophenotypic characterization of the bulk population of leukemic blasts in comparison with normal hematopoiesis. Leukemias arise from the (mono-) clonal expansion of a malignant transformed progenitor cell. Culturing of leukemic cell populations in vitro suggests that the disease is maintained by only a small number of clonogenic cells, so called leukemia initiating or leukemic stem cells (Griffin and Löwenberg 1986; Löwenberg and Bauman 1985; Sabbath et al. 1985). There has been uncertainty about the phenotype of leukemic stem cells and about the level of differentiation at which malignant transformation occurs. In about 50% of patients with acute leukemia and myelodysplastic syndromes (MDS) non-random genetic aberrations can be detected, either by metaphase analysis or by fluorescence in situ hybridization. Application of these techniques in peripheral blood samples and bone marrow aspirates from patients with AML and MDS have revealed that the genetic aberrations can be detected not only in the dominant myeloid blast population but frequently also in cells of megakaryocytic, erythroid, monocytic, T- and B-lymphoid lineage (Baurmann et al. 1993; Knuutila et al. 1994; Suciu et al. 1993; van Lom et al. 1993). These observations point to a very early, uncommitted progenitor cell as the target of malignant transformation.

Normal hematopoietic progenitor cells are characterized by the expression of the cell surface antigen CD34. Lineage commitment and differentiation is accompanied by the sequential acquisition of other cytoplasmic and cell surface antigens, e.g., CD38 and HLA-DR on early progenitors; CD117, CD33 and cMPO on myeloid progenitors; cCD79, cCD22 and CD19 on B-lymphoid progenitors; CD7 on T-lymphoid progenitors; c-mpl and CD61 on megakaryocytic progenitors; CD71 on erythroid progenitor cells. The earliest uncommitted progenitor cell is characterized by expression of CD34 and lack of expression of CD38, HLA-DR and lineage associated antigens (Huang et al. 1992). Two years ago, Mehrothra et al. and our group reported results on the genetic analysis of CD34+/CD38- subpopulations in bone marrow aspirates from patients with AML and MDS (Feuring et al. 1995; Haase et al. 1995; Mehrothra et al. 1995). Both groups observed that these cells with the stem cell-like immunophenotype regularly carried the genetic marker of the respective leukemia. While Mehrothra used FISH for identification of numeric aberrations, we also included metaphase analysis to study the whole complexity of leukemia cytogenetics. Analysis of further cases has substantiated this conclusion (Haase et al. 1997).

In parallel, several investigators have per-

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formed functional analysis of presumed leukemic stem cells. Methods include short-term colony assays, long-term bone marrow cultures and in vivo growth in immunodeficient mice. Clonogenic growth was almost exclusively detected in CD34 positive cells (Yin et al., 1993). Lapidot et al. showed that CD34+/CD38- cells from a child with newly diagnosed acute myeloid leukemia FAB M1 initiated leukemic growth in SCID-mice, while CD34+/CD38+ cells failed to generate leukemic colonies (Lapidot et al., 1994). In contrast, Terpstra et al. observed a patient in whom CD34+/CD38+ and CD34-/CD38+ were able to initiate leukemic growth in immunodeficient mice. In addition, the resulting leukemia was CD34-.

As part of a project on the characterization of leukemic stem cells, we have analysed the sequence of CD34 and CD38 aquisition in bone marrow aspirates from patients with acute myeloid leukemia with respect to synchronous or asynchronous expression, and have studies the clonogenic growth of isolated subpopulations in vitro.

Material and Methods

Immunophenotyping

Cell Preparation. Bone marrow aspirates of the patients were prepared for flow cytometric analysis using erythrocyte lysis. One volume of bone marrow was diluted with 14 volumes of the lysing solution (10^{-4} M EDTA, 10^{-3} M $KHCO_3$, 0.17 M NH_4Cl in H_2O, pH 7.3) and gently mixed. Cells were lysed for 3 to 5 min at room temperature and then centrifuged at 200 g for 5 min at room temperature. The pellet was resuspended in a volume of RPMI 1640 (Gibco) 14 times larger than the original bone marrow volume and centrifuged at 200 g for 5 min at 4 °C. This washing step was repeated twice and the cells were finally resuspended in phosphate buffered saline containing 1% bovine serum albumin and 20 mM Hepes (pH 7.3) (PBS). The cell concentration was adjusted to 1 x 10^7 cells/ml. Twenty µl of the monoclonal antibody CD38PE [Leu17 PE, Becton Dickinson Immunocytometry Systems, San Jose, CA (BDIS)] and CD34FITC [HPCA-2 FITC (BDIS)] at titer concentration was added to 100 µl of cell suspension. After an additional 15 min of incubation on ice, the cells were washed once with 2 ml of the PBS solution at 4 °C. The pellet of the immunofluorescent labeled cells was resuspended in 1 ml of 0.5% paraformaldehyde in PBS. In the control experiments, cells were incubated with fluorescence labeled isotype controls.

Flow cytometry. Flow cytometric analysis was performed on a FACS-can (BDIS). Data acquisition was performed using the FACS-can Research Software (BDIS). The instrument setup was standardized using T lymphocytes as a reference. This was achieved by gating on the fluorescence intensity of CD3+ lymphocytes, followed by an adjustment of the light scattering detectors to locate the CD3+ lymphocytes in a standard position in the correlative display of forward light scattering and orthogonal light scattering. The fluorescence detectors were adjusted using a tight light scattering gate, obtained from the light scattering of the CD3+ lymphocytes, followed by adjustment of the two fluorescence detectors of an unstained sample. Adjustment of the cross over of fluorescence signals of FITC and PE into other than the assigned detectors was obtained by compensation of samples stained with CD4FITC and CD8PE respectively. The forward light scattering and orthogonal light scattering signals and the tow fluorescence signals were determined for each cell and data of 20 000 events were stored in list-mode data files.

Hematopoietic Colony-Forming Assay

The colony-forming capacity of stem cells was investigated in methyl cellulose cultures. Cells were mixed with methyl cellulose medium consisting of 0.9% methyl cellulose (4 000 centipoises, Sigma, USA), 0,6% bovine serum albumine (Boehringer, FRG) and 30% pretested fetal calf serum (Gibco, FRG) in Iscove's modified Dulbecco's Medium (Gibco, FRG) containing 50 mM 2-mercaptoethanol and 2 mM L-Glutamin (Gibco, FRG). Final concentrations were 1.5 x 10^5 /ml for PBMC and 1 x 10^3 /ml for purified BM

cells. Cultures were plated in 35-mm petri dishes (Greiner, FRG) in the presence of growth factors: 50 ng/ml G-CSF (granulocyte colony stimulating factor) (Amgen/Roche, FRG), IL-3 and GM-CSF (granulocyte/macrophage stimulating factor) (Sandoz, FRG), 1 U / ml EPO (erythropoietin) (Boehringer, FRG) and 20 ng / ml SCF (stem cell factor)/ml (Genzyme, FRG). The plates were incubated at 37 °C under a fully humidified atmosphere and 8% CO_2. Colonies were scored after 10–14 days under a dissection microscope (Zeiss, FRG). Cell aggregates containing more than 50 cells were scored as colonies. Morphological analysis was performed on cytocentrifuge preparations of single colonies after Pappenheim staining.

Results

Co-expression of CD34 and CD38

Differentiation of normal hematopoietic progenitor cells is characterized by the coordinate expression of CD34 and CD38, see Fig. 1A. Maturation is characterized by gradual acquisition of CD38 and subsequently loss of CD34. Bone marrow aspirates from 340 patients with newly diagnosed acute myeloid leukemia were analysed for co-expression of CD34 and CD38 and classified into four different categories:

1. Coordinate co-expression (Fig. 1B)
2. Low or now expression of CD34 (Fig. 1C)
3. Asynchronous expression of CD34 (Fig. 1D)
4. Asynchronous expression of CD38 (Fig. 1E)

The distribution of the different expression patterns is summarized in Table 1. The do-

Table 1. Incidence of different antigen patterns in the co-expression of CD34 and CD38 in patients with acute myeloid leukemia

Pattern	n	%
normal differentiation CD34 high	231	68
normal differentiation CD34 low/negative	85	25
aberrant expression of CD34	17	5
aberrant expression of CD38	7	2
Total	340	100

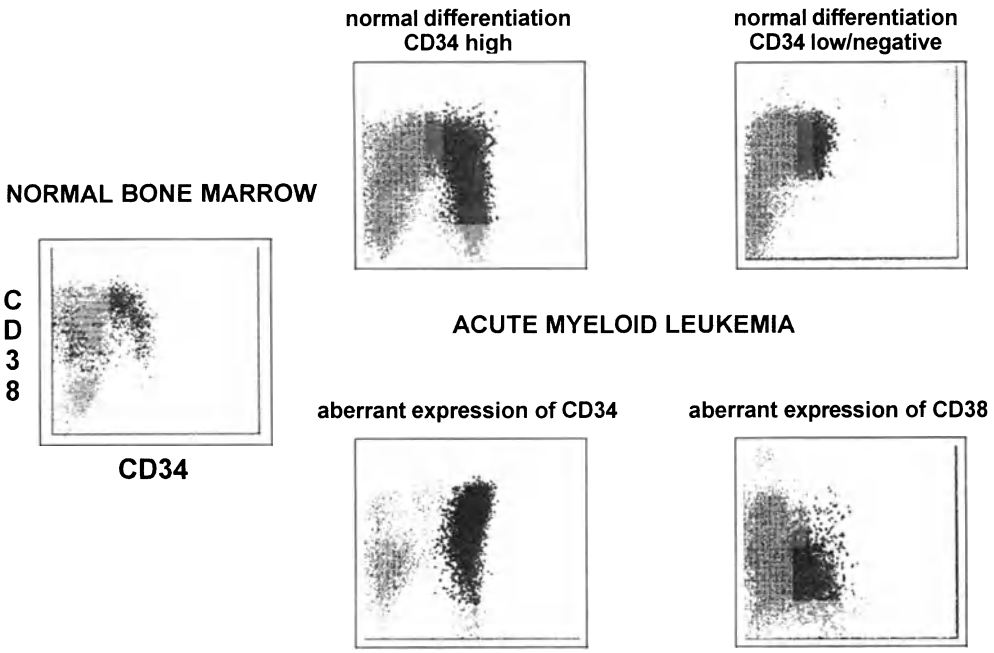

Fig. 1. Different patterns of co-expression of CD34 and CD38 in acute myeloid leukemia

minant pattern follows the coordinate expression of CD34 and CD38. Only half of these samples had a significant proportion of CD34+/CD38- cells; however, the remaining samples also followed the same expression pattern and were thus included into this group. Also the second category does not differ from the normal expression, except that CD34 is low or not expressed. These two categories account for 93% of all samples. The third category is characterized by the sequential acquisition of CD38, however, the gradual loss in CD34 of normal differentiation does not occur. There is even a tendency to overexpression of CD34 in CD38+ blasts; 17 of the analyzed samples (5%) were classified that category. The last category is characterized by a significant population of CD34+/CD38- cells, however CD38 is not acquired on the CD34+ cells, but differentiation "bypasses" the CD34+/CD38+ stage to directly go into the CD34-/CD38+ stage. Only 7 of all samples (2%) were classified in this group.

Clonogenic Growth

Leukemic blasts from ten samples were separated by fluorescence activated cell sorting into the categories CD34+/CD38-, CD34+/CD38+, and CD34-/CD38+. They were incubated in a short-term colony assay, developed for growth of normal hematopoietic progenitor cells. In this assay system, normal CD34+/CD38- cells do not grow. Comparison of the different patients reveals a heterogenous pattern, see Table 2. Analysis of the unsorted samples showed a high interindividual variability with growth ranging from 8 (patient 8) to 280 colonies (patient 6). In four of nine samples, the highest clonogenic potential resided in the population of CD34+/CD38- blasts. In four samples, a significantly higher proportion of colonies was generated from CD34+/CD38+ cells, while in additional two samples, highest cloning efficiency was obtained in the most mature samples of CD34-/CD38+ cells. In five samples (patients 1, 5, 7, 8 and 9) clonogenic growth occurred almost exclusively in one immunophenotypically defined compartment. In two of the remaining five samples, clonogenic growth was observed in two immunophenotypically related subpopulations (patients 2 and 6), in two further samples clonogenic growth was obtained only in the most immature and the most mature compartment (patients 3 and 4). In the last sample (patient 10) only clusters of leukemic cells were observed, no significant colony growth occurred.

Discussion

Research on the identification and characterization of leukemic stem cells has made significant progress within the past 15 years. Many points have been clarified, some are still open or controversial. There is agreement that the number of clonogenic cells is low (Griffin and Löwenberg 1986; Löwenberg and Baumann 1985; Sabbath et al. 1985). The clonogenic compartment resides in the CD34+ population. More definitively, it can be pinpointed to the compartment of CD34+/CD38-cells. This conclusion is soli-

Table 2. Clonogenic growth of CD34 subpopulations

No	unsorted	colonies CD34+/CD38–	CD34+/CD38+	CD34–/CD38+
1	93	450	–	3
2	44	–	162	28
3	183	160	–	25
4	156	154	–	61
5	68	9	–	117
6	288	95	297	–
7	24	0	10	0
8	8	0	34	0
9	–	80	1	–
10	–	1	2	–

dified by application of genetic analysis to sorted subpopulations by Mehrothra et al. and ourselves (Mehrothra et al. 1995; Feuring et al. 1995; Haase et al. 1995). They are further substantiated by functional analysis using either short-term colony assays, long-term bone marrow cultures (Terpstra et al. 1996) and immunodeficient mice (Lapidot et al. 1994). The observation by Terpstra et al. of a case with leukemia-initiating blasts in SCID-mice from a patient with CD34-/CD38+ blasts differs from that conclusion. However, analysis of the immunophenotype of this leukemic sample shows that it has to be classified into aberrant acquisition of CD38. According to our results in a large group of patients with newly diagnosed AML, presented in this paper, this group of leukemias is rare and accounts for only 2% of all samples. The function of CD34 has not been fully elucidated (Krause et al. 1996). However, there does not seem to be any antigen on the surface of leukemic cells, which may not in smaller or larger groups been found as aberrant or asynchronous (Terstappen et al. 1991). It would thus be very surprising, if CD34 or CD38 would be the only antigens not prone to disruption of either gene expression, transcription or post-translational modification induced by the malignant transformation. In addition, development of acute myeloid leukemia is considered to be the result of a multi-step pathogenesis in the majority of cases. It is also conceivable, that additional genetic aberrations at a more mature level of leukemic blasts may induce secondary autonomous proliferation in subsets of patients. Thus the observation of clonogenic growth from CD34-/CD38+ cells has to be considered as an exception to the above mentioned rule.

On clinical grounds, the localization of malignant transformation at this very early progenitor level is quite disturbing. It has to be postulated that relapse also stems from that cell population. Thus it is not surprising that M. Feuring-Buske et al. have described persistence of cells with the individual genetic marker in that compartment of early progenitors in patients in stable hematological complete remission (Feuring-Buske et al. 1997). An option for still distinguishing normal from leukemic progenitors by immunophenotyping lies in the analysis of the potential aberrant co-expression of markers of lymphoid lineage on CD34+/CD38- leukemic progenitors. Lymphoid-lineage associated markers are detected in up to 50% of all patients with newly diagnosed acute myeloid leukemia and may serve not only for detection of minimal residual disease in the bulk population of leukemic cells but also for the detection of minimal residual leukemic stem cells.

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Acute Leukemias VII
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Involvement of CD34+ Stem Cells in Malignant Transformation in AML and MDS – Genetic Analysis of Sorted Subpopulations by Classical and Molecular Cytogenetics

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Abstract. In this study we addressed the question whether hematopoietic stem cells characterized by the expression of CD34 are involved in the leukemogenic process in AML and MDS. For this purpose bone marrow cells were sorted according to their expression of CD34 and coexpression of CD38 or CD117 (stem cell factor receptor = CKIT). The sorted subpopulations were genetically analyzed either by cytogenetics or FISH. Successful cytogenetics of sorted CD34+ subpopulations could be performed in 24/54 pts. included in our study (AML: n = 18, MDS: n = 6). At diagnosis 18 displayed a wide variety of different clonal karyotype abnormalities in the unsorted bone marrow: add(2q); +4; 5q-; 5q- and complex anomalies; t(6;9); 6p-; -7; +8; t(8;21); -12; inv(16); i(17q); t(17;20); -21; i(21q). We analyzed sorted stem cell subpopulations with the immunophenotype CD34+/CD38± in 19 pts., in 5 pts. stem cells with the immunophenotype CD34+/ CD117± were examined. We found genetically abnormal stem cells in every subpopulation (CD34+/CD 38-, CD34+/CD38+, CD34+/CD117-, CD34+/CD117–) in every informative case (abnormal karyotype in the unsorted bone marrow and metaphases/interphase cells available for cytogenetic analysis). In 11 pts. a mosaic of normal and abnormal cells was observed in the sorted stem cell subpopulations. In five pts. secondary, progression-associated anomalies were present in the sorted stem cells. We conclude that in AML and MDS malignant transformation and disease progression occur at the level of immature hematopoietic stem cells independent from the phenotype of the leukemic bulk population and the type of genetic alteration.

Keywords: Stem cells, AML, MDS, chromosomes, cytogenetics, CD34, CD38, CD117

Introduction

The classification of acute leukemias suggests an arrest of maturation at a specific stage of differentiation, due to malignant transformation of the presumed normal counterparts [1]. In contrast in chronic myelogenous leukemia (CML) and MDS it is supposed that the transformation occurs at the level of pluripotent stem cells [2,3]. However, those inferences supporting a stem cell involvement were indirect – for instance by the demonstration of clonal genetic markers in different cell lineages – and not based on the examination of stem cells themselves [3, 4, 5]. A direct genetic analysis of purified and well-characterized stem cells was not possible as yet.

The very recently developed tools to identify and characterize stem cells by function [6] and specific surface molecules [7] have now provided an access to stem cell biology in normal hematopoiesis and malignant disorders.

Stem cells express a characteristic cell surface antigen classified as CD34 [8]. The po-

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pulation of CD34+ cells can be further subdivided by the expression of lineage-specific and/or lineage-associated cell surface molecules acquired on different maturational stages [9]. CD34+/CD38–/HLA-DR-cells are pluripotent hematopoietic cells and give rise to myeloid and B-lymphoid cells [10]. With increasing maturation the expression of CD34 gets lost. Immunophenotypical classification of acute leukemias is based on the comparison of leukemic cells with their presumed normal couterparts [11, 12, 13]. Fifty percent to 80% of newly diagnosed AML are CD34+ [14]. On leukemic blasts the immunophenotypic profile of normal immature hematopoietic progenitors characterized by the expression of CD34, CD38 and HLA-DR is well conserved [15]. Blasts from a patient with AML M1, injected into SCID mice were able to induce the clinical and hematologic features of AML [16] although it has been reported by others that also CD34– blasts have the potential for leukemia-induction in SCID mice [17]. However it has not been clarified, whether the functional behaviour of CD34+ cells differs between AML subtypes, especially in cases with no or only a minor population of CD34-alone positive cells. Characterization, discrimination, and isolation of normal and leukemic stem or progenitor cells are the basis for improvements in myeloablative chemotherapy with subsequent autologous retransfusion [18], for targeted gene therapy [19], and for monitoring of residual disease [20, 21]. By fluorescence-activated cell sorting (FACS) stem cells can be isolated up to a purity of >98%.

Fifty –80% of all patients with AML display clonal chromosome abnormalities [22, 23] which can be delineated by classical cytogenetic techniques in bone marrow, peripheral blood and other tissue specimens. Quantification of numerical and selected structural aberrations is facilitated by fluorescence in situ hybridization (FISH) [5, 24]. The majority of chromosomal abnormalities are leukemia-specific. While primary chromosomal changes are considered as initiating factors, secondary abnormalities are involved in disease progression.

We have developed techniques to further cultivate and process FACS-isolated stem cells for classical cytogenetics, fluorescence in situ hybridization (FISH) and molecular analysis [25, 26]. Thus we were able to analyze the genotypes of CD34+ stem cell subpopulations in MDS and AML.

For all questions addressed only patients whose bulk populations of leukemic blasts were CD34 negative were exmined.

Material and Methods

Patients

Bone marrow aspirates of 54 patients with AML or MDS at diagnosis or in relapse were selected for sorting procedures according to their immunophenotypes. Patients were admitted to the Department of Internal Medicine of the University of Göttingen (Göttingen, Germany). Diagnosis and classification of AML were based on light microscopy of Pappenheim-stained slides, and on cytochemical reaction with periodic acid schiff (PAS), myeloperoxidase, and esterase. Slides were reviewed by two independent hematologists according to the criteria of the French-American-British (FAB) classification. In 24 cases genetic analysis (classical cytogenetics, FISH or RT-PCR) of the sorted CD34+ cells was possible. Diagnosis was AML in 18 patients. Six patients suffered from MDS. The karyotype of the unsorted bone marrow was normal in 6 patients, one patient displayed a chromosomal instability and 17 patients had clonal, cytogenetic abnormalities. A total of 15 different clonal chromosomal aberrations could be detected: (2q+, +4, 5q–,5q– and complex anomalies, 6p–, t(6;9), -7, +8, t(8;21), –12, inv(16), i(17q), t(17;20), –21, i(21q)). The following highly purified stem cell populations were separated: CD34+/38–, CD34+/38+, CD34+/117–, CD34+117+. The patients' characteristics (age, sex, FAB-type, cytogenetic abnormalities and number of metaphases or interphases analyzed) are shown in the tables.

Immunophenotyping

Immunophenotyping was performed at diagnosis by multiparameter flow cytometry

using a whole blood lysis method and a set of monoclonal antibodies against myeloid and lymphoid lineage-associated antigens: CD2, 3, 4, 5, 7, 8, 10, 11b, 11c, 13, 14, 15, 16, 19, 20, 25, 33, 34, 38, 45. 56, 61, 71, HLA-DR and Glykophorin A (all antibodies directly conjugated with FITC, PE, PerCP or Cy5, all antibodies Becton Dickinson Mountain View, CA, USA).

Fluorescence-Activated Cell Sorting

Mononuclear cells [1-2 x 107] were isolated by Ficoll gradient centrifugation, washed twice in RPMI 1640 (GIBCO, Germany) and double-stained with the specific antibodies (CD34FITC, CD38PE (Becton Dickinson, San Jose, CA, USA)). The CD34+/CD38–, CD34+/CD38+, CD34+/CD117-, CD34+/CD117+ populations were sorted according to their light scatter properties and fluorescence intensity using a FACS Vantage (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm Argon laser, Lysis software. Sort purity was >98%. Drop drive frequency was below 2000/min, the sheath fluid was PBS.

The FACS Vantage is an expansion of a flow cytometer. It can separate subpopulations of cells on the basis of optical measurements. The particle stream is directed through a small orifice (50–100 μm diameter). The optical detection takes place in a square capillary channel after the particles leave the nozzle (called jet in air detection). Particles to be purified by sorting are recognized by their distinctive fluorescence and/or scattered light intensities in the same way as done in the flow cytometer. After passing the spot where the optical measurement is performed each particle continues to flow in the liquid jet to break up very reproducibly into small droplets, which all have the same size. An electric charge is induced on each droplet containing a particle to be sorted by applying an electric pulse (130 V) to the fluid inside the flow channel just before the droplet breaks off. The charged and uncharged droplets then pass through a high electric field (2000 V/m) where the charged droplets are deflected and separated from the uncharged droplets. The charged droplets are collected in a tube and can be used for further analysis.

Fluorescence in Situ Hybridization

For the FISH experiments cells were sorted onto glass microscope slides containing 100 μl 0.1% agarose and fixed with methanol:acetic acid, 3:1; vol/vol. Selection of DNA probes was individualized on the basis of the karyotype of each leukemic specimen. Centromeric probes for chromosome 8 directly labeled with SpectrumOrange were used (Vysis, Naperville, USA). Cellular DNA was denatured at 74 °C for 5 min in 70% formamide, saline sodium citrate (SSC 0.30 mol/l NaCL, 0.03mol/l sodium citrate, pH 7–8). After dehydration in ethanol series (70, 80, 90, 100%) and air-drying at 42 °C, the DNA probe was denatured in hybridization mixture (formamide 50%: dextran sulfate 10%; 2X SSC at 74 °C for 5 minutes and immediately applied to cells on slides that were hybridized overnight in a humidified chamber at 37°C. Slides were washed three times at 42 °C in 2X SSC, pH 7. Cell scoring was performed using a 100X Plan Neofluor objective mounted on an Axioscope (Zeiss, Wetzlar, Germany).

In Vitro Culturing and Cytogenetic Analysis

The FACS isolated cells were incubated at 37 °C for 24–72 h in RPMI 1640 (GIBCO, Karlsruhe, Germany) supplemented with 20% FCS (HyClone, Logan, USA), 100 U/ml recombinant human (rh) granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA), 100 U/mL glycosylated rh granulocyte-macrophage colony-stimulating factor (Behringwerke, Marburg, Germany), 100 U/ml rh interleukin-3 (Behringwerke), 1 U/ml erythropoietin (Boehringer, Mannheim, Germany), and 50 ng/ml stem cell factor (Genzyme, Boston, MA). Cells were incubated with 0.3 μg/ml colcemide (GIBCO) for 12 h. Chromosome preparation and staining by a modified GAG-banding technique have been described previously. The karyotypes were classified according to the International System of Chromosome Nomenclature [28].

Molecular Analysis of the AML1/ETO Fusion-Transcript

The AML1/ETO fusion transcript was analyzed in two patients with a known t(8;21) by rt-PCR. M-RNA of FACS-isolated CD34+ stem cells was extracted according to the protocol of InVitroGen. Reverse transcription was performed according to the Gibco protocol with Superscript-RT (recombinant M-MLV-RT RNAse H-) using ETO-Primer 1 at 42 °C. For the first amplification step ETO-Primer 1 and AML-1-Primer 2 with GIBCO Taq polymerase were used at 50 °C. The second amplification step was performed using ETO-Primer 3 and AML-1-Primer 4 at 50 °C. For hybridization oligo-probe 5 or KASUMI primer 3 and 4 amplification product as a probe were used. Labeling was performed with DIG dUTP by 3' TdT tailing. The localization of primers for the nested PCR was as published [29].

Results

We processed bone marrow biopsies of 54 patients with MDS or AML for FACS (fluorescence activated cell sorting) and subsequent genetic analysis.

Stem cells characterized by the co-expression of CD34 and CD38 were examined in 19 patients. In 5 patients CD34+ cells further subdivided by the expression of CD117, the receptor for the stem cell factor (CKIT) were studied. In detail we examined subpopulations with the immunophenotypes CD34+/CD38–, CD34+/CD38+, CD34+/CD117–, CD34+/CD117+.

In 24 cases genetic analysis (classical cytogenetics; FISH or RT-PCR) of highly-purified, FACS-isolated CD34+ cells was possible.

Eighteen patients had an AML (M0: 2 pts., M1: 1 pt., M2: 4 pts., M2baso: 1 pt., M4: 5 pts., M4eo: 5 pts.). A myelodysplastic syndrome was diagnosed in 6 patients (RA (secondary): 1 pt., RA: 2 pts., RAEB: 2 pts., CMML: 1 pt.). Twelve patients were of male, 12 of female gender. The age ranged between 8 and 81 years with a median of 59 years. Twenty-three patients were examined at diagnosis, one in relapse. The karyotype of the unsorted bone marrow was normal in 6 patients, 1 patient displayed a chromosomal instability and 17 patients had clonal, cytogenetic abnormalities. A total of 15 different clonal chromosomal aberrations could be detected in the following different highly-purified stem cell subpopulations: CD34+/CD38–, CD34+/CD38+, CD34+/CD117-, CD34+/CD117+.

Stem Cell Genetics in AML at Diagnosis

CD34+/CD38- Cells (Table 1)
The number of cells after the sort procedure ranged between 500 and 6×10^5 with a median of 2×10^4. A cytogenetic examination of this most immature stem cell population was possible in 10 of 16 patients. One case was examined by FISH and in a further patient rt-PCR was performed. The number of completely analyzed metaphases ranged between 1 and 36 with a median of 11. Four patients had a normal karyotype in their unsorted bone marrow. In one of them we failed to perform cytogenetic analysis of the stem cells. In the remaining three cases a normal karyoytpe was also present in the CD34+/CD38– cells. In seven patients we found clonal cytogenetic abnormalities [2q+, +4, t(6;9), 6p–, +8, –12, inv(16) in three patients] in the immature stem cell population which were identical to those delineated in the unsorted bone marrow.

In one case, trisomy 8 was demonstrable by FISH. Rt-PCR of the AML1/ETO fusion transcript was positive in one case. An AN-status (mosaic of normal and abnormal cells) could be observed in the sorted stem cell subpopulations of five patients. Secondary karyotypic changes were observed in two patients. In case no. 6 trisomy 8 occurred additionaly to translocation t(6;9) and in case no. 14 an inversion 16 was accompanied by trisomy 8 and monosomy 12.

CD34+/CD38+ cells (Table 1)
The cellular yield after cell sort ranged between 500 and 2×10^6 with a median of 1.5×10^5.

A cytogenetic examination of this population which is thought to represent committed stem cells was possible in 15 of 16 patients. In one case only rt-PCR was perfor-

Table 1. Stem cell genetics (CD34/CD38) in 16 pts. with AML at diagnosis

Pat. no./ age/sex	Diagnosis (FAB)	Karyotype, unsorted	Immuno-phenotype of sorted cells	Cells after short ($\times 10^3$)	Karyotype/ rt-PCR of sorted cells	Metaphases/ interphases analyzed
1/32/m	AML M0	Normal	CD34+/38– CD34+/38+	4.5 4.5	Normal Normal	5 2
2/27/f	AML M0	Normal	CD34+/38– CD34+/38+	2.3 215	No result Normal	0 12
3/46/m	AML M1	Normal/ add(2)(q37)	CD34+/38– CD34+/38+	55 60	2q+ Normal/2q+	2 3/8
4/8/m	AML M2 relapse	Not done	CD34+/38– CD34+/38+	12 50	No result Normal/–7	0 2/18
5/60/m	AML M2 MDS-prephase	Normal/+8	CD34+/38– CD34+/38+	30 70	No result Normal/+8 +8 Normal/+8	0 10/32 (FISH) 3 2/14 (FISH)
6/75/f	AML M2Baso	t(6;9),+8	CD34+/38– CD34+/38+	42 15	t(6;9),+8 t(6;9),+8	1 2
7/71/f	AML M4	Normal	CD34+/38– CD34+/38+	100 1,000	Normal Normal	9 17
8/73/m	AML M4 with PNH	Normal/ del(6p)	CD34+/38– CD34+/38+	5 100	Normal/del(6p) Normal/del(6p)	19/10 11/5
9/60/f	AML M4	Normal / +4	CD34+/38– CD34+/38+	45 52	Normal/+4 Normal/+4	2/11 13/1
10/58/f	AML M4	Normal	CD34+/38– CD34+/38+	50 500	Normal Normal	24 4
11/51/m	AML M4Eo	Normal/ inv(16)	CD34+/38– CD34+/38+	20 200	No result inv(16)	0 1
12/34/f	AML M4Eo	inv(16)	CD34+/38– CD34+/38+	30 100	Normal/inv(16) inv(16)	1/4 5
13/30/f	AML M4Eo	Normal/ inv(16)	CD34+/38– CD34+/38+	600 2,000	Normal/inv(16) inv(16)	2/34 36
14/81/m	AML M4Fo	+8,–12, inv(16)	CD34+/38– CD34+/38+	70 390	+8,–12,inv(16) +8,–12,inv(16)	23 19
15/59/m	AML M4Eo	+8,inv(16)	CD34+/38– CD34+/38+	10 400	No result +8,inv(16)	0 5
16/32/f	AML M2	t(8;21)	CD34+/38– CD34+/38+	0.5 0.5	PCR + PCR +	– –

med. The number of completely analyzed metaphases ranged between 1 and 36 with a median of 14. Four patients had a normal karyotype in the unsorted bone marrow as well as in the sorted CD34+/CD38- cells. We found clonal karyotype abnormalities in 11 patients. All of them had the same abnormalities in their unsorted bone marrow. In one case the AML1/ETO fusion transcript was detected by RT-PCR. An AN-status was observed in 7 patients. In this population secondary abnormalities were present in three patients. Trisomy 8 occurred additionaly to t(6;9) in patient no. 6, trisomy 8 and monosomy 12 secondary to inversion 16 were observed in patient no. 14, and in patient no. 15 inversion 16 was accompanied by trisomy 8.

CD34+/CD117-/+ cells (Table 2)

CD117, the receptor for the stem cell factor (SCF) was introduced into our analytical panel to examine whether this surface molecule can be used as a tool to separate normal from leukemic stem cells. CD34+ stem cells characterized by the coexpression of CD117 were examined in two patients with AML. The cellular yield was 3100 cells and 500 cells respectively in the CD34+/CD117- populations and 100000 cells and 300 cells respectively in the CD34+/CD117+ populations.

Table 2. Stem cell genetics (CD34/CD117) in 2 pts. with AML at diagnosis

Pat. no./ age/sex	Diagnosis (FAB)	Karyotype, unsorted	Immuno-phenotype of sorted cells	Cells after sort ($\times 10^3$)	Karyotype of sorted cells	Metaphases analyzed
17/70/m	AML M2	Normal/ i(21q)	CD34+/117- CD34+/117+	3.1 100	i(21q) No result	1 0
18/74/m	AML M4	Normal/ i(17q),s21	CD34+/117- CD34+/117+	0.5 3.0	i(17q),s21 i(17q),s21	2 7

We performed classical cytogenetics of sorted stem cells and detected clonal anomalies in both patients. In patient no. 17 an isochromosome of the long arm of chromosome 21 was present in the CD34+/CD117- subpopulation. We were not able to analyze the CD34+/CD117+ population in this case. In the second patient an isochromosome 17q and monosmy 21 was verifiable in both stem cell subpopulations.

Stem Cell Genetics in MDS at Diagnosis

CD34+/CD38-/+ Cells (Table 3)

Stem cells characterized by the expression of CD34 and CD38 were analyzed in three patients with MDS at diagnosis. The cellular yield after sort ranged between 900 and 3 x 10^4. The number of completely analyzed metaphases was 36, 10, and 15 in the respective subpopulations.

In patient no. 19 with a MDS secondary to ovarian cancer treated with treosulfan CD34+/CD38- and CD34+/CD38+ cells were sorted together because of the scarcity of cells in this special case. Cytogenetic analysis of the unsorted bone marrow revealed five different subclones with a 5q- chromosome as the primary abnormality related to each other by karyotype evolution. In the sorted CD34+ stem cell population all five subclones were verifiable.

The second patient had a cytogenetically normal unsorted bone marrow. In the CD34+/CD38- population karyotyping was not successful whereas in the CD34+/CD38+ population we likewise found only normal cells. In the third patient with MDS we verified a chromosomal instability known from the unsorted bone marrow within the CD34+/CD38+ progenitor cell subpopulation.

CD34+/CD117-/+ cells (Table 4)

In this group we analyzed sorted cells of three patients. The number of CD34+/CD117- cells after the sort was 4000, 10000 and 210 respectively. The yield of CD34+/CD117+ cells was 160, 400 and 50 respectively.

We were successful in performing genetic analysis of the CD34+/CD117- stem cell sub-

Table 3. Stem cell genetics (CD34/CD38) in 3 pts. with MDS at diagnosis

Pat. no./ age/sex	Diagnosis (FAB)	Karyotype unsorted	Immuno-phenotype of sorted cells	Cells after sort ($\times 10^3$)	Karyotype of sorted cells	Metaphases analyzed
19/62/f	MDS RA (S°)	5q-/ 5q-,17p-/ 5q-,17p-,der17/ 5q-,17p-,der17,-20/ 5q-,-7,17p-,der17	CD34+/38- together with CD34+/38+	25	All 5 abnormal clones as in unsorted cells, no normal cells	36
20/68/m	MDS RAEB	Normal	CD34+/38- CD34+/38+	8.0 30.0	No result Normal	0 10
21/65/m	MDS CMML	Normal/chromosomal instability	CD34+/38- CD34+/38+	0.9 4.0	No result Normal/chromosomal instability	0 15

Table 4. Stem Cell Genetics (CD34/CD117) in 3 pts. with MDS at Diagnosis

Pat/no./ age/sex	Diagnosis (FAB)	Karyotype unsorted	Immuno-phenotype of sorted cells	Cells after sort ($\times 10^3$)	Karyotype of sorted cells	Metaphases/ interphases analyzed
22/60/f	MDS RA	Normal/5q-	CD34+/117- CD34+/117+	4.0 0.16	Normal/q5- No result	1/10 0
23/71/f	MDS RA	Normal	CD34+/117- CD34+/117+	10.0 0.4	Normal No result	6 0
24/24/f	MDS RAEB	Normal/+8	CD34+/117- CD34+/117+	0.21 0.05	Normal/+8 No result	37/40(FISH) 0

population in all three patients. We thus were able to verify the 5q- anomaly of patient no. 22 and trisomy 8 of patient no. 24 within the immature stem cell population. The trisomy 8 was detected by FISH. Patient no. 23 had a normal bone marrow karyotype which was confirmed by us in her CD34+/CD117- cells. In both cases with abnormal stem cells we observed a mosaic of normal and abnormal cells (AN-status).

Discussion

We conclude that in AML and MDS the immature stem cells regularly are the target of primary, disease-initiating and secondary, progression-associated leukemogenic genetic defects. This can be infered from our observation that whenever a case was informative (clonal abnormality in the unsorted bone marrow and successful genetic analysis of sorted cells) we were able to detect abnormal stem cells. This seems to be independent from the type of leukemia (de novo vs. secondary), the FAB-subtype and the kind of (cyto-)genetic abnormality since 15 different genetic abnormalities were found in the stem cell populations.

Especially in the cases of M4Eo with inversion 16 these observations were quite unexpected, since it seemed obvious that in these patients. malignant transformation occurs at the level of myelomonocytic committed progenitors. Further remarkable are our findings in 5 pts (pts. no 6, 14, 15, 18, and 19) which argue for an occurrence not only of primary, transforming defects but also of secondary, progression-associated abnormalities at the level of immature progenitors. Case no. 19 is especially interesting since in this pt. with a secondary MDS all five abnormal cell clones consisting of a stem line with a 5q- chromosome as initial abnormality and four different depending subclones were demonstrable in the unsorted as well as the highly purified flow-sorted CD34+ stem cell population, providing clear evidence that karyotype evolution and thus the dynamic process of disease progression takes place in the stem cell compartment. This observation furthermore implies that genetic instability–the prerequisite for an accumulation of (cyto)genetic abnormalities–occurs at the level of CD34+ stem cells.

Our results demonstrate that even tiny stem cell subpopulations with a cellular yield after cell sort of under 1000 cells can be succesfully karyotyped. This observation can be explained by the outstanding response of the stem cells to the cytokine cocktail chosen by us [25]. The proliferative potential of FACS-isolated CD34+ lineage-negative stem cells is well compatible with the succesful initiation of leukemia in SCID mice by inoculation with highly-purified immature progenitor cells [16].

In ten patients with abnormal stem cells an accompanying population of normal cells was demonstrable. Further sort and genotyping research should focus on strategies for the discrimation between normal and abnormal stem cells. Our first attempt to follow this strategy was to use the coexpression of CD117 as putatively discriminating element. However, in this respect our results are disappointing since neither the CD34+/CD117- nor the CD34+/CD117+ populations were free of leukemia-associa-

ted abnormalities in the informative cases (Tables 2 and 4). Further data are needed to confirm our preliminary results especially for the CD34+/CD117+ populations which seem to be difficult to analyze.

The vast majority of published data from other groups is based on indirect evidence for a stem cell involvement in malignant transformation in hematologic malignancies. Most information was derived from FISH and/or cytogenetic analyses of immunophenotypically characterized cells from different cell lineages [4,5] or from combining morphology and FISH [29]. Another group examined Ig heavy chain gene rearrangements in lymphoid and myeloid cells from patients with mixed lineage de novo acute leukemias. Their results were compatible with a clonal gene rearrangement at the level of pluripotent stem cells [30]. Recently, independent from us two groups have analyzed highly-purified, flow-sorted stem cells. Turhan et al. [31] examined CD34+/CD38- and CD34+/CD38+ stem cell subpopulations for the presence of the PML-RARA fusion transcript in three patients with promyelocytic AML (M3-subtype) by reverse transcriptase-polymerase chain reaction (RT-PCR). In two patients clonality analysis was additionally performed using the BstXI polymorphic site of the PGK gene. It was concluded from the data that in AML M3 the leukemogenic event does not take place in the CD34+/CD38- stem cells but is demonstrable in the more committed CD34+/CD38+ population. Mehrotra et al. analyzed sorted stem cell subpopulations in 15 patients with AML harbouring numerical karyotype abnormalities in their unsorted diagnostic bone marrow biopsies by FISH. In 15/15 cases they were able to demonstrate the respective numerical abnormality in the sorted immature stem cells [32]. Together with our data 21 different karyotype abnormalities were found within CD34+ stem cell subpopulations up to now. This is a strong argument for the hypothesis that malignant transformation in AML at the level of immature stem cells is the rule rather than the exception which had been assumed as yet. The data of Turhan and colleagues however shade the light on the possiblity that distinct leukemias with a very special biology might be excluded from what seems to be a common pathway in leukemogenesis. However, data of this group on the lack of PML-RARA fusion transcripts in CD34+/CD38- cells need further confirmation.

Our findings have substantial implications for the pathogenetic concept of leukemogenesis. The conservative model of leukemogenesis is based on the dogma that the leukemic phenotype is a reflection of the level of the hematopoietic hierarchy where the leukemogenic defect occurs, mostly at the level of committed progenitors. We would like to suggest a revised model with the genetic defect regularly occurring at the level of immature stem cells. In this model the genetic defect itself is the determinant for the differentiational program of the affected cell clone. Clonal abnormalities are only detectable in an expanding cell clone of a distinct cell line, if they provide a survival benefit for the respective cell, e.g., t(8;21) in myeloblastic cells or t(8;14) in B-lymphoblastic cells. By PCR-techniques it seems to be possible to detect "myeloid" genetic abnormalities like t(8;21) in "non-myeloid" lineages like T-cells [28]. However, it can be speculated that the leukemogenic potential of distinct abnormalities depends on the characteristics of the affected cell lineage. As an example t(8;21) on the one hand is leukemogenic in myeloblastic cells and on the other hand is non-leukemogenic but compatible with cellular survival in T-cells. Thus, the paradoxon could be explained that most clonal abnormalities occur at the level of immature stem cells but not all of them can be observed in any cell lineage.

Our results furthermore have substantial implications for residual disease in leukemia, since immunophenotypically normal stem cells might harbour a reservoir for relapse-inducing cells. These findings also have to be taken into account if myeloablative therapy strategies in leukemia are combined with autologous stem cell transplantation.

Therapeutically our data have two consequences: firstly, there is accumulating evidence that in leukemia the stem cell has to be the target and not to be prevented from therapy. Secondly, our data show that in the stem cell pool normal and abnormal coun-

terparts coexist. Future efforts should focus on how to discriminate these two stem cell compartments.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Mutations of Mitochondrial DNA as an Early Event in the Pathogenesis of Myelodysplastic Syndromes

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Abstract. Chloramphenicol inhibits mitochondrial protein synthesis, and its prolonged administration produces pancytopenia and dysplastic changes in the bone marrow, including the formation of ring sideroblasts. These changes are comparable to the abnormalities observed in myelodysplastic syndromes (MDS). Another link between mitochondrial dysfunction and disordered hematopoiesis is provided by Pearson's syndrome, a rare congenital disorder presenting in infancy with a variety of symptoms, including refractory sideroblastic anemia, thrombocytopenia, neutropenia, and dysplastic changes in the bone marrow. Pearson's syndrome is caused by large deletions of mitochondrial DNA (mtDNA). Furthermore, point mutations of mitochondrial tRNAs also inhibit mitochondrial protein synthesis, as known from mitochondrial encephalomyopathies. We are screening bone marrow samples from MDS patients for point mutations of mtDNA, employing RFLP analysis and heteroduplex analysis with temperature-gradient-gel-electrophoresis (TGGE). Several point mutations have been identified that are heteroplasmic, i.e. they establish a mixture of normal and mutated mitochondrial genomes, which is typical of disorders of mtDNA. Mutations were found in mitochondrial tRNAs, in subunits 1 and 2 of cytochrome-c-oxidase (COX, complex IV of the respiratory chain) and in cytochrome b (redox center of complex III). Identification of two point mutations with very similar location in COX 1, as well as other lines of evidence, suggest that cytochrome-c-oxidase, the only copper-containing enzyme complex of the respiratory chain, is involved in mitochondrial iron uptake and conversion of Fe^{3+} to Fe^{2+} for heme synthesis. COX mutations may therefore play a pivotal role in the pathogenesis of sideroblastic anemia. Even though some mtDNA mutations are known to create a growth handicap for proliferating cells, the percentage of mutated mtDNA molecules in the bone marrow samples was as high as 40-60%. MtDNA mutations are therefore unlikely to be late events arising in a subclone, because in that case they would prevent their propagation in the bone marrow by putting their subclone at a disadvantage compared with other cells of the neoplastic population. This is incompatible with the observed high proportions of mutated mtDNA. It must be concluded that mtDNA mutations are early events, arising in a stem cell which later undergoes transformation through nuclear DNA mutations, thereby acquiring the capability of establishing clonal hematopoiesis and spreading the mtDNA mutation in the bone marrow.

Keywords. Myelodysplastic syndromes, sideroblastic anemia, heme synthesis, mitochondrial iron metabolism, respiratory chain, mitochondrial DNA

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Introduction

Mitochondrial DNA and Disease

Human mitochondrial DNA (mtDNA) is a small (16569 bp) double-stranded circular DNA molecule, which contains 13 protein genes, all of them coding for essential subunits of the mitochondrial respiratory chain, together with 2 ribosomal RNAs and 22 transfer RNAs that are necessary for the expression of the protein genes. Every mitochondrion has 5-10 copies of mtDNA, so that every cell contains hundreds or even thousands of copies of mtDNA. In 1988, it was discovered that mutations of mitochondrial DNA underlie mitochondrial myopathies and Leber's hereditary optic neuropathy. Since then, mtDNA mutations have been implicated in a number of neurological diseases, some forms of diabetes, and even aging (reviews: [1-8]). The preponderance of neurological symptoms in disorders of mtDNA is not surprising because the functioning of neurons and muscle cells depends very much on energy production by an intact respiratory chain. Up to now, there has been only one example of a mitochondrial DNA disorder featuring hematological symptoms. Pearson's syndrome is a rare congenital disorder, which is characterized by permanent lactic acidosis, pancreatic insufficiency, other metabolic derangements, and severe refractory sideroblastic anemia. The anemia is usually accompanied by neutropenia and thrombocytopenia. The bone marrow shows dysplastic changes, including ring sideroblasts and prominent vacuolization of precursor cells. A few years ago, it was found that Pearson's syndrome is caused by large deletions of mitochondrial DNA. Pearson's syndrome thus provides a strong link between mtDNA damage and bone marrow dysplasia with a sideroblastic phenotype. Another link between mitochondrial dysfunction and disordered hematopoiesis is provided by the well-known hematological toxicity of chloramphenicol [9]. Prolonged administration of chloramphenicol (CAP) produces pancytopenia and dysplastic changes in the bone marrow, including the formation of ring sideroblasts, which are comparable to the abnormalities observed in myelodysplastic syndromes. The hematological toxicity of CAP medication is mediated through inhibition of mitochondrial protein synthesis. Impairment of mitochondrial protein synthesis, on the other hand, is also the mechanism by which mutations of mitochondrial tRNAs exert their pathogenetic effect in mitochondrial encephalomyopathies. It is therefore tempting to speculate that mutations of mitochondrial tRNAs and other mtDNA mutations may give rise to hematopoietic dysplasia.

In Pearson's syndrome, investigation of many patients confirmed that mtDNA deletions are a consistent feature of the disease. However, the pathogenetic relation between mtDNA deletions and sideroblastic anemia has not been understood. We have recently developed a new pathogenetic model of sideroblastic anemia, which provides the missing link between mitochondrial DNA mutations and the formation of ring sideroblasts. This model not only applies to Pearson's syndrome but also to sideroblastic anemia in the context of myelodysplastic syndromes.

A New Pathogenetic Model of Sideroblastic Anemia

Sideroblastic anemia is characterized by inadequate formation of heme and excessive accumulation of iron in erythroblast mitochondria. For many years, investigators tried to clarify the disorder by searching for enzyme defects of heme biosynthesis (Fig. 1). A major shortcoming of this approach was its inability to explain why, at the terminal step of the heme synthetic pathway, protoporphyrin IX is elevated rather than decreased in most AISA patients [10]. This finding is incompatible with a significant enzyme defect upstream in the pathway, but it provides a strong argument for a possible defect in the last step of heme synthesis, namely insertion of iron into protoporphyrin IX. This step is catalyzed by ferrochelatase. However, this enzyme is unlikely to be responsible, because increased red cell protoporphyrin concentrations in sideroblastic anemia are not correlated with low ferrochelatase activities [11, 12]. With normal ferrochelatase activity, and with plentiful iron

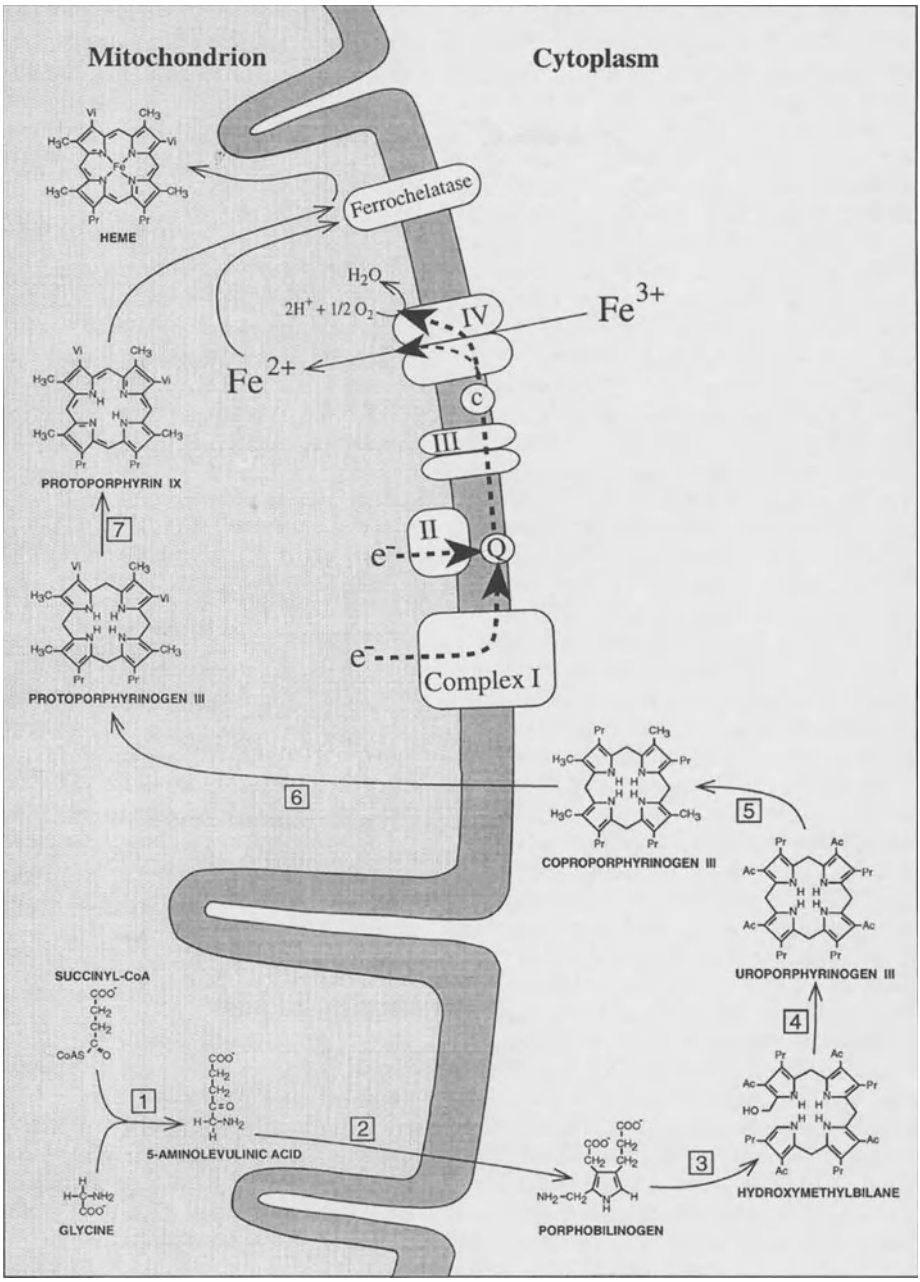


Fig. 1. Schematic representation of the heme biosynthetic pathway and its connection with the electron transport chain of the inner mitochondrial membrane. *1* 5-Aminolevulinic acid synthase; *2* 5-aminolevulinic acid dehydratase; *3* porphobilinogen deaminase; *4* uroporphyrinogen III synthase; *5* uroporphyrinogen III decarboxylase; *6* coproporphyrinogen III oxidase; *7* protoporphyrinogen III oxidase. *Complex I* NADH dehydrogenase; *Complex II* succinate dehydrogenase; *Complex III* b/c1-complex = ubiquinol cytochrome c reductase; *Complex IV* cytochrome c oxidase; *Q* coenzyme Q = ubiquinone; *c* cytochrome c

and protoporphyrin IX, iron is still not inserted into the heme precursor. The likely explanation is that iron is not in the right chemical form. Iron deposits in sideroblastic mitochondria contain iron in the trivalent ferric (Fe^{3+}) state [13], while ferrochelatase can only use ferrous iron (Fe^{2+}) for heme synthesis [14]. As Fe^{2+} is not stable under aerobic conditions, it is necessary for erythropoietic cells to have an enzyme system that can maintain a supply of Fe^{2+} as a substrate for ferrochelatase. About 20 years ago, reduction of Fe^{3+} was shown to be accomplished by the respiratory chain as the source of reducing equivalents [15-23]. At complete inhibition of mitochondrial respiration, heme synthesis becomes negligible. However, it is not known to which part of the respiratory chain the reduction of ferric iron is linked. We proposed that in sideroblastic anemia, one or several of the enzyme complexes of the respiratory chain are disturbed in such a way that efficient reduction of iron no longer occurs [24]. Under these circumstances, the imported iron cannot be utilized for heme synthesis and will accumulate in the mitochondrial matrix. The proposed malfunction could be due to mutations of nuclear DNA or mitochondrial DNA, because both genomes contribute to the assembly of respiratory chain complexes.

Our focus on mitochondrial DNA is partly explained by the precedent of Pearson's syndrome. However, there is another argument in favour of concentrating on the mitochondrial genome. Mutations of mtDNA are very useful to explain a peculiar feature of AISA, namely the heterogeneity in the degree of mitochondrial iron accumulation. Not all erythroblasts in AISA are ring sideroblasts, and not all ring sideroblasts show the same degree of mitochondrial iron overload. This heterogeneity has also been demonstrated within single erythroid colonies cultured in vitro [25-27]. Whereas such intraclonal heterogeneity is hard to explain in terms of mutations involving nuclear genes, mitochondrial DNA mutations could easily account for the phenomenon. Each cell contains hundreds of mitochondria and thousands of copies of the mitochondrial genome. Therefore, cells can harbour mixtures of mutant and normal mtDNAs (heteroplasmy), and each time a heteroplasmic cell divides, the mutant and normal mtDNAs are randomly segregated into the daughter cells [1]. A heterogeneous population will arise, consisting of grossly abnormal cells with a high percentage of mutated mtDNA, normal appearing cells with a low proportion of mutated mtDNA, as well as a spectrum of intermediate cells. In each cell, the proportion of mutated mtDNA determines the expression of the pathological phenotype. This mechanism provides a rational basis to explain the heterogeneity of the sideroblastic phenotype.

Having excluded large deletions of mtDNA as a cause of AISA [28], we started looking for short deletions and point mutations.

Materials and Methods

DNA Isolation

Total DNA was isolated from blood and bone marrow samples using the QIAamp Kit (Qiagen, Hilden, Germany), which is based on cell lysis in the presence of guanidine hydrochloride and subsequent binding of DNA to an anion-exchange resin in a spin-column. The procedure ensures extraction of mitochondrial DNA in addition to nuclear DNA.

MtDNA Amplification

MtDNA was amplified in 17 overlapping segments using the polymerase chain reaction. The exact position of segments according to the standard Cambridge sequence [29] and the respective primer sequences have been published [30]. DNA was amplified using 35 cycles of denaturation (1 min at 94 °C), annealing (30 s at 55-71 °C, depending on the pair of primers used), and extension (60 s at 72 °C). The 100 µl PCR reactions contained 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)2SO^4$, 4 mM $MgS0_4$, 400 µM of each dNTP, 50 pmol of each primer, and 1 unit of Vent DNA polymerase (New England Biolabs, Dreieich, Germany). Double stranded PCR products were puri-

fied with a commercial DNA purification system (Wizard PCR Preps, Promega, Heidelberg, Germany), separated in 1% agarose gels and visualized by ethidium bromide staining.

RFLP Analysis

Each amplified mtDNA segment was entered into 20 different restriction enzyme digestions using AluI, BamHI, CfoI, DdeI, EcoRI, HaeII, HaeIII, HincII, HindIII, HinfI, HpaI, MspI, PvuII, RsaI, SacI, ScaI, StuI, TaqI, Tru9I, and XbaI. Endonucleases were purchased from Boehringer Mannheim, Germany. All restriction enzyme reactions were performed according to manufacturer's recommendations, using approximately 100-200 ng of PCR-generated DNA for digestion with 5 units of enzyme. Restriction fragments were separated by electrophoresis in 3.5 % Visigel matrix (Stratagene, Heidelberg, Germany) and visualized by ethidium bromide staining and UV illumination.

Heteroduplex Analysis by TGGE

Point mutations in double-stranded DNA can be detected by denaturing-gradient gel electrophoresis (DGGE) [31] or temperature-gradient gel electrophoresis (TGGE) [32, 33] because mutations alter the denaturation pattern of double-stranded DNA at the site of the mutation and in its neighbourhood. The sensitivity of TGGE analysis is greatly enhanced through heteroduplex generation. During a denaturation/renaturation cycle, hybrids are formed between wild-type DNA fragments and homologous fragments carrying the mutation. The mismatch in the double helix at the site of the mutation leads to significant lowering of the midpoint melting temperature of the heteroduplex fragments. When exposed to increasing temperatures during electrophoresis (the TGGE procedure), heteroduplex hybrids show partial denaturation earlier than homoduplexes do. Because early partial denaturation causes early retardation in the gel, heteroduplexes can be resolved from homoduplex molecules by TGGE. We carried out intraindividual heteroduplex analysis, i.e., TGGE was employed to examine whether heteroduplexes formed after PCR-amplification of a mtDNA fragment are of interest. This only happens when there is coexistence of wild-type and mutated mtDNA in the patient's sample, which is also called heteroplasmy. PCR was followed by quantitative denaturation of the PCR product (heating in 4 M urea at 95 °C for 5 min) and subsequent renaturation (incubation at 50 °C for 20 min). Samples were then loaded onto thin polyacrylamide gels (5%) covalently bound to polyethylene gel support films. After electrophoresis, bands of heteroduplex- and homoduplex-DNA were visualized by silver staining. TGGE equipment was purchased from Qiagen, Hilden, Germany.

Sequencing

For sequencing of the region of interest, mtDNA from bone marrow was amplified using modified primers designed to introduce appropriate restriction sites for ligating the fragment with T4 DNA-Ligase into the sequencing vector pUC19 [34], which had been digested with the corresponding restriction enzymes. After transformation into E.coli strain DHaF', white colonies were selected on Luria broth (LB) medium containing 150 mg/l ampicillin and X-Gal [35]. Plasmid DNA from transformants was prepared using Tip20 columns (Qiagen, Hilden, Germany) and double-stranded DNA was sequenced with the Sequenase kit (USB, Cleveland, Ohio), using ^{35}S-dATP, according to the method of Sanger [36].

Cell Lineage-Involvement

MtDNA was extracted from unfractionated bone marrow samples and from peripheral blood cells of different lineages. For isolation of B and T lymphocytes from EDTA anticoagulated blood, immunomagnetic beads (Dynabeads M-450 Pan-B [CD19] and Dynabeads M-450 Pan-T [CD2], respectively) were used according to the recommendations of the manufacturer (Dynal, Oslo, Norway). Buccal mucosa cells were pelleted from

a vigorous mouthwash performed with 20 ml normal saline and depleted of contaminating granulocytes by density gradient centrifugation with Nycoprep 1.150 (Nycomed). Skin biopsies were obtained from patients A. M and H. L, and fibroblast cultures were established by outgrowth from these biopsies according to a standard method [37]. Granulocytes were enriched by depletion of peripheral blood mononuclear cells through density gradient centrifugation with Lymphoprep 1.077 (Nycomed, Oslo, Norway). A 200 µl sample from the granulocyte/erythrocyte pellet was used for DNA extraction. Erythrocytes do not contain mtDNA [38], because mitochondria are eliminated during the reticulocyte stage [39].

Results

As summarized in Table 1, mutations were found in mitochondrial tRNAs, in subunits of cytochrome c oxidase (complex IV of the respiratory chain), and in cytochrome b (redox center of complex III). At present, it is difficult to state the frequency of these mutations, because in many patients screening of the mitochondrial genome has not yet been completed. The whole mitochondrial genome has been screened by RFLP and TGGE in 20 patients (including 12 patients with sideroblastic anemia), and 4 mutations were found in those cases. The other mutations were identified in some 50 other patients whose mitochondrial DNA has been partially analysed. Detection of mtDNA mutations is probably far from complete even in the group of 20 patients who where examined fairly thoroughly. High-density RFLP analysis, as performed, can detect 10-20% of mutations. TGGE analysis can reach a sensitivy of 95%, but this requires a high degree of optimization, which has not yet been achieved.

Figures 2 and 3 show examples of TGGE heteroduplex findings which led to the detection and characterization of point mutations of mtDNA. The terms perpendicular and parallel refer the direction of the temperature gradient relative to the direction of migration during electrophoresis. Parallel TGGE permits analysis of the same mtDNA region in several patients simultaneously. Suspicious findings in parallel TGGE should always be confirmed by perpendicular TGGE to rule out extra bands resulting from unspecific PCR products.

Table 1.

Patient	FAB type	Gene	Nucleotide position	Nucleotide change	Amino acid change
K.A.	RARS	tRNA(Ala)	5631	G→A	
R.Sch.	RARS	tRNA			
A.M.	RARS	tRNA(Leu)	12301	G > A	Anticodon loop
		COX I			
H.L.	RARS	COX I	6742	T > C	Ile → Thr
H.W.	RARS	COX I	6721	T > C	Met > Thr
		COX II/tRNA(Lys)	8280	A > C	
R.P.	RARS	COX III			
H.H.	RARS	Cytochrome b	14774	C > A	Leu > Met
J.K.	RARS	Cytochrome b	14933	T > A	Phe > Tyr
J.B.	RARS	Cytochrome b	14900	G > A	Ala > Thr
H.K.	RA	tRNA(Lys)	8344	A > G	(MERRF)
E.HŸ.	RAEB	ATPase 8	8472	C > T	Pro > Leu
H.Ho.	RAEB	Cytochrome b	14762	A > G	Lys > Glu
B.Sm.	RAEB	Cytochrome b	14877	A > G	Gln > Arg
E.Sch.	RAEB-T	COX I			
E.H.	CMML	tRNA			
E.St.	MDS/AML	tRNA(Trp)	5548	A > C	
		tRNA(Lys)	8344	A > G	(MERRF)
		COX II			
		Cytochrome b	14793	A > G	His > Arg

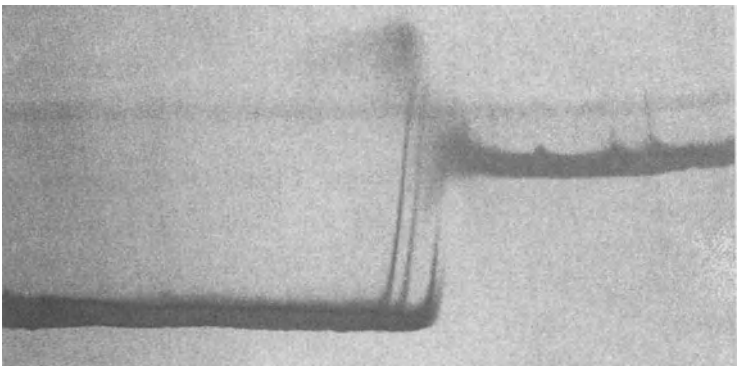


Fig. 2. Perpendicular TGGE. MtDNA fragment: nt 6504-6923; 5% polyacrylamide gel, silver staining. The typical heteroduplex pattern (two clearly separated heteroduplex bands on the *left*, two homoduplex bands not clearly separated on the *right*) indicates the presence of two homologous mtDNA species (wild-type and mutated), which after a cycle of denaturation and renaturation produce hybrids differing in their melting behaviour. Cloning and DNA sequencing revealed a point mutation at nt 6742 (T > C), producing an amino acid change (*i*soleucin > *t*hreonin) in subunit I of cytochrome c oxidase

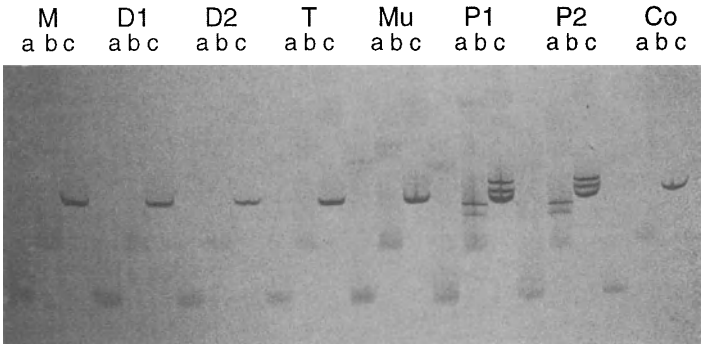


Fig. 3. Parallel TGGE. Same mtDNA fragment as in Fig. 2. After denaturation and renaturation, each PCR product was brought onto the gel in triplicate at time intervals of 15 minutes (lanes *a,b,c*, respectively). Running time was too long for samples designated a and b , so that bands became diffuse. Clear bands can be seen in lanes *c*. *M*, *D1*, *D2*: Normal bands in the mother and two daughters of patient 1; *T* and *Mu* normal bands in T-lymphocytes and buccal mucosa cells of patient 1; *P1* and *P2* heteroduplex bands in patient 1 (H. W.) and patient 2 (H. L.); *Co* normal bands in an additional control person

In three patients (A. M., H. W., and H. L.) cell lineage involvement was analyzed. An example is given in Fig. 4. A consistent pattern emerged, with the mutations being present in bone marrow and whole blood samples, in isolated platelets, and in granulocytes, but apparently being absent from T- and B-lymphocytes purified by immunomagnetic bead separation. The mutations were not detectable in buccal mucosa cells obtained by mouthwashes, and in cultured skin fibroblasts examined in two of the patients. This pattern of involvement suggests that the mtDNA mutations occurred in self-renewing bone marrow stem cells with myeloid determination (CFU-GEMM).

The proportion of mutated mtDNA molecules in the bone marrow and in different cell lineages was determined by assessing the relative intensities of bands in electrophoresis gels with an electronic camera and analyzing data with suitable software (Enhanced analysis system, EASY plus, Herolab GmbH, Wiesloch, Germany). After correcting for molecular weight, this analysis indicated that the proportion of mutated mtDNA in the samples ranged between 40 and 60%.

Discussion

Pathogenetic Relevance

The mutations that we detected fulfil the criteria that are generally applied to exclude non-pathogenic polymorphisms of mtDNA, namely

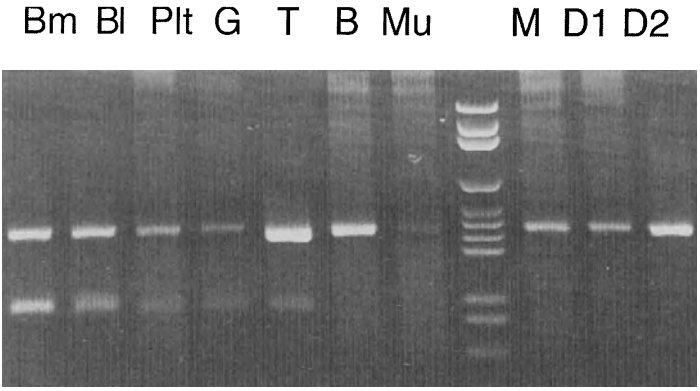


Fig. 4. Cell lineage involvement of the COX I mutation in patient H. W. Total DNA was extracted from different types of cells: *Bm* bone marrow; *Bl* peripheral blood; *Plt* platelets; *G* erythrocyte/granulocyte pellet; *T* T lymphocytes; *B* B lymphocytes; *Mu* buccal mucosa cells; *lane 8* DNA size marker; *M* peripheral blood from the mother of patient 1; *D1* and *D2* peripheral blood from the first and second daughter of the patient

(i) the observed nucleotide changes were absent in unaffected individuals,
(ii) they occurred in evolutionary conserved regions, and
(iii) the mutations displayed heteroplasmy.

When a mtDNA mutation arises, it creates an intracellular mixture of mutant and normal mtDNA molecules called heteroplasmy. Heteroplasmy is considered an indicator for pathogenic mutations because it is typical of disorders of mtDNA but generally absent in normal individuals.

Recently, we reported the first heteroplasmic point mutation of mitochondrial tRNA*Leu(CUN)* that we identified in the bone marrow cells of a patient with sideroblastic anemia [30]. However, mutations of mitochondrial tRNAs do not help to localize the crucial site of iron reduction and transport through the mitochondrial inner membrane, because mitochondrial tRNA mutations cause a general impairment of mitochondrial protein synthesis and can thus affect all respiratory chain complexes that possess components encoded by mtDNA. More informative in this respect are point mutations of genes coding subunits of cytochrome c oxidase. Identification of two point mutations with very similar location in COX I suggest that this enzyme complex is strongly related to the pathogenesis of sideroblastic anemia. Cytochrome c oxidase (COX) is a good candidate for playing a major role in mitochondrial iron metabolism. A strong hint in this direction comes from the "copper connection" [40], which refers to the close relation between copper and iron in hematopoiesis, including the role of copper in mitochondrial iron metabolism. COX is the only respiratory chain complex that contains copper, namely Cu_{A1} and Cu_{A2} in subunit II, and Cu_B in subunit I [41, 42]. Copper is an essential part of the metal redox centres of this enzyme complex. Intriguingly, copper deficiency can cause sideroblastic anemia, with usually moderate numbers of ring sideroblasts [9, 43-48]. Williams et al. [49] found that mitochondria isolated from copper-deficient animals were deficient in COX activity and failed to synthesize heme from Fe^{3+} and protoporphyrin at the normal rate. The rate of heme synthesis correlated with the COX activity. Through experiments with respiratory chain inhibitors, these authors confirmed that an active and intact electron transport chain is required for the reduction of Fe^{3+} on the inner mitochondrial membrane, and for a supply of Fe^{2+} as substrate for ferrochelatase. Williams et al. suggested that electrons may be donated to Fe^{3+} from cytochrome c oxidase. Because both COX I mutations that we identified are located in transmembrane helix no. VII in the immediate vicinity of the heme a_3-Cu_B centre [41], it is tempting to speculate that this metal redox centre is involved in supplying electrons to Fe3+. The copper connection has recently been strengthened by the finding that high-affinity transmembrane uptake of iron in Saccharomyces cerevisiae requires copper. This is explained by the involvement of a copper-containing oxidoreductase, FET3, in iron uptake [50, 51]. Yeast mutants defective in the fet3 gene are defi-

cient in high affinity Fe^{2+} transport, and copper depletion of wild-type S. cerevisiae also results in a selective reversible decrease in Fe^{2+} transporter activity. The FET3 protein exhibits extensive similarity to the familiy of blue multicopper oxidoreductases, an enzyme superfamily that also includes mitochondrial cytochrome c oxidase.

Mutations of cytochrome b probably disturb electron flow to cytochrome c oxidase and may thus also compromise iron reduction. In addition, those mtDNA mutations leading to significant impairment of respiratory chain function may decrease energy-dependent uptake of iron into mitochondria. This may be a key mechanism of impaired heme synthesis in refractory anemia without ring sideroblasts. In general, because heme synthesis strongly depends on the proper functioning of mitochondria, erythropoiesis can be expected to be particularly vulnerable to the effects of mtDNA mutations. This would explain why dyserythropoiesis and anemia are virtually always present in MDS patients, whereas dysgranulocytopoiesis and dysmegakaryocytopoiesis are often less prominent.

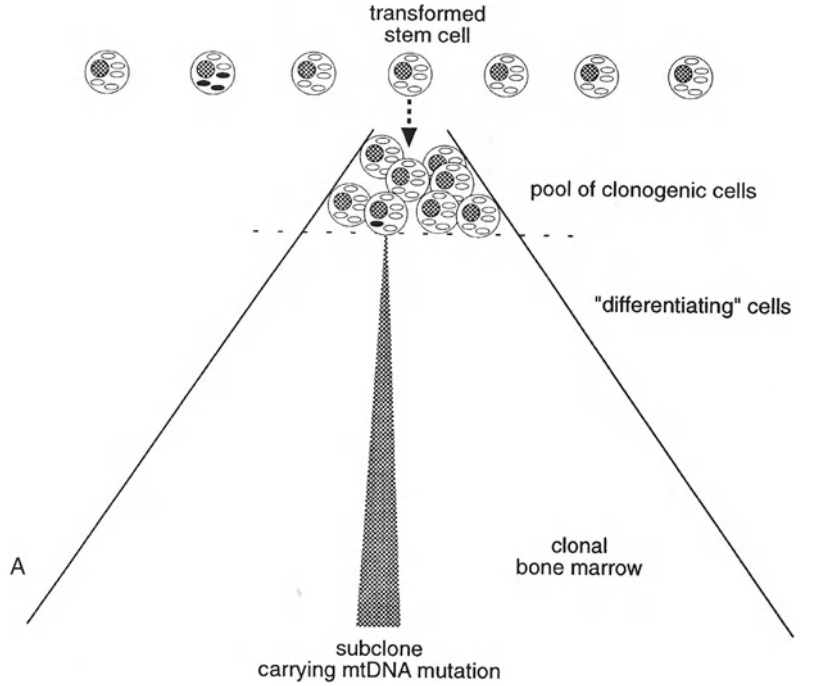
Why Are mtDNA Mutations Early Events in the Pathogenesis of MDS?

Figure 5 is to illustrate that a transformed stem cell will soon establish a pool of clonogenic cells which maintain the pathological clone. To be of any relevance at all, mtDNA mutations must arise in one of these cells with a high capacity for self renewal. Cells lower down in the hierarchy will die sooner or later, thereby eliminating the mutations they may incur. If a mtDNA mutation occurred as a secondary event in one of the cells of the clonogenic pool (Fig. 5A), this mutation would be confined to a small subclone, which would probably not be detectable with our methods. It is very unlikely that this subclone would become dominant, because it has been shown in cell culture experiments that mtDNA mutations confer a growth disadvantage rather than a growth advantage to proliferating cells [52-54]. Confinement of a mtDNA mutation to a subclone, however, is incompatible with our findings. We are finding mutations that affect up to 60% of all mtDNA molecules in the bone marrow. This is obviously too much to be derived from a small subclone. Therefore, it is very unlikely that these mutations occurred as a secondary events during the course of the disease.

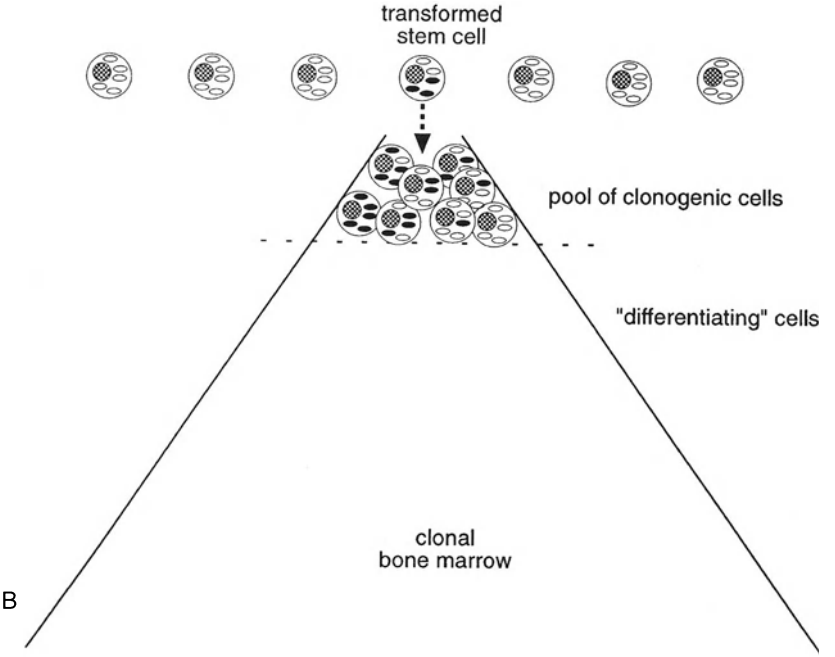
Much more likely is the following scenario (Fig. 5B): We propose that the mtDNA mutations that we identified were already present in a bone marrow stem cell before that stem cell became transformed. In that case, the random segregation of mitochondria during mitosis would result in some cells with a relatively high percentage and other cells with a relatively low proportion of mutated mtDNA, which would explain a lot of the intraclonal heterogeneity that can be observed in sideroblastic anemia and other types of MDS. When a stem cell carrying a mtDNA mutation acquires a growth advantage through clonal transformation, it is not hard to explain how the mtDNA mutation can spread in the bone marrow to reach a proportion of more than 50%. Clonal transformation is of course a matter of nuclear DNA. Accordingly, there is no conflict whatsoever between the mtDNA mutations that we found and the mutations of nuclear DNA, such as ras gene mutations, that occur in myelodysplastic syndromes.

How Do mtDNA Mutations Fit into the Whole Picture of MDS Pathogenesis?

With increasing age, hematopoietic stem cells probably accumulate mtDNA mutations, just as muscle or nerve cells do [54]. Some of the affected stem cells will not be able to cope with their mitochondrial damage and die, probably by apoptosis. This may contribute to the age-related shrinking of the bone marrow reserve. Other stem cells harbouring mtDNA mutations will produce progeny, which may show functional alterations or even morphological changes. However, this is not MDS because the bone marrow is not clonal. Only if a stem cell with mutated mtDNA becomes transformed by a nuclear gene mutation, a clonal bone marrow will be established and the clinical picture of MDS can arise. It is certainly temp-


transformed
stem cell
pool of clonogenic cells
"differentiating" cells
clonal
bone marrow
subclone
carrying mtDNA mutation

A


transformed
stem cell
pool of clonogenic cells
"differentiating" cells
clonal
bone marrow

B

Fig. 5A, B

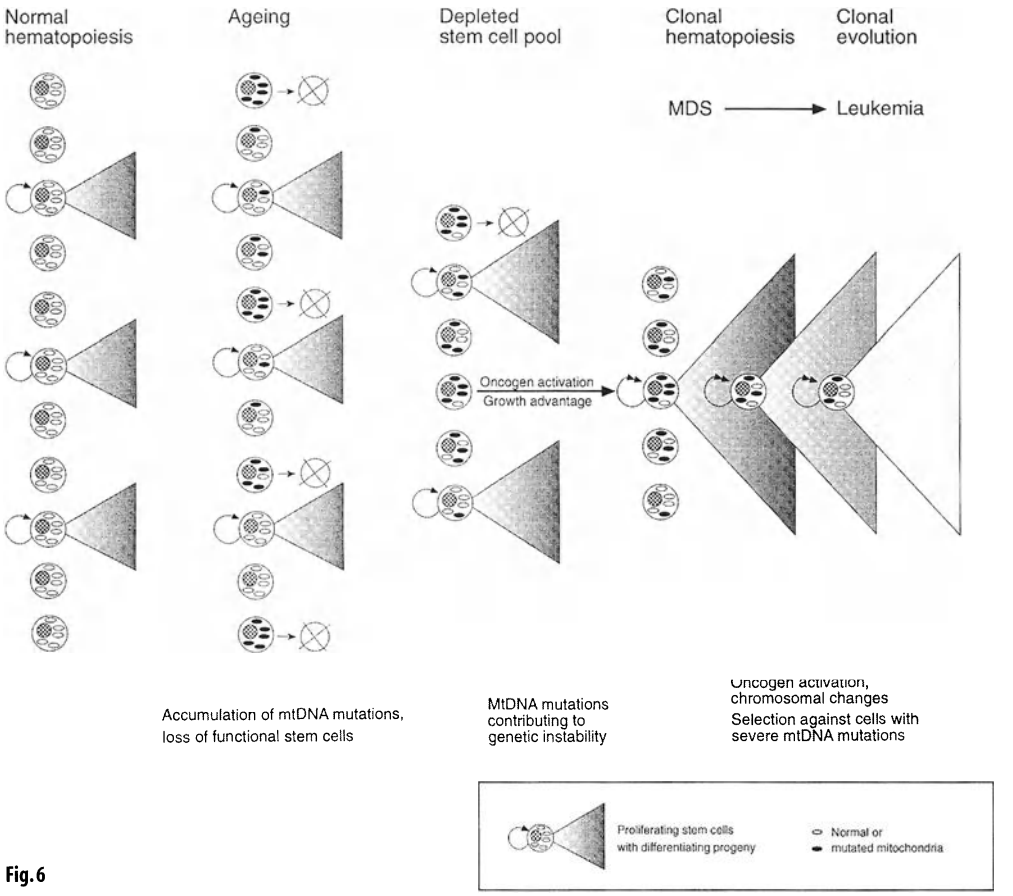


Fig. 6

ting to speculate that mtDNA mutations and mitochondrial dysfunction may not only contribute to the phenotype of the pathological clone, but may also contribute to genetic instability, thereby precipitating the transforming event as well as further nuclear gene mutations that characterize the path of clonal evolution towards leukemia.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Pilot Study of Pentoxifylline and Ciprofloxacin with or without Dexamethasone Produces Encouraging Results in Myelodysplastic Syndromes

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Abstract. Forty-three patients with myelodysplastic syndromes (MDS) were treated with a combination of pentoxifylline and ciprofloxacin (PC) with the addition of dexamethasone (PCD) in 18 patients who failed to respond to PC. There were 15 females and 28 males, and the median age was 67 years. A total of 18 patients either showed a hematopoietic improvement, a partial or complete cytogenetic response or a combination of both for an overall response rate of 42%. Seven PC only patients responded, four showing hematologic improvement, two cytogenetic responses and one patient showing a combined response. This 16% response rate to PC was increased to 61% by the addition of dexamethasone with 11/18 patients showing a response. Four of the 7 patients who responded initially to PC were given dexamethasone after at least 12 weeks of PC therapy, and only 1 showed a further improvement in response. Thus, we conclude that the combination of PCD provides an encouraging novel approach to treating MDS. The mechanism of action is probably related to the suppression of a veriety of cytokines which in turn attenuate the excessive intramedullary apoptotic death of hematopoetic cells in MDS, an observation which has been speculated to be the basis of the paradox of variable cytopenias despite cellular marrows in MDS. Larger numbers of patients need to be treated and followed for longer periods to determine the true efficacy of this therapy, especially the nature and duration of the cytogenetic responses.

Introduction

Hematologists regularly have to deal with patients suffering from myelodysplastic syndromes (MDS), a frustrating experience since therapeutic options for these unfortunate individuals are not only limited but also confusing, as they range from non-interventional approaches such as supportive care only to the aggressive use of bone marrow transplantation with its prohibitive risks [1, 2]. Furthermore, recent advances in the treatment of a number of hematopoetic disorders resulting from the introduction of growth factors combined with better uses of chemotherapies have not universally and/or consistently been applicable to MDS. The incidence of MDS appears to be on the rise not only because people are living longer and MDS is a disease of the elderly but also because of an increase in the incidence of MDS secondary to treatment for a prior malignancy [3]. New modalities for therapy are rather urgently needed. Novel insights into

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the biology of MDS have opened up at least one such area of therapeutic research which originated with the idea of suppressing a variety of cytokines believed to be operational behind the variable cytopenias frequently encountered in these syndromes and is therefore referred to as "anti-cytokine therapy."

Although MDS are clearly stem cell disorders marked by the presence of monoclonal hemopoiesis [4–7], the genesis of the clinical syndrome may have an additional component of cytokine mediated excessive apoptosis [8]. Our studies to address the paradox of cytopenias despite generally cellular marrows demonstrated the presence of large numbers of erythroid, myeloid and megakaryocytic cells actively engaged in DNA synthesis [9]. Unfortunately, this high rate of proliferation was often matched by an excessive apoptotic index [9]. We hypothesized that both the increased proliferation and apoptosis may be the result of dual acting cytokines which stimulate early $CD34^+$ progenitors to divide while inducing a programmed cell death in their maturing $CD34^-$ progeny [10]. Indeed, high levels of tumor necrosis factor alpha (TNF-α) [11], transforming growth factor beta (TGT-β) [11], and interleukin-1 beta (IL1-β) [12] have been demonstrated in a substantial number of MDS patients. If this new paradigm was correct, then suppression of these cytokines should yield dual benefits in that decrease in apoptosis should be accompanied by amelioration of cytopenias while reduction in stimulation of the transformed progenitors should allow some normal stem cells to be recruited back in cycle.

The ideal anti-cytokine approach would be to use a specific inhibitor for the factor identified as playing the predominant pathogenic role in individual patients such as an anti-TNF antibody, however, the pathology may be the result of a cascade of cytokines. An approach which could neutralize a variety of cytokines would be preferable. Fortunately, several of these proteins use the phosphatidic acid → diacylglycerol pathway and interruption of this step should theoretically attenuate their action simultaneously [13]. We chose to use pentoxifylline (PTX), a xanthine derivative which interferes with the lipid signalling pathway of several cytokines including TNF-α, TGF-β and IL1-β [14, 15]. Ciprofloxacin (Cipro) was given concomitantly to reduce the hepatic degradation of PTX by the P450 reductase system. After at least 12 weeks of this therapy, dexamethasone (decadron) was added off protocol to the regimen of 18 patients (PCD therapy). We found that 18/43 patients had either a hematopoietic, cytogenetic or a combined response. Among the 18 responders, 7 patients showed a response after PC therapy only while 11 responded only after the addition of dexamethasone. This chapter summarizes the clinical characteristics and response to therapy in these 43 MDS patients.

Materials and Methods

Forty-three patients with a confirmed diagnosis of MDS are the subject of this report. Of these, 13 patients were studied on protocol MDS 90–02 and 30 cases were treated on protocol MDS 95–01. Every patient received an infusion of Iodo- and/or bromodeoxyuridine (IUdR, BrdU) on MDS 90-02 for cell cycle studies prior to starting therapy. Informed consent for these studies was obtained in every case.

Clinical Studies: The first 13 patients were studied for biological characteristics on protocol MDS 90–02 and then started on PTX 800 mg tid po and Cipro 400 mg po bid. After completing at least 12 weeks of this therapy, dexamethasone 4 mg po q a.m. was added. The therapy had to be interrupted for the majority of cases receiving the dexamethasone eventually because of the long term side effects of steroids. The next 30 patients were treated on protocol MDS 95–01. On this protocol, PTX was given at a dose of 400 mg po tid for one week and then increased to 800 mg po tid. Cipro was started at 500 mg po bid from the third week on. Once again, at least 12 weeks of PTX + Cipro were given to all 30 patients. Dexamethasone was then added at a dose of 4.0 mg po q a.m. Of the 43 patients being reported here, 18 received the PCD therapy while 21 patients only received PTX + Cipro. Four patients who responded to PC were also given dexamethasone so that eventually a total of 22 MDS patients re-

ceived PCD. The therapy was well tolerated generally. Side effects of PTX included heart-burn and dyspepsia if taken on an empty stomach and occasional nausea and early satiety. With time, the symptoms decreased in the majority of patients, although the dose had to be decreased to 400 mg po tid in a few cases. There was one case of severe tendinitis observed as a Cipro side-effect. Dexamethasone, on the other hand, was associated with increased blood sugar levels, anxiety, hyperactivity, euphoria, weight gain and ultimately proximal muscle weakness.

Response Criteria. Restoration of normal hematopoiesis with normal PB counts was defined as complete remission (CR). Partial remissions (PR) were defined as a sustained improvement in one of the following parameters:

1. Decrease in monthly PRBC transfusion by 50%.
2. Increase in hemoglobin by 2 g m/dl over pre-treatment value was considered a good response (GR) while increase by 1 g/dl was considered a partial response (PR) and anything less as no response (NR).
3. Increase in platelet count by $> 30\,000/\mu l$ above pre-treatment value if pre-treatment count was $< 100\,000/\mu l$.
4. Increase in granulocyte count by $500/\mu l$ over pre-treatment value if pre-treatment value was $< 3500/\mu l$.
5. Sustained decrease in blasts in the BM by 50%.
6. Disappearance of one or more cytogenetic abnormalities.

Cytogenetic Studies. Standard karyotypic analysis using GTG banding was performed on every case prior to starting PCD therapy.

Statistical Section. Mann-Whitney tests were used for two sample comparisons of continuous variables. Contigency tables with chi square statistics or Fisher's exact test were used for analysis.

Results

Of the 43 patients with myelodysplastic syndromes being reported here, 27 were found to have refractory anemia (RA) according to the French-American-British (FAB) classification [16], 5 had RA with ringed sideroblasts (RARS), 8 had RA with excess blasts (RAEB) 1 had RAEB in transformation (RAEB-t) and 2 had chronic myelomonocytic leukemia (CMMoL). There were 15 females and 28 males. The median age was 67 years. Thirty had de novo myelodysplasia and 8 had documented secondary MDS. In addition, 20 patients gave a history of some form of toxic exposure. Once again, it must be noted that all patients received at least 12 weeks of therapy with just PTX and Cipro while dexamethasone was added after variable intervals to the treatment regimen of 18 patients. Among the 43 patients reported, 18 showed responses which were either hematopoietic, cytogenetic or both.

Hematologic and/or Cytogenetic Responses

There were no complete responders to either PC or PCD. Table 1 details the clinical and hematologic characteristics of the 18 patients who responded to the therapy. The median time to response for the entire group was 10 weeks, being 20 for PC and 9 for the PCD group. There were 16 males and 2 females among the responders and the median age of responders was 66 years. There were 10/27 RA, 1/5 RARS, 5/8 RAEB, 1/1 RAEB-t and 1/2 CMMoL patients among the 18 responders. Thus, 34% (11/32) of the RA + RARS patients responded while among the RAEB + RAEB-t groups 67% (6/9) responded. The responses were noted in 7 patients as they were receiving PTX + Cipro only (or 16% of the total patients who received PTX + Cipro) and in 11 after the addition of the dexamethasone (Table 1, last two columns identify these cases). Of the seven patients responding to PTX + Cipro only, there were four hematologic responses, two cytogenetic responses and one combined hematopoietic + cytogenetic response. Interestingly, three of the patients responding to PC did not receive dexamethasone,

Table 1. Clinical and hematologic characteristics of patients who responded to PCD therapy

Patient no.	Age	Sex	FAB	Original CBC			Response in			Median duration of response[a]	Response to PC only	Dexamethasone added/response
				WBC	Plt	Hb	WBC	Plt	Hb			
174/95	53	M	RAEB	2.70	70.00	6.10	Y	Y	N	5	No	Yes/Yes
163/95	60	M	RAEB-t	1.70	33.00	10.00	Y	Y	Gr	9	Yes	Yes/No
388/95	61	M	RARS	2.70	112.00	11.00	Y	NA	PR	8	Yes	No
164/95	48	M	RA	4.00	39.00	8.20	NA	Y	GR	10	No	Yes/Yes
329/94	77	F	RA	4.50	280.00	10.60	NA	NA	NA	26	Yes	Yes/No
331/95	73	M	RA	2.50	54.00	9.20	Y	Y	GR	12	No	Yes/Yes
149/95	48	M	CMMol	25.00	210.00	7.80	NA	NA	GR	10	No	Yes/Yes
86/94	72	F	RA	2.00	60.00	10.90	Y	Y	N	34	Yes	Yes/Yes
63/95	62	M	RAEB	0.60	25.00	8.50	Y	N	N	8	No	Yes/Yes
218/94	76	M	RAEB	3.50	22.00	10.20	N	Y	N	8	No	Yes/Yes
192/94	68	M	RAEB	3.20	78.00	8.60	Y	Y	PR		No	Yes/Yes
213/94	63	M	RA	3.50	130.00	9.90	N	N	N	28	Yes	Yes/No
277/95	26	M	RA	2.60	14.00	12.30	Y	N	PR	8	No	Yes/Yes
429/95	65	M	RAEB	1.60	160.00	8.90	Y	NA	N	8	Yes	No
441/95	69	M	RA	3.00	208.00	8.50	Y	NA	N	11	No	Yes/Yes
262/95	82	M	RA	4.60	59.00	12.40	NA	Y	PR	20	Yes	No
376/95	85	M	RA	3.20	99.00	10.60	Y	N	N	12	No	Yes/Yes
292/94	67	M	RA	3.20	95.00	9.00	Y	N	N		No	Yes/Yes

[a] Median duration of response is in weeks.

and of the four in whom it was added after at least 12 weeks of therapy, three did not show any further response while one patient showed further improvement. The median time to respond in this group of patients was 10 weeks, the range being 10 to 34 weeks.

Of the 11 patients who responded only after the addition of dexamethasone, 8 showed only a hematologic response and 3 a combination of cytogenetic and hematologic improvement (Table 1). The hematologic responses were in trilineage cytopenias in 2 patients, bilineage cytopenias in 3 and single lineage cytopenia in 6 patients (Table 1). The median time to response in PCD-treated patients was 9 weeks, the earliest response being noted at 5 weeks and the longest at 11 weeks. Among the 18 responders, irrespective of whether they received PC or PCD therapy, three patients had trilineage responses, 5 bilineage, 4 in monolineage with 2 having cytogenetic responses without a hematopoietic response. Among the remaining 4 patients, 1 had anemia as the only abnormality in the blood which improved by greater than 2 g/dl of Hb (PT # 149/95, Table 1). The other three patients had bilineage cytopenias and one showed improvement in both while the other two observed improvement in a single lineage only.

Among 16 hematologic responders, there were 15 patients who had anemia and 8/15 had an improvement in the hemoglobin levels, 4/8 showing an increase above 1.0 g/dl and 4/8 showing an increase of 2 g/dl. Fourteen patients had a WBC lower than 3500/μl and 12/14 showed an improvement of greater than 500/μl maybe reflecting a direct demargination effect of dexamethasone. Of the two patients whose WBC did not improve, one received only PTX + Cipro and one received PCD. Finally, 13 patients in this responding group had thrombocytopenia prior to starting therapy and 8/13 showed an increase of at least 30000/μl platelet count and it is of note that 3/8 responding patients received only PTX + Cipro while 5/8 received the dexamethasone also. Once again, there were no complete responders to either PTC + Cipro or PCD therapy.

Cytogenetic Response. GTG banding revealed normal pre-therapy karyotype in 22 patients while 21 had evidence of abnormalities. Among these 21 patients, 3 had trisomy 8, 6 had del 20q, 5 had abnormalities of chromosomes 5 and/or 7 while 7 patients had + 15, + 13, + 19, inv 16, t (2; 11), t (11) and add (9) abnormalities. Table 2 shows the cytogenetic characteristics of the 18 patients who re-

sponded to therapy. Of these, 8 had normal karyotypes and 10 showed cytogenetic abnormalities, 4 having del 20 (q), 1 del (5) (q22 q35) 1 der (7) t (1; 7), 1 add (9), 1 + 19, 2 + 8 and 1 isochroniosome 17 (Table 2). Six patients showed changes that could be considered as partial or complete cytogenetic responses. The median time for a cytogenetic response was 30 weeks, the range being from as little as 10 weeks to approximately a year (50 weeks) of therapy. Table 3 provides the detailed serial cytogenetic analyses on these individuals. Interestingly, the cytogenetic responses were not universally associated with a hematopoietic response. Most significantly, patient # 213/94 showed total disappearance of the del (20) (q11 q13) clone following PTX + Cipro therapy alone and has had the cytogenetics repeated 10 times on his BM in the last 2–1/2 years without reappearance of this clone, yet he did not have a significant hematologic response. Following the addition of dexamethasone, he continued with the same anemia as before. Patient # 86/94 showed disappearance of trisomy 8 confirmed by FISH analysis again while on PTX + Cipro therapy alone, without a hematologic response. The addition of dexamethasone in her case resulted in a hematologic response in the platelet (an increase from 10 000 to 140 000) and WBC counts but the patient continued to requiure blood transfusions. Patient # 329/94 showed disappearance of trisomy 8 but persistence of del (5) (q22 q35). Patient # 164/95 only showed disappearance of one clone (der (14) t (1; 14) (q11; p11)) while der (7) t (1; 7) (q11; q11) clone persisted in his BM throughout. Finally, patient # 218/94 had decrease of del (20) (q11; q13) clone. When treatment was temporarily interrupted, the clone reappeared in all metaphases examined. Treatment was restarted and the next BM showed a completely normal karyotype. Once again, treatment was halted due to dexamethasone side effects. The clone reappeared in all metaphases examined and persisted till the time of death from an intracranial hemorrhage. Patient # 149/95 had CMMoL and had a good hematologic response with Hb increasing from 8 to 12 g/dl and disappearance of add (9) (p22). Thus, six patients showed variable cytogenetic responses none of which were accompanied by a "complete" hematologic response. Of the six responders, three showed cytogenetic responses after therapy with PTX + Cipro alone while the other three responded after the addition of dexamethasone.

Summary of Hematologic + Cytogenetic Responses

In summary, 18/43 patients responded to this anti-cytokine treatment. A total of 7 patients out of 43 evaluable individuals responded to

Table 2. Cytogenetic characteristics of patients who responded to PCD therapy

Patient no.	Karyotype	Cytogenetic response
174/95	46XY, i/17q)(17)47, idem, +13(2)/46XY(1)	No
163/95	46XY	No
388/95	46XY	No
164/95	46XY,der(7)t(1;7)(q11;q11)(9)/46XYder(14)t(1;14)(q11;p11)	Yes
329/94	46XY(2)/46XXdel(5)(q22q35)(6)/47XX,+8(2)	Yes
331/95	46XY	No
149/95	46XY,add(9)(p22)(9),46XY(12)	Yes
86/94	46XX(33)/47XX+8(4)	Yes
63/95	46XY	No
218/94	46XYdel(20)(q11;q13)(19)/46XY(1)	Yes
192/94	46XY	No
213/94	46X,-Y(5)/46XYdel(20)(q11;q13)(6)/46XY(14)	Yes
277/95	46XdelY(q12),der(15)t(11;15)(q13;p11),del20(q11;q13)	No
429/95	46XY	No
441/95	46XY	No
262/95	46XY,del(20)(q11;q13)	No
376/95	47XY,+19(10)/46XY(10)	No
292/94	46XY	No

Table 3. Serial cytogenetic studies on MDS patients responding to PCD therapy

Patient no. 164–95	
Day 0:	46, XY, der(7)t(1;7)(q11;q11)[19]/46, XY, (14) t (?1;14) (?q11;p11)/46, XY[9]
Week 4:	46,XY,der(7)t(1;7)(q11;q11)[10]/46,XY,der(14)t(1;14)(q11;p11)[3]/46,XY[7]
Week 10:	46,XY,der(7)t(1;7)(q11;q11)[6]/46,XY[14]
Week 14:	46,XY,der(7)t(1;7)(p11)[5]/46,XY[15]
Patient no. 329–94	
Day 0:	46XX,[2]/46XX,del(5)(q22q35)[6]/47,XX,+8[2]
Week 10:	46,XX[2],46XX,del(5)(q22q35)[17]/47,XX,+8[1]
Week 26:	46,XX,del(5)(q22q35)
Patient no. 149–95	
Day 0:	46, XY, add (9) (p22) [9] / 46, XY [12]
Week 16:	46,XY, add (9) (p22) [10] / 46, XY [10]
Week 20:	46, XY, add (9) (p22) [7] / 46, XY [13]
Week 32:	46, XY
Patient no. 86–94	
Day 0:	46, XX
Week 16:	46, XX [33] / 47, XX, +8 [4]
Week 28:	46, XX [18] / 47, XX, +8 [4]
Week 34:	46, XX
Week 42:	46, XX
Week 54:	46, XX
Week 60:	46, XX
Patient no. 218–94	
Day 0:	46, XY
Week 8:	46, XY, del(20)(q11q13) [19] / 46, XY [1]
Week 16:	46, XY, del(20) (q11q13) [19] / 46, XY [2]
Week 28:	46, XY, del(20)(q11;q13) [12] / 46, XY [8]
Week 30:	46, XY, del(20) (q11;q13)
Week 50:	46, XY
Patient no. 213–94	
DAy 0:	45, X, -Y [5] / 46, XY, del(20) (q11;q13) [6] / 46, XY [14]
Week 8:	46, XY [7] / 46, del(20) (q11;q13) [10] / 45, X, -Y [3]
Week 28:	46, XY
Week 36:	46, XY
Week 40:	46, XY
Week 44:	46, XY [39] / 45, X, -Y [1]
Week 48:	46, XY
Week 52:	46, XY
Week 60:	46, XY
Week 68:	46, XY
Week 76:	46, XY
Week 112:	46, XY

PC only for a response rate of 16% and only 1 patient showed further improvement after dexamethasone amongst the 4 in whom it was eventually added. Of the 18 patients who received the PCD therapy, 11 had some type of a response for a response rate of 61%. Yet, there were no complete responders so that even in the patients showing combined hematologic and cytogenetic responses, some abnormality continued to persist. A prime example is patient # 329/94 (Table 1) who was treated with PTX + Cipro despite a relatively normal CBC to obtain a cytogenetic response. Interestingly, +8 disappeared from her marrow, but the del (5q) abnormality persisted. Thus the patient could not be considered a CR. Similarly, patient # 86/94 responded cytogenetically with disappearance of + 8 and improvement in WBC and platelet counts, but anemia persisted throughout therapy requiring transfusion support. In summary, 7 MDS patients responded to PC, 11 to PCD therapy and 1 patient initially responded to PC and improved further following the addition of dexamethasone.

Discussion

A novel anti-cytokine approach to treating myelodysplastic syndromes is described in this chapter. The therapy is unique in that it is neither cytotoxic [17] nor growth factor [18] based. Rather, an attempt is made to interfere with the lipid signalling pathway used by a variety of cytokines including TNF-α, TGF-β and IL1-β. The hypothesis is that one or more of these cytokines may contribute towards both the genesis of variable cytopenias in MDS by inducing apoptotic death in maturing cells as well as hypercellularity of the bone marrow by stimulating the proliferation of early progenitors. Suppression of the cytokine production should therefore result in a dual benefit as well. The present study confirmed several hypotheses. First, our hypothesis that excessive cell-death in MDS may be cytokine mediated was confirmed in vivo by the present study since suppression of the cytokine production with PCD therapy resulted in a reduction in apoptosis and a consequent amelioration of cytopenias in approximately 60% of the patients who received the three drugs. Second, our hypothesis that cytokine stimulation contributes of the proliferative advantage of the MDS clone in some patients was confirmed because disappearance of cytogenetically marked clones was seen in six cases of MDS following PC or PCD therapy. These results are quite impressive for several reasons. To start with, there were patients who showed either a hematologic improvement or a cytogenetic response or a combination of both when treated with PC only, therefore the responses cannot be ascribed only to the dexamethasone effect. Secondly, treatment of MDS patients in the past with steroids has produced rather low response rates, the best being in the range of 10% [19]. In addition, a previous study using PC therapy in MDS [20] did not show any clinical benefits for the patients probably because therapy was stopped at 28 days while our responders to PC show that longer treatment is needed. Clearly however, the higher response rates were observed in PCD-treated individuals establishing a synergistic effect of the drugs in vivo. This is not surprising since it has previously been shown that the combination of PTX + dexamethasone in vitro has a similar synergy [21] and in fact we added the dexamethasone to the PC regimen because of that observation. While PTX downregulates the transcription of TNF mRNA, dexamethasone suppresses the translation of any remaining message into the protein form [21]. Finally, the cytogenetic responses are of particular interest because this provides direct in vivo proof that at least some of the evolving MDS clones are cytokine-dependent. Such responses have not been commonly observed with growth factor therapies at least [22, 23].

As noted earlier, a variety of clinical responses were observed following therapy with this approach. While improvement of the hematologic profile was noted in the majority of responders, some patients only had a single lineage response while others had a cytogenetic response without a hematologic response and still others had both. What this suggest is that the therapy is having different biological effects in different patients. Does this mean that in some patients, cytokines are involved in the pathogenesis of the hematologic abnormalities while in others they are not? or is that cytokines are involved which are unaffected by the therapy we administered? A similar case can be made for the cytogenetic responses. For example, are these responses different because in some patients, the cytokines involved in the proliferative advantage of the BM precursor cells are suppressed by the treatment while in other patients, the cytokines are different than those affected by therapy? Alternatively, is it possible that in some MDS patients, cytokines do not play a role in conferring the growth advantage on the affected precursor, but the cells are autonomously proliferating? Does the anti-cytokine therapy we used affect the production of cytokines by the BM microenvironment differently from that produced by the MDS cells? We believe that these questions have allowed us to build a rather unique therapeutic program, unique especially in terms of using clinical response as a way of dissecting out different mechanisms underlying the differences in the manifestations of the disease among individual MDS patients. In sum-

mary, non-responsiveness to anti-cytokine therapy could be the result of:

1. Incomplete effect for pharmacokinetic reasons.
2. Involvement of cytokines other than those signaling through the phosphatidic acid → diacylglycerol pathway which is inhibited by PCD.
3. Predominance of stem cell defect without the confounding influence of cytokines.
4. Inadequate treatment duration.

Several other features of this pilot study are of note. Firstly, despite cytogenetic responses, there were no complete hematologic responders. Secondly, the side effects of long-term decadron were usually prohibitive so that proximal muscle weakness universally made the administration impossible beyond a few months and cessation of therapy was accompanied by a cessation in response as well. Finally, there was no increased incidence of transformation to acute myeloid leukemia which laid to rest the legitimate but as it turned out, only a theoretical concern that suppression of apoptosis may lead to an accumulation of blasts.

It appears that in a large proportion of the MDS patients, aberrant cytokine production may be responsible for the genesis of cytopenias. Anti-cytokine therapy seems to improve the cytopenias in these patients; however, since there were no complete remissions, it is clear that this therapy is probably not adequate by itself. By using PCD therapy, we are probably reversing only the cytokine-mediated part of the disease, leaving the stem cell defect part to be treated still. Given this scenario, a step-wise approach to therapy for the myelodysplastic syndromes can be visualized:

1. Optimize the anti-cytokine therapy and obtain the maximum improvement in cytopenias possible. This pilot study of anti-cytokine drugs employing PCD has been extremely useful for several reasons. Clearly, this approach is feasible in a certain group of MDS patients. Unfortunately, the drugs available to achieve an adequate suppression of cytokine levels are not the best. It has been shown that the first metabolite of PTX, lisophylline is 800 times more effective than PTX in suppressing the actions of a variety of cytokines [24]. Perhaps, the responses would be far better if lisophylline was substituted for PTX. On the other hand, a monoclonal anti-TNF antibody which has been shown to suppress TNF-α levels quite well [25] may be the best approach at least for cases where the predominant negative cytokine is TNF-α. Maybe PCD should be combined with other agents of proved efficacy in some MDS patients, agents such as amifostine [26], cyclosporin [27] or 5-azacytidine [28].

2. Once the peripheral blood counts normalize, consider addressing the stem cell defect using one of the following approaches:

a) Cyclical therapy with low dose cytokine arabinoside (araC) and/or a growth factor alternating with PCD therapy. Perhaps growth factors such as G-CSF and erythropoietin would be far more effective once the negative cytokine effects have been neutralized be PCD therapy.

b) Treatment with high dose chemotherapy alternating with PCD. This would be clearly much better tolerated when the PB counts are relatively normal as compared to when chemotherapy is administered to MDS patients with profound cytopenias. The situation would be analogous to using induction chemotherapy for AML with severe side effects as opposed to using the same drugs for consolidation with much fewer side effects due to the presence of a relatively more normal hemopoiesis in general.

c) Attempts could be made at this point to mobilize peripheral blood stem cells which could be harvested and stored for future use following chemotherapy and achievement of a complete hematologic and cytogenetic remission as well as polyclonal hemopoiesis wherever demonstrable.

Another possibility of improving therapeutic options for MDS patients is to employ a completely different strategy for suppressing excessive cell death. There may be many triggers responsible for this premature cell death including cytokines such as TNF-α, TGF-β, IL1-β or other as yet unidentified cytokines, the propensity of the transformed cell itself to undergo apoptosis or a combination of the above. However, if some or all the triggers initiate apoptosis via a common biochemical route, then the identification of

this common pathway becomes critical because it may serve as an excellent therapeutic target. We are presently trying to understand the biochemical pathways initiated cytokines such as TNF-α and several cysteine proteases have been found to be abnormally activated in at least a few MDS patients. Ultimately, specific protease inhibitors to suppress this common pathway may be a useful therapeutic choice in some MDS patients irrespective of the precise triggering mechanism. These areas are being investigated actively by our group presently.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Expression of Differentiation Antigens on Blast Cells of Acute Myelogenous Leukemia (AML, M0-M6)

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Abstract. Forty one patients with AML (M0[1] 4 patients, M1[1] 5, M2[1] 7, M4[1] 8, M5a[1] 16, and M6[1] one patient) have been studied. Cytochemical reactions (MPO, CAE, AcP, ANAE, NE, PAS) and immunological detection of antigens expression (indirect immunoperoxidase, APAAP methods and, in some cases, flow cytometry) were performed. The definition of typical phenotypical pattern for discrete FAB-variants AML was carried out. Significant heterogeneity of monoblasts was revealed. Cases of AML, M5a with unusual phenotypical features were discovered. Its origin from hierarchy of hematopoietic stem cells is discussed.

Keywords: acute myelogenous leukemia, monoblastic leukemia, hematopoietic progenitor cells, stem cells, antigen expression.

Introduction

Acute myelogenous leukemias (AML) are clonal diseases of hematopoietic cells. It is supposed that the malignant transformation occurs at the level of progenitors cells with myeloid differentiation and, in some cases, possibly pluripotent stem cells [1, 2].

Human hematopoietic progenitors cells can be identified on the basis of specific antigen expression. As the most primitive pluripotential cells were described $CD34^{2+}$ $CD38^{-}$ $CD90^{+}$ $CD45RO^{+}$ $HLA\text{-}DR^{-/low}$ $CD71^{-/low}$ cells [3, 4]. They may express some lineage-specific antigens–CD33, CD13, CD15, CD4, CD7 [5–9]. The process of commitment is accompanied by acquisition of CD38, HLA-DR and CD45RA antigens [10, 11]. A heterogenous population of linear committed progenitor cells contains polipotent precursors of granulocytes, monocytes, erythroid and megakaryocytic cells (CFU-GEMM) with phenotype $CD34^{+}$ $CD13^{+}$ $CD33^{+}$ $CD38^{+}$ $HLA\text{-}DR^{+}$ $CD45RA^{+}$; colony-forming units (CFU) of granulocytes-macrophages (CFU-GM) with phenotype $CD34^{+}CD13^{+}CD33^{+}CD38^{+}HLA\text{-}DR^{+}CD45RA^{+}$ $CD64^{+}CD15^{+}$; early erythroid cell-precursors–burst-forming units (BFU-E) with phenotype $CD34^{+}CD33^{+}$ $CD38^{+}HLA\text{-}DR^{+}$ $CD45RO^{+}$ $CD71^{low}CD41a^{+}$ $CD41b^{+}$; megakaryocyte colony-forming units (CFU-Meg) with phenotype $CD34^{+}CD33^{+}CD38^{low}$ $HLA\text{-}DR^{+}$ $CD61^{low}$; $CD34^{+}CD38^{+}HLA\text{-}DR^{+}CD10^{+}$ $CD19^{+}$ early precursors of B-lymphocytes and $CD34^{+}$ $CD33^{+}CD38^{+}$ CD4lowCD7^{+} $CD45RA^{+}$ $CD3^{-}CD8^{-}$ bipotent precursors of T-lymphocytes and natural killer cells. During following differentiation of hematopoietic progenitor cells the modification of antigen's expression (alteration of level of expression, the disappearance of some antigens and the acquisition of new function-significant, lineage-specific or lineage-associated antigens) take place.

It is supposed that the stage of malignant transformation, the stage of the acquisition of malignant phenotype and behavior, and the stage of accumulation of blast cells for

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AML cannot coincide [12]. In this situation, the definition of specific phenotypical signs of blast cells can reflect rather its residual capability to myeloid differentiation than indicate of malignant transformation.

The aim of our study was the definition of specific patterns of reactivity monoclonal antibodies (MoAbs) with blast cells of different FAB variants of AML and comparison of phenotypes of AML blast cells and cell precursors from the hierarchy of pluripotent and progenitor hematopoietic cells.

Material and Methods

Patients. Bone marrow aspirates and samples of peripheral blood taken for diagnostic purpose from 41 patients with AML were studied. The reference group was 71 patients with acute lymphoblastic leukemia (ALL). Diagnosis and AML variant were established according to morphological and cytochemical criteria of FAB-classification [13]. Cytochemical reactions (activity of myeloperoxidase (MPO), acid phosphatase (AcP), acid α-naphthyl acetate esterase (ANAE), α-naphthyl acetate esterase (NE), naphthol-AS-D-chloroacetate esterase (CAE), and PAS-reaction) were performed. The slides were reviewed by three independent hematologists.

Immunopenotyping Immunophenotyping was performed on diagnosis. Blood and bone marrow cells were Ficoll-separated to obtain a mononuclear population. In all patients included in the study, the blast cell content in the samples for immunophenotyping exceeded 60%. Antigen expression was detected by indirect immunoperoxidase and APAAP methods using a set of MoAbs: CD34, CD33, CD13, CD14, CD71, CD41, CD42b, CD61, CD45RO, CD56, CD19, CD20, CD22, CD10 (Dakopatts, Denmark), CD15 (clone PM-81, gift of E.D.Ball, USA), CD64 (clone 22.2, gift of N.Dinces, USA), CD16, CD7, CD3, CD5, CD2, CD4, CD8, CD45RA (clones LNK-16, LT7, LT3, LT5, LT2, LT4, LT8, LT45RA, gift of A.Filatov, Russia), CD38, HLA-DR (clones ICO-20, ICO-1, gift of A. Baryshnikov, Russia) and rabbit anti-mouse immunoglobuline conjugated with HRP or APAAP staining kit (Dakopatts, Denmark).

In some cases, immunophenotyping was performed by flow cytometry on FACSCAN using the blood lysis method and MoAbs against CD34 (HPCA-2, conjugated with phycoerythrin [PE]), CD14 (Leu-M3, conjugated with fluoresceinisothiocyanate [FITC]), CD15 (Leu-M1, conjugated with FITC), CD7 (Leu-9, conjugated with FITC), HLA-DR, conjugated with FITC, CD33 (Leu-M9, conjugated with PE) (all Becton Dickinson Immunocytometry System, San Jose, USA).

Results

General Characteristics of Immunophenotypical Data. AML patients were classified as M0 (4 patients), M1 (5), M2 (7), M4 (8), M5a (16) and M6 (1). The expression of the main myeloid antigens is presented in Table 1.

In cases of AML, M0 blast cells had morphological signs of lymphoblasts and were MPO, AcP, ANAE, CAE negative. A weak diffuse NE reaction and fine granular PAS reaction in a part of blast cells were detected in one patient. Blast cells were characterized by stable expression of CD34, CD33, CD13 and HLA-DR antigens, low expression of CD10 antigen (80–95% of blast cells in 4 patients), CD45RA (40–50% of blast cells in 2 patients) or CD45RO (30% of blast cells in one patient), the absence of CD38 and more late antigens CD64, CD15, CD14. In one patient the expression of the CD7 antigen and in one the low expression of the CD71 antigen was revealed.

In cases of AML, M1 14-90% of myeloblasts were MPO+, the intensity of reaction was weak in 4 and moderate in one patient. Blast cells of 4 patients had weak diffuse AcP and diffuse-granular PAS-reaction; activity of NE was revealed as isolated granules in 35% of blast cells in one patient. In all patients activities of CAE and ANAE were not detected. Typical immunophenotype of blast cells was $CD34^{-}CD33^{+}CD13^{+}HLA\text{-}DR^{+}CD38^{+}CD45RA^{+}$ $CD15+CD64^{+/-}$.

The expression of the CD16 antigen (45–65% of blast cells) was revealed in two, CD7 (80% of blast cells) in one, and CD19 (76% of blast cells) in one patient.

Myeloblasts in AML, M2 were more differentiated. In blast cells of all patients mode-

Table 1. Expression of the main myeloid antigens on blast cells in different *FAB*-variants of *AML*, % positive cells

Pt.No	FAB	CD34	CD33	CD13	CD14	CD64	CD15	CD71	HLA-DR
1.	M0	>95	>95,cy	>95,cy	0	0	0	31	80
2.	M0	56	80	ND	0	0	0	ND	69
3.	M0	>95	>95	>95	0	0	0	0	91
4.	M0	>95	>95	>95	0	0	0	0	97
5.	M1	0	90	0	0	ND	48	0	>95
6.	M1	0	95	95	0	95	50,cy	0	>95
7.	M1	0	70	84	0	0	95	0	0
8.	M1	0	0	76	0	ND	85	0	85
9.	M1	40	>95	>95	0	91	65	0	78
10.	M2	0	0	0	19	0	50	0	0
11.	M2	21	24	26	21	34	81	0	40
12.	M2	0	0	69	8	0	69	0	0
13.	M2	0	>95	>95	15	85	>95	0	91
14.	M2	0	0	82	0	0	>95	0	ND
15.	M2	6	30	15	5	15	26	0	32
16.	M2	67	>95	>95	4	78	>95	21	30
17.	M4	33	80	80	15	0	70	0	84
18.	M4	0	>95	>95	48	0	78	0	ND
19.	M4	0	>95	0	0	67	30	0	ND
20.	M4	0	>95	>95	35	ND	>95	0	87
21.	M4	0	>95	>95	40	82	90	0	>95
22.	M4	ND	ND	ND	60	ND	85	0	ND
23.	M4	0	>95	>95	50	ND	90	0	60
24.	M4	0	78	56	6	49	>95	0	60
25.	M5a	0	0	10	0	>95,cy	0	0	81
26.	M5a	0	0	0	0	ND	29	0	56,cy
27.	M5a	>95	0	0	0	ND	>95	0	>95,cy
28.	M5a	0	0	0	0	ND	>95	0	>95,cy
29.	M5a	30	54	42	51	ND	54	0	100,cy
30.	M5a	35	0	0	20,cy	0	80	0	69
31.	M5a	ND	ND	50	40	ND	>95	0	80
32.	M5a	74	32	71	30	ND	71	0	56
33.	M5a	31	43	0	28	ND	40	0	85
34.	M5a	95	0	0	19	60	43	0	82
35.	M5a	85	60	60	25	>95	>95	0	>95
36.	M5a	0	85	40	>95	54	80	0	63
37.	M5a	0	0	25	>95	35	ND	0	ND
38.	M5a	0	>95	70	60	73	85	0	ND
39.	M5a	0	80	65	68	ND	51	0	>95
40.	M5a	0	0	40	>95	ND	50	0	60,cy
41.	M6	10	ND	ND	0	ND	40	>95	>95

Abbreviations: ND, not done; cy, cytoplasmic reaction.

rate and strong MPO (75–95% of blast cells) and CAE (70–85% of blast cells in 6 patients and 25% in patient No16) activities, weak or moderate AcP (90–100% of blast cells) activity and weak diffuse PAS-reaction (60–80% of blast cells) were detected. Minor blast population (5–21%) contained strong NE and ANAE activity and was considered as monoblasts. The majority of blast cells were characterized by nonstabile weak/moderate expression of CD33, CD64 and HLA-DR antigens (in 4 of 7 patients), weak or moderate expression of CD13 antigen, strong expression of CD15 in comparison with CD13 and CD33 antigens, the presence of CD16 (40–50% of blast cells in 5 of 7 patients), the absence of CD38 and lymphoid antigens. CD14 antigens was detected on 4–19% of blast cells that corresponded to the quantity of ANAE+ cells. CD34 antigen was revealed only in three patients.

Blast cell population in AML, M4 patients was heterogenous and was represented by myeloblasts and monoblasts (in 7 patients). In patient no. 22 all blast cells were monoblasts, but in bone marrow pronounced

myeloid maturating component was revealed. Myeloblasts were MPO+, CAE+ and monoblasts were ANAE+ and/or NE+. The activity of NE was fluoride-sensitive. Blast cell immunophenotype was characterized by stable expression of CD33, CD15, CD13, CD64, CD14, and HLA-DR antigens. In some patients the percentage of CD14-positive and ANAE-positive cells was different with predominance of ANAE+ CD14-cells. The CD34 antigen was present on only 33% of blast cells in one patient and the CD16 antigen on 25–45% of the blast cells in two patients. In 3 patients 20–45% of blast cells coexpressed the lymphoid antigens CD7 and CD4.

AML, M5a FAB-variant was diagnosed in 16 patients. In 12 patients (nos. 29–40) the definition of the origin of the transformed cells was indisputable in view of monocytoid morphological signs, strong fluoride-sensitive NE and ANAE reaction. In 10 from 12 patients monoblasts were MPO negative, in 2 patients (nos. 34, 35) 90% and 93% of blast cells were MPO positive. Activity of AcP was weak or moderate diffuse, PAS-reaction was negative or as fine diffuse cytoplasmatic staining or isolated granules. In this subgroup of AML,M5a patients blast cells stably expressed CD15, CD14, HLA-DR, CD13, CD33, CD38 antigens. In 6 of 9 patients the coexpression of CD7 and CD4 antigens was revealed. Other lymphoid antigens were not determined. CD34 antigen was detected in 6 of 11 patients (54.5%) on 31–95% of blast cells. For these patients the high level of CD15 and HLA-DR expression was typical; expression of CD14 was detected in all patients but on smaller blast population. Among CD34 negative patients from this subgroup the level of CD14 expression exceeded the level of CD15 expression, expression of HLA-DR was low.

In 4 patients with AML,M5a blast cells were hardly distinguished from L1/L2 lymphoblasts, were only 3–9% of MPO-positive, CAE-negative, had negative or weak AcP, weak ANAE and/or NE activity; the PAS-reaction was negative or fine granular. Phenotypical markers were the expression of CD15 antigen, cytoplasmic expression of HLA-DR and CD7 (in 3 patients) antigens and the absence of antigens CD13, CD33, CD14, CD38. The CD34 antigen was revealed in one patient.

We observed only one patient with AML, M6. Erythroblasts were ANAE- and NE-positive, had fine granular PAS-reaction and local activity of AcP. Its origin was confirmed by the strong expression of CD71 and CD41 antigens.

Comparison of Phenotypes of AML Blast Cells and Cell-Precursors

Among patients with AML, M0-M2, M4 and typical M5a variants, we did not detect blast cells with phenotype of the most primitive pluripotent cells. Only in 2 patients from reference ALL group blasts cells were $CD34^{3+}CD45RO^{+}CD38^{low}HLA\text{-}DR^{low}$ in the absence of any lineage-specific antigens. Blast cells of one patient with unusual AML,M5a variant had close immunophenotype: $CD34^{3+}CD45RO^{+}CD38^{low}$ cyHLA-DR^{+} $cyCD7^{+}$ $ANAE^{weak}$.

Blast cells of 2 patients with ALL also had the immunophenotype of committed cell precursors ($CD34^{2+}CD45RA^{+}CD38^{+}$HLA-$DR^{+}$ without expression of lineage-specific antigens).

In cases of AML, M0 population of blast cells was monomorphic by cytochemical signs and antigen expression and resembled CFU-GEMM ($CD34^{+}CD33^{+}CD13^{+}$HLA-$DR^{+}CD38^{-}CD64^{-}CD15^{-}CD14^{-}$). We also discovered, using immunoperoxidase method, an unusual expression of CD10 antigen on the majority of AML, M0 blast cells, that had not been observed by other authors [14]. In other FAB variants of AML we found no CD10 expression on blast cells.

Blast cells in AML, M1 were heterogenic by MPO, but monomorphic by expression of main myeloid antigens. In one patient (no. 9) we revealed by flow cytometry two blast subpopulations, distinguished by CD34 expression; and in two patients (nos. 5, 6) by immunocytochemical method, two blast subpopulations, distinguished by CD15 expression. We believed that leukemic blast cell population in AML, M1 is presented by analogues of CFU-GM (or CFU-GM and CFU-G?) with different degrees of maturation, that reflected the expression of the CD15 and CD34 antigens. Because leukemia clo-

nogenous cells were revealed among a CD34-negative blast population [15], the absence of CD34 in most observed cases of AML, M1 did not contradict this supposition.

The majority of myeloblasts in 6 patients with AML, M2 and a minor population in patient no. 16 resembled the normal myeloblasts by phenotypical features ($CD34^-CD38^-$ HLA-DR^{low} $CD152^+$ $CD33^{low}$ $CD13^+$ $CD64^{low}$ $CD16^{+/-}$) and moderate and strong activity of MPO and CAE; just as the majority of myeloblasts in patient no. 16 and a minor population of blast cells in patient no. 11 were less differentiated ($CD34^+$ $CD33^+$ $CD13^+$) and corresponded to blast cells in AML, M1 FAB-variant. In 6 patients a small monoblastic component (relatively differentiated $CD14^+$ $ANAE^+$ blast cells) was revealed also.

In AML, M4 patients two distinct blast populations were detected: myeloblasts, corresponding to normal CAE+ myeloblasts and distingushed from AML, M2 myeloblasts by stable CD33, CD13 and HLA-DR expression; and monoblasts. The latter were heterogenous by CD14 expression ($CD14^+ANAE^+$ and $CD14^-ANAE^+$) that indicated a different degree of maturation. In one patient the myeloid component was presented by maturating cells. Its belonging to malignant clone did not appear. In one patient by flow cytometry a minor $CD34^+CD15^+$ and $CD34^+CD14^-$ blast subpopulation was revealed, that we considered may be common progenitor cells for more differentiated mono- and myeloblasts.

In 12 typical cases of AML, M5a monoblasts of individual patients were at different stages of maturation. In 6 patients with expression of CD34 antigens, some blast cells presented a CFU-M by expression of CD34, CD15, HLA-DR, ANAE and NE activity and the absence of CD14. We believe that a second, more differentiated $CD34^-$ $CD14^+CD152^+$ monoblast subpopulation was present in these patients, but unfortunately flow cytometry was not carried out. Data from other research suggested the impossibility of simultaneous expression of CD34 and CD14 antigens on the same cell [6]. A myeloid component was found in only 2 of $CD34^+$ 6 patients, represented by myeloblasts in one case and myeloblasts and promyelocytes in the second patient.

In CD34- patients with typical AML, M5a FAB-variant monoblasts were monomorphic relatively differentiated cells with high expression of ANAE, CD14, lower expression of CD15 ($CD142^+CD15^+$) and stabile CD13 expression. A myeloid component was detected in two patients as CAE+ strong MPO+ myeloblasts and in one patient as myeloblasts and promyelocytes.

In 4 patients with unusual AML, M5a FAB-variant monomorphic population of monoblasts gave minimal evidence of lineage-specific differentiation. In one patient blast cells may be considered as primitive pluripotent cells. In three another patients the phenotype was close but distinguished by the absence of CD34 antigen: $CD34^-CD33^-CD13^-$ $CD15^+CD14^-CD64^-$ $cyHLA\text{-}DR^+cyCD7^{+/-}$. The presence of activity of ANAE in some blast cells indicated the monocytic direction of its differentiation. A myeloid component was not revealed in any case. We found no published data on the existence of normal counterparts for these blast cells. In the absence of main lineage-specific antigen they may be estimated as primitive hematopoietic cells.

In one patient wih AML, M6 normal counterparts of blast cells might be erythroid CFU because of strong CD71 and CD41 expression and the absence of CD34.

Discussion

In summary, we cannot define the stage of malignant transformation in AML by immunophenotyping, but this method reveals the stages of the acquisition of malignant phenotype and blast accumulation. Obviously, it corresponds to blast cells in cases of AML, M0 (presumably CFU-GEMM) and AML, M1 (presumably CFU-GM or CFU-G), because we did not detect any signs of following differentiation. In cases of AML, M2; AML, M4 and typical cases of AML, M5a we believe that the stage of acquisition of malignant phenotype are the same, CFU-GM, but blast cells accumulated on the differentiation stages of monoblasts and myeloblasts. We found some evidence for this confirmation:

the simultaneous presence of myeloblasts and monoblasts in an individual patient; the presence of a less differentiated blast population like CFU-GM and AML, M1 blast cells in some patients. In cases of AML, M2; AML, M4, and most cases of AML, M5a malignant CFU-GM possesses a potential different to the development in monoblastic and myeloblastic direction. It is possible that differentiation in these directions proceeds independently. The discrepancy between stage of maturation of myeloid and monocytic blast components in individual patients indicates such a process. It may be the result of different genetic alterations distinguishing responsibility to the action of growth factors, different parameters of cell cycle during mono- and myelo-lineage-specific development, etc. The question remains, why in individual patients a malignant blast population may be at different discrete maturating stages (less and more differentiated), if the final arrest of development is at a more differentiated stage?

Actually, FAB classification based on morphological and cytochemical features of blast cells reflects functional abilities of blast cells and its residual potentials to development. Since in cases of AML, M1; AML, M2; AML, M4 and most cases of AML, M5a blast population represents the same cells (monoblasts, myeloblasts, blasts with immunophenotype of CFU-GM or CFU-G) but in different proportions, there are no specific immunophenotypical patterns for each FAB-variant of AML. We may only speak of a typical pattern of antigen expression (Table 2). We think also that in all cases the definition of antigen expression must be supplemented by cytochemical reactions for a more accurate detection of monoblastic (especially less differentiated $ANAE^+$ $CD14^-$ cells) and myeloblastic components.

Finally, we detected significant heterogeneity in monoblasts in AML, M5a patients. It may be presented by relatively differentiated blast cells ($CD34^-CD33^+$ $CD13^+$ $CD14^{++}$ $CD15^+$ $HLA\text{-}DR^+$ $CD7^+$ $ANAE^+$), monoblasts at a moderate stage of maturation ($CD34^-$ $CD33^{+/-}$ $CD13^{+/-}$ $CD14^+$ $CD152^+$ $HLA\text{-}DR^+$ $CD7^+ANAE^+$ and $CD34^{+/-}CD33^{+/-}CD13^{+/-}$ $CD14^-CD15^+HLA\text{-}DR^+$ $CD7^+$ $ANAE^+$) and blast cells with minimal evidence of monoblastic differentiation ($CD34^{+/-}$ $CD33^-$ $CD13^-$ $CD14^-CD15^{+/-}$ $cyHLA\text{-}DR^+$ $cyCD7^+$). The definition of esterase activities is to our mind the most characteristic feature of monocytic direction of development. We found no normal counterpart for the last-described monoblast population. It may be cells from the hierarchy of hematopoietic stem cells. Recently the existence of bipotential progenitor cell for monoblasts and B-lymphocytes has been discussed [11]. Discovered cases of unusual AML, M5a may be the result of malignant transformation of such cells. Blast cells with minimal evidence of monocytic differentiation had common morphological and cytochemical features with ALL B-cell origin and represented a problem for diagnosis. The main points for diagnosis were the presence of ANAE, 3-9% of MPO-positive blast cells, the absence of all B-cell lineage-specific antigens, and simultaneous cytoplasmic expression of HLA-DR and CD7.

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Table 2. Typical imunophenotype features of blast cells in different FAB-variants of AML

FAB	CD34	CD33	CD13	CD14	CD15	HLA-DR	CD10	CD71	T-cell antigens
M0	+	+	+	-	-	+	+	-	CD7
M1	-	+	+	-	+	+	-	-	CD7,CD16
M2	-	+/-	+	+/-	+	+/-	-	-	CD16
M4	-	+	+	+	+	+	-	-	CD7+CD4+
M5a	+/-	+	+	+	+	+	-	-	CD7+CD4+

Abbreviations: +, presence in the most cases; -, absence in the most cases; +/-, presence in part of cases.

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Experimental Approaches and Novel Therapies
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Cytoplasmatic Expression of CD34 in Acute Leukemia

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Abstract. CD34 is preferentially expressed on immature haematopoietic cells and also in transformed haematopoietic cells. Here we tested the surface (s) and cytoplasmatic (c) CD34 expression on bone marrow blasts in twelve cases of Acute Leukemia (AL). Intracellular CD34 showed a heterogeneous distribution in leukemia and there is a trend to correlate with the maturation status of leukemic cells.

Key words: cytoplasmatic CD34, acute leukemia, maturation status.

Introduction

CD34 is a surface glycosylated type I transmembrane protein expressed on developmentally early lymphohaematopoietic stem and progenitor cells [1, 2]. The function of CD34 glycophosphoprotein is still not elucidated, but preliminary evidence indicate its role in haematopoietic differentiation [2]. This study of cCD34 adds information of the role of CD34 in leukemogenesis and in the maturation process of normal stem cells [3,4].

Patients and Methods

Twelve patients with previously untreated acute leukemia , according to the French-American-British (FAB) criteria and to the immunological classification of the European group for immunological characterisation of leukemia (EGIL) were investigated for sCD34 (HPCA-2, Becton Dickenson) and cCD34 (HPCA-2-RPE) expression on bone marrow blasts using flow cytometry (FACscan Becton Dickenson).

Results and Discussion

Three groups are evident upon detection of sCD34 and cCD34 on bone marrow blasts from the different ALs.

The classically more mature AML (group I) do not express CD34 (Table 1). In group II, four AMLs (2 AMLs M2 and 2 AMLs M4 respectively) expressed CD34 on surface only, whereas all more immature ALs (i.e., AML M1 and AML M0) and ALLs displayed surface and cytoplasmic CD34.

It would seem that intracellular CD34 is restricted to the more immature blasts, albeit the small number of ALs investigated. In normal bone marrow CD34 expression on immature cells is dependent from the maturation stage and quantitative studies on sCD34 on leukemia have shown that the number of sCD34 antigens correlate with the maturation stage of the blasts [5]. Therefore, intracellular CD34 detection could be an alternative to distinguishing the phenotypic subtype of ALs. Intriguing was the presence of a small subpopulation expressing cC34 only in three cases (Fig. 1). If the $sCD34^-/cCD34^+$ expressing blasts characterize a more immature cell compared to the $sCD34^+/cCD34^+$ blasts, then which normal bone marrow stem cells do they derive?

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Table 1. Expression of sCD34 and cCD34 on AL bone marrow blasts

Group	AL type	$sCD34^+$ /$cCD34^-$	$sCD34^+$ /$cCD34^+$	$sCD34^-$ /$cCD34^+$
I	2 AML M5b	–	–	–
	1 AML M3	–	–	–
II	2 AML $M2^{++}$	+	–	–
	2 AML M4	+	–	–
III	1 AML M1	+	+	–
	1 AML M0	+	+	+
	2 com-B-ALL	+	+	+
	1 pre-T-ALL	+	+	–

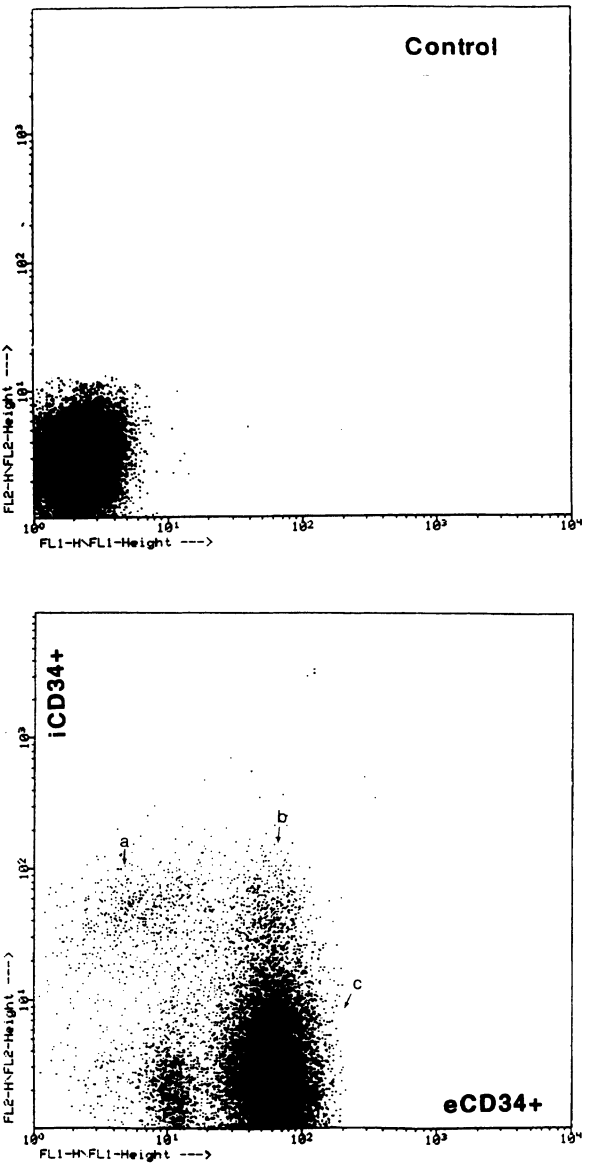

Fig. 1a, b. Expression of sCD34/cCD34 on bone marrow blasts from patient with a common-B-ALL. a $sCD34^-/cCD34^+$ cluster. b $sCD34^+/cCD34^+$ cluster. *c* $sCD34+/cCD34^-$ cluster

In order to answer this question we need to isolate $sCD34^-/cCD34^+$ blasts for better characterisation. Furthermore, a great number of ALs must be investigated to confirm these preliminary results and define the prognostic role of cCD34.

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Flow Cytometry of Leukocyte Alkaline Phosphatase in Human Hematopoietic Cells

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Abstract. Leukocyte alkaline phosphatase (LAP) is an enzyme expressed on the external aspect of the neutrophilic granulocyte plasma membrane, and it represents a specific marker for the fully differentiated granulocyte. In this report, we characterize 1B12.1, a monoclonal antibody raised against human bone alkaline phosphatase, for its ability to recognize the LAP protein. With this antibody, we developed a quantitative flow cytometry-based method for the determination of LAP. Double fluorescence flow cytometry demonstrates that the LAP protein is present in relatively high amounts in the neutrophilic granulocytes, but not in monocytes, natural killer cells, B- and T-lymphocytes of normal individuals. The protein is absent in granulocytes obtained from Chronic Myeloid Leukemia and Paroxysmal Nocturnal Hemoglobinuria patients. Higher than normal levels of LAP protein are evident in neutrophilic granulocytes of patients suffering from Polycythemia Vera, Essential Thrombocytemia and Severe Aplastic Anemia. However, the highest amounts of LAP protein are present in the granulocytes of normal individuals treated with G-CSF for the isolation of peripheral blood stem cells.

Introduction

Leukocyte alkaline phosphatase (LAP) is a plasma membrane protein; it belongs to the small family of alkaline phosphatase isoenzymes and is the product of the gene coding for the liver/bone/kidney-type (L/B/K-type) isoform [1–3]. LAP is a specific marker for the post-mitotic neutrophilic granulocyte [4, 5]. The amounts of LAP enzymatic activity are tightly regulated at various levels by different kinds of molecules [3, 6–10]. Granulocyte-colony-stimulating factor (G-CSF) causes the accumulation of LAP mRNA both in normal and in Chronic Myelogenous Leukemia (CML) granulocytes [5]. In Acute Promyelocytic Leukemia (APL) cells, combinations of all-trans retinoic acid (ATRA) and G-CSF or cAMP stable analogs induce the levels of the enzyme [3, 6–8]. In addition, expression of LAP enzymatic activity in the neutrophilic granulocyte is known to be altered in various physio-pathological conditions. High levels of the enzyme are observed in the neutrophilic granulocyte during infection, pregnancy, in Fanconi Anemia and in Polycythemia Vera (PV) [1]. By contrast, during the stable phase of CML and Paroxysmal Nocturnal Hemoglobinuria (PNH), a specific defect in the expression of neutrophilic LAP is observed [11]. Determination of LAP in peripheral blood neutrophils is diagnostically useful and it is a routine clinical labora-

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tory procedure, which is performed with a semiquantitative assay known as the LAP score [12, 13]. In this report we describe a flow cytometry-based method for the quantitative determination of LAP in peripheral blood neutrophilic granulocytes. The method was used for the determination of LAP expression in normal and pathologic blood samples.

Materials and Methods

Patients

Whole blood samples were obtained from patients and healthy volunteers after informed consent. Four cases of PNH, six cases of CML, eight cases of Essential Thrombocythemia (ET), eight cases of PV, and four cases of Severe Aplastic Anemia (AA) were analyzed. Diagnosis of each disease was made according to standard criteria [14–17]. In addition, samples from individuals treated with G-CSF for the mobilization of allogeneic [18] or autologous [19] peripheral blood progenitor cells (PBPC), were used. Informed consent was obtained from the patients and the donors using forms approved by the Institutional Review Board. A fully and detailed explanation of the potential risks and benefits concerning the collection of G-CSF mobilized PBPC for autologous and allogeneic transplantation was given to normal donors and the patients. Normal donors were more than 18 years old.

Reagents

Recombinant human G-CSF (specific activity 10^8 units/mg protein) was from Amgen Inc., (Thousand Oaks, CA). The 1B12.1 MoAb was obtained by immunization of mice with a highly purified preparation of human bone alkaline phosphatase as described elsewhere [20].

LAP Flow Cytometry and LAP Score Assays

Immunofluorescence analysis was performed on 50 µl of heparinized whole blood incubated for 30 min at 4 °C with isotypic matched negative controls (Becton Dickinson, Mountain View, CA) or with Fluorescein isothyocyanate conjugated (FITC) anti leukocyte alkaline phosphatase MoAb (1B12.1), and Phycoerythrin conjugated (PE) Leu15 (CD11b, Becton-Dickinson,) or 3G8 (anti CD16b, Serotec, Oxford, UK). After washing with medium containing 2.5% human AB serum, the erythrocytes were lysed with NH_4Cl buffer (NH_4Cl 8.99 g/l, $KHCO_3$ 1g/l, Na_4EDTA 0.037 g/l, pH 7.3) for 5 min at room temperature. The cells were then washed twice in phosphate-buffered saline (PBS) and then analyzed by flow cytometry using a FACScan analyzer (Becton-Dickinson) equipped with argon-ion laser tuned at 488 nm, power emission of 150 mW, and filter set for FITC color fluorescence. Double fluorescence staining of lymphocytes, monocytes, NK cells were performed as above using the following lineage-specific phycoerythrin-conjugated monoclonal antibodies: Leu15 (anti-CD11b) for granulocytes, Leu4 (anti-CD3) for mature T-cells, Leu12 (anti-CD19) for B-cells, LeuM3 (anti-CD14) for monocytes, Leu11c (anti-CD16a) for NK cells. All these monoclonal antibodies were from Beckton and Dickinson. FACS analysis on granulocytes, lymphocytes and monocytes was performed by specific gating off the cells on the basis of side and forward light scatter. LAP scores were cytochemically determined according to Kaplow as modified by Hayhoe and Quaglino [13].

Results

To establish that the 1B12.1 MoAb is directed against LAP, we performed two types of experiments. Firstly, we demonstrated that COS-7 fibroblasts transfected with the empty eukaryotic expression vector do not express significant amounts of 1B12.1 cross reactive protein, as assessed by Western blot analysis in non-reducing conditions. By contrast, COS-7 cells transfected with the same vector containing the full length cDNA coding for L/B/K-type alkaline phosphatase demonstrate the presence of a single 150 kDa protein specifically recognized by the 1B12.1 MoAb (data not shown). Secondly, we

showed that the 1B12.1 MoAb specifically recognizes LAP partially purified from peripheral blood neutrophils of G-CSF treated normal donors. Having demonstrated the specificity of our reagent, we developed a quantitative flow cytometry-based method for the determination of LAP in whole blood samples. As expected, double fluoresce assays performed with 1B12.1 and lineage-specific monoclonal antibodies demonstrated the presence of significant amounts of LAP on mature neutrophils but not on T-lymphocytes, B-lymphocytes, NK-cells and monocytes. Flow cytometry of normal neutrophils showed that these cells express significant amounts of LAP protein and CD11b. By contrast, granulocytes obtained from a PNH patient were characterized by a defective expression of LAP and CD16, which was accompanied by normal plasma membrane levels of CD11b. The data are in line with the fact that LAP [22] and CD16 [23] are pigtailed proteins and PNH is a disease associated with a defect in the assembly and surface export of this type of proteins [24]. Neutrophils obtained from the peripheral blood of patients with CML during the stable phase of the disease were devoid of LAP associated fluorescence. By contrast, PV and ET were usually associated with high levels of positivity for the 1B12.1 MoAb. Administration of G-CSF to mobilize PBPC resulted in the appearance of extremely high levels of 1B12.1 associated fluorescence. The results obtained by quantitative flow cytometry of LAP protein were compared to those obtained by the conventional cytochemical LAP score method and are summarized in Table 1. The flow cytometric data are expressed relative to the number of LAP positive cells and single cell mean associated fluorescence (CAMF). There is general concordance between the results obtained by the LAP score method and the number of cytofluorometrically determined LAP-positive cells and CAMF.

To further improve the flow cytometric assay of LAP, we devised a bi-parametric method for the specific determination of the antigen on the neutrophil plasma membrane. To do this, whole blood samples or bone marrow specimens from normal volunteers were subsequently stained with 1B12.1 MoAb, and-phycoerythrin conjugated anti-CD 16 or anti CD11b MoAbs. The results of this analysis are shown in Fig. 1. In the bone marrow, a gradient of positivity for CD16 and a relative homogeneous population of CD16/LAP double positive cells are evident. This suggests that CD16 is expressed at earlier stages of maturation along the myeloid maturation pathway than LAP. By contrast a sharp double-positive cell population, representing the terminally differentiated mature neutrophil, is present in the peripheral blood.

Table 1. Evaluation of LAP in human normal and pathologic neutrophilic granulocytes by cytochemistry and flow cytometric analysis using the 1B12.1 MoAb

	LAP score	1B12.1 positive cells (%)	1B12.1 CAMF (Arbitrary units)
Control	38 (25–125)	41 (21–60)	30 (24–60)
G-CSF	360 (200–400)	96 (85–98)	218 (114–315)
PNH	4 (0–15)	3 (1–17)	40 (12–56)
CML	12 (5–35)	7 (1–12)	26 (17–30)
PV	256 (78–360)	81 (57–98)	56 (46–118)
ET	70 (42–124)	76 (58–80)	48 (29–389)
AA	79 (46–150)	92 (72–96)	64 (60–81)

Heparinized whole blood samples were obtained from normal individuals and patients and subjected to the determination of LAP by LAP score and by flow cytometric methods. The flow cytometric analysis of LAP is expressed relative to the number of 1B12.1 MoAb-positive cells (1B12.1 positive cells) or relative to the cell associated mean fluorescence (1B12.1 CAMF). Results are the median value of each set of data with the intervals of confidence in brackets. Control = normal individuals; G-CSF = leukemic patients treated with G-CSF for the mobilization of hematopoietic stem cells; PNH = patients suffering from paroxysmal hemoglobinuria (PNH); CML = chronic myeloid leukemia; PV = polycythemia vera; ET = essential thrombocytemia; AA = aplastic anemia. CAMF = cell associated mean fluorescence.

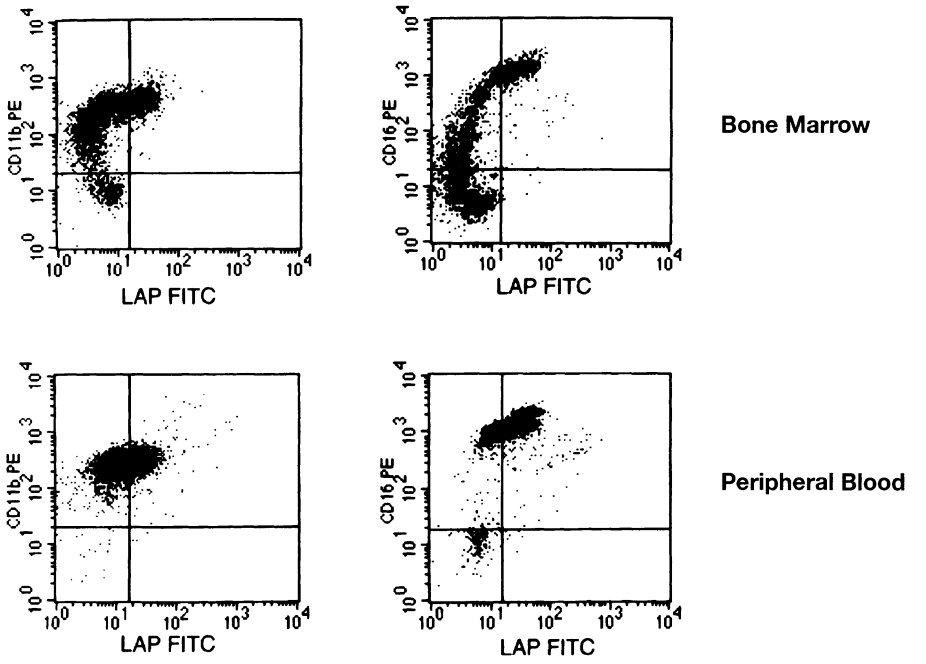

Fig. 1. Co-expression of LAP/CD16 and LAP/CD11b on normal peripheral blood or bone marrow granulocytes. Cells were stained with 1B12.1.1 MoAbb and with phycoerythrin-conjugated anti-CD16 (panels on *right*) or CD11b MoAbs (panels on *left*), and with isotype matched phycoerythrin or fluorescein conjugated control antibodies (not shown). Granulocytic cells were selected by specific gating on the basis of the forward (FSC-H/FSC-Height) and side (SSCH/SCC-Height) light scatter

Discussion

Determination of LAP activity is routinely used for the laboratory diagnosis of different hematological diseases such as CML, PV, idiopathic myelofibrosis and leukemoid reactions [12]. The test is based on the LAP-catalyzed formation of an insoluble dye readily visualized over neutrophilic leukocytes. The method is at best semi-quantitative and subject to great inter-laboratory variability. Introduction of a quantitative and reproducible assay that allows measurement of LAP would represent an important advance in the diagnostic hematological routine. In this report, we demonstrate that 1B12.1, a monoclonal antibody originally developed against human bone alkaline phosphatase [20], specifically recognizes both recombinant and natural LAP. In addition, we show that the antibody can be used to quantitatively determine LAP by flow cytometric analysis of unfractionated heparinized peripheral blood. With this method, we measured LAP protein in whole blood samples obtained from normal volunteers as well as hematologic patients. As expected, neutrophilic granulocytes obtained from PNH [11] and CML [5, 11] patients are characterized by a very small number of LAP-positive neutrophils. The data obtained in PNH granulocytes formally demonstrate that the defect in LAP enzymatic activity, typical of this disease, is due to a defective export of the corresponding protein to the plasma membrane and not to mutations affecting the catalytic activity of the polypeptide. This finding is in line with the fact that LAP is a PIG protein and PNH is characterized by a deficit in the assembly and the export of PIG proteins to the plasma membrane [23]. By contrast, the results obtained in CML granulocytes during the stable phase of the disease, are consistent with the lack of LAP mRNA observed in these leukemic cells [5, 11]. The high levels of LAP enzymatic activity observed in the granulocytes obtained from PV, ET and AA patients are the consequence of an increase in the levels of the corresponding protein and not due to activation of the enzyme. The

molecular mechanism underlying the higher than normal levels of LAP observed in these three myeloproliferative disorders are not yet known. By far the highest levels of LAP protein are preseent in the peripheral blood myeloid cells mobilized from the bone marrow upon in vivo treatment with G-CSF. In this condition, the peripheral blood is characterized by the appearance of a remarkable number of intensively stained LAP-positive cells. The data strongly suggest that in vivo treatment with G-CSF results in the rapid maturation of granulocytic precursors in which synthesis or plasma membrane export of LAP is dramatically induced. Interestingly, double fluorescence experiments demonstrate that LAP positive G-CSF mobilized cells are also CD16 positive, which indicates co-regulation of the two markers of the terminally differentiated granulocyte. The flow cytometric method for the determination of LAP in granulocytes has now been further improved by simulateneous staining of the whole blood preparations with 1B12.1 and anti-CD16 antibodies. The simplicity and reproducibility as well as the quantitative nature of the flow cytometric of this new assay is well suited for a routine hematologic laboratory and has already substituted LAP score in our laboratory.

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Biologic Determinants for AML Therapy

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Abstract. Cytomorphology as defined by the FAB classification still comprises the backbone of diagnosis of acute leukemias. Cytogenetics and molecular techniques have recently provided new insights into the biology of AML and were found to correlate with morphologic subgroups. Furthermore, the morphologic degree of dysplasia was found to be of prognostic significance although different investigators used different definitions for dysgranulopoiesis (DysG), dyserythropoiesis (DysE), dysmegakaryopoiesis (DysM), and trilineage dysplasia (TLD) and results are controversial.

The clinical significance of cytomorphological aspects and dysplasia was prospectively assessed in the AMLCG-92 trial. Dysplastic features were investigated in 272 patients with newly diagnosed AML. No dysplasia was observed in 34.5% of patients, TLD in 17.6%, all other patients had dysplasia in one or two cell lineages. The overall complete remission (CR) rate in all patients was 73%, no remission (NR) was achieved in 10%, 17% of patients suffered from early death (ED). Patients without any dysplasia achieved CR in 78%, NR in 7%, and ED was 15%. In contrast, TLD patients achieved CR in only 62%, but 23% NR and 15% ED was observed. Median overall survival for patients without any dysplasia was 20 months, but only 10 months for TLD patients (p=0.24). Cytogenetic analyses were available in 66% of patients. Unfavorable karyotypes were observed in 25% of patients with TLD, but in only 6% of patients without any dysplasia (p=0.017). Thus, morphology and cytogenetics provide the means to identify patients with different biology and prognosis in AML and may include informations to adapt treatment strategies accordingly.

Introduction

The prognostic significance of dysplastic features in de novo AML as a special aspect of cytomorphology had been investigated by several groups [1–7]. In some studies a poor outcome seemed to be associated with the detection of dysplastic features but the results remain controversial. Cytogenetics were also found to correlate with clinical outcome and may allow to group patients into favorable [t(8;21); t(15;17); inv(16)], intermediate (normal, others), and unfavorable risk groups (-5,5q-,-7, complex) [8]. In this prospective study we analysed the incidence and the prognostic significance of dysplasia in 272 patients with de novo AML

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treated in the AMLCG-92 trial. The results were then correlated with the cytogenetic analysis in these patients in order to define prognostic subgroups for treatment stratifications in the future.

Material and Methods

Between December 1992 and January 1997 de novo AML was diagnosed in 272 patients treated in the AMLCG-92 study [9, 10]. In this interim analysis of an ongoing study we only included patients when remission status and remission duration were available. Bone marrow and blood smears were sent for diagnosis and detailed cytomorphological examination to our reference center in Kiel. At the time of bone marrow analysis we had no knowledge of treatment decision or cytogenetic results and therefore avoided a bias by cytomorphological expectations.

Bone marrow smears were stained according to standard procedures [11]. For dysplasia the May-Grünwald-Giemsa and the myeloperoxidase-staining were examined. Dysplasia was defined according to standard criteria (4): Granulocytic dysplasia (DysG) was defined as >50% of at least ten polymorphonuclear neutrophils (PMN) being agranular or hypogranular, or with hyposegmented nuclei (pseudo Pelger-Huet anomaly). At least 25 cells were analysed but usually 100 cells were examined. Myeloperoxidase deficiency in the polymorphonuclear neutrophils (PMN) was defined as 50% or more myeloperoxidase-negative cells in at least 10 PMN after confirming strong positivity of eosinophils or other PMN. Erythroid dysplasia (DysE) was defined as 50% or more of the following dysplastic features in at least 25 erythroid precursors: megaloblastoid aspects, karyorrhexis, nuclear fragments or multinuclearity. A minimum of 25 cells were analysed but usually it was possible to examine 100 cells. Megakaryocytic dysplasia (DysM) was diagnosed when at least three megakaryocytes or >50% in at least six cells showed dysplastic features such as microkaryocytes or multiple separated nuclei. Trilineage dysplasia was diagnosed when DysG, DysE, and DysM was detectable.

Cytogenetic analysis was performed according to the standard protocol using fluorescence R-banding or G-banding and followed the rules of the International System for Human Cytogenetic Nomenclature, ISCN (1995) [12].

Results

272 patients (pts.) with de novo AML were analyzed (m = 136, f = 136). Median age was 50.5 years (range 17-76). According to the FAB-classification [13] five pts. were classified as AML M0, there were 60 M1 (22%), 91 M2 (33.4%), 4 M3 (1.7%, all others treated separatly since 1993 in an ATRA trial), 49 M4 (18%), 21 M4Eo (7.7%), 15 M5a (5.5%), 19 M5b (6.9%), 8 M6 (2.9%) and no M7. Remission rates according to FAB-types are shown in Table 1.

Dysplastic features were investigated in all patients. No dysplasia was observed in 34.5% of patients, TLD in 17.6%, all other patients had dysplasia in one or two cell lineages. The overall complete remission (CR) rate in all patients was 73%, no remission (NR) was achieved in 10%, 17% of patients suffered from early death (ED). Patients without any dysplasia achieved CR in 78%, NR in 7%, and ED was 15%. In contrary, TLD patients achieved CR in 62%, but 23% NR and 15% ED were observed (see Table 2). Median overall survival for pts. without any dysplasia was 20 months, but only 10 months for TLD pts. ($p = 0.24$, Fig. 1). At the time of this analysis we decided not to focus on patients with only one dysplastic cell lineage or a combination of two lineages because numbers were to small for valid evaluations.

Table 1. Remission rate according to FAB-type (n = 272)

	CR (%)	NR (%)	ED (%)
AML M0	60	20	20
AML M1	67	12	21
AML M2	75	11	14
AML M3	87	4	9
AML M4	63	14	23
AML M4Eo	95	0	5
AML M5	75	10	15
AML M6	50	25	25

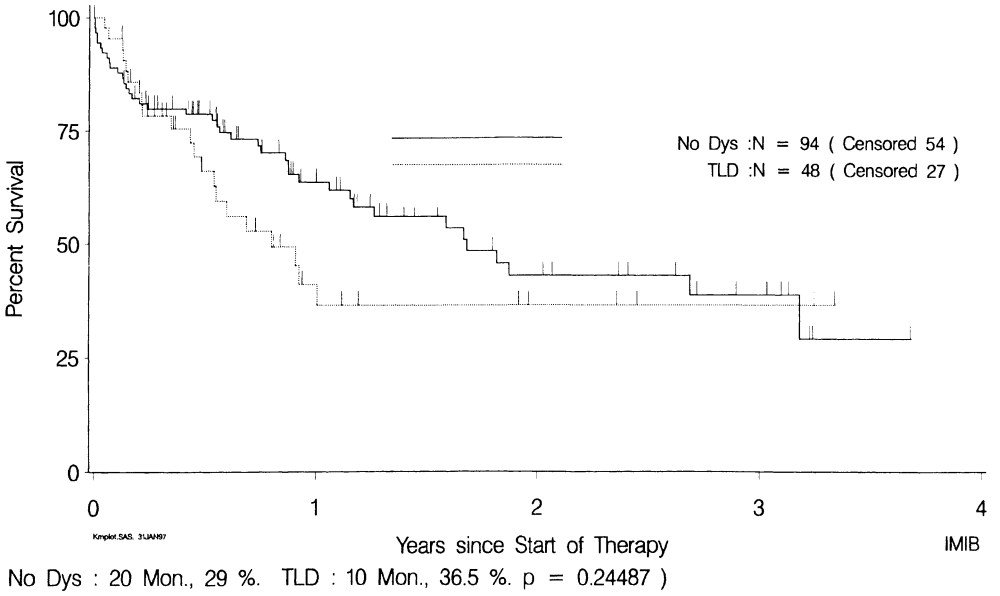

Fig. 1. Trilineage dysplasia (TLD) seems to be an unfavorable prognostic factor for remission and surival in patients with de novo AML The difference is not statistically significant so far.

Table 2. Dysplastic features and remission rate in de novo AML

	No Dys (n = 94)	All pts. (n = 272)	TLD (n = 48)
CR	78%	73%	62%
NR	7%	10%	23%
ED	15%	17%	15%

Table 3. Cytogenetic results in patients with de novo AML according to dysplastic features

	No Dys	TLD	p =
All patients (n)	94	48	
Results available	63%	69%	
Normal karyotype	58%	60%	n.s.
Favorable risk	21%	9%	0.16
Unfavorable risk	6%	25%	0.017
Others	15%	6%	n.s.
Not evaluable	10%	0%	

Cytogenetic analyses were available in 63% and 69% of patients without any dysplasia or TLD, respectively. Unfavorable karyotypes were observed in 25% of patients with TLD, but in only 6% of patients without any dysplasia ($p = 0.017$).

According to the cytogenetic subgroups, the median relapse free interval was still not reached for patients with favorable risk, it was 18 months for the intermediate group, but only 8 months for patients with unfavorable cytogenetics ($p = 0.0084$, Fig. 2).

Discussion

The identification of new prognostic factors in AML is needed to stratify patients into low risk and high risk group and to modify therapeutic approaches in the future. Some studies were able to demonstrate the significance of cytogenetic results for the outcome of patients [14–22], others identified age [19] or leucocyte count [20] as prognostic factors, but these results remain controversial. This is true also for the detection of myelodysplasia in de novo AML. The incidence of DysG, DysE or DysM differed widly in different studies [1–7]. Even if the same investigator analyzed the bone marrow smears variations of 100% were published in different trials [4, 5]. This demonstrates the complexity of the detection of dysplasia and one should avoid final conclusions from these retrospective studies. Only two studies could demonstrate the prognostic value of TLD for event-free survival [3, 7]. In one study DysM seems to influence the prognosis [5].

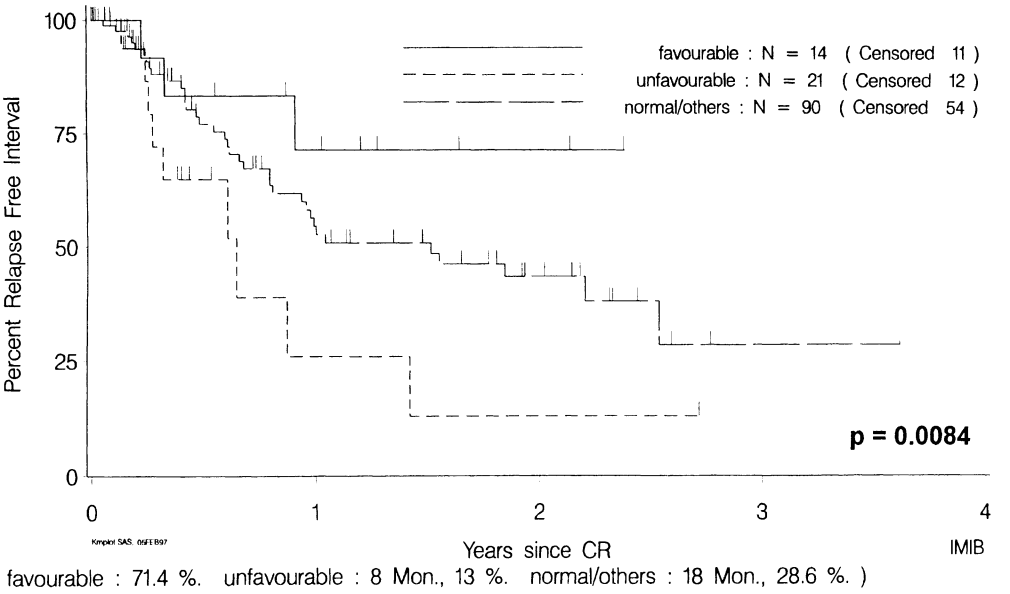

Fig. 2. Cytogenetics define different biological entities in de novo AML. Favorable: t(8;21); t(15;17); inv(16); intermediate (normal, others); and unfavorable risk group (-5,5q-,-7, complex aberrations).

In contrast, the incidence of patients without any dysplasia, or with TLD ranged in small limits in all studies published so far. Thus, it seems much easier to achieve reliable results especially for these two subgroups of patients.

We have investigated morphological dysplasia in 272 patients with de novo AML to clarify its clinical significance. All patients were treated in the AMLCG-92 trial. We were able to identify two subgroups of patients with a superior or a worse complete remission rate. Patients without any dysplasia in the bone marrow had a higher CR rate (78%) in comparison to all patients combined (73%) and especially in contrast to patients with TLD (CR 62%). Similar results were reported by some groups in retrospective studies [1,3,4,7]. Others failed to demonstrate a significantly lower CR rate for patients with TLD but used unconventional criteria for the definition of dysplasia so that it can not be compared to results of other studies [6].

We were not able to clarify the prognostic significance of dysplasia affecting only one or two cell lineages, because the number of cases was too small. Further investigations are on their way and may help to define smaller subgroups of patients with special combinations of dysplastic features. A multivariant analysis will help to grade the importance of other accompanying cytomorphological details.

Furthermore, we correlated the detection of dysplasia with karyotype. According to the actual definitions of favorable risk [t(8:21); t(15;17) and inv(16)], intermediate risk (normal karyotype, others), and unfavorable risk (-5, 5q-, -7, complex aberrations) we could demonstrate that the detection of TLD is highly correlated with unfavorable chromosomal aberrations ($p = 0.017$) and no dysplasia is much more often seen with favorable anomalies ($p = 0.16$). However, the most important difference for remission and survival was achieved comparing these three cytogenetic subgroups alone ($p = 0.0084$). Therefore, further investigations are necessary to define the prognostic impact of dysplasia in de novo AML and may lead to a reliable prognostic score in combination with cytogenetic data. This may hopefully allow treatment stratifications in order to avoid over-treatment in favorable risk patients such as allogeneic bone marrow transplantation in first CR. Furthermore, new treatment options for the unfavorable subgroup of patients have to be defined.

In conclusion, the detection of dysplasia and especially the leukemic karyotype at diagnosis are biological determinants for AML treatment results. After confirmation of these first prospective analysis, results should be implemented in further treatment stratifications in order to achieve best remission rates and survival for every single patient according to the biological entity of the individual leukemia.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Evaluation Of NOD-SCID-Mice for a Human B-Cell Precursor ALL Xenograft Model

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Abstract. To evaluate the newly bred NOD-SCID mice as recipients for human B-cell precursor ALL bone marrow or peripheral blood cells from 7 pediatric and one adult patient were transplanted onto irradiated NOD-SCID mice. Leukemic cells from 6 out of 8 patients (75%) successfully engrafted NOD-SCID mice and from 4 patients (50%) led to an extensive leukemic infiltration in the murine marrow. High level human cell engraftment could be demonstrated by flow cytometry, Southern blot analysis, PCR and cytology. By cytology and immunophenotype, the leukemia in the mice was indistinguishable from the original leukemia in the patients. Limiting dilution analysis with cells from one patient with pre-pre-B-ALL showed that as few as 10,000 cells were sufficient to transfer the leukemia onto NOD-SCID mice. The presence of human eosinophils in the marrow of highly engrafted mice indicated minimal coengraftment of rare residual normal cells. Development of leukemia in the mice varied between 1.5 and 7 months depending on cell dose and patient transplanted. Interestingly, conditioning of the mice by sublethal irradiation was not necessary for successful engraftment. These experiments demonstrated that NOD-SCID mice are sensitive recipients for human ALL xenografts.

Introduction

The original transplantation experiments by John Dick and coworkers had established that leukemic cells from patients with B-cell precursor ALL engraft immune-deficient SCID mice and proliferate in the murine microenvironment in a fashion similar to that seen in patients [1, 2]. This has been confirmed by several other groups [3–10]. In addition, SCID mouse models have been developed for normal human hematopoiesis and a variety of myeloid leukemias [11–17]. Recently, a new mouse strain has been bred by crossing the scid mutation onto the NOD mouse background [18]. Due to additional defects of the immune system, these NOD-SCID mice are better recipients for normal and leukemic human myeloid cells (reviewed in [12]). In this study we tested this new mouse strain for an ALL xenograft model.

Materials and Methods

Transplantation of human ALL onto NOD-SCID mice

The NOD-SCID mice were kept under defined flora conditions in individually ventilated (HEPA-filtered air) microisolator cages (Lab Products, Maywood, USA). 1×10^4–20×10^6 bone marrow or peripheral blood cells from seven pediatric and one adult patient with B-cell precusor ALL were transplanted

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via tail vein injection onto 8–14 week old NOD-SCID mice. Most mice were sublethally irradiated with a single dose of 3.75 Gy from a cobalt 60-unit one day prior to transplantation.

Multiparameter Flow Cytometry

To quantify and characterize the human leukemic cells in the transplanted mice the murine bone marrow was analyzed by three-color flow cytometry. Anti-CD45-FITC or anti-CD45-PerCP antibodies (Anti-HLe-1, Beckton Dickinson) were used to identify human cells in the murine marrow; expression of B cell markers was assessed by staining with anti-CD19-Cy5 or anti-CD19-PE (J4.119, Immunotech, Hamburg), anti-CD10-FITC (ALB2, Immunotech) and anti-CD20-PerCP (Leu16, Beckton Dickinson); anti-CD34-PE (QBend10, Immuntech) was used for the detection of immature cells and anti-CD13-PE (SJ1D1, Immunotech) und anti-CD33-PE (D3HL60.251, Immunotech) to assess coexpression of myeloid markers. Matched isotype controls were included in each experiment.

DNA Analysis

As previously reported [11] human cell engraftment was quantified by Southern blot analysis. 5 μg DNA were digested with EcoR1 (Boehringer Mannheim, Mannheim), separated on a 0.6% agarose gel, blotted onto a nylon membrane (Boehringer Mannheim) and hybridized with a luminescence-labelled human chromosome 17-specific alpha-satellite probe (p17H). Human cell engraftment was quantified by comparing the characteristic 2.7-kb band in the sample lanes with standard human:mouse mixtures. PCR-detection of human DNA in bone marrow of transplanted mice was performed by using primers specific for human intronic sequences of the muscular dystrophy gene according to our established protocol [16]: 7632 sense primer: 5'-AAT TCA CAG AGC TTG CCA TGC TG-3'; 6975 antisense primer: 5'-TGC CTC CCA GAT CTG ACT CCT GTA-3'. The PCR amplification results in a 230 bp product.

Results

Bone marrow or peripheral blood cells from seven pediatric and one adult patient with B-cell precursor ALL were transplanted onto NOD-SCID mice. The patient data and experiments are summarized in Table 1. By flow cytometry, human cells could be detected in the bone marrow of mice transplanted with cells from six out of the eight patients (75%) (Table 1) and in the marrow of mice transplanted with cells from four patients an extensive leukemic infiltration with 18 to 84% human leukemic blasts was observed (as an example see Fig. 1).

The human cells in the bone marrow of transplanted mice had the same immunophenotype as the leukemic cells from the original patient sample [19]. In addition to the B-cell precursor immunophenotype, coexpression of myeloid makers on the leukemic blasts confirmed that leukemic cells and not mature B-cells had engrafted these mice.

Little variability in the engraftment kinetics were observed when comparable numbers of cells from the same patient were transplanted in different experiments (data not shown). However, marked differences were seen when cells from different patients were transplanted. Time to the development of leukemia in transplanted mice varied from 1–7 months depending on cell dose and patient sample (Table 1).

Transplantation experiments with decreasing numbers of cells demonstrated the sensitivity of this ALL-NOD-SCID mouse model. In limiting dilution experiments with leukemic blasts from patient #1 as few as 1×10^4 cells were sufficient to successfully reinitiate the leukemia in transplanted mice: 2 out of 5 mice transplanted with 1×10^4 cells were successfully engrafted with 81 to 93% leukemic blasts infiltrating the murine marrow 70 to 134 days after transplantation.

Human cell engraftment could also be confirmed by Southern blot analysis and PCR. There was a good correlation between the level of human cell engraftment as quantified by flow cytometry and Southern blot analysis (data not shown).

Touch preparations of the murine marrow showed extensive infiltration with lymphatic blasts. In addition to human ALL

Table 1

ALL subtype	Clinical course	Cell dose	Time to engraftment	Llevel of engraftment by flow cytometry
ALL #1	> 3 years	$10 - 20 \times 10^6$	41–43 days	11–83% (n = 8)
pre-pre-B	in CCR	1×10^6	43–80 days	3–64% (n = 4)
ALL with		1×10^5	16–134 days	0–65% (1 of 5)
del(11)(q23)		1×10^4	70–134 days	0–93% (2 of 5)
ALL #2	> 1 year in	20×10^6	43 days	0% (n = 1)
pre-pre-B ALL	CCR	$5–9 \times 10^6$	190 days	59–84% (n =3)
with t(4;11)				
ALL #3	death	5×10^6	35 days	0% (n = 3)
first cALL	2 months after		124 days	3% (n = 1)
relapse	relapse		143 days	45% (n = 1)
ALL #4	> 1.5 years in	4×10^6	34 days	0% (n = 2)
pre-B ALL	CCR			
ALL #5	> 1.5 years in	5×10^6	40 days	< 1% (n = 1)
cALL with t(9;22)	CCR			
ALL #6	Death 1 year	2×10^6	101–185 days	0% (n = 3)
2nd cALL	after second			
relapse	relapse			
ALL #7	No data	4×10^6	52 days	6% (n = 1)
adult BCP-ALL	available			
ALL #8	> 7 months in	8×10^6	86–112 days	5–29% (n = 3)
cALL	CCR			

blasts and residual mouse hematopoiesis, low numbers of human eosinophils could be detected in highly engrafted mice [19]. This indicated minimal coengraftment of rare residual normal human hematopoietic stem cells.

An interesting oberservation was that irradiation of NOD-SCID mice prior to transplantation proved to be unnecessary for successful engraftment of B-cell precursor ALL. This was shown with cells from patients #1, 7, and 8.

Discussion

This study established that NOD-SCID mice are good recipients for human ALL xeno-

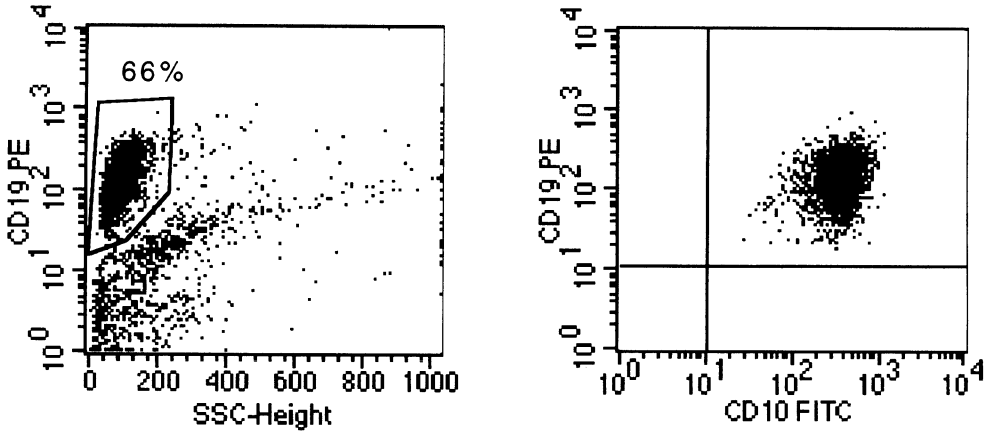

Fig. 1. Bone marrow of a NOD-SCID mouse transplanted with leukemic blasts from patient ALL #3. The cells in the murine marrow are pregated by their forward and site scatter profiles. Like the cells from the original patient the human leukemic blasts in the murine marrow coexpress CD19 and CD10.

grafts. 75% of the transplanted leukemias engrafted and in most cases caused overt leukemia in the mice. By bone marrow cytology and immunophenotype the leukemia in the mice was indistinguishable from the leukemia in the original patient.

The sensitivity of the NOD-SCID mouse assay could be demonstrated by the observation that 1×10^4 bone marrow cells from patient #1 contained enough malignant stem cells to reinitiate the leukemia in transplanted mice. Most published studies with conventional SCID mice use much higher cell doses, i.e., $5–10 \times 10^6$, for transplantation. In addition, the presence of human eosinophils in ALL-transplanted NOD-SCID mice indicated minimal coengraftment of residual normal stem cells which are only present at very low frequency in human marrow at the time of diagnosis [20].

Transplantation experiments with cells from patient #1, 7 and 8 with non-conditioned mice showed that irradiation of the mice was not necessary for successful engraftment. This is in accordance with a recent study using unirradiated SCID mice as recipients for ALL transplants [6]. As there are no competing murine B-lymphoid cells and as all B-stimulatory growth factors are most likely upregulated in SCID and NOD-SCID mice human leukemic B-cell precursor cells unlike human myeloid cells might already find an optimal microenvironment in unconditioned mice.

This ALL-NOD-SCID mouse xenograft model will be a valuable tool to characterize candidate normal and leukemic stem cell populations in B-cell precusor ALL and to develop and evaluate new antileukemic treatment strategies.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Flow Cytometric Analysis of Immature Cell Populations in B-Cell Precursor ALL

J. Vormoor, G. Baersch, M. Baumann, J. Ritter, and H. Jürgens

Abstract. A flow cytometric protocol was developed that allowed identification and analysis of rare CD34+CD19- cells in the bone marrow of patients with B-cell precursor acute lymphoblastic leukemia (ALL). Within this CD34+CD19- cell population an expression of CD38 was observed that was compatible with differentiation from primitive CD34+CD38- to more mature CD34+ CD38+ cells. Lack or very low levels of CD45 expression on the leukemic cells of 8 patients was used as a marker for the leukemic cell clone. In 3 of these 8 patients, all CD34+CD19- cells did express CD45; in the other 5 patients, some or most of the CD34+CD19- population were CD45-. In normal bone marrow, CD34+CD19- cells were always CD45+ (n= 6). This suggests heterogeneity of stem cell involvement in childhood B-cell precursor ALL. In some patients the leukemic transformation may arise from a lymphoid-committed cell already expressing CD19, while the CD34+ CD19- cells may represent residual normal cells. To identify early differentiation events within the leukemic cell clone CD24 and CD31 expression was analyzed. Our preliminary data are compatible with the most immature leukemic blasts being CD34+ CD31+ CD24-. These data need now to be confirmed by molecular methods and transplantion of these populations onto immune-deficient mice.

Introduction

Flow cytometric analysis in acute myeloid leukemia (AML) had shown that although there are abnormal patterns of antigen expression in the bulk population of AML cells, the earliest differentiation events seem to be highly conserved between normal and leukemic stem cell populations [1]. In accordance with these findings it could be shown that the most immature CD34+CD38- cells in the bone marrow of AML patients contain the genetic alterations specific for the leukemic cell clone [2]. Moreover, only AML cells with this stem cell-like CD34+CD38- phenotype and not more mature leukemic cells could transfer the human AML onto immune-deficient SCID mice [3]. Together these data established that like in normal hematopoiesis there is a small population of leukemic stem cells in AML which maintains the malignant growth in vivo. Like normal stem cells these malignant stem cells loose their self-renewal and proliferative capacity during differentiation.

In this study we investigated if unlike in AML it would be possible to identify candidate residual normal stem cell populations in patients with B-cell precursor ALL that could be distinguished from the leukemic cell clone by cell surface marker expression. In addition, we tried to characterize early differentiation events within the leukemic cell clone.

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Materials and Methods

To characterize rare CD34+ cell populations in B-cell precusor ALL, bone marrow or peripheral blood cells were stained with the following antibody combinations: anti-CD38-FITC / anti-CD34-PE / anti-CD19-PECy5 (J4.119, Immunotech)/(T16, Immunotech)/(QBend10, Immunotech) anti-CD45-FITC / anti-CD34-PE / anti-CD19-PECy5 (Anti-HLe-1, Becton Dickinson (BDIS))/(T16, Immunotech)/(QBend10, Immunotech) [4]. 5×10^6 cells (0.5 - 1 ml whole blood or bone marrow) were incubated with 30 µl of the antibodies for 20 min at room temperature. Subsequently, the red cells were lysed (cell lysis buffer, BDIS) and the sample washed twice with PBS buffer. Data acquisition and analysis was performed on a FACScan (BDIS) using CellQuest software (BDIS). After calibration and compensation, 1×10^6 events were acquired but only CD34+CD19- events were stored (storage gate). Using the same gate, 1×10^6 events from the same sample stained with matched isotype control antibodies were analyzed to exclude amplification of unspecific events. To reduce background, the acquisition speed was kept below 2000 events/s and the cytometer was flushed for 10 min with 5% hypochloride and for 10 min with pure water before each acquistion.

Expression of CD24 and CD31 was analyzed according to standard flow cytometric protocols as described before [4, 5].

Results

To identify rare CD34+ cell populations in the bone marrow of patients with B-cell precursor ALL at diagnosis high numbers of cells had to be analyzed and several steps to be performed to avoid amplification of unspecific background events as described under Methods.

With this flow cytometric protocol it was possible to identify a CD34+CD19- cell population in the bone marrow of 18 BCP-ALL patients at diagnosis, i.e. an immature CD34+ cell population that lacked expression of lymphoid cell surface markers. This population represented only about 0.1% of all bone marrow cells. A small minority of these cells were CD38- while the majority of CD34+CD19- cells expressed CD38.

In 8 patients with CD45- ALL, CD45 expression was analyzed on this CD34+CD19- cell population. In 3 patients, all CD34+CD19- cells expressed CD45 (Fig. 1A). In 5 patients, two distinct populations were detected in the CD34+CD19- compartment, one expressing CD45 and one lacking expression of CD45 (Fig. 1B). In 6 normal bone marrow samples the CD34+CD19- cells always expressed CD45.

Twentythree patients were analyzed for CD24 expression within the leukemic cell clone. In 16 patients CD24 expression was inconclusive as the leukemic cell clone was either CD24- (4 pat.) or CD24+ (12 pat.). In 7 patients there was a small minority of CD24- cells while the majority of cells expressed varying amounts of CD24. When CD34 expression was downregulated with differentiation within the leukemic cell clone (2 pat.), CD24 expression was inversely correlated with CD34 expression (Fig. 2).

So far, 10 patients have been analyzed for CD31 expression. In 4 patients, CD31 expression was inclusive as the leukemic cell clone was CD31+. In 6 patients, there was a small minority of CD31+++ while the majority of leukemic cells expressed low levels to no CD31. In 5 of these 6 patients there was a direct correlation between the level of CD34 and CD31 expression (CD34+++CD31+++ -> CD34+/-CD31+/-).

Discussion

One of the open questions concerning leukemogenesis of acute lymphoblastic leukemia is if the transformation event/events like in AML occur/s in a pluripotent stem cell or rather in a lymphoid-restricted progenitor cell.

To address this question we developed a flow cytometric protocol to characterize rare CD34+CD19- immature cell populations in patients with B-cell precursor ALL at diagnosis [4]. Using this protocol we were able to identify immature CD34+ cells which lacked expression of lymphoid cell surface markers. When these cells were analyzed for

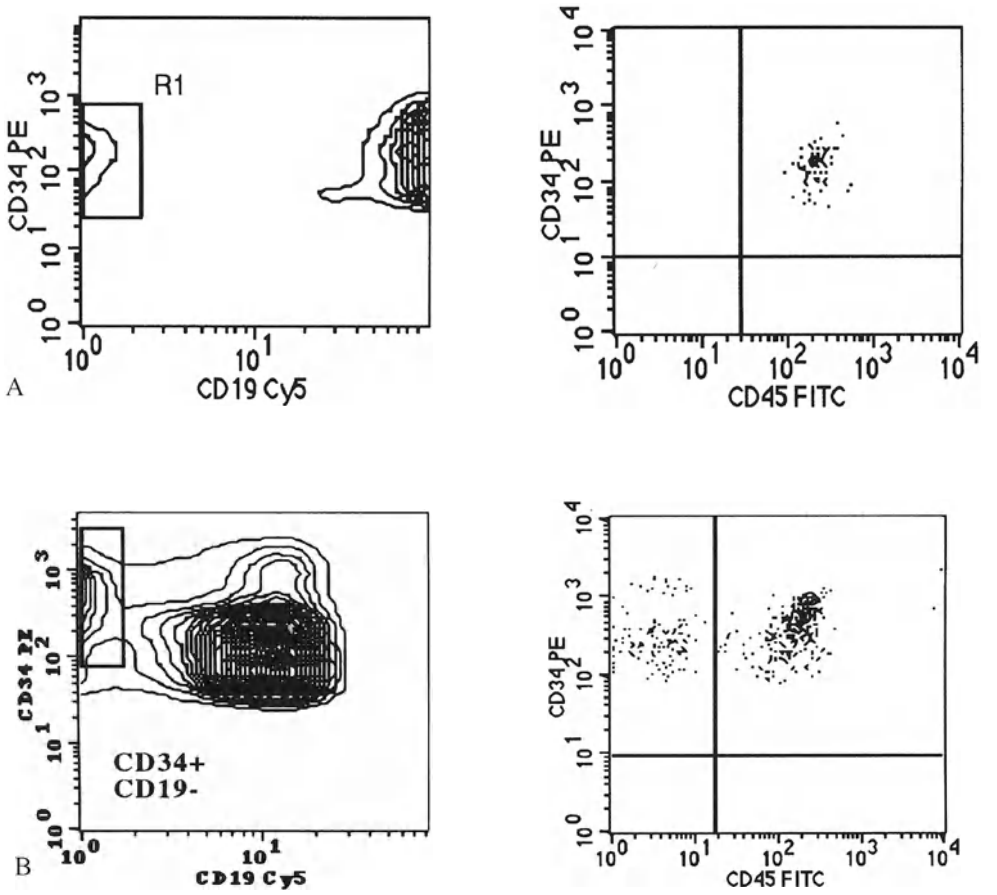

Fig. 1. Expression of CD45 on CD34+CD19- cells in the bone marrow of patients with CD45- B-cell precursor ALL. In the right dot plot only the CD34+CD19- cells (gate R1) are displayed

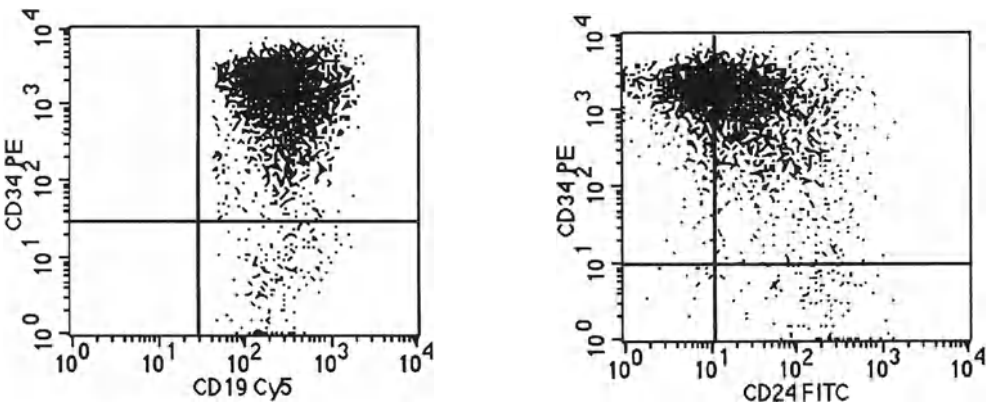

Fig. 2. Expression of CD24 on the leukemic blasts of a patient with B-cell precursor ALL

CD38, a pattern of CD38 expression was seen that was compatible with differentiation from immature CD34+++CD38- to more mature CD34+CD38+. The lack of expression of lymphoid cell surface markers and the pattern of CD38 expression suggested that these cells may represent residual normal cells.

Therefore, 8 patients with CD45- ALL were further analyzed. In CD45- ALL, absence of CD45 expression provides a unique marker for the leukemic cell clone. In 3 of these patients, all the CD34+CD19- cells unlike the leukemic cells expressed CD45 (Fig. 1A). In the other 5 patients, 2 separate populations could be identified within the CD34+CD19- compartment, one presumably normal CD45+ and one presumably leukemic CD45- (Fig. 1B). CD34+CD19- cells from normal bone marrow always expressed CD45.

These data suggest that in a third of the CD45- ALL patients analyzed in this study the leukemic transformation occured in a lymphoid progenitor cell already expressing CD19. This also indicates that at least in some patients with B-cell precursor ALL it might be possible to identify and potentially purify residual normal stem cell populations. However, CD45- ALL is considered to be a prognostic favourable subtype [6] and it has to be seen if these findings also apply to other ALL subtypes.

In the other 5 patients, a CD45- population could be identified within the compartment of immature CD34+CD19- cells. This suggests that in these cases the leukemia arises from a cell not yet expressing CD19. Further analysis of these cells is necessary to determine if these cells have a CD34+CD38- stem cell-like phenotype or if they are CD34+CD38+ progenitor cells. Future studies also have to identify additional markers which may allow to distinguish between candidate leukemic and residual normal stem cell populations.

The next question we tried to address is if we could identify early differentiation events within the leukemic cell clone. Aim of this approach is to identify candidate ALL stem cell populations. In analogy to normal B cell development [7,8] expression of CD24 and CD31 was assessed on CD34+CD19+ leukemic blasts. The expression patterns found were compatible with the differentiation of CD34+CD31+CD24- to more mature CD34+/-CD31-CD24+ leukemic cells (Fig. 2).

However, analysis of larger numbers of patients is necessary and these data need to be confirmed by molecular methods and transplantation of theses subpopulations onto immune-deficient mice before final conclusions can be drawn from these studies.

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Immunophenotypical Subtype of Childhood T-All with Favourable Outcome in the COALL 1985-1992 Study Group

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Abstract. Physiologically, T-cell differentiation involves passage through the thymic cortex, where approximately 90% of the T-cells will be eliminated by apoptosis. This thymic cortical T-cell population is called selection-related (SR) and can be identified by a distinct pattern of surface marker expression ($CD1^+CD4^+8^+$).

Aims of the study: as malignant transformation can occur at different stages of T-cell differentiation we asked the following questions:

1. What percentage of T-ALL cases are of SR phenotype?
2. Does SR phenotype confer a different prognosis under standard leukemia therapy?

Patients and methods: immunophenotypes of 87 ALL cases diagnosed as T-ALL and treated between 1985 and 1994 according to the high-risk arm of the COALL protocols were reviewed.

Results: 37/87 (42.5%) patients presented as SR phenotype; 39 cases (44.8%) were classified as non-selection related (NSR) consisting of subsets with pre- (n=12) and post-selection (n=13) phenotype or coexpression of myeloid markers (n=14). 11 cases (12.6%) did not meet the criteria for either subgroup. EFS of SR phenotype T-ALL (0.92±0.04) was significantly higher compared to the NSR group (0.59±0.08, log rank test, p=0.0018). Because of small patient numbers the subsets of the NSR group showed no significant differences between each other.

Conclusion: T-ALL with a SR phenotype is a distinct group of patients with excellent prognosis under a standard high risk treatment protocol.

Introduction

Immunophenotyping has been very useful to identify prognostic subgroups in ALL deriving from B-lineage precursors. The knowledge about T cell differentiation and its associated immunophenotype is rapidly increasing (reviewed in [1, 2]). A key maturation process of immune system cells is selection of T-lymphocytes that are capable to distinguish between self and non-self. Based on the current understanding of the immunophenotype of T-cells undergoing selection we used a novel classification. We differentiated between thymocytes undergoing selection with a selection-related phenotype (SR) and thymocytes/lymphocytes before or after selection with a non-selection related phenotype (NSR) and applied this to T-ALL blast phenotypes.

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Material and Methods

Patients

From January 1985 to August 1994 patients were recruited in the German COALL 85/89/92 multicenter trial. Patients had to be over 1 year and under 18 years of age. Patients were excluded from the study if they were treated for more than 14 days with chemotherapy other than vincristine, daunoblastin and prednisone, if dosage was reduced or therapy discontinued in an other way than stated in the study protocol, if death occured before initiation of therapy or if parents denied to give consent to participate in the study.

Diagnosis and Therapy

T-ALL was diagnosed when there were more than 25% lymphoblasts in the BM by microscopy and blasts exhibited classical T-cell markers that are usually not present on B-/myeloid cells by immunophenotyping; 87 of the recruited cases were diagnosed as T-ALL. At diagnosis the size of lymph nodes, liver and spleen as well as the mediastinal mass and testicular involvement was determined by physical examination and routine full blood counts were recorded. If there were more than 5 lymphoblasts /µl in CSF patients were diagnosed to have CNS involvement. Patients with T-ALL received the high risk arm of the protocol COALL-85/89/92 [3–5]. Complete remission (CR) was defined as absence of leukemic blasts in peripheral blood, BM (< 5%) and CSF. Relapse was defined as reoccurence of leukemic blasts in any location.

Immunophenotyping and Immunological Subclassification

Immunophenotyping was done by standard immunoflourescence microscopy and FACS analysis using directly labelled, fluorescinated monoclonal antibodies. The most lineage specific, classical T-cell markers CD1, CD2 surface sCD3, CD4, CD5, CD7, CD8 were used to define T-ALL. Due to retrospective analysis double binding studies were not available in all cases. To differentiate from B-cell lineage blasts were also stained with the classical B-cell markers CD19, CD20, CD22. T-ALL was defined as SR if at least one or both of the following two criteria were fulfilled. CRITERION I: CD1 expression of more than 10% of leukemic blasts in PB and BM irrespective of CD3 expression. CRITERION II: Evidence for CD4/CD8 double-positive (DP) leukemic blasts in PB/BM. The sum of percentages of CD4+ and CD8+ blasts is > 30% and exceeds the percentage of sCD3 cells by a factor >3 (%CD4+%CD8 / %CD3 = >3) or exceeds 150% in total (%CD4 + %CD8= >150%). Percentages of CD4+ and CD8+ blasts have to differ less than 6-fold from each other (%CD4 / %CD8 = > 6 or < 0.16). CD24 is a B-cell marker. However CD24 is thought to be the human analogue of heat-stable antigen which in the mouse is transiently expressed and appears to be involved in T-cell development [1]. Therefore in combination with the above criteria CD24 expression of up to 50% was still included in the SR phenotype. T-ALL with Pre-Selection (PRS) phenotype, with post-selection (POS) phenotype and My+/B+ phenotype with co-expression of B-cell lineage or myeloid markers were summarized under non-selection-related (NSR). T-ALL with PRS phenotype is defined by CD7 expression and no expression of CD1, sCD3, CD8. In all cases more than 65% of blasts were CD7 positive. In most cases CD5 and CD2 are expressed. CD4 can be expressed in pro-Tcells [1], therefore expression of CD4 up to 50% can be found in rare cases of the PRS group. T-ALL with POS phenotype is defined as expression of sCD3 in more than 15% of blasts. The sum of CD4 and CD8 does not exceed the percentage of total sCD3 cells by >10% (CD3/CD4+CD8= >1.1). CD1 has to be >10%. CD2, CD5, CD7 are usually expressed >50%. T-ALL with My+/B+ phenotype is defined as expression of one or more of the classical B-cell/myeloid markers >20% in BM/PB. CD24 was only counted as a B-cell marker, if CD24 was >50% (see above) and there was a NSR phenotype or if there was additional evidence of B-lineage (CD19, CD20 > 20%).

T-ALL were not classified if there were no data for >5 classical T-cell markers or if

Table 1. Classification criteria used for immunophenotypical subtypes of T-ALL.

T-ALL immunophenotypical subtype	Criteria
SR	CRITERION I: CD1 > 10% irrespective of CD3 expression.
	CRITERION II: CD4+ and CD8+ blasts is > 30% and %CD4 + %CD8 / %CD3 = >3 or %CD4 + %CD8 = >150% and %CD4 / %CD8 = > 6 or < 0.16
NSR	
a) PRS	CD7+ (>65%) CD1-,sCD3-,CD8-
b) POS	sCD3 > 15% and CD3/CD4+CD8 = >1.1, CD1 <10%
c) My+/B+	one or more >20%: CD13, CD14, CD33, CD19, CD 20, CD22, CD24 (>50%)

SR = selection-related, NSR = non selection-related, PRS = pre-selection, POS = post-selection, My+/B+ = coexpression of myeloid/B-cell markers

there were no data on the most useful markers CD1 and CD3.

Statistical Analysis

The Kaplan Meier method was used to estimate event-free survival rates. Events were defined as failure to get into complete remission, relapse and death during CR. Differences between the immunological subgroups SR and NSR were calculated by the two-sided log-rank test.

Results

Immunological Classification and Immunophenotype of subgroups

37 (42.5%) cases were classified as SR phenotype. In 12 cases peripheral blood, in 20 cases BM and in 5 cases both were used for immunophenotyping. In 16 cases classification as SR based on criterion I, in 13 cases on II and 8 cases on both criteria. Data on CD3, CD4, CD8 were available in all patients. In 5 patients there were no data on CD1 expression. In these patients criterion II was fulfilled and patients were thereby classified as SR. 39 cases (44.8%) were classified as NSR phenotype. Immunophenotyping was done in 7 cases on PB, in 28 cases on BM and in 4 cases on both BM and PB. At least one classical My+/B+ marker was stained in the 14 My+/B+ cases, in 4 cases no classical myeloid markers were investigated, so that in these 4 cases aberrant marker expression might have been missed. 3 T-ALL cases coexpressed 1 myeloid marker (My1+), 2 cases expressed 2 myeloid markers (My2+) and in 9 cases at least one B cell marker was detected. In the remaining NSR patients 12 were classified as PRS and 13 as POS. 11 cases (12.6%) could not be classified at all. Either too few markers were stained for (3 cases) or cases did not fit into any of the subgroups (8 cases).

Clinical characteristics of patients with SR and NSR phenotype at diagnosis

Children in the SR group presented at a a younger age with a higher initial leucocyte count and percentage of blasts and had more often a mediastinal mass compared to the NSR group. However these differences were only minor. Size of liver and spleen were almost identical in both groups. Sex, hemoglobin and platelets at presentation, CNS involvement as well as presence of lymph nodes did not differ considerably between the groups. There was no case of testicular involvement in either group.

Table 2. Patient and clinical characteristics of the SR and NSR subgroups

	SR	NSR
Age	7.05 years	8.92 years
Sex	24 M, 13 F	28 M, 11 F
Deaths	3 DR, - CR	14 DR, 2 CR
Leucocyte count/µl	168.027	125.182
Platelet count/µl	80300	97400
Pecentage Blasts	65.1%	55.4%
Hemoglobin	12.2	11.1
Liver size in cm	3.75	3.87
Spleen size in cm	4.43.	4.43
Presence of a thymic mass	21/37 cases	16/39 cases
CNS involvement	2/37 cases	3/39 cases
Lymph nodes present	28/37 cases	28/39 cases
Testicular involvement	0 cases	0 cases

DR=disease-related CR=complication-related

Clinical Outcome

Outcome was observed over 11.56 years in the SR and 11.62 years in the NSR group. In the SR group 16 patients were treated with the COALL-85, 6 patients with the COALL-89 and 15 patients with the COALL-92 protocoll. Deaths were due to disease in the 3 cases of the SR group with 2 deaths occuring in the COALL-92 and 1 in the COALL-85 protocoll. In the NSR group there were 14 deaths due to disease and 2 due to complications with 10 deaths occuring in 24 patients treated with the COALL-85, 2/3 in the COALL-89 and 4/12 in the COALL-92 protocoll. Figure 1 gives the standard Kaplan-Meier analysis for SR (n=37) vs. NSR (n=39):

Event-free survival in the NSR subgroups PRS (0.64, SE=0.14, n=12), POS (0.58, SE=0.14, n=13) and My+/B+ (0.57, SE=0.13, n=14) was not significantly different from each other. Events in the SR group were 3 relapses. In the NSR group 15 events occurred and consisted of deaths by complication, 1 NR and 12 relapses. One patient in the SR and 3 patients in the NSR group were lost to follow-up.

Discussion

Physiological Phenotype of T-Lineage Cells During Differentiation as the Basis for a Novel Classification

Originating from lymphoid stem cells in BM T precursor cells migrate to the thymic subcapsular region. These cells are called pro-T cells/subcapsular thymocytes and have a CD7+ CD3- CD1- CD4-(+) CD8- phenotype. We refer to these cells as pre-selection (PRS) phenotype. From the thymic subcapsular region thymocytes move to the thymic cortex. The cortex contains 80-85% of all thymocytes. Here most of the processes resulting in selection take place. Cortical thymocytes have unique phenotypic and biological properties. Biologically cortical thymocytes first undergo massive proliferation and then apoptosis with 98% of the thymocytes generated per day dying [6]. Phenotypically a hallmark for cortical thymocytes is the CD4+CD8+ double-positive (DP) phenotype, which is absent in PB or BM or any other lymphoid organ. Evidence for such a CD4 and CD8 DP phenotype on T-ALL blasts was called selection-related (SR). Similarly CD1a on lymphocytes is never expressed in peripheral blood or lymphoid organs under normal circumstances. Intrathymically CD1a is almost exclusively expressed on cortical thymocytes and a role for CD1 in T-cell selection has recently been postulated [2]. CD1 expression of cortical thymocytes preceeds expression of CD3. In contrast to previous studies we therefore already consider >10% cells of this phenotype (CD1 and DP) in peripheral blood or bone marrow as significant for selection related leukemic blasts. In addition surface expression of CD3 begins during selection [2] and is first observed on cortical thymocytes. Thus cases with CD3 expression were inclu-

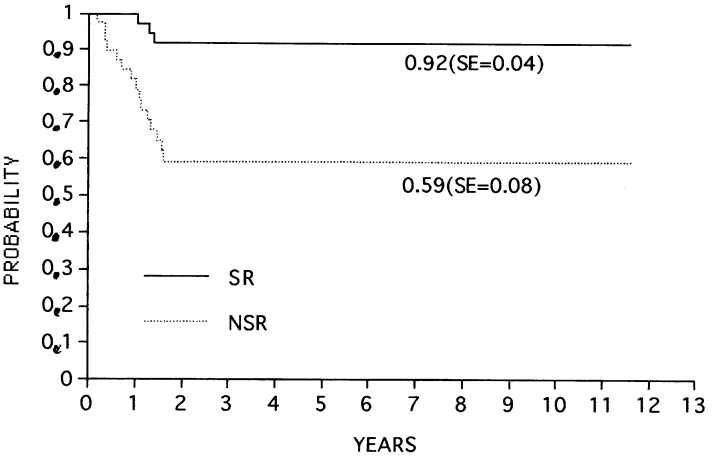

Fig. 1. Standard Kaplan-Meier analysis. The event free survival (EFS) for the immunological subgroup selection-related phenotype (SR) was 0.92 (SE=0.04) with 34 CCR in 37 patients. Survival in the non-selection-related phenotype (NSR) group was 0.59 (SE=0.08, with 24 CCR in 39 patients). Logrank comparison for EFS between subgroups showed a highly significant differences for SR vs NSR ($p = 0.001845$).

ded into the SR phenotype if there was coexpression of CD1 or if there was evidence for DP blasts. They were, however, referred to as POS, if they expressed >15% CD3 and were CD1 negative. This phenotype is found on cells that have left the thymic cortex and moved to the thymic medulla. Under physiological circumstances these medullary thymocytes/peripheral CD3+ cells express either CD4 or CD8. With the exception of minor subpopulations of monocytes (CD4) and NK cells (CD8) CD4 and CD8 are always expressed concomitantly with CD3. A condition (%CD4 / %CD8 = >6 or < 0.16) was introduced into criterion II to exclude the possibility of cases with NK (CD3-CD8+)/monocyte (CD3-CD4+) transformation. Unselected T precursor cells with the pre-selection phenotype and mature selected T-cells with post-selection phenotype are fundamentally different from cortical thymoctes and were summarized under the non-selection-related phenotype (NSR). It is a common observation that some leukemic blasts coexpress markers of other lineages. These T-ALL cases were referred to as My+/B+ phenotype and also accounted for as NSR.

Rationale for a Novel Classification and its Differences to Former Classifications

The allocation of T-ALL subgroups based on the classification defined by Reinherz more than 15 years ago showed limited prognostic significance [7]. More recently Campana et al. have investigated TCR expression in 40 T-ALL cases [8] in the age group from 6-65 years. Classification by TCR was found to be highly specific for T-ALL and obviously the process of rearrangement and surface expression of the T-cell-receptor most accurately reflects T-cell selection. However there was no detailed clinical and prognostic information on the different subgroups. Still there is ample evidence that there are subgroups of T-ALL cases with a different prognosis [9–13]. We modified criteria from former classifications: Differential expression threshold for a marker to be positive, evidence for CD4+CD8+ (DP) blasts and introduction of a separate subgroup with aberrant marker expression. Clearly our SR phenotype is related to the intermediate or common or midthymocyte phenotype, which constituted 20–43% of T-ALL cases in former classifications [7, 9, 10] and was already connected with a favourable, but not significantly better prognosis [10]. The above changes have the following impact on classification and outcome.

Threshold for CD1 and CD3 Expression

In the classification used thus far more than 20% [8, 11], 25% [12] or 30% [9, 10] expression of any given marker were required for a marker to be positive. We lowered this threshold to 10% for CD1a expression (SR) and >15% CD3 (POS) as these markers are both very lineage specific and helpful in determination of maturational stage. It is clear to us that by lowering the CD3 threshold we increase the likelyhood of including normal CD3+ cells that might be present in a heterogenous blast population. However false positive expression of CD3 in our classification will not affect false allocation to the NSR group as long as the majority of blasts express CD1 and/or are DP. In our classification low CD1 (10-30%) or CD3 (15-30%) expression was found in 8 SR and 3 POS (NSR) cases. Many of these cases would have been classified either as mature or as early in former classifications that used thresholds of >30% staining for a marker to be positive.

Evidence for DP Blasts

In univariate analysis CD4 and CD8 as single parameters confer a better prognosis in T-ALL [13]. Rather than looking at single CD4 or CD8 expression we correlated the sum of percentages of CD4 and CD8 to the percentage of CD3 cells We are aware of the fact that evidence for DP blasts is only indirect due to the lack of double-staining for DP blasts in the routine immunophenotyping of T-ALL. In 5 cases of our SR group either CD4, or CD8 or both were below the 30% threshold. In 11 SR cases there was evidence for DP blasts when at the same time CD1 was negative or not available. In the former classifications these CD1-CD4+(-)CD8+(-)CD3- cases were not included or classified as early thymocyte.

Aberrant Marker Expression

It is thought that leukemogenesis is occuring at distinct differentiational stages and that leukemic clones phenotypically reflect a distinct maturational stage (frozen leukemogenesis, [14]). Our classification into SR, PRS and POS is based on this concept. We incorporated the fact, that in a number of T-ALL cases there is expression of markers (e.g., myeloid, B-cell) not associated with T-cell lineage. We call these cases T-ALL with My+/B+ phenotype. The definition of this phenotype is difficult and our thresholds for B-cell/myeloid markers are arbitrary. In the case of B-cell marker expression we cannot entirely rule out the possibility of `contamination` with normal B-cells as double staining for both T- and B-cell markers was not performed. It seems unlikely, however, because in most cases the percentages of staining with T and B markers added up to >100% of the blast population making coexpression more likely than contamination. By our classification we find a percentage of cases with My+/B+ phenotype (14/87= 16%) which is less than that cited in the literature (24% [15], 16% [16] for myeloid coexpression only). In a considerable number of SR cases (8/37) there was no staining for myeloid markers. It can therefore not be excluded that My+ cases were missed. The prognostic significance of myeloid coexpression in T-ALL remains controversial (15). In our group a My+/B+phenotype appears to confer a poorer prognosis, but more data on prospectively studied My+/B+ T-ALL cases are needed to confirm this observation.

Patient Data

Clinically one would expect that due to cell kinetics of cortical thymocytes and their location in the thymus children with SR T-ALL should present with a high leucocyte count and a mediastinal mass. Compared to the NSR group we find only a slight elevation of leukocyte numbers and almost the same number of mediastinal masses. One has to consider the possibility that blasts (in particular PRS) might retain the ability to home to the thymus resulting in a similar number of medistinal tumors in the SR group. Further it is conceivable–due to the possible biology of the SR blasts–that SR cases might be detected earlier in the disease course than the NSR counterparts resulting in similar leucocyte numbers at diagnosis.

Why Should a Selection-Related Phenotype Confer a Better Prognosis than the NSR?

It seems possible that a majority of T-ALL cases derive from this SR population either due to a defective control of proliferation or a defective process of apoptosis. It is conceivable that leukemic cells deriving from actively dividing thymocytes might be more susceptible to chemotherapy. Clearly cortical thymocytes are extremely sensitive to glucocorticoids [6]. In the BFM-86 trial a T-ALL subset with excellent prognosis (comparable to the SR-phenotype) was defined by early response to prednisone [11]. Furthermore cortical thymocytes express high levels of FAS [17]. Induction of FAS-L has recently been shown to be a mechanism (autocrine suizide) by which doxorubicin and MTX induce FAS-mediated apoptosis in T-leukemia cell lines in vitro at concentrations that matched doxorubicin/MTX patient levels measured in vivo [18]. It will be interesting to correlate the SR phenotype with FAS expression. Alternative, Fas-independent pathways of apoptosis are currently discovered, that might also play a role for the improved response of SR T-ALL to chemotherapy or might on the other hand explain why in some cases there is no sufficient response of the leukemia to chemotherapy. Apoptosis induced by glucocorticoids or FAS/FAS-L mediated signals via chemotherapy might be more easily achieved in a cortical thymocyte-derived cell than in a clone derived from a precursor T-cell or a more mature, physiologically long-lived T-cell. To our knowledge CD1 has not been tested in univariate analysis yet. In an adult series on T-ALL CD1+T-ALL showed a significantly better response to induction therapy, however not improved overall survival [19]. In our data a CD1+ phenotype clearly seems to confer a favourable prognosis. Whether this is due to CD1 expression on thymocytes undergoing selection or

whether the molecule CD1 itself has a beneficial function is at present unclear. Phenotypically our pre-selection phenotype group (PRS) is related to the phenotypes defined as pro/pre/stem-cell T-ALL. It has already been shown that in children [10, 19] as well as adults [20, 21] pro/pre/stem-cell T-ALL is associated with a poorer prognosis compared to more mature T-ALL. Our data on the PRS phenotype is consistent with these observations. The post-selection phenotype group was found to have a poor prognosis. This is not surprising as membrane CD3 expression is known to confer a poorer prognosis [12]. It is possible that more SR cases would have relapsed if treated with a less intense treatment protocoll. Using more intensive chemotherapy recent data of the Childerns Cancer Group [22] and our CO-ALL treatment protocoll indicate an overall outcome for T-ALL that is better than in former studies on T-ALL (reviewed in [23]) that have tried to correlate T-ALL subgroups with prognosis. So improved overall outcome itself might have facilitated the recognition of different prognoses between NSR/SR groups.

Outlook

In order to validate the results of this retrospective analysis the classification should be applied to newly diagnosed T-ALL cases and studied prospectively.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Acute Leukemias with Hybrid Features in Children and Adolescents from the Kiev Region (Ukraine)

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Abstract. From Apr. 94 to Sept. 96, 41 patients (11 months – 18 years) with acute leukemias (Al) were diagnosed in Kiev Regional Hospital using immunocytochemical [29] and/or cytogenetic [23] methods. Two patients showed "hybrid" immunophenotype: the first presented B-ALL with expression of the two myeloid markers $CD14^+$ and $CD15^+$. The second had phenotype $CD14^+$, $Cd33^+$, $CD34^+$, $CD5^+$, $CD38^+$, $CD45RA^+$, and cytogenetic analysis showed the presence of 3 clones: t [2; 11] (q33; q13) / t [2; 11] (q33; q13), del [16] (q22) / ∽ 4n; the patient suffered very early relapse. Another patient, initially diagnosed as ALL with phenotype $HLA\text{-}DR^+$, $CD1O^+$, $CD7^+$, $CD15^+$, had del [20] (q1; q13) which persisted also in CCR. Another two children showed discrepancy between the morphocytochemically established diagnosis and the result of immunophenotyping: in a case of M5a $HLA\text{-}DR^+$, $cyCD7^+$, $CD15^+$, in an ALL-case $HLA\text{-}DR^+$, $CD45RA^+$, $CD15^+$. ALL-specific cytogenetic abnormalities were registered in 2 cases of AML: t(11;14) (p13;q11) in M5a and del (6) (q27) in M5b. AML-spezific aberrations were seen in 4 cases of ALL: del (16) (q22); del [2] (p23); t [15, 17] (q22; q11), t [6; 11] (q27; q23). Among 6 cases with atypical karyotype 5 had cytogenetic marker of induced disease (∽3n, ∽4n, 6q-/∽4n, (17), and 4 patients were resistant to therapy or suffered early relapses. Thus, the incidence of "hybrid" features among 41 non-selected AL-patients was 31% (13 cases). This unusual frequency in patients from the Kiev Region could be a result of the Chernobyl catastrophe.

Introduction

According to data of different therapeutical studies the incidence of hybrid leukemias are about 10% of total cases of acute leukemias [1, 2, 3]. It is supposed that the initial malignant transformation in these cases takes place in the hematopoietic stem cell [4]. Comparatively bad results in treatment patients with hybrid leukemias have been reported by some authors [2, 5]. Attempts to develop special chemotherapeutical protocols for leukemias with hybrid features have not yet improved the survival of patients, therefore they need allogeneic bone marrow transplantation in first remission.

In response to their standard chemotherapy, hybrid leukemias are similar to induced or secondary leukemias. It may be that their development is also caused by exogenous leukemogenous factors including ionizing radiation [6].

At present no conventional classification of hybrid leukemias exists because the criteria of "hybrid" nature have not yet been standardized [7].

The aim of this study was to evaluate:

1. The incidence of "hybrid" features in immunophenotype and karyotype in blast

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cells of children and adolescents with acute leukemias from the Kiev region
2. Possible prognostic significance of these features.

Material and Methods

Patients

Forty one non-selected patients with average age 6.9 years (range 11 months – 18 years; 17 female, 24 male) with acute leukemias diagnosed at the pediatric oncohematologic department in Kiev regional hospital from April 1994 to October 1996 were included in the study.

The diagnosis of ALL and AML variants was based on the morphologic and cytochemical criteria according to FAB classification [8,9].

Patients with diagnosed non-B-cell-ALL were treated according to protocols ALL-GPLU-93, -95 (modifications of protocols ALL-BFM-90 and -95); children with B-cell-ALL received chemotherapy according to a modified NHL-BFM-90 protocol; patients with diagnosed AML were treated by a modified protocol AML-BFM-93. Patients with proven hybrid features of blast bells received initial chemotherapy in accordance to dominative immunophenotypical and/or cytogenetic signs.

Immunophenotyping

Bone marrow cells were separated on a Ficoll-hypaque gradient. Cell surface antigens were detected by standard indirect immunofluorescence and/or immunocytochemical methods using the representative panel of monoclonal antibodies for the clusters of differentiation (CD1a; CD2; CD3; CD4; CD5; CD7; CD8; CD9; CD10; CD13; CD14; CD15; CD19; CD20; CD22; CD24; CD32; CD33; CD34; CD38; CD45RA; CD45RO; CD61; CDw65; CD71; HLA-DR; GLY-A; TDT) defined by the VIth International Workshop of human leukocyte differentiation antigens (Kobe, Nov. 1996). Leukemic cells were also tested for surface (sIg) and cytoplasmic immunoglobulin (cyIg). Immunocytochemical labelling was performed by immunoperoxidase (PAP technique) and/or immunoalcaline phosphatase (APAAP technique).

Cytogenetic Investigation

Chromosome analysis was carried out on unstimulated bone marrow cells which were incubated in RPMI 1640 and 20% FCS for 24 h culture. The cell suspension was brought to hypotonicity with KC1 (20 min) and fixed in methanol-acetic acid (3:1). Then the suspension was washed four to five times. The cells were pipetted onto cold wet slides to spread metaphases. G-banding was done after a tripsin pretreatment (1–3 s) 7 days later. The subsequent karyotyping followed the recommendations of ISCN (1991).

Results

Among 41 patients, 30 (73%) were initially diagnosed as ALL and 11 (27%) as AML.

In ALL, 9 children (30%) had hybrid features such as aberrant expression of surface markers and/or typical for AML karyotypic abnormalities. Aberrant immunophenotype was found in 6 patients with ALL: coexpression of one myeloid antigen in 3 children (cases 2, 4 and 5); two or more myeloid antigens in 3 (cases 1, 3, 8). In one of these cases, initial lymphoid phenotype with typical for AML del (16) (q34) was replaced by myeloid phenotype at the time of very early relapse (6 months after achieving remission). In general, combinations of karyotypic abnormalities, typical for AML, with aberrant expression of myeloid antigens were registered in 3 cases (1,2 and 3). In case number 1 very early relapse (after 6 months remission) was also seen. A patient with ALL/L1 (case 2), co-expression CD15 and del(20) in karyotype was transfered from ALL-treatment to AML-protocol and is stable in CCR (> 1.5 years).

Two children with B-cell ALL also had blasts with hybrid features: one patient (case 8) revealed coexpression of CD14 and CD15; the second (case 9) had two translocations in the same cells, t [8, 14] and t [15, 17]. In both cases, blasts had typical L3 morphology but

did not express sIg. Both patients have been in stable remission for > 1.5 years.

In cases 4, 5, 6 and 7, patients are in stable remission from 1 to 2 years after standard ALL-therapy.

In AML 4 patients had hybrid features: two revealed coexpression of lymphoid antigens (bases 10 and 11) and two were typical for ALL cytogenetic abnormalities (cases 12 and 13). These hybrid features in cases 11 and 12 were seen only in relapse.

One child with AML/M1 (case 11) was resistant to standard induction course and, in relapse after 6 months of remission, leukemic cells had coexpression of 4 lymphoid antigens.

In case 12, during the 4 week after induction, the patient had persistent enlargement of tonsilla, hepar and spleen; after 2 months he achieved combined relapse (bone marrow + CNS + lymphnodes + skin) and cytogenetic investigation revealed typical T-ALL t [11; 14].

One patient (case 13) died before protocol treatment and the last one (case 10) is in stable remission (> 1.5 years).

In general, aberrant immunophenotype was revealed in 8 patients from 29 immunophenotyped bases (27.6%). A discrepancy between type of leukemia and variant of karyotypic abnormaly was seen in 8 children from 23 who underwent cytogenetic investigation (34.8%) Seven patients from 11 with hybrid features and cytogenetic abnormalies also had karyotypic markers of induced leukemia (Table 1, 2).

Thus, hybrid characteristics were registered in 13 children (31.7%) among 41 patients with acute leukemias. Eight from 13 (61.5%) are in CCR from 1 to 2 years; 4 had early relapse (3 of them were partially resistant to induction therapy); 1 died before intensive treatment.

Case 9

The patient, a 4-year-old girl, was admitted to the hospital in June, 1995 due to fever, hepatosplenomegaly, generalized lymphadenopathy. In peripheral blood severe anemia (hb 3,2 g/l), thrombocytopenia

Table 1. Cases of acute leukemias with hybrid features (FAB-ALL)

Patients FAB-AL	Immunophenotype	Karyotype
Case 1 ALL L1	$CD34^+, CD33^+, CD14^+, CD5^+, CD38^+, CD45RA^+$	46; XY, t(2; 11) (q33; q13)/ 46, XY, t (2; 11) (q33; q13), del (16) (q22) / ∽4n
Case 2 ALL L1	$HLA\text{-}DR^+, CD10^+, CD7^+, CD15^+$ Not made	Initial: 46, XY, del (20) (q11; q13) In remission: 46, XY, del (20) (q11; q13)/ 92, XXYY
Case 3 ALL L1/L2	Initial: $HLA\text{-}DR^+, CD19^+$ In relapse: $CD34^+, CD33^+, CD13^+, CD15^+, CD4^+$	46, XY / 46, XY, del (16) (q22) / ∽4n Not made
Case 4 ALL L2	$HLA\text{-}DR^+, CD45RA^+, CD15^+$	46, XX/∽4n
Case 5 ALL L1	$CD34^+, CD33^+, CD38^+, CD45RA^+, CD10^+$	Not made
Case 6 ALL L1	Not made	46, XY, 46, XY, del (2) (p23)
Case 7 ALL L1	$HLA\text{-}DR^+, CD19^+, CD10^+, CD3^+, CD40^+$	46, XY, t(6; 11) (q27; q23)/~4n
Case 8 ALL L3	$HLA\text{-}DR^+, CD45^+, CD10^+, CD14^+, CD15^+, CD19^+$, sIg	Not made
Case 9 ALL L3	$HLA\text{-}DR^+, CD19^+, CD10^+$, sIg	46, XX, t (8; 14) (q24; q32), t (15; 17) (q22; q11)

Table 2. Cases of acute leukemias with hybrid features (FAB-AML)

Patients FAB-AL	Immunophenotype	Karyotype
Case 10 AML M5a	HLA-DR$^+$, cyCD7$^+$, CD15$^+$	26-32-42
Case 11 AML M1 Auer +	Initial: not made In relapse: HlA-DR$^+$, CD64$^+$, TR$^+$, CD7$^+$, CD38$^+$, CD19$^+$, CD56$^+$	46, XX, t (9, 22) (q34; q11) Not made
Case 12 AML M5a	HLA-DR$^+$, CD33$^+$, CD15$^+$ Not made	Initial: 46, XY, i (17q)/⌣3n/46, XY, 13? In relapse: 46, XY/⌣3n/51, XY, +5, +8, +12, +16, +17, +18, +20, +21 −7, −9, −13, t (11, 14) (p13; q11)
Case 13 AML M5b	Not made	45, XY, del(6) (q27)/⌣4n

(29 000/mkl), leukocytes 12 400/mkl and 6% blasts were registered. Bone marrow puncture showed nearly 100% blasts with L3 morphology and positive PAS reaction. The conclusion after immunocytochemical analysis was "common-ALL" (immunophenotype see Table 1). Cytogenetic investigation revealed the presence of two abnormalities at the same clone: t (8; 14) and t (15; 17).

The treatment was begun according to the NHL-BFM-90 protocol (arm B-NHL/ALL) with very good clinical response to prephase with cyclophosphamide, vincristin and prednison. Then the child was taken out of the department by the parents, who refused further protocol treatment. From July, 1995 the patient received no intensive chemotherapy, only short courses of low-dose ARA-C, but no signs of leukemic proliferation have been seen to the present.

In our opinion, the specific features of this case are:

- discrepancy in data from cytomorphochemistry, immunophenotyping and cytogenetic investigation;
- lack of dic despite presence of t (15; 17);
- favourable clinical course, which could be connected with the presence of t (15; 17) as a factor for promoting cell differentiation.

Discussion

The incidence of hybrid features in our group of patients with acute leukemias is high in comparison with reports from other centers (1, 2, 3): aberrant coexpression of surface markers and/or presence of cytogenetic abnormalities not corresponding to the cell line were seen in 31.7%.

The hybrid type of acute leukemia was not always combined with bad response to standard chemotherapy and/or early relapse, so the data on the prognostic significance of these features in our series of cases are controversial, despite the fact that some patients with proven hybrid character of disease were partially/completely resistant to therapy or suffered very early relapses, as was also reported by different authors [1, 2, 4, 5, 10].

Patients with hybrid leukemias in 7 of 11 cases had additional karyotypic signs of induced disease; remembering the fact that they came from regions of Chernobyl catastrophe, we could suppose the role of inducive factors (especially ionizing radiation) in the development of hybrid acute leukemias, but, of course, these data are preliminary because of the small number of cases in our study.

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Experimental Approaches and Novel Therapies
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CD95(Fas/APO-1) Antigen Expression on the Cells of Myelodysplastic Syndromes, Acute Myelogenous Leukemia, and Chronic Myelogenous Leukemia Patients

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Abstract. CD95(Fas/APO-1) antigen is a member of TNF/NGF receptor superfamily that includes the TNF-RI and TNF-RII tumour necrosis factor receptors, nerve growth factor receptor, the T-cell activation marker CD27, the Hodgkin disease-associated antigen CD30, B-cell surface antigen CD40 and some other mammalian and viral homologues. In this study we analyzed the expression of CD95(Fas/APO-1) antigen on bone marrow cells of myelodysplastic syndrome patients (MDS), on peripheral blast cells of acute myelogenous leukemia patients (AML) and bone marrow and peripheral blood cells of chronic myelogenous leukemia (CML) patients in chronic phase (CP) and blastic crises (BC). Antigen expression was studied by indirect immunofluorescence assay using flow cytometry (FACScan, Becton Dickinson) and apoptosis was investigated using flow cytometric method of measurement of hypodiploid DNA, labeled with propidium iodide. CD95(Fas/APO-1) antigen was found on 38.1±19.2% bone marrow cells of 8 of 19 (36.8%) MDS patients, on 45.5±22.8% of blast cells in 6 (45%) of 15 AML patients, Fas/APO-1 antigen was totally absent in CML chronic stage; and its expression was found in 34% (12 of 35) of our patients with CML BC on peripheral blood blasts and in 45% (5 of 11) on bone marrow blasts. According to our data at studing the expression of CD95(Fas/APO-1) antigen in MDS patients we suggested that the absence of CD95(Fas/APO-1) antigen on bone marrow cells of MDS patients is an unfavourable sign and CD95- MDS patients represent a risk group for both short overall survival and progression to acute leukemia. In AML patients we also noted a negative influence of CD95(Fas/APO-1) absence on overall survival, though this conclusion needs to be confirmed. In the case of CML BC patients remissions were achieved in the lymphoid and mixed variants. No remissions were obtained in $CD13^+$ and $CD95^+$ groups regardless of CD10 expression. Fas antigen has no prognostic value in CML BC.

Introduction

CD95(Fas/APO-1) antigen was independently discovered by two laboratories which used monoclonal cytolytic antibodies for a variety of human cell lines. They observed a very distinct pattern of cell death [1,2]. CD95(Fas/APO-1) antigen is a member of a superfamily of proteins that includes the nerve growth factor receptor, the tumour necrosis factor receptors I and II (TNF-RI and TNF-RII), the OX-40 antigen and the CD27, CD30 and CD40 glycoproteins [3]. CD95(Fas/APO-1) antigen can mediate apoptosis of various human cells, T- and B-lymphoblastoid cells and diploid fibroblasts.

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With the help of monoclonal antibodies (Mabs) the CD95(Fas/APO-1) antigen was found in a wide variety of normal and tumour cells, but the information concerning the expression of CD95(Fas/APO-1) antigen on leukemic cells is very contradictory. It has been reported in the materials of the 5-th International Workshop and Conference on Differentiation Antigens of Human Leukocytes that neoplastic cells from most patients with acute lymphoblastic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia and multiple myeloma appear to lack CD95(Fas/APO-1) antigen [3]. However, approximately 30% of cases of acute myelogenous leukemia and other leukemias, examined by Munker et.al., expressed the Fas antigen [4]. Min et.al. suggested that low expression of Fas antigen in bone marrow blasts was associated with a low complete remission rate after induction chemotherapy [5].

In this report we analyzed the expression of CD95(Fas/APO-1) antigen on bone marrow cells in patients with myelodysplastic syndromes (MDS), on peripheral blast cells in acute myelogenous leukemia (AML) and on the cells in patients with chronic myelogenous leukemia(CML) in chronic phase and blastic crises. We also assessed prognostic significance of the CD95(Fas/APO-1) antigen expression.

Materials and Methods

Monoclonal Antibodies. For evaluation of CD95(Fas/APO-1) antigen expression and immunophenotyping of blast cells, the following monoclonal antibodies (Mabs) against cell surface antigens were used (Table 1).

Anti-CD95 mouse Mabs IPO-4 were characterized at Fifth International Workshop and Conference on Human Leukocyte Differentiation Antigens. Mabs against human leukocyte differentiation antigens gave the opportunity to characterize various differentiation stages of blast cells from haematopoietic precursors to the mature cells. Each marker was regarded as diagnostically valuable if it was detected on $\geq$ 20% of the cells.

Table 1. Monoclonal antibodies used in the study.

Antibody	Antigen	Main distribution
IPO-4[a], ICO-160[b]	CD95	Broad expression
ICO-115[b]	CD34	Progenitor cells
Anti-CD33[c]	CD33	Myelomonocytic progenitors
My32	CD13	Myelomonocytic progenitors
My1[c]	CD15	Myeloid cells
Anti-CD14[c]	CD14	Monocytes
ICO-124[b]	CD10	Lymphoblastic cells
ICO-1[b]	HLA-Dr	HLA-Dr
ICO-150[b]	CD24	B cells, granulocytes

[a] Mabs kindly provided by Dr D.Gluzman.
[b] Mabs kindly provided by Medbiospectr, Moscow, Russia.
[c] Mabs kindly provided by Dr T.Trishman, Johns Hopkins Oncology Center, Baltimore, USA.

Immunofluorescence Assays. Antigen expression was studied by indirect surface immunofluorescence assay on freshly isolated cells using flow cytometry (FACScan, Becton Dickinson). Blood and bone marrow samples were isolated by sedimentation in 1% gelatin solution for 45 min at 37 °C and leukocytes were resuspended in 0.5 ml of lysing solution (Becton Dickinson) for total lysis of erythrocytes, then washed three times in phosphate-buffered saline (PBS) and resuspended in PBS.

Indirect Immunofluorescence Technique. With 20 µl of monoclonal antibodies (Mabs) 5×10^5 cells were incubated at room temperature for 30 min. After one washing with PBS they were stained with 20 µl FITC-conjugated sheep antiserum against mouse immunoglobulins for 30 min at 4 °C. After that the cells were washed twice and resuspended in PBS with 1% formalin and 0.1% sodium aside to be tested for reactivity by indirect immunofluorescence.

Two-colour Immunofluorescence. Analysis. Two-colour flow cytometry analysis was performed using a combination of Mabs IPO-4 and isotype-specific anti-mouse IgM FITC-conjugate (Sigma) and then the second Mabs directly conjugated with phycoerythrin(PE) (CD34PE).

Measurement of Apoptosis. Apoptosis was assessed with the cytofluorometric analysis of hypodiploid DNA-labeled with propidium iodide (PI); 2×10^5 cells were washed in PBS and resuspended in 70% ethanol for 1 h, after that they were centrifuged at 500 rpm for 7 min. Then the cells were resuspended in 1.0 ml of hypotonic fluorochrome solution (5 μg/ml PI, 0.1% sodium citrate, 0,1% Triton X100). After gentle mixing, cells were incubated at room temperature for 15 min. in the darkness. The PI fluorescence of stained DNA was measured with FACScan without further washings. FACS settings were designed to identify a distinct hypodiploid DNA region below the diploid G0/G1 DNA peak.

Cell Treatment. To evaluate apoptosis, the cells were incubated in standard medium RPMI-1640 (Flow Laboratories), containing 2 mmol/l glutamine, 1 mg/ml gentamycin, 20% fetal calf serum in the presence of 10 μg/ml Mabs IPO-4 or ICO-160 for 24 h at 37 °C in 5% CO_2.

The expression of CD95(Fas/APO-1) antigen was studied on bone marrow cells of 19 MDS patients, peripheral blood and bone marrow blast cells of 15 acute myeloid leukemia (AML) patients, blast cells and granulocytes of 68 patients with chronic myeloid leukemia (CML) - 24 in chronic, 9 in accelerated phase and 35 in blastic crises (BC).

CD95(Fas/APO-1) antigen was revealed on bone marrow cells of 8 out of 19 (36.8%) MDS patients; the percentage of antigen-positive cells was 38.1±19.2%. CD34 antigen, a stem cell marker, was expressed on 18% of antigen-positive cells in 7 of 19 patients studied. We found no statistically significant correlation between CD95(Fas/APO-1) and CD34 or between CD95(Fas/APO-1) and CD11b antigens.

According to presence or absence of CD95(Fas/APO-1) antigen on bone marrow cells, all MDS patients can be divided into 2 groups. In the $CD95^+$ group the antigen was revealed on 38.1±19.2% cells. According to FAB classification, this group included 5 patients with refractory anemia (RA), 1 patient with RA with excess of blasts (RAEB) and 2 patients with RAEB in transformation (RAEBt). The $CD95^-$ group consisted of 3 RA patients, 3 RAEB, 3 RAEBt and 2 patients with chronic myelomonocytic leukemia (CMML). In all except one $CD95^-$ patients at the beginning of the disease or during the follow up time the percentage of blast cells increased up to 10–25%, and 2 patients developed frank AML. At present 87% patients of the $CD95^+$ group are alive (survival duration from 6 to 49 months, median not reached), while only 18% patients of the $CD95^-$ group are alive (median survival 16.7 months). No correlation between CD95(Fas/APO-1) antigen expression and age, FAB variant and CD34 expression was found. Therefore, we can speculate that the absence of CD95(Fas/APO-1) antigen on bone marrow cells of MDS patients is an unfavorable sign and $CD95^-$ MDS patients represent a risk group for both short overall survival and progression to acute leukemia.

The study of immunological phenotype of AML blast cells included the CD34, CD33, CD13, CD11b, CD15, CD24, HLA-DR antigens expression; in all cases we also studied the expression of CD95(Fas/APO-1) antigen. CD95(Fas/APO-1) antigen was revealed on 45.5±22.8% of cells in 6 (45%) of 15 AML patients. We have revealed a significant correlation between CD34 and CD95(Fas/APO-1) both on peripheral blood ($r=0.54$, $p<0.05$) and bone marrow blast cells ($r=0.85$, $p<0.01$). We have also found a correlation between the expression of CD95(Fas/APO-1) and CD33 ($r=0.58$) and CD95 (Fas/APO-1) and CD13 ($r=0.55$), but it was not significant because of the small number of patients under observation. There were no correlation between CD95(Fas/APO-1) and CD15 and between CD95(Fas/APO-1) and CD11b antigens representing further stages of myeloid cells differentiation. We have also tried to divide all AML patients in accordance with CD95(Fas/APO-1) antigen expression. The $CD95^+$ group included 6 patients, among them 3 patients with FAB variant M2, 1 with M1, 1 with M3 and 1 with M4. In this group complete remission was achieved in 3 (50%) of 6 cases and at present the median remission duration is not achieved (life duration 2, 7, and 26 months); 50% of patients in this group are alive, median survival 3 months. The $CD95^-$ group consisted of 9 patients, among them 3 patients with M2, 3 with M1 and 3 with M4. Complete remission

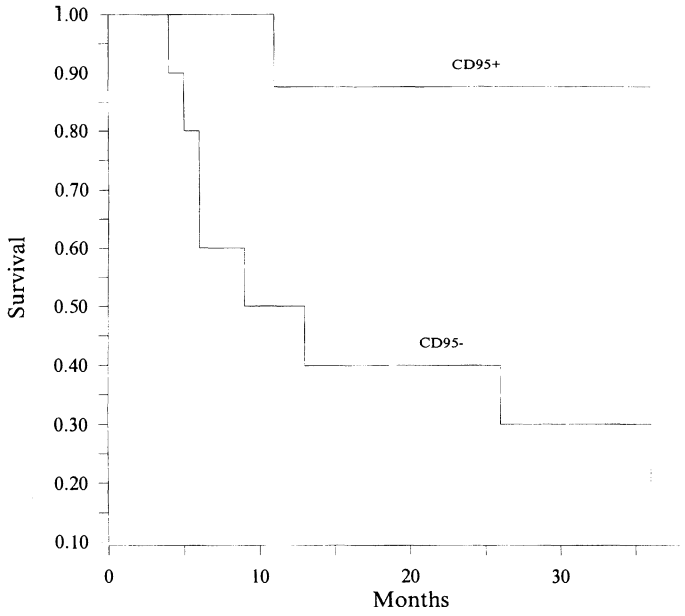


Fig. 1. Overall survival of MDS patients, according to CD95(Fas/APO-1) antigen expression

was achieved in 6 (66.7%) of 9 patients, median remission duration 11 months. At present 88% patients have died (median survival 7 months). Comparing $CD95^+$ and $CD95^-$ groups, one can see that percentage of complete remissions is approximately equal, but in the $CD95^+$ group 3 patients remain in complete remission, while in $CD95^-$ only 1 and the others have relapsed. Thus we can suggest a negative influence of CD95(Fas/APO-1) absence on overall survival, though this conclusion needs to be confirmed.

Fas/APO-1 antigen was totally absent in CML chronic stage; its expression was found in 34% (12 of 35) of our patients with CML BC on peripheral blood blasts and in 56% (5 of 9) on peripheral blasts CML accelerated phase patients (Table 2).

In the present study we have found that Fas-antigen, expressed on the CML BC blast cells is functionally active, that is proved by results of apoptosis induction by Mabs ICO-160 and IPO-4 against CD95(Fas/APO-1) antigen using flow cytometric method of measurement of hypodiploid DNA, labeled by propidium iodide. The percentage of apoptosis, induced by Mabs in CD95(Fas/APO-1)-positive, cultivated with Mabs (10 mg/ml) for 24 h, cells of 6 of 7 CML BC patients was 17-60% (Fig. 2).

There was a slight tendency to a decrease of this antigen expression after polychemotherapy (41% in "primary" vs. 31% in pretreated patients in peripheral blood and 50% vs. 40% respectively in bone marrow), but the difference was not significant ($p>0.05$).

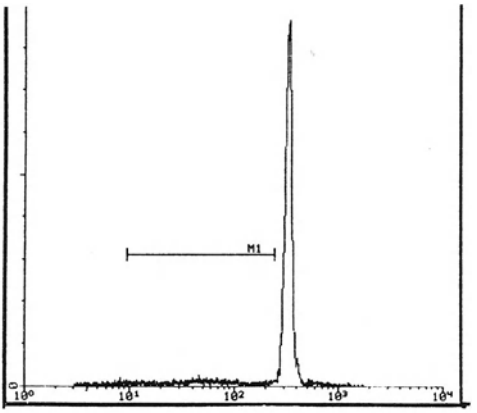
We also studied the associations of CD95(Fas/APO-1) antigen and other differentiation antigens and found a direct correlation between the percentage of $CD34^+$ and $CD95(Fas/APO-1)^+$ cells both in peripheral blood (r=0.44, p=0.05) and bone marrow (r=0.83, $p<0.05$) of newly diagnosed CML BC. With the use of the two-color immunofluorescence we have shown that these antigens are coexpressed on the part of cells (Fig. 3).

Table 2. Fas-antigen expression at different stages of myeloid cells maturation in chronic myelogenous leukemia

Cells	Fas-positive patients	Total number of patients	Frequency
Mature granulocytes[a]	0	24	0%
Blast cells[b]	5	9	56%
Blast cells[c]	12	35	34%

[a] in chronic phase
[b] in accelerated phase
[c] in blastic phase

Control



Apoptosis induction by anti-CD95 Mabs

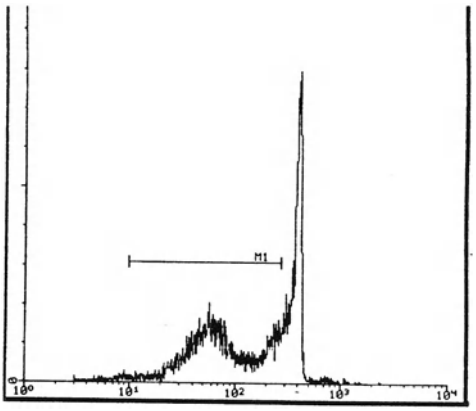


Fig. 2

More detailed search revealed a significant correlation rate (r=0.7, $p<0.05$) in peripheral blood of $CD10^-$ untreated patients. Besides CD34, CD95(Fas/APO-1)-antigen also correlated with CD13 and CD14 (r=0.48 and r=0.47, p<0.05), but not CD15 and CD11b. We have also found a strong positive correlation between CD95(Fas/APO-1) and CD10 in bone marrow (but not in peripheral blood) of "primary" patients (r=0.82, $p<0.05$). In patients, who have already received polychemotherapy, there was no significant correlation (r=-0.47, $p>0.05$).

Remissions in CML BC are infrequent. We have obtained only 5 (3 complete and 2 partial) remissions in our patients. All of them were achieved in the lymphoid and mixed variants. The phenotype of these patients was $CD10^+CD13^-Fas^-$. No remissions were obtained in $CD13^+$ and $CD95^+$ groups regardless of CD10 expression; CD34 expression had no influence on survival (4 vs. 3 months, $p>0.05$). In the mixed variant (i.e. positive for both CD10 or CD22 and one or more of the myeloid antigens), where 6 of 9 patients were CD95-positive, the remissions were obtained only in Fas-negative subgroup. Surprisingly, the median survival in the Fas^+ and Fas^- patients was nearly equal: 3.5 vs. 3 months in the whole group, and 8 vs. 9 months in the $CD10^+$ patients (all differences not significant). Only CD10-positivity had prognostic significance for the survival in CML BC (8 vs. 3 months, $p<0.05$). Interestingly, the $CD10^+$ group had also significantly shorter acceleration phase (0 vs. 3.5 months, $p<0.01$) (Table 3).

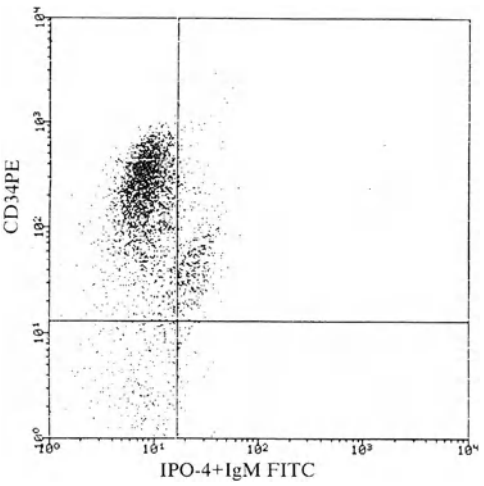


Fig. 3

Discussion

The study of the expression of CD95(Fas/APO-1) antigen, mediating apoptosis on early hemopoietic precursors has shown that this antigen is absent on the primitive $CD34^+$ stem cells , but is found on the $CD34^+$ stem cells, activated by interferon-gamma and/or tumour necrosis factor-alpha [6]. We have shown before that both CD34 and CD95 are expressed on the blast cells in the CML BC [7]. In the present

Table 3. Median survival and remission rate of CML BC patients, according to antigen expression.

Variant	Patients		Remissions	Median survival		
	Total	Followed-up	complete/partial	CP	AP	BC
CD10+	13	10	3/2	22.0	0.0[a]	8.0[a]
CD10-	18	16	0/0	27.0	3.5	3.0
CD34+	16	15	2/1	25.0	1.0	4.5
CD34-	14	12	1/0	12.0	1.0	3.0
Fas+	11	10	0/0	30.0	4.0	3.0
Fas-	20	19	3/2	21.0	0.5	6.0
CD13+	9	7	0/0	10.0[a]	0.5	3.0[b]
CD13-	19	16	2/1	24.5	3.5	5.0
CD10+Fas-	7	6	3/2	24.0	0.0	9.0[b]
CD10+Fas+	5	4	0/0	15.5	2.0	5.0

[a] $p<0.05$
[b] Median not reached

study we have shown that these antigens also are coexpressed on the myeloblasts from patients with AML. The frequency of expression CD95(Fas/APO-1) antigen were the same in newly diagnosed CML BC and AML, 41 and 45%, respectively. The frequency of CD34 antigen expression was in "primary" CML BC was 55% and 36% in AML. Double staining procedure has shown that both antigens coexpressed on the same cells. The expression of CD95(Fas/APO-1) antigen on the blast cells from patients with CML BC correlated also with the expression CD13 and CD14, but not CD33 antigens. In contrast, in AML the expression CD95(Fas/APO-1) antigen correlates with the expression of CD33 and CD13 antigens. There was no correlation between CD95(Fas/APO-1) and CD15 and between CD95(Fas/APO-1) and CD11b antigens representing further stages of myeloid cells differentiation. The analysis of Fas expression according to FAB-classification has shown that it was rarely seen in M1 variant, corresponding to more earlier stages of differentiation and was more frequently found in M2 variant. CD95(Fas/APO-1) antigen was not expressed on granulocytes of patients with CML in chronic stage of the disease. Therefore, CD95(Fas/APO-1) antigen is present only at early myeloid cells differentiation stage (late $CD34^+$ and $CD33^+$/$CD13^+$) and is absent at later stages. The CD95(Fas/APO-1) antigen on blastic cells from patients with CML BC and AML is functionally active. Mabs against CD95(Fas/APO-1) are inducing apoptosis in positive cases. So, it is possible that CD95(Fas/APO-1)-mediated apoptosis is the part of differentiation process on some discrete stages of differentiation of myeloid cells.

The CML BC is heterogeneous by immunophenotype of blast cells and is represented by cells of different lines and at different stages of differentiation - myeloid, monocytic, lymphoid, erythroid, megakaryocytic. We have previously described different immunological variants of CML BC - lymphoid (positive for 1 or more of lymphoid antigens (CD10, CD19, CD20, CD22) and negative for all of myeloid (CD33, CD13, CD14, CD15, CD11b)), myeloid (positive for 1 or more of myeloid antigens and negative for all of the lymphoid), mixed (positive for 1 or more of both lymphoid and myeloid antigens), primitive (expressing only CD34) and undifferentiated (expressing no differentiation antigens) [8]. The highest frequency of Fas-antigen expression was found in mixed variant, which probably represents the partially committed bone marrow precursors. In less differentiated variants (primitive and undifferentiated) Fas-positivity was less frequent (25% vs. 67%, $p>0.05$), as well as in more differentiated lymphoid and myeloid variants (28% vs. 67%, $p>0.05$). (Table 4). In patients with AML, which represents the next differentiation stage we have found that Fas antigen is expressed in 40% of cases (it correlates with published data).

Table 4. Fas-antigen expression in different immunologic variants of CML BC.

Variants	Number of patients	Fas-positive patients	Frequency (%)
Lymphoid	5	1	20
Myeloid	11	3	27
Mixed	9	6	67
Undifferentiated	4	0	0
Primitive	3	1	33
Total	32	11	34

We have also estimated the prognostic value of Fas-antigen expression. Our data about overall survival of $CD95^+$ MDS patients suggest that the presence of Fas antigen is a favourable prognostic sign for patients with MDS. The patients from $CD95^-$ group represent a risk group both for survival and AML transformation. In the AML patients we can also suggest an unfavourable influence of Fas-antigen absence on overall survival, though this hypothesis needs further confirmation, because the results of Munker et. al. [6] indicate that Fas positivity in patients with AML had no obvious clinical relevance. The same tendency of survival prolongation was described [9] in 27 ALL children, studied in 1988. Median survival in patients with Fas-positive blast cells was 96 months, while in others only 18 months. At present 91.2% of children in Fas-positive and only 16.2% in Fas-negative group are alive. In this group there was a direct correlation (r=0.56) between expression of Fas and CD10 antigens. Conversely, in CML BC group the survival does not depend upon Fas-antigen expression.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

The Premyelodysplastic State and the Secondary Hematologic Disorders

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Abstract. The secondary hematologic disorders consist of the progressive appearance of increasingly abnormal marrow cell populations with increasingly abnormal hemopoiesis culminating in the appearance of secondary acute myelogenous leukemia. At the biological level the secondary hematologic disorders result from the accumulation of molecular genetic lesions which confer a proliferative advantage on a stem cell and its progeny which in turn results in the successive overgrowth of increasingly abnormal monoclonal cell populations. We have identified 4 of 15 female patients cured of lymphoma by intensive cytotoxic therapy as having monoclonal hemopoiesis. Despite the presence of normal peripheral blood counts, the bone marrows of the 3 patients who were studied intensively manifested evidence of grossly abnormal hemopoiesis. We have named this state of normal blood counts in combination with monoclonal hemopoiesis in patients previously exposed to hemopoietic toxins "preMDS." This "preMDS" state either represents an earlier stage of the secondary hematologic disorders or represents a transient preclinical manifestation of myelodysplasia. Longitudinal patient follow up is needed to distinguish between these two possibilities.

Introduction

The secondary hematologic disorders (SHD), myelodysplasia and the acute myelogenous leukemia which evolves from MDS (sAML), are the end stages of a process which is initiated by a genotoxic insult to the marrow stem cell. The earliest clinically recognized state, MDS, is a complex state which may be characterized by cytopenias of one or more cell lineages, together with hyper, hypo, or normocellular bone marrow with morphologic evidence of differentiation abnormalities. Further, at the laboratory level MDS can be associated with no apparent or with complex cytogenetic abnormalities, and a variety of molecular genetic lesions, and with a reduced capacity of marrow cells to proliferate in vitro. The highly complex nature of MDS suggests that there should be one or more steps distal to the genotoxic insult but proximal to clinically apparent MDS during the evolution of SHD.

Detection of a "PreMDS" State

The Cardif group studied a cohort of "hematologically normal" individuals who had been cured of lymphoma by means of intensive cytotoxic therapy. This group reported the presence of ras and fms mutations in 13-16% of the patients studied [1, 2] and also that monoclonal hemopoiesis was present in as many as 20% of the patients [3]. It should be noted that the bone marrows of the patients who were studied apparently were not examined nor were the "normal" hematologic parameters described in the papers.

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We recently investigated a comparable group of 50 patients believed to be hematologically normal and cured of lymphoma one or more years previously. Using blot hybridization [4] we were unable to detect any ras of fms mutations in the peripheral blood cells of this group of patients. Among 20 female patients in this patient cohort 15 were found to be informative on the HUMARA clonality assay [5] and among these 15 monoclonality was found to be present in 4 patients (the patients own T cells served as the controls for these studies). Further investigation demonstrated that one of these patients had recurrent lymphoma in the marrow. This patient was not studied further. Two of the remaining three patients had received combined chemo/radiotherapy for their lymphoma at least 3 years prior to study and the third patient had received an autologous marrow transplant for recurrent lymphoma 3 years prior to study.

These studies strongly suggest that monoclonal hemopoiesis is common in patients cured of lymphoma by intensive cytotoxic therapy.

The PreMDS State

To the present time we have conducted a variety of studies of the three patients with monoclonal hemopoiesis. The peripheral blood counts of two of the three patients are completely normal including rbc indices and reticulocyte counts. The blood counts of the post-transplant patient are also normal save for a hemoglobin of 10 g. As described in Table 1 the bone marrows of all 3 patients were grossly abnormal. Two were hypocellular and one hypercellular with relative erythroid hyperplasia, morphologic abnormalities, and relative myeloid hypoplasia present in all 3 marrows. These changes were most evident in the marrow biopsies. While cytogenetic studies were normal in all three marrows and peripheral blood specimens, FISH analysis suggested the presence of a very small population of cells containing monosomy 7 (approximately 4% of the cells present) in two of the three marrows. These data are only suggestive since the size of the population, if present, is in the region of questionable reliability of the FISH methodology. FISH studies with the EGR probe failed to reveal any evidence of del 5q. Only time will tell if the populations which appear to be present will increase in size to the point that their presence can be confirmed.

In vitro cloning demonstrated good growth by marrow aspirate cells from all 3 patients but the number of BFUe and CFUgm is at the lower end of normal. Preliminary data suggest that there is an increase in telomerase activity in all 3 marrow cell populations. The cytokine transcript profile of the marrow aspirate cells is basically normal with the cells containing transcripts for IL1b, IL1 receptor antagonist, stem cell factor, and flt-3 ligand. It does appear, however, that the number of TNFa transcripts is increased in PreMDS marrow cells in compa-

Table 1. Summary of laboratory findings in 3 preMDS patients.

Pt #	CBC	Bone Marrow Characteristics	In Vitro Cloning	Cytokines	Telomerase	FISH
1	norm	Hypocell, erythroid hyperplasie with megalo or dysplastic Δ s; decr. myeloid ser;	norm	TNFa	?inc	?subpop of cells with momosomy 7; no evidence del 5q
2	norm	Hypercell; dysplastie normoblasis; decr late myeloid ser;	norm	TNFa	?inc	?subpop of cells with momosomy 7; no evidence del 5q
3	IIb–10	Hypocell; erythroid hyperplas; rare megalo Δ s; myeloid hypoplasis	norm	TNFa		N.D.

rison with the number present in normal marrow cells.

It is thus apparent that the bone marrows of these three patients is neither normal nor is hemopoiesis in these patients sufficiently abnormal to be classified as myelodysplastic. We propose to call the syndrome which is present "PreMDS" since it appears likely that these patients are at extremely high risk to develop MDS. The absence of detectable ras of fms mutations in the PreMDS marrows and their presence in many florid MDS and sAML marrows [6,7] suggests that these molecular lesions are not responsible for the early stages of the SHD. The same can be said for p53 mutations and for monosomy 7 should the latter be ultimately shown to be definitely present as the diseases evolve in the future.

The Development of Monoclonality and Genetic Instability

Figure 1a illustrates the theoretical stepwise evolution of the SHD. The development of monoclonal hemopoiesis represents the overgrowth of a normal polyclonal hemopoietic cell population by the progeny of a single stem cell. Similarly, the successive appearance of new and increasingly abnormal monoclonal cell populations must represent the successive appearance of cell populations which have a proliferative advantage over their predecessor cell populations (Fig. 1b). While most speculation regarding the basis for the development of other monoclonal states such as CML postulate disordered maturation and/or increased proliferative rates, a simpler mechanism can accomplish the same thing. On the basis of computer modeling we have found that an increase in the self-renewal probability of the marrow stem cell population from the normal value of $p = 0.5$ to $p = 0.56$ would result in monoclonal hemopoiesis [8]. A further increase beyond $p = 0.6$ in time would lead to stem cell (acute leukemia or blastic transformation of CML) overgrowth of the marrow.

Monoclonal hemopoiesis, or hemopoiesis resulting from the proliferation efforts of a single stem cell and its progeny, must place a substantial proliferative burden on the founding stem cell and its immediate progeny. Since the monoclonal hemopoiesis in SHD which occurs prior to the appearance of clinically apparent MDS in all likelihood persists for several years, it is our opinion that the proliferative stress exceeds that which a normal stem cell can maintain. We therefore postulate that in PreMDS the initial step which permits successful monoclonal hemopoiesis also involves in increase in telomerase activity, a change which would increase the proliferative potential of the abnormal stem cell. As noted above, our preliminary observations are compatible with this concept. We also postulate that if a population appears with a proliferative advantage but without a commensurate increase in telomerase activity, monoclonal hemopoiesis can be established but it would be of limited duration because the founding stem cell will eventually become "exhausted." Perhaps some of the myeloproliferative states which end in marrow fibrosis are the end result of such a discordance between proliferative advantage and proliferative potential.

Undoubtedly the appearance and evolution of the SHD represent the biological consequences of a progressive accumulation of genetic abnormalities. Given this accumulation it is likely that an element of genetic instability facilitates the continuous appearance of genetic abnormalities with the selection of those abnormalities which confer a

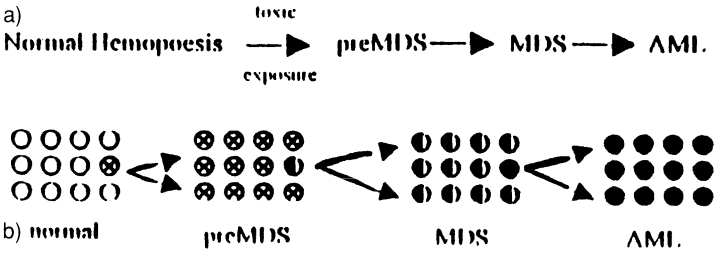

Fig. 1a, b. Stepwise evolution of the secondary hematologic disorders. **b.** Sequential evolution of progressively abnormal populations of cells resulting from the appearance of cells with a proliferative advantage over the populations in which they arise

proliferative advantage of the cell population. One obvious question relates to the origin of the genetic instability. It is possible that the initial genotoxic insult itself results in genetic instability with the lag time between the insult and the appearance of biologically detectable abnormalities being dependent upon the appearance of the genetic lesion [s] which confers the proliferative advantage. This scenario seems intuitively unlikely in the majority of cases although some MDS cases in which there is no history of toxic exposure may be initiated by the appearance of genetic instability.

One can speculate on three possible sources of the genetic instability present in the majority of SHD:

1. The proliferative burden associated with monoclonal cell populations might in and of itself lead to genetic instability
2. Abnormal cytokine stimulation might lead to functional inactivation of the p53 gene [9] since continuous cytokine stimulation might result in the wild type p53 protein continuously assuming a mutant configuration [10]
3. Some combination of the above. What ever the cause, evidence has been presented for genetic instability being present in patients with MDS [11] and in patients with secondary AML in which p53 mutations are present [12]. Hence it is clear that genetic instability contributes to the evolution of the SHD. The primary question is whether the instability appears during the PreMDS state or perhaps even earlier.

The Development of the Secondary Hematologic Disorders

It is generally believed that MDS results from a stem cell defect. While the monoclonality of MDS makes this undoubtedly the case, secondary phenomena such as abnormal cytokine production make significant contributions to the disease state [13]. In fact in many patients the clinical symptoms and the evolution of the disease are heavily influenced by the abnormal production of TNFa. Figure 2 summarizes observations that the abnormal production of TNFa has a dual effect resulting in marrow apoptosis which contributes to the cytopenias present in MDS patients and stimulating the proliferation of the abnormal clones [14]. The observation that the suppression of TNFa production can lead to the disappearance of cytogenetically marked MDS clones [15] demonstrates quite clearly that in many situations the inherent proliferative advantage of abnormal cell populations is dependent on cytokine stimulation for the potential proliferative advantage to become an actual advantage. The biological and clinical significance of these observations are readily apparent.

Is PreMDS the Earliest Abnormal State in the SHD?

The abnormalities detected in the marrows of patients with PreMDS lead to at least two possibilities:

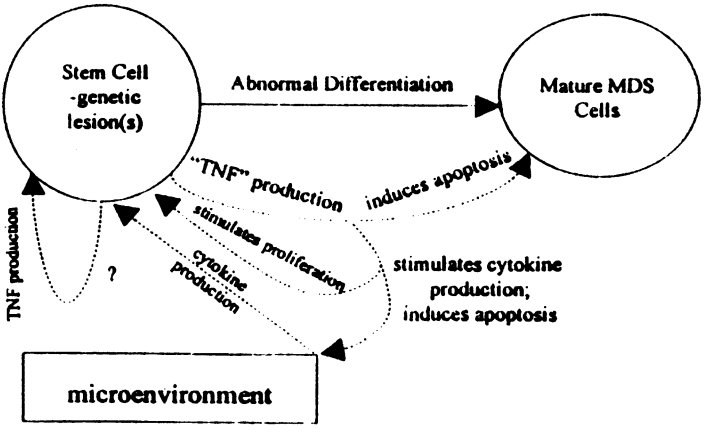

Fig. 2. Interaction between the abnormal stem cell and increase environmental levels in MDS patients resulting in the clinical features of MDS

1. There is a still earlier state of abnormal hemopoiesis in which monoclonal hemopoiesis is present but in which hemopoiesis is otherwise normal. If this were the case then one might expect that the appearance of the PreMDS state described above would be dependent upon the appearance of additional molecular genetic abnormalities or upon the effects of prolonged abnormal cytokine effects on the marrow. Whether either or both of these suppositions are valid is unknown;
2. That given the morphologic similarity between the marrows of the PreMDS patients and those of patients with clinically apparent MDS, the PreMDS state as described here is merely preclinical MDS. Implicit in this concept would be the fact that every patient with PreMDS would eventually evolve to clinically apparent MDS. While this may indeed be the case, the relatively normal proliferative capacity of the marrow cells in vitro suggests that additional abnormalities are needed for the appearance of florid clinical MDS. If this latter interpretation were correct, then not every PreMDS patient will evolve to clinically apparent MDS just as not every MDS evolves to sAML. Time will tell as to which of these possibilities is correct.

Conclusions

The presence of normal peripheral blood counts in patients who have been heavily treated with cytotoxic agents cannot be used a proof that hemopoiesis is normal. The detection of monoclonal hemopoiesis in 20% of patients cured of lymphoma is compatible with the estimates that as many as 10% of patients cured of lymphoma by cytotoxic therapy and as many as 20% of patients who receive autologous peripheral blood stem cell transplants as part of their therapy will develop a SHD [16,17]. The development of relatively nontoxic therapies for MDS, such as the combination of pentoxyfillin/ ciprofloxacin/dexamethasone [18] makes it important to identify patients with SHD before they become clinically apparent since it is likely that early intervention can prevent evolution to the more malignant forms of this disorder.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Physiological Events during Ara-C Toxicity Mapped Using Multiparametric Flow Cytometry

D.W. Hedley and E.A. McCulloch

Abstract. The events leading to ara-C-induced apoptosis have been investigated using flow cytometry and viable cell probes for: reactive oxygen generation, glutathione, intracellular calcium, surface membrane phosphatidylserine, and mitochondrial membrane potential. This approach defines a sequence of physiological changes during ara-C treatment that starts with low levels of reactive oxygen generation and an increase in glutathione. This is followed by the loss of glutathione and depolarization of the mitochondrial inner membrane. Cells then go through a phase of high reactive oxygen generation, probably due to uncoupling of oxidative phosphorylation. Phosphatidylserine exposure on the cell surface occurs synchronously with the loss of mitochondrial membrane potential. Later intracellular calcium regulation fails, and finally surface membrane integrity is lost. Drug resistance associated with increased bcl-2 expression is manifested as an interruption in this death sequence prior to the loss of cellular GSH. These experiments show that the mitochondrial permeability transition occurs during ara-C toxicity, and clarify its relationships with oxidative stress. Oxidative stress appears to be a biphasic phenomenon, with the permeability transition taking place between a depletion of reduced glutathione and a phase of high reactive oxygen production. Several molecular mechanisms have been identified that can influence apoptosis in model systems. We suggest that the definition of apoptosis in physiological terms provides a framework into which their complex interactions might be fitted.

Introduction

Previous work investigating the mechanism of ara-C resistance has shown that distal mechanisms occurring downstream of initial DNA damage play a major role determining whether cells undergo apoptosis, or are able to repair the damage [1,2]. The action of bcl-2 and its family members can protect cells by reducing the probability of cell death [3–6]. This appears to be relevant to drug resistance in AML patients [3, 6]. Indirect evidence was obtained suggesting that oxidative stress might be relevant to ara-C toxicity. For example, cells could be protected by the antioxidant N-acetylcysteine, and leukemic blasts transfected with the bcl-2 gene were resistant to a direct oxidative stress using hydrogen peroxide, as well as being resistant to ara-C and daunorubicin [2]. These findings are consistent with earlier reports suggesting that the action of bcl-2 might involve some form of antioxidant mechanism, or protect against increases in intracellular calcium [7–9]. In order to study this effect directly, we developed complex flow cytometry methods that are capable of mapping the alterations in cellular physiology that take place during drug-induced cytotoxicity.

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Mechanisms of Cell Injury

Oxidative Stress

Recently there has been considerable interest in the idea that oxidative stress is an important mediator of programmed cell death [7,8,10,11]. The internal redox environment of cells is normally maintained in a reducing state by antioxidants; particularly the glutathione redox cycle. Oxidizing species such as reactive oxygen intermediates (ROI) can be generated by normal metabolic processes, by redox cycling xenobiotics, and in response to some types of cell injury. Oxidative stress can damage critical macromolecules such as lipids, proteins, and nucleic acids; cells are therefore dependent on antioxidant defences for survival [11-12].

Mitochondrial Permeability Transition

An alternative viewpoint is that oxidative stress occurring during apoptosis is not directly responsible for cell death, but is rather the consequence of more relevant mediators of cell injury, particularly the mitochondrial permeability transition [13]. The permeability transition is an active process that involves the opening of pore complexes in the mitochondrial inner membrane, resulting in the loss of the mitochondrial membrane potential and the cessation of mitochondrial ATP generation. Since respiration is feedback inhibited by cellular ATP levels, the permeability transition causes increased electron transport through the respiratory chain [13]. This in turn may result in the increased generation of reactive oxygen intermediates, since these are byproducts of mitochondrial respiration [14,15]. The mitochondrial permeability transition appears to be a critical event in apoptosis, regardless of whether this is induced physiologically or in response to cell injury [16-18]. Failure of normal ionized calcium regulation would be expected to ensue, due to inactivation of ATP-dependent calcium pumps or to oxidative damage to membrane sites of calcium regulation. Activation of calcium-dependent endonucleases would then lead to internucleosomal cleavage of DNA [19]. Kroemer and colleagues have recently proposed an alternative mechanism by which the permeability transition might mediate cell death. This mechanism involves the release from the mitochondria of a death effector molecule that is capable of producing the nuclear features of apoptosis [16,20].

Experimental Procedures

Most of the experiments were done using the well-characterized OCI/AML-2 line, established in our own institution, or with variants that had been transfected with the bcl-2 gene [4]. This is a growth factor independent line that grows in suspension culture with a doubling time of approximately 24 h. Cells were treated with ara-C by continuous exposure for various times; usually at a concentration of 7 μM that produces approximately 90% loss of clonogenic survival after 24 h. In addition, we investigated the changes occurring during ara-C toxicity in fresh patient samples maintained in short term culture. End points for ara-C toxicity were clonogenic survival, and flow cytometric assays of live cell function. The flow cytometry was done using a multilaser cell sorter capable of measuring up to five fluorescence parameters simultaneously, in addition to forward and orthogonal light scatter.

Results

Oxidative Stress During Ara-C Toxicity and the Effects of Bcl-2

Measurement of Reactive Oxygen Generation

The rate of generation of reactive oxygen intermediates in live cells was measured using either dichlorofluorescin diacetate or dihydrorhodamine 123 (DHR-123). These compounds are oxidized by ROI to the brightly fluorescent dichlorofluorescein and rhodamine 123, and retained inside the cell.

Fig. 1 shows frequency distribution histograms of log ROI generation following 24 h treatment with 7 uM ara-C compared to untreated control cells. There was a modest increase in ROI generation in the bulk of the ara-C-treated cells, with a much larger increase seen in a subpopulation. Similar results were obtained using the two fluores-

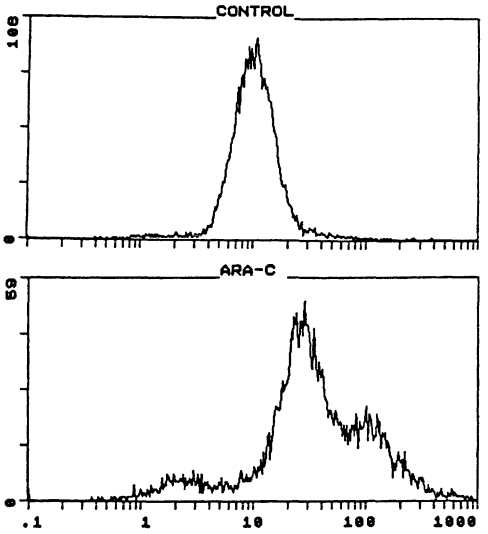

Fig. 1. Effects of ara-C on ROI (log scale). *Top panel* is control

cent ROI probes. This is unlikely to be due to activation of the respiratory burst oxidase system, which is functional in phagocytes and in some myeloid leukemia cell lines, since OCI/AML-2 cells were unable to mount a respiratory burst in response to treatment with phorbol ester.

Effects of Ara-C on Cellular Glutathione

Because ara-C toxicity was associated with increased ROI generation, it was anticipated that this would result in exhaustion of cellular glutathione (GSH), the main cellular antioxidant. A double labeling method was developed, using monobromo-bimane to measure cellular GSH [21]. As shown in Fig. 2, the earliest change was an increase in glutathione content that coincided with a modest increase in ROI generation, seen as a shift upwards and to the right in the position of control cells shown on the left. The most likely explanation for this is that cells are initially capable of countering oxidative stress by increasing the synthesis of GSH. A similar protective response has been reported following acute oxidative stress caused by hyperthermia or transient hypoxia [22, 23]. The population of cells with high levels of ROI, seen in Figure 1, was markedly depleted in GSH, indicating that they can no longer defend against ROI and have shifted their cellular redox balance to an oxidizing state. There was then a progressive decline in the intensity of staining with monobromobimane and DHR-123, and eventually loss of surface membrane integrity as shown by inability of the cells to exclude propidium iodide. As described below, during these later phases the cells lose the ability to regulate their intracellular calcium ion concentration.

Time Course of Ara-C Toxicity

The heterogeneity seen in the two parameter plot of ROI generation versus GSH content following 24 h ara-C treatment suggests that a sequence of events is occurring; this sequence is asynchronous because of the cell cycle phase specificity of ara-C toxicity. In order to confirm this we did time course experiments in which OCI/AML-2 cells were treated continuously with 7 mM ara-C, and sampled at 6-hourly intervals for flow cytometry analysis and clonogenic survival. Results for the flow cytometry measurements are shown in Fig. 3. The expected sequence of events is clearly seen, with the earliest

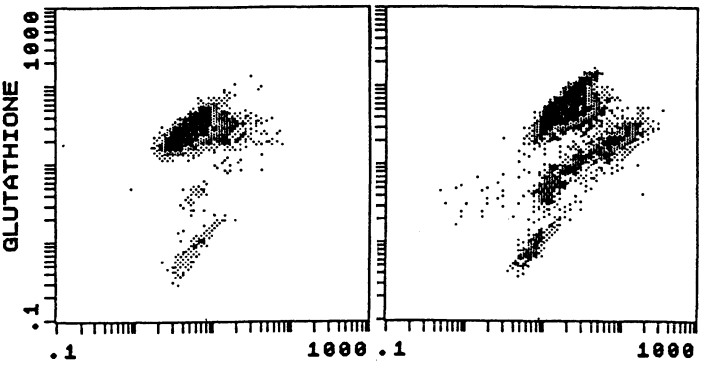

Fig. 2. Correlations between ROI generation and GSH content following ara-C (log/log scale)

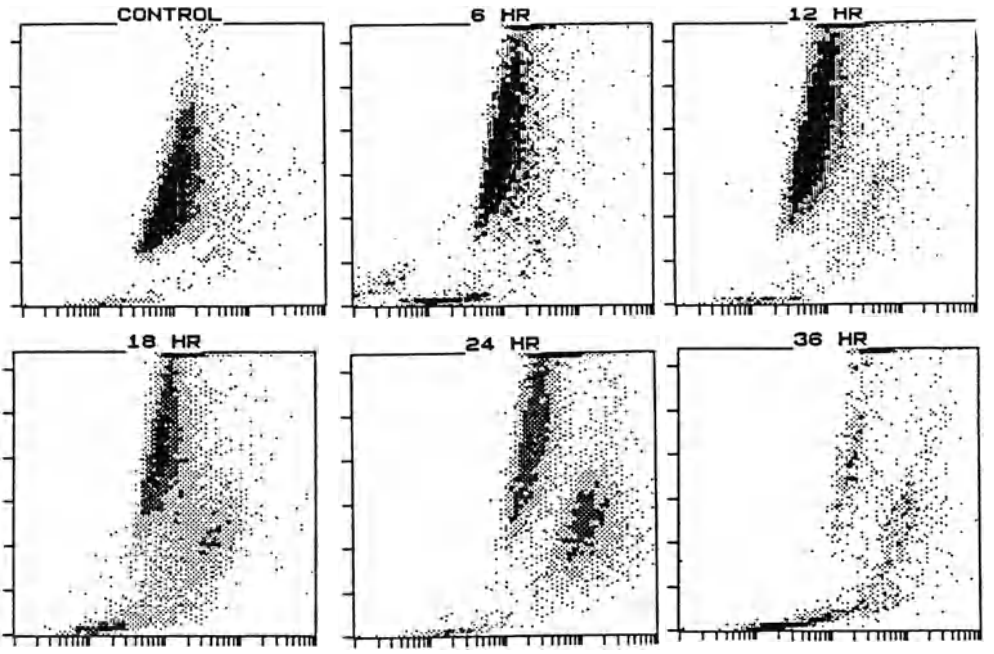

Fig. 3. Time course of ara-C exposure, with times indicated on each panel. Glutathione content (vertical axis) is shown on a linear scale, which exaggerates the effects of ara-C. Gated to exclude cells that have lost surface membrane integrity

changes being an increase in ROI and GSH seen at 6-hours. At the later time points cells accumulated in the low GSH/low ROI state characterized by increased intracellular calcium and loss of surface membrane integrity.

The sequence of events following ara-C treatment is consistent with the idea that DNA damage by this agent in some way results in the increased generation of ROI. The mechanism for this is unknown, although similar results have been observed following cell stress or exposure to a wide range of DNA damaging agents [22–24]. Initially the cells are able to counter this oxidative stress by increasing the level of reduced glutathione, but later they undergo a transition to a state in which GSH is greatly depleted, and ROI generation is very high. The failure of antioxidant defences has serious metabolic consequences, including lipid peroxidation damage to membranes, and disulphide bonding of critical intracellular proteins.

We considered the possibility that oxidative stress is a significant mediator of ara-C toxicity, and that the ability to maintain glutathione levels is associated with ara-C resistance. There is an extensive literature showing that increases in glutathione activity can cause resistance to alkylating agents or platinum compounds, but GSH is not normally considered to be relevant to antimetabolite resistance (25). We were therefore interested to know whether similar changes to those seen in OCI/AML-2 cells occurred during ara-C toxicity in AML patient samples.

Effects of Ara-C on AML Patient Samples ex Vivo

Peripheral blasts obtained from three AML patients were grown in short term culture, using medium conditioned by 5637 bladder carcinoma cells. Ara-C sensitivity was determined using a clonogenic survival assay, and the cells were then treated with an IC90 concentration of ara-C for 48 h and examined by flow cytometry using the dual ROI/GSH labeling method.

As shown in Figure 4, there was a striking heterogeneity in the effects of equitoxic doses of ara-C. The blasts from Patient 1 showed a large increase in ROI in a subpopulation that then lost GSH and finally entered

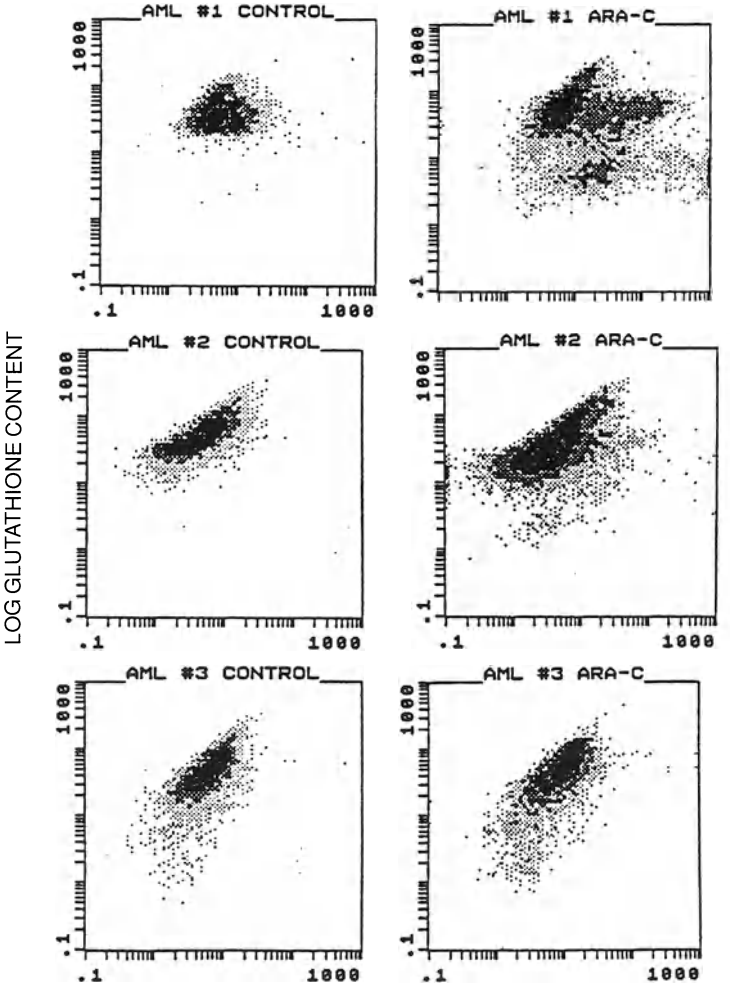

Fig. 4. Log/log plots of GSH vs. ROI in ara-C treated AML patient samples

a phase where there was an approximately 100-fold increase in ROI generation compared to untreated control cells. In contrast, ara-C toxicity produced minimal changes in the sample from Patient 3, while the pattern obtained from Patient 2 was midway between these two extremes. These preliminary results using fresh patient samples indicate that oxidative stress can occur during ara-C toxicity.

Effects of Bcl-2 Over Expression

Over expression of bcl-2 protein has been reported to protect against oxidative stress, and is associated with ara-C resistance in AML patients. The OCI/AML-2 variant 2bcl-2, that has been transfected with the bcl-2 gene, is resistant to ara-C [4]. We therefore used the flow cytometry method in order to identify the point in the "death sequence" at which bcl-2 over expression enhances cell survival. Results of a typical experiment are shown in Figure 5. Following 24 h ara-C treatment a small increase in ROI and GSH levels was observed, similar to the early changes seen in the parental line. However, cells transfected with bcl-2 were able to maintain this state, and did not enter the next stage of the sequence that is characterized by depletion of GSH and very high levels of ROI production (indicated by an amorphous region drawn in top right panel). Results from these experiments are described in a recent paper [26].

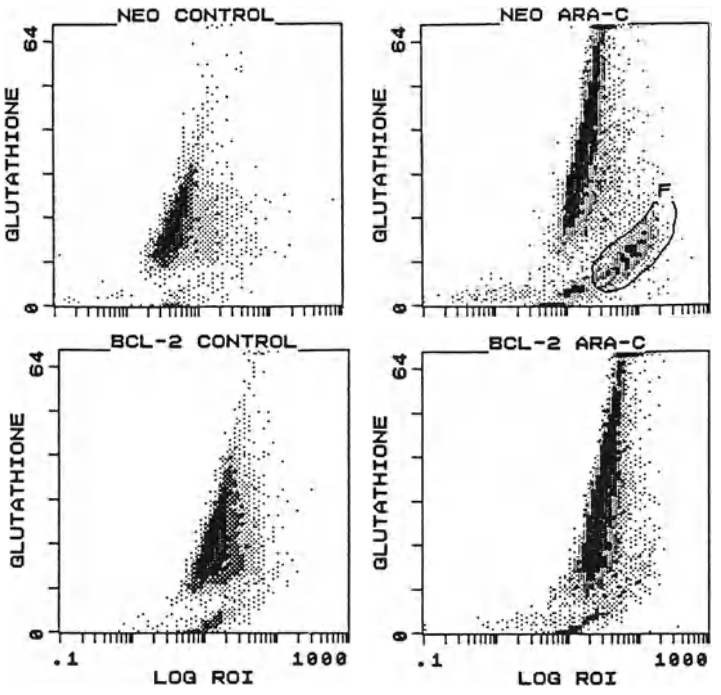

Fig. 5. Effects of ara-C on GSH (linear) and ROI (log) in control *(top panels)* and bcl-2 transfected

Relationships Between Oxidative Stress and the Mitochondrial Permeability Transition

Background

Our results investigating the role of bcl-2 in the context of ara-C-induced oxidative stress are consistent with the idea the bcl-2 in some way has an antioxidant role. Recently an alternative viewpoint has been proposed, in which bcl-2 acts to inhibit the mitochondrial permeability transition [20]. The permeability transition is an active process, in which complex pores, or "megachannels", open in the inner mitochondrial membrane. The detailed structure of the pore complex is unknown, but it probably includes the ADP/ATP translocase and a voltage dependent anion transporter [17, 20]. The bcl-2 protein also appears to associate with the pore complex, and may act to stabilize this in the closed position [20]. Opening of the pore complex is an early and apparently critical event during apoptosis, regardless of the initiating factor [13]. Because the mitochondrial membrane potential is lost, the permeability transition uncouples oxidative phosphorylation. Respiratory chain activity then increases due to loss of feedback inhibition. Since superoxide is generated as a byproduct of the respiratory chain, the effect of bcl-2 transfection on ROI generation might mediated via inhibition of the permeability transition. We were therefore interested to know if the permeability transition occurs during ara-C toxicity, and to determine whether this precedes the onset of high ROI generation.

Experimental Setup

A direct relationship between the permeability transition and the intensity of labeling with mitochondrial membrane potential sensitive dyes can be shown using confocal microscopy. The permeability transition can therefore be demonstrated by flow cytometry, using fluorescent probes that are sensitive to the mitochondrial membrane potential. The most frequently used probe is the cyanine dye DiOC6(3). Because this has similar spectral properties to the ROI probes, we modified the method by substituting the analogue DiIC6(5) which can be excited using a 633 nm helium neon laser. The com-

Table 1

	Probe	Staining conditions	min	λ Excitation/emission (nm)
ROI generation	DHR-123	1 μM	30	488 / 525
GSHutathione	Monobromobimane	40 μM	5	325 / 450
Mitochondrial membrane potential	DiIC6(5)	40 μM	30	633 / 675
Surface membrane integrity	Propidium Iodide	5 μg/ml	5	488 / 640

bined labeling method used for these experiments consisted of (Table 1):

The flow cytometer setup consisted of a 488 nm argon laser as the reference beam, and a 325nm HeCd laser and a 633 nm HeNe laser running colinearly with a 40 ms time delay. Because all four parameters were collected simultaneously, it was possible to examine their correlates directly at the single cell level.

Results

Following ara-C treatment a population of cells appeared with reduced DiIC6(5) fluorescence labeling, indicating loss of mitochondrial membrane potential (Fig. 6).

This finding is similar to previously reported results [27], and indicates that the permeability transition occurs during ara-C toxicity. Figure 7a is a correlated two parameter plot of mitochondrial membrane potential versus ROI generation. High ROI generation is seen in cells that have undergone the permeability transition, indicates that this occurs later than the permeability transition. This is consistent with the uncoupling of oxidative phosphorylation causing increased ROI production in the respiratory chain. Our results using ara-C treated leukemic blasts are similar to those obtained Kroemer et al. using a wide range of cell types undergoing apoptosis [16, 27, 28]. However, when the results for cellular GSH content are plotted against mitochondrial membrane potential, it can be seen that the permeability transition occurs only in cells from the lower range of the distribution for GSH values (Fig. 7). Experiments in which correlated measurements of mitochondrial membrane potential, GSH, and ROI were made at 2-h intervals show that this overall pattern is maintained during the course of ara-C treatment, with the percentage of cells showing low GSH and low mitochondrial membrane potential increasing over time. This indicates that the permeability transition is preceded by a decrease in cellular GSH content.

Glutathione is needed to maintain the protein sulphydryls inside cells in their normally reduced state. It has been previously shown that disulphide bonding in the mitochondrial matrix space can trigger the permeability transition [29, 30]. Mitochondria contain a pool of GSH that protects against reactive oxygen intermediates generated by the respiratory chain [31, 32]. The decrease in whole cell GSH content that is observed prior to the loss of mitochondrial membrane potential might reflect a more significant depletion of mitochondrial GSH during ara-C toxicity. The interrelations between oxidative stress and the permeability transition could therefore be explained on the basis that oxidative stress is a biphasic phe-

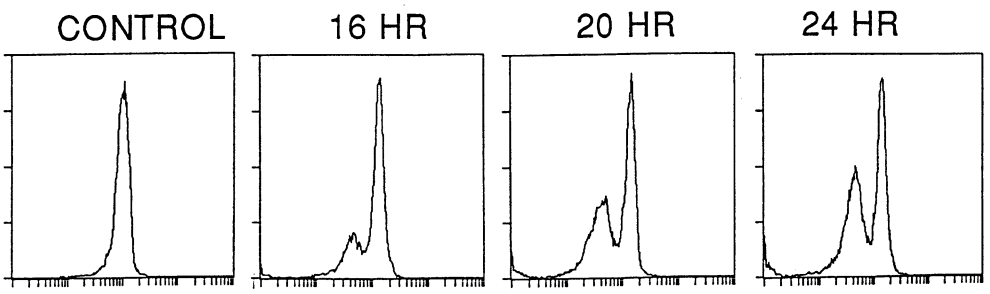

Fig. 6. Appearance of cells with low DiIC6(5) fluorescence over time

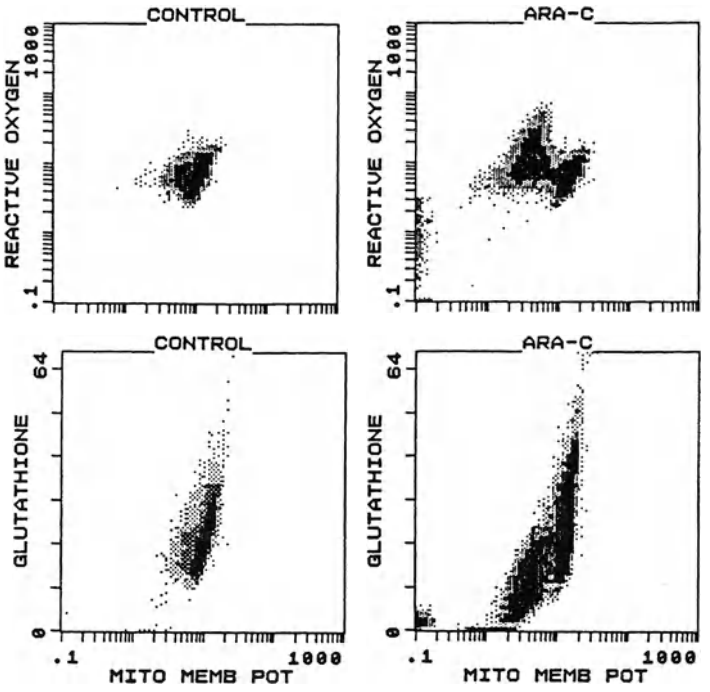

Fig. 7. Relationships between mitochondrial membrane potential *(horizontal axis)* and: ROI generation *(upper panels)* and GSH content *(lower panels)*

nomenon, with the permeability transition resulting from loss of reduced GSH, and preceding high levels of ROI generation due to the uncoupling of oxidative phosphorylation.

The Physiology of Apoptosis Mapped by Multilaser Flow Cytometry

Introduction

Increases in ionized calcium occur during apoptosis, and can produce classical internucleosomal fragmentation of DNA through the activation of calcium-dependent endonucleases [19]. More recently, however, alternative death effectors have been proposed that may not depend on calcium regulation. Of particular relevance are the ICE-like cysteine protease CPP-32, which can be activated during ara-C toxicity [33], and a less well characterized Apoptosis Initiating Factor that appears to be released by mitochondria following the permeability transition [16,20].

Recently there has been interest in alterations in the surface membrane lipid composition that take place during early apoptosis. These changes involve the translocation to the cell surface of phosphatidylserine, an aminophospholipid that is normally confined to the inner leaflet of the lipid bilayer [34, 35]. This is an active process that probably involves the combined action of a specific aminophospholipid translocase and a non-specific lipid scramblase [36]. It allows cells that are undergoing apoptosis to be recognized by phagocytes, and it appears to be a ubiquitous event that takes place in a wide variety of cell types in response to a wide range of stimuli. Therefore we developed flow cytometry methods to investigate whether phosphatidylserine exposure or increases in calcium occur during ara-C toxicity, and to establish their temporal sequence relative to oxidative stress and the mitochondrial permeability transition.

Methods

Phosphatidylserine exposure was detected using a fluorescein conjugate of the natural ligand annexin V [34], and ionized calcium concentration was measured using the fluorescent calcium indicator. A combined flow cytometry method was developed as follows (Table 2):

Table 2

	Probe	Staining conditions	min	l Excitation/emission (nm)
Ionized calcium	Indo-1	3 μM	30	325/405:525
Phosphatidylserine	Annexin V-FITC	5 μl/ml	5	488/525
Mitochondrial membrane potential	DiIC6(5)	40 nM	30	633/675
Surface membrane integrity	Propidium iodide	5 μg/ml	5	488/640

The instrument setup was similar to that used to measure oxidative stress and mitochondrial membrane potential. Intracellular ionized calcium [Ca^{2+}]i was measured by taking the ratio of the calcium bound (405 nm) and calcium free (525 nm) emissions of indo-1, using electronic ratio circuitry. Therefore this method required collection of five fluorescence signals, in addition to forward and orthogonal light scatter. It was designed to investigate events taking place downstream of the mitochondrial permeability transition, with the mitochondrial membrane potential measurements allowing results to be linked to those obtained using the oxidative stress method.

Results

A population of annexin V positive cells appeared during the course of ara-C treatment, indicating loss of the normal asymmetric distribution of phosphatidylserine in the surface membrane. Fig. 8 shows two parameter dot plots of annexin V labeling versus mitochondrial membrane potential.

There was a striking correlation between the two membrane events, so that almost all cells showed either loss of mitochondrial membrane potential and phosphatidylserine expression, or else they had energized mitochondria and were annexin V negative. This strong correlation was seen at all time points examined, from 8-26 hr of drug treatment, and shows that these apparently independent membrane events occur simultaneously. Furthermore, this must take place rapidly, since very few cells were observed in transitional stages.

Measurement of [Ca^{2+}]i showed that large increases occurred during ara-C toxicity. Fig. 9 shows correlated dot plots of surface membrane integrity, as shown by propidium iodide exclusion, versus intracellular calcium. A sequence of events can be seen in which cells initially exclude propidium iodide and have low [Ca^{2+}]i. They then move to a state of high [Ca^{2+}]i, following which they lose the ability to exclude propidium iodide. Beyond this point [Ca^{2+}]i can no longer be monitored reliably, since the indo-1 begins to leak out of the cells.

Combined measurement of [Ca^{2+}]i and mitochondrial membrane potential shows that all of the high calcium cells have depolarized mitochondria, whereas a proportion of the low MMP cells still have low [Ca^{2+}]i values (Figure 10).

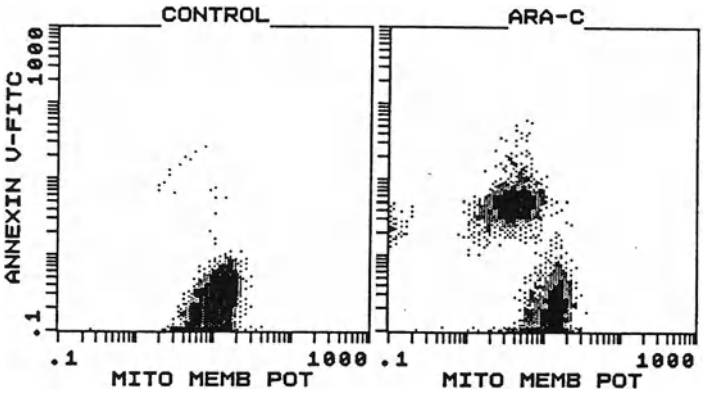

Fig. 8. Relations between phosphatidylserine expression and mitochondrial membrane potential following 24h ara-C treatment.

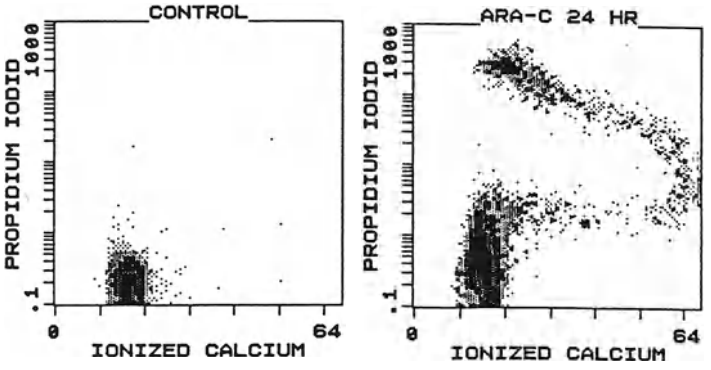

Fig. 9. Increase in intracellular calcium during ara-C treatment, followed by loss of membrane integrity (and leakage of indo-1)

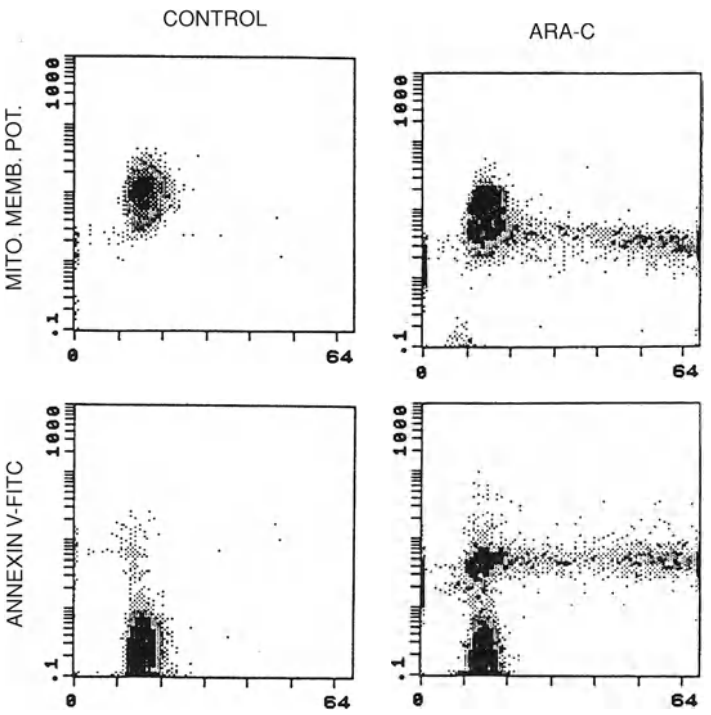

Fig. 10. Relations between intracellular calcium *(horizontal axis)* and: mitochondrial membrane potential *(upper panels)* and phosphatidylserine expression *(lower panels)*

This result clearly demonstrates that the loss of calcium regulation occurs at a later time than the permeability transition. Because phosphatidylserine exposure is strongly linked to mitochondrial membrane potential, the correlated plot of annexin V labeling versus ionized calcium resembles the inverse of the MMP versus $[Ca^{2+}]i$ plot (Fig. 10).

Discussion

Programmed cell death is a complex phenomenon that can be influenced by a large number of gene products, including the many members of the bcl-2 gene family, p53, stress activated protein kinase, and various death effectors such as the ICE-like proteases [5]. It is difficult to fit all of these molecules into a single unifying scheme, and it is possible that individually their relevance depends on which particular model of apoptosis is under study. In the work described here we have taken the alternative approach of describing cell death in terms of alterations in cellular physiology; an integrative discipline that is concerned with the functional integrity of complex systems. Important

physiological changes that occur during apoptosis include oxidative stress and the depletion of cellular GSH, increased $[Ca^{2+}]i$, phosphatidylserine exposure, and the mitochondrial permeability transition. These functional changes appear to be fundamental, regardless of the specific molecular processes that are controlling apoptosis.

Flow cytometry allows correlated measurements of multiple parameters in cell populations. It has been widely used to infer the normal differentiation sequences of blood cells from patterns of surface antigen expression, using a logical approach to data analysis [37]. Here we describe an analogous process using fluorescent probes for live cell function. For example, during ara-C treatment virtually all cells are either annexin-V negative/high MMP, or annexin-V positive/low MMP. Therefore the permeability transition and the exposure of phosphatidylserine are synchronous events. Increased $[Ca^{2+}]i$ was only seen in cells that were annexin-V positive/low MMP, whereas low $[Ca^{2+}]i$ values were also found in some of this population; therefore the loss of calcium ion regulation is the later event. Activation of calcium-dependent endonucleases is probably

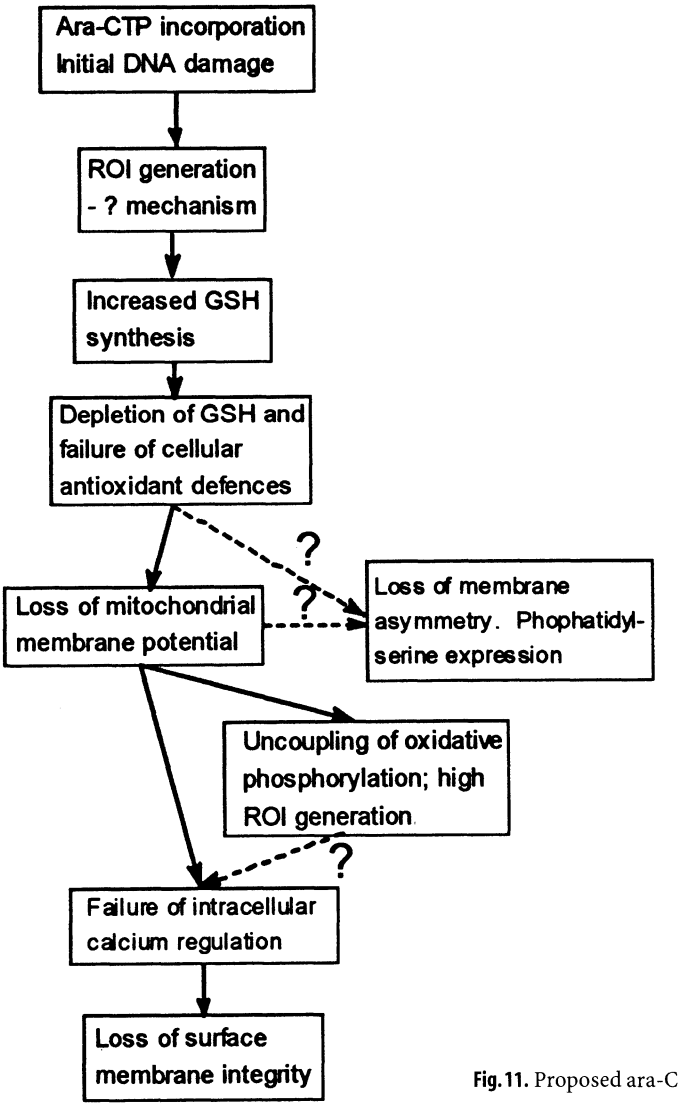

Fig. 11. Proposed ara-C "death sequence"

responsible for the DNA fragmentation that has been shown to occur in ara-C-treated OCI/AML-2 cells.

Increased reactive oxygen generation and increased glutathione content occur early during ara-C toxicity. This likely results from DNA damage following incorporation of ara-CTP, although the exact mechanism for this early onset of ROI generation is uncertain. The increase in GSH is probably a cellular response to oxidative stress that protects by maintaining cellular redox balance in a reducing state. However, later there is an abrupt transition to a state in which most of the reduced GSH is lost, and very high levels of ROI are produced. This transition indicates the collapse of cellular antioxidant defences, and a shift towards an oxidizing intracellular environment. The increase in $[Ca^{2+}]i$ that subsequently occurs is likely caused by either lipid peroxidation damage to membrane sites of calcium regulation, or to the failure of ATP-dependent calcium pumps. Fig. 11 proposes a sequence for the physiological changes associated with ara-C toxicity, based on the data shown here.

Transfected OCI/AML-2 blasts that over express bcl-2 are resistant to ara-C [4]. Mapping the sequence of physiological changes that occur during ara-C toxicity, we have located the action of bcl-2 at a point prior to the loss of reduced GSH and the onset of high ROI production [26]. Initial increases in ROI and GSH were seen, as with the non-transfected controls. Our results are therefore compatible with earlier suggestions that bcl-2 acts as an antioxidant. However, we were concerned about the lack of any obvious source of metabolic energy that would allow bcl-2 to maintain the intracellular reducing state in the face of a sustained oxidative stress.

It has been proposed that the mitochondrial permeability transition is a crucial event in apoptosis, regardless of the cause [13, 16]. Because the mitochondrial membrane is depolarized by the permeability transition, oxidative phosphorylation is uncoupled, ATP generation is lost, and there is increased respiratory chain activity due to loss of feedback control. Since superoxide anion is generated as a byproduct at the respiratory chain complexes, this results in increased ROI generation. This effect of the permeability transition has been reported by others [27, 28], and is seen in our results (Fig. 7). The loss of mitochondrial ATP generation and the increase in ROI generation might then be expected to impair the ability to regulate intracellular calcium, causing endonuclease activation.

Two parameter plots of GSH content versus mitochondrial membrane potential show that the permeability transition occurs in cells that are at the lower end of distribution of GSH values. This suggests that the permeability transition results from loss of GSH. There is a distinct pool of mitochondrial GSH which cannot be identified using the flow cytometry method [31]. Because mitochondria are a source of ROI, mitochondrial GSH plays an important role in maintaining reducing conditions within the matrix space. Oxidative stress in mitochondria, manifested as protein thiol oxidation or the production of lipid peroxidation products, is a potent initiator of the permeability transition [30, 38]. We propose that the depletion in total cellular GSH that precedes the permeability transition is associated with a more profound loss of mitochondrial GSH. Oxidative damage that occurs within the mitochondria as a consequence of this then triggers the permeability transition. Oxidative stress during ara-C toxicity is therefore a biphasic phenomenon, with the permeability transition occurring between the two phases. It has been suggested that bcl-2 is physically linked to the mitochondrial membrane pore complex, and acts to reduce the probability that the permeability transition will occur. This mechanism would be compatible with our findings, and explain why bcl-2 appears to exert an antioxidant effect in the absence of any obvious input of metabolic energy.

Use of flow cytometry and multiple fluorescent probes for live cell functions has enabled us to define a sequence of physiological changes that occur during drug-induced apoptosis. We propose that this provides a framework into which the actions of specific molecules such as bcl-2 can be fitted. This approach might be helpful for defining the roles of the various mechanisms that appear to be capable of regulating apoptosis during

the treatment of leukemias with chemotherapy.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

CD95-Mediated Apoptosis in Acute Myeloid Leukemia (AML): Dependence on Maturational Stage and Growth Characteristics in Vitro

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Abstract. The CD95 (APO-1/Fas) receptor, which is able to induce apoptotic cell death upon oligomerization by its natural ligand or by anti-CD95 antibodies, is broadly distributed in normal and malignant hematopoietic cells. However, its functionality could be demonstrated only in a limited number of cell types, and molecular events responsible for the resistance of cells to CD95-mediated apoptosis remain to be elucidated. The bcl-2 protein which has been associated with increased resistance to various apoptotic stimuli has been shown to block partially the CD95-mediated apoptosis in murine cell lines, but not in human lymphocytes. In AML, a limited number of cases has yet been tested as to their susceptibility to CD95-induced apoptosis. Using flow cytometry, we investigated both expression and functionality of CD95 and bcl-2 in untreated leukemic cells collected from 46 de novo AML patients, and correlated the results with the expression of the progenitor cell associated CD34 antigen and with growth characteristics of leukemic cell samples in vitro. Our study revealed that almost all (>90%) AML samples expressed both bcl-2 and CD95. The level of bcl-2 expression, quantified as mean fluorescence intensity, was significantly higher in $CD34^+$ than in $CD34^-$ AML ($p<0.001$) while that of CD95 was significantly lower in $CD34^+$ leukemic cell samples ($p<0.001$). The apoptotic effect of crosslinking of CD95 by anti-CD95 antibody was observed in 88% of $CD34^-$ AML but only in 29% of $CD34^+$ AML samples ($p<0.01$). No correlation between the extent of CD95-induced apoptosis and the level of CD95 and bcl-2 expression was found. Autonomous growth was observed in both $CD34^+$ and $CD34^-$ samples (67 and 84% of cases, respectively). In $CD34^+$ AML, all cell samples susceptible to CD95-mediated apoptosis exhibited autonomous growth. By contrast, $CD34^-$ samples were highly sensitive to $CD95^-$triggering independent of their growth characteristics. In conclusion, our data reveal a high susceptibility to $CD95^-$ triggering of AML with a $CD34^-$ phenotype, and suggest that this subgroup of AML may represent an appropriate target for therapeutic strategies based on tumor-specific T-cell cytotoxicity involving CD95-CD95L interactions.

Introduction

Programmed cell death or apoptosis is one of the central control mechanisms which counterbalances cell proliferation and differentiation during normal tissue development and cellular homeostasis [1-3]. It is a morphologically defined process of cell sui-

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cide initiated within cells as response to withdrawal of growth factors, DNA damage and metabolic distortions [4]. Apoptosis can be also induced by triggering of specific cell surface molecules, such as CD120a (TNFR I) or CD95 (APO-1/Fas) [5]. The CD95 protein that belongs to the superfamily of tumor-necrosis-factor (TNF) and nerve-growth-factor (NGF) receptors was demonstrated to induce apoptosis upon trimerization by its natural cognate, CD95 ligand (CD95L), or oligomerization by monoclonal antibodies (moAbs) of IgG3 and IgM class [5-7]. Recent studies revealed a broad distribution of the CD95 in a wide variety of cells including normal and malignant hematopoietic cells [8-11]. However, its functional capacity to induce apoptosis could be observed only in a limited number of cell lineages. Thus, CD95-induced apoptosis was demonstrated in activated peripheral T- and B-lymphocytes [12, 13], mature neutrophils [9] and, as to malignant hematopoietic and lymphoid cells, in adult T-cell leukemia [14, 15] and, partially, in T-lineage ALL [16] and myeloma [17] cells. Susceptibility to CD95-triggering was also found in a small subgroup of AML cases [18]. Most malignant cells failed to undergo CD95-mediated apoptosis, and additional treatment of the cells was nessessary to overcome their resistance to CD95 triggering [16,18-20].

Resistance to induction of apoptosis depends on various factors involved in apoptotic intracellular signalling pathways. Recent studies suggest that members of the bcl-2 family play a central role in apoptosis regulation [21, 22]. The founding member of this steadily expanding gene family, the proto-oncogene bcl-2, was originally discovered at the chromosomal breakpoint of t [14, 18] in human follicular lymphoma [23], and its overexpression has been shown to prevent or delay apoptosis induced by growth factor withdrawal, gamma-irradiation, glucocorticoids and several chemotherapeutic drugs [21, 24]. In malignant cells, the bcl-2 is thought to contribute to oncogenesis by suppressing signals that induce apoptotic cell death and, therefore, to be one of the factors contributing to the malignant transformation [25-27]. In AML but not in ALL, a high percentage of bcl-2 positive cells has been associated with a poor response to chemotherapy and a shortened overall survival [28, 29]. The involvement of bcl-2 in molecular pathways of CD95-induced apoptotic signalling is not fully elucidated. The bcl-2 dependent inhibition of CD95-induced apoptosis was demonstrated in mice cells forced to overexpress human CD95 and bcl-2 [30]. However, this inhibition was not complete. Moreover, Strasser et al. [31] found that bcl-2 provided little protection against CD95-transduced apoptosis in B-lymphoid cell lines, thymocytes and activated T-cells.

In normal hematopoiesis, the expression of CD95 and bcl-2 as well as the susceptibility to CD95-triggering strongly depends on the maturational stage of the hematopoietic cells [32-34]. Thus, the most immature, CD34 antigen positive and lineage marker negative ($CD34^{+}lin^{-}$) cells were found to be CD95 negative or to express very low levels of CD95, high levels of bcl-2 and to be refractory to CD95-triggering [32, 33, 35, 36]. By contrast, more mature, $CD34^{-}$ myeloid cells exhibited high levels of CD95 expression and susceptibility to CD95-induced apoptosis [32, 34, 36].

In AML, expression of CD34 has been demonstrated in about 30 to 60% of cases, often associated with other markers of cellular immaturity, such as CD7, HLA-DR and TdT [37]. In this study, we investigated the expression and functionality of CD95 in leukemic cells collected from de novo AML patients in relation to the expression of CD34 antigen. Additionally, we determined a possible involvement of bcl-2 protein in CD95-mediated signalling.

Materials and Methods

Patients

Leukemic blasts from 46 adult patients with newly diagnosed AML, either positive (n= 21) or negative (n= 25) for CD34 expression, were studied. The diagnosis and FAB subtype of AML was determined from Papenheim-stained bone marrow and blood smears [38, 39]. Immunophenotyping was carried out on leukemic blasts isolated by

standard Ficoll-Hypaque density gradient centrifugation (Pharmacia, Uppsala, Sweden), and cell-surface as well as cytoplasmic antigens were detected using direct or indirect immunofluorescence (IF) techniques as described elsewhere [40, 41]. AML cell samples were considered positive for CD34 antigen expression if more than 20% of the cells expressed fluorescence intensity greater than 98% of the negative control cells.

Cell Purification and Cell Culture

Leukemic cells were recovered from either fresh or cryopreserved patient samples and purified by density gradient centrifugation using Ficoll-Hypaque separation. Viability of cells was always more than 90% as determined by trypan blue or propidium iodide (PI; Sigma, Deisenhofen, Germany) exclusion. All samples contained more than 90% leukemic cells based on morphological criterias. Functional assays with freshly isolated or thawed leukemic cells of the same patient gave similar results. Cells were maintained in RPMI 1640 (Biochrom, Berlin, Germany) standard medium (SM) containing 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco BRL, Paisley, Scotland).

Assessment of CD95 and bcl-2 Expression

CD95 antigen was detected by conventional immunofluorescence staining of intact cells using phycoerythrine (PE)-conjugated anti-CD95 antibody (clone DX2; Pharmingen, San Diego, CA) [32]. To evaluate the expression of the intracellular bcl-2 protein, leukemic cells were fixed and permeabilized using the fixation-permeabilization kit (Fix & Perm; An-der Grub, Kaumberg, Austria) as recommended by the manufacturer. Bcl-2 antigen was detected by the FITC-conjugated anti-bcl-2 antibody 124 (Dako, Glostrup, Denmark) which was added with the permeabilization medium. After incubation for 30 min at room temperature cells were washed twice and analyzed for immunofluorescence. FITC- and PE-conjugated irrelevant mouse antibodies of the appropriate subclasses (Immunotech, Marseille, France) were used as negative controls to determine background fluorescence. Immunofluorescence analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) using CellQuest software (Becton Dickinson). Expression of antigens was quantified as relative fluorescence intensity, RFI, determined by the ratio of mean fluorescence intensity of cells stained for either CD95 or bcl-2 to mean fluorescence intensities of the corresponding negative controls.

Assessment of Cell Apoptosis and Growth by Flow Cytometry

To assess spontaneous apoptosis, 0.5x106 leukemic cells/well were cultured in 96-well microtiter plates (Nunc, Roskilde, Denmark) using SM for 20 h at 37 °C in a humidified atmosphere of 7% CO_2 in air. To investigate the CD95-mediated effects, leukemic cells were cultured for 20 h in the presence of the anti-CD95 moAb CH-11 (IgM; Immunotech, Marseille, France; 1 mg/106 cells). As a negative control, cells were preincubated with irrelevant control antibodies. After the incubation, leukemic cells were fixed, permeabilized and stained with PI as described elsewhere [42]. Briefly, a pellet of 5x105 leukemic cells was fixed by addition of 2 ml of ice-cold 70% ethanol for 1 h at 4 °C. After washing the cells were resuspended in 0.5 ml PBS containing 1mM EDTA, 0.05% Triton X-100 (Serva, Heidelberg, Germany), and 50 mg/ml PI, pH 7.5. Following the treatment with 10 ml of 10 mg/ml RNase (type I-A; Boehringer Mannheim, Mannheim, Germany) for 30 minutes at room temperature in the dark, the cells were stored at 4 °C until analysis by flow cytometry. Growing cells were identified using flow cytometric dot plots of DNA content versus forward scatter (CellQuest software, Becton Dickinson) as cells with increased scatter and/or increased DNA content. Cells undergoing apoptosis were identified by analysis of DNA histograms or dot plots of DNA content versus forward scatter and quantified as percentage of cells with subdiploid DNA content [42]. The extent of the CD95-mediated apoptosis

(%) was assessed by formula as described elsewhere [16]:

$$\frac{(\text{CD95-mediated apoptosis} - \text{spontaneous apoptosis}) \times 100}{(100 - \text{spontaneous apoptosis})}$$

The CD95-mediated apoptosis is the percentage of apoptotic cells in the presence of the anti-CD95 moAb, and the spontaneous apoptosis is the percentage of apoptotic cells in the presence of the irrelevant control moAb or medium alone.

Cell samples were regarded as CD95-sensitive when the extent of CD95-mediated apoptosis was higher than 20%.

Statistical Analysis

Results are given as mean ± SEM. Differences were evaluated using the two-tailed, non-parametric Mann-Whitney U-test for continuous variables and c2-test for categorical variables. Differences were considered to be significant for P values < 0.05.

Results

Expression of the CD95 and bcl-2 in $CD34^+$ and $CD34^-$ AML

The surface expression of the CD95 antigen and intracellular expression of the bcl-2 protein was analyzed by direct immunofluorescence in 46 cases of AML which were subgrouped according to their CD34 expression (21 and 25 of cases positive or negative for CD34, respectively). All cases, with exception of one $CD34^+$ AML sample, were found to express CD95. The level of expression, quantitated as relative fluorescence intensity (RFI) of the CD95 staining, ranged from 1.9 to 10.5 for $CD34^+$ samples and from 3.1 to 19 for $CD34^-$ cells. Overall, as summarized in Table 1, the CD95 surface expression in $CD34^+$ cases was significantly lower ($p<0.01$) as compared with that in $CD34^-$ samples.

Table 1. Expression of CD95 and bcl-2 in leukemic cells from de novo AML patients.

	n	CD95 (RFI)[a]	bcl-2 (RFI)[a]
$CD34^+$	21	4.4±0.5	4.2±0.4
$CD34^-$	25	9.6±0.9	2.6±0.2

[a] RFI is the ratio of mean fluorescence intensity of cells stained for CD95 or bcl-2 to mean fluorescence intensity of the corresponding negative controls. Average values (mean ± SEM) of RFI estimated for the $CD34^+$ and $CD34^-$ AML samples are presented.

Bcl-2 could also be detected in almost all samples except for one $CD34^-$ case. The bcl-2 expression was significantly higher ($p<0.01$) in $CD34^+$ than in $CD34^-$ samples (Table 1). This difference was also reflected in the range of bcl-2 expression: from 2.0 to 8.6 in $CD34^+$ samples and from 1.2 to 5.7 for $CD34^-$ cells.

CD95-Mediated Apoptosis in AML Cells

To assess the CD95-mediated specific apoptosis, AML cells were cultured for 20 h in the presence of the anti-CD95 IgM MoAb, and apoptotic cells were detected by flow cytometry. A representative example of the CD95-mediated apoptosis is shown in Fig.1. The apoptotic cell fraction (subset a) can be seen as a subpopulation of cells with lower forward scatter and lower DNA content compared with normal cells (subset n).

As shown in Table 2, the CD95-mediated apoptosis exceeding a 20% level was detected in 22 cases of $CD34^-$ (88%), but only in 6 cases of $CD34^+$ AML (29%) samples. These results demonstrate a considerably higher susceptibility of $CD34^-$ cells to CD95-mediated apoptosis as compared with that of $CD34^+$ cells. The statistically significant ($p<0.001$) difference of mean values of the extent of CD95-specific apoptosis (45% in CD34- versus 17% in $CD34^+$ samples) substantiates this observation (Table 2).

To analyze whether CD95-induced apoptosis depends on the intensity of CD95 cell surface expression by the leukemic cells, the specific CD95 RFI surface expression was correlated with the extent of CD95-mediated apoptosis. We did not find a correlation between the level of CD95 surface expression and the CD95-mediated effects (data not shown). Similarly, there was no quantitative correlation with the extent of bcl-2 expression.

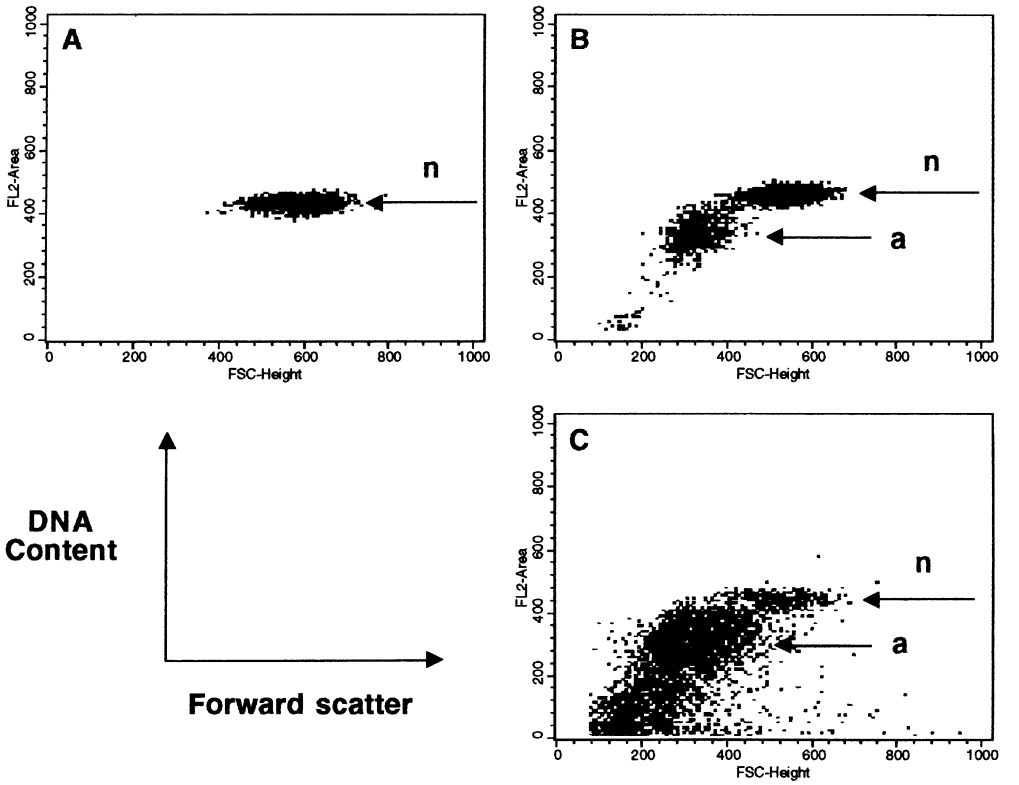

Fig. 1 A–C. CD95-mediated apoptosis of AML cells detected by flow cytometry. The cells were measured either immediately after isolation *(A)* or cultured in standard medium in the absence *(B)* or presence *(C)* of anti-CD95 moAb for 20h at 370 C. A subpopulation of apoptotic cells *(a)* can be readily distinguished from normal, nonapoptotic cells *(n)* in the density plot of linear DNA content versus forward scatter. In the example shown, the spontaneous apoptosis rate was 28% and the CD95-specific apoptosis 83%.

Table 2. Differential effect of CD95-triggering on $CD34^+$ and $CD34^-$ AML cells.

CD95-mediated apoptosis	CD34+ samples	CD34- samples
samples with extent of apoptosis <20%	15/21 (71%)b	3/25 (12%)
samples with extent of apoptosis >20%	6/21 (29%)	22/25 (88%)
mean extent of apoptosis (± SEM)	17.4 (± 4.3)%	44.6 (± 4.4)%
range of apoptosis (min - max)	0 - 69%	0 - 83%

[a] Cells were incubated with anti-CD95 IgM moAb for 20 h at 37 °C, assessed for apoptosis by flow cytometry and the specific CD95-mediated apoptosis was calculated as described in Materials and Methods. Experiments were performed at least twice for each leukemic cell sample.
[b] Number of cases /total number of cases (percent of cases).

Comparison of AML Samples in Relation to Their Growth Characteristics in Vitro

Growing cells were identified as cells with increased scatter and/or increased DNA content. Autonomous growth of AML cells was observed in 14 cases of $CD34^+$ AML (67%) and 21 case of $CD34^-$ AML (84%). Expression levels of CD95 and bcl-2 were found to be not significantly different in samples exhibiting autonomous and non-autonomous growth (data not shown). Furthermore, we considered susceptibility to CD95-triggering of AML samples in relation

Table 3. Susceptibility to CD95-mediated apoptosis in $CD34^+$ and $CD34^-$ AML samples in dependence on their growth characteristics in vitro.

	n	Susceptibility to CD95-mediated apoptosis	
		yes	no
$CD34^+$ AML samples with autonomous growth	14	6/21	8/21
$CD34^+$ AML samples without autonomous growth	7	0/21	7/21
$CD34^-$ AML samples with autonomous growth	21	18/25	3/25
$CD34^-$ AML samples without autonomous growth	4	4/25	0/25

to their growth characteristics in vitro (Table 3). Interestingly, there was no significant correlation between CD95-susceptibility and growth parameters in $CD34^-$ AML. By contrast, in $CD34^+$ AML all samples susceptible to CD95-mediated apoptosis exhibited autonomous growth while the samples without autonomous growth were refractory to CD95-triggering.

Discussion

In the present study, we investigated CD95 (FAS/APO-1) and bcl-2 expression in leukemic blasts from 46 adult patients with de novo AML. In addition, AML cell samples were tested for their susceptibility to CD95-mediated apoptosis. We found that almost all samples studied (45/46) expressed surface CD95 antigen with expression levels, however, varying considerably between low (RFI=1.9) and high (RFI=19). These results are in line with data published by Komada et al. [18] demonstrating a similar range of CD95 expression levels (1.8-14.8 RFI) in a smaller series of 20 AML patients. In contrast to these findings, Min et al. (43) observed a lower incidence (47%) of CD95 positivity (samples containing >20% CD95 positive cells were considered as positive) in 30 AML samples, and Munker et al. [11] found an even lower proportion of CD95 positive AML cases detected by fluorescence microscopy (28%, 13/47 cases). These discrepancies could be at least partially due to differences in sensitivities of the detection methods applied and in criteria used to define CD95 positivity. Our findings, indicating a broad distribution of CD95 in AML are in line with recent reports on CD95 expression in malignant hematopoietic cells. Thus, CD95 was detected on the majority of myeloma cells [17, 20, 44], in adult T-cell leukemias [14], and in T-lineage ALL [16]. However, its functional activity could be observed only in a limited number of hematopoietic cell lineages. Thus, the crosslinking of CD95 by antibody was demonstrated to be highly effective in adult T-cell leukemia cells [14, 15] and, to a lower extent, in T-lineage ALL [16], while myeloma and B-lineage ALL cells [17, 20, 44] failed to undergo CD95-mediated apoptosis, and additional treatment of the cells was nessessary to overcome their resistance to CD95-triggering [16, 18-20]. In the present study, a CD95-induced apoptosis was observed in 61% of AML cases (28/46 cases). However, there was no quantitative correlation between CD95 expression and sensitivity to CD95 in individual samples. Similar results were reported by Komada et al. [18], who found 8 of 14 samples (57%) to be sensitive to CD95-triggering, without a clear-cut correlation between CD95 density on the cell surface and the extent of apoptosis.

The most interesting findings emerged from subgrouping of AML samples according to their CD34 antigen expression. In normal hematopoiesis, hematopoietic stem cells are believed to reside within the $CD34^+$ cell subpopulation and immature, CD34 positive and lineage marker negative ($CD34^+lin^-$) cells were found to be CD95 negative or to express very low levels of CD95, and to be refractory to CD95-triggering [36]. In myeloid progenitor cells with a $CD34^+CD33^+$ phenotype low levels of CD95 expression and susceptibility to CD95-triggering have been found [34, 36]. By contrast, the more mature, $CD34^-CD33^+$ myeloid cells exhibited high levels of CD95 expression and susceptibility to CD95-induced apoptosis [34]. In addition, mature granulocytes from peripheral blood are known to be extremely sensitive to CD95-triggering [9].

In AML, expression of CD34 has been demonstrated in about 30 to 60% of cases, often associated with other markers of cellular immaturity, such as CD7, HLA-DR and TdT [37]. In the present study, leukemic cells in $CD34^-$ AML samples were found to express significantly higher levels of CD95 and lower levels of bcl-2, as compared with $CD34^+$ samples, and revealed significantly higher susceptibility to CD95-mediated apoptosis. These data suggest that $CD34^+$ AML cells are refractory to CD95-triggering comparable with normal myeloid progenitors. In contrast, leukemic cells from the more differentiated AML subtypes with a $CD34^-$ phenotype might acquire susceptibility to CD95-triggering found in normal mature myeloid cells.

AML blast cells reveal various extents of proliferation when cultured in vitro, and autonomous growth in AML samples has been reported to be an important indicator of a poor response to chemotherapy in AML [45]. Consistent with previous reports [28, 33], we observed a considerable number of AML samples which exhibited autonomous growth in vitro. Since a close association between cell cycle progression and CD95-mediated apoptotic cell death has been demonstrated in AML cells [18], an increased sensitivity of autonomously growing blasts to CD95-triggering could be expected. Indeed, in $CD34^+$ AML, we found that all samples which were susceptible to CD95-mediated apoptosis also exhibited autonomous growth while the samples without autonomous growth were refractory to CD95-triggering. However, in $CD34^-$ AML, leukemic samples were found to be highly sensitive to CD95-triggering independent of their growth characteristics. It can be speculated that CD95-mediated apoptotic signalling pathways might be cell cycle dependent in immature, $CD34^+$, but not in more mature, $CD34^-$, AML cells. In this context, it is interesting to note that the data reported by Komada et al. [18] were mainly obtained using a cell line established from a patient with immature AML of M1 subtype. It is obvious that further studies should be undertaken to investigate cell cycle specificity of the CD95-mediated apoptosis in myeloid cells dependent on their maturational stage.

Intracellular pathways of apoptotic signalling involve various gene products, such as the members of the Bcl-2 gene family [21, 22]. In AML, the expression of the bcl-2 proto-oncogene was extensively investigated, and its increased expression was shown to be associated with resistance of AML cells and myeloid cell lines to various apoptosis-inducing cytotoxic drugs, as well as with a low complete remission rate after intensive chemotherapy of AML patients [28, 33, 35]. In addition, a significantly higher bcl-2 level in $CD34^+$ AML cells compared with that of $CD34^-$ has been reported [28]. This difference between $CD34^+$ and $CD34^-$ AMLs, which was also observed in our study, might account for the different CD95-sensitivities characteristic of these two AML subgroups. However, the absence of a quantitative correlation between the expression level of bcl-2 and sensitivity to CD95-triggering in our study as well as other reports [16, 17] suggests that the expression and complex interactions of other members of the bcl-2 family, including bak, bax, bcl-XL and bcl-XS, involved in the intracellular apoptotic signalling process, have to be taken into consideration [22].

In conclusion, our data reveal differential expression and functionality of CD95 protein in $CD34^+$ and $CD34^-$ AML cells. Our finding that $CD34^-$ AML samples are highly sensitive to apoptotic cell death induced by CD95 crosslinking might have some clinical relevance. Thus, innovative immunotherapeutic strategies involve application of cytotoxic T-lymphocytes (CTLs), e.g. by expanding and adoptive transfer of tumor-specific T-lymphocytes, by redirection of CTLs against cancer cells by bispecific reagents or activation of T-cells at the tumor site [46-48]. Since, according to recent studies on T-cell mediated cytotoxicity, one of the main killing mechanisms used by effector T-cells involves CD95-CD95L interactions [49, 50], CD95-sensitive cells should be more susceptible to T cell-mediated cytotoxicity than CD95-resistant cells [51]. In acute leukemia, this effect was recently demonstrated using CD95-sensitive and -resistant AML cell lines [52]. In this regard, our results suggest that AML with the CD34- phenotype might be an appropriate target for T-cell mediated cytotoxicity.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Structural Properties of the Cell Membrane of Apoptosis-Induced Cells and Leucemic Blast Cells Studied by Electrospinresonance-Spectroscopy

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Dedicated to Prof. Dr. Dr. h. c. Bernhard Kornhuber on his 65th birthday

Abstract. The application of Electrospinresonance-Spectroscopy (ESR) allows the study of the structural properties of the cell membrane of intact live cells. The spectra obtained provide profound information on the fluidity, as given by the order parameter S, and the polarity (h_{-1h}/h_{-1P}) of the cell membrane. The signal reduction gives rise to the approximate oxidative activity. In this study, the determination of the order parameter yielded S = 0.092–0.153 for leukemic blast cells (n = 18) and leukemia-derived cell lines (MOLT-4, KG-1, U 937, H 9, K 562) compared to S = 0.155–0.172 for lymphocytes and granulocytes of healthy donors. The results show, that the cell membrane of malignantly transformed cells is more fluid compared to the group of healthy donors. The polarity of the cell membrane is low for cell lines with (h_{-1h}/h_{-1P}) = 1.5–10, high for the group of healthy donors with (h_{-1h}/h_{-1P}) = 0.35–1.7 and highest in case of leukemic blast cells with (h_{-1h}/h_{-1P}) = 0.1–1.3. Cell lines, lymphocytes and granulocytes show no signal reduction (SR), while leukemic blast cells show considerable SR. PMA-activated granulocytes show distinctive, PHA-activated lymphocytes show no SR.

Apoptosis, or programmed cell death, is a common feature of the differentiation and maturation of cells in all kinds of tissue in many organisms, ensuring a controlled pattern of cell growth. This form of cell death is also assumed to play an important role in protection from pathologic cell growth, such as in malignant tumors. The starting point of apoptosis lies, in contrast to necrosis, in the nucleus, not affecting the integrity of the cell membrane at this early stage. In this study, leukemia derived cell lines (MOLT-4, U 937, H 9) were induced to perform apoptosis with the alkaloid Camptothecin. Intact cells were observed for alterations in the properties of the cell membrane using ESR. Apoptosis-induced cells showed a characteristic ESR-spectrum 12–18 h after induction with camptothecin, consisting of a distinctive increase in membrane fluidity (S = 0.091–0.104) and polarity [(h_{-1h}/h_{-1P}) = 0.16–0.4] compared to non-induced cells [S = 0.12–0.14, (h_{-1h}/h_{-1P}) = 1–1.8]. These findings are significantly different from that in case of necrosis and current studies aim at confirming the possibility to determine the fraction of cells performing apoptosis in a population of live cells under certain conditions (i.e., cytostatic drugs) using ESR.

Introduction

Modern techniques of cellular biology enable the scientist to study a great variety of processes concerning life and death of cells. Among these, many processes involve the cellular membrane.

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Electronspinresonance spectroscopy (ESR) is a powerful technique to study the properties of the cellular membrane of intact live cells. The evaluation of ESR spectra allows to determine different parameters, such as membrane fluidity, polarity [1,2] and signal reduction [3].

Equipped with a characteristic set of proteins, every cellular membrane is capable of performing highly specialized functions. Many of them can be studied specifically.

In contrast, every cellular membrane consists basically of the same phospholipid bilayer structure as described by Singer and Nicolson [4]. The physical and structural properties of this cellular backbone are of great interest.

The bilayer in its entirety shows the properties of a liquid and gel-like substance respectively. In this context, the term fluidity describes the quality of ease of molecular movement in a macromolecular environment. Fluidity is essential for the cellular plasticity and seems to be crucially important for the regulation of membrane proteins [7]. Previous studies have pointed out the possible role of changes in membrane fluidity in correlation to malignancy [5,6].

The fluidity of a cellular membrane is determined by its lipid composition to a great extent. Accordingly, changes in fluidity are mainly caused by alterations of the phospholipid bilayer. Several studies have shown the influence of membrane proteins on the fluidity to be negligible [1, 2], although the percentage by weight in eukaryotic cells amounts 50% and more [7].

Another structural property of great interest is the polarity of the cellular membrane. Studies of the polarity may provide important information on lipid-protein interactions and different patterns of perturbation in the phospholipid bilayer.

The aim of this study is to systematically investigate and characterize physical and structural properties of the cellular membrane of intact live cells obtained from hematologic patients.

Following the encouraging results of our investigations, our interest was then focused on the process of apoptosis and its influence on the properties of the cellular membrane as observed by ESR-spectroscopy. Attention was paid especially on the dynamics and the time dependence of alterations within the phospholipid bilayer and a comparison to the process of necrosis.

Materials and Methods

Materials

Leukemic blast cells at the onset of childhood leukemia and different leukemia-derived cell lines formed a group of malignantly transformed cells. Due to the lack of experience in the systematic study of leukemic cell material by ESR, our first interest aimed at the observation of blast cells from the onset of the disease, were no specific drug effects would complicate the interpretation of the results.

Cell lines represent a suitable control for the group of malignantly transformed cells. Following good conditions in the cultivation, they are available at any time. Their cellular membrane properties were being studied frequently in order to rule out systematic errors.

Lymphocytes and granulocytes from healthy donors represented the control group.

Methods

Cell Separation

Cell separation was performed using a density-gradient centrifugation. This yielded a purity of mononuclear and polymorphnuclear cells of more than 90%. An amount of at least 10^6 cells is required per sample.

After separtion, a cell suspension with a concentration of 10^6 cells/ml is established, and portions of 1 ml are distributed to Eppendorf cups.

Spin Labeling

Eppendorf cups are centrifugated 2 min with approximately 300 g. The pellet is then resuspended in 50 µl phosphate buffered saline (PBS). Spin labeling is performed by the addition of 1 µl of a 5-mmol solution of 16-doxyl-stearic-acid in pure ethanol. The sample is vigorously stirred for 5–10 s and after-

wards incubated for 3 min. The spin label is then washed out by the addition of 1 ml PBS, stirring and centrifugation with 300 g. After resuspension of the pellet in 50 µl PBS, the labeled cells are transferred in a 50-µl capillary which is placed in the ESR spectrometer.

Induction of Apoptosis

To study the influence of apoptosis, leukemia derived cell-lines MOLT-4, U 937 and H 9 were induced to perform apoptosis by use of the alkaloid camptothecin. To confirm a successful induction, we performed gel electrophoresis, an acridin-orange dye and a morphologic control by the use of Pappenheim dye.

ESR Spectroscopy

ESR-Spectroscopy is based on the detection of magnetic spin moments as presented by free electrons. Consequently, ESR-Spectroscopy requires the presence of molecular structures with stable free electrons. Biological membranes usually do not fulfill this requirement, why spin label molecules are being introduced into the cellular membrane. These molecules provide a paramagnetic reporter group with a free electron (Fig. 1).

The similarity of the basic structure of these amphipathic molecules, consisting of an apolar fatty acid chain and a polar carboxyl head group, compared to the structure of the phospholipids, enables the spin label to be readily incorporated into the cellular membrane (Fig. 2).

Subsequently to the labeling of the cells (2.2.2), the sample is placed in the ESR spectrometer and simultaneously exposed to a magnetic field of increasing strength and microwave radiation of constant frequency. Increasing the magnetic field strength over a well-defined narrow range, leads to energy absorption by the free electrons, resulting in a characteristic resonance spectrum.

The form of this spectrum depends mainly in the degree of mobility of the spin label molecules within the cellular membrane as well as of the polarity of the latter. The mobility is related to the degree of order of the cellular membrane and its fluidity respectively. An increase in the degree of order leads to a decrease in fluidity. Figure 3 shows an exemplary ESR spectrum with the characteristic triplet form, consisting of three distinct peaks (h_{+1}, h_0, h_1), as obtained by a solution of spin label 16-doxyl-stearic acid (16-DSA) in water without cellular material.

Fig. 1. Spin label 16-doxyl-stearic-acid with paramagnetic reporter group

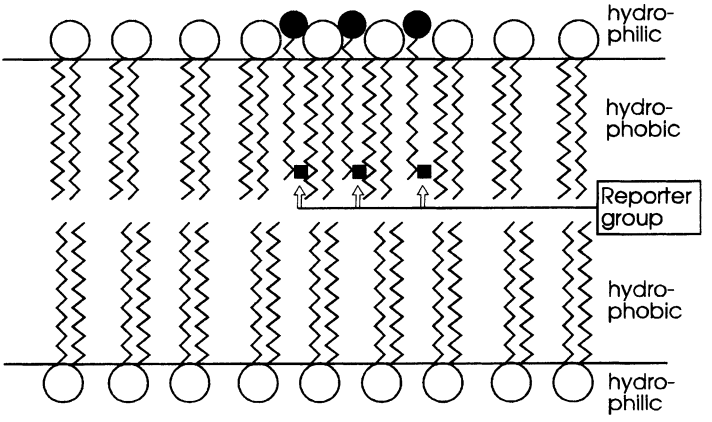


Fig. 2. Simplified biological membrane with incorporated spin label 16-DSA

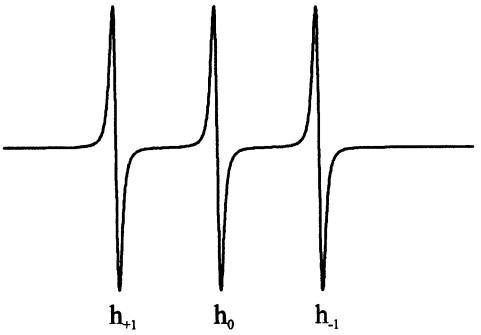


Fig. 3. Watery solution of spin label 16-DSA without cell material

In this case, spin label molecules are freely mobile in all directions of space.

Figure 4 shows an ESR-Spectrum of a cell suspension. In contrast to Figure 3, spin label molecules are incorporated into the cellular membrane and their mobility is restricted. Increasing restriction of mobility corresponds to a broadening and flattening of the spectrum.

ESR-spectra allow to determination of different parameters quantitatively:

1. Order parameter S is calculated by the values A_{II} (parallel) and A_I (perpendicular) (Fig. 4). S measures the degree of order of the cellular membrane. An increase in the degree of order corresponds to a decrease in fluidity. S ranges from 0, most fluid, to 1, least fluid.
2. The partition coefficient f can be determined, when splitting of the third peak occurs (Fig. 4). It is given by the ratio of the two components of the h_{-1}-peak: f = hs1H/hs1P. f is a measure for the membrane polarity. It ranges from 0, highest polarity, to ∞, lowest polarity.

Results

Differentiation Between Malignant and Non-Malignant Cells

The evaluation of the obtained ESR-Spectra has confirmed our assumption that each type of cell investigated in the course of this study, can be assigned to a group with characteristic properties of the cellular membrane. Among these, the differentiation between malignant and non-malignant cells by means of the order parameter S, was most significant. Within the groups "malignant" and "non-malignant", the determination of the polarity shows significant differences between leukemic blast cells and cell lines.

Table 1 shows the results of the calculation of the order parameter S and the determination of the partition coefficient f.

The results indicate, that the cellular membrane of non-malignant cells has a higher degree of order and is less fluid compared to the malignant group. Within both groups, with exception of cases of AML, no significant differences were observed. A tendency to relatively low fluidity was seen in 4 out of 5 cases of AML.

The cellular membrane of leukemic blast cells shows a significantly higher polarity compared to the cell lines (KG-1, MOLT-4, K 562, HL 60). No significant differences in po-

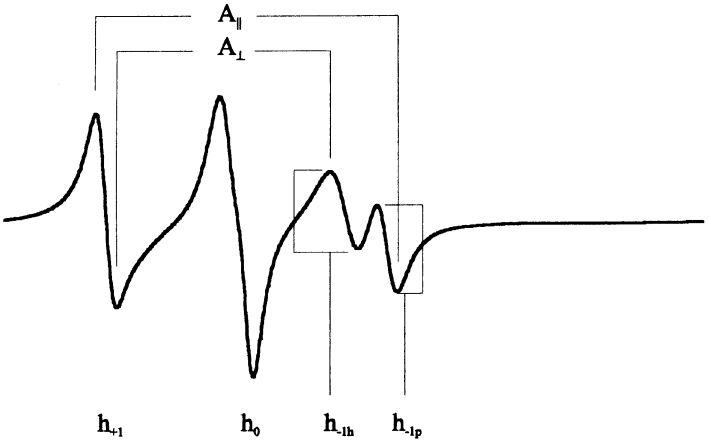


Fig. 4. ESR-Spectrum of cell suspension, criteria for evaluation

Table 1. Values of order parameter S and partition coefficient f

Cell material		Order parameter S	Partition coefficient f
I. Non-malignant	(n = 23)		
a) Lymphocytes		S = 0.155–0.172	f = 0.35–1
b) Granulocytes		S = 0.163–0.172	f = 0.47–1.7
II. Malignant	(n = 43)		
a) Cell lines		S = 0.092–0.149	f = 1.5–10
b) Leukemic blasts		S = 0.100–0.152	f = 0.1–1.3

larity could be observed in the group of non-malignant cells.

Cellular Membrane Characteristics of Apoptosis and Necrosis

The study of the process of apoptosis yielded further interesting results. According to expectations, cellular necrosis revealed early changes indicating a perturbation of the phospholipid bilayer integrity.

This corresponds to a strong increase in cellular membrane polarity, whereas the membrane fluidity is not significantly altered.

Incidentally, it is a remarkable phenomenon, that in the hydrophobic core of the cellular membrane, necrosis does not seem to have a significant influence on the membrane fluidity. Thus, fragments of the phospholipid bilayer do obviously have the same fluidity as the intact bilayer.

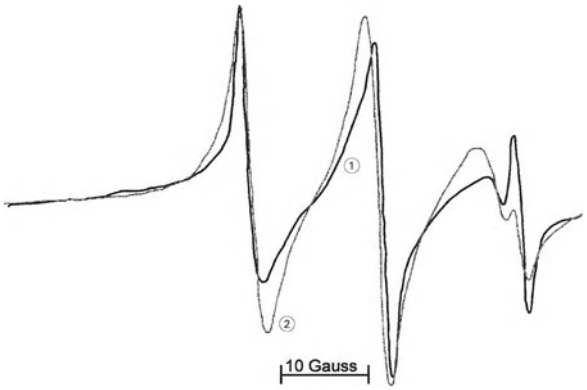
In apoptosis-induced cells, the starting of DNA-fragmentation can be monitored in gel electrophoresis after 2 h and it is completed after approximately 8 h. Accordingly, in the Acridin-Orange dyed samples, apoptosis is clearly recognizable in more than 90% of the cells.

ESR-Spectroscopic studies after 2–8 h do not show any significant changes concerning the cellular membrane. This is confirmed with the dye Trypan Blue, which is able to prove the vitality of the cellular membrane. In more than 90% of the cells, the cellular membrane was found to be intact.

Earliest significant changes observed by ESR, were recognized after 12–14 h. This resulted in a strong increase of membrane fluidity as well as membrane polarity. Maxima were reached after 16–18 h (Fig. 5).

Continuing ESR-spectroscopic observation leads to additional interesting results. After approximately 24 h fluidity decreases again, finally reaching a level that is characteristic for the intact membrane and necrotic fragments, respectively.

This result is of course not surprising as the process of apoptosis finally leads to a pattern of damage that does no longer allow



① Grey: U 937, Control
② Black: U 937, apoptosis-induced

Fig. 5. ESR-Spectra, control and apoptosis-induced sample

the differentiation between apoptosis and necrosis. In this state, the cellular membrane is disintegrated to a large extent.

Discussion

In the course of our ESR-spectroscopic studies, we were able to reliably discriminate different cell types by means of characteristic properties of the cellular membrane. A reliable differentiation between intact live malignant and non-malignant cells could be established, although, to the present stage, the systematic study of cellular membrane properties within the group of leukemias did not yield an obvious correlation to clinical or immunological aspects in the case of ALL. The tendency of AML to show relatively low fluidity is subject to further confirmation. The considerable increrase in membrane fluidity observed during the process of apoptosis is an interesting phenomenon. Our results suggest that the fluidization of the cellular membrane is an effort, that is bound to the integrity of the phospholipid bilayer. To the present, we have not been able to report fluidity of this extent in a case other than in apoptosis.

Moreover, these findings indicate that fluidization of the hydrophobic core of the cellular membrane can not simply be put on a level with membrane damage.

Future studies will be focused on the changes in the cellular membrane properties caused by drug treatment, especially by cytostatic drugs.

Acknowledgments. Supported by the Hessisches Ministerium für Wissenschaft und Kunst, 1995

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Simultaneous Detection of Leukemia Blast Cells on PB/BM – Smears Combining May-Gruenwald Giemsa- and APAAP Staining

U. Ebener, and S. Wehner

Dedicated to Prof. Dr. med. Dr. h. c. B. Kornhuber on his 65th Birthday.

Abstract. For subtyping acute leukemias the French-American-British (FAB) classification system has been the most widely used system. The system uses morphological and cytochemical criteria to initially define myeloid and lymphoid groups. Since the recent availability of monoclonal antibodies (MoAbs) recognizing various differentiation antigens the diagnostic precision has increased. Immunologic assessments of cell-surface antigen expression have now been recognized as providing an additional set of criteria upon which to base a more comprehensive description of leukemic cells.

In the present study we describe the combination of Pappenheim staining with an immunocytochemical method (APAAP-complex-technique), providing the visualization of morphological features as well as cell surface antigens, e.g., CD2, CD3, CD7, CD10, CD13, CD14, CD19, CD20, CD24, CD34, HLA-DR and TdT in air-dried cell smears from different subtyps of leukemic blasts (ALL/AML) form children and from leukemia-derived cell lines (Molt-4, REH, U937).

Following May-Gruenwald Giemsa staining, the morphological structure of cells was examined under an optical microscope, photographed, destained, fixed and immunolabeled by a modified alkaline phosphatase anti-alkaline phosphatase (APAAP) complex technique with repeated incubation steps using Silicone-Chamber-System (SCS).

After finishing the immunoenzymatic colour reaction by the application of naphthol AS-MX-phosphate and Fast Red TR Salt as chromogenic substrate the marked cell areas were visualized and photographed again.

Performing the proposed schedule, it was still possible to classify acute leukemias according to their surface marker expression and to distinguish simultaneously between myeloid and lymphoid cells by morphological criteria.

In conclusion, the application of simultaneous staining procedure allows the direct comparison of morphology by light microscopy and the marker expression at single leukemic blast cell level.

Introduction

Acute leukemias are traditionally classified according to their morphological and cytochemical features, according to the French-American-British (FAB) classification system [2–5]. The initial FAB classification was particularly limited in defining differences among lymphoid leukemias. Criticisms of the FAB classification system have been raised because one cannot distinguish between immunologic subgroups by morphological criteria [6, 15]. Immunological assessments of cell surface antigen expression have now been recognized as providing

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an additional set of criterion upon which to base a more comprehensive description of leukemic cells [16, 17]. Since the recent availability of monoclonal antibodies (MoAbs), recognizing various differentiation antigens (CD) using immunofluorescence analyses on microscope or flow cytomer (FACS) the diagnostic precision has increased [1, 13, 15, 17–19].

There are, however, still cases (e.g., AML-M0 and AML-M7) that cannot be judged by morphological nor by immunological features alone [16–18]. In these circumstances it would be very helpful, to be equipped with a technique that allows simultaneous analysis of cells for hematological characteristics and immunological cell marker profils [12, 14]. The additional detection of antigens could help to come to an accurate diagnostic decision [14].

Problems

The Pappenheim-staining procedure (May-Gruenwald Giemsa) is the most common technique for morphological and cytodiagnostic studies in hematology. On the other hand, a morphological reviewing of cells applying panoptical staining procedures alone, is dependent on the investigators experience as well as on the quality of the cells to be analysed. Perhaps the interpretation remains to be difficult and not in any case a reliable and satisfactory reason can be given to decide the diagnosis ALL or AML. An additional characterization of such undefinable cells using monoclonal antibodies should support one to find an exact diagnosis.

Aim

Therefore in the present study our intention was the development and standardization of a practicable method, that adds an immunological marker profile to cells that are still morphologically characterized by conventional panoptical Pappenheim stain. The new combined staining procedure (CSP) should close the gap between conventional hematology and immunology. CSP should enable one to additionally proof antigens that are of relevance in acute leukemias and therefore lead to a more detailed diagnosis.

Material and Methods

Patients

Samples were collected from peripheral blood or from bone marrow aspirates of patients with acute leukemias and from healthy donors. The diagnoses were based on criteria of the FAB classification system completed by immunophenotyping using flow cytometry (FACScan/BD) to assess leukemic cell differentiation to characterize the profile of cell surface marker expression [1, 13, 18].

For immunophenotyping peripheral blood and bone marrow the smears were prepared as for conventional hematological examinations. Smears were air dried at room temperature for 12–24 h or partly stored at −20 °C for retrospective analyses [8, 11]. Most slides were stained immediately after preparation. Frozen smears were thawed for 5 min, before fixation.

Cell Cultures

Likewise the new staining procedures was performed to cytospin preparations and smears of various leukemia derived cell lines (Molt-4: T-lineage-ALL, REH: B-lineage-ALL, U937: Promyelocytic AL): The cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

Fixation

Immediately before immunolabelling control slides applying standard APAAP-complex system, cell smears were fixed with glutaraldehyde (Sigma Diagnostics) for 5 min to demonstrate surface markers, or with acetone for 90 s for staining intracytoplasmic as well as nuclear antigens. Following fixation and before labelling, slides were rinsed in

Dulbecco's phosphate buffered saline (PBS; Gibco). In the present study we have examined an additional number of fixatives (methanol, acetone, paraformaldehyde, methanol/paraformaldehyde).

Silicone Chamber System (SCS)

In order to determine a different number of antigens on one and the same slide, we used the application of a self-developed Silicone Chamber System [9]. The reaction fields are prepared with a commercially available silicone-sealant. This system offers the advantage that each slide can accomodate several different monoclonal antibodies separately on the reaction fields, without the risk of uncontrolled spreading of reagents. After completion of the immunoreaction, the silicone is easily removed using a scalpel. Counterstaining with hematoxylin and covering of the smears can be carried out, thus enabling a light microscopic evaluation.

Monoclonal Antibodies

Monoclonal antibodies (MoAbs) against T- and B-lineage antigens as well as MoAbs against myeloid and precursor cells were used to investigate the applicability of demonstrated procedure. Details of monoclonal antibodies used in this study are given in Table 1. Soluble Alkaline phosphatase anti-alkaline phosphatase (APAAP) complexes and secondary antibodies (rabbit anti-mouse immunoglobulins) were obtained from DAKO. Working dilutions of APAAP-complexes and bridging antibody were 1:40.

Standard APAAP-Complex Technique

To be able to evaluate antigen expression of cells, we additionally from each case performed preparations to conventional APAAP procedure, as described previously by Cordell et al. 1984 [7]. The intensity of colour reaction of these internal controls

Table 1. CD cluster of differentiation

Monoclonal antibodies used for combined staining procedure (CSP)			
Antibodies CD no.	Cellular distribution of antigens	Working dilution	Source
CD2	T-cells; most NK-cells; (SRBC receptor, LFA-2 receptor, LFA-3 (CD58) ligand	1:100	DAKO
CD3	T-cells; (CD3 complex associated with T-cell receptor TCR -α/β or -γ/δ)	1:50	DIANOVA
CD7	T-cells; NK-cells; immature myeloid cell subsets (involved in T- and NK-cell activation)	1:50	DIANOVA
CD10	Pre-B cell subsets; B-cell subset; cortical thymocyte subset; granulocytes; (CALLA; neutral endopeptidase)	1:20	DIANOVA
CD13	Most cells of myeloid origin (gp 150; aminopeptidase N)	1:20	DIANOVA
CD14	Monocytes, macrophages, granulocytes, dendritic cells, B-cells; (PI-linked); Langerhans' cells; expression in AML but not in ALL	1:50	BD
CD19	Precursor B cells and B cells	1:20	DAKO
CD20	Precursor B cell subset; B cells; follicular dendritic cells	1:50	BD
CD24	B-cells; granulocytes and epithelial cells (PI-linked)	1:100	DIANOVA
CD34	Hematopoietic precursor cells; endothelial cells	1:20	BD
HLA-DR	B-lymphocytes, activated T-cells; monocytes macrophages, thymic epithelial cells, blast bells of acute myelogenous leukemia	1:20	DIANOVA
TdT	Most lymphoblastic leukemia and lymphoma; cortical thymocytes and a minor subset of bone marrow pre-lymphocytes	1:20	DAKO

can be compared with those maintained by the presented combined staining procedure.

Each incubation step of standard APAAP-method lasted 30 min, with 3 min phosphatase buffer (PBS) washes between each step. Negative control staining was performed by omitting the primary MoAb and substituting with buffer solution. In order to increase the labeling intensity, the incubation with the secondary antibody and the APAAP complexes was repeated. To inhibit irreversible endogenous alkaline phosphatase activity, 1 mM levamisole (Sigma Diagnostics) was added to the substrate-chromogen reaction (naphthol-AS-MX-phosphate / Fast Red TR–salt). Following indicator-reaction (10–20 min) preparations were counterstained using hematoxylin. During all incubation steps, slides were placed horizontally and all stages were performed in a plastic humidity chamber. Smears were mounted with Kaiser's glycerol gelatine (E. Merck) and examined microscopically.

Combined Staining Procedure (CSP)

For simultaneously demonstrating morphology and immunological profile of cells, firstly the prepared cell smears were processed applying standardized Pappenheim method. Panoptically stained smears were examined under an optical microscope (Olympus BH) and representative areas were selected and photographed. For the immunophenotyping procedure the smears were prepared as follows. In order to place selected MoAbs to defined areas on cell smears we applied our Silicone Chamber System. Subsequently smears were destained and fixed again. The APAAP technique was performed as described above. For best staining results all incubation steps were repeated. The primary MoAb was incubated at room temperature for 12 h. As a chromogenic substrate likewise naphthol AS-MX phosphate and Fast Red TR salt was used. After finishing the immunoenzymatic color reaction the previously specified cell areas on the smears were visulaized under an microscop and photographed again. Both pictures (photo 1: Pappenheim staining; photo 2: APAAP labeling) made from the identical cell area are compared and evaluated for morphological and immunological features.

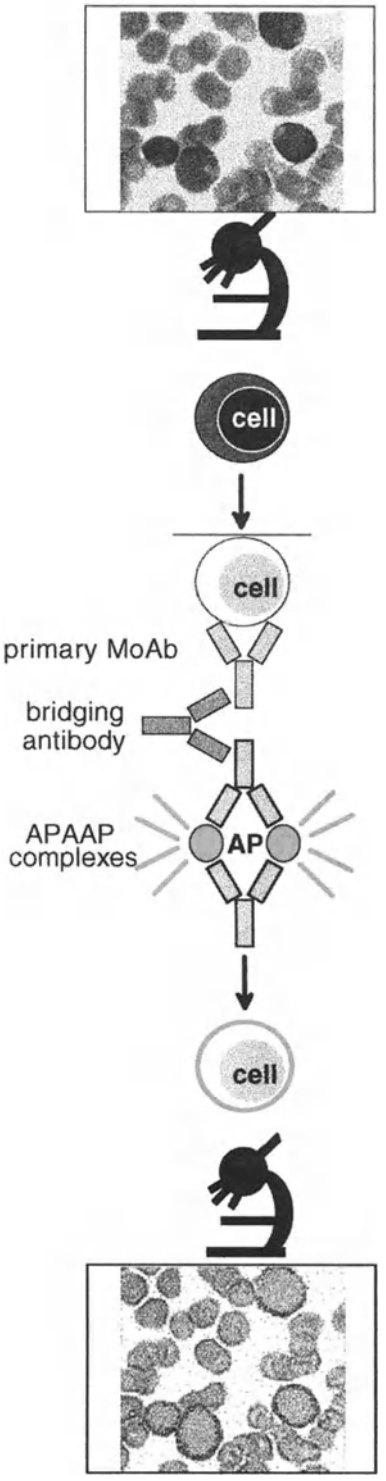
The standardized conditions of the presented combined staining procedure are given in detail in Figure 1.

Results

Peripheral blood and bone marrow samples from children suffering from different subtypes of acute leukemias as well as leukemia-derived cell lines were analysed. The APAAP complex technique was shown to be potential value for the immunological characterization of human leukemia since it could be applied directly to smears of blood and bone marrow cells [8, 10, 11, 14].

Fixatives

In immunochemistry fixation is an important aspect, for immunocytochemical techniques are always dependent on fixed tissues or cell preparations. Any fixation is shown to affect the results of the immunocolour reaction. Ideally a number of criteria should be fullfilled when incubating mainly organic solvents. The procedure of fixing should cause minimal denaturation of the antigens and on the other hand preserve morphology, too. In the present study we compared the intensity of the antigen expression performing standard APAAP omitting May-Gruenwald Giesma stain. Our results maintained by CSP in relation to the fixation are comprised in Table 2. Among different fixatives routinely applied in immunohistochemistry, methanol, acetone and PFA used exclusively, seemed not to be very suitable revising the topic question. The mentioned fixatives, separately inspected, resulted in significant loss of immunoreactivity of antigens. Although especially acetone is known to preserve of antigenic structure of most cells surfaces, it was not satisfactory in CSP. A combination of methanol and PFA immediately before immunolabeling preparations proved to the adequate for evaluating antigenic pattern.



May-Gruenwald-Giemsa staining ("Pappenheim"-procedure)

1st Photo - Documentation

Destaining / Fixation

APAAP-Complex-technique

2nd Photo - Documentation

Fig. 1. Combined staining procedure (CSP)

Table 2. Influence of different CSP conditions on the intensity of antigenic pattern of acute leukemic blast cells

Antigen	Conventional APAAP control reaction (fixation: aceton)	Combined staining procedure (CSP): May-Gruenwald-Giemsa / APAAP			
		Methanol	Acetone	PFA	Methanol/PFA
CD2	+++	+	+	+	+
CD3	+++	+	+	+	++
CD7	+++	±	++	+	++
CD10	++	−	−	−	±
CD13	−	n.t.	−	±	
CD14	++	n.t.	n.t.	+	±
CD19	++	−	±	+	+
CD20	++	−	±	±	+
CD24	+++	+++	+	+	+++
CD34	++	++	−	+	++
HLA-DR	++	−	n.t.	±	±
TdT	+	−	−	−	−

− negative +/− maybe positive or negative ++ strongly positive
\+ positive +/− very strong positive n.t. not tested

Immunocytochemistry

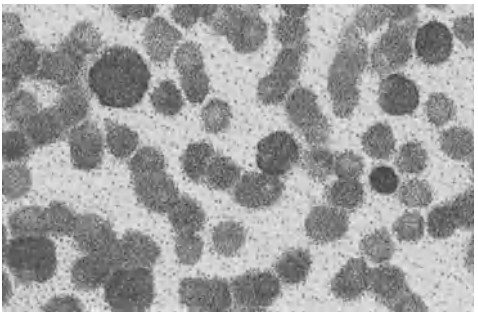
The application of monoclonal antibodies by APAAP-complex technique on cell smears allowed an accurate identification and characterization of leukemic blast cells [11]. A subtype associated marker profile of acute lymphoblastic leukemias was assigned initially by flow cytometry reviewed on routinely prepared smears with a highly sensitive immunoenzymatic labeling system (APAAP), firstly described by Cordell et al. [7]. Standardized APAAP technique at the same time served as a control in comparison to demonstrated combined staining procedure (CSP). The antigenic profile of the leukemic cells and intensity of color reaction was determined under an optical microscope. Antigen-positive blast cells were easily identified in blood and bone marrow smears and in cytospin preparations, since they showed a vivid red reaction. Antigenic refractivity in cell smears is still possible within 1 week after storage at room temperature or for at least 1 year at −20 °C or 4 years at −80 °C [8].

Combined Staining Procedure (CSP)

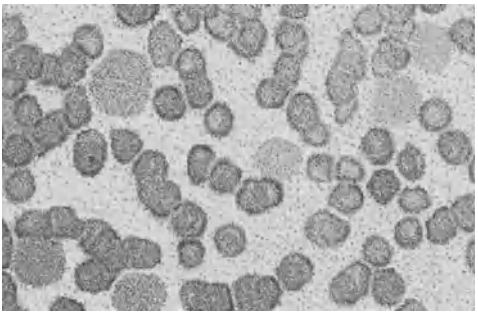
The present chapter reports an evaluation of simultaneous demonstration of morphological and immunological features of the blast cells on single cell level, based on the application of a combined staining procedure (CSP). The improved staining procedure was performed on routinely prepared air dried cell smears. The principle of the CSP we established is shown in detail in the flow-chart (Fig. 1).

Figure 2 exemplarily illustrates photographs of bone marrow smears from patients with common acute lymphoblastic leukemia. These pictures document our results to be found by the described CSP. The last picture demonstrates the control reaction immunolabeling cell smears by conventional APAAP system. The success of CSP depended on a multitude of different single parameters (fixative, density of antigens). The fixation procedure following the panoptical staining proved to be the most critical step in the demonstrated system. These effects are summarized in Table 2. The addition of usually applied fixatives (methanol or PFA) prior to immunostaining resulted in a reduction or loss of antigenic activity. Better results were partly retained by using acetone. No detection of antigens like CD10, CD13, CD34 and terminal transferase (TdT) was observed. It was surprising that approximately acceptable staining results were given by a combination of PFA and methanol. Nevertheless a

May-Gruenwald-Giemsa procedure

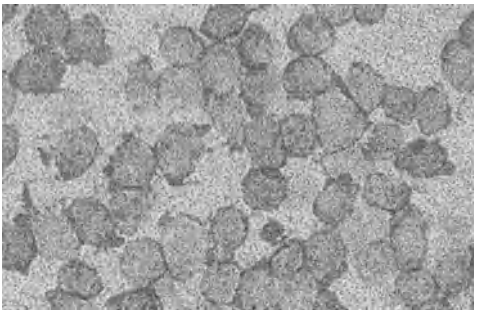


cALL; bone marrow smear:



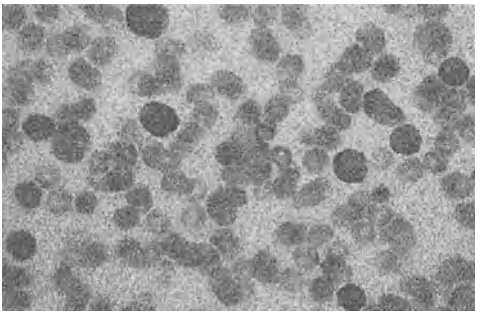
cALL; bone marrow smear:

conventional APAAP-procedure:

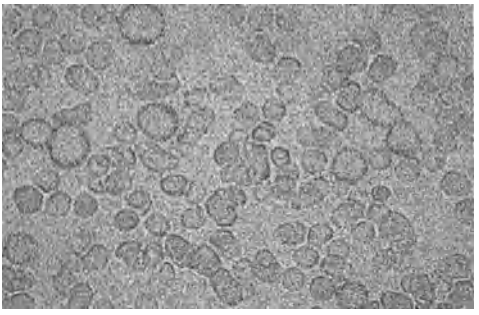


cALL; bone marrow smear:
labeling of leukemic blast cells: CD24

Immunological detection of antigens



labeling of T-lymphocytes: CD7



labeling of leukemic blast cells: CD19

Fig. 2. Demonstration of staining results providing combined staining procedure (CSP)

more moderate colouring in contrast to the conventional APAAP-technique was unavoidable. Performing the proposed schedule, the immunoenzymatically detection of TdT, an intracellular located antigen, wasn't possible till now.

No staining results were seen by changing the sequence of CSP schedule, because no acceptable panoptical stain was reached.

Discussion

According to the French-American-British (FAB) classification system panoptical staining procedures usually enable the classification of acute leukemias, based on morphological and cytochemical criteria. Usually the information maintained by these techniques is sufficient to discriminate between

ALL and AML. Especially atypical leukemias as well as very undifferentiated leukemic blast cells (e.g., AML-M0, AML-M7) often cannot be classified definitely. Additionally immunological phenotyping is provided today, particularly in acute lymphoblastic leukemias (ALL) to subclassifity these malignancies.

Likewiese the infiltration of minimal residual tumor cells (i.e., MRD) in bone marrow in hematological complete remission is very difficult to identify by morphological and immunological criterion alone. A combination of these both methods may close this diagnostical gap and improve the diagnostic accuracy in selected cases. It is suggested that immunotyping, when added to morphological assessments, provide additional information about the nature of the leukemia.

To reveal antigens that constitute important diagnostic markers we studied the reliability of our established CSP. Some modifications of the immunostaining were necessary to obtain an efficient result. It has been suggested that the fixation procedure is the key element in the combined visualization of morphological and immunological aspects. Among different fixatives examined, PFA, following destaining procedure using a short time incubation with methanol, proved to be adequate for immunologic staining. Nevertheless fixation is always a comprise and the requirements of an ideal and adequate fixative vary according to the different antigens, staining techniques and topics. For quantitative measurements of cell-surface antigen expression, however, the precise effects of the fixatives on the detection of the studied antigen should be determined for each antigen individually. To reach sufficient staining results, with CSP it is absolutely indispensable to strictly keep the sequence of the reaction schedule.

The CSP provides the possibility to analyse different cells from peripheral blood, bone marrow or from culture simultaneously for hematological characteristics and immunological cell marker profiles. A sequence of staining procedures maintaines components of the May-Gruenwald Giemsa staining followed by the highly sensitive APAAP-complex system.

Further studies, performed on air dried cell smears or cytospin preparations from acute leukemias and other hematological disorders, are underway in our division in order to detect the expression of additional relevant markers using the combined staining procedure.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Anomalistic Signaling as a Possible Biochemical Explanation for Discordant Maturation in Chronic Myelogenous Leukemia (CML)

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Abstract. The 9;22 chromosomal translocation characteristic of CML results in a fused bcr/abl gene and an abnormal fusion protein, $p210^{bcr/abl.}$ Relative to normal c-abl, $p210^{bcr/abl}$ has elevated tyrosine kinase activity that is essential for its transforming activity. We recently reported a prominent 62 kDa GAP-associated P-tyr protein and 5 additional consistent but less prominent P-tyr proteins as well as 5 more minor P-tyr proteins that are constitutively tyrosine phosphorylated in primary primitive lineage negative (lin) chronic phase CML blasts but not in comparable primary lin-normal blasts. The GAP-associated p62 protein has now been purified, sequenced and its gene has been cloned; it is a previously unidentified protein and is currently being characterized.

In analyzing P-tyr proteins in primary lin- normal blasts in response to various hematopoietic cytokines, we found a striking similarity in the tyrosine phosphorylation of 4 major and 3 minor proteins after stimulation with c-kit ligand (KL) and the P-tyr proteins that are constitutively phosphorylated in primary primitive lin- chronic phase CML blasts. Other cytokines tested (ie, GM-CSF, G-CSF, IL-3, FLT3 ligand, TPO, EPO) were much less active or stimulated phosphorylation of other proteins. KL/c-kit and bcr/abl have some similar activities including enhancing survival and expansion of hematopoietic progenitor cells, probably acting primarily on early progenitors at the time of lineage commitment rather than on self-renewing stem cells.

Activation of growth factor receptors promote a cascade of protein phosphorylations that can ultimately result in a wide range of cellular responses. Sustained activation of discrete signaling pathways in some types of cells results in differentiation, whereas transient activation instead causes a proliferative response; in other cell types, the converse is true. It may be postulated that stem cells and primitive progenitors are at a particularly susceptible stage of development that renders them especially responsive to sustained bcr/abl-induced phosphorylation of a number of signaling proteins that are components of critical regulatory pathways, including c-kit. The affected pathways control and coordinate multiple diverse cell processes including proliferation, differentiation, maturation, and apoptosis, processes that are normally tightly regulated and integrated. Perturbation of these key pathways in primitive progenitors would be expected to seriously disrupt orderly hematopoiesis and could also explain the multiple subtle pleiotropic biological abnormalities charac-

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teristically observed in later maturing CML compartments that we have collectively designated "discordant maturation." The true situation is undoubtedly very complex and involves interaction of multiple cytokines and signaling pathways that we are now trying to define. Constitutive down-stream activation of critical pathways in susceptible early progenitors that normally require KL or other factors for activation could explain most if not all features of the disease.

Introduction

Ever since chronic myelogenous leukemia (CML) was first described as a specific disease entity 150 years ago [1-5], various theories have been proposed to explain the most prominent feature of the disease, namely expansion of the myeloid compartment and especially of the granulocytic lineage.

In the ensuing years, CML was gradually distinguished from other types of leukemia and other myeloproliferative diseases on the basis of differing clinical and pathological features, but the first real clue as to its pathogenesis was the landmark discovery in 1960 of an abnormally small chromosome in the leukemic cells, thereafter designated the Philadelphia (Ph) chromosome [6-9]. A decade later the Ph chromosome was identified as a modified 22 chromosome [10], and a few years later Rowley showed that the Ph anomaly resulted from translocation of the distal segment of the long arm of chromosome 22 to the distal portion of the long arm of chromosome 9 [t(9;22) (q34; q11)] [11]. In this translocation, the c-abl oncogene is transposed from its normal position on chromosome 9 (q34) to a 5.8 kb major breakpoint cluster region (M-bcr) on chromosome 22q11, forming a fusion bcr/abl gene [12-15]. The bcr/abl gene transcribes a novel 8.5 kb mRNA which in turn encodes a p210 kDa protein which, unlike the normal c-abl protein, is phosphorylated on tyrosine, and has increased intrinsic protein tyrosine kinase activity compared to that of the normal c-abl protein [16, 17]. Because the tyrosine kinase activity of the Abelson murine leukemia virus product, $p160^{v\text{-}abl}$, is known to be necessary for cellular transformation [18], it was suggested that the $p210^{bcr/abl}$ tyrosine kinase may have an important role in the pathogenesis of CML [17, 19-21]. A great deal of experimental data have now accumulated lending support for this suggestion [22-26].

Three major forms of BCR-ABL fusion proteins are now recognized, and it appears that the inclusion or exclusion of BCR exons is largely responsible for determining the disease phenotype caused by these proteins [27, 28]. The smallest fusion protein, $p190^{bcr/abl}$ (m-bcr breakpoint), predominantly causes acute lymphoblastic leukemia (ALL) and is only rarely associated with CML, AML or other diseases. $p210^{bcr/abl}$ (M-bcr) is the commonest fusion protein and most frequently causes classical CML but can also be associated with ALL, AML (usually FAB M4 or M5) or rarely other diseases such as essential thrombocythemia. The largest BCR-Abl fusion protein, $p230^{bcr/abl}$ (μ-bcr), includes over 90% of BCR amino acids, lacking only the C-terminal two-thirds of the GAP^{rac} domain, and appears to cause chronic neutrophilic leukemia (CML-N), a rare indolent myeloproliferative disease [28-31].

The evidence is now quite compelling that CML is a clonal neoplastic disease resulting from an acquired mutation in a single multipotent hematopoietic stem cell [15, 24, 32-37]. The bcr/abl fusion gene is probably the usual sole initial causative event and is responsible for all the manifestations of the early chronic phase of the disease [38, 39]. The primary 9;22 translocation somehow induces further genetic instability, and subclones with additional secondary genetic abnormalities arise. One or more of these subclones, which have lost their capacity to mature, eventually becomes dominant, giving rise to the accelerated or blastic phase of the disease [40].

Theories to Explain Myeloid Expansion

While the underlying genetic abnormality in CML is now quite well defined, the critical biochemical aberrations caused by $p210^{bcr/abl}$ that can result in the expansion of the myeloid compartment are still largely

unknown. Until the middle of the present century, the original suggestion that CML might be some type of "suppurative" or infectious disease was seriously considered; as recently as 1947, the leading *Textbook of Medicine* was still hedging as to whether leukemia was an infectious or neoplastic disease [41]. The seminal observations that viruses can cause tumors in chickens were made over 85 years ago [42, 43], and, beginning in the 1950s, the infectious hypothesis was revitalized in a modified form with the demonstration that viruses can cause cancer in rodents, cattle, cats and other animals [44-47] and by numerous reports of the occurrence of "clusters" of human leukemia [48, 49]. However, despite an intensive worldwide search and the clear demonstration that both DNA and RNA viruses can induce leukemia and other neoplasms in experimental systems [50-54], no virus has yet been identified that causes spontaneously occurring human leukemia; moreover, extensive epidemiological studies have failed to validate that leukemic clusters have an infectious origin, or even to provide convincing evidence that they do not occur merely by chance.

Other theories that have been proposed over the years to explain the myeloid expansion in CML have been more rapid proliferation of the leukemic cells, unregulated proliferation or increased self renewal of Ph-positive stem and/or progenitor cells [55, 56], prolonged life span of CML leukocytes [57-59] and, more recently, reduced cell death by apoptosis according to some [60, 61] but not all investigators [62, 63].

Before methods were available to quantitatively measure cell kinetic parameters, most observers assumed as an article of faith that the leukemic cells proliferated more rapidly than their normal counterparts. However, essentially all kinetic studies performed during the chronic phase of CML have shown that, on the average, while the DNA synthesis time of both blood and marrow myelocytes in CML is about the same as that of normal marrow granulocyte precursors [64, 65], the leukemic precursors have lower mitotic indices, a smaller fraction of cells in DNA synthesis, longer generation times, and longer transit times in the maturation compartments than do comparable normal precursors (reviewed in [39]). However, the proliferative parameters return towards normal when the leukocyte count is reduced by treatment [66, 77] and no consistent difference in proliferation between normal and CML progenitors are found in vitro [39, 68, 69]. Thus it is likely that the slower proliferative parameters found in vivo are probably related to the high cell density of the CML marrows and that the cell cycle parameters are similar at comparable cell densities.

Life Span and Programmed Cell Death

Using a variety of isotopic labeling procedures, it has been found consistently that the mature granulocytes in chronic phase CML have a markedly slower rate of disappearance from the blood than do normal mature granulocytes [57-59, 70-77]. Interpretation of the slow granulocyte disappearance rate in CML is confounded by the presence of many circulating immature granulocytes, by the abnormal granulocyte traffic and distribution patterns, and by the premature release of incompletely mature segmented neutrophils into the blood from the marrow and spleen in CML [38, 39, 67, 73-81]. Although the complexity of the system and limitations of the data do not permit calculation of precise values, these studies strongly suggest that CML mature granulocytes have a significantly increased life span, but that this is not of sufficient magnitude to account solely for the huge expansion in the granulocyte mass in CML and that therefore production of granulocytes must also be substantially increased.

Once committed to differentiation, all hematopoietic cells have finite life spans and normally undergo programmed cell death at prescribed times depending on the lineage and environmental factors [82-85]. There are numerous reports demonstrating that apoptosis is inhibited under a variety of conditions in cell lines expressing $p210^{bcr/abl}$ [86-92], in v-abl transfected cells with activated tyrosine kinase activity [93], as well as in progenitors and granulocytes obtained directly from CML patients [60]. Bedi et al. [60,

61] have suggested that reduced programmed cell death may be the primary mechanism responsible for expansion of the leukemic clone in CML. On the other hand the older cytokinetic data cited above strongly suggests that prolonged life span cannot solely account for the expansion of the CML population, and moreover, other reports have not supported apoptosis as being the main mechanism responsible for the myeloid expansion [62, 63].

Morphologic, Biochemical and Functional Changes

It must also be recognized that delayed apoptosis alone cannot explain all the other abnormal features that have been observed in CML such as the aberrant lineage distribution, frequent basophilia, asynchronous maturation of the nucleus and cytoplasm [94-100] and the increased incidence of dysplastic changes that have been observed in CML cells during the chronic phase. Rare but conspicuous dysplastic changes in CML include hyposegmentation or an acquired Pelger-like anomaly of neutrophils [101-103] hypogranulation of neutrophils [103], neutrophils with ring-shaped nuclei [104]; multinuclearity of erythroblasts [103], large mononuclear forms of megakaryocytes, and both immature and mature chimeric granulocytes containing mixed basophil/eosinophil granules [105, 106]; according to Mlynck et al, such bigranulated cells are not found in normal subjects and are thought to demonstrate lineage infidelity in CML. As the disease undergoes progression to the blastic phase, the incidence and severity of the dysplastic changes increases and the cells develop additional abnormalities such as micromegakaryocytes [103, 107]. In the chronic phase of the disease the leukemic cells retain the capacity to differentiate almost normally, and the biochemical and functional defects exhibited by the leukemic cells are not of sufficient severity to prevent them from carrying out their essential functions in supporting life in the absence of normal mature cells. Nevertheless, in addition to the kinetic and dysplastic changes noted above numerous subtle biochemical and functional abnormalities of the leukemic cells have been described in CML which have been summarized in several recent reviews [38, 39, 108, 109]. Most of these abnormalities appear to be mutually linked, are quantitative rather than qualitative, and tend to return towards normal when the disease is brought into hematologic remission by treatment; many of the abnormalities may simply reflect the premature release of incompletely mature marrow bands and polymorphs into the blood in CML.

Discordant Maturation Hypothesis

In attempting to correlate the molecular, biochemical and biological changes induced by the bcr-abl protein, it is important to recognize that the biological changes are pleiotropic, usually quite subtle, often interconnected, and that in the aggregate, they cannot all be explained by simply focusing on one mechanism such as dysregulation of the cell cycle, reduced programmed cell death, increased self-renewal of stem cells, or impairment of any single property such as cell adhesion, anchorage-dependence, or responsiveness to natural inhibitory factors. The signaling pathways controlling apoptosis, differentiation, maturation, proliferation and other cell processes are intimately interrelated, and considered in this context, it is quite reasonable to expect that the biological abnormalities would be both pleiotrophic and mutually linked. We have reviewed the multiple biological abnormalities in CML cells that have been well-described in some detail in recent publications and have collectively designated these abnormalities discordant maturation [38, 39, 109, 110]. If the discordant maturation hypothesis is indeed valid, it should provide a comprehensive explanation for all of the consistent major biological abnormalities that characterize the disease. Our prior studies have shown that increased cell production in the intermediate and later maturation compartments is responsible for a major portion of the myeloid expansion in CML, but that the initial expansion originates in a primitive progenitor cell compartment [38, 39, 68, 69, 109-113]. In this brief review we will show representative experimental data to illustrate

some of the most prominent and consistent biological abnormalities in CML, and then present a tentative biochemical explanation that can account for all the abnormalities including the enormous myeloid expansion.

Cytokinetic Studies Conducted in Vivo

We will first show two ^{3}H-thymidine (^{3}HTdR) continuous labeling experiments conducted in vivo in two patients, one in chronic phase and the other with Ph$^+$ ALL during early relapse from a brief hematologic remission. Both these studies have previously been reported in detail and the key results are presented here in summary form [74, 114, 115].

The first patient was a previously untreated 16 year old young woman who presented with progressive weakness and abdominal discomfort, splenomegaly, WBC 175 000/μl, platelets 800 000/μl, and hemoglobin 8 g/dL Fig. 1. A sternal aspirate showed a hypercellular marrow, a differential consistent with chronic phase CML (1% blasts), and Ph$^+$ marrow metaphases. Prior to any treatment, a continuous intravenous infusion of ^{3}HTdR was given for 10 days. She was then treated with splenic irradiation, following which she had a hematologic remission lasting 8 months before her disease relapsed, still in chronic phase.

The results of the ^{3}HTdR autoradiographic study are shown in Fig. 2. The pulse ^{3}HTdR labeling index (LI) of the marrow blasts determined in vitro was ~20% (not shown). The labeling patterns and labeling intensities of granulocyte precursors and mature cells in the marrow, spleen, and blood were

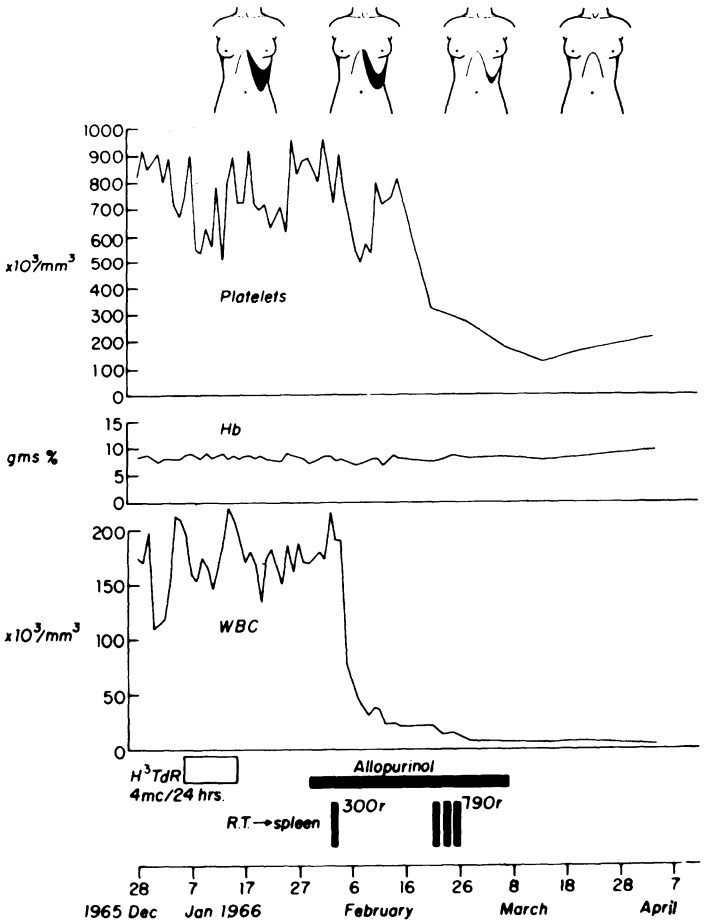

Fig. 1. Clinical course of a 16-year-old female patient with newly diagnosed chronic phase CML [74]

quite similar during the in vivo infusion of ^{3}HTdR. Measurement of additional kinetic parameters indicate that the rates of cell production were similar in the spleen and marrow, that there was continuous cell traffic between those 2 organs, and that cells rarely divided in the blood. All of the granulocyte precursors were labeled in all three compartments by the end of a week and 99-100% of the mature granulocytes were labeled by the end of the 10-day infusion or a few days later. This study demonstrates that essentially all of the recognizable granulocyte precursors are actively proliferating and is unlike the situation in most patients with acute leukemia in whom significant numbers of blasts may remain dormant for many weeks or months [80, 81, 114-122].

The kinetic data in this and other CML patients also demonstrate that the CML precursors generally proliferate more rapidly than in acute leukemia but more slowly than the corresponding normal precursors when the marrow is densely populated, although the proliferative parameters of normal and CML precursors are similar at comparable cell densities. The emergence times of labeled non-dividing CML granulocytes (i.e., metamyelocytes, bands and segmented neutrophils) are very similar to comparable normal cells, indicating that the minimum maturation time is approximately normal in CML [74]. However, the disappearance times of labeled cells and the median grain count halving times after discontinuing exposure to ^{3}HTdR (which reflects the median generation or intermitotic time) are generally longer than normal [74]. This is consistent with other cell labeling studies noted earlier and indicates that CML cells have a longer life span than comparable normal cells.

The second illustrative study was carried out in another young woman who presented with Ph+ acute leukemia. She was asympto-

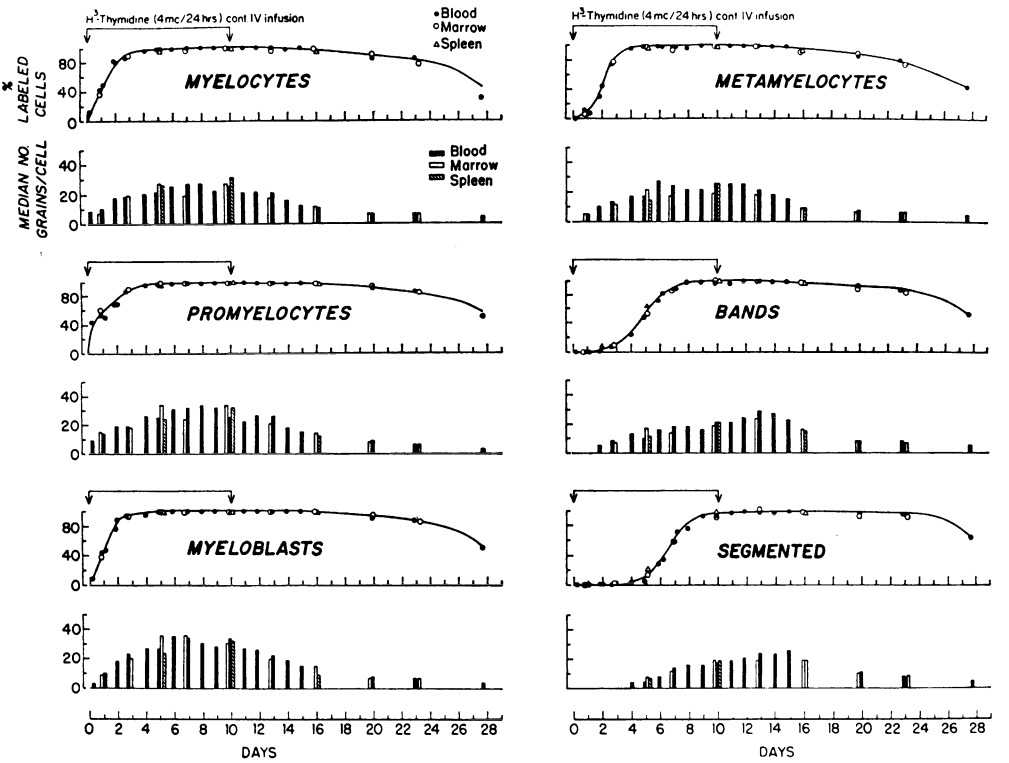

Fig. 2. Labeling pattern of granulocyte precursors and mature granulocytes in a young woman with newly diagnosed chronic phase CML during and after a continuous intravenous infusion of ^{3}H-thymidine for 10 days. *Segmented* includes all polymorphonuclear neutrophils with 2, 3 and 4 segments [74]

matic until about a month prior to admission when she developed progressive weakness, anorexia, night sweats, exertional dyspnea, abdominal discomfort, and cutaneous ecchymoses. On admission she had a fever of 102 °F, a massively enlarged spleen, slight hepatomegaly, a large mediastinal mass, sternal tenderness and scattered ecchymoses. Her hematologic parameters are shown in Fig. 3. Following treatment with vincristine and prednisone she had a nearly complete hematologic remission except for slight persistent splenomegaly which lasted about 6 months when relapse occurred rapidly. Karyotypic analysis of the marrow cells both at presentation and relapse showed that the majority of metaphases had a Ph chromosome and about half of the cells had additional chromosomal abnormalities, not specified. Unfortunately no chromosome studies were performed during remission so it is not known how many karyotypically normal cells were present in the marrow prior to the first relapse.

The pulse ^{3}HTdR labeling indices (LI) of the marrow lymphoblasts (in vitro) at presentation and again after full relapse (10/28) were consistently low (4.8 to 5.9%) whereas immediately after induction of remission when the marrow only contained 1.5% blasts (4/22), the LI had risen to 23% (Fig. 3). Based on their morphological features as previously described [114], 10.8% of the 1.5% "blasts" on 4/22 were classified as leukemic lymphoblasts and their LI was 5.5% whereas the LI of the other 89.2% "normal" blasts was 26%; most of the latter appeared to be myeloblasts with possibly some early pronormoblasts.

After induction of remission, the patient was treated with a variety of drugs [114]. The first evidence of relapse was noted on 10/7 when 15% blasts were found in the marrow, most of which appeared to be leukemic; the most recent previous marrow on 9/17 had only 1% blasts. After relapse was first detected on 10/7, the disease progressed quickly; the spleen enlarged rapidly, and numerous blasts appeared in the marrow and blood (Fig. 3). A continuous intravenous infusion of ^{3}HTdR was started on 10/13 and continued for 8 days. No treatment was given for 7 days after ending the infusion when vincristine and prednisone were started, the latter initially in a dosage of 100 mg/day. Except for arresting the rising number of circulating blasts there was no noticeable improvement, and it was not until the dose of prednisone was increased to 5 g/day followed by arabinosylcytosine and splenic irradiation that a second remission was reinduced. This only lasted 3 months; there was less and less response to subsequent treatment and the patient died as a result of extensive leukemic infiltration and multiple complications in November 1965, 13 months after her first relapse.

The results of the ^{3}HTdR continuous infusion study during her first relapse are summarized in Fig. 4. The ^{3}HTdR infusion was started on 10/13, 6 days after reappearance of increased blasts was first noted in the marrow. Essentially all of the blasts in mitosis were labeled after one day, but, consistent with their lower fraction in S phase (i.e., in vitro pulse ^{3}HTdR LI = ~5.6% vs ~20% in previous patient), the interphase leukemic blasts labeled more slowly than the blasts in the chronic phase patient shown earlier in Fig. 2. Nevertheless, after 8 days, 93% of the marrow blasts, 95% of the circulating blasts, and 87% of the blasts in the spleen were labeled. In contrast to the relatively slow labeling of the leukemic blasts, the remaining erythroblasts labeled very rapidly, even as the majority of erythroblasts were rapidly disappearing as the marrow quickly filled with leukemic blasts (Fig. 4); the labeled erythroblasts also disappeared much more rapidly than the leukemic blasts after ending the infusion [115].

Key cytokinetic parameters of the leukemic blasts and erythroblasts are compared in summary form in Table 1. Virtually all of the surviving red cell precursors were cycling very rapidly with an estimated average intermitotic time (i.e., median grain count halving time) of 19-27 h compared to 85-95 h for the leukemic blasts [114, 115]. After vincristine and prednisone was started the latter increased to 195 and 190 hours in the marrow and blood, respectively; the effect of this treatment on the erythrocyte precursors could not be measured since they had already lost their label.

It is not known for certain whether the erythrocytes and granulocytes were normal

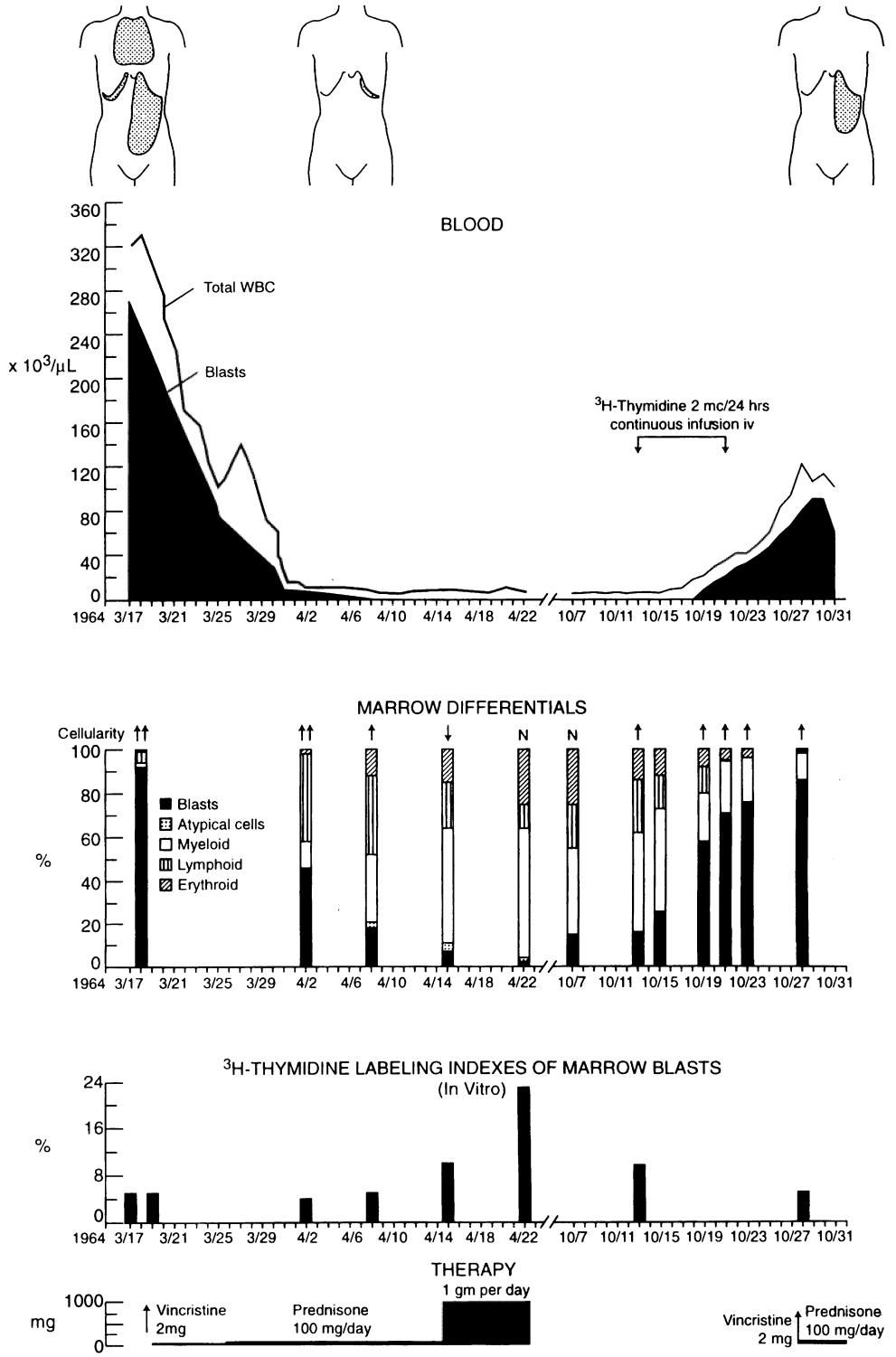

Fig. 3. Clinical course of a newly diagnosed young woman with Ph⁺ ALL at her initial presentation and at time of her first relapse [114]

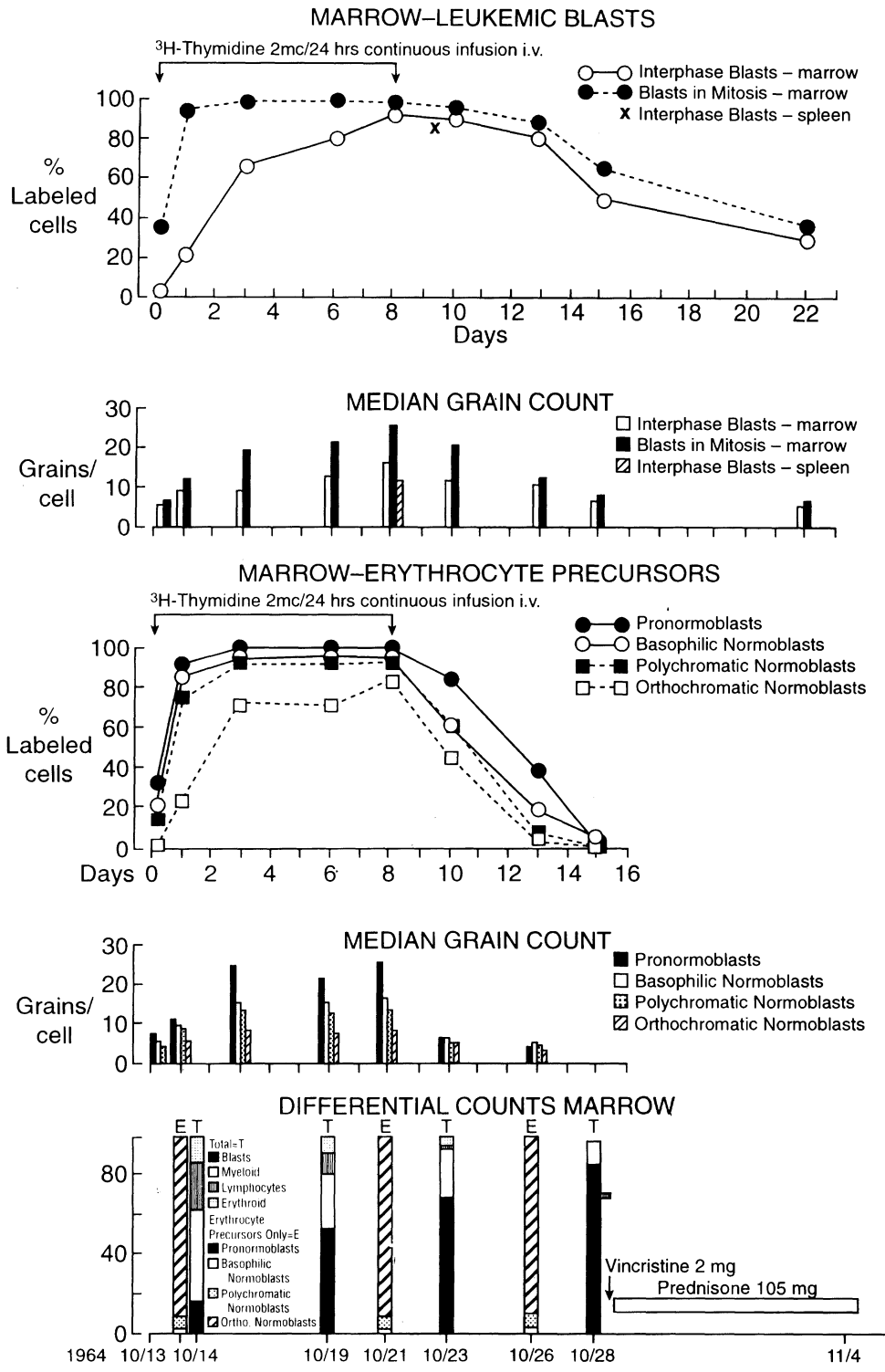

Fig. 4. Comparison of labeling pattern of leukemic blasts and erythrocyte precursors in patient with Ph^+ ALL shown in Fig. 3. during and after continuous infusion of 3H-thymidine at time of first relapse [114, 115]

Table 1. Comparative measurements of cytokinetic parameters of leukemic blasts and morphologically identifiable erythrocyte precursor cells during and after ^{3}H-thymidine (^{3}HTdR) continuous intravenous infusion

	Pulse ^{3}H-TdR L1 (in vitro) %	Maximum ^{3}H-TdR L1 (at end of infusion in vivo) %	Maximum median grain count (at end of infusion in vivo)	Median grain count halving (after end of infusion in vivo) (h)	Distribution E1:E2:E3:E4 cells
Leukemic (Marrow)	6	93	16.5[a]	95	–
Blasts (Blood)	3	95	15	85	–
Proerythroblasts = E1	100	100	26[a]	19	1
Basophilic Erythroblasts = E2	88	95	17[a]	24	2
Polychromatophilic erythroblasts = E3	89	96	14	27	14
Orthochromatic erythroblasts = E4	6	85	9	27	180

[a] The pronormoblasts were larger than the leukemic blasts (mean nuclear area 144 versus 114 μ^2), while the basophilic normoblasts were smaller (mean nuclear area 78 vs. 114 μ^2)

or belonged to a chronic leukemic clone in this patient, but we suspect they were normal. The experiment was performed before methods had been developed to permit accurate identification of specific chromosomal rearrangements or other abnormalities in subpopulations of marrow cells [10, 40]. Ph$^+$ ALL had not yet been defined as a disease entity [123] and it is of course also unknown whether the Ph chromosome was due to a M-bcr or m-bcr breakpoint. Whereas one would expect the erythrocyte and granulocytic lineages to be part of the leukemic clone if the disease was a blastic phase of CML with an M-bcr breakpoint [32,34], de novo Ph$^+$ ALL is heterogeneous with respect to the level of commitment of the target cell, both in M-bcr and m-bcr cases [124-131]. In some cases a multipotent stem cell is the target and the myeloid lineages are involved, whereas in others the Ph chromosome is restricted to the lymphoid (usually pre-B or C-ALLA) lineage. Paradoxically lymphoid-restricted cases appear to have a worse prognosis than those arising in a pluripotent stem cell (although both are poor), whereas the breakpoint does not seem to affect prognosis [128,130].

Granulocyte kinetics were also studied [115] (not shown). There were 47% granulocytes (myelocytes and later forms) on 10/13 at the start of the ^{3}HTdR infusion, but like the erythrocyte precursors they rapidly decreased to 12% by 10/28. Their labeling pattern was similar to that shown in the first patient including the emergence times of labeled bands and polys and the median grain count halving times of myelocytes (105 hours) and metamyelocytes (90 hours). While these intermitotic times are similar to that of the leukemic blasts, in contrast to its effect in prolonging the grain count halving times of the leukemic cells and normal lymphocytes (not shown), prednisone had little effect on the halving time of the granulocyte precursors [115].

Regardless of whether the erythrocyte and granulocyte precursors were normal or part of an (unrecognized) chronic phase leukemic clone, these data provide a striking illustration of how quickly a relatively slowly proliferating leukemic blast cell population is capable of dominating a granulocyte precursor population growing at about the same rate, as well as replacing a much faster growing erythrocyte precursor population. The high ratio of E1:E4 cells (Table 1) probably reflects curtailment of production beginning at the earliest committed erythroid progenitor cell as a result of inhibition by the rapidly increasing leukemic blasts [115],

rather than an increased number of divisions occurring during maturation as happens in microcytic (e.g., iron deficiency) anemia [38, 109, 112]. The mechanism of inhibition is unknown, but erythropoiesis was arrested so abruptly that it likely reflects a potent direct effect of acute leukemic blasts in suppressing erythropoiesis rather than a differential response to more generally acting inhibitory cytokines such as TGF- or MIP-1 [132].

The two in vivo kinetic studies summarized in Figs. 2 and 4 illustrate several important points about the behavior of CML cells. Chronic phase CML granulocyte precursors have similar cell cycle times but a lower growth fraction, at least at higher cell densities, than corresponding normal precursors. During acute transformation one or more subclones arrested at the blastic stage with an even lower growth fraction are capable of rapidly replacing normal or CML chronic phase precursors. The CML chronic phase precursors are capable of fully maturing and their rates of maturation are similar to normal. The mature granulocytes have a longer life span than normal but this is not sufficient to account for the huge myeloid expansion in CML. Because of obvious limitations to the types of experiments that can be conducted in vivo, it is necessary to turn to in vitro studies to examine finer differences in the biological behavior of the leukemic and normal cells.

Short-Term in Vitro Clonogenic Studies Comparing Normal and CML Progenitors

Although hematopoietic cells do not develop entirely normally in any liquid or semi-solid culture conditions so far devised, their growth and maturation in short-term cultures are sufficiently comparable to that in vivo to provide much valuable information. We will summarize some representative results of our in vitro studies comparing the behavior of normal and CML progenitors to illustrate the most prominent and consistent abnormalities that have been observed [38, 39, 68, 69, 74, 81, 109-113]. As noted in the original papers, our findings are generally consistent with those of many other investigators.

Total GM Progenitors

The main hematologic parameters in 4 newly diagnosed, previously untreated patients with CML in chronic phase are summarized in Table 2. Patient #1 had the least and patient #4 the most advanced disease while the other two patients were intermediate. All marrow metaphases examined were Ph^+ and no additional cytogenetic abnormalities were noted. We compared the clonogenic data in these 4 patients with those of 6 healthy normal volunteers who had entirely normal hematologic parameters; the cell counts of these normal marrows were similar (mean = 74×10^9/l) so that the cellularity of the CML marrows ranged from 2.9 x normal in patient #2 to 5.6 $\times$ normal in patient #4. The methods employed and the results have previously been reported in detail, and we will just present some representative examples for illustrative purposes here [39, 113].

Figure 5 shows the 3- and 14-day cloning data in these 6 normal subjects and 4 CML patients for the granulocyte/monocyte

Table 2. Hematologic parameters of previously untreated CML patients in chronic phase at time of study

Patient no. age/sex	WBC count ($X10^9$/l)	Platelet count ($X10^9$/l)	HGB (g/l	HCT (%)	PB blasts (%)	Spleen size (cm below costal margin)	Marrow cell count ($X\ 10^9$/l)	Marrow blasts (%)	Marrow cytogenetics Ph^+/Total metaphases	
no. 1 42/M	26	229	14.4	45	0	0	255	1.3	18/18	46, xy, t(9q;22q)
no. 2 28/F	54	423	13.2	39	0	1	217	3.0	23/23	46, xx, t(9q;22q)
no. 3 41/M	80	243	13.0	40	0	0	228	2.6	25/25	46, xy, t(9q;22q)
no. 4 24/M	496	521	8.2	32	5	Huge, below pelvic brim	411	1.2	8/8	46, xy, t(9q;22q)

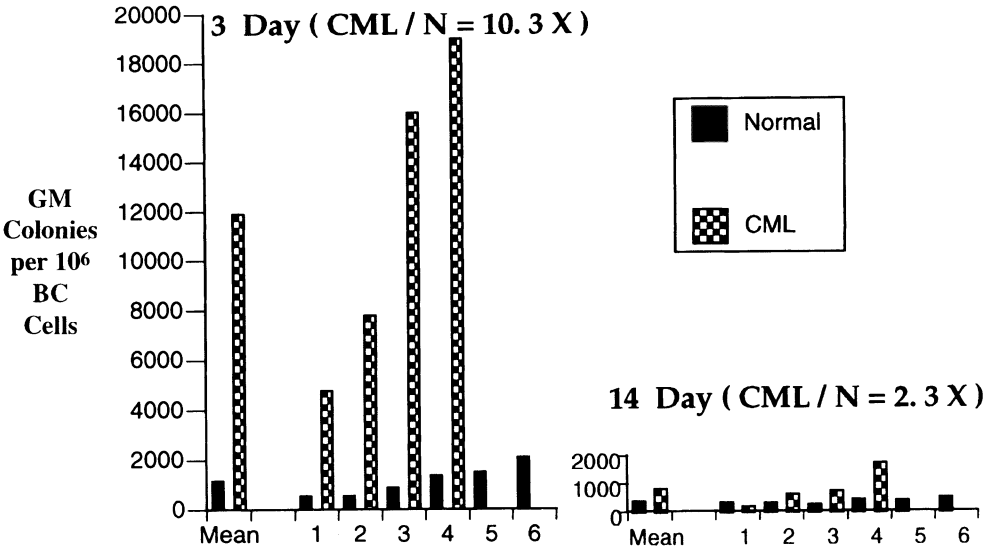

Fig. 5. Comparison of 3- and 14-day cloning data for 6 normal subjects and 4 newly diagnosed CML patients listed in Table 2 for total GM progenitors per 10^6 marrow buffy coat (BC) cells plated [39, 113]

(GM) progenitors per 10^6 marrow buffy coat cells. As we have previously shown, the light density fraction of both normal and CML marrow buffy coat cells contain essentially all of the progenitors capable of forming CFU-GM and BFU-E colonies of any size, so this fraction was used for assaying the total progenitor population. The CML marrows produced on the average 10.3 × as many 3-day colonies as the normal marrows per million light density buffy coat cells, but only 2.3 × as many 14-day colonies. If one calculates the number of colonies per ml of marrow based on the marrow cell counts in individual CML patients and normal subjects, the differences are even more striking: The 3 and 14 day CML/normal ratios are respectively 43× and 10×.

Comparison of the sizes of the GM colonies produced by normal and CML progenitors provides additional information on differences in their biological properties. Because none of the 3-day GM colonies, either normal or CML, contained more than 20 cells, the data shown in Fig. 6 shows the mean number of 7- and 14-day colonies arranged according to size. While the CML progenitors produced 5.3× and 2.3× the total number of GM colonies per 106 buffy coat cells as the normal progenitors at 7 and 14 days respectively, the normal progenitors produced about the same number of colonies containing over 100 cells at both time points. If one assumes that all 14-day colonies arose from 3-day colonies that continued growing (Fig. 5), it can be calculated that 21.4% of the normal 3-day colonies grew to >100 cells at 14 days whereas only 1.8% of the CML 3-day colonies did so [113].

Enriched GM Progenitors

During the past 15 years we have used various negative selection and velocity sedimentation procedures to partially or highly enrich subpopulations of normal and CML progenitor cells in order to compare their proliferative characteristics and other properties and determine how they might differ from the total progenitor populations [68, 69, 109-112, 133-135]. The progenitors are enriched by negative selection using panels of monoclonal antibodies to remove cells committed to differentiation along any of the major lineages; the enriched lineage-negative (lin-) blast population usually comprises about 0.1-0.7% of the initial marrow buffy coat cells and consists almost entirely of Type I blasts plus a few Type II blasts or very early promyelocytes. In some experiments the enriched progenitors were further

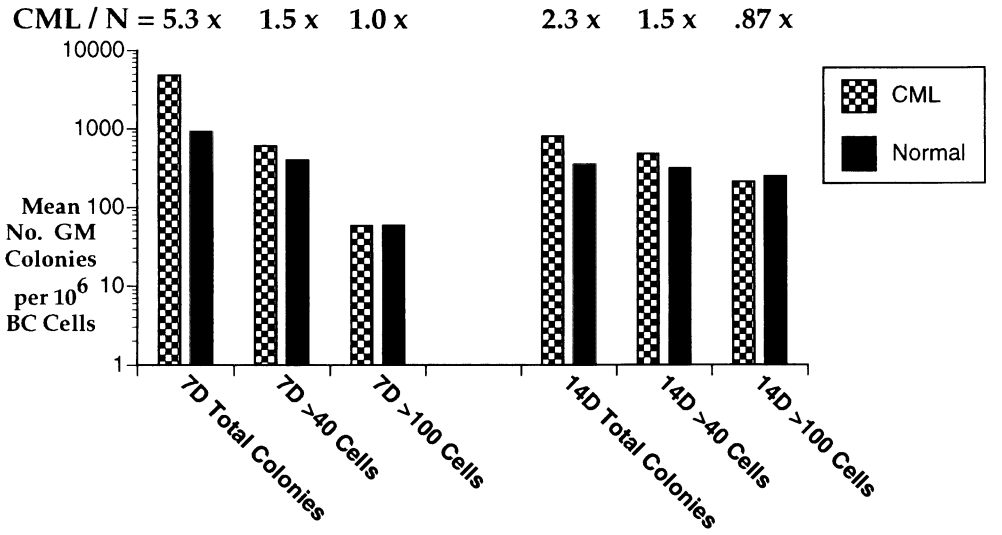

Fig. 6. Comparison of size distribution of 7- and 14-day GM colonies produced by normal and CML total GM progenitors per 10^6 buffy coat marrow cells plated. See Fig. 5

separated on the basis of size into primitive, intermediate, and late progenitors by velocity sedimentation in an isokinetic gradient [69, 109-111, 133].

Identical cloning experiments using enriched normal and CML progenitors were carried out simultaneously for comparison with those of the total progenitors shown in Figs. 5 and 6. The relative numbers of Types I and II blasts in the enriched normal and CML progenitor populations are shown in Table 3. As previously described in detail [39], Type I blasts are undifferentiated while Type II blasts show early evidence of granulocytic maturation including non-specific azurophilic granules; Type II blasts cannot be distinguished purely on morphological grounds from early promyelocytes.

The mean percentages of Type I and Type II blasts in the 6 enriched normal progenitor populations were 73% Type I blasts and 9% Type II blasts, and in the 4 enriched CML progenitor populations 72% Type I blasts and 23% Type II blasts (the remaining cells were diverse cell types which varied in different experiments). Of the total number of Type I blasts initially present in the marrow buffy coat, an average of 68% of the normal Type I blasts and 48% of the CML Type I blasts were recovered in the enriched pro-

Table 3. Mean numbers of type I and type II blasts + promyelocytes in normal (n = 6) and CML (n = 4) ehriched and total marrow GM progenitor populations

	Normal		CML	
Mean % present in enriched progenitor populations	Type I blasts 73	Type II blasts + promyelocytes 9	Type I blasts 72	Type II blasts + promyelocytes 23
Mean no. progenitors per 10^6 Marrow buffy coat cells[a]	2028	285	3560 CML/N = (1.8×)	1033 (3.6×)

[a] Total enriched cells recovered as % of marrow buffy coat cells: N = 0.29%; CML = 0.47%

genitor populations, but an average of only 7% of the total Type II blasts (plus early promyelocytes) initially present were recovered in both the normal and CML enriched populations. Based on the total and differential cells counts of the marrow buffy coat cells and the recovery values cited, the number of progenitors per 10^6 buffy coat cells were calculated; on the average the CML marrows contained 1.8× more Type I blasts and 3.6× more Type II blasts than the normal marrows. This is consistent with all of our other morphological, phenotyping, and cloning data, and demonstrates that using identical cell separation and enrichment procedures, CML progenitors are more mature than comparable normal progenitors.

The mean cloning efficiencies (CE) of the enriched normal and CML GM progenitors was 5.0 and 12.1% respectively compared to mean CEs of 0.526% and 3.99% respectively for the total normal and CML GM progenitors present in the light density fraction of the marrow buffy coat. As in the case of the total progenitors, the maximum CE values for both normal and CML enriched progenitors occurred at 3 days [113].

Figure 7 shows the number of 3- and 14-day GM colonies produced by the enriched progenitors, calculated per 10^6 buffy coat cells for comparison with the total progenitors shown in Fig. 5. On the average, the enriched CML progenitors produced 4x as many 3-day colonies as the normal progenitors compared to 10.3× for total progenitors; the reason for this difference is that the CML total progenitor population contains many more later committed progenitors with limited proliferative potential than the normal total progenitor population, and the majority of these late progenitors are missing in the enriched populations. The average ratio of 14-day CML/normal colonies was the same (2.3) for the enriched progenitors as for the total progenitors, but there was again considerable variability in the number of 14-day colonies produced in different subjects (Fig. 7).

The data on the size of 7- and 14-day colonies produced by enriched normal and CML enriched GM progenitors (Fig. 8) are similar to that of the total progenitors (ct. Figs. 6 and 8), again demonstrating that a lower proportion of CML progenitors are capable of producing large colonies compared to normal. If the data are presented as the number of 14-day GM colonies produced per 104 enriched lin-blasts plated, it can be seen that there is

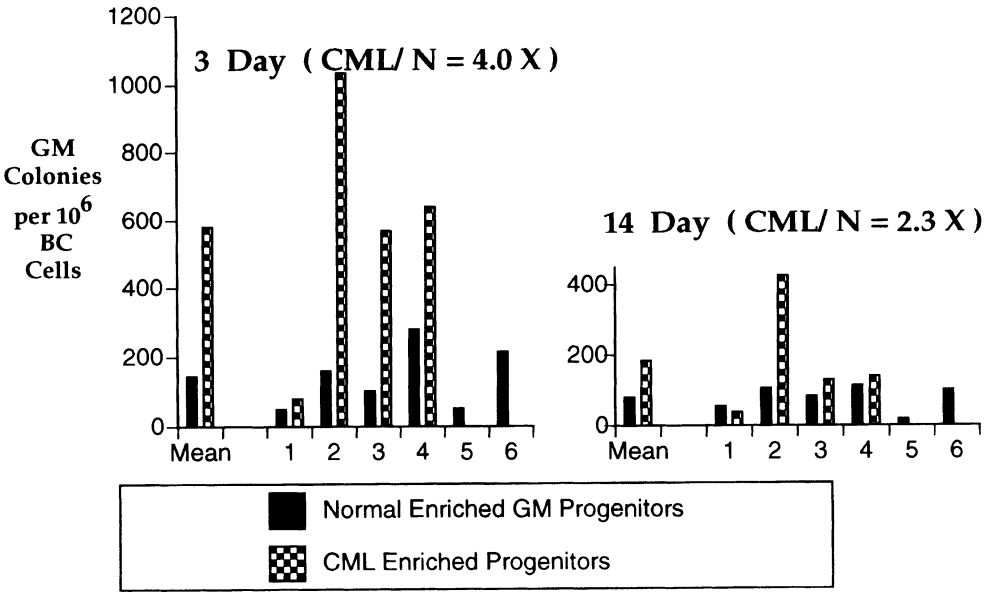

Fig. 7. Comparison of 3- and 14-day cloning data for 6 normal subjects and 4 newly diagnosed CML patients listed in Table 2 for enriched GM progenitors per 10^6 marrow buffy coat *(BC)* cells plated [39, 113]

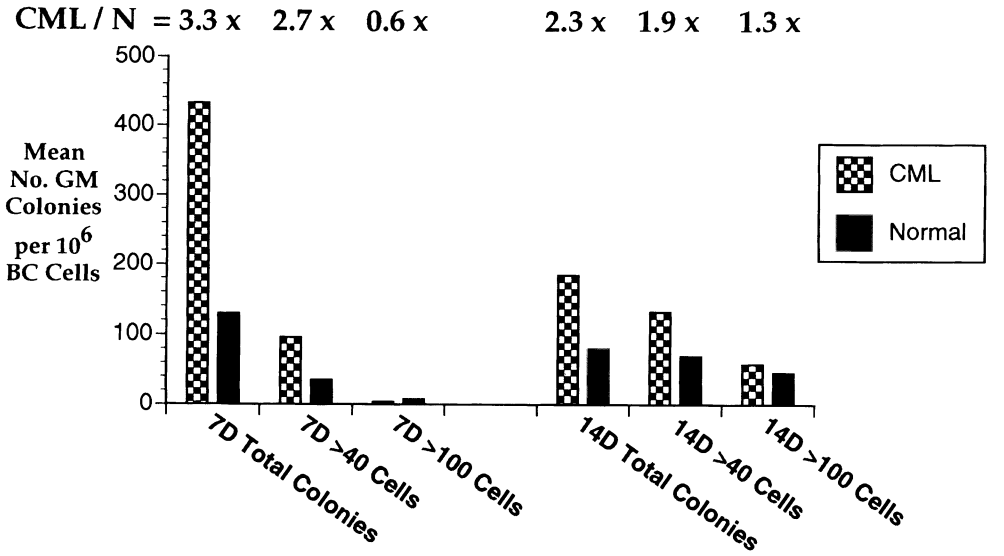

Fig. 8. Comparison of size distribution of 7- and 14- day GM colonies produced by normal and CML-enriched GM progenitors per 10^6 buffy coat marrow cells plated. See Fig. 7

considerable variability and overlap among both normal subjects and CML patients (Fig. 9). It is well known that cloning efficiencies vary widely among normal donors, and the variability is also probably partly attributable to the complexity of the enrichment procedures as well as kinetic differences among individuals. Overall, in this and other studies comparing normal and CML progenitors [39, 69, 113], and unlike the highly consistent greater cloning efficiency (CE) of more mature CML progenitors in producing 3-day GM colonies, we have found no consistent difference in the CE of

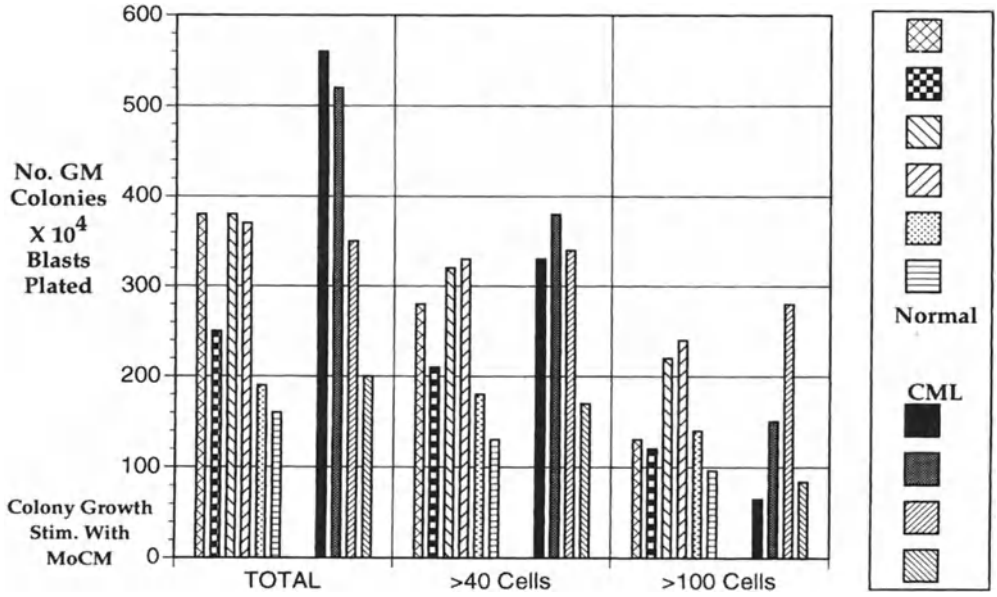

Fig. 9. Generation of 14-day GM colonies per 104 enriched lineage negative blasts plated in 6 normal subjects and 4 CML patients to illustrate variability and overlap [39, 113]

more primitive normal and CML progenitors capable of producing 14-day GM colonies.

In terms of the total numbers of cells generated by the normal and CML progenitors, we have calculated that high proliferative progenitors (i.e., those generating > 100 cells per colony) comprise 24% of the total normal GM progenitors and these produce 85% of the GM cells in normal marrow [113]. In contrast, high proliferative progenitors comprise only 2% of the total CML progenitors and these produce only 50% of the CML GM cells. Thirty-five percent of the normal enriched progenitor population is comprised of high proliferative progenitors and these produce 90% of the normal GM cells, whereas only 10% of the enriched CML progenitors are high proliferative progenitors and they produce 68% of the CML cells. Because many more of the CML than normal progenitors with low proliferative potential have been removed by the cell separation procedures, the differences are less marked when comparing highly enriched normal and CML progenitors than when comparing normal and CML total progenitors. These results emphasize the need to consider the total GM progenitor populations in comparing normal and CML cell production in order to obtain an accurate picture of the cytokinetic abnormalities in CML.

Separation of Normal and CML Lin- Blasts Into Mature and Primitive Subpopulations by Velocity Sedimentation and Characterization of Their Properties

In other experiments we used a linear Ficoll gradient, which separates cells mainly on the basis of size as previously described [68, 69, 109-112, 133], to further fractionate the enriched lin- blast populations in order to compare the characteristics and proliferative potential of the most primitive and more mature normal and CML lin-blast subpopulations. The smallest, most primitive blasts are concentrated in the earlier fractions (Fractions 8-10, designated Fx 8); intermediate blasts are contained in Fractions 11-12 (Fx 11); and the largest, more mature blasts are concentrated in the later Fractions 13-15 (Fx 13). In the experiments illustrated here, the cell cycle parameters of the CML and normal enriched total lin-blast populations prior to separation on the gradient were similar (mean % in S + G2/M = 21 and 26% respectively) [69], and these values are also similar to those found in other experiments with total enriched blast populations. As might be expected and as we have consistently found in previous autoradiographic studies in which blast cell size and 3HTdR LI 3HTdR labeling frequency and intensity were measured simultaneously [114-117, 136], cell cycle analysis of both the normal and CML fractions showed that the percentage of cells in S + G2/M increases with increasing size of blasts (i.e., Fx 8: N=5% and CML=13%; and Fx 13: N=52% and CML 45%) [69].

In the experiments shown here, colony number and size were determined at 14 days. PCR analysis was performed on representative individual GM colonies from CML patients to determine how many might be derived from normal progenitors. Consistent with our previous experience [39, 137], chimeric bcr/abl mRNA was detected in the great majority of colonies derived from CML patients (i.e., overall 94% of GM colonies were Ph^+; rare Ph-negative colonies were found in all 3 fractions) [69, 113].

The cellular composition of the three pooled fractions obtained from linear ficoll gradients are shown in Table 4. All the CML fractions contained higher proportions of more mature Type II blasts (including linearly promyelocytes) than comparable normal fractions; if all 3 fractions are taken together (i.e., total fractions 8-17), the results are very similar to those shown previously in Table 3. A higher percentage of the total enriched CML blasts was present in the small (primitive) cell Fx 8 compared to normal (57% vs. 32%), and this fraction contained 24x more Type II blasts per 106 marrow buffy coat cells than the normal Fx 8 subpopulation of primitive progenitors. The percentages of normal and CML Fx 8 blasts expressing CD34, CD38, H25/H366 and DR were similar, but consistent with the morphological evidence that they are more mature, higher percentages of the CML blasts in both Fx 8 and Fx 11 expressed CD33 than the comparable normal blasts (mean values =

Table 4. Mean numbers of a type I and type II blasts + promyelocyted in normal (n = 3) and CML (n = 3) enriched lineage negative blast subpopulations

	Normal			CML		
Mean % present in enriched progenitor populations	% Total enriched cells per fraction	Type I blasts	Type II blasts	% Total enriched cells per fraction	Type I	Type II
Fractions 8–10	32	94	2.5	57	82	15
Fractions 11–12	26	90	8	17	49	44
Fractions 13–17	34	77	21	11	55	39
Mean no. progenitors per 10^6 marrow buffy coat cells[a]						
Fractions 8–10		608	14		1648	333
				CML/N =	(2.7×)	24×)
Fractions 11–12		460	45		282	274
Fractions 13–17		480	132		212	157
Total fractions 8–17		1548	191		2143	764
				CML/N =	(1.4×)	4×)

[a] Total enriched cells recovered as % of marrow buffy coat cells: N = 0.19%; CML = 0.35%.

Fx 8 CML 41% vs. N 8%; Fx 11 CML 41% vs. N 11%) [69].

Comparison of Normal and CML GM Progenitors and Their Response to Kit Ligand (KL)

No consistent differences in expression of c-kit (as identified by the YB5.B8 monoclonal antibody) were observed between the small, intermediate and large blast fractions, nor between the normal and CML blasts in any of these fractions; 25% or fewer of the blasts in any of the fractions expressed detectable c-kit [69]. As reported by numerous investigators including ourselves [69, 112, 138-142], KL by itself has little effect in stimulating colony growth, but acts synergistically with other growth factors. In combination with G-CSF, GM-CSF, or both, KL had the greatest stimulatory effect in increasing both the number and size of colonies derived from normal primitive and intermediate lin- blasts in Fx 8 and Fx 11. KL had less stimulatory effect on the large mature blasts concentrated in Fx 13; there was little increase in the total number of GM colonies in Fx 13 but an increased number of larger colonies. The data for normal Fx 8 and Fx 13 are shown in Figs. 10 and 11; the effect of KL on Fx 11 was similar to that on Fx 8 (not shown).

In contrast to its major synergistic stimulatory effect on normal GM progenitors, KL had very little effect in enhancing the growth of comparable CML progenitor subpopulations (Figs. 12, 13). Generation of the majority of large (> 500 cells) GM colonies, both normal and CML, required the presence of both G-CSF + GM-CSF which together had a synergistic effect. However, normal progenitors had a greater requirement than CML for KL plus additional growth factors in order to generate the maximum numbers of these large GM colonies. G-CSF alone was sufficient to initiate proliferation of the maximum total number of small, primitive (Fx 8) CML progenitors and a mean of 76% of the maximum number of small blast progenitors capable of generating colonies > 100 cells (Figure 12). In contrast, comparable normal small, primitive blasts in Fx 8 required multiple growth factors (i.e., KL + G-CSF + GM-CSF) for stimulation of the maximum number and size of colonies (Fig. 10).

Cell counts on pooled GM colonies > 500 cells showed normal colonies had 14000-30000 cells/colony while CML colonies had 4000-14000 cells/colony [69, 112]. All of the above observations are compatible with the conclusion that a greater proportion of the CML progenitor subpopulations have characteristics associated with a more advanced

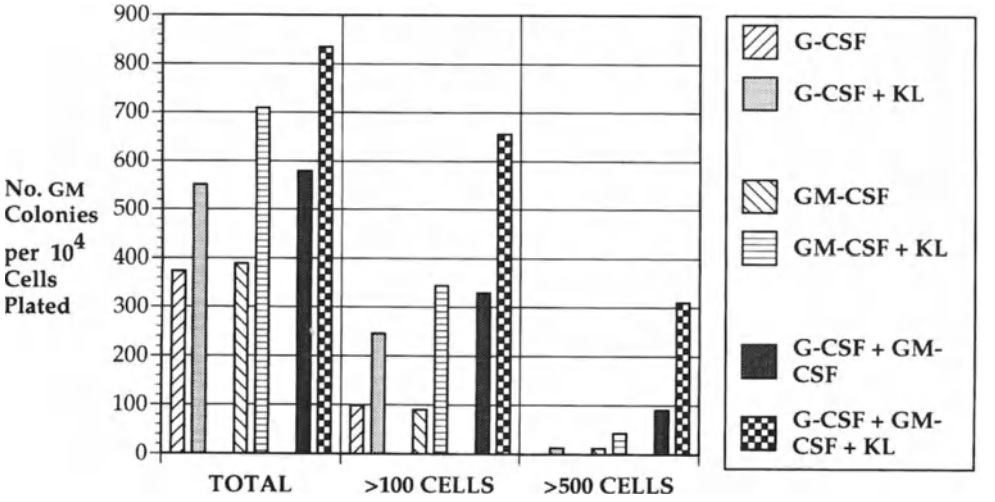

Fig. 10. Effect of *kit* ligand *(KL)* 1000 pmol/l in combination with G-CSF, GM-CSF, and both (each 400 pmol/l) in stimulating primitive normal progenitors (Fx 8–10) to form GM colonies at 14 days [69]

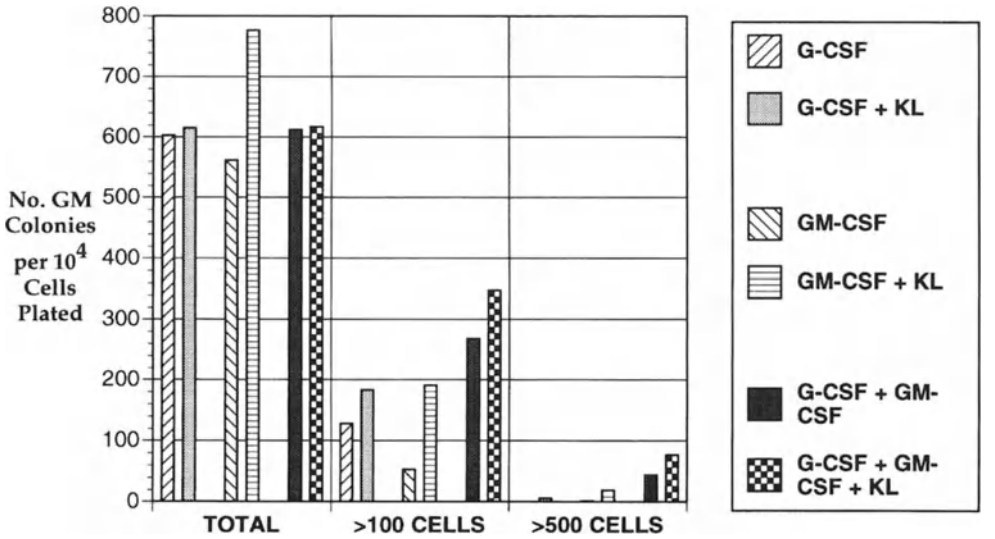

Fig. 11. Effect of *kit* ligand *(KL)* in combination with G-CSF, GM-CSF, and both in stimulating more mature normal progenitors (Fx 13–17) to form GM colonies at 14 days. See Fig. 10 for cytokine concentrations [69]

stage of maturation and correspondingly have less proliferative potential than comparable normal progenitor subpopulations.

Comparison of Normal and CML Erythrocyte Progenitors and Their Response to KL

Experiments similar to those conducted for GM progenitors were also conducted to compare the proliferative capacities of normal and CML BFU-E using the same enriched progenitor populations from normal subjects and CML patients [39, 110, 112, 113]. To evaluate their proliferative capacity as quantitatively as possible, the BFU-E colonies were divided into 4 subpopulations (XL = extra large, L = large, M = medium and S = small). Representative colonies were aspirated and cell counts per-

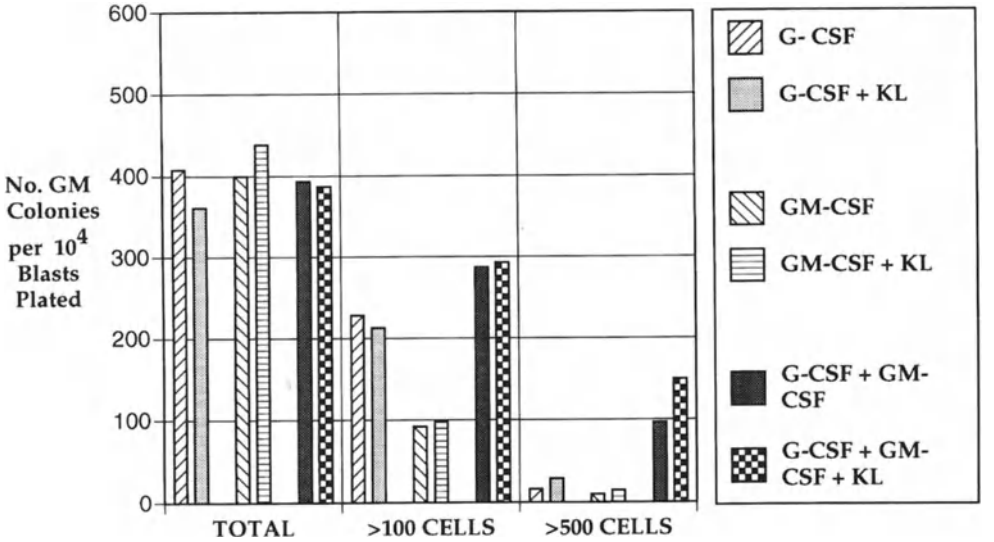

Fig. 12. Effect of *kit* ligand *(KL)* in combination with G-CSF, GM-CSF, and both in stimulating primitive CML progenitors (Fx 8–10) to form GM colonies at 14 days. See Fig. 10 for cytokine concentrations [69]

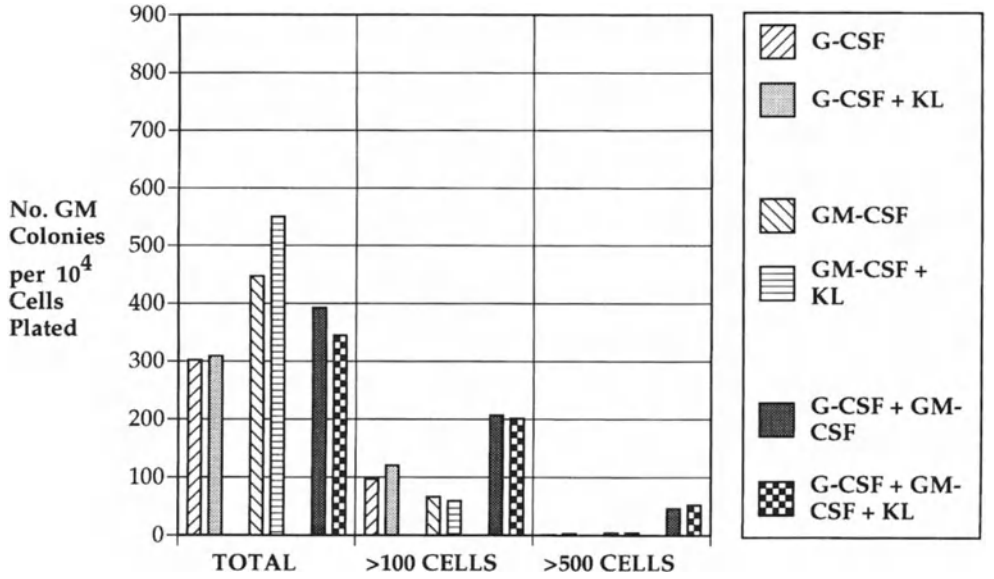

Fig. 13. Effect of *kit* ligand (KL) in combination with G-CSF, GM-CSF, and both in stimulating more mature CML progenitors (Fx 13–17) to form GM colonies at 14 days. See Fig. 10 for cytokine concentrations [69]

formed on individual XL BFU-E or pooled BFU-E from each of the smaller categories. The approximate mean numbers of cells per BFU-E in different size categories at 14 days were: XL=10^5 to >4×10^5, L=5×10^4 to 105, M=5×10^3 to 5×10^4, and S= ~10^3 to 5×10^3. The progenitor populations were grown in 1.3% methyl cellulose instead of the more commonly used 0.8% methyl cellulose; under the former conditions the BFU-E remain more compact, thus facilitating sizing of colonies since they only

break up into multiple subunits at later culture times.

Like the GM progenitors, the ratio of more mature erythrocyte progenitors with low proliferative potential to primitive progenitors with high proliferative potential is also increased in CML. However, unlike granulopoiesis, there is no comparable expansion of the erythrocyte population in CML, probably at least in part because enucleation prevents the more mature progenitors from undergoing additional divisions as takes place in the granulocytic lineage [109, 110, 112].

In the experiments illustrated here, the normal BFU-E populations were comprised of 21.3% (16-24%) high proliferative BFU-E (XL+L), whereas CML BFU-E populations had only 4.7% (4-5%) high proliferative BFU-E (L only; no XL CML BFU-E were observed). As a result of this difference, 67% (63-71%) of normal erythroblasts were generated by high proliferative BFU-E (L+XL) whereas CML high proliferative BFU-E (L only) generated only 17% (13-22%) of CML erythroblasts [112].

The CML and normal marrows had similar numbers of BFU-E per 106 buffy coat cells (mean values 338 and 282 respectively), but because the majority of CML BFU-E (i.e., 83%) were only capable of generating small or medium sized colonies, they only generated about 1/3 as many erythroblasts as normal (i.e., CML 3.76×10^6 vs. N 10.7×10^6 cells per 10^6 buffy coat cells) [112, 113]. However, because of the increased cell densities of the CML marrows, the numbers of cells generated on the average per ml of normal or CML marrow are almost equal. These findings are consistent with the clinical observation that at the time of diagnosis the majority of CML patients have either normal erythrocyte counts or are only slightly anemic, except for patients presenting with very elevated leukocyte counts and more advanced disease (e.g., Table 2). [38, 39].

The effects of KL when added to erythropoietin (EPO) alone, Mo T-cell conditioned media (MoCM), and to GM-CSF + IL3 on the growth of normal and CML BFU-E are shown in Figs. 14 and 15 respectively. Unlike the pronounced differences in KL's effect on normal and CML early and intermediate GM progenitors described earlier, normal and CML BFU-E responded similarly to KL. Whereas KL had only a slight or modest effect in increasing the total number of BFU-E, it caused a marked increase in the size of both normal and CML BFU-E. However as

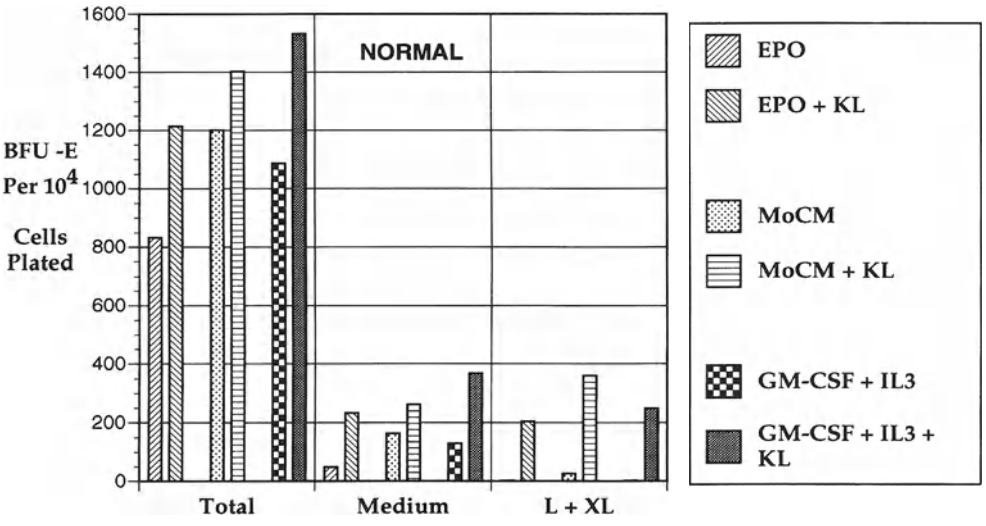

Fig. 14. Response of normal BFU-E to *kit* ligand *(KL)*. Erythropoietin (EPO) 1 unit/ml was added to all cultures initially. KL (1000 pmol/l) was added to EPO alone, to MoT conditioned media (MoCM), and to GM-CSF (400 pmol/l) plus IL3 (2600 pmol/l) to test its effect in stimulating the total highly enriched normal progenitor population to form BFU-E at 14 days. Medium-sized colonies contained 5000–50 000 cells, large *(L)* 50 000–100 000; and extra large *(XL)* > 100 000 cells [112]

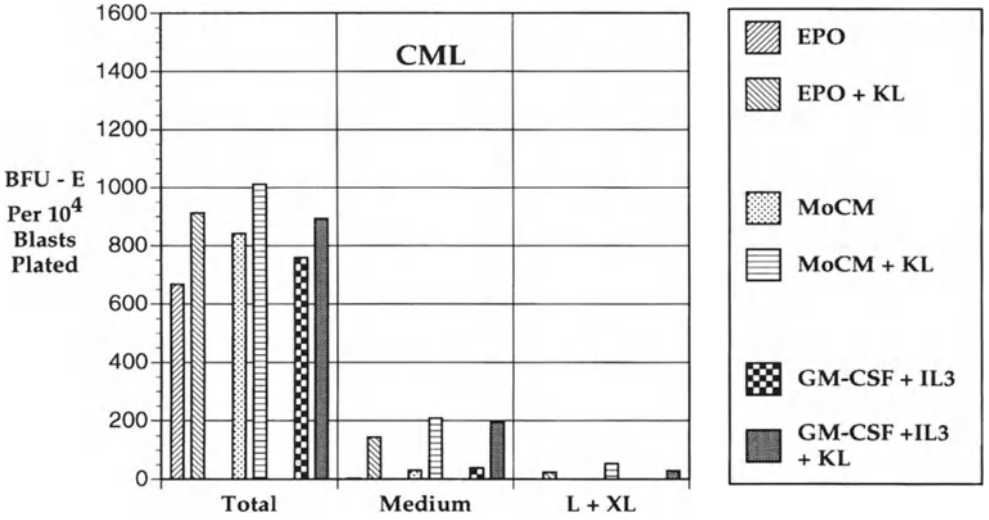

Fig. 15. Response of CML BFU-E to *kit* ligand *(KL);* the experimental conditions were identical to those described for Fig. 14 [112]

highlighted in Fig. 16, the number of large CML BFU-E was far less than the number of large normal BFU-E and the latter contained twice as many cells on the average; moreover no XL CML BFU-E were observed.

Thus, like CML GM progenitors, the majority of CML erythrocyte progenitors are also more mature and incapable of as extensive proliferation as comparable normal erythrocyte progenitors. This conclusion is in accord with the findings of several other investigators [143, 144], but one recent report using quite different methodology for estimating proliferative potential reported that CML BFU-E did not have reduced proliferative capacity [145]. However, the latter studies are not comparable to ours since KL and other purified growth factors were not used to maximally stimulate the normal cells; one would not expect to find the same difference that we observed with suboptimal stimulation.

Summary of Similarities and of Differences in Biological Behavior of Normal and Chronic Phase CML Progenitor Cells

The foregoing experiments shown above in summary form were selected from a large body of experimental data to illustrate important similarities and differences in the biological behavior of normal and CML progenitors. These similarities and differences have been described in detail in recent reviews [38, 39], but can be summarized under four categories as follows:

Morphologic and Phenotypic Abnormalities

Chronic phase CML cells retain their capacity for complete maturation, but a greater proportion of CML progenitors have premature cytoplasmic maturation than comparable normal progenitors (e.g., higher proportions of Type II blasts with non-specific granules, increased CD33 positivity, higher expression of EPO receptors, etc.). Despite their ability to undergo near normal maturation, CML chronic phase progenitors and maturing cells may exhibit a number of subtle abnormalities which tend to increase with disease progression. These include ultrastructural evidence of asynchronous maturation of the nucleus and cytoplasm with the latter maturing more rapidly, an increased incidence of dysplastic changes, and appearance of chimeric basophil/eosinophil granules which are not seen in normal granulocytes.

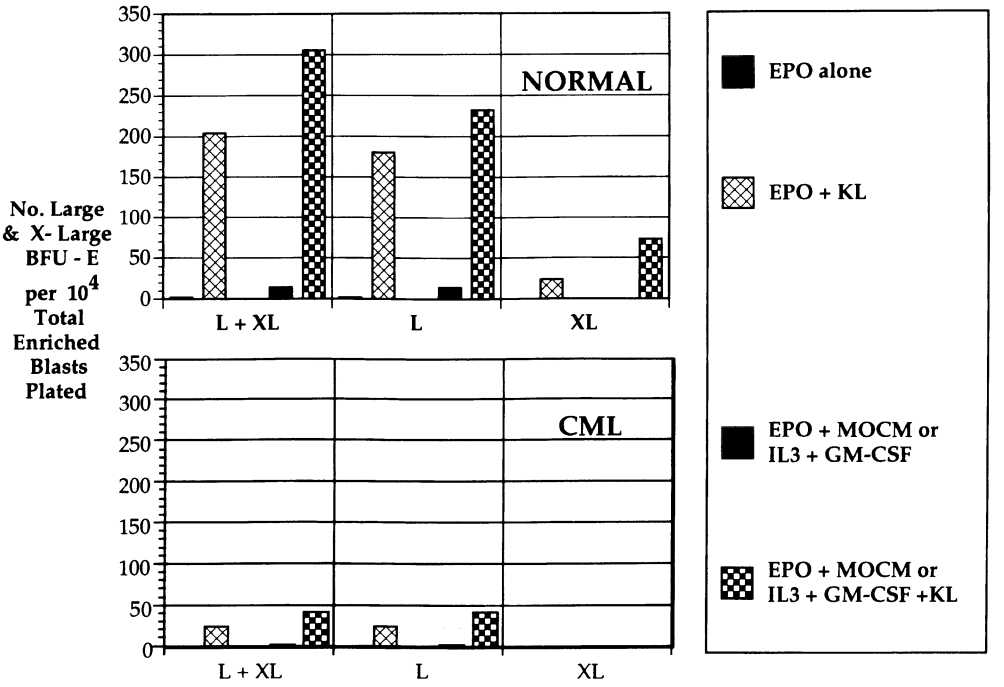

Fig. 16. Effect of *kit* ligand *(KL)* in stimulating growth of large *(L)* and extra large *(XL)* normal and CML BFU-E at 14 days. The mean numbers of cells in the large normal and BFU-E colonies were 94 000 and 46 000 respectively and in the XL normal 211 000 (129 000–431 000); no XL CML BFU-E colonies were found. The experimental conditions are as described for Fig 14 with EPO added initially [112]

Functional abnormalities

In developing and/or mature granulocytes, these include altered motility and chemotaxis, reduced phagocytic and bacterial killing activities, and subnormal adhesiveness to glass, nylon, fibronectin, and other stromaal elements.

Among the various functional abnormalities attributed to the $p210^{bcr/abl}$ fusion protein (reviewed in [39]), McWhirter and Wang [146] found that bcr sequences not only deregulate abl tyrosine kinase, but also activate an actin filament binding function associated with c-abl. They proposed that the normal function of bcr is related to maintenance of the cytoskeleton, and that the chimerization of bcr and abl permits abl to bind to actin microfilaments. Since actin fibers are vital elements involved in maintaining cell shape and in regulating many cellular functions and interactions, disregulation of actin could have a critical role in altering cell growth and maturation.

Cytokinetic Abnormalities

The cell cycle parameters of normal and CML chronic phase progenitors and later precursors are similar when the cells are at comparable cell densities (both in vivo and in vitro). As in other cell populations, the proportions of actively proliferating CML cells (i.e., in $S+G_2/M$) falls with increasing cell density, and in both normal and CML progenitors the growth fraction is lower in primitive than in more mature progenitors. The growth fraction of CML progenitors decreases further after blastic transformation. Crucial kinetic abnormalities in CML include:

1. The normal lineage apportionment is unbalanced with disproportionate committal to granulopoiesis and thrombopoiesis.
2. CML mature granulocytes have prolonged life spans and circulation times compared to normal cells, but the magnitude of the differences is not sufficient to account for the enormous expansion of the granulocyte compartment; increased production is also required.

3. In CML there is a failure to adequately curtail cell production after exceeding cell densities in the marrow at which cell production normally is reduced more completely under steady state conditions. Moreover anomalous cell production often takes place in the spleen at a similar rate to that in the marrow, and sometimes hematopoiesis also occurs in other extramedullary sites. The mode of density dependent inhibition of normal progenitors and the specific abnormalities in CML progenitors that allow them to reach higher-than-normal cell densities are not well understood although various explanations have been proposed (reviewed in [39]).
4. In CML, unlike normal hematopoiesis, large numbers of immature cells are released into the blood from the marrow, spleen and sometimes other sites, and continuous cell traffic occurs between these sites of production. Except in patients with extreme leukocytosis, the cells rarely divide in the blood, but must return to the marrow or spleen to undergo mitosis. However, a significant fraction of immature CML precursors in S phase are released into the blood. As in acute leukemia, the S phase fraction of the circulating blasts and later precursors is usually lower than that in the marrow except in patients with very elevated WBC when it may be the same or even higher due to release of cells in S phase from grossly enlarged spleens. The abnormal release of immature cells into the blood has variously been attributed to space limitations and overcrowding in the marrow, to decreased adherence to stromal elements, to increased motility or invasiveness or to some combination of these factors.
5. In accord with their more advanced state of maturation, the ratio of more mature progenitors with limited proliferative potential to primitive progenitors with high proliferative potential is substantially increased in chronic phase CML compared to normal progenitors; this results in the majority of CML cells being generated by more mature progenitors which are no longer under tight regulatory control.
6. Whereas a number of human cell lines expressing $p210^{bcr/abl}$ are growth factor independent, like normal progenitors, primary CML progenitor cells are absolutely dependent on hematopoietic growth factors for survival, proliferation, differentiation and maturation. However, we have found certain differences in the response of normal CML progenitors to cytokine stimulation which we believe may be important in understanding their abnormal behavior.

CML GM progenitors are less responsive than normal GM progenitors to the synergistic effect of KL in combination with other cytokines, especially in increasing the size of colonies produced by primitive progenitors with high proliferative potential. As a corollary, normal primitive GM progenitors have a much higher requirement than comparable CML GM progenitors for the synergistic effect of KL with other cytokines in causing an optimal proliferative response; conversely, CML progenitors have a reduced requirement for multiple growth factors including KL to attain their maximal response.

Biochemical Abnormalities

As reported in CML cells, these include low neutrophil alkaline phosphatase (NAP) activities, subnormal lactoferrin and lysozyme contents, hypersialylation of membrane protein, reduced total gangliosides and neutral glycosphingolipid content of the cell membrane, and quantitative changes in granule and other cellular proteins and in membrane constituents (reviewed in [38, 39, 108, 109]). Many of the biochemical and functional abnormalities in CML cells appear to be mutually linked, quantitative rather than qualitative, and probably reflect different maturation stages of normal and CML cells; they tend to return towards normal when the disease is brought into remission by treatment.

Recently we have reported constitutive tyrosine phosphorylation of a number of phosphotyrosyl (P-tyr) proteins in early CML progenitors that are not detectible or are phosphorylated at a greatly reduced level in comparable normal progenitors [147-149] as we will describe in more detail below.

Differences in Protein Tyrosine Phosphorylation in Normal and CML Progenitors and Correlation with Biological Abnormalities

Because of the probable causal relationship between constitutive $p210^{bcr/abl}$ protein tyrosine kinase activity and the manifestations of chronic phase CML, we initiated studies in primary primitive lineage negative (lin) chronic phase CML progenitors and comparable normal lin primitive progenitors to identify differences in proteins constitutively phosphorylated on tyrosine. We subsequently reported a prominent 62 kDa P-tyr protein that is constitutively phosphorylated in chronic phase CML primary primitive blasts whose tyrosine phosphorylation is greatly reduced in comparable unstimulated normal blasts [147]. We established [147, 148] that this P-tyr p62 protein associates with GAP and is not immunologically related to the RNA binding GAP-associated p62 protein previously identified by Wong et al [150] which is now referred to as Sam 68 [151]. Our novel p62 protein ($p62^{dok}$) has now been sequenced and its gene (Dok) cloned [149]; the equivalent murine gene has also now been cloned and is highly homologous to the human gene [152]. $p62^{dok}$ has no significant homology to any existing protein and does not contain any catalytic domain. However, it contains 15 potential tyrosine phosphorylation sites, it is proline-rich with ten PXXP motifs, and sequence alignment studies show it to contain a pleckstrin homology (PH) domain at its amino-terminus (Fig. 17). Using a rabbit polyclonal antibody raised against a peptide at the carboxyl-terminus of $p62^{dok}$, we have identified the prominent P-tyr p62 protein in primary CML primitive blasts to be this novel protein and have further determined that when tyrosine phosphorylated it does complex with ras-GAP. However, we have found that the stoichiometry of this complex is rather low and estimate that only a small fraction of total ras-GAP is associated with tyrosine phosphorylated $p62^{dok}$. $p62^{dok}$ appears to be a docking protein that is a major substrate of numerous tyrosine kinases, but its function in different cell types is still uncertain [149, 152].

The evidence to date suggests that $p62^{dok}$ is a substrate of the constitutive tyrosine kinase activity of $p210^{bcr/abl}$ and that it probably functions as a signaling molecule [149]. PH domains are thought to mediate protein-protein interactions and interactions with cellular membranes, possibly by binding to inositol phosphate components of the lipid bilayer. If the function of the PH domain at the N-terminal region of $p62^{dok}$ is to bind components of the membrane or other proteins, then the C terminal portion which contains the majority of tyrosines would likely be positioned to interact with other proteins within the cytosol. The tyrosines may well be targets for neighboring tyrosine kinases including $p210^{bcr/abl}$, and after being phosphorylated they could then serve as docking sites for proteins containing SH2 domains. As noted above, $p62^{dok}$ also contains ten PXXP motifs which are the core conserved sequence of proline-rich regions recognized by molecules containing SH3 domains. Thus while its true biological function is still unknown, $p62^{dok}$ has several prominent features suggesting it may have an important role in signal transduction networks, probably as a component of a signaling cascade initiated by receptor or membrane-associated tyrosine kinases.

In addition to $p62^{dok}$, we have detected in whole cell lysates the presence of additional clearly consistent but less prominent P-tyr proteins with molecular weights of ~155, 140, 110, 55-56, and 45 kDa as well as more minor P-tyr proteins of ~190, 85, 51-53, 42

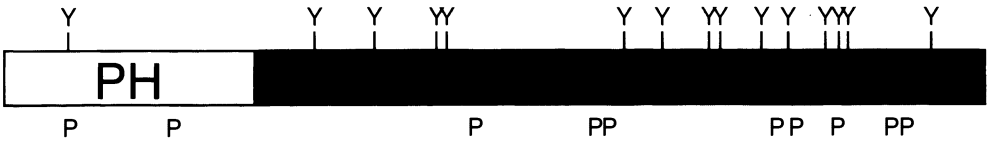

Fig. 17. Schematic diagram of $p62^{dok}$. The pleckstrin homology *(PH)* domain at the amino terminus is indicated as are the locations of the 15 tyrosines *(Y)* and 10 PXXP motifs

and 39 kDa that are constitutively tyrosine phosphorylated in primary primitive lin chronic phase CML blasts but not, or to a much lesser extent, in comparable primary lin normal blasts [148, 153] (Fig. 18). We are presently trying to identify and characterize all these P-tyr proteins and to define their inter-relationships in the signaling pathways involved.

In analyzing proteins tyrosine phosphorylated in primary primitive lin normal blasts in response to various hematopoietic cytokines, we found a striking similarity in the phosphorylation of at least four major (~140, 110, 62 and 55/56 kDa) and at least three minor (~51/53, 45 and 42 kDa) P-tyr proteins after stimulation with KL and the P-tyr proteins that are constitutively phosphorylated in primary primitive lin chronic phase CML blasts (Fig. 19). Other growth factors tested (i.e., GM-CSF, G-CSF, IL-3, FLT3 ligand, EPO and TPO) were much less active and/or stimulated phosphorylation of other proteins. Thus at least seven proteins that are rapidly and transiently phosphorylated on tyrosine in the KL/c-kit signal transduction pathway in lin normal blasts may be constitutive substrates for the $p210^{bcr/abl}$ activated tyrosine kinase in comparable lin chronic phase CML blasts [148, 153].

Tyrosine phosphorylation is known to not only be important in transmission of mitogenic signals [154-156], but also in transduction of signals regulating differentiation and maturation in many types of cells [157-160], including normal hematopoietic and leukemic cells [161-167]. With regard to the seven P-tyr proteins that are rapidly and transiently phosphorylated on tyrosine in the c-kit signaling pathway in primary primitive lin normal blasts that may be constitutive substrates for the $p210^{bcr/abl}$ activated tyrosine kinase in comparable chronic phase CML blasts [148], it is worth noting that KL and bcr/abl have sever-

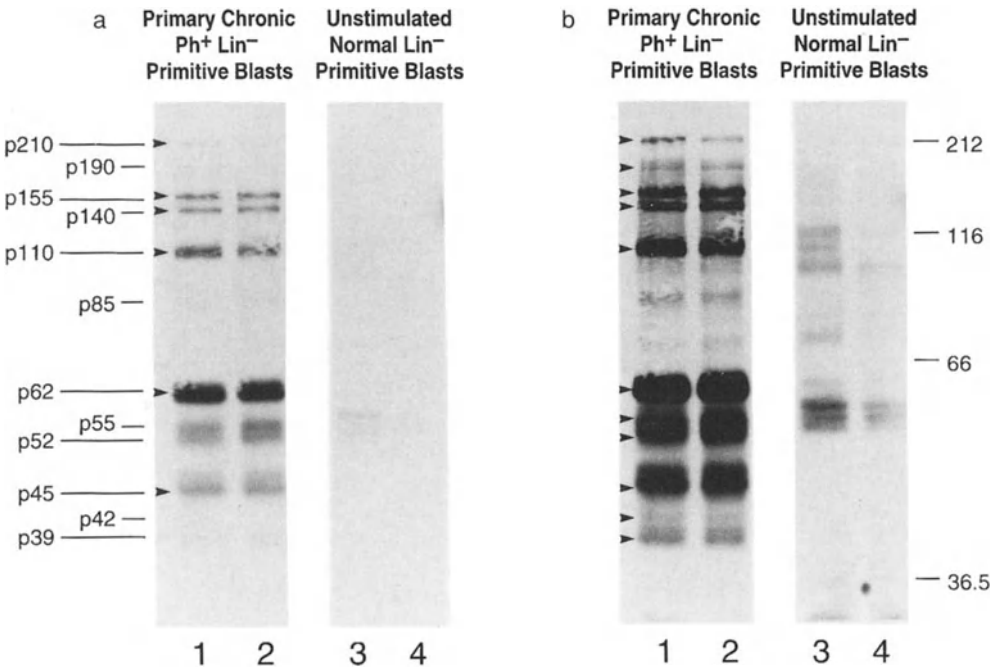

Fig. 18 a, b. Immunoblot analysis of constitutive P-tyr containing proteins derived from primary primitive lin-normal blasts and Ph^+ chronic phase CML blasts. 5×10^5 blasts were lysed in hot SDS sample buffer. Cell lysates were separated by 8% SDS-PAGE, immunoblotted with an α-P-tyr moAb, developed with the ECL detection system, and exposed to Hyperfilm for *a* 1 min and b *4* min. Constitutive appearance or enhancement of P-tyr proteins in CML blasts are indicated by *arrows* on the *left* and molecular weight standard in kilodaltons (kDa) are indicated on the *right* [148]

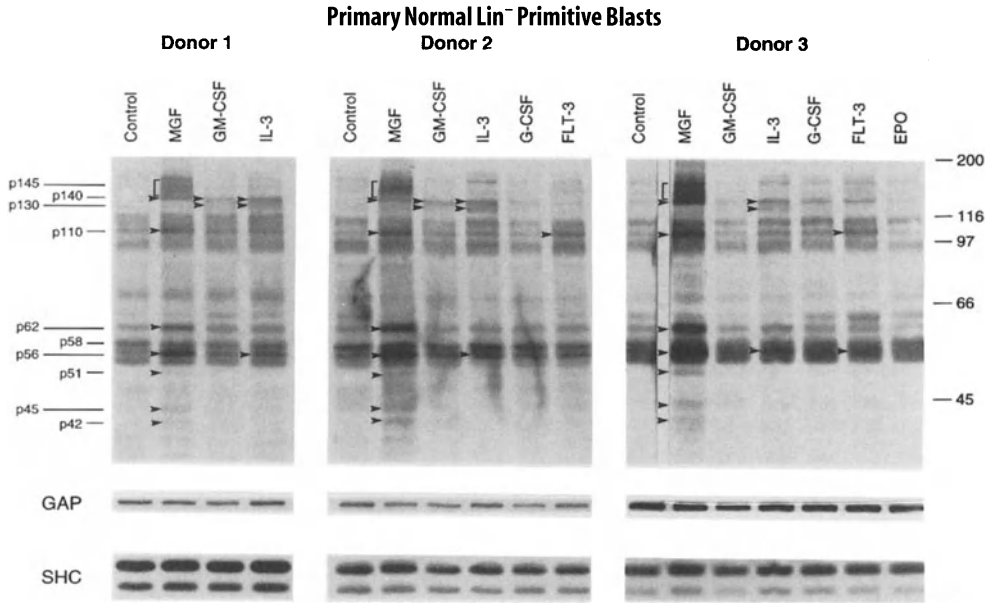

Fig. 19. Analysis of proteins phosphorylated on tyrosine in response to hematopoietic factors. Primary primitive lin⁻ normal blasts ($3–5 \times 10^5$) from three normal donors were exposed for 5 min at 37 °C to saturating amounts of growth factors shown. Cells were immediately lysed in hot SDS sample buffer. Cell lysates were separated by 8% SDS-PAGE and immunoblotted with an α-P-tyr moAb. The appearance or enhancement of P-tyr proteins, relative to the unstimulated controls, are indicated by *arrows* on the *left* and molecular weight markers are indicated on the *right*. α-P-tyr blots were stripped and reprobed simulataneously with α-SHC and α-GAP antibodies to demonstrate equivalent amounts of proteins in the cell extracts [148]

al important similarities in their biological effects including enhancing survival of early hematopoietic progenitor cells as well as stimulating their expansion (i.e., KL in synergy with other growth factors). The KL/c-kit receptor system mediates numerous diverse cellular processes in different cell types, including cell proliferation, differentiation, maturation, survival, migration, adhesion to extracellular matrix and secretion of cellular proteins [168-171]. Numerous investigators including ourselves have shown that while KL enhances the short-term survival of hematopoietic stem cells and early progenitor cells it has only limited effect by itself on inducing their proliferation or differentiation. However, when combined with other hematopoietic growth factors, KL can have pronounced synergistic effects on proliferation, differentiation, maturation, and distribution [39, 69, 112, 113, 172-175]. In examining the effects of KL on subsets of human progenitor cells, Olweus et al [176] found that KL alone had a greater effect in inducing proliferation of erythroid progenitors than GM progenitors and, as we also found, a stronger synergistic effect on primitive than on more mature GM progenitors, but despite these differences there were no differences in expression of the KL receptor between the progenitor subsets.

Our own studies are generally in accord with the observations of most other investigators. In addition, as shown earlier we have found that primitive normal granulocyte/macrophage (GM) progenitors are more responsive than comparable CML GM progenitors to KL's synergistic stimulatory effect on proliferation and that a higher proportion of CML GM progenitors compared to normal no longer require KL for a maximum proliferative response. In contrast to the GM progenitors and in keeping with the lesser disturbance of the erythroid lineage in CML, there is no appreciable differential effect of KL on normal and CML erythrocyte progenitors (ct. Figs. 10-13 with Figs. 14-16). However higher proportions of both normal GM and erythrocyte progenitors are capable of more extensive proliferation when opti-

mally stimulated than the corresponding CML progenitors; moreover the CML high proliferative early progenitors in both these lineages are generally incapable of producing as large colonies as the comparable high proliferative normal progenitors.

Our studies also support the view that the primary expansion of the CML population takes place in a primitive progenitor compartment at the time of lineage commitment rather than at the level of earliest self-renewing stem cell. If the latter were true, one would expect an acute rather than a chronic form of leukemia. There are also strong theoretical reasons why the day-to-day fine-tuning regulation of hematopoiesis operates at a later stage than the true (mostly quiescent) stem cell. It is well established that the majority of normal stem cells are quiescent in steady state conditions [38]. If large numbers of stem cells were simultaneously activated to meet every demand, the system would rapidly become chaotic and ultimately break down. Moreover, there is no need to invoke extensive expansion of the stem cell compartment since an essential property of both normal and CML stem cells is their enormous self-renewal and repopulating potential [39]. However, since there is no reliable in vitro assay to distinguish stem cells from early committed progenitors, it is uncertain whether the initial step in the myeloid expansion involves constitutive activation of more stem cells with enhanced input into the early committed progenitor compartment or if the primary expansion begins in this compartment.

Tentative Hypothesis Linking Biochemical and Biological Abnormalities

In attempting to integrate the biochemical and biological observations, we would like to propose a tentative simple model comparing normal and CML production. The available evidence so far leads us to postulate that the increased tyrosine kinase activity of $p210^{bcr/abl}$ causes untimely or inappropriate constitutive tyrosine phosphorylation of a number of proteins involved in critical signaling pathways, especially including the c-kit pathway. It is known that sustained activation of discrete signaling pathways in some types of cells results in differentiation, whereas transient activation is not associated with differentiation but rather leads to a proliferative response; in other cell types, the converse is observed [177-179]. Different cell types may respond differently depending on prior developmental events such as their content of specific responsive transcription factors.

Thus in CML it may be postulated that the stem cells or primitive committed progenitors are at a particularly susceptible stage of development that renders them especially responsive to constitutive, sustained bcr/abl-induced downstream hyperactivation of components of critical regulatory pathways that are ordinarily activated by low-level, transient extracellular stimulation by KL and other cytokines. The affected short-circuited pathways presumably control and coordinate multiple diverse cell processes including proliferation, differentiation, maturation and programmed cell death, processes that are normally tightly regulated and highly integrated. Perturbation of these key pathways in stem cells or primitive progenitor cells would be expected to seriously disrupt orderly hematopoiesis and could also explain all of the subsequent subtle, pleiotropic biological abnormalities characteristically observed in later maturing cell compartments that we have collectively designated "discordant maturation" [38, 39, 109, 110].

Fig. 20 shows a hypothetical model comparing normal granulopoiesis and granulopoiesis in an early stage of chronic phase CML, illustrating how either increased activation of stem cells with enhanced input into the primitive committed progenitor compartment, an increased number of divisions in the primitive progenitor compartment, or a reduced fraction of cells undergoing programmed cell death can lead to a slight growth advantage and increased cell production. Our own data and that in the literature are not sufficiently precise to be able to distinguish between these possibilities, and it is quite possible that all three mechanisms are operative. Moreover, since the transition from stem cells to primitive multipotent progenitors to the next stages of

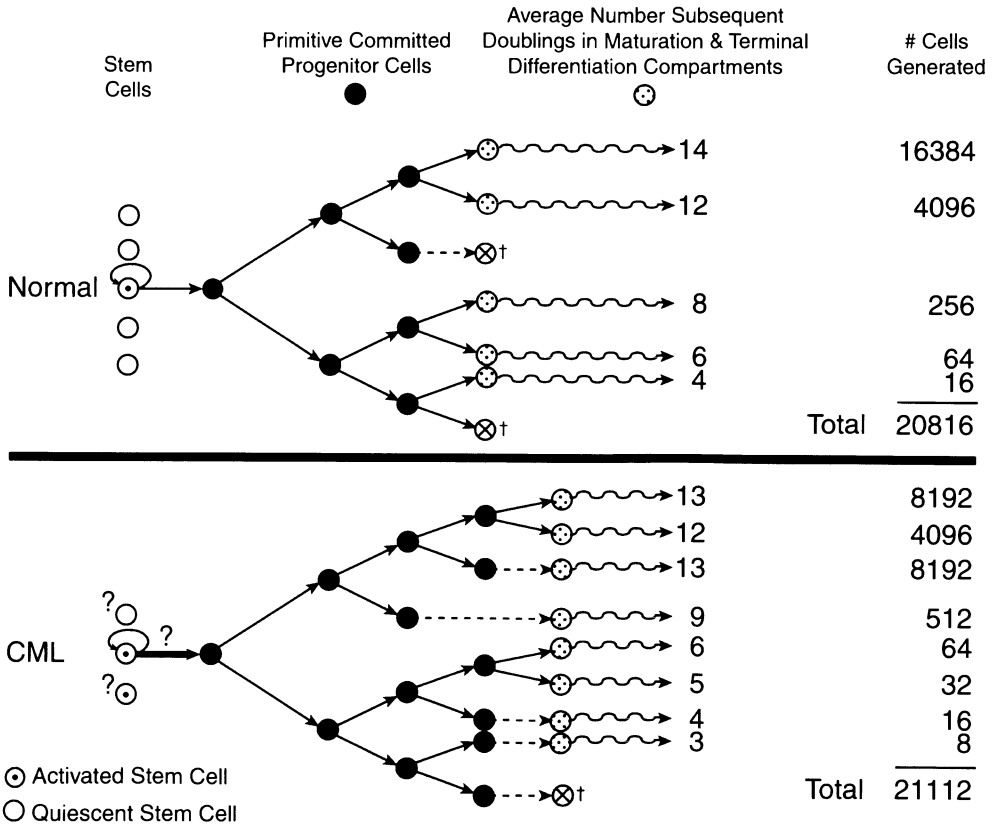

Fig. 20. Hypothetical model comparing primitive GM Progenitors in normal granulopoiesis and in early chronic phase CML. *Question marks* indicate uncertainty about the state of activation of CML stem cells and extent of input into primitive committed progenitor compartment. ⊗ † indicates cells undergoing programmed cell death

commitment is undoubtedly a continuum, compartmental distinction in this context is largely arbitrary in any case. The expansion of early progenitors is of course eclipsed in kinetic studies by the subsequent greatly amplified expansion of later progenitor and precursor cells.

While the model assumes that the initial expansion of the CML population begins in primitive committed progenitor cells, it rejects the notion that simple increased self-renewal in this compartment can by itself explain all the subtle kinetic and other abnormalities that occur in CML cells. One can easily envision that if $p210^{bcr/abl}$ simultaneously induces premature and discordant maturation of early progenitors, the ultimate proliferative potential of the affected progenitors could well be limited rather than enhanced as would be expected if increased self-renewal was the sole mechanism. Thus, in accord with the experimental data cited earlier, a higher proportion of CML later progenitors and precursors have more limited proliferative capacity than the corresponding normal cells.

The two models in Fig. 21 are intended to compare normal and leukemic granulopoiesis in overview form. The CML model suggests that one or more extra divisions may take place in a primitive committed progenitor compartment; alternatively, as stated above, one could postulate activation of more stem cells or early progenitor cells. It is implied in the model that a smaller fraction of the CML progenitors than normal undergo programmed cell death as indicated by a cross; however, reduced apoptosis alone cannot explain all the abnormalities such as the dysplastic changes, asynchronous matu-

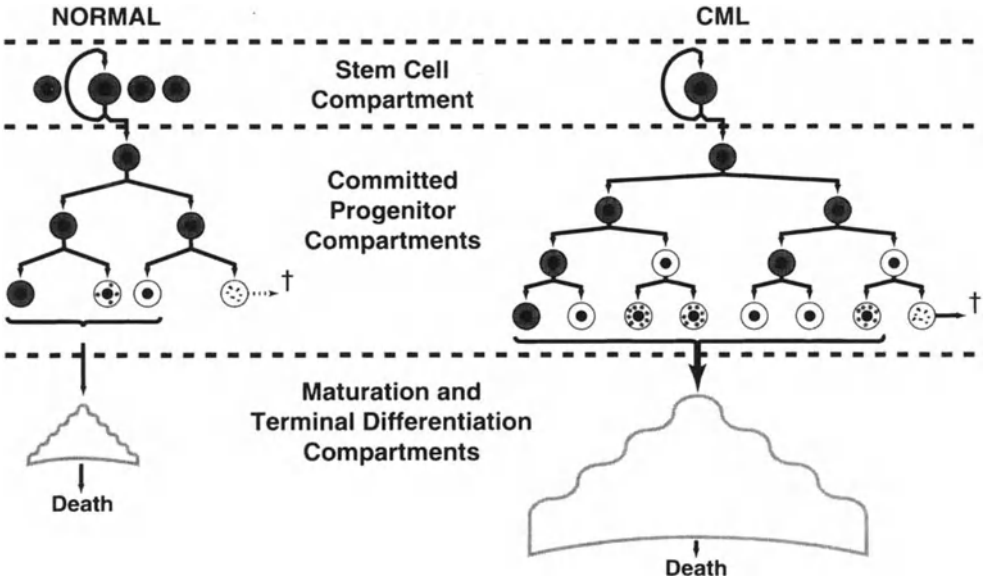

Fig. 21. Hypothetical models comparing overview of granulocyte production in normal state and CML. The *crosses* indicate progenitor cells undergoing apoptosis

ration and aberrant lineage distribution noted earlier. The signaling pathways controlling differentiation, maturation, proliferation, and programmed cell death are closely interconnected, and it is only to be expected that constitutive activation of one or more of these pathways in a primitive progenitor could seriously derange what is normally a highly integrated and exquisitely coordinated regulatory system. As we have shown, the CML progenitors are slightly more mature and on the average have less proliferative capacity (as indicated by the number of squiggles) than their normal counterparts, thus resulting in reduced proliferative capacity and a higher proportion of smaller colonies. Based on cell labeling studies, it has been known for many years that mature CML granulocytes live longer than normal, thus further contributing to expansion of the maturing CML compartments.

The control of hematopoiesis is very complex and involves interaction of multiple cytokines and signaling pathways. Constitutive downstream activation of the c-kit pathway by $p210^{bcr/abl}$ is undoubtedly only part of the story, but it is not yet known what other pathways may be involved. As we have noted, there are some striking similarities in the protein tyrosine phosphorylation pattern found constitutively in CML progenitors and in KL-stimulated normal progenitors, but also some differences. Likewise, as mentioned earlier, while bcr/abl and KL/c-kit have some notable similar biological effects such as in enhancing cell survival and expansion, there are also differences. If bcr/abl does in effect cause downstream activation of components of the normal c-kit pathway, it must be incomplete activation because normal and CML progenitors respond similarly to KL, the main differences being that (a) a higher proportion of CML GM progenitors compared to normal no longer require KL for a maximum proliferative response, and (b) greater proportions of both CML CFU-GM and BFU-E are incapable of as great a proliferative response as the corresponding normal progenitors.

Using highly purified progenitors and subpopulations of progenitors, we are currently trying to determine more precisely possible differential effects of KL and other cytokines on normal and CML progenitors. We are also trying to define as precisely as possible the level of progenitor cell development at which the expansion first occurs in

CML, and whether the expansion is primarily due to activation of more stem cells or early progenitors rather than additional divisions or reduced apoptosis.

In summary, we propose that $p210^{bcr/abl}$-induced intracytoplasmic short-circuiting of critical regulatory pathways, including c-kit, results in constitutive and sustained hyperactivation of important components of these pathways, and further that the involved pathways are particularly important in determining the fate of primitive progenitor cells. If one considers how such a disruptive effect can be amplified and modified in ensuing phosphorylation cascades, it seems quite plausible that such a disturbance in a stem cell or early progenitor cell may have sufficiently broad ranging consequences to explain all of the pleiotropic features of chronic phase CML. It will be important to define the involved signaling pathways more completely and precisely, not only in order to better understand the disease but to seek possible ways to develop specific therapies.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Transformation of Myeloid Cells by the BCR/ABL Oncogene

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Abstract: BCR/ABL transforms hematopoietic cells in vitro and in vivo and exerts a wide variety of biological effects, including induction of factor-independence, reduction of apoptosis, and altering adhesion of CML cells to marrow stroma. However, at a biochemical level, the mechanisms by which BCR/ABL transforms myeloid cells remain poorly understood. p210BCR/ABL has elevated ABL tyrosine kinase activity, relocates to the cytoskeleton, and phosphorylates many cellular signaling proteins. Recent advances in identifying the substrates of the ABL tyrosine kinase have led to a better understanding of the mechanism of transformation. A variety of signaling pathways are activated by p210BCR/ABL, including those involving p21ras, PI3K, and CRKL. Considerable progress has been made linking each of these pathways to specific biological and clinical abnormalities in CML.

Chronic Myeloid Leukemia

CML was the first neoplasm associated with a specific chromosomal translocation, the Philadelphia chromosome, t(9;22)(q34;q11) [1]. The molecular basis for CML has been worked out in elegant detail by a number of investigators (for reviews see [2,3]). Virtually all cases are the result of fusion of the c-ABL tyrosine kinase with BCR, a ubiquitously expressed gene probably normally involved in regulating the activity of small G-proteins such as p21rho [4,5]. Two major translocations exist, producing two related fusion proteins, a shorter form associated with Ph(+) ALL, $p190^{BCR/ABL}$, and the more common $p210^{BCR/ABL}$ associated with CML. The difference between the two forms is in the amount of BCR fused with ABL. The $p190^{BCR/ABL}$ form has several fold higher tyrosine kinase activity than $p210^{BCR/ABL}$, and both forms have higher tyrosine kinase activity than endogenous c-ABL [6]. Interestingly, a third breakpoint has recently been described which fuses additional sequences of the BCR gene to ABL, and some of these cases are clinically quite mild and have been referred to as chronic neutrophilic leukemia [7].

CML is characterized by massive expansion of myeloid cells in the marrow and blood, splenomegaly, extramedullary hematopoiesis, basophilia, hyper metabolism, and a high propensity to convert to acute leukemia. In many respects, the "stable" phase of CML is a preleukemic disorder, but it appears that virtually all patients with CML will evolve to acute leukemia if they do not succumb to an intervening illness. CML "blast crisis" is an aggressive acute leukemia with uniformly short survival. The blast crisis phase is characterized by the emergence of poorly differentiated primitive subclones, which typically have acquired new genetic mutations in addition to the Ph chromosome.

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The BCR/ABL Oncogene

BCR/ABL is unique to CML and Ph(+) ALL, and does not effectively transform most non-hematopoietic cells, even in tissue culture [8]. For example, the NIH3T3 fibroblast cell line is not generally transformed by BCR/ABL, although sublines can be isolated which are transformable. There has been a perception by many investigators that the BCR/ABL gene product is toxic to many types of cells, and it is not known why myeloid cells are permissive to BCR/ABL transforming functions. The activation of the tyrosine kinase of ABL is critical for transformation [9], and there is growing evidence that this activation is due to dimerization or oligomerization of the ABL kinase by a domain in the N-terminus of BCR. This domain, which has a coiled-coil structure is likely to spontaneously form tetramers or oligomers [10]. We have recently tested the hypothesis that oligomerization is sufficient to activate the ABL tyrosine kinase by constructing a chimeric receptor/oncogene by fusing the ligand-binding domain of the erythropoietin (EPO) receptor with c-ABL. The resulting protein has no kinase activity in the absence of added erythropoietin, but is rapidly activated after the addition of EPO, and has biological effects similar to BCR/ABL in model systems [11].

$p210^{BCR/ABL}$ is exclusively cytoplasmic, with about 70% of the protein associated with the cytoskeleton [12]. Two groups have shown that $p210^{BCR/ABL}$ has a specific actin binding domain [13-15], and studies from our lab show that $p210^{BCR/ABL}$ co-localizes with vinculin and paxillin in focal adhesion plaque-like structures in myeloid cells [16, 17]. Many domains in $p210^{BCR/ABL}$ have been shown to play a functional role in transformation, dependent on the type of assay used to measure function. BCR has been shown to be important for cytoplasmic localization, multimer formation (10), and activation of the ABL kinase [18]. The SH3 domain of ABL is inhibitory to kinase activity, and deletion of the SH3 domain makes c-ABL transform, suggesting that an unknown cellular protein(s) bind to the SH3 domain and down regulate c-ABL. Several potential ABL-SH3 binding proteins have been identified, including Abi-1, MENA, EVL, and VASP [19,20], although none have been clearly shown to inhibit BCR/ABL transformation. Interestingly, the function of MENA has been linked to control of cytoskeletal functions [19]. The SH3 domain is deleted in v-ABL. Tyr 177 has been shown to be part of a binding site for the SH2 domain of GRB2, and this site has been shown to be important for transformation in some assays [21,22]. The major phosphorylation site within the kinase domain of p190 is important for transformation [9,23]. Finally, recent studies by Sawyers and colleagues suggest that a proline rich domain in ABL which is believed to be the binding site for CRKL may also be important for transformation in some assays [24].

c-ABL

c-ABL is ubiquitously expressed, both as a nuclear and a cytoplasmic protein [25,26]. Older studies suggested that nuclear c-ABL is likely to function as a negative regulator of growth, possibly through binding to known growth regulatory proteins in the nucleus such as Rb [27]. More recent studies suggest that c-ABL is part of a response to certain types of genotoxic stress, such as X-irradiation or UV light exposure [28]. Interestingly, G1 arrest induced by DNA damaging agents has been shown to require c-ABL, further implicating c-ABL in negative growth regulation. It is not known if BCR/ABL affects the function of c-ABL with regards to responding to DNA damage, and this will be an important area to explore in the future.

Biological Effects of BCR/ABL

It has been a challenge to link the signaling activities of BCR/ABL with specific biological effects. The stable phase of CML is remarkable in that there is no apparent block in cell differentiation. In this phase, CML might more accurately be called a clonal myeloproliferative disorder. There is a striking tendency of CML to evolve, however, and acute leukemias will eventually develop in

most patients. The actual causes of the massive accumulation of myeloid cells characteristic of CML remains unclear. CML progenitor cells are actively cycling, probably more so than normal progenitor cells, but are entirely factor-dependent for proliferation in vitro [29,30]. In tissue culture, introduction of BCR/ABL into factor-dependent cell lines or murine marrow cells typically generates cell lines which evolve from hypersensitivity to growth factors to full factor-independence [31]. Even modest hypersensitivity to growth factors is not a common feature of CML progenitor cells, except in more advanced stages of the disease (Griffin, J.D., unpubl.).

Several other biological effects have been reported, and may be important. First, BCR/ABL may affect the sensitivity of CML cells to apoptosis [32-35]. Normal hematopoietic cells are in a delicate balance of life and death. Most of the known hematopoietic growth factors induce cells to proliferate, differentiate, and or activate, but almost all factors also support viability. Most factor dependent cell lines, such as 32Dcl3 or FDCP1 cells, also die rapidly, starting at about 18 hours after removal of growth factors such as IL-3 or GM-CSF. BCR/ABL has a strong anti-apoptotic activity in myeloid and lymphoid cell lines, and recent studies from several groups, including our own, suggest that this is a primary and potentially important activity of this oncogene [32-36].

Several model systems have been created in which the functions of the BCR/ABL oncogene can be regulated, and these are starting to be used to improve our understanding of both biology and signal transduction. For example, our group made mutants of BCR/ABL which were temperature sensitive for ABL kinase activity [36]. The best mutant, p210BCR/ABLts-1 has two point mutations (aa 457 R-H and aa 469 Y-H) and has low kinase activity at the non-permissive temperature (39 °C) and high kinase activity at the permissive temperature (33 °C). At the non-permissive temperature, the cells are wild-type (IL-3-dependent for growth and viability) while at the permissive temperature the cells require IL-3 for optimum proliferation, but no longer need IL-3 for viability. At the permissive temperature, the cell lines will also proliferate slowly in the absence of IL-3. Thus, BCR/ABL dramatically reduces cell death in the absence of growth factors, at least in cell lines. Similar results were obtained by several groups with ts mutants of v-ABL [33] and with primary CML neutrophils and progenitor cells [32]. We have recently created a second model in which ABL kinase activity can be regulated by fusing the external, ligand binding, domain and the transmembrane domain of the EPO receptor to c-ABL [11]. In this chimeric receptor, the ABL kinase is inactive until EPO is added to the cells. After adding EPO, the kinase activity of ABL is regulated in a dose-dependent manner. Our studies so far indicate that low level activation of ABL is sufficient for prolonging viability and high level activity of ABL is sufficient for proliferation and altering adhesive properties [11].

The adhesive abnormalities of CML cells are particularly interesting. CML cells have altered adhesion to marrow stromal cells and some extracellular matrix proteins, notably reduced adhesion to fibronectin [37]. It has been suggested that this property contributes to early release of CML cells from the marrow. CML cells leave the marrow when immature, circulate in the blood in high numbers, and proliferate actively in tissues such as the spleen and liver which are not normally hematopoietic in adult humans. There is growing evidence that this event is related to altered integrin expression and function.

It has recently been proposed that IFN-a may overcome the defective adherence of CML progenitors to stromal cells. Verfaillie et al have shown decreased adhesion to stroma and fibronectin but increased adhesion to the basement membrane components laminin and collagen type IV of malignant progenitors in CML [37]. Defects in b1 integrin function have been described and also shown to be corrected by IFN-a [38]. These observations suggest the premature release of malignant CML progenitors may be caused by loss of adhesive interactions with stroma and/or fibronectin and acquisition of adhesive interactions with basement membrane components.

Integrins and Adhesion of Progenitor Cells

Integrins are expressed on almost every cell type and are important in linking the extracellular matrix to the cytoskeleton [39]. Each integrin consists of noncovalently linked, heterodimeric a and b chains. Integrins are typically grouped according to the b subunits. The b2 leukocyte integrins (CD18 b chain) have been demonstrated to be important in neutrophil functioning [40]. There are several ligands for b2 integrin family members, including ICAM-1 (intercellular adhesion molecule 1), fibrinogen, coagulation factor X, and iC3b. CD11b/CD18 is upregulated by tumor necrosis factor, C5a, platelet activating factor, fMLP, and phorbol esters.

Interestingly, integrins send signals back to the cell when they are in contact with an appropriate ligand [39,41-43]. For example, crosslinking of CD11b/CD18 in normal neutrophils leads to rapid tyrosine phosphorylation of several new cytoplasmic proteins. Recent studies have suggested that one biological effect mediated by integrin-initiated signaling may be inhibition of apoptosis for some cell types, and augmentation of apoptosis for others. Integrin signaling, and control of integrin expression and function are poorly understood events. In adherent cells such as fibroblasts, the integrins congregate in areas which are in contact with ECM proteins. Such cells have developed specializes structures on the inside of the cell membrane termed "focal adhesions " ("FA") that contain a unique set of proteins which are believed to be involved in linking the cytoskeleton to integrins and in sending and receiving messages from integrins [44-46]. Many of these specialized focal adhesion components have been identified [41, 45, 46]. These components are either extracellular (such as the extracellular matrix (ECM) proteins fibronectin and vitronectin), transmembrane (integrins), or cytoplasmic (such as actin, vinculin, tensin, paxillin, and other molecules). Focal adhesions function as anchors for actin fibers, and play a role in regulating actin assembly. Recent studies have shown that cross-linking of integrins sends signals into the cell through FA components [41,43,44,47,48]. For example, after ECM molecules bind to integrins, there are several proteins which have been shown to be phosphorylated, including tensin, talin, and p125FAK, and RAFTK. p125FAK and RAFTK are tyrosine kinases which are concentrated in FAs, and may be responsible for phosphorylating some of the other FA proteins after activation. It is likely that these phosphorylations are associated with transient alterations in FA structure and function. In summary, FA structures appear to be important in structural support of the cell, and transmitting signals from cell receptors and various oncogenes to the cell membrane.

BCR/ABL and the Actin Cytoskeleton

Several tyrosine kinase oncogene products (such as $p60^{v\text{-}Src}$, $p160^{v\text{-}ABL}$, $p60^{v\text{-}YES}$, and $p210^{BCR/ABL}$) induce phosphorylation of one or more cytoskeletal proteins such as vinculin, talin, and tensin [49,50]. The interaction of oncogenes with FAP proteins has been implicated in transformation and particularly in changing adhesion to ECM proteins, decreased contact inhibition, and ability to metastasize [46,51]. BCR/ABL localizes to the cytoskeleton and causes a pronounced disorganization of the actin microfilaments associated and a significant increase in the phosphotyrosine content of cytoskeletal proteins. For example, both paxillin and $p130^{CAS}$ are also prominent substrates of BCR/ABL [52]. Paxillin is one of the major tyrosine phosphoproteins in cells transformed by v-Src or v-Crk [53,54]. Paxillin interacts directly with both v-Src and v-Crk proteins, through the SH2 and SH3 domains of Src, and the SH2 domain of Crk, based on in vitro binding studies. Binding of v-Src and v-Crk to paxillin may be important in concentrating both of these oncogenes in focal adhesions, and therefore could be important in the altered cytoskeletal structure and adhesive properties that accompany transformation by both viral oncogenes. Interestingly, one of the major targets for BCR/ABL in the cytoplasm is an adapter protein termed CRKL, or CRK-like [55-57]. CRK and CRKL each have one SH2 domain, an SH2' domain, and two SH3 domains, without oth-

er known functional domains [58,59]. Like GRB2, CRK and CRKL are likely to function as "adapter proteins," linking different proteins in a regulated manner. We and others have recently identified a series of signaling proteins linked to BCR/ABL through CRKL, and many of these proteins are also involved in integrin signaling in normal cells. These proteins include paxillin, $p130^{CAS}$, $p120^{CBL}$, and HEF1. Each of these proteins bind to CRKL through the CRKL SH2 domain, which recognizes Y-X-X-P motifs, particularly Y-D-X-P [16,17,60-64].

Defects in Cytoskeletal Function in Cells Transformed by BCR/ABL

We have recently demonstrated that the cytoskeletal abnormalities of cell lines transformed by BCR/ABL are more significant than previously realized [17]. In addition to the well known adhesive defects to fibronectin, BCR/ABL affects the morphology of the cytoskeleton and has a profound effect on motility of myeloid cell lines on certain surfaces [65]. Cell lines such as Ba/F3 tend to be quite motile when cultured on plastic surfaces or surfaces coated with some ECM proteins such as laminin or vitronectin. However, on fibronectin, they tend to stop their spontaneous crawling, round up and adhere loosely. Cells containing BCR/ABL are unable to control their motility on such surfaces and tend to have hypermotility at all times. This is likely to reflect the altered integrin signaling that occurs in these cells in the presence of BCR/ABL.

Summary

The biological defects of CML cells are becoming clearer and are likely to include subtle defects in the cell cycle, apoptosis, adhesion, motility, and DNA repair. The BCR/ABL gene product is a large protein with many signaling motifs, many different substrates, and a variety of cellular effects that depend, in part, on the model system being studied. So far, a number of murine models of CML have been identified, but many are closer to acute leukemias than chronic myeloproliferative disorders. Interestingly, a mouse with a myeloproliferative disease similar to CML was recently created by knocking-out the ICSBP gene, a transcription factor belonging to the interferon regulatory factor family. [66]. It will be of interest to determine if the clinical effects of interferon are related to this pathway. Presumably, all of the biological effects of BCR/ABL are initiated from its cytoskeletal location, and these are likely to include effects on transcription factors such as c-myc [23,67], cell cycle regulatory proteins such as cyclin D1 [68], and cytoplasmic signaling proteins involved in p21ras regulation [69-72], and proteins that are involved with integrin signaling or cytoskeletal function [16,60,61,64,73,74].

The effects of BCR/ABL on integrin function are particularly interesting. BCR/ABL may block the signals that regulate either expression or affinity, thus altering the ability of the cell to change its adhesive profile. This could easily change the ability of the cell to bind to critical extracellular matrix components. BCR/ABL may also interfere with signals that are initiated by integrins when they are either bound to their ligand or aggregated. Such signals are known to involve rapid activation of the focal adhesion kinase FAK, and tyrosine phosphorylation of FA proteins such as paxillin. The role of integrin signaling into the cell is not clear, but several themes are emerging. First, in addition to simply providing adhesion to the proper substratum, some cells are likely to need to know that they are in the right microenvironment. One way in which this could occur is to have integrins (and/or other adhesion molecules and cell surface receptors) send signals into the cell when they are appropriately activated by ligand. In this way, cells would sense when they are in the right microenvironment for proliferation or different functions. Such a sensing mechanism may be particularly important for highly migratory hematopoietic cells. Although immature hematopoietic stem cell are found in the blood and circulate through all organs, proliferation of these cells is observed almost exclusively (in humans) in the bone marrow. It is likely that multiple signals through cell surface receptors are required

to permit initiation of proliferation to occur, and one reasonable set of receptors would be integrins. If so, BCR/ABL could cause the cell to grow under conditions which inhibit proliferation of normal progenitor cells. The data cited above, in fact, would suggest that BCR/ABL would initiate signals that could indicate that integrins are "activated," even when they are not. These new avenues of research on BCR/ABL biology will likely be extremely informative in the near future.

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New Isoforms of the ZFM1 Gene: a Growing Family of Signal Transduction and Activator of RNA (STAR) Proteins

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Abstract. Two novel isoforms of the recently described ZFM1 gene were identified by differential screening of a cDNA library obtained from a GM-CSF dependent human myeloid leukemia cell line (GF-D8). The transcription pattern of the ZFM1 gene is complex and characterized by four transcripts of approximately 3.9, 3.7, 3.2 and 2.9 kb which are apparently up-regulated upon stimulation with GM-CSF. The same pattern of expression is also observed in freshly isolated myeloid leukemia cells and human normal $CD34^+$ stem cells. In light of these data, and since GM-CSF is known to stimulate signal transduction pathways, it become relevant that all the different isoforms of ZFM1 contain the KH module which is a sequence motif present in proteins playing a major role in regulating cellular RNA metabolism. Search for functional domains demonstrates that ZFM1 belongs to a new and growing family of genes including Sam68, a target of Src, Fyn and Grb2 and the newly clone mouse quaking proteins (QKI) necessary in early embryogenesis and myelination. All these molecules are thought to play a down stream role in cell signaling and RNA binding and were defined as signal transduction and activator of RNA (STAR) proteins. ZFM1 is highly conserved from yeast to humans and this might imply that this new pathways has significant function.

Introduction

Leukemic cell lines established from patients with acute myeloid leukemia have been largely used as useful models to identify and clone genes controlling myeloid differentiation and proliferation [1]. We established a new growth factor-dependent acute myeloid leukemia cell line named GF-D8 [2] whose long-term survival and proliferation in vitro are strictly dependent upon the presence of GM-CSF or IL-3. Many other cytokines including G-CSF, IL-6 and Flt ligand are unable to support the proliferation of GF-D8 making this experimental model attractive to search for GM-CSF regulated genes. A number of growth factor induced genes were identified by differential hybridization of cDNA libraries using probes obtained from activated compared with quiescent cells [3]. Although this method is limited in its sensitivity and detects only those transcripts that are expressed in relatively high abundance, it allowed the identification, in cultured vascular endothelial cells, of a novel IL-1β inducible pentraxin-related gene called PTX-3 [4]. Thus we use this strategy, to characterize new genes whose transcription could be induced or regulated by GM-CSF.

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Material and Methods

Cells

The establishment and characterization of the human GM-CSF dependent myeloid leukemia cell line GF-D8 has been reported previously [2]. Human normal hematopoietic stem cells ($CD34^+$ cells) were obtained from the peripheral blood of healthy donors treated with G-CSF to mobilize progenitor cells for allogeneic transplantation [5]. A highly purified fraction of $CD34^+$ cells (> 95% pure) was obtained by positive selection using CD34 magnetic microbeads, XS^+ column and a SuperMACS separator (Miltenyi Biotec, Germany) which allows collecting up to 1×10^9 positive cells. Acute myeloid leukemic cells were obtained from bone marrow aspirates from patient at diagnosis. The leukemic blast population was purified by Ficoll gradient sedimentation and was more than 90% pure as assessed by conventional morphology and flow cytometric analysis using a FACScan apparatus (Becton Dickinson, Mountain View, CA) and stem cell and myeloid specific monoclonal antibodies (HPCA-2, anti CD34), (Leu M7, anti CD13) and (Leu M9, anti CD33) (Becton Dickinson).

cDNA Libraries and Differential Screening

Total RNA was extracted and purified by guanidinium isothyocyanate and cesium chloride gradient as previously described [6] from GF-D8 cells cultured for 2 h in the presence or absence of both GM-CSF (50 ng/ml) and cycloheximide (10 μg/ml). $Poly(A)^+$ RNA was further purified by affinity chromatography on oligo(dT)-cellulose and a cDNA library was constructed in the γ-ZAPII vector (Stratagene, La Jolla, CA) as described [4]. The cDNA probes were obtained from resting and GM-CSF activated GF-D8 cells and the membranes were hybridized and washed as described [4]. Plaques showing a stronger hybridization signal with the cDNA probes obtained from GM-CSF-stimulated relative to untreated GF-D8 were picked. These were rescreened three times differentially to obtain single clones. Phage inserts were rescued in the Bluescript vector and sequenced with the dideoxynucleotide chain termination method [7].

Northern Blot Analysis

Northern blot analysis was performed using either total cellular RNA or $poly(A)^+$ selected mRNA. as described [6]. Equal RNA loading was checked by hybridization with the cDNA coding for the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [8]. Probes were labeled to a specific activity of 10^9cpm/mg by using hexanucleotide primers and ^{32}P-dCTP [9].

RT-PCR

RT-PCR reactions were performed using cDNAs synthesized with 1 μg of total denaturated RNA (from GM-CSF activated GF-D8 cells) in a 20 μl reaction mixture containing 100 μM dNTPs, 1X First strand Buffer, 1 μl Superscript RT RNase H^- Virus Reverse Transcriptase (Gibco BRL, Gent, Belgium), 20U RNase inhibitor and random hexamers, using a commercial kit (Perkin Elmer-Cetus). All PCR experiments were performed using an automated heat block (PCR system 9600, Perkin Elmer-Cetus). cDNA clones containing the whole coding sequences of the new ZFM1 isoforms were obtained in vitro by Expand High Fidelity PCR System (Boehringer, Mannheim). The PCR reaction was performed by adding 5 μl of the cDNA product to a PCR mixture containing 1.5 mM $MgCl_2$, 1X Expand HF buffer, 400 μM dNTPs, 2.6 U of a mixture of Taq and Pwo DNA Polymerases, 300 nM of a 3′ amplimer common to all ZFM1 isoforms (nucleotides 2501–2520 [10]) and 300 nM of a 5′amplimer corresponding to the 5′ untranslated sequence of ZFM1 (nucleotides 328 to 347 [10]). After an initial denaturation at 95 °C for 1 min, 12 cycles of amplification were performed each consisting of a denaturation step at 95 °C for 30 s, and annealing step at 56 °C for 30 s, an extension at 72 °C for 3 min; 16 cycles each consisting of a denaturation step at 95 °C for 30 s, an annealing step at 56 °C for 30 s and an extension at 72 °C for

3 min + 20 s/cycle. A final extension at 72 °C for 10 min (1 cycle) and cooling at 4 °C was done at the end. The amplified DNAs wre directly subcloned from the PCR reaction into a pMOS plasmid (Amersham, Buckinghamshire, UK).

A specific region of the B74b4 cDNA was subcloned by RT-PCR using as 5′ primer the oligonucleotide CTCCCATGGACCCTTCTA and as 3′ primer the oligonucleotide TCCCAAGCGAATCCTCAG (both derived from the B74b4 sequence). Five μl of the cDNA product were added to a PCR mixture containing 1.5 mM $MgCl_2$, 1X PCR Buffer (Perkin Elmer-Cetus), 200 μM dNTPs and 2.5 U of Taq DNA Polymerase. After an initial denaturation at 95 °C for 1 min, 35 cycles of amplification reactions were performed, each consisting of a denaturation step at 95 °C for 30 s an annealing step at 54 °C vor 30 c and an extension at 72 °C for 30 s. The 5′ portion of ZFM1 was similarly cloned by PCR using as 5′ primer an oligonucleotide from position 45 to 66 and as 3 primer an oligonucleotide from position 1024 to 1046 (both derived from the published sequences of ZFM1) [10] using the same amplification protocol as described above.

In Vitro Transcription and Translation

The full-length cDNA clones B3OS (subcloned into the pBluescript II SK ± vector downstream from the T3 promoter) and B4OS (cloned into the pMOS vector) containing the complete ORF of the two new isoforms of the ZFM1 gene, were used for the in vitro transcription and translation assay. One microgram of each recombinant plasmid was used in a rabbit reticulocyte lysate assay with a commercially available kit (Promega) in the presence of ^{35}S-labeled methionine. The final products were run in a standard 8.5% SDS-polyacrylamide gel, fixed in 40% methanol-10% acetic acid, dried and autoradiographed.

Computer Methods

All sequences were obtained from the Swissprot WWW and GeneBank servers. Multiple sequence alignment was performed using the software package Camelon (Oxford Molecular Ltd., Oxford, UK) and Clustal W 1.6. The mutation data matrix [11] was used as similarity matrix. Similar results were obtained with other matrices (data not shown). All sequences were scanned against the Prosite database [12] to look for sites and patterns.

Results

By differential screening of the cDNA library constructed from the GF-D8 cell line stimulated with GM-CSF, we obtained three clones (B74c1, B74b4 and B74b3) showing a partial identity with the ZFM1 nuclear protein recently described by Toda and coworkers [10]. Although the ZFM1 sequence showed a high degree of identity with the sequence of our cDNA clones, we noticed some important differences. The B74b3 cDNA clone differs only for a four nucleotide insertion at nucleotide 1724 of the ABCDF isoform of ZFM1 [10], resulting in a frame shift of the ORF. Clone B74b4 contains the insertion of 383 additional nucleotides at position 2137 of the nucleotide sequence for the ABCDEF isoform of ZFM1 [10]. Again, this insertion determines a different sequence of the putative protein product towards the COOH terminus. By RT-PCR amplification using RNA from GM-CSF activated GF-D8 cells the whole coding sequences of these two new isoforms of ZFM1 were cloned (B4OS, a 2576-bp-long sequence with an ORF of 1917 bp coding for 639 aa and B3OS, a 1943-bp-long sequence which presents an ORF of 1713 bp coding for 571 aa). The B3OS and B4OS cDNAs were therefore considered representative of two new isoforms of the ZFM1 gene and will be thereafter referred to as B3 and B4, respectively.

The cDNA clones B3OS and B4OS can effectively initiate protein synthesis, as demonstrated by experiments of in vitro transcription and translation performed using the reticulocyte lysate assay. As shown in Figure 1, we could obtain two protein products of 62 and 68 kDa, respectively, in total agreement with the molecular weight predicted by their ORF.

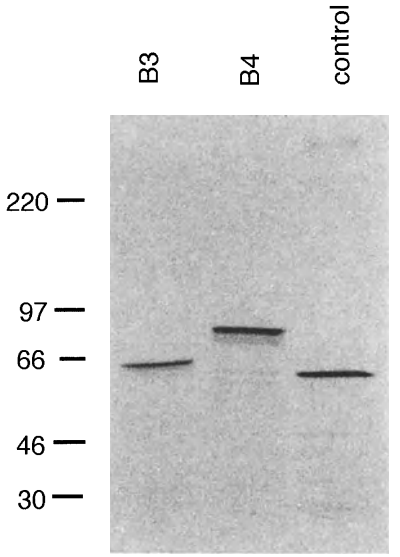


Fig. 1. In vitro transcription and translation of the B3 and B4 ZFM1 isoforms. The B3OS and B4OS cDNAs, cloned into pBluescript and pMos vectros, respectively, were in vitro transcribed and translated using a reticulocyte lysate assay with ^{35}S-methionine and are identified as *B3* and *B4* in the figure. Luciferase protein *(control)* was used as positive control of the reaction as well as molecular weight standards (61 kDa). The additional molecular weights indicated in the figure were calculated using Rainbow coloured proteins (Amersham, Buckinghamshire, UK) run in parallel in the polyacrylamide gel

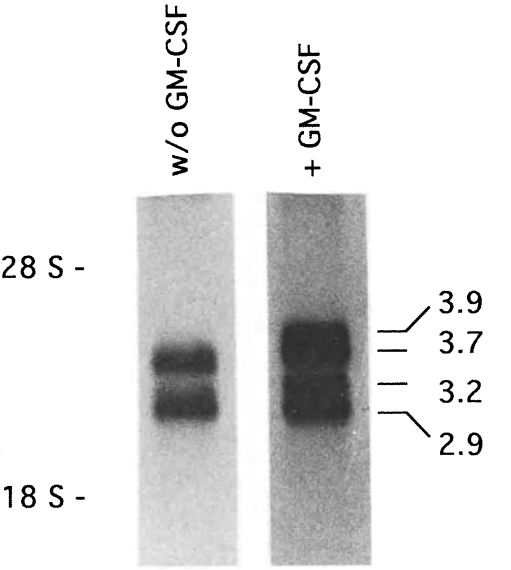


Fig. 2. Northern blot analysis of poly(A)$^+$ RNA from resting *(w/o GM-CSF)* and GM-CSF stimulated *(+ GM-CSF)* GF-D8 cells, probed with the B74cl cDNA clone

Expression of ZFM1 is Regulated by GM-CSF

To confirm ZFM1 expression in myeloid cells and its inducibility by GM-CSF, we used the B74c1 cDNA clone (919 bp long, corresponding to the 3′ end of ZFM1) as a probe on northern blots experiments. Using poly(A)$^+$ RNA, we show that the pattern of ZFM1 transcription is highly complex since in basal conditions, two bands with an apparent molecular weight of 2.9 and 3.7 kb are evident whereas upon treatment with GM-CSF the intensity of these two bands is consistently increased and accompanied by the appearance of two new bands of 3.2 and 3.9 kB (Fig. 2).

Moreover, by RT-PCR, we were able to generate a cDNA probe specific for the 383 nucleotide sequence exclusively present in the B4 isoform and identified as X in Figure 3. When used as a probe in Northern blot experiments with total cellular RNA obtained from GF-D8 cells, this B4 specific sequence hybridized only to the high molecular

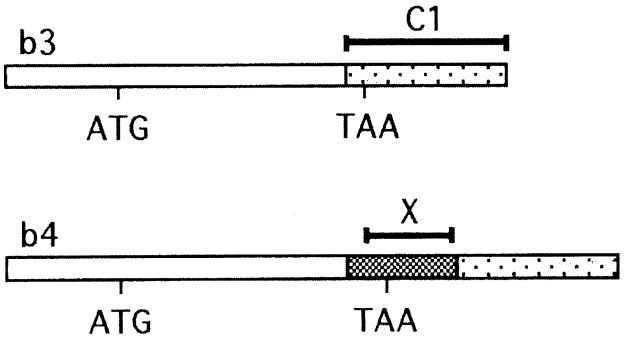


Fig. 3. Northern blot analysis of total RNA from GF-D8 cells sequentially hybridized on the same filter with the B74cl cDNA clone *(C1)* and, upon removal of this probe by high stringency washing, with a B4 specific sequence *(X)* which hybridizes only to the high molecular weight transcripts

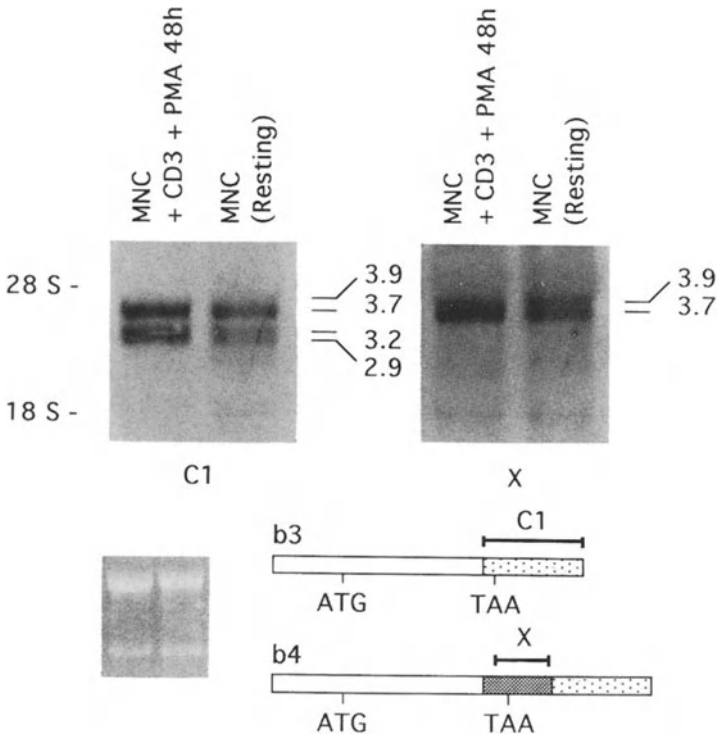


Fig. 4. Expression of ZFM1 in normal peripheral blood mononuclear cells in resting condition or upon in vitro stimulation of cell proliferation by mitogens. The same filter was sequentially hybridized with the B74c1 cDNA clone *(C1)* and, upon removal of this probe by high stringency washing, with a B4-specific sequence *(X)* which hybridizes only to the high molecular weight transcripts

weight transcripts, as compared to the B74c1 cDNA probe (indicated as C1 in Figure 3) which recognizes both high and low molecular weight transcripts.

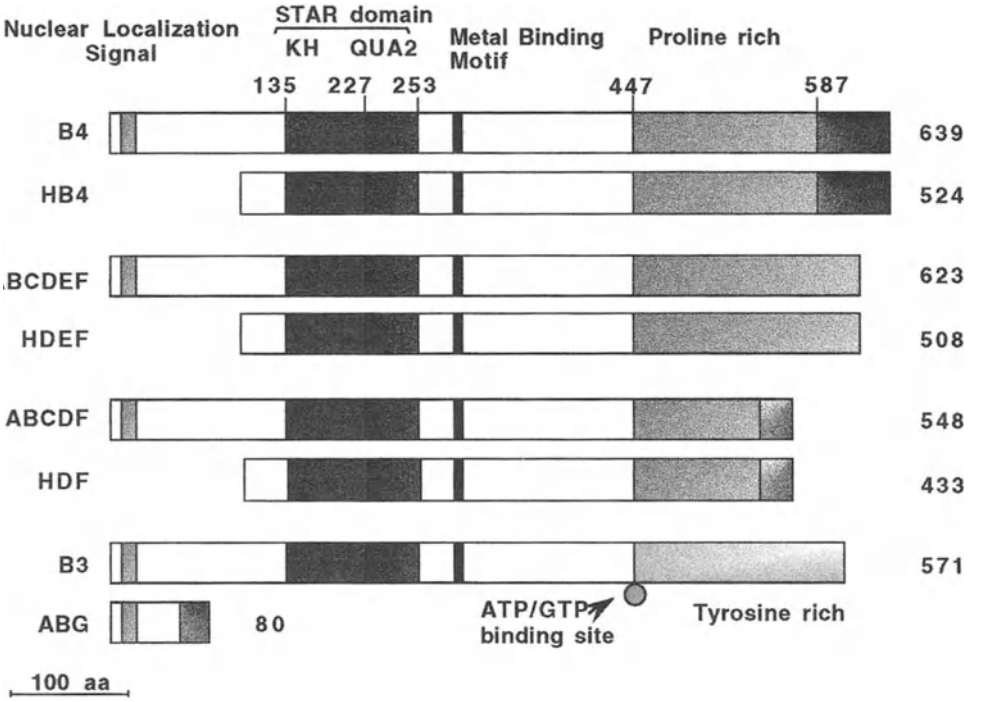
The peculiar hybridization pattern of the B4 specific nucleotide sequence was confirmed using RNA obtained from resting and mitogen activated normal peripheral blood mononuclear cells. In this case, however, no evidence of transcription induction was obtained upon stimulation of cell proliferation (Fig. 4).

Interestingly enough, ZFM1 expression was found partially regulated by GM-CSF also in highly purified normal human hematopoietic stem cells ($CD34^+$ cells) obtained from the peripheral blood of normal volunteers treated with G-CSF for collection of allogeneic stem cells (data not shown). Similarly, ZFM1 was found constitutively expressed in primary human myeloid leukemia cells obtained from AML patients at diagnosis. In vitro exposure to GM-CSF of leukemic blasts was able to prevent the spontaneous down modulation of ZFM1 gene expression observed in control samples (data not shown).

ZFM1 Belongs to the STAR Protein Family

The protein products of these two novel isoforms of the ZFM1 gene (B3 and B4) code for 571 and 649 aa, respectively. When compared to the ABCDEF and ABCDF isoforms of ZFM1 previously reported by Toda and coworkers [10], we notice that B3 and B4 encode for the same protein for the first 447 aa (Fig. 5).

Three amino acids (E at position 269, R at position 348 and R at position 377), are present in B3 and B4 sequences substituting for G, A and W residues, respectively, in the ABCDEF and ABCDF isoforms [10]. The B3 sequence diverges from the others starting at position 448. Interestingly, the Prosite screening revealed the presence of an "A type" ATP/GTP binding site motif overlapping the site of divergence from residue 443 to residue 450 (GPPPMGKS) of the B3 sequence. Also noteworthy, the GTAA additional nucleotides were previously considered the first four nucleotides of the intron lying between exon 10 and 11 [10]. Furthermore, the new carboxy terminus of B3 ap-



ig. 5. Schematic representation of ZFM1 isoforms. Numbers above the boxes indicate the site of divergence etween different proteins. Numbers on the right indicate the length in aminoacids

ears to be relatively rich in tyrosine residues (8/164). B4 diverges from ABCDF at position 528 and from ABCDEF at position 587. As previously described [10], the common C-terminal region of these isoforms (aa position 420 to 586) is rich in proline residues. Interestingly, the B4 specific C-terminal sequence starting at position 587 adds a further proline-rich region (23/53).

Using the Blast server at EMBL we noticed that very recently two new sequences had been identified which may represent the mouse (CW17R mouse accession number 1083269) and the *Saccharomyces cerevisiae* (CW17R SacCer, accession number 1256857) ZFM1 orthologous genes.

Furthermore, we noticed a significant alignement between ZFM1 protein and several other gene products containing a KH domain. This domain was originally identified in the human heterogeneous nuclear ribonucleoprotein (RNP) K protein [13–15] and now demonstrated to be able to bind single stranded RNA [16]. in ZFM1 the KH domain extends from position 135 to position 227 according to the exact definition of the boundaries of the KH domain recently obtained with the three dimensional solution structure [17].

Although a very large group of proteins that bind non specifically to ssRNA show the KH domain [17] a subgroup containing the ZFM1 protein has been identified for structural reasons and shown to include the ZFM1 gene, the Grp33 from *Artemia salina* (brine shrimp) [18], the human Sam68 (previously and erroneously identified as p62) [19, 20], the quaking gene (gkI) from mouse [21], the Gld-1 Cele from *Caenorhabditis elegans* [22] and the YKCA-Cele gene also from *C. elegans* (accession number P42083). We now update this growing family showing the alignement also with the CW17R from mouse (accession number 1083269) and CW17R SacCer from *Saccharomyces cerevisiae* (accession number 1256857), the mouse orthologous of Sam 68 (accession number 602251) and yet another *C. elegans* gene, the F54D1.1 Cele [23]. Interestingly, the alignement extends beyond the 3′ boundary of the

KH domain and confirms what had been hypothesised as the presence of another region of homology called QUA2 domain [21]. QUA2 was shown common to qkI, the human Sam68 and the Gld-1 genes [21].

Discussion

We cloned two novel isoforms of the ZFM1 nuclear protein which shows interesting structural properties including a nuclear transport domain, a metal binding motif and glutamine and proline-rich regions. ZFM1 maps on chromosome 11q13 where genetic linkage studies and analyses of loss of heterozigosity in multiple endocrine neoplasia type 1 (MEN1) associated tumors have localized the candidate genes for MEN1 [24, 25]. While the main common structural feature described so far for all the alternative COOH tails is their high proline content [26] it has to be noted that one of the isoform we cloned, because of four additional nucleotides, contains an ATP/GTP binding site and is also rich in tyrosine residues even though an abundant presence of potential phosphorylation sites is evident in all isoforms.

We also show that GM-CSF is able to regulate the expression of ZFM1 not only in the GF-D8 cell line, but also in human $CD34^+$ stem cells and freshly isolated myeloid leukemias. However, different ZFM1 isoforms have been identified from cDNAs libraries prepared from human brain and fetal liver [10]. In agreement with these data we also observed expression of ZFM1 in non-hematopoietic tissues, such as endothelial cells and placental preparations (data not shown) and expression of this gene has been shown in other tissues of mouse and man [27]. On the other hand, in human hematopoietic cells, we have been able to isolate only the B3 and B4 isoforms thus suggesting that ZFM1 might be differentially expressed in different tissues. Interestingly, a ZFM1 related sequence has been recently cloned as a gene activated during p53-induced apoptosis in mouse M1 myeloid leukemia cells [28].

A comparison of the sequence of the ZFM1 product with known proteins showed significant structural homologies with 9 other different genes. The homology is mainly due to the presence in all these genes of one KH domain, recently crystallised [17] and shown able to bind single stranded RNA [16, 27]. Proteins sharing a KH domain may act as docking proteins for molecules involved in signal transduction and gene expression [29] and we noticed that all such genes present another domain of homology immediately adjacent to the 3′ end of the KH domain and called QUA2 [21], which may take up an α helical conformation (data not shown). Intriguingly, for three genes of the family genetic lesions have been described; the ZFM1 may be a likely candidate for the genetic lesion responsible for the multiple endocrine neoplasia type 1 (MEN1) syndrome; the Gld-1 is subject to three different mutations which can give two different phenotypes, one is a loss of function and involves the RNA binding ability of the KH domain, the other two, more interestingly, give a change in phenotype (MOG for masculinization of germline) [22]. In addition, the recently cloned qkI gene, expressed in the earliest cells of the embryonic nervous system and in the myelinating tracts of the neonatal brain, is responsible for the quaking mutation in mouse and can suffer for truncations or ENU induced point mutations in a region immediately upstream the KH domain [21].

The presence of Sam68 gene which has been demonstrate to be a target of Src, Fyn and Grb2 phosphorilation [30] and the clear identification of a subclass of KH genes led to the proposal to identify such genes as STAR (signal transduction and activator of RNA) indicating the association of QUA and KH domains as a marker for this family. We now further substantiate this proposal by the demonstration that ZFM1 is a GM-CSF inducible gene and with the alignement of 10 different genes which clearly indicate the contemporary presence of KH and QUA2 domains in all members. This new group of STAR proteins has been proposed as a general cellular pathway analogous to the signal transduction and activation of transcription pathway JAK-STAT [21]. Preliminary experiments with a polyclonal antibody raised against the B4 isoform allowed to demonstrate that ZFM1 mainly localizes in the nucleus of GF-D8 cells exposed to GM-CSF (manuscript in preparation). On the other

hand, the B3 sequence presents several tyrosine residues at the COOH terminus which might be suitable substrates for phosphorylation induced by tyrosine kinases which are typically phosphorylated when cells are exposed to appropriate signals like GM-CSF in the case of hematopoietic progenitor cells [31]. Since ZFM1 belongs to this emerging family of potential signal transduction molecules, experiments are currently ongoing to elucidate whether ZFM1 is a protein target of phosphorylation.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Expression of Tie Receptor Tyrosine Kinase in Leukemia Cell Lines

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Abstract. The *tie* gene encodes a receptor tyrosine kinase that together with its thus far unidentified ligand appears to play a distinct role in the regulatory pathway of early hematopoiesis and angiogenesis. We analyzed the possible involvement of *tie* in the pathobiology of hematopoietic malignancies by examining *tie* mRNA expression in human leukemia and lymphoma cells. We used a panel of 93 human leukemia-lymphoma cell lines as model systems for the various hematopoietic cell lineages. At the Northern blot level, 0/27 lymphoid leukemia or lymphoma-derived cell lines (originating from 4 B-precursor leukemia, 4 B-cell leukemia, 4 B-cell non-Hodgkin´s lymphoma, 2 myeloma, 2 Burkitt lymphoma, 4 T-cell leukemia, 5 Hodgkin lymphoma, 2 anaplastic large cell lymphoma) expressed *tie* transcripts, whereas 23/42 (55%) of the myeloid cell lines expressed *tie* mRNA: in detail, 15/20 (75%) megakaryocytic, 5/11 (45%) erythroid, 3/7 (43%) myelocytic and 0/4 monocytic cell lines were *tie* mRNA positive. In the reverse transcriptase-polymerase chain reaction analysis, detecting low levels of mRNA expression, all 12 myeloid cell lines and 19/39 (48%) lymphoid cell lines were positive. In experiments aimed at inducing cellular differentiation, the phorbol ester PMA strongly enhanced *tie* mRNA expression in one erythroid and in one myelocytic cell line, but (like thrombopoietin) down-regulated *tie* mRNA expression in two megakaryocytic cell lines. These results indicate that *tie* is predominantly expressed in leukemia cells derived from the myeloid cell lineages (and here in particular in megakaryoblastic cells) and not in lymphoid leukemia cells. These observations provide some evidence for the hypothesis that *tie* is a receptor for a regulatory factor involved in normal and plausibly also leukemic hematopoiesis.

Introduction

Proliferation and differentiation of hematopoietic cells is controlled by a series of soluble and membrane-bound regulators and their receptors. Protein tyrosine kinases are involved in the transduction of a variety of these extracellular signals into distinct cellular responses [1]. The complexity of intracellular signaling is reflected by the diversity of the tyrosine kinases that comprise a large gene family [2]. The so-called receptor tyrosine kinases (RTKs), for instance *c-fms, c-kit, flt-3 and ufo/axl* [3, 4], function directly as receptors for hematopoietic factors.

Reverse transcription-polymerase chain reaction (RT-PCR) with degenerate oligonucleotide primers had been used to clone the *tie* RTK gene isolated from the chronic myeloid leukemia (CML)-derived cell line K-562 treated with the phorbol ester PMA [5, 6]. *tie* and the related *tek* (also termed *tie2*) constitute a distinct family of RTKs sharing cer-

DSMZ-German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures, Braunschweig, Germany

tain structural properties [7, 8]: two immunoglobulin-like domains, three epidermal growth factor-like domains, and three fibronectin type III-like repeats in the extracellular region and a kinase domain with a short kinase insert sequence in the intracellular region. *tie* and *tek* are broadly expressed in endothelial cells from different murine and human tissues, including differentiating angioblasts and vascular endothelial cells of embryos [9]. Studies on knockout mice showed that they have critical, but distinct roles in blood vessel formation [10, 11].

The detection of *tie* in the developing blood islands of mouse embryos and in murine bone marrow stem cell fractions suggested that *tie* is also expressed in primitive human hematopoietic cells [8, 9]. Recent reports described the expression of *tie* in human hematopoietic stem/progenitor cells [12, 13].

To begin to understand the possible pathobiological involvement of the *tie* RTK in leukemogenesis we examined its expression at the mRNA level in a panel of human cell lines representing the major hematopoietic cell lineages.

Materials and Methods

Culture of Cell Lines and in Vitro Stimulation

The continuous cell lines were taken from the stock of the DSMZ cell bank (German Collection of Microorganisms and Cell Cultures) [14] or were generously provided by the investigators who established the cell lines for research purposes. They were grown at 37 °C in a humidified atmosphere of air containing 5% CO_2. The basal growth media (Gibco BRL, Eggenstein, Germany) were supplemented with 5-20% fetal bovine serum (FBS) (Sigma, Deisenhofen, Germany). Several cell lines are constitutively growth factor-dependent for survival and proliferation; these cell lines were cultured with 10-20% (vol) of conditioned medium from the human bladder carcinoma cell line 5637 known to contain several cytokines including granulocyte colony-stimulating factor, granulocyte-macrophage CSF and stem cell factor. The adherent cell line 5637 was detached with trypsin/EDTA. The cell counts and viabilities were examined by trypan blue (Sigma) dye exclusion. Cells were harvested for DNA and RNA isolation in their logarithmic growth phase and with viabilities of more than 90%. Cell pellets were stored frozen at -20 °C or were further processed immediately.

In vitro stimulation was performed with the protein kinase C activator phorbol 12-myristate 13-acetate (PMA; Sigma) and the vitamin A-analogue all-trans retinoic acid (ATRA; Sigma). These reagents were first dissolved in ethanol at an initial concentration of 10^{-3} M, subsequently diluted in RPMI 1640 medium and used at a final concentration of 1×10^{-7} M, so that the final concentrations of the solvent was maximally 0.01%. Some cell lines were also treated with 100 U/ml thrombopoietin (TPO; from Zymogenetics, Seattle, WA, USA). Kinetic analyses were performed by harvesting cells after different incubation times (after 0, [12], 24, 48, 72 or [96] h).

Isolation of RNA and Northern Blot Analysis

Total RNA was isolated by guanidinium isothiocyanate and cesium chloride gradient centrifugation. 10 µg total RNA were separated in a denaturing 1.0% agarose formaldehyde gel and transferred to a nylon membrane. The RNA was fixed to the membrane by baking the filter at 120 °C for 30 min. Filter hybridization was carried out after 2 h of prehybridization by addition of the probe that was labeled with [α-^{32}P]dCTP (Amersham-Buchler, Braunschweig, Germany) by random-priming (Stratagene, Heidelberg, Germany) or nick translation (Gibco BRL). The membranes were hybridized overnight at 62 °C. After stringent washing the membranes were exposed to X-ray films with intensifying screens for several days. For the detection of the β-actin housekeeping gene the membranes were stripped and rehybridized with a hamster β-actin probe. The following probes were used: a 1.7 kb partial *tie* cDNA cloned into the HindIII/SacI site of the GEM3Zf(+)-vector (from Dr. K. Alitalo, Helsinki, Finland). The β-actin probe

was a 1.25 kb fragment of the cDNA of the hamster β-actin cloned into the *Pst*I site of pBR322.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Five μg total RNA were used for the synthesis of first strand cDNA applying a RT preamplification kit (SuperScript; Gibco BRL). The RT was carried out with 50 ng of random hexamers in a final volume of 20 μl RT buffer (containing 20 mM Tris-HCl of pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin). The mixture was incubated at 70 °C for 10 min; 200 U of Moloney murine leukemia virus RT and 1 μl of 10 mM dNTP mix were added to the reaction and incubated at 42 °C for 50 min. The reaction was stopped by heating to 90 °C for 5 min and then quickly chilled on ice. After brief centrifugation, 2 U of RNase H were added to the reaction mixture and incubated for 20 min at 37 °C. 2 μl first strand cDNA (corresponding to 0.5 μg of RNA) were diluted with PCR buffer (10X 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) containing 20 pmol of each upstream and downstream primer, 10 nmol of dNTP mix and 1 U of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany) for the PCR reaction. To detect the *tie* mRNA the primers used were sense F-TIE (5′-TCGGCTCCTGACCAAGCAGAG-3′) (nt 1395-1415) and antisense R-TIE (5′-AGTCCCGTCAGGAGGGCAGT-3′) (nt 1865-1846). To determine the quality of the RNA, the RT reaction and the PCR amplification, the following primers were used to amplify β-actin: sense F-ACTIN (5′-ATGGATGATGATATCGCCGCG-3′) and antisense R-ACTIN (5′-CTAGAAGCATTTGCGGTGGAC-3′). The PCR reactions were performed with a DNA thermal cycler (Perkin Elmer Cetus, Heidelberg, Germany) under the following conditions: denaturation for 7 min at 95 °C; 3 min at 75 °C and addition of the Taq polymerase to perform a hot-start PCR; 2 min at 60 °C and 10 min at 72 °C for one cycle. The amplification was carried out for 35 cycles of 30 sec at 94 °C, 30 sec at 60 °C and 60 sec at 72 °C with 2 sec of extension time per cycle. Nine μl of the reaction mix were electrophoresed on an ethidium bromide-stained 1.3% agarose gel, blotted with 20X SSC onto nylon filters (GeneScreen), and hybridized with radioactively-labeled probes using standard methods.

Results

Constitutive Expression of tie mRNA in Leukemia-Lymphoma Cell Lines

We examined the tie mRNA expression in 69 human leukemia- or lymphoma-derived cell lines using standard Northern blot analysis (Table 1). A 4.4 kb mRNA signal (Fig. 1) was detected in 23/42 (55%) cell lines assigned to the myeloid lineages, but in none of the 27 cell lines derived from lymphoid leukemias or lymphomas (4 B-precursor cell leukemia, 4 B-cell leukemia, 4 B-cell NHL and 2 Burkitt lymphoma, 2 myeloma, 4 T-cell leukemia, 5 Hodgkin and 2 ALCL). The following results on the *tie* mRNA expression of myeloid cell lines were seen: 15/20 (75%) megakaryocytic, 5/11 (45%) erythroid, 3/7 (43%) myelocytic, and 0/4 monocytic. In addition, RT-PCR was performed to detect very low level expression of *tie* (Table 1). Here, all myeloid cell lines tested (3/3 megakaryocytic, 5/5 erythroid, and 4/4 myelocytic) were positive; however, a large percentage of lymphoid lines (7/10 B-precursor cell, 7/14 B-cell, 1/5 plasma cell, and 4/10 T-cell) also expressed signals (Fig. 2).

Modulated Expression of tie mRNA in Leukemia Cell Lines

In order to analyze the regulation of *tie* transcription during induced in vitro differentiation, six leukemia cell lines were incubated with the biomodulators PMA (phorbol ester), ATRA (vitamin A-analogue) or TPO (cytokine) for up to 96 h (Table 2). A strong increase in *tie* mRNA expression was seen in the erythroid K-562 cells and in the myelocytic MUTZ-2 cells during treatment with PMA. This phorbol ester down-regulated *tie* mRNA expression in the myelocytic TI-1 and the megakaryocytic M-07e and M-MOK

Table 1. Human *tie* mRNA expression in leukemia-lymphoma cell lines

Cell line	Type/ origin[a]	*tie* mRNA[b] NB	PCR	Cell line	Type/ origin[a]	*tie* mRNA[b] NB	PCR
MEGAKARYOCYTIC CELL:[c]				ALL-1	pre B-ALL		+
CHRF-288-11	AML M7	+		EU-1	ALL		(+)
CMK	AML M7	++		LILA-1	pre B-ALL		(+)
ELF-153	AML M7	–		LK-63	pre B-ALL		–
HIMEG	CML	–		NALM-6	ALL		–
HU-3	AML M7	+++		TAHR-87	AUL		(+)
KH-184	AML M7	–		B-CELL:			
M-07e	AML M7	++	+++	BJAB	Burkitt	–	–
MC-3	CML-BC	++		BONNA-12	HCL	–	(+)
MEG-01	CML-BC	(+)		DOHH-2	B-NHL	–	
MEG-A2	CML-BC	++		EB-1	Burkitt	–	+
MEGAL	AML M7	–	++	EHEB	B-CLL	–	+
MKPL-1	AML M7	+++		ESKOL	HCL	–	(+)
M-MOK	AML M7	++		HBL-1	B-NHL	–	
MOLM-7	CML-BC	(+)		HC-1	HCL	–	+++
NS-MEG	CML-BC	(+)		KARPAS-422	B-NHL	–	
SAM-1	CML-BC	(+)		MC-116	B-NHL	–	
T-33	CML-BC	(+)		1E8	B-ALL		–
TS9;22	CML-BC	–		BALM-1	B-ALL		–
UT-7	AML M7	(+)		DAUDI	Burkitt		–
YS9;22	CML-BC	+	+	JVM-2	B-PLL		(+)
ERYTHROID CELL:[c]				KARPAS-353	B-ALL		–
AP-217	CML-BC	(+)		MN-60	B-ALL		(+)
F-36EGM	AML M6	–	+	NAMALWA	Burkitt		–
F-36P	AML M6	–		WIEN-133	B-ALL		–
HEL	AML M6	++	+++	PLASMA CELL:			
JK-1	CML-BC	–		EJM	myeloma	–	–
K-562	CML-BC	–		MM-S1	myeloma	–	++
KH-88	CML-BC	–		KARPAS-707	myeloma		–
KMOE-2	AML M6	+	+++	L-363	PCL		–
LAMA-84	CML-BC	(+)	(+)	OPM-2	myeloma		–
OCI-M1	AML M6	+++	++	T-CELL:			
TF-1	AML M6	–		BE-13	T-ALL	–	
MONOCYTIC CELL:				CCRF-CEM	ALL	–	+
KBM-3	AML M4	–		LOUCY	T-ALL	–	++
MOLM-13	AML M4	–		MOLT-16	T-ALL	–	(+)
THP-1	AML M5	–		HPB-ALL	T-ALL		–
U-937	hist. lymphoma	–		JURKAT	ALL		–
MYELOCYTIC CELL:				KE-37	ALL		–
HL-60	AML M2	–	+	MDS	CMML		–
KG-1	AML	++	+++	P12/ICHIK.	ALL		+++
KG-1a	AML	–	++	PF-382	ALL		–
KOPM-28	CML-BC	+++		SKW-3	CLL		–
KYO-1	CML-BC		(+)	HODGKIN-ALCL:			
MUTZ-2	AML M2	–		CO	Hodgkin	–	
TI-1	AML M2	++		DEL	ALCL	–	
B-PRECURSOR CELL:				FE-PD	ALCL	–	
697	cALL	–	+	HDLM-2	Hodgkin	–	
BV-173	CML-BC	–	–	HD-MY-Z	Hodgkin	–	
HAL-01	ALL	–	++	L-428	Hodgkin	–	
LAZ-221	ALL	–	++	L-540	Hodgkin	–	

Abbreviations: ALCL = anaplastic large cell lymphoma; ALL = acute lymphoblastic leukemia; AML = acute myeloid leukemia; AUL = acute undifferentiated leukemia; B = B-cell; Burkitt = Burkitt's lymphoma; cALL = common ALL; CLL = chronic lymphocytic leukemia; CML-BC = chronic myeloid leukemia in blast crisis; CMML = chronic myelomonocytic leukemia; HCL = hairy cell leukemia; hist. lymphoma = histiocytic lymphoma; Hodgkin = Hodgkin's lymphoma; NHL = Non-Hodgkin's lymphoma; PCL = plasma cell leukemia; PLL = prolymphocytic leukemia; pre B = pre B-cell; T = T-cell.

[a] Cell lines were assigned to the respective cell type categories based on their origin and their phenotypic and functional characteristics; subtypes are given as indicated in the original publications (for details on cell lines see ref. [14-16]).

[b] tie expression was examined by Northern blot (NB) and/or RT-PCR (PCR) analysis; , negative; (+), barely detectable; + - +++, faint to very strong.

[c] The differentiation between erythroid and megakaryocytic cell lines is occasionally arbitrary as these lines often share expression of surface antigens (e.g. glycophorin A, CD41, CD42, CD61), transcription factors (e.g. GATA-1, NF-E2, SCL) and other parameters (e.g. platelet peroxidase, hemoglobin) of both lineages.

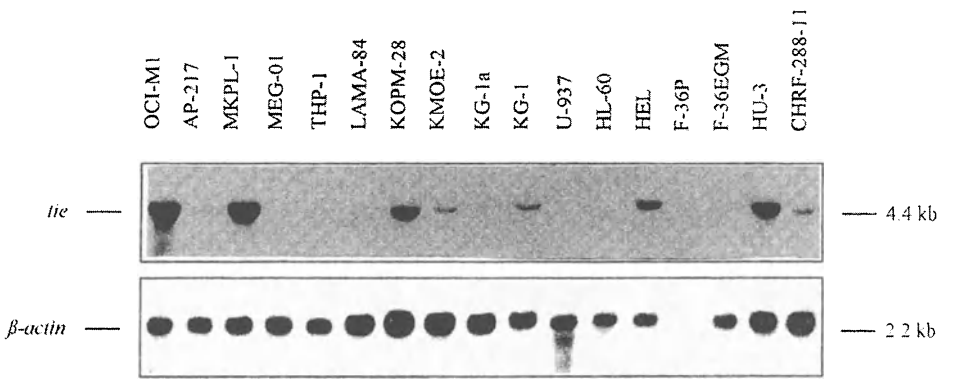

Fig. 1. Northern blot analysis of *tie* mRNA expression in leukemia cell lines. Isolated RNAs were separated by formaldehyde-agarose gel electrophoresis, Northern-blotted and hybridized with the *tie* cDNA probe. Hybridization with the ß-actin probe was used as an internal control to assure the loading of equal amounts and the integrity of RNA (see Table 1 for details on the cell lines).

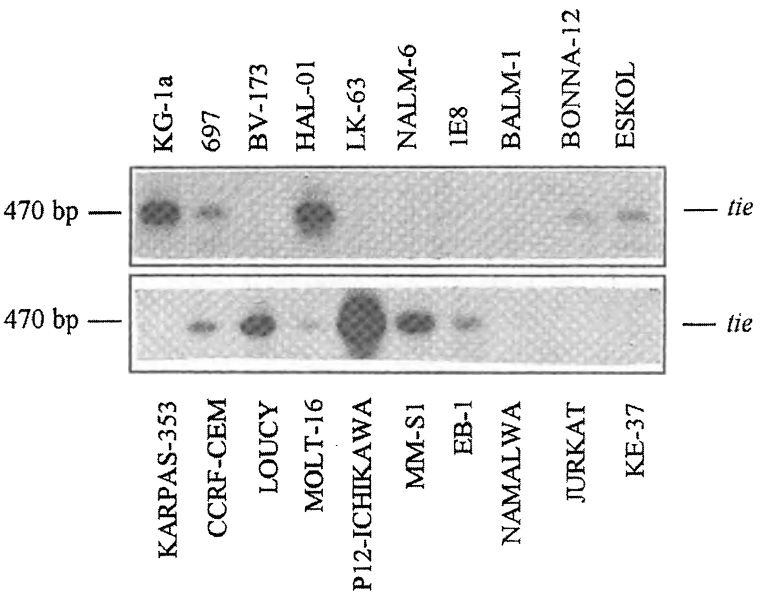

Fig. 2. RT-PCR analysis of *tie* mRNA expression in leukemia cell lines. The PCR products were separated by agarose gel electrophoresis, Southern-blotted and visualized with a radiolabeled *tie*-specific probe (see Table 1 for details on the cell lines).

cell lines. TPO also down-regulated *tie* mRNA levels in M-07e and M-MOK. KG-1 cells were unresponsive to PMA or ATRA in this regard.

Discussion

The pattern of tie expression in normal and malignant cells indicated that this RTK is involved in angiogenesis and hematopoiesis [17]. Endothelial cells and hematopoietic stem cells are thought to originate from a common precursor cell, the hemangioblast. tie does not appear to be absolutely required for hematopoietic stem cell function as targeted mutations of the gene did not completely abolish hematopoiesis [10, 11, 18]. Still, tie-deficient mice died of hemorrhaging during their development.

To identify the nature of tie expression in hematopoietic cells, we have determined here the specificity and expression pattern of the tie gene among the various hematopoietic cell lineages. A total of 93 cell lines derived from the various hematopoietic lin-

Table 2. Tie mRNA expression in cell lines treated with differentiation-inducing agents

Cell line	Cell type	Modulator [a]	Regulation of tie mRNA expression [b]			
K-562	Erythroid	PMA	Up-regulation:	-	→	++/+++
MUTZ-2	Myelocytic	PMA	Up-regulation:	-	→	++
TI-1	Myelocytic	PMA	Down-regulation:	++	→	+/(+)
M-07e	Megakaryocytic	PMA	Down-regulation:	++	→	(+)
		TPO	Down-regulation:	++	→	+
M-MOK	Megakaryocytic	PMA	Down-regulation:	++	→	+
		TPO	Down-regulation:	++	→	+
KG-1	Myelocytic	PMA	Unchanged:	++		
		ATRA	Unchanged:	++		

[a] Cells were exposed for 72-96 h to these reagents at concentrations indicated above.
[b] *tie* mRNA transcripts were visualized by Northern blotting; results are expressed as up- or down-regulation of expression in comparison with the untreated control cells; , negative; (+), barely detectable; + - +++, faint to very strong.

eages were screened with a *tie* cDNA probe. Using Northern blotting to detect mRNA expression, none of the 27 lymphoid leukemia or lymphoma-derived cell lines that were established from patients with B-precursor cell leukemia, B-cell leukemia, B-cell non-Hodgkin´s or Burkitt's lymphoma, myeloma, T-cell leukemia, Hodgkin's or anaplastic large cell lymphoma expressed *tie* transcripts. On the other hand, 23/42 (55%) of the myeloid cell lines investigated displayed *tie* mRNA: 15/20 (75%) megakaryocytic, 5/11 (45%) erythroid, 3/7 (43%) myelocytic and 0/4 monocytic cell lines were *tie* mRNA positive. RT-PCR analysis, to also probe for very low levels of mRNA expression, detected *tie* expression in all 12 myeloid cell lines and in 19/39 (48%) lymphoid cell lines. These findings confirm and greatly extend data reported by others [13, 19] who detected *tie* mRNA transcripts by Northern blot analysis in (on aggregate, both previous studies taken together) 0/5 T-cell, 0/4 pre B-/B-cell, 0/4 myelocytic, 1/2 monocytic, 1/2 erythroid and 4/4 megakaryocytic leukemia cell lines.

Recently, two groups reported that *tie* is strongly expressed in primitive hematopoietic cells, namely the $CD34^+$ cells isolated from umbilical cord blood and bone marrow [12, 13]. While up to 80% of the most immature hematopoietic stem cell, $CD34^+$ $CD38^-$ cells, were tie^+, all peripheral blood mononuclear cells, thus mature terminal cells, were tie^-. Surprisingly, some $CD34^-$ $CD19^+$ $CD20^+$ B-cells were found to express *tie* [13]. Stem/progenitor cell culture assays demonstrated that the tie^+ fraction contained more primitive cells than the tie^- fraction suggesting that *tie* is expressed physiologically in primitive stem cells rather than in committed progenitor cells.

Furthermore, tie^+ cells contained more granulocyte-macrophage colony-forming cells than tie^- cells [13], supporting our finding that *tie* mRNA was expressed in a significant percentage of myelocytic leukemia cell lines. Under culture conditions allowing for myeloid, erythroid and/or megakaryocytic differentiation the $CD34^+$ normal cells lost tie expression concomitantly with that of CD34; also the number of tie^+ cells induced by TPO to differentiate decreased over the treatment period of 10 days. These results are in accordance with our experiments in which we found down-regulation of *tie* mRNA expression in megakaryocytic or myelocytic cell lines induced to differentiate by the phorbol ester PMA or the cytokine TPO. However, we also observed up-regulation of *tie* mRNA transcription in two cell lines which has also been reported by others [13, 19]. It has been argued that during megakaryocytic differentiation *tie* mRNA expression can be increased by exposure to phorbol esters [12, 13, 19]. Moreover, *tie* seems to be considerably down-regulated in normal megakaryocytes in comparison with (some) leukemia cell lines [12]. Further studies are required to establish whether the

up- or down-regulation of *tie* mRNA expression correlates with a specific stage of differentiation and/or a defined cell lineage.

In summary, the structure of the orphan *tie* RTK indicates that it functions as a receptor for a thus far unknown ligand. This factor might physiologically affect myeloid proliferation and/or differentiation, in particular differentiation along the megakaryocytic-erythroid cell axes. Given its strong and widespread expression in myeloid leukemia cell lines, an involvement of *tie* in myeloid leukemogenesis is plausible, but further experimental evidence is required to support this notion.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Acute Erythroblastic Leukemia is a Rare, but Distinct Subtytpe of Acute Myeloid Leukemia

M. Saito, K. Kuriyama, H. Tsusima, Y. Miyazaki, and M. Tomonaga

Abstract. We described four cases of de novo acute leukemia, which were initially diagnosed as undifferentiated leukemia or AML-M0 because of negative myeloperoxidase (MPO) activity, absence of B/T lymphoid markers and positive myeloid markers (CD13 and/or CD33). They were finally diagnosed as acute erythroblastic leukemia by the specific reaction to EPO in in vitro cultures. The leukemic blasts showed proerythroblast-like morphology characterized by highly basophilic cytoplasm with some vacuoles, positive acid phosphatase reaction and CD36. In semi-solid cultures of blast cells with EPO, all cases showed colony formation and we could establish an EPO-dependent leukemic cell line from them. These cases can be distinguished from AML-M0 and AML-M6 or erythroleukemia, based on some characteristics such as pure population of erythroid precursor cells and absence of MPO(+) myeloblasts. These cases could be categorized as a distinct subtype of AML as French Group recently proposed this category as M6 variant. This subtype seems to belong to refractory AML such as AML M0 and M6.

Introduction

We have experienced four cases of de novo acute leukemia, which were initially diagnosed as AML-M0 or acute undifferentiated leukemia (AUL) [1], but an erythroid colony growth of leukemic origin was observed in all cases. The blasts were characterized by proerythroblast-like morphology, presence of CD36 antigens, negative T/B lymphoid markers, and their responsiveness to erythropoietin(EPO). According to these results, we concluded that their leukemic blasts were erythroid precursors arrested at CFU-E or BFU-E level, and not classifiable by FAB criteria. Similar cases were previously reported as early erythroblastic leukemia or AML-M6 variant [2-7].

Material and Methods

Four cases are selected from de novo acute leukemias for the following reasons:

1. <3% leukemic cells had myeloperoxidase (MPO) activity,
2. surface T/B markers were not present, and CD13 and/ or CD33 were present,
3. in vitro colony formation in response to EPO.

Smears were stained with May-Grunwald Giemsa, myeloperoxidase, alpha-naphthyl acetate esterase, alpha-naphthyl butyrate esterase, acid phosphatase, and periodic acid Schiff.

Mononuclear cells of the bone marrow were separated with Ficol Conray. The surface immunophenotype was assessed by immunofluorescence with flow cytometer (CYTRON ABSOLUTE, Ortho Diagnostic

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System, Tokyo, Japan). Monoclonal antibodies (MoAbs) were purchased from multiple commercial sources. The mononuclear cells were cultured in Iscove's modified Medium supplemented with 20% heat inactivated fetal calf serum(FCS) in the presence of 2U/ml of EPO and observed the colony formation after 2-3 weeks. After washing with Iscove's medium, mononuclear cells were fixed with diaminobenzidine and embedded in Epon. Thin sections were examined with an electron microscope. Cytogenetic analyses was performed using the G-band method.

Results

Clinical and Laboratory Features

The results are shown in Table 1. All of them were male, and above 50 years old. All cases had anemia, and the percentage of blasts in the peripheral blood was relatively low. Many of them showed hypercellular marrow and organomegaly.

Leukemic Cell Morphology and Cytochemistry

The morphology of leukemic cells is shown in Fig.1. The leukemic cells appeared as medium to large size (15–30 μm), with an extremely basophilic cytoplasm. A clear zone was present as a thin pale crescent near the nucleus, and several peroxidase negative azurophilic granules or small vacuoles were present in the Golgi zone. The nucleus was large with one or two nucleoli. Some of them showed a proerythroblast-like morphology. The myeroperoxidase activity was totally negative, but acid phosphatase was positive and showed dot-like activity at the Golgi area.

Immunophenotype

Results of immunophenotyping are shown in Table 2. Three of four cases had positive myeloid markers(CD13 and/or CD33), and two had CD36 antigen(ag).

Cytogenetics

Cytogenetic results are shown in Table 3. All but one showed abnormal karyotype, and the abnormalities are complex and multiple.

In Vitro Culture of Leukemic Cells

All cases showed colony formation in EPO-containing semi solid culture, and we could establish an EPO-dependent leukemic cell line from them.

Ultrastructural Study

Ultrastructural studies were performed in two cases. Platelet peroxidase-like activity and ferritin-containing granules were observed in one of them.

Review of Similar Cases in the Literature

Sixteen cases of de novo acute erytheoblastic leukemias have been previously reported. Six were infant (4 Down's syndrome), and ten adults. All of adult cases were above 50 years old, and five of them were above 70 years old. Morphological and cytochemistlical characteristics of blast cells were similar to those of our cases. All cases showed negative MPO activity, and 90% showed positive acid-phosphatase with a dot-like pattern. Immunophenotyping showed more than 30% had positive myeloid markers (CD13 and/or CD33) and 90% had positive CD36 ag. In vitro culture was performed in 8 cases, and colony formation in the presence of EPO was observed in 7 of them, and EPO dependent cell line had established in three of them. Electron microscopic study was performed in 7 cases, and platelet peroxidase activity without CD41 ag and/or ferritin molecule was present in six.

Discussion

Acute erythroblastic leukemia(AEL) or early erythroblastic leukemia is a rare subtype of acute leukemia which were previously

Table 1. Patients' characteristics at initial diagnosis

	Age (yr)	Sex	Peripheral blood RBC;mal10 (/micro 1)	Hb, (g/dl)	Hct. (%)	WBC (μl)	Blast (%)	Plt.×10 (/micro 1)	Bone marrow NCC	Blast (%)	G/E	LDH unit	Outstanding signs
Case 1	60	M	220	7.1	21.8	7800	12.0	8.1	16 100	72.2	1.97	347	Anemia, splenomegaly
Case 2	50	M	181	5.5	16.4	4200	2.0	3.3	Hyper	27.4	5.90	719	Anemia, hepatomegaly, edema
Case 3	72	M	191	6.9	18.9	3500	4.0	2.2	Hyper	67.6	1.38	5211	Anemia
Case 4	77	M	164	6.2	16.6	2300	2.0	7.1	55 000	38.8	2.41	2500	Anemia, hepatosplenomegaly

NCC: nucleated cell count.

Table 2. Surface immunophenotype analysis

	CD3	CD5	CD7	CD9	CD10	CD13	CD14	;d33	CD34	CD41a	CD71	HLA-DR	CD36	GPA	TdT
Case 1	1.8	Nd	5.5	18.3	1.1	33.7	26.6	47.7	8.4	18.0	ND	11.3	ND	ND	–
Case 2	ND	–	ND	ND	–	ND	ND	ND	ND	ND	ND	+	ND	ND	–
Case 3	0.8	1.5	ND	0.4	ND	6.0	ND	39.4	11.6	3.1	84.9	48.2	95.5	0.2	ND
Case 4	47.8	19.8	20.2	9.5	5.3	25.9	24.1	35.9	ND	4.3	48.2	67.4	40.4	12.0	–

ND: not done −: negative +: positive TdT: terminal deoxynucleotidyl transferase.

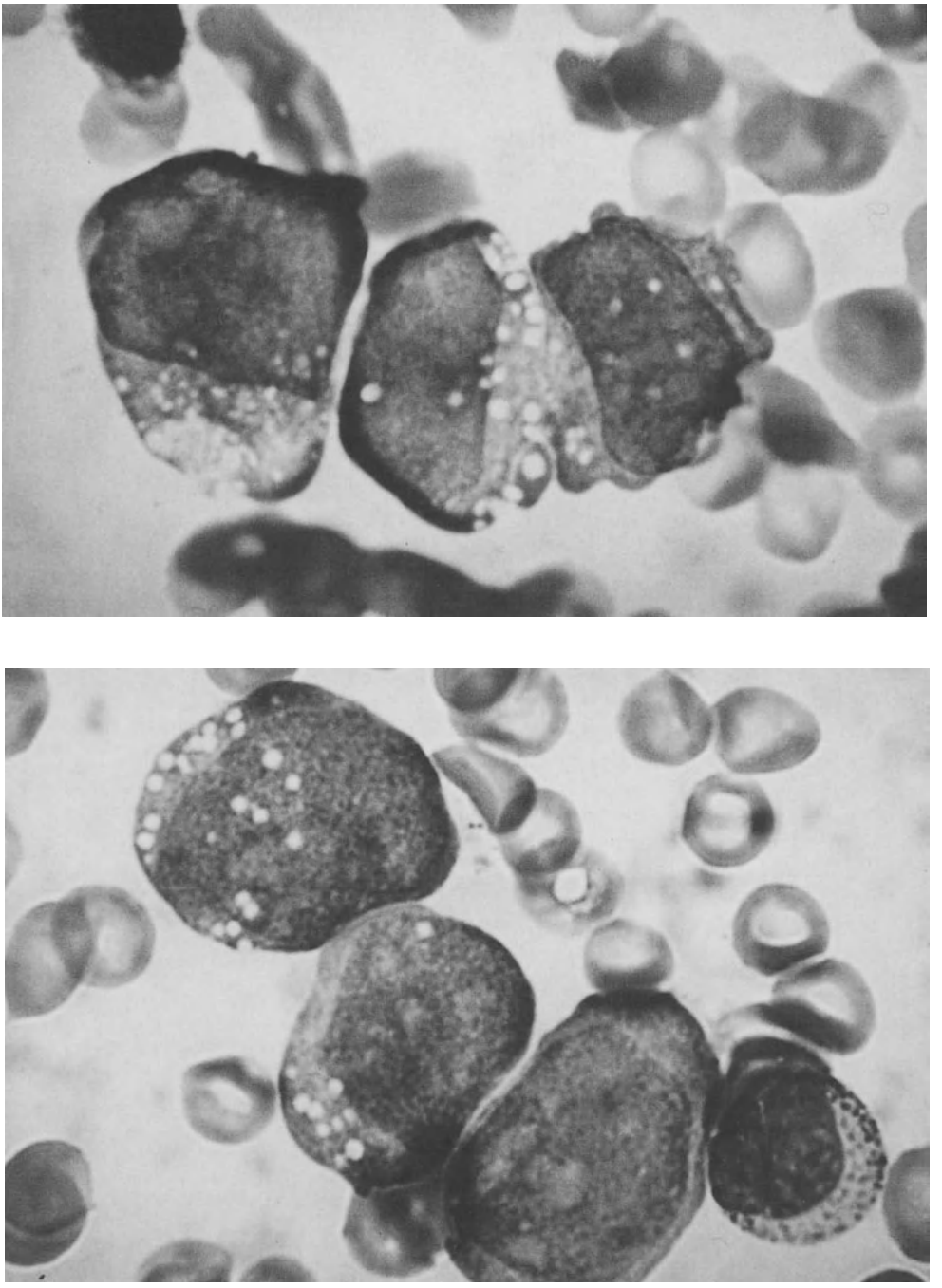

Fig.1. The morphology of leukemic cells (case 1 and case 3)

Table 3. Kyryotypic analysis

	Karyotype
Case 1	46, XY [20]
Case 2	Hypote traploid [11/13], 46 XY [2/13]
Case 3	38–43, XY, 1q+, 5p+, 6p+, −7, −13, −16, 19p+, −20, −22, +mar, +mar [cp20]
Case 4	43–46, X, -Y, -8, 14p+, 15p+, 16q+, -16, 19p+, 21p+, +mar [cp18]

categorized as AUL or AML-M0. These cases are different from AML-M6 or erythroleukemia, which consists of morphologicaly identifiable erythloblasts in more than 50% of nucleated cells and myeloblasts in more than 30% of non-erythroid cells. According to immunophenotyping and colony formation with EPO, the blast cells of AEL are likely to be erythroid precursors arrested at CFU-E or BFU-E level.

A French group has previously proposed the cases of leukemia with a proerythloblast-like or undifferentiated morphology and HLA-DR(-), CD36 (++), B (-), T(-), myeloid (+/-) immunophenotype as "AML-M6 variant" [7]. They and others included secondary leukemia and CML crisis among the cases they described. In this report, we describe four cases of de novo AEL, which show relatively homogeneous characteristics like the cases previously reported as early erythrolblastic leukemia or FAB-M6 variant. These characteristics are as following: (1) morphology of blasts is relatively homogeneous, showing features of erythroid precursor cells, (2) negative surface T/B antigens, and often positive myeloid markers (CD13 and/or CD33), (3) many of them are positive for CD36 antigens, and some shows positive glycophorin A, (4) in vitro colony formation in response to EPO, (5) positive PPO-like activity without megakaryocytic maturation(CD41 negative) and ferritin-containing granules with an electron microscope. Further heterogeneity should be present in this subtype, depending on the level that the blasts are arrested. Many of the cases showed poor prognosis, suggesting that the subtype belong to refractory AML.

We propose acute erythroblastic leukemia as a distinct subtype of de novo acute myeloid leukemia.

Acknowledgment. We thank Dr. Miyawaki S, Saiseikai Hospital, Gunma, for presentation of case 3 and case 4.

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Experimental Approaches and Novel Therapies
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Plasma Cell Leukemia in a 7-month-old Infant

M. ZEGADLO, M. MATYSIAK, and M. OCHOCHKA

Abstract. We report a case of a very rare malignant disease of the haemopoietic system. The clinical picture was characterized by anaemia, manifestations of haemorrhagic diathesis and marked hepatosplenomegaly. The child died 9 months after the diagnosis from progression of leukemia and haemorrhage into the central nervous system.

Introduction

Plasma cell leukemia is a rare type of malignant disease of the haematopoietic system. It occasionally occurs in adults and accounts for 1.65% of all diagnosed cases of plasma cell myeloma [1]. A careful review of the literature did not reveal any reports on this type of leukemia in children. Our patient was a 7-month-old infant and probably it is the first case ever described in the world medical literature. We present the case report not only because of a particularly rare occurrence of the disease, but also because of the interesting clinical picture, course of the disease and haematological and biochemical findings.

Case Report

A 7-month-old infant was born on 13 August 1993; he was a term product of the 4th normal pregnancy and delivery with a birth weight of 3300 g and length of 55 cm. He was breast-fed until the age of 4 weeks, when he was weaned. Until the age of 7 months, the child developed properly and was in good health. He was subcutaneously vaccinated with bacille Calmette-Guerin (BCG), but was not given other recommended immunizations because his parents neglected to consult a doctor.

At the age of 7 months, the boy was admitted to the Department of Ped. Haematology and Oncology, Warsaw University Medical School after a 5-day stay in a regional hospital. On admission, he was severely ill and unconscious. The physical examination revealed pallor of the skin, manifestations of haemorrhagic diathesis such as subcutaneous extravasation and the enlarged liver and spleen 10 and 8 cm respectively. There was no lymphadenopathy.

The initial examination of the peripheral blood revealed the following values: hemoglobine 7 g/dl, leukocytes 18 600 /mm3 and blood platelets 56 000/mm^3. The peripheral blood film demonstrated myelocytes 1%, metamyelocytes 3%, band cells 2%, polymorphonuclear cells 30%, eosinophils 1%, lymphocytes 37%, monocytes 6% and plasma cells 20%. ESR 38 mm/h. CSF NAD. Chest X-ray: slight parahilar consolidation on the right side, trace pleural effusion bilaterally and slight lateral enlargement of the cardiac silhouette. Since the diagnosis of leukemia was considered, bone marrow aspiration was performed which demonstrated uniform blast proliferation, i.e., plasmablasts

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and plasma cells with numerous polynuclear (3-4 nuclei) cells. Antigen phenotype assay: CD19 (-); CD7 (-), single cells (+); HLA-DR (-), single cells (+); CALLA rare cells (+); UL38 (Biotest) single cells (±); LCA 50% of cells (+), some cells (±); IgM single cells (+); Lambda numerous cells (±); Kappa single cells (+). Most cells demonstrated weak expression of epithelial membrane antigen (EMA). Serum M.-component was absent and Bence Jones protein was not detected in the urine. X-ray of the skull and long bones was normal. The remaining findings:

Karyotype 46, XY normal; blood group O Rh (-); HbsAg (-); total protein 50/l; albumin 57%; alpha 1 7.5%; alpha 2 9.0%; beta 11.3%; immunoglobulins: IgG 3.2 g/ l, IgA 0.58 g /l, IgM 0.48 g/l; CMV ELISA total (-) IgM (-); indirect fluorescent antibody (IFA) reaction for toxoplasma gondii (-); complement fixation (CF) reaction for mycoplasma (-); latex rheumatoid factor (RF) (-); antinuclear antibodies (ANA) (-). Renal function tests were normal. Also, iron , total bilirubin, urine,uric acid and glucose concentrations remained within normal limits. There were increases in the levels of aspartate transaminase (AST): 213 IU /l (normal value < 40 IU/ l) and ALT: 91 IU/l (normal value < 40 IU/l) as well as elevated concentrations of cholesterol at 298 mg/dl and of triglycerides at 433 mg/dl .

Ultrasonography of the abdominal cavity revealed the markedly enlarged liver and spleen, which were uniformly echogenic. The lymph nodes were not enlarged.

Plasma cell leukemia was diagnosed on the basis of serious general illness, clinical picture and bone marrow examination. Intensive supportive treatment was instituted, which consisted of antibiotics (cefotaxime and netilmicin) and symptomatic treatment for respiratory tract infection. The patient was also given blood and blood products. The main problem, however, was the selection of cytostatic treatment. After the child's general condition had improved, chemotherapy was started employing the regimen used in acute lymphoblastic leukemia (ALL-BFM-86). The boy (10.5 kg b.w.; 0.3 m^2 body surface area) was given dexametasone iv. 1 mg/kg/24 h followed by prednisone 2 mg/kg in combination with vincristine 1.5 mg/m^2 and daunorubicin 30 mg/m2/24 h q.i.d. every 7 days. At that time, the patient did not require blood or blood products. Blood test results varied: Hb 8,7-11,2 g/dl; leukocytes 4300-14500 /mm^3. Peripheral blood films revealed single plasma cells.

A bone marrow aspirate after 4 weeks of treatment demonstrated slight improvement. Plasma cells were found (64%) as well as erythroblasts (c. 13%) and granulocytes at different stages of developement (c. 23%). Response to treatment was also confirmed by the peripheral blood film which demonstrated an increase in the Hb concentration while plasma cells were absent.

However, as complete remission had not been induced , two-drug therapy , which is usually employed in adult patients, was introduced (melphalan 0.2 mg/kg b.W./24 h and prednisone 2 mg/kg b.W./24 h), administered for 4 days every 4 weeks. After the second course the child's general condition returned to normal and there was a marked decrease in the size of the liver (+ 4 cm) and spleen (+1 cm) as compared to the findings on diagnosis. At that time, no plasma cells were found in the peripheral blood. The improvement lasted for 6 weeks and just before the fourth course of chemotherapy the patient's general condition deteriorated with aggravation of the underlying disease (anaemia, thrombocytopenia, plasma cells in the peripheral blood, hepato- and splenomegaly). From that time on, in spite of treatment, the child's general condition continued to deteriorate. The leukemia progressed, complicated by haemorrhage into the central nervous system with the resulting right-sided hemiparesis. As a last resort, we instituted another regimen using a combination of four drugs (prednisone, cyclophosphamide, vincristine and adriamycin) given for 5 consecutive days. However, the patient did not respond to treatment.

The patient died in hospital after a nearly 9-month stay from progression of lekemia and multiorgan insufficiency. Postmortem examination revealed generalized haemorrhagic diathesis with intracranial haemorrhage, bleeding into the gastrointestinal tract and peritoneal cavity, and extensive involvement of the liver in the course of plasma cell leukemia.

Discussion

Plasma cells (plasmacytes) are rarely encountered in the peripheral blood of adults and children. They are also absent in the bone marrow or their proportion there is negligible. In some viral infections, such as rubeola, infectious mononucleosis (atypical lymphocytes) and measles, plasmacytes are present in the peripheral blood as single cells , which are a kind of reaction to the infection. Plasma cells are more numerous in the peripheral blood and bone marrow of patients suffering from connective tissue disease (rheumatoid arthritis) and liver cirrhosis.

Malignant proliferation of plasma cells is characterized by increased localized or generalized multiplication of immunocompetent cell clones. The most typical and common example of malignant plasma cell proliferation is multiple myeloma (plasma cell myeloma). Multiple myeloma occurs mostly in the elderly and its incidence increases with age [2, 3, 4]. Multiple myeloma accounts for ca. 1% of all tumours and ca. 10% of tumours of the haematopoietic system [5]. It is characterized by widespread osteolytic skeletal lesions (long bones, vertebral column, skull) , secretion of an M. component and Bence Jones proteinuria. Spread to extraosseous sites (lymph nodes, thorax and nasopharynx) may occur. Plasma cell myeloma is extremely rare in children. In all the world literature only a few cases have been reported in children and adolescents [6-10]. In all of them the diagnosis was confirmed by the finding of abnormal proteins.

In our patient, the pathological line of plasma cells was manifested as acute plasma cell leukemia with the total involvement of the bone marrow as indicated by the initial manifestations of anaemia, haemorrhagic diathesis, marked hetatosplenomegaly and the presence of plasma cells in the peripheral blood. Bone marrow examination was the essential investigation to confirm the diagnosis of leukemia; 99% of the cells were plasmacytes in different stages of development, ranging from plasmablasts to mature plasma cells, often having 3-4 nuclei. Only single normal bone marrow cells were found among abundant malignant cells. The erythroblasts and mature granulocytes accounted for ca. 4% of all bone marrow cells. No megakaryocytes were found.

We did not detect any osteoporotic foci in either the long bones or the skull which are characteristic of multiple myeloma. Also, investigations for the M protein and Bence Jones protein were negative. There were no features of renal failure or calciuria. All these findings favoured the diagnosis of acute plasma cell leukemia rather than plasma cell myeloma.

It should be emphasized that in our patient the disease was associated with severe damage to the liver, which was markedly enlarged while the levels of transaminases, cholesterol and triglycerides were elevated. Postmortem examination revealed extensive plasma cell infiltrations which involved the whole liver.

The prognosis had been very unfavourable since the very beginning. As in adults, this form of leukemia is characterized by a dramatic course and a fatal outcome.

The patient responded well to melphalan and could be discharged home for a few weeks. He died 9 months after admission from the progression of leukemia, relapse of heamorrhagic diathesis and haemorrhage into the central nervous system .

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Experimental Approaches and Novel Therapies
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Myelodysplastic Syndromes in Children: Clinical and Morphological Analysis

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Abstract. Six children (range of age 6 month to 11 years) with diagnosis of myelodysplastic syndrom (MDS) based on established FAB criteria were observed between 1990 and 1995. The diagnosis and classification of MDS were made on the basis of peripheral blood smear and bone marrow examination. Three children were classified as refractory anemia (RA), two children as anemia with excess of blasts (RAEB) and one with RAEB in the phase of transformation (RAEB-t). In the peripheral blood smear and in the bone marrow study significant dyserytropoiesis with some dysmyelopoietic and dysmegakaryopoietic changes were observed. Cytogenetic study revealed karyotype abberrations in two children. The transformation to acute myeloblastic leukemia was observed in all children initially classified as RAEB, and RAEB-t. All these children had been treated according to the protocol BFM 83 and died during induction of remission or after a short duration of achieved remission. In two children with RA progression of the disease was not observed during 2 years of study. They are still alive and being treated symptomatically. We conclude that MDS in children are characterized by rapid progression, worse results of treatment and shortened life span than the same disease in adult.

Introduction

Myelodysplastic Syndromes (MDS) are clonal disorders of hematopoietic system characterized by irreversible damage of the bone marrow stem cells. They cause morphological and functional disorders of the bone marrow cells which in turn lead to ineffective hematopoietic function. In 95% of cases the presence of hypercellular bone marrow as well as cytopenia in the peripheral blood were determined [1].

In 1982 French/American/British (FAB) cooperative group categorized MDS into five morphological subtypes: refractory anemia (RA), refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess of blasts (RAEB), chronic myelomonocytic leukemia (CMML), refractory anemia with excess of blasts in transformation (RAEB-t) [2].

Myelodysplastic syndromes can be congenital or acquired [1]. The latter can be further divided into primary and secondary. Primary myelodysplastic syndromes occur most frequently and 90% of them appear after 40 years of age. The frequency of occurrence of primary myelodysplastic syndromes in children below 15 years of age is rare and appears in 3.4/1 000 000 [3]. That is why majority of findings on myelodysplastic syndromes refers to adults. Studies on MDS in children are rare especially with regard to their differences in morphological picture and clinical course [4].

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Material and Methods

Six children treated in the Department of Pediatric Hematology and Oncology at the Medical School of Warsaw in the years 1990-1996 fulfilled FAB diagnostic criteria for the MDS classification. All children had had bone marrow aspirates performed and some of them trephinel biopsies. Four children had chromosome analysis performed by direct and short-term culture methods with the use of standard techniques. Simultaneous evaluation of the peripheral blood specimens and histopathological examination were performed by the same doctor and classified according to FAB criteria. None of the six children examined had received chemotherapy or radiotherapy.

Clinical Evaluation

The initial data for peripheral blood and the preliminary diagnosis as well as the progression of the disease are presented in Table 1.

Patients 3-6 obtained inductive treatment according to BFM 83 protocol for acute myeloblastic leukemia. No long-term remission has been obtained in any of the children and all of them died after a few months. Two of the children who survived (patient 1 and 2) obtained only symptomatic treatment and remained in a stable period of the disease for 1–2 years.

Morphological Evaluation

Peripheral blood specimen revealed thrombocytopenia, anemia and leukopenia. In the peripheral blood dysplastic changes referred mainly to granulocytes (lack of cytoplasmic granules, pseudo Pelger-Huet anomalies) as well as red cells (anisocytosis of red cells) (Fig. 1).

Bone Marrow Aspirate

In the bone marrow aspirates dysplastic changes were observed mainly in the erythropoietic colonies (significant megaloblastic renewal) and also in the myeloid colonies as well as megakaryoblasts.

Blurring of the normal morphological architecture of the bone marrow (disturbed normal distribution of cells in the bone marrow cavity) was observed in all cases in which bone marrow biopsy was performed and the so-called ALIP (abnormal localization of immature precursors) was determined in two children.

Discussion

Myelodysplastic syndromes occur rarely in children and according to the newest data constitute not more than 3% of all cancer diseases in children [5]. Some studies revealed that MDS preceded acute leukemia in 20% of children with acute myeloblastic leukemia [1]. Due to the fact that more or less intensive dysplastic changes in the bone marrow cells occur in the majority of bone marrow leukemias it becomes sometimes impossible to determine whether an acute proliferation of cells was preceded by a myelodysplastic syndrome or not.

In our studies the most frequently occurring syndrome was RA and RAEB. RAS and CMML/JCML (juvenile chronic myeloge-

Table 1. Clinical characteristics of MDS patients

Patient no.	Sex	Age at diagnosis (years)	Hb (g/l)	WBC ($x10^9$/l)	Platelets ($x10^9$/l)	Karyotype	FAB diagnosis	Progression of the disease
1.	F	5	98	2.9	70	?	RA	-
2.	F	11	114	4.4	64	?	RA	-
3.	F	1/2	48	1.8	15	?	RA	AML-M2
4.	M	1	49	20.1	19	del(13)del(5)	RAEB-t	AML-M6
5.	M	1	91	7.8	38	46 XY	RAEB	AML-M2
6.	M	11	56	1.6	65	16q-	RAEB	AML-M2

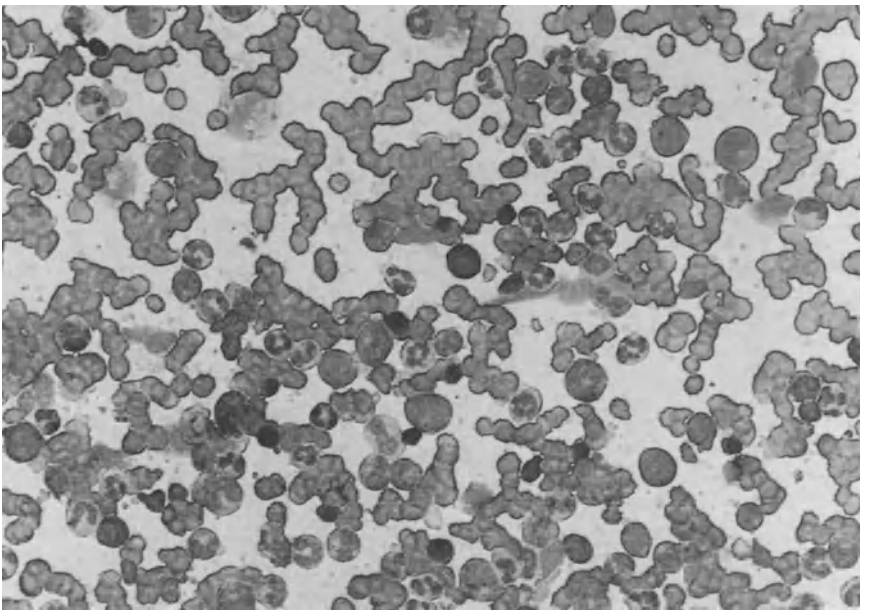

Fig. 1

nous leukemia) were not observed in the study group. The most frequently determined disturbance of the peripheral blood were thrombocytopenia as well as displastic changes in granulocytes. Displastic changes in the bone marrow which were of the character of the megaloblastic renewal referred mainly to the erythropoietic colonies.

Appearance of ALIP was determined in two children and their presence caused faster progression of the disease. Cytogenetic study revealed karyotype abberations in two children.

Transformation of MDS to acute myeloblastic leukemia appeared in four children with the preliminary diagnosis of RAEB, RAEB-t and RA. Fast progression (6 months from the diagnosis) and resistance to conventional treatment were striking. Two children with RA remain in the stable stage of the disease. In one of them, however, progression of the disease with RA was observed after 23 months.

Myelodysplastic syndromes in children constitute a group of diseases which despite the undoubtful progress in their diagnosis still constitute a therapeutic challenge for the doctors [4, 5].

Our treatment attempts with intensive BFM 83 protocol ended in failure. The only effective treatment which can cause a long-term remission would be bone marrow transplantation, but none of our patients had a familiar compatible donor [5, 6].

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Molecular-/Cytogenetics

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Molecular Screening of Acute Myeloid Leukemia Using Interphase Cytogenetics

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Abstract. The objective of this study was to design DNA probe sets that enable the detection of chromosome abnormalities in AML by interphase cytogenetics using fluorescence in situ hybridization (FISH), and to compare the results of interphase cytogenetics with those of conventional chromosome banding analysis. 170 consecutive patients with adult AML entered on the German multicenter treatment trial AML HD93 were studied with a comprehensive set of DNA probes recognizing the most relevant AML-associated structural and numerical chromosome aberrations: translocations t(8;21), t(15;17), t(11q23), inversion inv(16); chromosomal deletions [5q-; 7q-; 9q-; 12p-; 13q-; 17p-; 20q-]; and numerical chromosome abnormalities. The incidence of clonal chromosome aberrations was 51% (87/170) both by banding analysis and by FISH. Interphase cytogenetics was more sensitive for detecting AML-specific chimeric gene fusions, especially inv(16) and t(8;21) as well as some partial trisomies. Interphase cytogenetics provides a powerful technique complementary and, with further development of diagnostic DNA probes, even alternative to chromosome banding studies for the cytogenetic analysis of AML, in particular in a reference laboratory for a multicenter treatment trial.

Introduction

In recent years, a large number of chromosome aberrations have been identified in acute myeloid leukemia (AML) which are associated with specific clinical, morphological and immunological features [1, 2]. The molecular characterization of these aberrations has confirmed that genes important in cellular proliferation and differentiation are affected by these chromosome aberrations [3]. Both, retrospective and prospective treatment trials showed that these chromosome aberrations are one of the most important predictive factors for response rates and remission durations. Thus, cytogenetic analysis has become of great importance for the management of patients with AML.

Chromosome banding analyses are still the standard method for the identification of chromosome aberrations. However, chromosome banding studies may be hampered by poor chromosome morphology, low proliferative activity of the leukemic cells, complex karyotypes or reduced cell viability after transport to the reference laboratory. More recently, molecular genetic techniques, such as Southern blot analysis or reverse-transcriptase polymerase chain reaction (RT-PCR), have been used for the detection of the AML-specific translocations or inversions but these techniques are restricted to the detection of a few aberrations.

Fluorescence in situ hybridization

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(FISH) techniques using genomic DNA probes have been increasingly applied for the cytogenetic analysis of leukemia [4, 5]. In comparison to conventional cytogenetic analysis, FISH allows the identification of chromosome aberrations in interphase cells, referred to as interphase cytogenetics. With the developments in the Human Genome Project, a large number of DNA probes have now become available that permit hybridization to DNA sequences of different complexity. So far, the majority of cytogenetic studies using FISH have been limited to the detection of chromosome aneuploidies using centromere-specific DNA probes [4, 5].

The objective of this study was to design DNA probe sets that enable the detection of AML-relevant structural and numerical chromosome aberrations and to compare this interphase cytogenetic approach with conventional chromosome banding analysis in a large series of consecutive patients with adult AML enrolled in the German multicenter treatment trial AML HD93.

Material and Methods

Patients

Between December 1993 and March 1997, 170 consecutive patients with newly diagnosed AML were studied by metaphase and interphase cytogenetic analysis. All patients were entered on the multicenter treatment trial AML HD93 in which postremission therapy is stratified according to the karyotype [6]. 83 patients were male and 87 were female. The and 13 patients had secondary AML after a primary malignancy. Leukemias were classified according to the French-American-British (FAB) criteria. G-banding analysis was performed using standard methods and the karyotypes were designated according to the International System for Cytogenetic Nomenclature (ISCN).

DNA Probes

Table 1 gives a summary of the DNA-clones which were used for the detection of AML-associated chromosome aberrations by interphase cytogenetics.

Translocations/Inversions: t(8;21). For the detection of the t(8;21) we used a pool of two partially overlapping yeast artificial chromosome (YAC) clones (464H8 and 72H9), recognizing AML1 and AML1-flanking sequences at 21q22, and a differently labeled P1-phage clone (P1 164) containing the ETO gene. Two criteria were required for the identification of the t(8;21) in interphase nuclei: (i) splitting of one AML1 YAC signal; and (ii) colocalization of one AML1 YAC and one P1 signal. t(15;17): for the diagnosis of the t(15;17) we used YAC 365C12 recognizing the PML gene, and a pool of two overlapping cosmids (RARAcos121 and 124) spanning the breakpoint region in the RARA gene. Both the YAC and the two cosmids are split by the translocation. t(11q23): YAC 13HH4; this YAC spans the breakpoint cluster region in the MLL gene. Since 11q23 translocations are in some cases associated with interstitial deletions, we applied a pool of two YACs (785C6 and 856B9) in parallel, which spans MLL and approximately 1.0 Mb of distally flanking sequences at 11q23. To identify the most frequent translocation partner, the AF9 gene in the t(9;11), cohybridization was performed with two overlapping cosmids (48 and 55), recognizing AF9 sequences distally flanking the breakpoint. inv(16): YAC 854E2; this clone spans the breakpoint cluster region in the MYH11 gene at 16p13. The CBFβ-MYH11 gene fusion was shown by cohybridization of YAC 854E2 with two cosmids (LA2-2 and LA4-1) spanning the breakpoints in the CBFβ gene at 16q22.

Aneuploidies/Deletions. The following monosomies, trisomies and deletions were detected by interphase cytogenetics (clone designation, chromosomal localization, and gene/DNA marker are given in parenthesis): ±X (A24CH4; Xq28; DXS304); ±Y (yOX-57; Yq11); -5/5q- (773D3; 5q31; D5S89; and yPR411; 5q33; CSF1R); -7/7q- (HSC7E506; 7q22; D7S240; and HSC7E124; 7q35; D7S688); +8/8q+ (935A12; 8q24; D8S508); -9/9q- (253F8; 9q13-21; D9S15); 12p- (964C10; 12p13; TEL); 13q-/+13q (Rb phage clones; 13q14; Rb); -17/17p- (TP53 cosmid

Table 1. DNA clones, chromosomal localization and gene/locus for the detection of acute myeloid leukemia-associated aberrations by interphase cytogenetics

Aberration	DNA-clone	Localization	Gene/Locus
-5/5q-	yPR411	5q33	CSF1R
	773D3	5q31	D5S89
-7/7q-	HSC7E506	7q22	D7S240
	HSC7E124	7q35	D7S688
+8/+8q	935A12	8q24	D8S508
t(8;21)	464H8/72H9 pool	21q22	AML1
	P1 164	8q22	ETO
-9/9q-	253F8	9q13-21	D9S15
t(11q23)	13HH4	11q23	MLL
	785C6/856B9 pool	11q23	MLL, Thy1
t(9;11)	+ 48/55 cosmid pool	9p22	AF9
t(12p13)/12p-	964C10	12p13	TEL
13q-/+13q	RB-1 phage pool	13q14	RB-1
t(15;17)	365C12	15q22	PML
	RARAcos121/124 cosmids	17q21	RARA
inv(16)	854E2	16p13	MYH11
	LA2-2/LA4-1	16q22	CBFß
-17/17p-	TP53 cosmid pool	17p13	TP53
-20/20q-	808C05	20q13	D20S99
+21q	464H8/72H9 pool	21q22	AML1
+22q	D107F9	22q11	BCR
-/+Xq	A24CH4	Xq28	DXS304
-/+Yq	yOX-57	Yq11	sY73

pool; 17p13; TP53); -20/20q- (808C05; 20q13; D20S99); +21/+21q (464H8/72H9 pool; 21q22; AML1); +22/+22q (D107F9; 22q11; BCR).

Preparation of DNA Clones

Cosmid and P1-phage DNA was prepared according to the plasmid MIDI KIT protocol (Qiagen, Germany). Human sequences from YAC clones were generated by a PCR protocol using primers directed against Alu-sequences [7]. The probes were labeled by nick translation with biotin-16-dUTP or digoxigenin-11-dUTP.

For interphase cytogenetic analysis methanol/acetic acid-fixed cell pellets from bone marrow or peripheral blood were used [8,9]. Fluorescence signals were enumerated in 200-300 interphase nuclei using single or dual band-pass filters. Images were captured by a cooled charged coupled device (CCD) camera (Xillix Technologies Corporation, Richmond, Canada). To define the cut-off levels for the diagnosis of specific chromosome aberrations, hybridization experiments for each DNA-probe or probe set were performed (i) to blood specimens from 5 probands, and (ii) to 12 leukemic samples which on chromosome banding analysis exhibited a normal karyotype. The latter experiments were done to consider the hybridization properties of the test cells. The cut-off level was defined by the mean + 3 standard deviations (SD).

Results

Comparison of Metaphase and Interphase Cytogenetic Analysis

Evaluable metaphase spreads for G-banding analysis were obtained in 159 of the 170 (94%) cases. In contrast, all 170 cases were evaluable by interphase cytogenetics. The incidence of chromosome aberrations was 51% (87/170) both by banding analysis and by FISH. A comparison of the results between metaphase and interphase analysis for specific aberrations is shown in Table 2.

By interphase cytogenetics, more cases of inv(16), t(8;21), t(11q23), -7/7q-, +8q, +11q, +21q, +22q and -Y were detected. These additional aberrations were identified in cases

Table 2. Comparison of metaphase and interphase cytogenetic analysis according to specific aberrations in 170 consecutive patients with acute myeloid leukemia

	G-Banding no. of cases	FISH no. of cases
inv(16)	16	20
t(8;21)	10	12
t(11q23)	14	15
t(15;17)	8	8
-5/5q-	14	12
-7/7q-	13	14
+8/8q+	8	14
Pseudodiploid	14	10
Normal	72	83
No evaluable metaphases	11	—

in which no evaluable metaphase spreads or only metaphase chromosomes of poor quality were obtained on G-banding, or in cases with very complex karyotypes. In contrast, two additional cases of 5q-, three additional cases of 9q- and one additional case of 20q- were identified by chromosome banding analysis because the DNA probes we used obviously do not map to the critical region of these deletions.

Fourteen cases of t(11q23) were detected by banding analysis: 8 cases of t(9;11)(p21;q23); and one case each of t(1;11)(q21;q23), t(10;11)(p11;q23), and t(11;17)(q23;q21). All t(9;11) and the t(1;11) were identified using YAC 13HH4. The translocation t(11;17) and t(10;11) were only detected when using the YACs 785C6/856B9. By FISH, an additional case of an 11q23 aberration was identified, not by the mechanism of translocation but by insertion of part of the sequences recognized by YAC 13HH4 into chromosome 6. Using the YAC 13HH4 and YACs 785C6/856B9, three signals were identified in interphase nuclei; cohybridization with a whole chromosome 6 painting probe showed a clear YAC pool signal on the terminal part of 6q in metaphase FISH. All cases of t(15;17) were detected by G-banding analysis and by FISH.

Eighty-three cases had a normal FISH karyotype. In 5 of these cases, clonal chromosome aberrations were detected by banding analysis: ins(2;3)(p22;q21q26); t(6;9)(p23;q34); t(3;8)(q13.2;q24.1); der(13;14)(q10;q10); and 48,XX,+2mar. All 5 aberrations occurred as single aberrations and could not be detected by the DNA probe sets selected for this study.

Seventy-two cases had a normal G-banding karyotype. In 3 of these cases, chromosome aberrations were detected by interphase cytogenetics: inv(16), nullisomy Y, and del(12p).

In 11 cases, no evaluable metaphase cells were found on banding analysis. In 6 of these cases, chromosome aberrations were detected by interphase cytogenetics, i.e. 2 cases with +8q as sole aberration, one case with t(8;21), and 3 cases with a complex karyotype: +8q,+9q,inv(16); +Y,+7q,+11q,13q-; 7q-,8q+,11q+,17p-,+21q.

Discussion

For the identification of chromosome abnormalities in AML rapid and sensitive methods are needed because molecular based classifications and treatment strategies are becoming increasingly important [10]. The objective of this study was to establish DNA probe sets that allow for a precise molecular diagnosis of the AML-specific numerical and structural chromosome aberrations by interphase cytogenetics.

Interphase cytogenetics was particularly sensitive for the detection of the AML-specific translocations and inversions. Using YAC 854E2 which was originally isolated for positional cloning of the p-arm breakpoint [11], 4 additional cases of inv(16) were identified by FISH. In a recent study of 29 leukemias carrying the inv(16), FISH was performed using YAC 854E2: all cases of inv(16) were detected using this diagnostic probe [12]. Interphase cytogenetics may even be more sensitive than RT-PCR in detecting the inv(16), because in recent studies leukemias were identified that cytogenetically showed the inv(16) but lacked a CBFβ-MYH11 fusion transcript detectable by the PCR assay [13].

By analogy, interphase cytogenetics allowed sensitive detection of the t(8;21) and t(15;17). Two additional cases of AML1-ETO fusion were only detected by FISH. Retrospective analysis of one of these cases showed that the AML1-ETO fusion resulted from a variant t(8;21) that was not identified by

banding analysis. In the second case, no evaluable metaphase cells were available. Using RT-PCR, AML1-ETO fusion transcripts have also been identified in leukemias with morphologic features characteristic of the t(8;21) but without the cytogenetic presence of the t(8;21) [14]. The identification of such cases, which presumably result from interstitial insertions, by FISH would depend on the size of the inserted genomic fragment. Such an insertion has recently been shown by FISH in a case of microgranular variant of acute promyelocytic leukemia, which on banding analysis lacked the t(15;17) [15].

Interphase cytogenetics was also more sensitive for the detection of aberrations involving chromosome band 11q23. One additional case of 11q23 aberration was found by FISH resulting from an insertion ins(6;11) that could not be detected by chromosome banding analysis. When screening for 11q23 aberrations by FISH it is important to use a contig of YAC probes which span approximately 1 Mb of DNA sequences distal to MLL since interstitial deletions distal to the breakpoint, which may be several hundred kilobases in size, have been reported in up to 30% of the t(11q23) [16]. Accordingly, two of our cases, a t(11;17) and a t(10;11), were only detected by using the YAC pool 765C6/856B9 but not by the 300 kb sized YAC 13HH4 alone. Thus, FISH using the 11q23 YAC pool is an alternative to chromosome banding analysis for the diagnosis of 11q23 translocations, however, it will not allow for the detection of more subtle rearrangements such as the partial MLL gene duplications. In a recent series of 93 AMLs exhibiting a normal karyotype on banding analysis, 10 (11%) cases showed MLL rearrangement on Southern blot analysis [17]. All these rearrangements resulted from partial MLL duplications as shown by a PCR assay.

For deletion screening, we chose DNA probes that recognize DNA sequences which map to the critical regions of the deletions. However, the selected probes were not informative in all cases. Three cases of 9q- and two cases of 5q aberrations were only diagnosed on chromosome banding analysis. In the latter two cases, we could show by metaphase FISH that a translocation breakpoint had occurred at 5q31. The breakpoints in both cases were located between the two markers which we used for deletion screening, D5S89 at 5q31 and CSF1R at 5q33. Thus, for the diagnosis of both 5q and 9q aberrations more informative probes have to be selected based on the precise molecular deletion mapping [18].

Finally, more cases of trisomy 8q, 11q, 21q and 22q were detected by FISH. These aberrations were either identified in cases without evaluable metaphases on banding analysis or in cases where partial trisomies were presumably masked by complex karyotypes.

The DNA probe-sets selected in our study identify specific targeted chromosome regions and therefore will not detect all possible chromosome aberrations due to the molecular heterogeneity of AML. Accordingly, the aberrations identified by banding analysis could not be detected by FISH in 5 cases. Nevertheless, our study demonstrates the power of interphase cytogenetics for detecting the most important chromosome aberrations in AML. Interphase cytogenetics proved to be more sensitive for detecting AML-specific translocations and inversion and provided a technique for the detection of chromosome aberrations in 6 of 11 cases where no evaluable metaphases were found on banding analysis. Furthermore, these DNA probe sets are currently being tested for the monitoring of residual disease. Compared to PCR-based tests, FISH is less sensitive, however, it allows the quantification of cells carrying the aberration and the detection of chromosome abnormalities for which no PCR assays are available.

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Experimental Approaches and Novel Therapies
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Routinely Prepared Cells for Cytogenetic Analysis Stored at -20 °C for Several Years can be Used for RT-PCR-Based Detection of Chromosomal Aberrations in Leukemias

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Abstract. Reverse transcriptase polymerase chain reaction (RT-PCR) is a well established method to detect chromosomal abnormalities in hematological malignancies. Because RT-PCR requires RNA of high quality, freshly obtained cells must be processed immediately or have to be frozen in liquid nitrogen or at –80 °C to prevent RNA degradation. However, in many cases no material has been stored for RNA extraction. Therefore, it would be of great advantage, if RT-PCR could be applicable to methanol/acetic acid fixed material, stored in many cytogenetic laboratories. We have applied RT-PCR to methanol/acetic acid fixed cells from 28 patients with acute and chronic leukemias. Cytogenetically prepared bone marrow cells had been stored frozen in methanol/acetic acid for up to 5 years. RNA was extracted by a modified guanidinium thiocyanate phenol/chloroform protocol without prior manipulation of the cells. The patients had the following diagnosis: 23 patients (pts.) suffered from acute leukemia including 3 pts. with t(4;11), 9 pts. with t(8;21), 5 pts. with t(15;17) and 6 pts. with inv(16). Five pts. suffered from chronic myelogenous leukemia (CML) with t(9;22). RT-PCR was performed for the intact cABL gene to verify the integrity of the RNA and for the respective fusion gene using a nested or semi-nested primer system. In all cases the intact cABL transcript was amplified. In 3/3 cases with t(4;11), 8/9 cases with t(8;21), 3/5 cases with t(15;17), 6/6 cases with inv(16) and 3/5 cases with t(9;22) the specific fusion genes could be detected (82.1%). Our results demonstrate that routinely stored cells in cytogenetic laboratories can be a useful source for RT-PCR based detection of chromosomal aberrations in many cases.

Introduction

For the most common chromosomal abnormalities in acute myeloid leukemia (AML), the t(8;21) [1-4], the t(15;17) [5,6], and the inv(16) [7,8] PCR tests are available. The PCR based identification of the t(4;11) in acute lymphocytic leukemia (ALL) [9] and of the t(9;22) in Philadelphia (Ph)-positive ALL and in CML is also well established [10-12]. The detection of these chromosomal abnormalities is based on RNA analysis by RT-PCR, revealing a specific mRNA fusion transcript. Generally, cells have to be frozen at -80 °C or in liquid nitrogen within 24-48 h to obtain good quality RNA. Previously, we demonstrated that RNA for RT-PCR analysis can also be obtained from air-dried bone marrow smears [13]. Other authors showed that RNA for the RT-PCR based amplification of the BCR/ABL transcript can be obtained from bone marrow cells prepared for cytogenetic analysis and stored at -20 °C [14].

In the present study we have applied RT-PCR based detection of the most common

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chromosomal abnormalities in acute leukemias and in CML to methanol/acetic acid fixed bone marrow aspirates from 28 patients. Cells were routinely stored at -20 °C for up to 5 years. The use of this source gives the opportunity to perform RT-PCR at the time of the diagnosis in cases where cells have not been prepared for RNA extraction.

Material and Methods

Patients and Cytogenetics

Bone marrow samples were collected at diagnosis for cytogenetic analysis and chromosome karyotyping was performed by routine methods. The samples included nine cases of AML M2 with t(8;21), five cases of AML M3 with t(15;17), six cases of AML M4Eo with inv(16), three cases of ALL with t(4;11) and five cases of CML with t(9;22). Cells were stored at -20 °C in methanol/glacial acetic acid (3:1 v/v) according to standard procedures for up to 5 years before thawing for the RNA-extraction for RT-PCR.

RT-PCR

Fifty to 500 µl of the methanol/acetic acid cell-suspension was isolated by a brief centrifugation-step. The RNA was extracted from the cell fraction with a buffer containing guanidinium thiocyanate and phenol (RNAzol, WAK-Chemie, Germany) followed by chloroform purification [13, 15]. The RNA-pellet was precipitated once in isopropanolol and a second time in ethanol and afterwards dissolved in 15 µl DEPC treated water. The first-strand cDNA synthesis was carried out with 7 µl of the RNA solution and AMV-reverse transcriptase (cDNA-Kit Serva) for 45 min. at 42 °C using random primers in a total reaction volume of 15 µl. For each PCR amplification 1 µl of the cDNA product was used. Every test was carried out with one primer pair for the cABL gene as a control that RNA has been extracted. A second primer pair was used to detect the specific fusion transcript: the BCR-ABL fusion transcript for t(9;22) [10], the PML-RARα fusion transcript for t(15;17) (5), the AML-ETO fusion transcript for t(8;21) [1,3], the CBFβ-MYH11 fusion transcript for inv(16) [7] and the MLL-AF4 fusion transcript for t(4;11) [9]. PCR was performed with Goldstar-polymerase (Eurogentec, Seraing, Belgium) in a total reaction volume of 13 µl at standard conditions with an annealing temperature between 50 and 60 °C, depending on the primers used (Thermocycler 9200, Perkin Elmer, Foster, City, CA). The PCR product was diluted 1 : 100 and 1 µl of this solution was used for a second step of amplification with a second set of primers. The PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and visualised under UV-light. All samples were investigated twice and numerous precautions were made to avoid cross contaminations.

Results

We studied a total of 28 archival cell samples stored frozen at -20 °C in methanol/acetic acid. The time of storage ranged from 3 to 46 months. As illustrated in Table 1, the extraction of intact mRNA was successful in all 28 samples yielding the correct amplification product of the cABL mRNA. Twenty of these samples showed the amplification product of the cABL gene in the first round of amplification (71%).

The specific mRNA fusion transcript for each chromosomal abnormality was amplified in 3 out of 3 cases with t(4;11), in 8 out of 9 cases with t(8;21), in 3 out of 5 cases with t(15;17), in 6 out of 6 cases with inv(16) and in 3 out of 5 cases with t(9;22) (82.1% of the total number of samples). The sizes of the amplification products corresponded to the expected sizes deduced from mRNA sequences and primer locations for each fusion gene and were in accordance with the cytogenetic findings (see Table 1).

Discussion

The aim of the present study was to determine, whether routinely stored cells in cy-

Table 1. Diagnosis according to the FAB classification, the cytogenetic and the PCR results for each patient. PCR results are indicated as (+) for positive and (-) for negative. The symbols before and after the slash correspond with the first PCR step or the second PCR step, respectively.

Patient no.	Diagnosis	Cytogenetic analysis	RT-PCR (cABL)	RT-PCR (specific fusion transcript)	Molecular rearrangement
1	ALL	t(4;11)	+/+	-/+	MLL-AF4
2	ALL	t(4;11)	+/+	+/+	MLL-AF4
3	ALL	t(4;11)	-/+	-/+	MLL-AF4
4	CML	t(9;22)	-/+	-/+	BCR-ABL
5	CML	t(9;22)	-/+	-/+	BCR-ABL
6	CML	t(9;22)	-/+	-/-	BCR-ABL
7	CML	t(9;22)	-/+	-/-	BCR-ABL
8	CML	t(9;22)	+/+	+/+	BCR-ABL
9	AML M2	t(8;21)	+/+	+/+	AML-ETO
10	AML M2	t(8;21)	+/+	-/-	AML-ETO
11	AML M2	t(8;21)	+/+	-/+	AML-ETO
12	AML M2	t(8;21)	+/+	+/+	AML-ETO
13	AML M2	t(8;21)	+/+	+/+	AML-ETO
14	AML M2	t(8;21)	+/+	+/+	AML-ETO
15	AML M2	t(8;21)	+/+	+/+	AML-ETO
16	AML M2	t(8;21)	-/+	-/+	AML-ETO
17	AML M2	t(8;21)	+/+	-/+	AML-ETO
18	AML M4Eo	inv(16)	+/+	+/+	CBFB-MYH11
19	AML M4Eo	inv(16)	+/+	-/+	CBFB-MYH11
20	AML M4Eo	inv(16)	+/+	-/+	CBFB-MYH11
21	AML M4Eo	inv(16)	+/+	+/+	CBFB-MYH11
22	AML M4Eo	inv(16)	+/+	+/+	CBFB-MYH11
23	AML M4Eo	inv(16)	-/+	-/+	CBFB-MYH11
24	AML M3	t(15;17)	+/+	-/-	PML-RARA
25	AML M3	t(15;17)	+/+	-/-	PML-RARA
26	AML M3	t(15;17)	-/+	-/+	PML-RARA
27	AML M3	t(15;17)	+/+	+/+	PML-RARA
28	AML M3	t(15;17)	+/+	-/+	PML-RARA

togenetic laboratories could be used for RT-PCR based molecular studies. In all of the 28 investigated cases the extraction of mRNA was successful. The presence of the cABL gene product in the first round of amplification, as it is usually seen when fresh material is used, was observed in a considerable number of cases and indicates a good quality of RNA. A brief washing step of the stored cells with phosphate-buffered saline (PBS), as it is usually processed with freshly aspirated marrow cells, significantly decreased the RNA yield (data not shown). The methanol/acetic acid fixed cells were therefore used without prior manipulation in our routinely applied protocol for the extraction of RNA from bone marrow smears [13].

Furthermore, we could demonstrate that the specific fusion transcripts of the most common chromosomal aberrations in acute leukemias can be detected by RT-PCR analysis in the majority of cases. The fact that amplification has failed in some samples might be due to the small amount of material or degradation of RNA in these samples.

According to our results, the application of stored cytogenetic cells might gain further importance for retrospective studies, but it is important to be aware that cross contaminations might give false positive results due to the handling of the specimens in cytogenetic laboratories. Further investigations to clarify this problem are in progress.

In conclusion, fusion genes can be detected with a high sensitivity when methanol/acetic acid fixed samples were analysed after up to 5 years of storage. The use of this method could increase the precision in identifying the genetic changes in the malignant cells in large amounts of archival material.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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RT-PCR-Based Detection of TEL/AML1 Fusion Transcript in Childhood B-Cell Precursor Acute Lymphoblastic Leukemia – a Czech Childhood Leukemia Working Group Experience

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Abstract. Chromosomal translocations involving chromosome 12p are rarely detected by conventional cytogenetic analysis in patients with acute lymphoblastic leukemia (ALL). Recently, fluorescence in situ hybridization (FISH) and molecular methods revealed that the t(12;21)(p13;q22) translocation is the most common genetic aberration in pediatric ALL. We established a reverse transcriptase-polymerase chain reaction (RT-PCR) protocol for the detection of TEL/AML1 fusion mRNA resulting from this translocation. We analyzed bone marrow and/or peripheral blood from 54 pediatric patients with B-lineage ALL at diagnosis or relapse (pre-pre-B, n=3; common ALL, n=32; pre-B, n=18; B, n=1). Cytogenetic analyses did not reveal the t(12;21)(p13;q22) translocation in any of the patients. Patients with T-cell immunophenotype and those with RT-PCR proven hybrid genes BCR/ABL and MLL/AF4 were excluded. For the detection of TEL/AML1 fusion transcript, we performed two-round RT-PCR using primers in exon 5 of the TEL gene and exon 3 of the AML1 gene. Two different PCR products were identifiable – either a 464 or 425-bp product in the first round, thus implying the presence or absence of the 39-bp-long exon 2 of the AML1 gene. The one step amplification of ABL exon a2/a3 was used as a quality control. Out of 32 patients with common ALL , 12 were positive for TEL/AML1 rearrangement (34.4%). In addition, we identified this genetic lesion in 4 out of 18 (22.2%) pre-B ALL patients. All pre-pre-B and B-cell leukemic samples were negative. We conclude that TEL/AML1 fusion can be demonstrated in one fourth of the children with a B lineage ALL and is associated with CD10+ immunophenotype. The high frequency of the t(12;21) provides a new tool for the study of minimal residual disease.

Introduction

Non-random chromosomal translocations are generally thought to be stable markers of leukemic cells. The most commonly tested translocations in pediatric ALL, t(9;22), t(1;19) and t(4;11), have each been shown to define a subgroup of patients with distinct clinical features. Patients whose leukemic blasts carry the t(9;22) or t(4;11) respond poorly to therapy, with long-term event-free survival ranging only from 10 to 30% [1, 2]. Moreover, RT-PCR based methods for detection of these translocations provide us a very convenient and powerful tool for monitoring of minimal residual disease (MRD). However, all these translocations are detected in less than 15% of pediatric cases [3].The recurrent t(12;21)(p12;q22) translocation has recently been recognized as the most frequent chromosomal aberration in childhood ALL (4,5) despite its apparent rarity shown by routine karyotypic analysis. The

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frequency of the t(12;21) in B-cell precursor ALL (BCP-ALL), estimated by FISH and/or RT-PCR and published in current literature, ranges from 16 to 28% [4–7]. The t(12;21) fuses the helix-loop-helix (HLH) domain of the TEL gene to the DNA-binding and transactivation domains of AML1 [8, 9]. Both these genes have been previously shown to be independently involved in myeloid malignant proliferation and in physiologic regulation of cell development [10, 11].

In this chapter, we evaluated the frequency of TEL/AML1 fusion gene in childhood BCP-ALL in the Czech population and assessed the use of TEL/AML1 RT-PCR detection for the MRD monitoring.

Material and Methods

Patients

Cryopreserved bone marrow (BM) and/or peripheral blood (PB) samples were available for 54 children aged 2 to 15.5 years. All children with newly diagnosed ALL (n=49) were treated according to ALL BFM (Berlin–Frankfurt–Münster) 90 or 95 protocols, relapsed patients (n=5) on ALL REZ 90 protocol in one of the Czech Childhood Leukemia Working Group centers.

Immunological Analysis

Flow Cytometry

The immunological analysis was performed in the center where the diagnosis was established (seven different laboratories within the Czech Republic). Fresh BM specimens were stained using directly labeled monoclonal antibodies. Red blood cells were subsequently lysed, the cell suspension was washed and analyzed by flow cytometry. Intracellular staining was performed according to manufacturer's recommendation using the Fix and Perm Kit (An der Grub).

Immunophenotype Classification

Leukemic blasts were classified as pre-pre-B (CD10-, 19+, cIgM-, sIg-), cALL (CD10+, 19+, cIgM-, sIg-) or pre-B (CD10+, 19+, cIgM+, sIg-). Patients with sIg+ were classified as mature B-cell ALL. In several patients, cIgM was not assessed; these children were classified according to presence or absence of other B-lineage differentiation specific antigens.

RT-PCR Amplification

Total RNA was extracted from cryopreserved BM or PB cells using modified method described by Chomczynski and Sacchi [12]. The integrity and quantity of extracted RNA was controlled on ethidium bromide stained 1% non-denaturating agarose gel. Complementary DNA was prepared using MMLV Reverse Transcriptase (Gibco BRL) under conditions recommended by the manufacturer. The one step amplification of ABL exons a2/a3 was used as a quality test. The sequences of these primers were: A2 - 5′- TTCAGCGGCCAGTAGCAT CTGACTT - 3′ and A3 - 5′- TGTGATTATAGCCTAA-GACCCGGAGCTTTT- 3′. For the detection of TEL/AML1 transcript, primers used in the first amplification step were: TL667 - 5′-GCTGAGAGAGCTCAGGGACC -3′ and AM1130 5′- ATCTTGCCTGGGCTCAGCGC - 3′. PCR products from the first round were then 100x diluted and subsequently used in the second step amplification with primers TL841 5′- ATCATGCACCCTCTGATCC -3′ and AM1109 5′- GTGGAAGGCGGCGT GAAGC -3′. The amplification profile on a DNA thermal cycler (Perkin Elmer 9600/2400) was as follows: denaturation at 94.5 °C for 5 min, then 40 cycles of 30 s at 94 °C, 20 s at 63 °C, 60 s at 72 °C, with a 10-min final extension at 72 °C [13].

Sequencing of RT-PCR Products

RT-PCR products were directly sequenced using Silver Sequence DNA Sequencing System kit (Promega). Sequencing primer was AM1109.

Dilution Experiments and Minimal Residual Disease Detection

To assess the sensitivity of the RT-PCR, cells from diagnosis samples lyzed in guanidin-

ium thiocyanate were serially diluted in cell lysates from healthy volunteer donors. Total RNA was extracted and then subjected to RT-PCR as described above. Complete remission samples were analyzed at least twice.

Statistical Aanalysis

The distribution of clinical and biological features for patients with or without expression of the TEL/AML1 chimeric transcript were compared by unpaired t-test.

Results

RT-PCR Detection of TEL/AML1 Chimeric Transcript

We excluded all patients with previously detected BCR/ABL and MLL/AF4 rearrangements and children with T-lineage phenotype. In the remaining cohort of 54 B-lineage ALL patients, RT-PCR analysis revealed TEL/AML1 fusion transcripts in 15 patients (27.8%). One TEL/AML1 positive and 4 negative patients were analyzed at relapse and the first diagnosis samples suitable for RT-PCR detection were not available. In all but 1 patient we found identical PCR product of 464 bp, representing fusion of TEL exon 5 to AML1 exon 2. The remaining 1 sample exhibited the 425-bp fragment, representing TEL exon 5 and AML1 exon 3 fusion. In the vast majority of cases expressing 464 bp product, we were repeatedly able to amplify less abundant 425 bp fragment, representing an alternative splicing variant (Fig. 1). The specificity of the RT-PCR products was confirmed by direct sequencing.

Biological and Clinical Characteristics of the Patients

In the group of TEL/AML1 positive patients, 11 children were classified as cALL and 4 as pre-B ALL (Table 1). None patient was classified as pre-pre-B ALL. Three children from the negative group were classified as pre-pre-B, 21 as cALL, 14 as pre-B ALL and 1 as mature B-cell ALL. The patient with mature B-cell and children analyzed only at relapse were excluded from the comparison of clinical and biological features.

There were no statistically significant findings with respect to the sex, age, white blood cell (WBC) count and response to Prednison among TEL/AML1 positive children as compared with negative childhood BCP-ALL. However, comparison of the distribution of favorable presenting features showed a trend towards younger age ($p = 0.065$), lower leukocyte counts ($p = 0.077$) and better response to Prednison ($p = 0.16$) in patients with the fusion transcript. Since no fresh patient of both groups has relapsed

Table 1. Biological and clinical features of the TEL/AML1 positive ALL children at presentation

No.	CD19	CD10	CD20	cIgM	sIgM	Age at dg (years)	WBC x109/l	Sex	PR	follow up (months)
1	+	+	+	–	–	4.5	0.8	M	+	19
2	+	+	–	–	–	4	49.9	F	+	18
3	+	+	–	+	–	4	7.7	F	+	17
4	+	+	–	ND	–	12	1.4	F	+	17
5	+	+	–	–	–	3.5	4.4	F	+	15.5
6	+	+	–	+	–	4	0.7	M	+	12
7	+	+	ND	ND	–	6	5.2	M	+	11
8	–	+	–	ND	–	4	8.2	F	+	9.5
9	+	+	–	–	–	4.5	10.0	F	+	9
10	+	+	–	ND	–	8	0.7	F	+	9
11	+	+	+	ND	–	3.5	23.7	F	+	6
12	+	+	–	+	–	3.5	8.5	F	+	5.5
13	+	+	ND	ND	ND	3.5	5.4	F	+	4
14	+	+	ND	+	–	4.5	11.9	M	+	0.5
15[a]	+	+	–	–	ND		8.0	M		16

[a] Patient examined at relapse; ND – not done; PR – Prednison response; WBC – white blood cell count at diagnosis

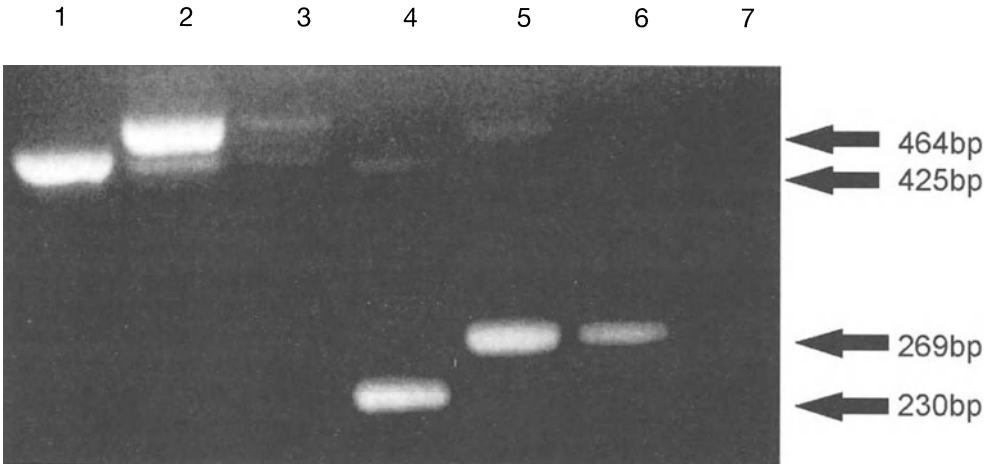

Fig. 1. Agarose gel electrophoresis of TEL/AML1 RT-PCR products. *Lane* 1 Patient no. 13 expressing fusion transcript lacking 39 bp exon 2 of AML1 (1st round amplification product); *lane* 2 patient no. 12 at diagnosis (1st round amplification product); *lane* 3 patient no. 12 at the end of induction (1st round amplification product); *lane* 4 patient no. 13 (2nd round amplification product); *lane* 5 patient no. 12 (2nd round amplification product); *lane* 6 patient no 12 at the end of induction (2nd round amplification product); *lane* 7 negative control – no cDNA added (2nd round amplification product)

till now (with follow-up ranging from 0.5 to 19 months), we have found no difference in disease-free survival between TEL/AML1-positive and t negative children.

Detection of MRD

Limiting dilution experiments revealed, that two-round RT-PCR approach allowed us to detect TEL/AML1 transcript up to a dilution of 10^{-5}.

Of the 15 TEL/AML1 positive cases, BM samples at remission were available in three cases (nos. 12, 13 and 15). Two newly diagnosed patients out of these three expressed detectable, but low levels TEL/AML1 transcript at the end of induction therapy (Fig. 1). One of them (no. 12), became negative 5 months after diagnosis, before starting ALL BFM 95 Protocol II. Patient no. 15 underwent the allogeneic bone marrow transplantation in the second complete remission and had two negative results over 10- and 12-month followup, respectively.

Discussion

In the present study, we used RT-PCR for the detection of TEL/AML1 chimeric transcript in 54 children with B-lineage ALL. We confirmed previous observations demonstrating that TEL/AML1 fusion is the most frequent genetic abnormality in childhood ALL. The overall frequency of 27.8% in cases of B-lineage ALL is similar to 28% in the retrospective study performed by Harbott et al. [14], 22% reported by Romana et al. [4], 28% found by Shurtleff et al. [5] and 23% in the work of Cayuela et al. [6]. Lower frequencies were found in studies analyzing the Chinese and Japanese populations – 19 and 16%, respectively [7, 13]. Our study also confirmed variant forms of TEL/AML1 transcripts – one patient out of 15 exhibited only the transcript representing a fusion between exon 5 of TEL gene and exon 3 of AML1 gene. All other patients displayed, except for this variant, a major fragment representing TEL exon 5 and AML1 exon 2 fusion [4, 13].

Although it was not definitively proven, TEL/AML1 positive patients are likely to represent a homogenous subset of B-lineage ALL with a favorable prognosis [5, 7, 14]. Our data, limited both in the number of the patients and the length of followup, did not allow us to establish the independent prognostic implication of this abnormality. However, a trend toward younger presenting age, lower leukocyte count at diagnosis and better response to Prednison seems to pre-

dict better-than-average outcome for the patients with TEL/AML1 chimeric transcript. A longer followup and larger size of the patient group may clarify whether TEL/AML1 positive cases really overcome the disease.

Among the methods for detection of MRD, RT-PCR based approaches are the most accurate and sensitive [15]. Unfortunately, their use in pediatric B-lineage ALL was till now limited by the low frequency of the most common targets, the fusion genes BCR/ABL, E2A/PBX1 and MLL/AF4 [3]. The high prevalence of TEL/AML1 chimeric gene will simplify the strategy in MRD evaluation in childhood ALL. Our data suggest that the two-round RT-PCR detection of TEL/AML1 mRNA is a reliable tool for MRD detection with adequate sensitivity and specificity.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Occurrence of TEL-AML1 Fusion Resulting from (12;21) Translocation in Human Early B-Lineage Leukemia Cell Lines

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Abstract. The recurrent (12;21)(p13;q22) translocation fuses the two genes *TEL* and *AML1* that have previously been cloned from translocation breakpoints in myeloid leukemias. Using mainly reverse transcriptase-polymerase chain reaction (RT-PCR), the *TEL-AML1* chimeric transcript has been observed in 22-27% of pediatric patients with acute lymphoblastic leukemia (ALL), in particular in the early B-lineage ALL subtype, making it the most common genetic lesion in these patients. The vast majority of acute myeloid leukemias, other ALL subtypes and even adults with early B-lineage ALL were *TEL-AML1*-negative. We determined whether the *TEL-AML1* fusion gene can also be observed in leukemia cell lines with an early B-lineage phenotype. Twenty-nine cell lines established from children (n = 13) or adults (n = 13) with early B-lineage ALL and five cell lines derived from chronic myeloid leukemia in blast crisis or B-cell non-Hodgkin's lymphoma were investigated for the occurrence of the *TEL-AML1* rearrangement by RT-PCR. While all 13 adult early B-lineage ALL cell lines and the five cell lines from other leukemias or lymphomas were negative, 1/13 pediatric cell lines (cell line REH) was found to be positive for *TEL-AML1;* though neither reciprocal *AML1-TEL,* nor normal *TEL,* mRNA was detectible by RT-PCR in this cell line. These findings agreed with the results of conventional cytogenetic and FISH analysis of REH which carries the der(21) partner only of t(12;21)(p13;q22), probably resulting from a complex translocation, t(4;12;21;16) (q32;p13;q22;q24.3). Hybridization with flanking cosmid clones, covering exons 1 and 8 respectively of *TEL,* confirmed a rearrangement accompanying the t(12;21), and showed cryptic deletion of the residual allele resulting from an apparently reciprocal t(5;12)(q31;p13). These findings in REH provide a further example of, and possible cytogenetic mechanism for, the paradigm of *TEL-AML1* fusion accompanied by deletion of the residual *TEL* allele. The low rate of early B-lineage ALL cell lines carrying this translocation contrasts clearly with the relative high frequency of *TEL-AML1*-positive cases in primary material. It is possible that expression of the fusion product hampers the in vitro growth and establishment in culture of such leukemic cells. The cell line REH represents a useful tool for the further molecular characterization of this unique breakpoint and can serve as a positive control in routine PCR reactions.

Introduction

Acquired chromosomal translocations have long been recognized in association with de-

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fined subtypes of leukemias [1]. Identification of these chromosomal abnormalities has aided in characterizing patient subgroups with specific clinical features and prognosis. Additionally, cloning of the genes involved in these translocations has provided important insights into the mechanisms of leukemogenesis and has allowed for the development of molecular approaches for diagnostic procedures and for monitoring impending relapse.

The most common translocations in pediatric acute lymphoblastic leukemia (ALL), t(1;19), t(4;11), and t(9;22), have each been shown to identify subgroups of patients with distinct clinical features; however, overall these translocations are detected in less than 15% of patients [2]. While some patients might have either normal karyotypes, nonrecurrent structural abnormalities or rare recurrent translocations, some of these latter cytogenetic lesions might not be appreciated due, in part, to inherent technical difficulties in identifying certain aberrations involving specific chromosomes. For instance, the t(12;21) is only rarely identified by classical cytogenetics in pediatric leukemia, accounting for less than 0.05% of cases [3]; fluorescence in situ hybridization (FISH) analysis indicated that cytogenetics may clearly underestimate its prevalence [4-7].

Recently, the t(12;21)(p13;q22) breakpoint was cloned and shown to fuse the helix-loop-helix (HLH) domain of the *TEL* gene on 12p to the DNA-binding and transactivation domains of the *AML1* gene on 21q [8,9]. *AML1* is the DNA-binding component of the *AML1/CBFβ* tanscription factor complex which is most frequently involved in myeloid-associated translocations including, for instance, the t(3;21), and t(8;21) [1]. The *TEL* gene was first identified after cloning of the t(5;12)(q33;p13) that is associated with one subtype of the myelodysplastic syndromes (MDS), namely chronic myelomonocytic leukemia. This translocation leads to expression of a fusion transcript in which the tyrosine kinase domain of the *PDGFRβ* gene on ch5 is coupled to the *ETS*-like gene *TEL* on ch12 [10]. *ETS* proteins constitute a family of DNA-binding proteins acting as transcriptional activators. Also here the HLH domain of *TEL* is retained in the fusion protein. *TEL* is also involved in other fusion-type translocations such as *TEL/ABL*-t(9;12) in ALL and AML, in *MN1/TEL*-t(12;22) in AML and MDS, and in t(10;12)(q24;p13) in MDS [11-14].

To investigate the frequency of *TEL-AML1* rearrangements, several groups analyzed panels of children with acute lymphoblastic leukemia (ALL) or acute myeloid leukemia (AML) using Southern blotting, FISH and reverse-transcriptase polymerase chain reaction (RT-PCR) [3, 15-25]. Only very few adult ALL cases were found to carry the *TEL-AML1* fusion (Table 1). The reciprocal *AML1-TEL* transcript was detected in a subset of patients only suggesting that only the protein product encoded by *TEL-AML1* plays a role in leukemogenesis [3, 17]. It was further noted that the *TEL-AML1* fusion is

Table 1. Incidences of TEL-AML1 fusions in childhood and adult acute leukemias

Leukemias		Range of frequencies (in different series) %	Total no. of cases (on aggregate)
Children:	AML	0	0/67 (0%)
	ALL	10–27	145/746 (19%)
	BCP-ALL	12–32	126/574 (22%)
	B-ALL	0	0/4 (0%)
	T-ALL	0	0/68 (0%)
Adults:	ALL	0–3	8/455 (2%)
	BCP-ALL	0–3	2/224 (1%)
	B-ALL	0	0/2 (0%)
	T-ALL	0	0/34 (0%)

Data were compiled from the literature (references 3,15-25; and unpublished data, Borkhardt et al.). BCP-ALL (B-cell precursor acute lymphoblastic leukemia) includes the various immunologically defined subtypes (e.g., pre-pre B-ALL/pro B-ALL, common ALL, pre B-ALL).

accompanied in many cases by loss or deletion of the residual (non-translocated) *TEL* allele (= loss of heterozygosity, LOH); however, none of the patients with *TEL* LOH had mutations in the residual *TEL* allele, suggesting that *TEL* is not a classical tumor suppressor gene [3, 9, 16, 17, 26-28] – the tight association between loss of one allele and rearrangement of the other in *TEL-AML1* suggesting a novel tumorigenic mechanism. Co-immunoprecipitation experiments demonstrated that *TEL-AML1* formed homodimers in vitro, and heterodimerized with the normal *TEL* protein when the two proteins were expressed together [16]. *TEL-AML1* transcripts were never found associated with any other fusion transcripts or typical cytogenetic translocation [20, 22]. *TEL-AML1*-positive leukemic children appeared to have a better treatment outcome when compared to the other B-lineage cases [3, 15, 16]. One study found a slightly higher incidence of the *TEL-AML1* fusion in relapsed B-lineage ALL cases compared with the untreated cases [25].

It can be concluded that

1. *TEL-AML1* is the most common gene rearrangement in childhood ALL, despite the t(12;21) not being evident cytogenetically; this finding may have implications for rapid diagnosis and minimal residual detection.
2. 12p deletions and *TEL* LOH subsequent to the t(12;21) are likely to represent permissive events, possibly unmasking the activity of the *TEL-AML1* product and providing a proliferative advantage to leukemic cells.
3. the *TEL-AML1* fusion identifies a subgroup of pediatric ALL patients with a good prognosis.
4. the *AML1* transcription factor is clearly a frequent target of aberrations in both pediatric ALL as well as in de novo AML.

Continuous cell lines provide permanent sources of material for researchers in several areas, but may be of particular interest for studies on the molecular changes involving the recurrent breakpoints. In the event that no adequate cell line exists, investigators are restricted to using the limited amount of material available from patient samples. In the present study, we attempted to define the frequency of the t(12;21) in a series of continuous leukemia cell lines derived from patients with early B-lineage ALL pinpointing cell lines with this unique chimeric mRNA transcript.

Materials and Methods

Leukemia Cell Lines

The continuous cell lines were taken from the stock of the cell bank of the DSMZ (German Collection of Microorganisms and Cell Cultures) or were generously provided by the investigators who established the cell lines for research purposes (Table 2). The cell lines were grown at 37 C in a humidified atmosphere of air containing 5% CO_2. The basal growth media (Gibco BRL, Eggenstein, Germany) were supplemented with 5-20% heat-inactivated fetal bovine serum (FBS) (Sigma, Deisenhofen, Germany). Cells were harvested in their logarithmic growth phase with viabilities of more than 90%.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated using guanidinium thiocyanate and ultracentrifugation methods. 5 μg total RNA were used for the synthesis of first strand cDNA applying a RT preamplification kit (SuperScript; Gibco BRL). The RT was carried out with 50 ng of random hexamers in a final volume of 20 µl RT buffer (20 mM Tris-HCl of pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin). The mixture was incubated at 70 °C for 10 min; 200 U of Moloney murine leukemia virus RT and 1 ml of 10 mM dNTP mix were added to the reaction and incubated at 42 °C for 50 min. The reaction was stopped by heating to 90 °C for 5 min and chilled on ice. After centrifugation, 2 U of RNase H were added to the reaction mixture and incubated for 20 min at 37 °C. Two µl of the first strand cDNA (corresponding to 0.5 µg of RNA) were diluted with PCR buffer (10X 500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) contain-

Table 2. Early B-lineage leukemia cell lines analyzed

Cell line 1	B-type [a] of cell line	Age/ sex	Sample site[b]	Original disease[c]	Stage of disease[d]	Trans-locations[e]	*TEL-AML1* mRNA
Cell lines from children with BCP-ALL:							
IARC-318	B-II/III	?/?	?	pre B-ALL	?	n.d.	–
KOPN-8	B-III	1 F	PB	ALL	?	t(11;19)	–
LILA-1	B-II/III	?/?	PB	pre B-ALL	?	t(1;19)	–
LK-63	B-III	?/?	PB	pre B-ALL	?	t(1;19)	–
MHH-CALL-2	B-II	15 F	PB	pre B-ALL	D	n.d.	–
MHH-CALL-3	B-II	11 F	BM	pre B-ALL	D	t(1;19)	–
PRE-ALP	B-III	6 F	BM	pre B-ALL	?	t(1;19)	–
RCH-ACV	B-II	8 F	BM	cALL	?	t(1;19)	–
REH	B-II	15 F	PB	ALL	R	t(12;21)	+
SEM	B-I	5 F	PB	pre B-ALL	R	t(4;11)	–
SUP-B15	B-III	9 M	BM	pre B-ALL	R	t(9;22)	–
SUP-B27	B-III	15 M	?	pre B-ALL	?	t(1;19)	–
UOC-B9	B-II	9 F	?	pre B-ALL	D	n.d.	–
Cell lines from adults with BCP-ALL:							
HPB-NULL	B-III	47 M	?	ALL	?	n.d.	–
LAZ-221	B-III	24 F	PB	ALL	?	n.d.	–
NALM-6	B-III	18 M	PB	ALL	?	t(5;12)	–
NALM-26	B-III	24 M	PB	pre B-ALL	D	n.d.	–
NALM-27/-28	B-II	38 M	PB	pre B-ALL	D	t(9;22)	–
NALM-30/-31/-32	B-II	38 M	PB	pre B-ALL	R	t(9;22)	–
OM9;22	B-II	19 F	BM	ALL	?	t(9;22)	–
PC-53	B-II	33 M	BM	cALL	R	n.d.	–
RS4;11	B-I	32 F	BM	ALL	R	t(4;11)	–
TAHR-87	B-II	27 M	?	pre B-ALL	?	n.d.	–
TOM-1	B-II	54 M	BM	pre B-ALL	?	t(9;22)	–
Z-119	B-II/III	25 F	BM	pre B-ALL	R	t(9;22)	–
Z-181	B-II/III	33 M	BM	pre B-ALL	R	t(9;22)	–
Cell lines from other leukemias or lymphomas:							
JM-1	B-II/III	?/?	PB	B-NHL	?	n.d.	–
MHH-PREB-1	B-III	7 M	LN	B-NHL	D	n.d.	–
MUTZ-1	B-III	6 F	BM	MDS	D	n.d.	–
NALM-1	B-III	3 F	PB	CML-lymBC	?	t(9;22)	–
SMS-SB	B-III	16 F	PB	B-NHL	?	n.d.	–

[a] Cell lines carry an early B-lineage immunophenotype based on marker expression (CD19, CD22, CD10, cyIgM, HLA-DR, CD24) in the absence of typical T-cell or myelomonocytic-erythroid-megakaryocytic markers and lack of surface immunoglobulin expression. Subcategorization into pro B-type (= B-I; CD19+ HLA-DR+), common B-type (= B-II; CD19+ HLA-DR+ CD10+), pre B-type (= B-III; CD19+ HLA-DR+ CD10+ cyIgM+), mature B-type (= B-IV); CD19+ HLA-DR+ sIg+). The cyIgM-status is not known for cell lines classified as B-II/III (but cells are always CD19+ CD10+ sIg-).

[b] According to original publication; BM: bone marrow; LN: lymph node; PB: peripheral blood; ?: not given.

[c] According to original publication; B-NHL: B-cell Non Hodgkin´s lymphoma; cALL: common ALL; CML-lym BC: chronic myeloid leukemia in lymphoid blast crisis.

[d] According to original publication; cell lines were established prior to therapy (at diagnosis, D) or at relapse (R); ?: not indicated.

[e] Cell line known to carry chromosomal translocations involving possibly these fusion genes; t(1;19)(q23;p13): *PBX1-E2A;* t(4;11)(q21;q23): *AF4-MLL;* t(5;12)(q33;p13): *PDGFRß-TEL;* t(9;22)(q34;q11): *ABL-BCR;* t(11;19)(q23;p13): *MLL-MLLT1* (for details see ref. 1). n.d.: none described (which also means that this cell line might not have been analyzed at all in this regard).

ing 20 pmol of each upstream and downstream primer, 10 nmol of dNTP mix and 1.25 U of Taq DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). For the *TEL-AML1* fusion primers used were sense *TEL969* (5′-GAACCACATCATGGTCTCTG-3′) and antisense *NAML1* (5′-AGCGG-CAACGCCTCGCTCAT-3′) [8, 17]. For the reciprocal product *AML1-TEL,* the primers sense *PG4* (5′-GGAGGAAGCGATGGCTT-CAGACAGC-3′) and antisense *NTEL* (5′-AGCGGCAACGCCTCGCTCAT-3′) were employed. To determine the quality of the RNA, the RT reaction and the PCR amplification,

the following primers were used to amplify β-actin: sense F-ACTIN (5′-ATGGATGATGATATCGCCGCG-3′) and antisense R-ACTIN (5′-CTAGAAGCATTTGCGGTGGAC-3′). The PCR reactions were performed with a DNA thermal cycler (Perkin Elmer Cetus, Heidelberg, Germany): denaturation for 7 min at 95 °C; 3 min at 75 °C and addition of the Taq polymerase; 2 min at 60 °C and 10 min at 72 °C for one cycle. The amplification was carried out in 35 cycles of 30 s at 94 °C, 30 s at 60 °C and 60 s at 72 °C with 2 s of extension time. Nine µl of the reaction mix were electrophoresed on an ethidium bromide-stained 1.4% agarose gel and blotted with 20X SSC onto nylon filters (Gene Screen; DuPont, Bad Homburg, Germany) using standard methods. Digoxigenin-labeled oligonucleotides (*TEL-AML1:* 5′-AGAATAGCAGAATGCATACT-3′; *AML1-TEL:* 5′-TTCATGAGAGACTGTAGACTG-3′) were used for the detection of the amplification products [17]. The hybridization was carried out overnight at 42 °C. The detection was performed using an alkaline phosphatase-conjugated anti-Dig antibody and the substrate solution CSPD (Boehringer Mannheim, Germany).

Cytogenetic Analysis

For conventional cytogenetic analysis, standard procedures were used. Briefly, metaphase cells were arrested from growing cell cultures of REH by colcemid blockade (0.1 µM for 3.5 h) and swollen for 7 min in an hypotonic solution comprising 0.075 M KCl and 0.9% Na-citrate (1:1). After centrifugation, cells were fixed in ice-cold methanol/acetic acid (3:1) and refrigerated overnight. After two washes in fixative, suspensions were dropped onto chilled, precleaned microscope slides. Whole chromosome painting probes were obtained from Cambio (Cambridge, UK): chr 3, 4, 5, 12, 16, 21 labelled with biotin, and chr 4, 12 and 21 labelled with fluorescein isothiocyanate (FITC). A probe specific for the telomere on chromosome 12q (D12S399, D12Z5) labelled with digoxigenin was purchased from Appligene-Oncor (Heidelberg, Germany). Commercial probes were hybridized and washed according to manufacturers' instructions. Cosmid clones covering exons 1 (179A6) and 8 (148B6) of *TEL* (donated by Prof. P. Marynen, Leuven, Belgium) [29] were contrastingly labelled with digoxigenin-11-dUTP and biotin-16-ATP by nick translation. For the cosmid probes FISH was performed using probe DNA at a concentration of 4 ng/µl with 100-fold excess of Cot-1 DNA (Boehringer Mannheim) in 2XSSC, 10% dextran sulphate, 50% deionized formamide (Sigma). In all cases standard chromosome slide preparations were denatured (by incubation in 70% formamide, 2XSSC, pH 7.0, at 74 °C for 2 min), and then dehydrated in an ethanol series. After denaturing and prehybridizing probe mixtures (72 °C for 5 min and 37 °C for 30 min), hybridizations were performed over 48 h at 42 °C in a moist chamber. Standard "sandwich" detection was used to visualize biotin (avidin-Texas Red) and FITC (anti-FITC). Digoxigenin was visualized using a murine monoclonal antibody against digoxin, haptenized with FITC (Sigma).

Results and Discussion

RT-PCR analysis was performed on mRNA specimens from 34 leukemia cell lines derived from 31 patients (Table 2). The specificity of the PCR products was assessed with an internal probe after Southern blotting. The amplification of the house keeping β-actin transcript was used as a quality control. Samples from pediatric patients with early B-lineage ALL carrying a t(12;21) were employed as positive controls (Fig. 1).

All cell lines display an early B-lineage phenotype based on immunophenotyping and genotyping data. The cell lines were assigned to three different categories based on their origin from early B-lineage ALLs in children, from early B-lineage ALLs in adults, or from other leukemias (i.e. CML in blast crisis) or B-cell type non-Hodgkin's lymphomas (Table 2). None of the cell lines established from adults with early B-lineage ALL or from other leukemias/lymphomas showed the *TEL-AML1* fusion in the RT-PCR. Out of 13 pediatric cell lines only the cell line REH carried the *TEL-AML1* fusion

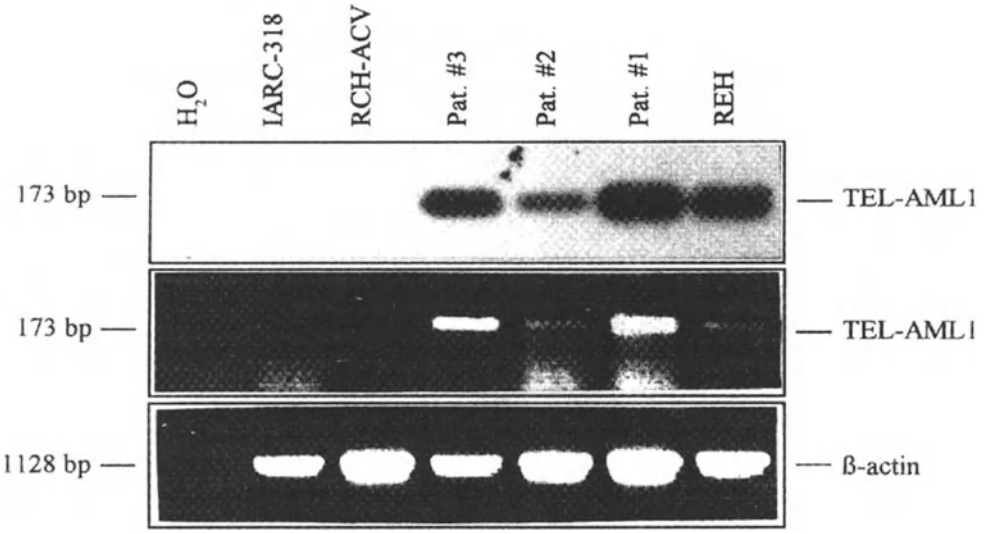

Fig. 1. Identification of TEL-AML1 Fusion by RT-PCR. RNAs derived from three positive patients (#1-3 used as positive controls) and from three early B-lineage ALL cell lines were used in an RT-PCR with TEL/AML1-specific primers. Aliquots of the amplification products were separated by gel electrophoresis and visualized with ethidium bromide *(middle panel)*. This gel was blotted and hybridized with a breakpoint-spanning probe *(top panel)*. Amplifiable RNA and fidelity of the PCR reaction were controlled using primers specific for ß-actin (bottom panel). The predicted 173-bp TEL-AML1 fragment was amplified in the cell line REH, but not in IARC-318 and not in RCH-ACV

gene (Fig. 1). While all three primary samples used as positive controls for the *TEL-AML1* fusion mRNA detection were also positive for the *AML1-TEL* PCR product, REH does not appear to have this reciprocal chimeric transcript (data not shown). RT-PCR with the *TEL969-NTEL* or *NAML1-PG4* primers showed that the non-rearranged *AML1* allele was invariably expressed in cases displaying the *TEL-AML1* fusion mRNA; on the other hand, while the patients were positive for the second non-rearranged *TEL* mRNA, this *TEL* allele was not expressed by the cell line REH suggesting a deletion.

The consensus karyotype of REH was found to be: 46, X, -X, del(3)(p21.3p24), der(4)(4pter→q32::?16q24.3→qter), t(5;12)(q31.2;p13), der(12)(12qter→q23::12p13→q23::4q32→qter), +16, der(16)-t(16;21)(q24.3;q22)x2, der(21)t(12;21) (p13;q22). Apart from the unrelated del(3), rearrangements fell into two groups, each involving 12p13 from either homologue. The first group responsible for the t(12;21), appeared to involve a complex four-way translocation, t(4;12;21;16)(q32;p13;q22; q24.3), with inversion at 12p13 and q23 in the der(12) partner. Signal from cosmid 179A6, but not 148B6, colocalized to the medial long-arm region of the der(21), detected by simultaneous hybridization with painting probes for chromosomes 12 and 21, confirming translocation of *TEL* effected by the t(12;21). Colocalization of signals from the telomere 12q and chromosome 12 painting probes showed that the t(12;21) and t(4;12) accompanied an inversion at 12p13 and 12q23. Though conventional G-banding suggested the t(5;12) involved a straightforward reciprocal exchange of material between 5q31.2 and 12p13, neither participating chromosome yielded signal from either *TEL* cosmid indicating deletion of exons 1 to 8 of the residual *TEL* genomic region (spanning 240 kb), possibly as a direct result of the translocation.

While the absence of any *TEL-AML1* fusion product in the early B-lineage ALL cell lines established from adults accords with the data on primary material [18,19], the low percentage (8%) of *TEL-AML1-positive* pediatric early B-lineage ALL cell lines is sur-

prising. This might be simply due to statistically random sampling of negative cell lines. Alternatively, as the rate of success in attempts to establish leukemia cell lines is relatively low, certainly below 5% for lymphoid leukemias and probably closer to 1% for myeloid leukemias, it is possible that the *TEL-AML1* fusion product hampers further the in vitro growth of such *TEL-AML1*-positive ALL cells and consequently also the establishment of a permanent cell line. It may be relevant that both examples of cell lines with t(12;22), namely MUTZ-3 and UCSD/AML1, are dependent on growth factor supplementation - a procedure rarely undertaken by those attempting to establish cell lines.

Furthermore, some cell lines may have alternative breakpoints that result in a chimeric product that could not be amplified by our oligonucleotide primers. In some lines, *TEL* may be fused to genes other than *AML1* or may be partially deleted. Further studies are required to clarify any possible molecular lesions in our cell lines panel.

Finally, it is interesting that 9/13 childhood ALL cell lines studied here have other known recurring translocations: 6 cell lines with t(1;19), one cell line each with t(4;11), t(9;22) or t(11;19). To our knowledge, the *TEL-AML1* fusion has not yet been detected in patients who also have one of these common recurring translocations. So, consideration of cell lines without these translocations increases the frequency from 1/13 to 1/4 (25%). One might speculate that the t(12;21) is one of the critical events in the leukemogenesis of these cells.

Two independent sets of complex chromosome rearrangements are responsible for the respective rearrangement and deletion of TEL in REH:

1. *TEL-AML1* fusion by formation of der(21)t(12;21) with concominant loss of the reciprocal *AML1-TEL* fusion gene due to the surrounding, complex four-way t(4;12;21;16) translocation; and
2. loss of the residual *TEL* allele by cryptic deletion accompanying translocation in the t(5;12).

Despite their complexity, these cytogenetic mechanisms in REH effect the same combination of *TEL-AML1* fusion/residual *TEL* deletion consistently observed in patient studies [14, 17]. It is thus likely that the rearrangements in REH arose in vivo. Thus, REH provides an insight into possible cytogenetic mechanisms underlying the paradigm of *TEL* rearrangement and deletion in t(12;21) seldom affordable by the more limited studies possible on primary leukemia cells.

In summary, we detected a cell line, the pediatric early B-lineage ALL-derived line REH, that carries the t(12;21) fusion gene *TEL-AML1* accompanied by deletion of the residual allele. As leukemia-lymphoma cell lines have been instrumental in the study of many breakpoints, this cell line represents a powerful tool for the characterization of this unique breakpoint and can be used as a permanently available (standardizable) positive control in the PCR analysis of *TEL-AML1* fusion transcripts.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Frequent Loss of Heterozygosity on Chromosomes 6q, 9p, 11q or 12p in Childhood Acute Lymphoblastic Leukemia

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Abstract. Chromosomal abnormalities on 6q, 9p, 11q and 12p have been reported frequently in acute lymphoblastic leukemia (ALL). In order to define regions that may contain tumor suppressor genes more precisely, the loss of heterozygosity (LOH) was analyzed on respective chromosome arms in childhood ALL. Using highly informative microsatellite markers, LOH was found in 17 of 112 (15%) ALL samples on 6q; in 29 of 54 (54%) on 9p; in 14 of 112 cases (13%) on 11q; in 33 of 100 (33%) on 12p. The commonly deleted region on 6q was flanked by the markers D6S468 and D6S283/D6S449 at 6q21. In 27 of the 29 cases with LOH on 9p the critical region was characterized by D9S1747 and D9S1748. Homozygous deletions of the CDKN2/INK4A/p16 gene residing in this region were found in 14 of the 27 patients. Two cases revealed LOH at the IFNA locus. Two distinct commonly deleted regions were identified on 11q and 12p, respectively. One region at 11q22 was flanked by D11S901 and D11S1391, and the other at 11q23 by D11S614 and D11S924. On chromosome 12p, one critical region was flanked by the markers D12S77 and D12S98 including the TEL gene, and the other was localized around the p27/kip1 locus. Our data narrow down regions on chromosomes 6q, 9p, 11q and 12p containing putative tumor suppressor genes which may play an important role in leukemogenesis of childhood ALL.

Introduction

Cytogenetically, non-random chromosomal aberrations have been frequently observed in acute lymphoblastic leukemia (ALL) [1,2]. Cytogenetic deletion mapping revealed that the loss of entire chromosomes most commonly affects nos. 7, 20, 21, X and Y in ALL. Structural aberrations resulting in the deletion of distinct chromosomal regions have been reported at 6q15-27, 7p13-22, 9p11-24, 11q22-25, 12p12-13, 19p13 and 22q11-13 with frequencies of 8-17, 3-4, 7-16, 3-5, 9-10, 9 and 4%, respectively. These observations seem to indicate the presence of tumor suppressor genes residing in these regions that may play an essential role in leukemogenesis. The application of polymerase chain reaction (PCR) strategies to analyze the loss of heterozygosity (LOH) using highly informative microsatellite markers allows a detailed deletion mapping of chromosomes since this method permits the identification of small interstitial deletions which are beyond the sensitivity of conventional cytogenetic anal-

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ysis. Our previous allelotype analysis of childhood ALL using 101 microsatellite markers that covered all autosomal chromosomes pinpointed LOH on chromosome arms 6q, 9p, 11q and 12p with frequencies of 13, 40, 8 and 26%, respectively [3].

In this study, we performed detailed deletion mapping by means of LOH analysis, focusing on these four chromosome arms, and determined the smallest commonly deleted regions.

Material and Methods

Cells

After informed consent, bone marrow (BM) and peripheral blood (PB) samples were obtained from 113 children with ALL (93 precursor-B ALL and 20 T-ALL) enrolled in the multicenter ALL trial ALL-BFM 90 of the German Berlin-Frankfurt-Münster (BFM) study group, as well as from healthy individuals. These patients were selected based on the availability of cryopreserved cell samples from initial diagnosis and complete remission. Methods and criteria for the definition of immunophenotypes have been previously described [4, 5]. The blast content of the leukemia cell samples was more than 90%.

Analysis of LOH Using Microsatellite Markers

The LOH analysis was performed by PCR-amplification of microsatellite sequences. 79 markers (31 on chromosome 6q, 18 on 9p, 14 on 11q and 16 on 9p) were used in this study. The genetic map of chromosome 6, 9, 11 and 12 was compiled mainly from the Genethon microsatellite map [6-8]. High molecular weight genomic DNA was prepared from cryopreserved BM and PB cells. Each PCR reaction contained 5-20 ng of DNA, 10 pmol of each of the primers, 2 nmol of each of the four deoxyribonucleotide triphosphates, 0.5 units of Taq DNA polymerase, 3 μCi of [α-^{32}P]dCTP, in 20 μl of the specified buffer with 1.5 mM $MgCl_2$. Samples were amplified by 30-35 cycles of denaturing for 40 s at 94 °C, annealing for 30 s at 55 °C and extending for 1 min at 72 °C in a programmable thermal controller. After amplification, PCR samples were diluted 5-fold in loading buffer containing 20 mM EDTA, 96% formamide and 0.05% of both bromophenol blue and xylene cyanol. The products were heated to 95 °C for 5 min and 1.5 μl of each sample was electrophoresed through a 6% polyacrylamide gel containing 8.3 M urea for 3-4 h at 85 W. Subsequently, the gels were dried and exposed on X-ray film at -80 °C. Loss of heterozygosity was inferred when visible reduction was measured in the ratio of allele radiographic signal intensities in the tumor sample relative to those in the corresponding normal sample.

Southern Blot Analysis

Ten μg of DNA were digested with an appropriate restriction enzyme, separated on 0.6% agarose gel and transferred onto nylon membranes (Nytran 13N; Schleicher & Schuell, Dassel, Germany). After hybridization with radiolabeled probes the filters were washed and exposed on X-ray film using intensifying screens as previously described [9].

Results

Analysis of LOH on Chromosome 6q

We first screened 48 ALL samples for LOH of chromosome 6q using 22 microsatellite markers. Six samples showed LOH at least at one locus on chromosome 6. Based on the results from the 6 patients, a commonly deleted region on 6q was determined between markers D6S284/D6S286 and D6S267/D6S287/D6S303/D6S3024 (Table 1). In order to identify the smallest commonly deleted region in the above determined region, we examined 112 ALL samples for LOH of the region between D6S284/D6S286 and D6S262 using 19 highly informative microsatellite markers. LOH was found at least at one locus in 17 of 112 (15%) ALL patients (2/19 T-ALL, 15/93 precursor-B ALL). Six cases showed LOH at all informative loci. Five samples revealed relatively large interstitial deletions (D6S275/D6S417-D6S268). Three very informative samples lost alleles at D6S468 or

Table 1. Microsatellite and polymorphic markers used for the LOH analysis on chromosome arms 6q, 9p, 11q and 12p. Markers are sorted according to their positions on respective chromosomes and the smallest commonly deleted regions are indicated

q	9p	11q	12p
Centromere	9pter	Centromere	12pter
D6S313	D9S168	D11S901	D12S91
D6S280	D9S156	D11S2179	D12S100
D6S284	D9S157	D11S1391	D12S93
D6S286	D9S162	D11S897	D12S99
D6S275	IFNA	D11S29	D12S77
D6S417	D9S1749	D11S1356	D12S89
D6S300	D9S1747	D11S1341	D12S98
D6S424	(p16)	D11S976	p27/Kip1
D6S468	D9S1748	D11S614	D12S358
D6S283	(p15)	D11S1364	D12S320
D6S449	D9S1752	D11S924	D12S364
D6S268	D9S171	D11S528	D12S269
D6S278	D9S126	D11S925	D12S308
D6S302	D9S265	D11S933	D12S310
D6S261	D9S259	11qter	D12S363
D6S266	D9S169		D12S87
D6S267	D9S270		Centromere
D6S287	D9S161		
D6S303	D9S165		
D6S304	D9S163		
D6S262	Centromere		
D6S270			
D6S292			
D6S308			
D6S310			
D6S314			
D6S290			
D6S305			
D6S264			
D6S297			
D6S281			
6qter			

D6S300/D6S424, but maintained heterozygosity at the distal neighboring locus D6S283/D6S449. The other two samples lost alleles at D6S268, but maintained heterozygosity at the proximal neighboring locus D6S468. Thus, the smallest commonly deleted region is flanked by the markers D6S468 and D6S283/D6S449, comprising 2cM. A deletion at this region was identified in 16 of 17 samples with 6q LOH.

Analysis of LOH on Chromosome 9p

Using 18 highly polymorphic markers, LOH was found at least at one locus on the short arm of chromosome 9 in 29 of 54 (54%) ALL samples. Sixteen cases showed LOH at all informative loci on 9p. Four samples revealed terminal deletions and 9 cases showed interstitial deletions. Twenty-seven of the 29 samples lost alleles in the region flanked by D9S1747 and D9S1748. This area contains the CDKN2/INK4A/p16 gene [10]. In 2 cases LOH was restricted to the IFNA locus [11] while heterozygosity was retained at the CDKN2/INK4A/p16 locus.

Analysis of the CDKN2/INK4A/p16 and CDKN2B/INK4B/p15 Gene by Southern Blot Hybridization

Southern blot analysis showed that 14 of the 29 patients with 9p LOH had homozygous deletions of the CDKN2/INK4A/p16 gene, and 7 of the 14 cases had homozygous deletions of the CDKN2B/INK4B/p15 gene [12],

as well. Not a single case showed a homozygous deletion of just the CDKN2B/INK4B/p15 gene. All of the 14 patients exhibited large LOH surrounding the CDKN2/INK4A/p16 and CDKN2B/INK4B/p15 loci. This result suggests that homozygous deletions of the putative tumor suppressor genes result from the loss of a large segment on one allele and a small deletion on the other.

Analysis of LOH on Chromosome 11q

Using 14 highly informative microsatellite markers located between 11q14 and 11q24, LOH was found at least at one locus in 18 of 113 (16%) ALL samples (3/20 T-ALL, 15/93 precursor-B ALL). Eight cases showed LOH at all informative loci. Two samples revealed relatively large deletions between markers D11S901 and D11S925. Four cases showed small deletions between markers D11S614 and D11S924. Two very informative cases revealed small deletions at D11S2179. The other two patients showed deletions around D11S2179 but not at D11S614. Thus, two distinct commonly deleted regions were identified on chromosome 11q. The first region is flanked by markers D11S901 and D11S1391 at 11q22, containing the ATM gene [13], and the second region by D11S614 and D11S924 at 11q23, including the MLL gene [14].

Analysis of the MLL Gene by Southern Blot Hybridization

Alterations of the MLL gene were examined by Southern blot analysis in 14 ALL samples that showed LOH at 11q23. A PstI/SacI fragment from exon 8 of the MLL gene was used as a probe (kindly provided by Dr. P. Domer). All 14 patients revealed a germline configuration of the MLL gene on the second allele.

Analysis of LOH at Chromosome 12p

Using 16 highly polymorphic markers, LOH was found at least at one locus on the short arm of chromosome 12 in 33 of 100 ALL samples (33%) (Fig. 1). It was observed more frequently in precursor-B ALL (32/80; 40%) than in T-ALL (1/20; 5%) ($p = 0.0027$). Four cases showed LOH at all informative loci on chromosome 12p. Six cases revealed large terminal deletions. Ten samples showed large interstitial deletions between D12S89 and D12S308. Four very informative samples lost alleles at D12S89, but maintained heterozygosity at the distal neighboring locus D12S77. Two of these samples also maintained heterozygosity at the proximal neighboring locus D12S98. LOH at D12S89 was identified in 21 of 82 (26%) informative patients. Thus, this smallest commonly deleted region is flanked by D12S77 and D12S98, and has a size of 4cM.

Seven other informative samples showed LOH at p27/Kip1 [15, 16], but one of these samples retained both alleles at the proximal neighboring locus D12S358. Another sample maintained heterozygosity at the distal neighboring locus D12S98. Moreover, five of these samples retained heterozygosity at D12S89, which lies in the smallest commonly deleted region as defined above. Thus, a second smallest commonly deleted region resides around the p27/Kip1 locus. LOH at the p27/Kip1 locus was found in 15 of 34 (44%) informative patients.

Analysis of the TEL Gene and the p27/Kip1 Gene by Southern Blot Hybridization

Using a PCR amplified exon 5 of the TEL gene as a probe, alterations of the TEL gene [17] were examined in 21 ALL samples that showed LOH at 12p13 by means of Southern blot hybridization of BamHI-digested DNA samples. A rearrangement was detected in 8 cases. One common ALL revealed a homozygous deletion of the TEL gene. The deleted regions on chromosome 12p in 8 of the 9 ALL cases with TEL rearrangements or homozygous deletion covered the TEL locus.

Alterations of the p27/Kip1 gene were analyzed in the 100 ALL cases by Southern blot hybridization. All of the patients revealed a germline configuration of the p27/Kip1 gene.

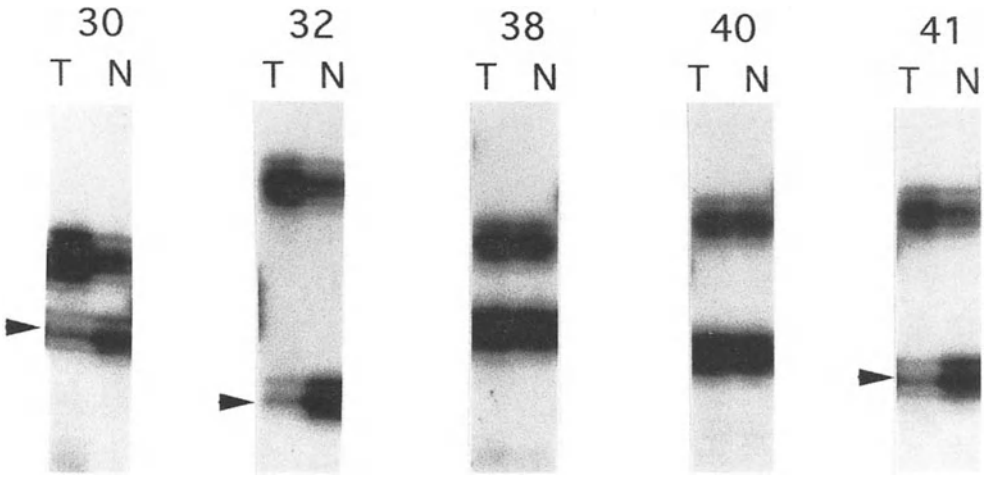

Fig. 1. Representative autoradiograph showing loss of heterozygosity at D12S89. LOH is found in samples 30, 32 and 41 (indicated by *arrowhead*). The weak radiographic signals at deleted positions in tumor DNA samples stem from contaminating normal cells. The sample numbers are shown *above the diagrams.* T tumor DNA, N normal DNA

Discussion

Detailed deletion mapping of four chromosome arms identified in previous studies [3] was performed by usage of highly informative microsatellite markers in childhood primary ALL. In 15% of cases LOH was found on 6q; in 54% on 9p; in 16% on 11q; in 33% on 12p. The frequency of deletions in this study is higher than that reported for those chromosome arms in previous cytogenetic analyses, suggesting that the PCR-mediated approach allows a more sensitive detection of deletion events.

On the long arm of chromosome 6, the smallest commonly deleted region is flanked by D6S468 and D6S283/D6S449 at 6q21. The genetic distance between these 2 loci is approximately 2cM.

On the short arm of chromosome 9, the region was localized between markers D9S1747 and D9S1748, including the putative tumor suppressor gene CDKN2/INK4A/p16 at 9p21. Twenty-seven of the 29 patients with 9pLOH lost alleles in this region. Fourteen of the 27 cases exhibited homozygous deletions of the CDKN2/INK4A/p16 gene. However, the other 13 cases showed a retention of both CDKN2/INK4A/p16 and CDKN2B/INK4B/p15 genes, suggesting that an additional tumor suppressor gene resides in this region. The relationship between homozygous deletions of the CDKN2/INK4A/p16 gene and LOH on 9p was analyzed in 14 cases with homozygous deletions. A surrounding larger LOH on the second allele was found in all 14 cases. These results show that homozygous deletions at 9p21 occur after a loss of a large chromosome segment on one allele. Two cases showed LOH at the IFNA locus and no LOH in the above-determined region. Deletions of the IFN genes have been reported in 29% of ALL patients [18]. These results suggest that a second commonly deleted region resides around the IFNA locus.

On the long arm of chromosome 11, one critical region is flanked by D11S901 and D11S1391 at 11q22 and the other by D11S614 and D11S924 at 11q23, suggesting that in this area two different tumor suppressor genes are localized on chromosome 11q. A rearrangement of the MLL gene residing in the critical region at 11q23 was not found in patients with LOH at this area. This finding indicates that the MLL gene is probably not the critical target for the deletion event in 11q23.

On the short arm of chromosome 12, one regions is flanked by D12S77 and D12S98 containing the TEL gene, and the other is around the p27/Kip1 locus at 12p13. Rearrangements of the TEL gene were observed

in 8 of the 21 patients with 12p LOH around the TEL locus, suggesting that the TEL is an actual target for some ALL cases with 12p LOH, and in one patient, the TEL gene was homozygously deleted. However, 12 cases showed a germline configuration of the TEL gene in spite of 12p LOH. Furthermore, all patients with LOH at the p27/Kip1 locus revealed a germline configuration of the gene. These results pinpoint the existence of two novel tumor suppressor genes on chromosome 12p.

In summary, our detailed mapping efforts provide important information toward the cloning of novel tumor suppressor genes that may play an essential role in leukemogenesis of childhood ALL.

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Deletions of Human Chromosome Region 6q23-24 in B-Lineage Neoplasias

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Abstract. Deletions of the long arm of chromosome 6 (6q) are among the most frequent chromosome aberrations in B-lineage leukemias and lymphomas and often occur as secondary changes in addition to typical translocations, such as t(14;18). Using fluorescence in situ hybridization (FISH) with two YAC-DNA probes hybridizing to human chromosome region 6q23-24 and with the centromeric probe D6Z1 as internal control, we studied 31 low-grade and 8 high-grade B-cell lymphomas. Deletions in 6q23-24 were detected in 21 cases (54%) by FISH, compared to 13 cases (33%) by chromosome analysis. Deletions in 6q23-24 were found by FISH in 5/13 small lymphocytic lymphomas, 2/3 mantle cell lymphomas, 10/14 t(14;18)-positive follicular lymphomas and in 4/8 centroblastic lymphomas, three of the latter carrying a translocation t(14;18). This study demonstrates that in B-cell lymphomas deletions in 6q23-24 are present more frequently than suggested hitherto and that they can be detected more sensitively by FISH than by chromosome analysis. In contrast to previous reports suggesting the region 6q23-24 to be preferentially deleted in low-grade lymphomas lacking the translocation t(14;18), our results indicate that deletions in 6q23-24 appear to be also common in other pathological subsets of B-cell lymphomas, especially in follicular lymphomas carrying the translocation t(14;18).

Introduction

Deletions of the long arm of chromosome 6 (6q) are among the most frequent chromosome aberrations in malignant lymphomas, in acute leukemias as well as in some solid tumors, e.g. ovarian cancer and melanoma [1]. Therefore, the existence of one or more tumor suppressor genes involved in the pathogenesis or progression of these malignancies is presumed in 6q. In non-Hodgkin's lymphomas (NHL), deletions of 6q frequently occur as secondary changes in addition to typical translocations, e.g. the translocation t(14;18), and define a subgroup of patients with a poor prognosis. By loss of heterozygosity (LOH) analyses, three regions of minimal molecular deletions (RMD) in 6q have been defined in NHL [2, 3]. These RMDs were reported to be associated with certain pathological subtypes of NHL, namely RMD1 at 6q25-27 with intermediate-grade NHL, RMD2 at 6q21 with high-grade NHL and RMD3 at 6q23 with low-grade NHL lacking the translocation t(14; 18). In order to determine the frequency of deletions of this latter region in different subtypes of NHL we investigated 39 low-grade and high-grade B-cell lymphomas with and without translocation t(14; 18) by means of interphase fluorescence in situ hybridization (FISH) using two 6q23-24-specific YAC-DNA probes.

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Material and Methods

Patients

39 patients (18 male, 21 female) with B-cell NHL were studied. Thirteen patients were diagnosed with small lymphocytic lymphomas including nine cases of B-CLL and four cases of lymphoplasmocytic lymphomas, three patients with mantle cell lymphomas, one patient with a marginal B-cell lymphoma, 14 patients with follicular lymphomas (corresponding to follicular center lymphoma, follicular type, grade II in 13 patients and grade III in one patient) and eight patients with high-grade centroblastic lymphoma (corresponding most likely to diffuse large B-cell lymphoma). At the time of chromosome analysis, one B-CLL had already underwent transformation into a high-grade immunoblastic lymphoma; one patient presented with a low-grade follicular lymphoma which evolved to a high-grade lymphoma within two months.

Cytogenetic Analyses

Chromosome analyses and interphase cytogenetics were performed on short-term cultures of lymph node biopsies or peripheral blood. For chromosome banding, a fluorescence R-banding technique was applied.

Fluorescence in Situ Hybridization (FISH)

As probes for interphase cytogenetics two YACs, yWPR108 and yWPR 255 mapping to human chromosome region 6q23-24, were kindly provided by Dr. D. Schlessinger (Center for Genetics in Medicine, MI, USA). YAC-DNA was amplified by Alu-PCR and biotinylated using a random primed labelling kit (GibcoBRL, Eggenstein, Germany). In order to determine the copy number of chromosome 6 the digoxigenin-labelled alpha-satellite probe D6Z1 (Oncor, USA) specific for the centromere of chromosome 6 served as internal control in each experiment. Dual-color FISH was performed with one of the YAC-DNA probes and D6Z1 as previously described [4]. Biotinylated YAC probes and digoxigenin-labelled D6Z1 were visualized by AMCA (blue) and FITC (green) fluorescent dyes. More than 200 cells for each YAC probe were analysed per case. Evaluation of slides was performed in a blind fashion, i.e., the persons who evaluated the slides did neither know the diagnoses of the patients, nor the results of previous chromosome studies.

Results

Cytogenetic Analyses

By cytogenetics, clonal chromosome aberrations were detected in 32 patients. A translocation t(14;18)(q32;q21) was found in all 14 tumor samples classified as follicular lymphoma and in 5 of 8 samples classified as centroblastic lymphoma. A translocation t(11;14)(q13;q32) was observed in two of three mantle cell lymphomas. By chromosome analyses, structural alterations of chromosome 6 were seen 17 lymphoma samples. In 12 of these samples, the clones contained deletions affecting 6q. Single cells with a deletion in 6q were found in two patients. In three patients, the whole long arm of chromosome 6 was deleted due to the formation of an isochromosome i(6)(p10). Except for two tumor samples containing clonal deletions of 6q13-?21 and 6q27-qter, respectively, the deletions affecting the long arm of chromosome 6 always encompassed the region 6q23-24. Thus, by cytogenetics a clonal loss of chromosome region 6q23-24 was detected in 13 patients (33.3%).

Control Studies for the Detection of a Deletion 6q23-24 by Interphase FISH

By our FISH assay for the detection of deletions in human chromosome region 6q23-24, interphase nuclei of diploid cells without alteration of chromosome 6 should display two green hybridization signals for D6Z1 and two blue hybridization signals for either yWPR 108 or yWPR 255. Deletions in chromosome region 6q23-24 are indicated by lack of one of the two blue signals (Fig. 1).

In order to define the thresholds for the detection of a deletion 6q23-24 by interpha-

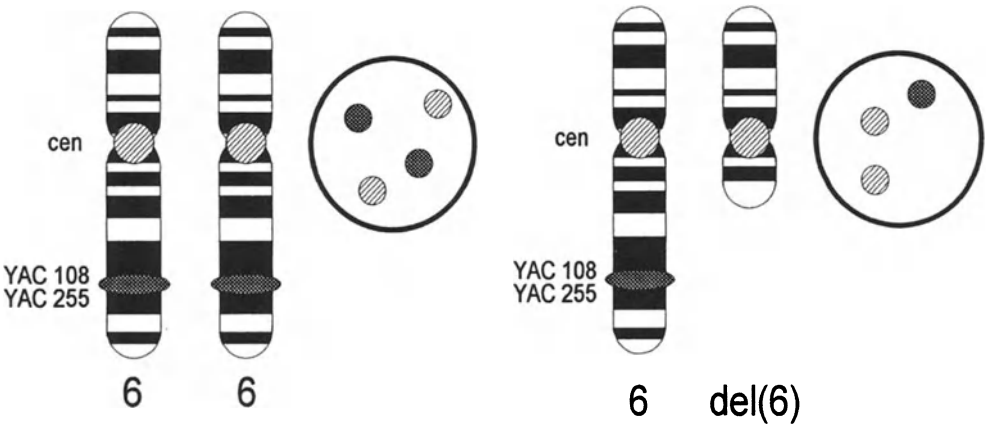

Fig. 1. Schematic illustration of the interphase cytogenetic approach for the detection of deletions in human chromosome region 6q23-24
Metaphase chromosomes and interphase nuclei without *(left)* and with *(right)* deletion in human chromosome region 6q23-24. The interphase nucleus carrying a deletion in 6q23-24 displays two signals derived from the probe D6Z1 hybridizing to the centromere of chromosome 6 *(bright dot)*, but only one signal derived from the respective YAC *(dark dot)*.

se FISH 1200 peripheral lymphocytes from five healthy donors were analysed. Among these cells, 12 nuclei (1.1%) contained one hybridization signal for D6Z1, 9 nuclei (0.8%) and 11 nuclei (0.9%) displayed only one signal for the yWPR 108 and yWPR 255 probes, respectively. Based on these control experiments, the cut-off limit for detecting deletions of 6q23-24 was set at 5%. Thus, a deletion in 6q23-24 was diagnosed, if more than 5% of lymph node cells showed two hybridization signals for D6Z1, but only one hybridization signal for YAC DNA probe, or - in case of triploid or tetraploid cells- three or four hybridization signals for D6Z1, but only two or three hybridization signals for the YAC DNA probes.

Detection of Deletions 6q23-24 in Lymphoma Specimen by Interphase FISH

By interphase FISH, a deletion in chromosome region 6q23-24 was detected in 21 patients (57%). In each case the percentages of cells displaying a deletion in 6q23-24 agreed completely for both YAC-DNA probes used. A deletion in 6q23-24 was observed in five of 13 patients with small lymphocytic lymphoma (4 B-CLL, one lymphoplasmocytic leukemia), in 2 of 3 patients with mantle cell lymphoma, in 10 of 14 patients with follicular lymphoma and in 4 of 8 patients with centroblastic lymphoma. In all 13 patients with a clonal deletion of 6q23-24 identified by chromosome analysis, these deletions were confirmed by FISH. In the two patients with single cell deletion in 6q, interphase FISH revealed deletions in 6q23-24 in 27.7 and 20.1% of cells, respectively. In the two lymphoma samples, which were thought to carry deletions of 6q13-?21 and 6q27-qter by chromosome analysis, respectively, FISH detected loss in 6q23-24 in 18.7% and 46.9% of cells. Four patients without cytogenetic alterations of 6q were found to have a deletion in 6q23-24 by FISH. Among the 19 lymphomas carrying a translocation t(14; 18), deletions in 6q23-24 were detected by FISH in 13 samples. Of theses, 10 samples were classified as follicular lymphoma and three as centroblastic lymphoma. A deletion in 6q23-24 was also present in one centroblastic lymphoma without a translocation t(14; 18).

In the different cases, 16.1 to 91.0% of cells contained deletions in 6q23-24. The mean percentages of cells with the deletion were 31.1% in small lymphocytic lymphomas, 39.2% in follicular lymphomas and 45.1% in centroblastic lymphomas. The percentages of cells with the deletion was higher in the cases with cytogenetically visible de-

Table. 1. Deletions of 6q23-24 detected by cytogenetics and FISH

	No. of cases	Cytogenetics	FISH
Small lymphocytic lymphoma	13	4 (31%)	5 (39%)
Mantle cell lymphoma	3	2 (67%)	2 (67%)
Marginal cell lymphoma	1	0	0
Follicular lymphoma	14	5 (36%)	10 (71%)
Centroblastic lymphoma	8	2 (25%)	4 (50%)
Total	39	13 (33%)	21 (54%)

letion in 6q, as compared to the cases without a cytogenetically detectable deletion (46.8% vs. 20.7%, p=0.016), suggesting that in these cases deletions in 6q had occured in a minority of the tumor cells, probably as secondary changes.

Discussion

Cytogenetically detectable deletions of the long arm of human chromosome 6 have been reported in 13% to 20% of B-cell lymphomas with a translocation t(14; 18) [5, 6]. LOH analyses revealed deletions of chromosome regions 6q21-23 and 6q25-27 in 22.5% and 20.5% of NHLs, respectively [2, 7]. By FISH, deletions of human chromosome regions 6q16.3-21 and 6q23.1-27 have been reported in 4/7 and 7/7 NHL cases [8, 9]. Moreover, in 7/7 Burkitt's lymphoma cell lines deletions of 6q25-27 have been described [10]. In the present study, FISH revealed deletions in 6q23-24 in 54% of B-cell lymphomas, compared to 33% detected by chromosome analysis. Therefore, deletions in 6q23-24 appear to exist even more frequently than suggested hitherto. Additionally, FISH turned out to be much more sensitive than chromosome analysis in detecting deletions in 6q.

Based on cytogenetic analyses, Offit et al. [3] suggested deletions of human chromosome region 6q23-24 to characterize a subset of low-grade small lymphocytic lymphomas lacking the translocation t(14;18). In contrast to these karyotype studies, our FISH analyses revealed deletions in 6q23-24 in 71% of low-grade follicular lymphomas with t(14;18) as compared to 39% of small lymphocytic lymphomas. In total, 13 of 19 t(14;18)-positive lymphomas contained cells carrying a deletion in 6q23-24. Thus, in the present study deletions in 6q23-24 were observed even more frequently in follicular lymphomas carrying a translocation t(14;18) than in other low-grade B-cell lymphomas. In order to define the critical regions of deletion in different subtypes of B-cell lymphomas further studies with additional YAC DNA probes spanning other regions of 6q are in progress. These analyses will provide the prerequisite for the identification of putative tumor suppressor genes involved in the pathogenesis or progression of B-lineage neoplasias.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Excessive Differentiation of Myeloid Cells in AML-M2 with Translocation 8;21

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Abstract. t(8;21) is a chromosomal abnormality found in a part of AML-M2 cases and exceptionally in other blood disorders. According to literature data, CR in this form of M2 is easy to obtain and therefore BMT is not recommended in the first remission. We report here on 3 patients observed in the past 3 years, in whom blood disease was firstly diagnosed as blast crisis/acceleration of CML, because of an excessive differentiation of their leukemic cells and low FAG scores. These results prompted us to diagnose BC/ACC-CML and to administer a proper therapy. However, cytogenetic examination of BM cells of the patients did not confirm the diagnosis: t(8;21) and no Ph chromosome was found in all samples examined, which enabled us to change the diagnosis. During the course of the disease a disappearance of t(8;21) in remissions and reappearance in relapses was revealed. t(8;21) is very rarely seen in BC-CML of M2 type and was never described in Ph(–) CML. Our observation confirm that of Yamasaki and co. that t(8;21) M2 may show an excessive differentiation of the myeloid cell line.

Introduction

t(8;21)(q22;q22) giving rise to AML 1/ETO fusion gene formation is an acquired chromosome aberration seen in about 1.5% of acute myeloid leukemia (AML) cases, especially of M2 type [M2/t(8;21) according to MIC] [1, 2]. The leukemic cells often have a characteristic morphology (large, often monocytoid blasts with Auer rods, with greater tendency to maturation to neutrophils, and also to eosinophils, than in M2 without t(8;21)), cytochemistry (high MPO and low NAP activity) and immunophenotype ($CD13^+$, $CD15^+$, $CD19^+$, $CD34^+$, $HLA\text{-}DR^+$ and often $CD56^+$) [3, 4, 5, 6, 7].

t(8;21) is rarely seen as the sole abnormality, in 80% of cases it is accompanied by secondary aberrations, mainly a loss of a sex chromosome [1].

t(8;21) is considered as a good prognostic factor in AML M2, 96% of patients achieve complete remission (CR) and have a long overall survival time [1, 8, 9]. They relapse, however, frequently during the first year of CR. Moreover, an occurrence of a granulocytic sarcoma, seen in > 35% of patients, in probable association with $CD\ 56^+$, as the first manifestation of the disease or of the relapse worsens the prognosis of this type of AML [5, 8, 10].

t(8;21) is not restricted to AML M2, it is also found in some cases of other types of AML e.g. M4, M1 [1, 11]. Recently, t(8;21) was described in MDS RAEB-t, similar to M2/t(8;21) with respect to cytomorphology, immunophenotype and clinical features, and different from it only with respect to percentage of blasts (< 20%) [9, 12, 13], and the authors proposed to name this form of

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MDS t(8;21) positive smoldering leukemia [9]. There are only single observations of the presence of t(8;21) in chronic myeloproliferative disease [14, 15] and Ph-positive CML [16, 17]. The presence of t(8;21) in blast phase of Ph-negative CML was described only once in a patient in whom molecular investigation showed BCR positivity [11].This spectrum of diseases and laboratory features suggests an occurrence of t(8;21) at multipotential stem cell level [7, 11]. M2/t(8;21) shares with CML several features, such as leukemic cells maturation, low levels of NAP in mature granulocytes, frequent loss of a sex chromosome; the differential diagnosis between both diseases may be sometimes difficult [10].

We describe here 3 patients with M2/t(8;21) and an excessive differentiation of their leukemic cells as compared to other M2/t(8;21) patients. In all three patients an initial haematological diagnosis was blastic crisis or acceleration of CML (CML-BC), but subsequent laboratory studies (e. g., cytogenetics) and clinical course of the disease enabled us to change this diagnosis.

Material and Methods

Patients

Among 14 patients with final diagnosis of AML-M2 cytogenetically examined in the years 1993–1996 at diagnosis/admission to the Department of Haematology there were 4 patients with t(8;21). Among other patients 8 had normal karyotype, one had trisomy 3 and one add(11)(p15). One of the patients with t(8;21), a 19 years old female, had a typical form of AML, with > 30 % of blasts, hiatus leukaemicus and features of M2, myeloid differentiation, leukemic cells with Auer rods, low NAP activity etc. Three others had cytomorphology, hematogram and myelogram typical of blastic or accelerated phase of CML and were first diagnosed as having this disease. Clinical and haematological data of the patients are summarized in Tables 1 and 2.

Case Reports

Patient 1. Forty three-year-old woman not exposed previously to any environmental or professional mutagens fell ill in Nov. 1993, when weakness, headache, sweating as well as bruising appeared. On admission to our Dept. of Haematology muco-cutaneous haemorrhagic diathesis was present, but without organomegaly. Because of elevated leukocyte count (20G/l) with 26 % of blasts and Ph chromosome absence in microscopic cytogenetic analysis Ph(–) CML in blastic phase was diagnosed and therapy with hydroxyurea (HU) was started. The myeloblasts contained Auer rods, and a part of neutrophils showed Pelger-Huet anomaly. In the subsequent detailed cytogenetic analysis a t(8;21) was shown. Because no remission was obtained the cytostatic therapy was

Table 1. Clinical data of AML-M2 patients

No.	Date of symptoms/ diagnosis sex/age	Initial diagnosis	Therapy	Course of the disease	Symptoms at diagnosis	FAG at diagnosis	Remarks
1.	11.1993 F/43	BC-CML	HU Epi-DX, Ara-C, 6TG	Only short-lasting amelioration Ex.02.94	Weakness Haemorrhagical diathesis	28	Not fitting criteria of AML
2.	10.1995 F/36	Acc-CML	Epi-DX Ara-C	PR Relapse Alive (16 months)	-„-	2	-„-
3.	01.1995 M/23	BC-CML	HU DNA, Ara-C	CR (5 months) Relapse Ex.04.96	Weakness, oral mucositis, nausea, vomiting	27	-„-

changed to DAT protocol, but it did not result in remission. The patient died in Feb.1994 after a 4-month course of the disease.

Patient 2. Thirty six-year-old woman, a chemist, was referred to the Dept. in Nov.1995 because of suspicion of leukaemia. The disease presented with weakness, dyspnoe, bone aches, tachycardia. On admission a high degree of anaemia was found, with highly elevated leukocyte count and thrombocytopenia. The differential WBC count with 8% of blasts and 15% of monocytoid cells was consistent with accelerated phase of CML (Table 2). Immunophenotyping of the cells showed HLA DR(+) and CD15(+). Cytogenetic examination revealed t(8;21). Ph chromosome was absent. The patient was treated with epirubicin (Epi-DX) and Ara-C and a partial remission (PR) was obtained in April 1996, sustained with LD Ara-C applied subcutaneously. During the PR Hb level was within the normal limits, the platelet count increased to 516G/l,but the granulocytic lineage did not recover and showed an elevated percentage of BM myeloblasts (22%) with stable leukopenia in the peripheral blood. Cytogenetic analysis did not show at that time the presence of t(8;21). Because of the resistance of leukaemia to the applied cytostatics, chemotherapy was changed to etoposide and amsacrine (VP-16 + AMSA), which appeared to be uneffective. In October 96 the PR finished and the percentage of blasts (M2 type) increased to 93% and the leukocyte count to 56G/l. The karyotype was t(8;21)-positive. Intermediate-dose Ara-C with Epi-DX was administered with only minimal effect. The patient is still alive, karyotype of her leukemic cells shows the presence of t(8;21).

Patient 3. Twenty seven-year-old man, an electrotechnicist, was admitted to our Dept.in March 1995 because of recently (in Feb.1995) diagnosed CML presented with an elevated WBC count, prominent anaemia and thrombocytopenia and low NAP activity. Chromosome Ph was absent in microscopic cytogenetic analysis. The patient was treated with HU with a good, but only short-lasting response. In May 1995 the leukocyte

Table 2. Haematological data at diagnosis of patients suffering from M2/t(8;21) with excessive differentiation

Pts no.	Total blood cells counts at diagnosis					Myelogram												
	Differential %																	
	Hb g%	WBC G/l	Plts G/l	Blast	Prom	Mye	Meta	Band	Neutr	Lymph	Mon	Erytrbl	Mbl	Prom	Myel	Met	Band	Neutr	Total % of granulocytic line
1.	7.8	20.0	20.0	26	2	3	2	2	36	25	2	14	15	1	35	4	1	21	77
2.	5.5	81.5	21	8	9	14	6	6	22	8	15	3	22	9	17	2	10	13	76
3.	6.1	118	26	17	22	6	–	–	12	43	–	10.5	8	18	24	10	13	15	92

count rose to 168G/l with 92% of M2 blasts.The karyogram from March 1995 showed t(8;21). Cytostatic therapy, consisting of daunorubicin (DNR), Ara-C and VP-16, was started resulting in PR in July 1995 and CR with a cytogenetic remission a month later. In spite of consolidating cytostatic courses the disease relapsed 3 months later. During the relapse there was evidence of CNS leukemia, treated intrathecally, as well as of hepatic infiltrations. The patient died in April 1996 because of bilateral pneumonia, his survival time was 16 months.

Methods

Cytogenetic examinations were performed on bone marrow cells from 24 h unstimulated cultures at 37 °C under 5% CO-2. A culture medium was RPMI 1640, supplemented with 15% FCS and antibiotics. Cells were harvested with colcemid, treated with hypotonic KCl (0.075 M) and fixed with 75% methanol / 25% acetic acid. Five-day-old airdried slides were treated with trypsin solution and stained with Giemsa stain. At least 20 GTG-banded metaphases were analysed according to ISCN'91 and ISCN'95 [18, 19].

Results

Cytogenetic analyses at diagnosis did not reveal the presence of chromosome Ph. A BC/acceleration of Ph-negative CML was thus suspected, suggested by haematological, cytomorphological and cytochemistry data. In patients 1 and 3 the first microscopic cytogenetic analyses did not reveal the presence of t(8;21) because of a poor quality of metaphases. The abnormality was found only on subsequent analysis made 1–2 months later (Fig. 1). In patient 2 the first analysis already showed the presence of t(8;21). There was neither cytogenetic nor clinical remission in patient 1, and the aberration was present in in each examination until the patient's death. In patient 2, still alive, partial clinico-haematological remission was accompanied by a cytogenetic remission. Subsequent cytogenetic analyses showed normal karyotypes of bone marrow cells. The analyses done at relapse of the disease disclosed again t(8;21). In the patient no. 3 CR was achieved along with a disappearance of t(8;21). The translocation reappeared at relapse. Surprisingly, the last cytogenetic examination, done during the relapse of the disease, showed a disappearance of the aberration.

No additional chromosome aberrations were found in the patients karyotypes. All cytogenetic data are shown in Table 3.

Discussion

For many years t(8;21) was regarded as connected with AML M2, although it was also found in other AML types, especially M1 and M4 (1,11). Recent data showed the presence of t(8;21) in MDS and chronic myeloproliferative disorders including CML. Multilineage involvement of the cells bearing t(8;21) and heterogenous immunophenotypic character of the cells are in favour of the occurrence of this aberration at the multipotent stem cell level [3, 7, 14]. Probably a next genetic or regulatory "hits" (e.g., CSF-s action) are necessary to push a cell to a particular cell compartment, preferentially the myeloid one [11]. Recent data directly proved that t(8;21)-positive cells can undergo granulocytic as well as monocytic differentiation [3].

Our patients primarily diagnosed as having BC/acceleration of CML showed an excessive differentiation of myeloid cells along with low NAP activity. The percentage of myeloblasts was insufficient to diagnose an overt leukemia as well as a blastic crisis in patient no. 2, so an acceleration was suspected. The following clinical course – a progression of the disease to an overt leukemia, as well as permanent lack of Ph chromosome and the presence or absence of t(8;21) according with clinical state – enabled to change the diagnosis, which was supported by the fact that t(8;21) is extremely rare in BC of Ph-positive CML [16,17] and practically absent in Ph-negative CML [11]. An adverse suggestion was given by the lack of additional aberrations, what is rare in AML M2 and frequent in CML

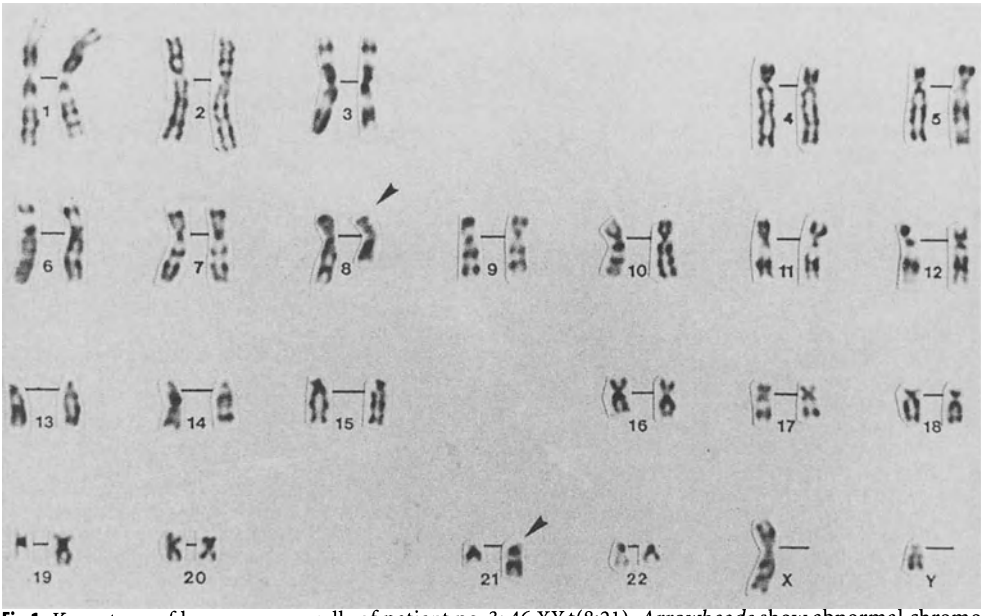

Fig.1. Karyotype of bone marrow cells of patient no. 3: 46,XY,t(8;21). *Arrowheads* show abnormal chromosomes

Table 3. Sequential cytogenetic data in patients with AML-M2 with excessive myeloid differentiation

No. of patient		Clinical course		Karyotype of bone marrow cells
1.		Diagnosis	- 11.1993	Ph(-) - microscope analysis
			- 01.1994	46,XX,t(8:21)[26]
			- 02.1994	46,XX,t(8;21)[22]
2.	PR	Diagnosis	- 11.1995	46,XX,t(8;21)[26]
			- 04. 1996	46,XX[29]
			- 07.1996	46,XX[21]
		Relapse	- 11.1996	46,XX,t(8;21)[33]
			- 01.1997	46,XX,t(8;21)[20]
3.		Diagnosis	- 02.1995	Ph(-) - microscope analysis
	PR		- 03.1995	46,XY,t(8;21)[16]/46,XY[6]
	CR		- 05.1995	46,XY,t(8;21)[32]
			- 09.1995	46,XY[39]
		Relapse	- 11.1995	46,XY,t(8;21)[16]/46,XY[4]
			- 12.1995	46,XY[20]

PR - partial remission, *CR* - complete remission

[1]. Some other clinical data of our patients - young age, easily obtained remission, but with rapid relapse - were consistent with those concerning M2/t(8;21), described by other authors [8, 9, 10].

Although AML M2 shares some haematological, cytomorphological, cytochemical and cytogenetic features with CML-BC [10], a differential diagnosis of both diseases is possible on the basis of cytogenetic, molecular and haematological changes during the course of the disease. Presented results showed that having such an approach it was possible to diagnose properly our patients.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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The Prognostic Impact of Trisomy 8 in Acute Myeloid Leukemia: Do Accompanying Aberrations Influence Prognosis?

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Abstract. Trisomy 8 is the most common numerical chromosome aberration in acute myeloid leukemia (AML). It occurs either as the sole anomaly or together with other clonal chromosome aberrations. Since interphase cytogenetics with centrometric probes for chromosome 8 have become more popular as a diagnostic tool in AML, we addressed the question whether the information of the existence of trisomy 8 alone is sufficient for the prediction of prognosis. Since 1986 847 patients with AML were successfully karyotyped within the AMLCG-trials. The overall incidence of trisomy 8 was 8.5%. For 51 patients complete follow-up data were available. Patients were divided into three different categories: group 1: trisomy 8 as the sole cytogenetic anomaly (n = 20), group 2: trisomy 8 in addition to favorable chromosome aberrations (t(8;21)(q22;q22), t(15;17)(q22;q21), inv(16)(p13q22)) (n = 10) and group 3: trisomy 8 accompanied by other anomalies, in most cases of complex type (n = 21). Event-free survival (EFS) at 3 years differed significantly between patients with trimosy 8 as the sole cytogenetic anomaly (EFS 37.5%), patients with trisomy 8 in combination with t(8;21)(q22;q22), t(15;17)(q22;q21) or inv(16)(p13q22) (EFS 55%) and patients with trisomy 8 and other accompanying anomalies, mostly complex chromosome aberrations (EFS 9%). Therefore, patients with trisomy 8 as the sole cytogenetic anomaly have an intermediate prognosis. Patients with favorable chromosome aberrations and trisomy 8 maintain a good clinical outcome. Trisomy 8 in combination with complex anomalies leads to the worst prognosis. The detection of trisomy 8 using interphase cytogenetics is not sufficient to determine prognosis. A complete karyotype analysis is necessary to define the biological entities in AML.

Introduction

Chromosome aberrations have been shown to constitute markers of diagnostic and prognostic value in acute myeloid leukemia (AML). For several cytogenetic anomalies a correlation with patients outcome was observed in larger series. Under currently used treatment protocols patients with t(8;21)(q22;q22), inv(16)(p13q22) or t(15;17)(q22;q21) show high complete remission rates and achieve long-term disease-free survival [1, 2]. In contrast, patients

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with -5/5q-, -7/7q- or complex karyotype anomalies respond badly to therapy resulting in short survival times [3, 4].

Although trisomy 8 is the most common numerical chromosome aberration in acute myeloid leukemia (AML) only few data are available concerning its prognostic implication. Trisomy 8 occurs as a solitary change in 5–6% of all cytogenetically abnormal acute myeloid leukemias. If those with +8 and additional aberrations are counted, this frequency doubles [5]. Most studies show an intermediate [6–10] or bad prognosis [11–15] of patients with +8. Due to the small number of cases reported in each study, patients were not further subdivided according to the chromosome aberrations that occurred in addition to trisomy 8. Since interphase cytogenetics with centromeric probes for chromosome 8 became more popular as a diagnostic tool in AML, we adressed the question whether the only information of the existence of trisomy 8 in AML using FISH is sufficient to define prognosis.

Material and Methods

Since 1986, 847 patients were sucessfully karyotyped within the multicenter trials of the German cooperative AML study group. The overall incidence of trisomy 8 was 8.5%. Twenty-one patients were excluded due to various reasons (secondary AML, contraindications against therapy protocol, protocol violations). complete follow-up data were available for 51 patients with de novo AML.

All patients were treated according to the protocols of the German cooperative AML study group AMLCG using 6-thioguanine, cytosine arabinoside, daunorubicin and mitroxantrone [16, 17]. Chromosome analyses were performed according to standard protocols using G- or R-banding. The chromosomes were classified according to the ISCN [18].

Results

Clinical, Morphological and Cytogenetic Data

Fifty-one patients with complete follow-up data and trisomy 8 were divided into three different categories: group 1: trisomy 8 as the sole cytogenetic anomaly, group 2: trisomy 8 in addition to favorable chromosome aberrations and group 3: trisomy 8 accompanied by other anomalies.

Group 1. In 20 patients the gain of one chromosome 8 was the only chromosome aberration observed. Fourteen patients were male, 6 female. The median age was 57 years (range 24 to 76 years). According to FAB-criteria bone marrow specimen of five patients each were classified as AML M1, M2 and M5, in three patients AML M4 and in one patient AML M6 was diagnosed. Figure 1 shows a karyotype of a patient with trisomy 8 as the sole anomaly.

Group 2. In ten patients trisomy 8 was observed in addition to a favorable chromosome aberration, in two patients each in addition to t(8;21)(q22;q22) and (15;17)(q22;q21) and in six patients together with inv(16)(p13q22). The sex ratio was balanced. The median age was 47 years (range 24 to 53 years). AML of both patients with t(8;21) was classified as AML M2. Both cases with t(15;17) showed AML M3 and in all six patients with inv(16) AML M4eo was diagnosed. In Fig. 2 a karyotype of a patient with t(15;17) and +8 is presented.

Group 3. In 21 patients trisomy 8 was accompanied by other anomalies. In 18 cases trisomy 8 occured within complex karyotype (three or more numerical and/or structural chromosome anomalies). Seven patients were male, 14 female. The median age was 57 years (range 26 to 75 years). All FAB-subtypes were observed in this group with the exception of AML M0 and AML M3. Figure 3 shows a complex aberrant karyotype including trisomy 8.

Patients with +8 and favorable chromosome aberrations were significantly younger than patients with only +8, or +8 and other abnormalities (median age 47 vs. 57 vs. 57 years) ($p < 0.05$). While the sex ratio was ba-

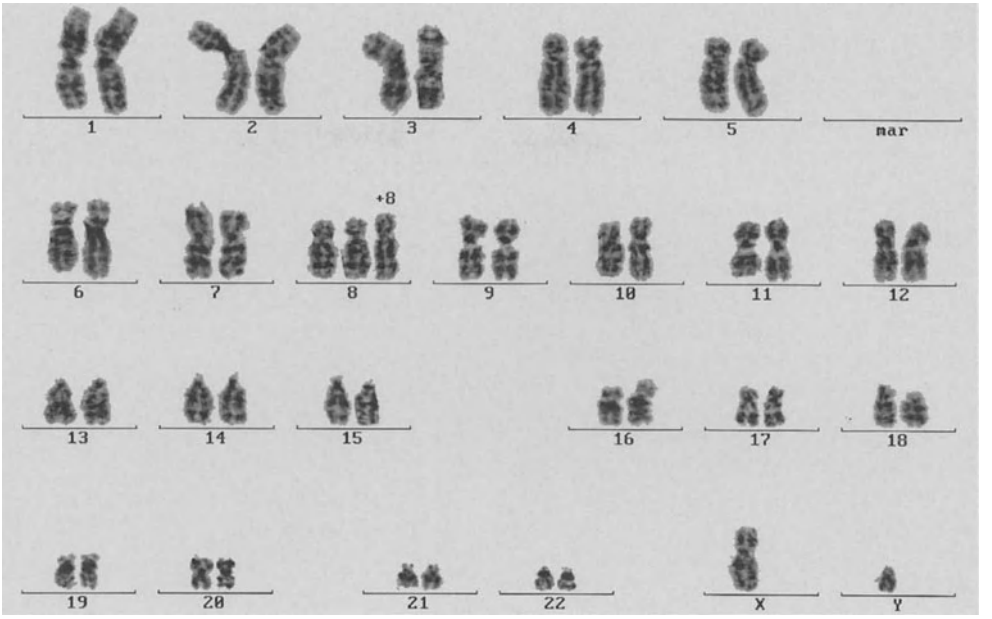

Fig. 1. G-banded karyotype of a patient with AML M2: 47,XY,+8 (example for group 1)

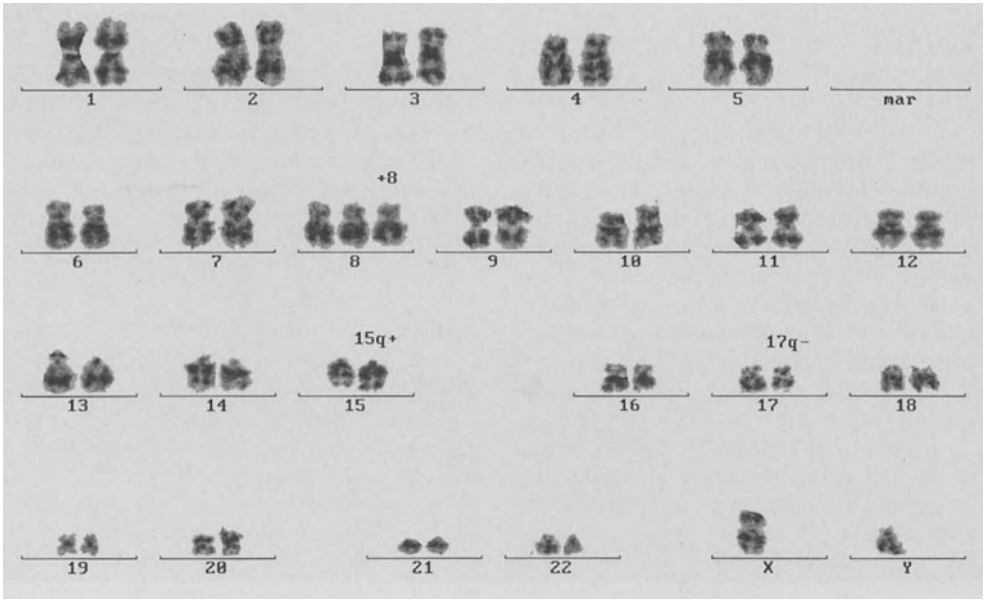

Fig. 2. G-banded karyotype of a patient with AML M3: 47,XY,+8,t(15;17)(q22;q21) (example for group 2)

lanced in the group of patients with trisomy 8 in addition to favorable anomalies male gender predominated in the group with only +8 (male : female = 2.3:1) and female gender in the group with +8 and other anomalies (male : female = 1:2). No significant differences between the groups were observed concerning leukocyte count, platelet count and hemoglobin concentration at diagnosis.

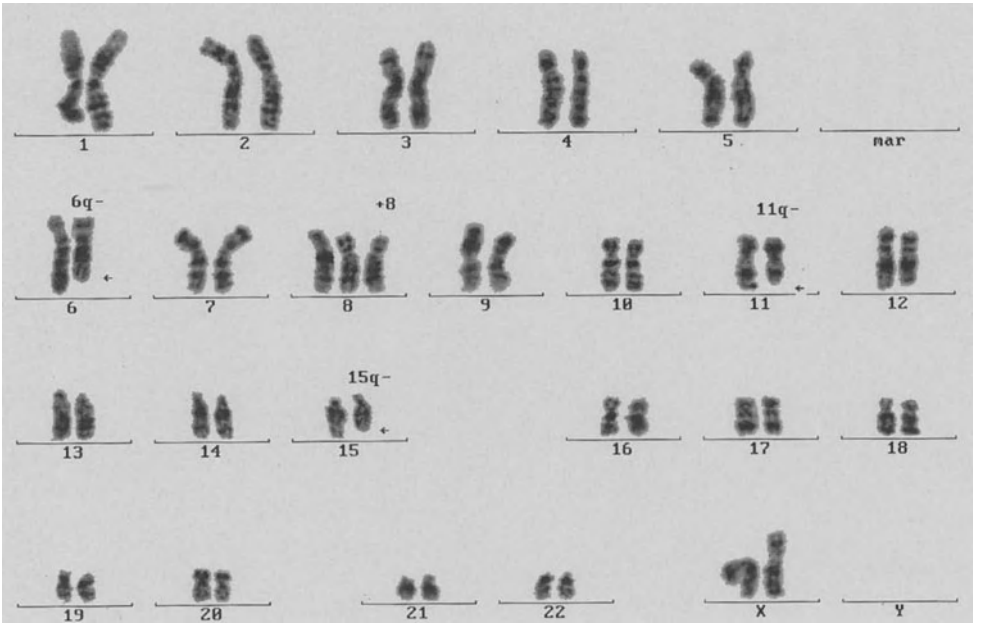

Fig. 3. G-banded karyotype of a patient with AML M4: 47,XX,del(6)(q21q25), +8, del(11)(q21q25), del(15)(q24) (example for group 3 with complex anomalies)

Clinical Outcome

Patients with +8 and favorable anomalies (group 2) had the highest complete remission rate (90%) and the lowest relapse rate (11%) compared to patients with only +8 (group 1: CR 70%, relapses 50%) and patients with +8 and other abnormalities (group 3: CR 67%, relapses 78.6%). Median event-free survival differed significantly between group 1 and group 3 (5 vs. 2 months, $p = 0.005$) and between group 2 and group 3 (not reached vs. 2 months, $p = 0.05$).

Figures 4 and 5 show the event-free and the overall survival curves of 51 patients with trisomy 8 divided in three prognostic subgroups.

Therefore, patients with trisomy 8 as the sole cytogenetic anomaly have an intermediate prognosis. Patients with favorable chromosome aberrations and trisomy 8 maintain a good clinical outcome, while trisomy 8 in combination with complex anomalies determines the worst prognosis.

Discussion

Data on clinical outcome of patients with AML and trisomy 8 are reported from several large cytogenetic studies. Percentages of patients with +8 who achieved CR varied between 29% and 91% (Mrozek et al. 1997). These differences may be related to the very

Table 1. Clinical outcome

Group	n	CR	EFS (median)	EFS (at 3 years)	OS (median)	OS (at 3 years)
Only +8	20	70%	5 months	37.5%	20.5 months	49.0%
+8 and favorable	10	90%	Not reached	55.0%	Not reached	56.0%
+8 and other/complex	21	67%	2 months	9.0%	8 months	15.0%

EFS: event-free survival, OS: overall survival.

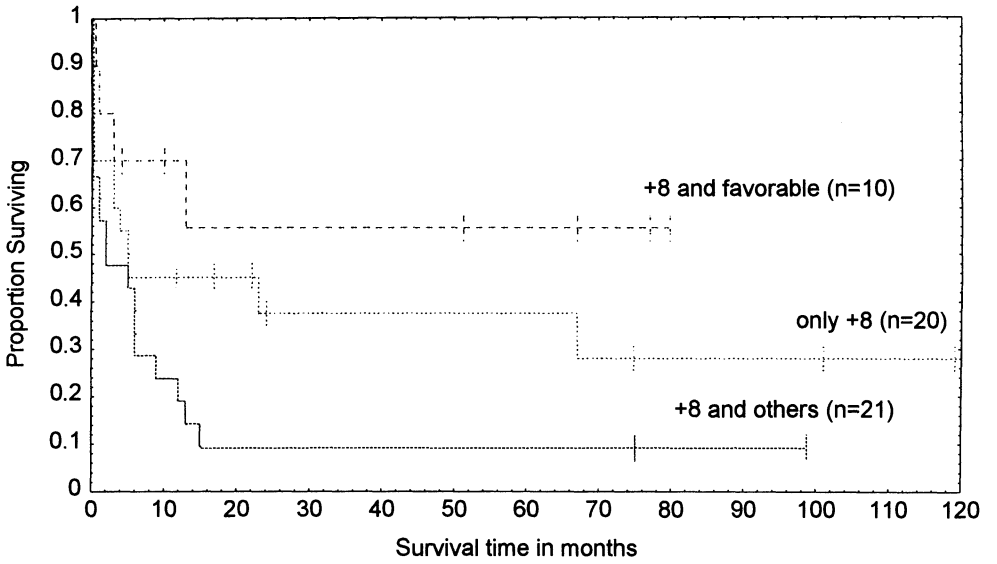

Fig. 4. Event-free survival of 51 patients with trisomy 8

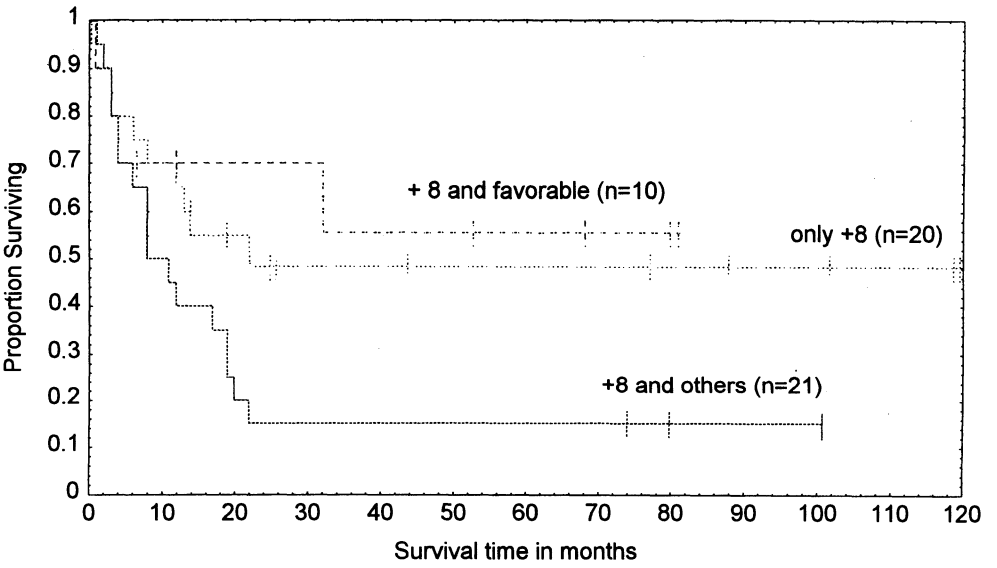

Fig. 5. Overall survival of 51 patients with trisomy 8

small number of patients in some reports and to the cytogenetic heterogeneity of the cases. In most studies not only cases with isolated trisomy 8 were included but also patients who had other anomalies in addition [6, 9, 11, 14, 19, 20, 21]. Only four studies report on survival data with +8 as the sole anomaly [7, 8, 10, 15]. Complete remission rates vary from 51 to 91% and long term survival in first complete remission from 5 to 37.5%. These variances maybe either due to differences in patient characteristics or in treatment protocols. Using a therapy protocol with double induction combined with consolidation and bone marrow transplantation or maintenance therapy as in our study yields a high remission rate of 70% resulting in an overall survival of 49% at 3 years.

Furthermore, our study shows that the chromosome anomalies accompanying tri-

somy 8 have a crucial influence on clinical outcome. Therefore, the detection of trisomy 8 alone using interphase cytogenetics (FISH) is not sufficient to determine prognosis. A complete karyotype analysis also detecting the accompanying anomalies is necessary to define the biological entities in AML.

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Experimental Approaches and Novel Therapies
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G-CSF Increases the Number of Trisomy 8 Metaphases in Vitro in Patients with Acute Myeloid Leukemia or Myelodysplastic Syndrome

C. Schoch, E. Cremer, D. Haase, B. Wörmann, and W. Hiddemann

Abstract. G-CSF (granulocyte colony stimulating factor) is either used to shorten the duration of neutropenia after cytotoxic therapy or as an agent to induce proliferation in malignant blasts to increase their susceptibility to chemotherapy ("priming" concept). We addressed the question, whether in vitro G-CSF stimulation of bone marrow cells of patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML) induces proliferation of normal cells and/or the malignant clone. Therefore, we incubated bone marrow samples of ten patients with trisomy 8 for 24, 48 or 72 h in parallel in medium with or without addition of G-CSF (Neupogen, Fa. Amgen). G-CSF increased the total number of mitosis significantly 1.3- to 13-fold. Furthermore analyses by fluorescence in situ hybridization with a chromosome 8 specific centromer probe showed an increase of the percentage of aberrant metaphases. The absolute number of aberrant metaphases increased significantly in patients with AML after G-CSF-stimulation ($p = 0.02$). In the interphase compartment no shift was observed between the normal cell population and the aberrant cell clone. Our in vitro data show that G-CSF in patients with trisomy 8 preferentially stimulates the aberrant cell clone to proliferate and therefore a G-CSF application before cytotoxic treatment may have the potential to recruite malignant blasts into a susceptible state in this subgroup of patients.

Introduction

Several clinical trials showed that G-CSF decreased the duration of neutropenia in patients treated for acute myeloid leukemia (AML) [1, 2, 3]. No significant increase in the incidence of drug-resistent leukemia was observed in trials using G-CSF after completion of chemotherapy. Other trials administered G-CSF or GM-CSF [1, 2] either before or simultaneously with chemotherapy to enhance chemosensitivity and decrease drug resistance ("priming" concept). There are data from 10 clinical studies using growth factors before or simultaneously with chemotherapy and results are contradictory [1, 2]. This may be due to treatment protocols and heterogenous patient groups leading to the speculation that some subgroups of patients may benefit and some do worse.

Heil et al. [4] supported this hypothesis, they observed an age-dependent effect of GM-CSF on treatment outcome in their trial. While outcome of patients younger than 50 years appeared to be improved by GM-CSF, the survival rate of patients aged over 50 years seemed to be impaired under GM-CSF. Büchner et al. [5] concluded from their data that age and karyotype may determine the therapeutic or adverse effects of GM-CSF multiple course priming in patients with AML. In vitro data from Jahns-Streubel et al. [6] also strengthen the hypothesis that only certain subgroups of patients may benefit from pretherapeutic stimulation with GM-CSF.

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Therefore, we decided to investigate G-CSF stimulation of bone marrow cells in vitro in a defined subgroup of patients with myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML).

Material and Methods

Bone marrow samples of ten patients with trisomy 8 and five patients with normal karyotype were incubated for 24, 48 or 72 h in medium with or without addition of G-CSF (Neupogen, Amgen).

Fluorescence in situ hybridization (FISH) was performed on bone marrow cells prepared as for conventional cytogenetics. An alpha satellite chromosome 8 specific DNA probe detecting the highly repeated alphoid sequences on the centromeric region of chromosome 8 was used (CEP 8 labeled with spectrum orange, VYSIS, Stuttgart, Germany). The hybridization was performed according to the protocol of the manufacturer.

Specimens from 5 patients with AML and normal karyotype (controls) and from 10 patients with trisomy 8 were analysed. In eight patients trisomy 8 was the sole anomaly (2 MDS, 2 AML de novo, 4 secondary AML), in two cases trisomy 8 was accompanied by other clonal aberrations. Signals were counted in 500 interphase nuclei in controls and in 1 000 interphase nuclei in patients with +8. All detectable metaphases were analysed as well.

Results

Controls

In the five control patients two signals were detected in 94.0–97.0% (mean 95.6%), one signal in 1.6–4.1% (mean 2.5%) and three signals in 0.2–2.5% (mean 1.1%). No differences were observed in specimens prepared from cultures with or without G-CSF stimulation.

Patients with Trisomy 8

Clinical and cytogenetic data of ten patients with trisomy 8 are shown in Table 1.

Table 1. Clinical and cytogenetic data

Patient no.	Diagnosis	Karyotype
1	MDS RAEB	47,XX,+8[15]/46,XX [2]
2	MDS RAEB	47,XY,+8[25]
3	AML after MDS	47,XX,+8[19]/46,XX[5]
4	AML after MDS	47,XY,+8[5]/46,XY[20]
5	AML after MDS	47,XY,+8[20]/46,XY[3]
6	AML after chemotherapy	47,XY,+8[21]/46,XY[4]
7	AML	47,XY,+8[8]/46,XY[17]
8	AML M5a	47,XY,+8[13]/46,XY[11]
9	AML M4eo	46,XX,inv(16)(p13q22)[19] 47,idem,+8[3]
10	MDS RA	46,XY,t(20;21)(q10;q11)[1]/ 47;idem,+8[9]/47, idem,t(7;8)(p22;q22),+8[3]

In the interphase compartment the ratio of normal and aberrant cells did not differ between cultures with or without G-CSF. Specimens obtained from cultures without G-CSF showed two signals in 28.3 to 90.9% (mean 62.3%) and three signals in 6.6 to 71.2% (mean 36.3%). Cells after G-CSF stimulation showed two signals in 25.5 to 95.3% (mean 63.5%) and three signals in 3.4 to 73.4% (mean 35.3%).

G-CSF increased the total number of detectable metaphases significantly 1.3- to 13-fold. In unstimulated cultures 3 to 20 metaphases (mean 11) were detectable compared to 4 to 70 (mean 35.9) in the stimulated cultures ($p = 0.026$).

The mean percentage of aberrant metaphases increased in G-CSF stimulated cultures. The mean propotion of aberrant metaphases was 42.5% in unstimulated cultures and 55% in G-CSF stimulated cultures. The absolute number of aberrant metaphases also increased significantly after G-CSF stimulation ($p = 0.02$). The mean number of aberrant metaphases generated from unstimulated cultures was 6.25 and 12 in G-CSF stimulated cultures, respectively.

Discussion

G-CSF is administered to patients with AML in order to overcome two limitations of chemotherapy, myelotoxicity as well as chemoresistance. The latter concept is based

Table 2. Results of FISH-analysis

Patient no.	Cultivation	Interphase nuclei			Metaphases		
		Total	2 signals (%)	3 signals (%)	Total	2 signals (%)	3 signals (%)
1	Without G-CSF	1000	68.7	29.0	3	0	100
	With G-CSF	1000	75.0	23.0	4	25	75
2	Without G-CSF	1000	42.1	56.5	5	40	60
	With G-CSF	1000	33.0	65.9	70	0	100
3	Without G-CSF	1000	58.3	40.2	15	67	33
	With G-CSF	1000	56.7	41.4	23	48	52
4	Without G-CSF	1000	90.3	6.6	16	100	0
	With G-CSF	1000	95.3	3.4	39	95	5
5	Without G-CSF	1000	38.7	59.7	20	5	95
	With G-CSF	1000	25.5	73.4	28	3	97
6	Without G-CSF	1000	28.3	71.2	0	No data	No data
	With G-CSF	1000	34.7	64.2	0	No data	No data
7	Without G-CSF	1000	90.8	8.7	10	90	10
	With G-CSF	1000	90.8	8.9	35	80	20
8	Without G-CSF	1000	41.6	57.1	0	No data	No data
	With G-CSF	1000	64.5	31.6	0	No data	No data
9	Without G-CSF	1000	90.9	7.1	14	79	21
	With G-CSF	1000	86.6	12.0	75	71	29
10	Without G-CSF	1000	72.9	26.4	5	80	20
	With G-CSF	1000	70.5	29.2	13	38	62

Table 2 gives detailed data on the results of FISH-analysis.

on the asumption that G-CSF recruites chemoresistant resting leukemic cells into sensitive phases of the cell cycle. In vitro studies showed that growth factors are able to promote growth of AML cells. In vivo effects of G-CSF on AML cells were examined by Baer et al. [7]. In 27 of 28 analysed patients an increase in at least one of the following parameters after 72-hour intravenous infusion of G-CSF was observed: blood blasts, bone marrow blasts, leukemia cells in S-phase or interphase cells with leukemia-specific markers shown by FISH. The authors conclude that the percentage of leukemic cells in S-phase increases and that leukemic cell populations undergo expansion during short-term administration of G-CSF in almost all AML patients.

Although experimental data seem to suggest that therapy protocols using "priming strategies" may be more effective than conventional treatment protocols, data obtained from clinical trials are controversial [1, 2]. This may be due to the fact that the beneficial effects of priming regimens in certain subgroups of patients may be balanced or outweighed by deleterious effects in certain other subgroups. The aim of our study was to establish an in vitro system that allows to analyse, whether G-CSF stimulates preferentially the proliferation of leukemic or of normal hematopoietic cells in a cytogenetically defined subgroup of patients. Our data show that G-CSF in patients with trisomy 8 stimulates the proliferation of the aberrant cell clone to a greater extent than the normal hematopoietic cell population detected by metaphase analysis. Therefore, a G-CSF application before cytotoxic treatment may have the potential to recruite malignant blasts into a susceptible state in this subgroup of patients. Correlation with clinical data are needed to determine whether an in vitro test could help to differentiate between patients who might benefit from G-CSF use in a priming setting and those who might not.

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The Karyotype in MDS – Cytogenetic Profile, Clinical Correlations and Prognostic Implications in 362 Patients with MDS

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Abstract. Successful cytogenetic examinations were performed in 362 pts. with MDS, diagnosed according to the FAB-criteria. Only cases with unequivocal morphology and cytogenetics were included in this study. Evaluation, classification and documentation of karyotype abnormalities were performed according to the ISCN. A total of 535 examinations was performed. 161 pts. were of female-, 201 of male gender. Patients' age ranged between 0.2 and 86 years with a median of 59 years. Three age groups were examined: <16 years, 16 - 60 years and >60 years. The distribution of MDS-subtypes was: RA (65 pts./18%), RARS (28/7.7), RAEB (68/18.8), CMML (41/11.3), RAEB-T (48/13.3), AML after MDS (61/16.9), ALL after MDS (3/0.8), biphenotypic AL after MDS (1/0.3), no classification (47/13). 21 pts. had a secondary MDS. The median number of metaphases analyzed was 27 (range 3 to 194). 191 (52.8%) of pts. showed clonal chromosome abnormalities. Frequency of clonal anomalies in MDS-subgroups was 43.1% in RA, 28.6% in RARS, 52.9% in RAEB, 41.5% in CMML, 68.8% in RAEB-T, and 63.1% in akute leukemias following MDS (MDS-AL). Non-clonal karyotype instability occurred in 47 (13.0%) pts.. The mean number of abnormalities per case was 1.5 in RA, 2.5 in RARS, 3.8 in RAEB, 1.6 in CMML, 3.3 in RAEB-T, 5.1 in MDS-AL. The most frequent karyotype abnormalities were: 5q- in 58 pts. (30.5% of abnormal cases), -7 in 43 (22.6%) pts., +8 in 39 (20.5%), -20/20q- in 19 (10.0%) pts., trisomy of 1q in 18 (9.5%) pts., 12p-abnormalities in 18 (9.5%) pts., -18/18q- in 17 (8.9%) pts., 3q21/3q26-abnormalities in 15 (7.9%) pts.. Complex chromosome anomalies occurred in 58 (16.0%) pts.. An evolution of karyotype was observed in 48 (13.3%) pts.. Seven pts. displayed independent clones. Median survival was > 60 months in pts. with a normal karyotype, 18.6 months in pts. with one or two abnormalities and 3.4 months in pts. with complex karyotype abnormalities; differences were highly significant (normal vs. complex $p<0.0001$; non-complex abnormal vs. complex abnormal $p < 0.0001$. Further cytogenetic findings and their clinical correlations are discussed.

Introduction

Myelodysplastic syndromes (MDS) comprise a heterogenous group of clonal disorders affecting hematopoietic stem cells [1, 2, 3]. The clinical picture of bi- or pancytopenia is caused by qualitative and quantitative bone marrow dysfunction. MDS are classified on a morphological basis [4] and are considered preleukemic disorders since in one third of cases a transformation to acute

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leukemia occurs [5]. Clonal chromosome abnormalities have been observed in up to 80% of patients with MDS [6] and prove these syndromes to be real neoplastic. In clinical studies it was demonstrated that some karyotypic changes are strongly correlated with a high risk of leukemic transformation [6, 7] irrespective of the FAB-subtype. In multivariate analyses distinct karyotype abnormalities like chromosome 5- and 7-anomalies turned out to be the most important factors for remission rate and survival [8, 9, 10]. However, since karyotype abnormalities in MDS show a marked heterogeneity little is known about the prognostic impact of distinct single abnormalities which can be regarded as delineating genetic entities.

It was our aim to examine the prognostic impact of general karyotypic changes and distinct cytogenetic subgroups in MDS. Their relation to the FAB-classification, age and clinical course was analysed in a population of 362 patients with MDS.

Material and Methods

Patients

In this study, cytogenetic findings of 362 patients with MDS were evaluated. Only patients with unequivocal diagnosis of MDS according to the FAB -criteria were taken into account. Patients' blood and bone-marrow specimens were sent for cytogenetic examination to the Medical University of Lübeck and the University of Göttingen between 1982 and 1996. Patients' age ranged between 0.2 and 86 years with a medium of 59 years. Based upon the age, they were categorized into three groups : < 16 years , 16 - 60 years , > 60 years. 201 patients were of male gender, 161 of female gender. The distribution of MDS-subtypes was : RA (65 pts. / 18.0%), RARS (28 / 7.7), RAEB (68 / 18.8), CMML (41 / 11.3), RAEB-T (48 / 13.3), AML after MDS (61 / 16.9), ALL after MDS (3 / 0.8), biphenotypic AL after MDS (1 / 0.3), no classification (47 / 13). Patients with MDS-AML, MDS-ALL and MDS-biphenotypic AL were summarized in one group : MDS-AL (65 pts. / 18%). 21 patients had a secondary MDS after radiation, chemotherapy and / or other mutagen exposure.

Cytogenetics

Chromosome analyses were performed on short-term (0.5, 24, 48 and 72 h) cultures of bone marrow and unstimulated peripheral blood. The number of completely analyzed metaphases ranged between 3 and 194 with a median of 27 metaphases. Evaluation and documentation of cytogenetic findings was performed according to the ISCN [11]. A total of 535 examinations were completed. The first succsessful one of each patient was taken into account. A successful examination was defined as a minimum of 10 evaluated normal metaphases or the proof of a clonal abnormality in abnormal metaphases. In patients with a normal karyotype, deletions, translocations, gain of whole chromosomes and double minutes in single cells as well as chromosome- and chromatid breaks were classified as non-clonal karyotype instability. Abnormalities per case were counted as published [12].

Statistics

For the calculation of survival-propability the Kaplan-Meier method [13] was used. Significance testing was performed using the Mantel-Haenszel-Chi-square test.

Results

General Karyotypic Changes

The overall rate of clonal chromosomal - aberrations was 52.8% (191 of 362 pts.). There were significant differences within the age- and FAB-subgroups. The highest rate of aberrations was found in secondary MDS, where 17 of 21 pts. had clonal aberrations (81%). Within the FAB-subgroups, the highest incidence was found in RAEB-T (68.8% aberration rate), followed by MDS-AL (63.1%), RAEB (52.9%), RA (43.1%), CMML (41.5%) and finally, RARS (28.6%). In the age groups, older patients had a high-

Table 1. General cytogenetic findings in FAB and age groups

	n	Aberr.[a]	NN[a]	NA[a]	AA[a]	Com.[a]	Evol.[a]	N-cl.I.[a]	A / case
RA	65	43.1	56.9	33.8	6.2	4.6	12.3	27.7	1.5
RARS	28	28.6	71.4	17.9	10.7	3.6	3.6	17.9	2.5
RAEB	68	52.9	47.1	36.8	16.2	20.6	16.2	14.7	3.8
CMML	41	41.5	58.5	22.0	19.5	4.9	7.3	7.3	1.6
RAEB-T	48	68.8	31.3	41.7	27.1	27.1	14.6	4.2	3.3
MDS-AL	65	63.1	36.9	30.8	32.3	33.8	21.5	6.2	5.1
No class.	47	59.6	40.4	36.2	17.0	6.4	8.5	10.6	2.2
s-MDS	21	81.0	19.0	28.6	52.4	52.4	23.8	9.5	4.8
< 16 years	35	42.9	57.1	25.7	14.3	0.0	2.9	5.7	1.3
16-60 years	152	48.7	51.3	30.3	17.8	12.5	11.2	11.8	2.7
> 60 years	167	56.9	43.1	36.5	20.4	22.8	17.4	16.2	3.9
All pts.	362	52.8	47.2	32.6	18.8	16.0	13.3	13.0	3.2

[a] Percentage of all patients; Aberr: Aberrations; NN: Only normal cells; NA: Mosaic of normal and abnormal cells; AA: Only abnormal cells; Com: Complex aberrations; Evol: Evolution of karyotype; N-cl.I: Non-clonal-karyotype instability; A/case: Aberrations per case (mean value).

er frequency of aberrations as compared to younger ones. Aberration rates were 42.9% (<16 years), 48.7% (16-60 years) and 56.9% (>60 years). Complex abnormalities were absent in pts. <16 years, amounted to 12.5% in pts. between 16 and 60 and showed the highest frequency with 22.8% in pts. >60 years. Comparable correlations were observed for the phenomenon of karyotype evolution. In the MDS-subtypes the percentage of karyotypic evolution ranged between 3.6 % in RARS and 21.5 % in MDS-AL. More details are shown in Table 1. A mosaic of normal and abnormal cells (NA) was observed in 118 pts., 68 pts. had only abnormal cells (AA) in their cytogenetic examination. Complex aberrations occured in 58 pts. (16.0%). Due to the overrepresentation of NN-karyotypes, non-clonal karyotype instability was high in early stages of MDS. Aberrations/case ranged between 1.5 in RA and 5.1 in MDS-AL (mean value).

Distinct Cytogenetic Abnormalities in FAB and Age Groups

Most frequent distinct chromosome abnormalities in our study were 5q- (30.5% of abnormal cases), -7 (22.6%), +8 (20.5%), -20/20q- (10%), +1q (9.5%), abnormal 12p (9.5%), -18/18q- (8.9%), 3q21/q26-abnormalities (7.9%).

The deletion of parts of the long arm of chromosome 5 (5q-) with different breakpoints but the commonly deleted band 5q31 is the most frequent abnormality in patients with MDS, affecting all FAB-subgroups with the highest relative frequencies in RA (39.3%), RAEB (33.3%), RAEB-T (39.4%), secondary MDS (47.1%), patients aged 16-60 (29.7%), and patients aged > 60 (33.7%). This abnormality is considered to have a good prognosis as single abnormality. In RA, it was mostly present as a sole abnormality (7 of 11 pts. / 63.6 % of all RA-pts. with 5q- ; data not shown) while in advanced stages of MDS 5q- occurs mainly within complex abnormal karyotypes (9 of 13 pts. / 69.2% of all RAEB-T- patients with 5q-).

As published, [14, 15] monosomy of chromosome 7 is the most frequent clonal abnormality in pediatric patients suffering from MDS. In this study, –7 occurred in more than one half of patients aged younger than 16 years (53.3%). For further details, see Table 2, in which the percentage of the most frequent clonal abnormalities within age- and subgroups is given :

Correlation of Cytogenetic Findings with Survival

Followup data of 166 pts. were correlated with cytogenetic findings. In 70 pts. with a normal karyotype the mean survival was not reached as yet, 5 years survival was 52%. Pts. with a mosaic of normal and abnormal cells (NA) had a mean survival of 16.4

Table 2. Distinct cytogenetic abnormalities in subgroups

	n	5q-(%[a])	-7(%[a])	+8(%[a])	-20/20q-(%[a])	+1q(%[a])
RA	28	39.3	7.1	14.3	21.4	10.7
RARS	8	25.0	25.0	25.0	12.5	0.0
RAEB	36	33.3	27.8	22.2	13.9	19.4
CMML	17	5.9	35.3	17.6	0.0	17.6
RAEB-T	33	39.4	21.2	12.1	12.1	6.1
MDS-AL	41	29.3	19.5	34.1	7.3	7.3
s-MDS	17	47.0	29.4	0.0	11.8	0.0
< 16	15	6.7	53.3	20.0	0.0	6.7
16-60	74	29.7	13.5	23.0	10.8	9.4
> 60	95	33.7	25.3	20.0	11.6	10.5
All pts.	191	30.5	22.6	20.5	10.0	9.5

[a] Percentage of patients with clonal chromosomal abnormalities.

months as compared to 8.2 months in pts. with only abnormal cells (AA). The differences between NA and AA were significant and highly significant between AA and NN. The presence of an evolution of the karyotype (KE) lead to a mean survival of 3.9 months as compared to 19.3 months in pts. without KE, the difference was highly significant. Pts. with complex abnormalities (CA) had a mean survival time of 3.4 months while mean survival in pts. with abnormalities other than complex was 18.6 months. The differences between normal karyotype and complex abnormalities and complex abnormalities and other than complex abnormalities were highly significant. More details are given in Table 3, see also figs. 1 to 7.

Discussion

The overall rate of clonal chromosomal abnormalities (52.8%) in this study is in good confirmity with those from other extended studies [10, 16]. Within the FAB groups, the highest rates were found in advanced stages of MDS (RAEB : 52.9% RAEB-T : 68.8%), while in RARS only 28.6% of pts. had an abnormal karyotype. This is in line with the assumption that RARS seems to own the highest (cyto)genetic stability [17]. We observed a correlation between the frequency of complex abnormalities and clonal evolution on the one hand and advanced MDS-stage and pts. age >60 on the other hand with the exception of CMML. Remarkably, none of the pediatric patients had complex cytogenetic changes. The assumption that cytogenetic abnormalities accumulate in old patients is furthermore supported by the finding that not only complex abnormalities and clonal evolution but also non-clonal-instability is increased pts. >60 years as compared to pediatric patients (16.2 vs. 5.7%). The prognostic impact of a mosaic of normal and abnormal cells (NA-karyotype) is discussed controversally [18, 19]. We found a statistically significant survival benefit for pts. with NA- vs. AA-karyotype ($p = 0.0387$). An evolution of karyotype highly significant worsened the prognosis ($p = 0.00031$) as compared to pts. without this cytogenetic finding.

We furthermore examined the prognostic impact of the most frequent abnormalities as sole changes, as changes accompanied by other single abnormalities and as part of complex abnormalities. As yet we could not find statistically significant differences for 5q- (data not shown) and monosomy 7 under the above mentioned conditions, which might possibly be due to the low number of pts. examined. However, differences were significant for trisomy 8 alone vs. trisomy 8 + complex abnormalities ($p = 0.0402$). The prognostic impact of a 20q- abnormality is discussed controversally [20, 21]. We found a significant survival benefit for pts. with -20/20q- alone vs. -20/20q- and complex abnormalities possibly explaining the discrepancies found in the literature of the outcome of pts. with this abnormality. Comparing the extent of cytogenetic changes measured as aberrations per case a significant disadvantage was observed whenever more than 4 abnormalities were present.

Table 3. Prognostic impact of cytogenetic subgroups

Category (n = number of pts.)	Mean survival (months)	Comparison	Significance
NA (n=59)	16.4	NA vs. NN	No
	8.2	NA vs. AA	Yes (p=0.0387)
AA (n=34)		AA vs. NN	High (p=0.0002)
Karyotype evolution (k.e.) (n=26)	3.9	k.e. vs no k.e.	High (p=0.00031)
No k.e (n=139)	19.3		
Complex anomalies (n=31)	3.4	NN vs. complex	High (p<0.0001)
		complex vs. not	High (p<0.0001)
Not complex (n=65)	18.6		
+8 alone (n=8)	12	+8 vs. +8-complex	Yes (p=0.0402)
+8 and complex (n=5)	3.7		
-20/20q- alone (n=5)	36.7	-20/20q- vs. -20/20q- comp	Yes (p=0.0491)
-20/20q- and complex (n=9)	2.2		

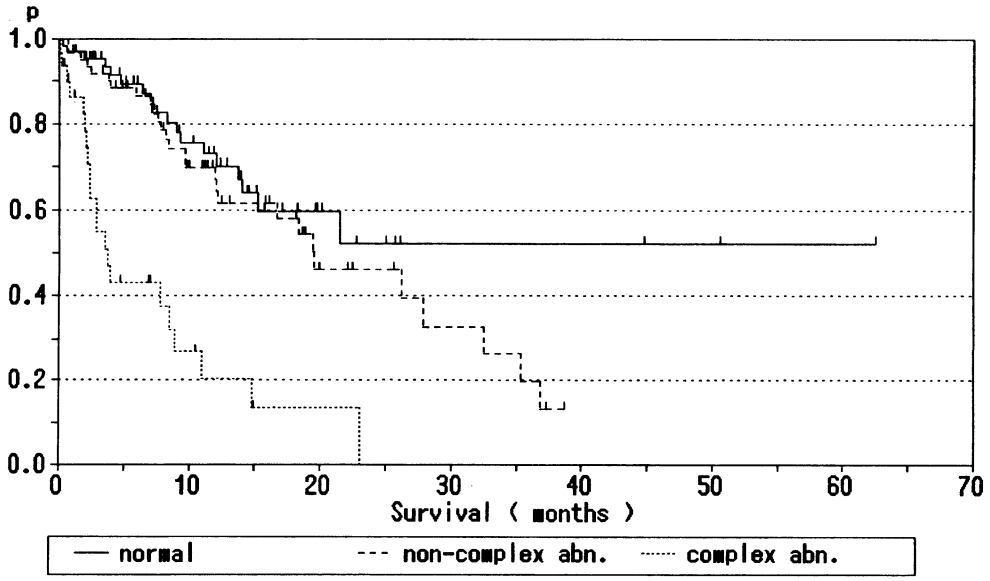

Fig. 1. Survival curves of patients with normal, non-complex and complex abnormal karyotype Median survival was >60 months in patients with normal karyotype (n=70), 18.6 months in pts. with one or two aberrations (n=65) and 3.4 months in pts. with complex (n=31) abnormalities. Differences were highly significant for normal vs. complex ($p< 0.0001$) and for non-complex vs. complex abnormalities ($p< 0.0001$)

We conclude that cytogenetics can provide decisive prognostic informations which might lead to therapeutic decisions. The aim to calculate the clinical course in the individual patient however can only be achieved if the impact of real single abnormalities (genetic entities) is delineated within large scale studies which are rare as yet. From our results it can be derived that an individual profile of prognostic factors in each patient has to consider general and distinct cytogentic findings.

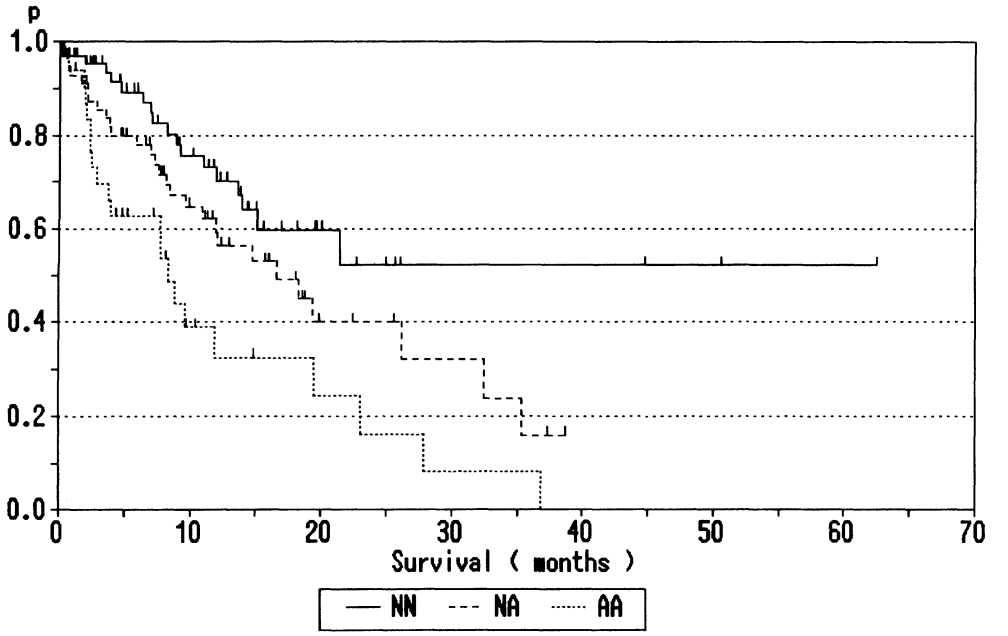

Fig. 2. Survival curves of patients with NN, NA and AA karyotype. Median survival was >60 months in NN karyotype (n=70), 16.4 months in pts. with NA-karyotype (n=59) and 8.2 months in pts. with AA-karyotype (n=34). Differences between NN and AA were highly significant (p=0.0002) and significant between NA and AA (p=0.0387)

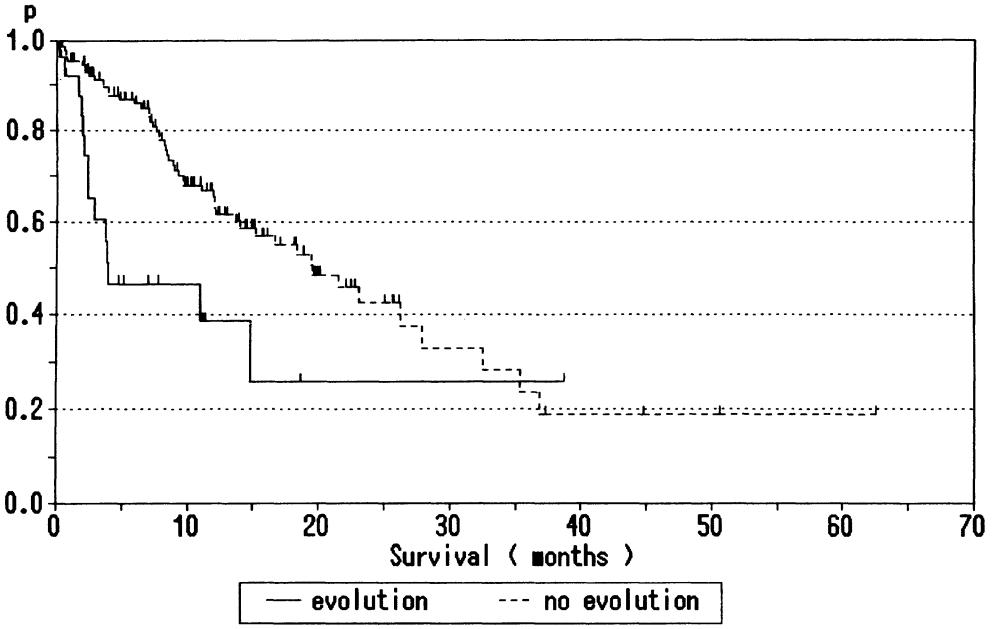

Fig. 3. Survival curves of patients with and without karyotype evolution. Median survival was 19.3 months in pts. without (n=139) and 3.9 months in pts. with evolution (n=26). The difference is highly significant (p=0.00031)

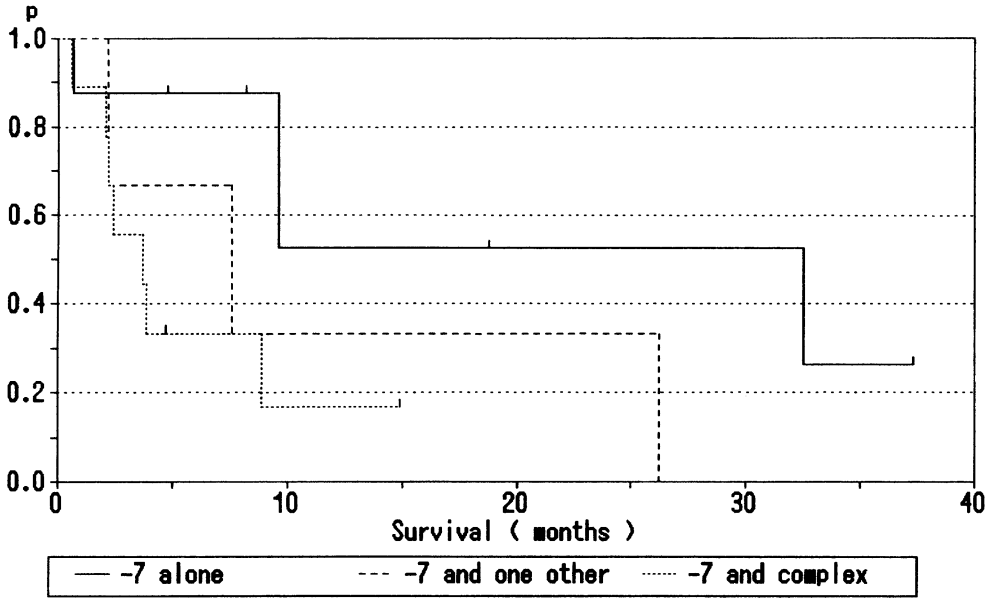

Fig. 4. Survival curves of pts. with monosomy 7 as the sole abnormality, with one other abnormality and within a complex abnormal karyotype. Median survival was 32.4 months in pts. with -7 alone (n=8), 7.5 months in pts. with one other abnormality (n=3) and 3.7 months in pts. with -7 within a complex abnormal karyotype (n=9). Differences were not significant

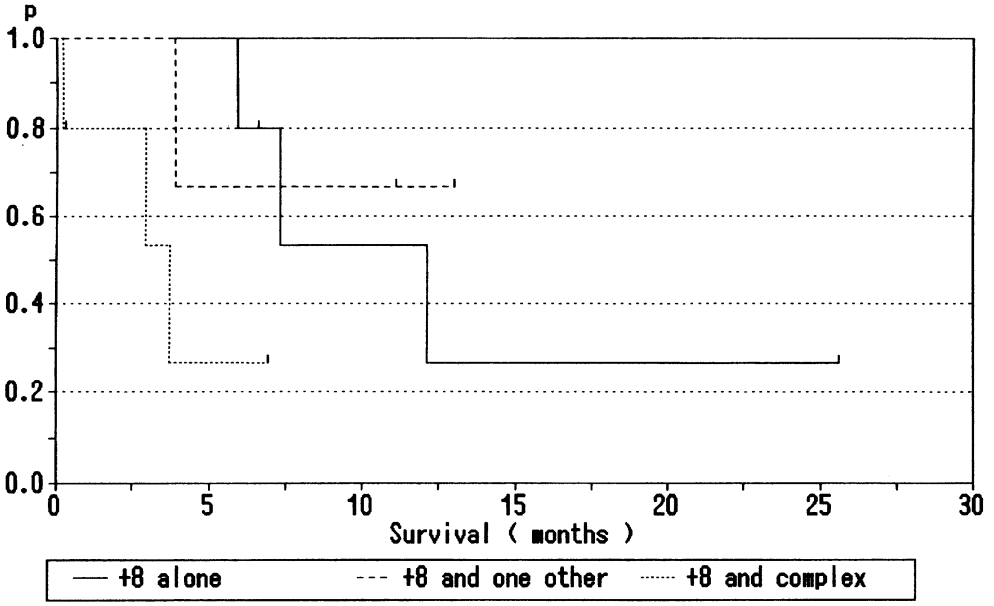

Fig. 5. Survival curves of pts. with trisomy of chromosome 8 as the sole abnormality, with one other abnormality and within a complex abnormal karyotype. Median survival was 12 months in pts. with +8 alone (n=8), >13 months with one other abnormality (n=5) and 3.7 months in pts. with +8 within a complex abnormal karyotype (n=5). Differences were significant between +8 alone vs. +8 and complex abnormalities (p=0.0402)

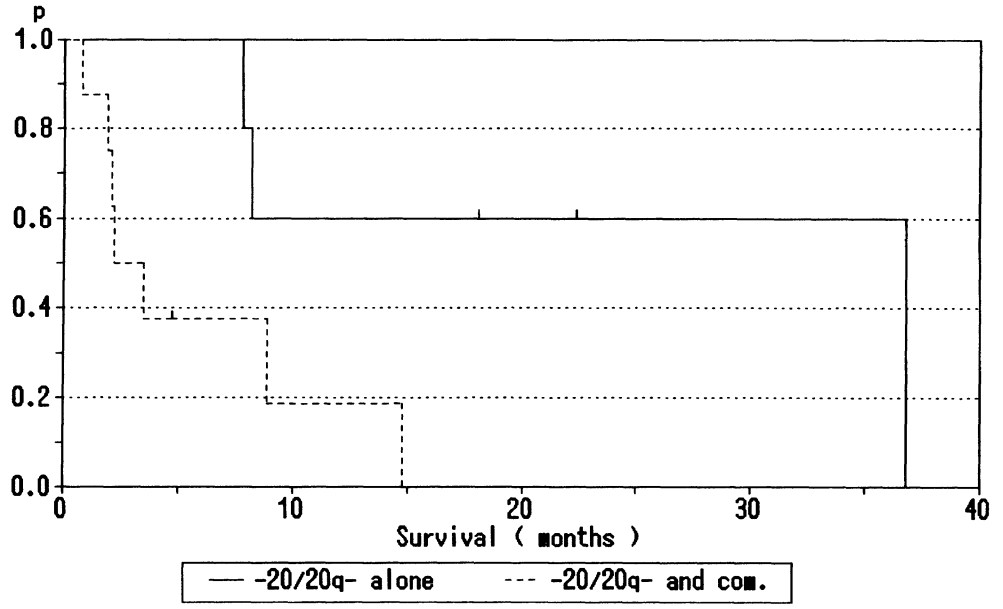

Fig. 6. Survival curves of patients with -20/20q- as the sole abnormality and within a complex abnormal karyotype. Median survival was 36.7 months in pts. with -20/20q- alone (n=5) and 2.2 months in pts. with -20/20q- within a complex abnormal karyotype (n=9). The difference was significant (p=0.0491)

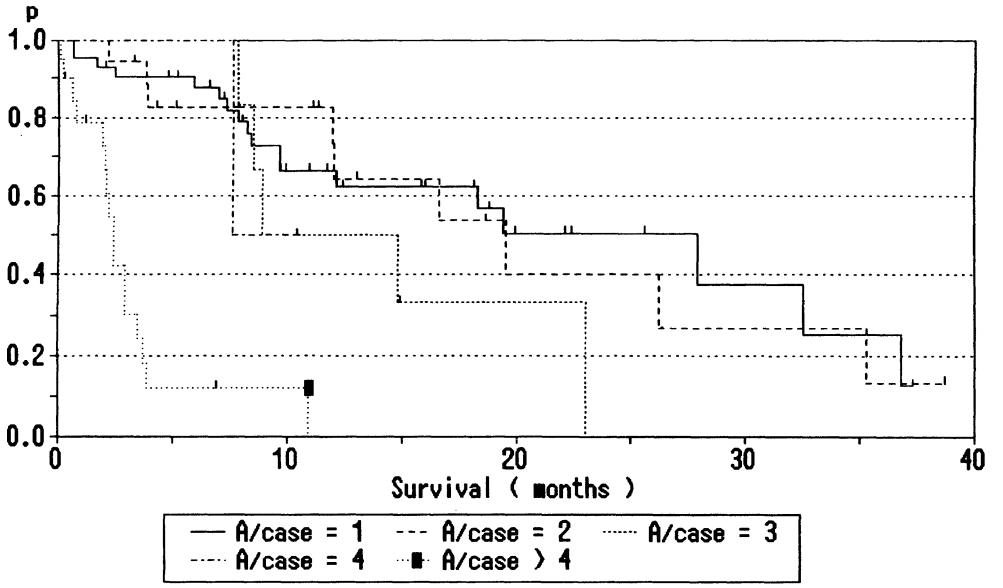

Fig. 7. Survival curves of pts. with 1, 2, 3, 4 and more than 4 aberrations per case. Median survival was 27.8 months in 1 A/case (n=44), 19.2 months in 2 A/case (n=20), 8.8. months in 3 A/case (n=6), 7.5 months in 4 A/case (n=6) and 2.4 months in patients with more than 4 aberrations per case (n=20). Differences between 1, 2, 3, 4 aberrations/case and more than 4 aberrations per case were all significant (1 vs. > 4: $p< 0.0001$, 2 vs. > 4: $p< 0.0001$, 3 vs. > 4: p=0.0069, 4 vs. > 4: p=0.0200)

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Cytokines and Biomodulation

Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Vitamin D_3 and Retinoid-Mediated Inhibition of Leukemic Cells

M. J. CAMPBELL and H. P. KOEFFLER

Abstract. An attractive therapeutic goal in the treatment of acute leukemias is to induce relatively immature and rapidly growing malignant cells to undergo differentiation towards a more mature quiescent phenotype. Various agents have been considered for the potential to initiate this process. Two related compounds that have potent differentiation-inducing activities in many cell types are the seco-steroid hormones vitamin D_3 and retinoids. These compounds bind to specific nuclear receptors that act as ligand inducible transcription factors and thereby initiate a cascade of genomic responses that include inhibition of proliferation and promotion of differentiation. All-trans retinoic acid has proved remarkably successful in the clinical treatment of acute promyelocytic leukemia. The use of vitamin D_3 analogs and conformationally restricted retinoids have resulted in many advances in the understanding of the cellular and molecular biology of these potent compounds. Many of these developments have great clinical potential for the treatment of myeloid proliferative disorders.

Introduction

Human acute myeloid leukemia (AML) cells have a growth advantage as a result of neoplastic transformation at the stem cell level. The resulting cells do not achieve terminal differentiation, but rather are interrupted in their normal differentiation and remain in an immature, proliferative stage, thus rapidly accumulating. This results in a variety of abnormalities in the peripheral blood which include increased susceptibility to anemia, infection and abnormal bleeding. Various strategies for the treatment of AML have been utilized including cytotoxic drugs to kill aberrantly proliferative clones, chemotherapy and bone marrow transplants [1]. However, the high incidence of disseminated intravascular coagulation, which is often made worse by chemotherapy, initiated a search for alternative therapies. In the 1970s, phenotypic reversion by induction of differentiation was proposed [2].

Promotion of differentiation of immature malignant clones to a mature, quiescent phenotype and possibly initiating programmed cell death (apoptosis) may eliminate the malignant clones. Studies with either fresh leukemic cells and cell lines derived from these cells have identified many initiators of differentiation. Differentiation of these cells can be stimulated along various lineages that include the granulocytic or monocytic pathways. These pathways have various morphological, antigenic, histochemical and functional markers that serve as signposts to monitor differentiation.

Various agents have been recognized for inducing differentiation to a mature pheno-

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type, including polar-planar drugs, hexamethylene bisacetamide, prostaglandin E_2, interferon [3,4,5,6]. Other physiologic compounds include vitamin D_3, the vitamin A derivatives known as retinoids, and tumor necrosis factor alpha.

Vitamin D and Retinoids Are Seco-Steroid Hormones

1α,25 dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] and the natural retinoid ligands (All trans retinoic acid (ATRA) and 9 cis retinoic acid (9cRA)) mediate their activity by binding with specific nuclear receptors; for instance, 1α,25$(OH)_2D_3$, interacts exclusively with the vitamin D_3 receptor (VDR); ATRA and 9cRA with the retinoic acid receptor (RAR), and 9cRA also binds with the retinoid X receptor (RXR). Furthermore, each of the RAR and RXR receptors has three isoforms; α,β and γ [7]. The exact function of the various retinoid receptor isoforms are being elucidated by murine receptor-knockout studies which have shown that certain combinations, for example a double knockout of RAR α and β, are lethal [8]. An examination of various cancer cell lines where abnormal patterns of expression are often observed has also been informative[9]. These nuclear receptors are part of the steroid hormone superfamily and act as ligand inducible transcription factors. They exert specific cellular effects by binding to hexameric response elements within the promoter/enhancer regions and thereby regulating transcription of target genes some of which are associated with inhibition of proliferation and induction of differentiation (reviewed in [7,9,10]).

The genes for the steroid hormone receptors share a high degree of homology and each displays a characteristic structure with five to six separate regions; A/B, C, D, E, and F. The function of the A/B region is unclear, it encodes the autonomous activity function (AF-1) which can activate transcription in the absence of the ligand binding domain. Region C encodes the DNA-binding domain containing two zinc fingers; D is a hinge region; and E encodes the ligand-binding and the heterodimerization surface [11]. In general, the DNA-binding domains tend to be the most conserved amongst the steroid hormone receptors and the ligand domain show less homology [12]. Very high homology between the RAR alpha and beta isoforms and the thyroid hormone receptors alpha and beta has been shown suggesting an evolutionary link by duplication from a common ancestor.

The receptors readily undergo dimerization principally with the RXR receptor which thus plays a pivotal role in this family as a co-factor in regulation of target gene transcription [13]. For example, in studies of keratinocytes [14] as well as other tissues [15], the RAR-RXR has been demonstrated to be the major functional retinoid receptor-dimer and VDR-RXR the major functional vitamin D_3 receptor-heterodimer [16]. Others have demonstrated the combination of 9cRA and 1α,25$(OH)_2D_3$ is able to synergistically up-regulate a murine osteoponitin promoter reporter gene construct that had been transfected into Drosophila SL-3 cells [17]. Co-operation between these two receptor pathways has been the basis for combinational therapy; and we have previously demonstrated synergistic inhibition of proliferation of AML cells by a potent vitamin D_3 analog in combination with 9cRA [18].

The RXR does not exclusively control seco-steroid hormone mediated gene expression as up-regulation of the human osteocalcin gene by 1α,25$(OH)_2D_3$ has been shown to occur in an RXR- independent manner, possibly through the action of VDR homodimers [19]. VDR homodimer activity has also been suggested in several in vitro [20] and in vivo [21] studies; and heterodimerization of VDR with other steroid hormone receptors, such as with RAR [22] or the thyroid receptor [23], has been observed experimentally, although the biological significance of these receptor-dimers is as yet unclear. Other mechanisms by which retinoids and vitamin D_3 might additively or synergistically control target genes include multiple response elements in the gene; for example, the gene encoding $p21^{waf1}$, a cyclin-dependent kinase inhibitor (CDKI), contains both a vitamin D3 response element (VDRE) and a retinoic acid

response element (RARE) within its promoter/enhancer region [24, 25].

A wide variety of transcriptional events are mediated through the steroid hormone receptors. Recent research has shown that nuclear proteins (CBP, p300, TFIID) act to link the steroid hormone receptor, bound to its cognate response element, to the basal transcription machinery [26, 27]. These very large nuclear proteins have many binding sites to which different steroid hormone receptors and other transcription factors can compete and co-operate for binding and thus regulate gene transcription. For example, factors such as cytokines [28] can effect activity of seco-steroids; and the steroid hormone receptors can act to repress AP-1 protein (c-Jun/c-Fos) mediated events [29]. Response elements of different receptors which are apparently widely spaced on a promoter can have the capacity to display "crosstalk" thus allowing for interaction between different classes of transcription factors [30].

This apparent flexibility of receptor dimerization coupled with their abilities to interact with other transcription factors results in greater spatial and temporal specificity of gene regulation and thus provide fine-tuning of biological responses to allow for the possibility of synergistic positive and negative effects.

Vitamin D_3 and Retinoids in Normal Hematopoieses

Vitamin D_3

The $1\alpha,25(OH)_2D_3$ plays a fundamental role in the regulation of calcium metabolism in humans and the major source of production of $1\alpha,25(OH)_2D_3$ is the kidney in responce to serum calcium levels [10]. Extrarenal synthesis of $1\alpha,25(OH)_2D_3$ can occur under both physiological and pathological conditions. For example, during pregnancy serum levels of $1\alpha,25(OH)_2D_3$ frequently rise due to ectopic production by the placenta and decidua. We have shown that normal human macrophages can be stimulated by gamma-interferon (γIFN), or other agents to produce $1,25(OH)_2D_3$ [31]. Furthermore, $1\alpha,25(OH)_2D_3$ inhibits the production of cytokines by normal human lymphocytes [32]. These findings together with the results obtained from expression studies of VDR in macrophages and T-lymphocytes would suggest a possible interaction between macrophages and T-lymphocytes in a paracrine fashion using $1\alpha,25(OH)_2D_3$ and γIFN as signals [10, 31]. Interestingly, pulmonary alveolar macrophages (PAM) from patients with sarcoidosis synthesize $1\alpha,25(OH)_2D_3$ in culture and probably are the source of elevated serum levels of $1\alpha,25(OH)_2D_3$ associated with hypercalcemia in these patients [33, 34].

Cells of the hematolymphopoietic tissue contain VDR, for example it is expressed constitutively in monocytes and certain subsets of thymocytes [35, 36]. Bone marrow-derived stromal cells express VDR and exposure to $1\alpha,25(OH)_2D_3$ results in their reduced proliferation. The $1\alpha,25(OH)_2D_3$ can induce normal human committed myeloid stem cells selectively to differentiated to macrophages in soft agar culture or in liquid culture [37, 38]. In further studies, these macrophages were functionally competent, and were able to release large amounts of tumor necrosis factor-α (TNF-α), IL-6 and showed reduced production of IL-1 [39]. Furthermore, the terminal differentiation of monocytes into mature macrophages can be obtained in vitro by culturing these cells in the presence of serum or in a serum-free medium with the addition of vitamin D3 compounds [39, 40]. Both T-helper and -suppressor lymphocytes express similar concentrations of VDR. In particular, T-lymphocytes express high levels of VDR mRNA, while resting B-lymphocytes express very low or non-detectable levels of VDR transcripts [35, 41], but stimulation of B-Lymphocytes by the lectin phytohemagglutinin-A (PHA) for 24 hours results in their upregulation. This suggests that in lymphocytes transcriptional control of VDR expression is the principal route of their upregulation [35, 42].

Indeed, $1,25(OH)_2D_3$ is able to regulate many lymphokines. For example, we [43] showed that expression of the lymphokine GM-CSF is regulated by $1\alpha,25(OH)_2D_3$ through cellular VDR by a process indepen-

dent of IL-2 production. In particular, we demonstrated that $1,25(OH)_2D_3$ is able to inhibit both GM-CSF mRNA and protein expression in PHA-activated normal human peripheral blood lymphocytes (PBL) by concentrations of $1\alpha,25(OH)_2D_3$ similar to those reached in vivo. A 50% reduction of GM-CSF activity occurred at 10^{-10} M $1\alpha,25(OH)_2D_3$ In addition, IL-2 did not affect the modulation of GM-CSF production by $1\alpha,25(OH)_2D_3$ in these PBLs co- cultured with $1\alpha,25(OH)_2D_3$ (10^{-7} - 10^{-10} M) and high concentrations of IL-2. Interestingly, $1\alpha,25(OH)_2D_3$ decreased the accumulation of GM-CSF mRNA; and this occurred at least in part by destabilizing and shortening the half-life of the GM-CSF mRNA [43].

Retinoids

Retinoids as group of substances are important for many biological processes that involve differentiation, for instance embryonal development where it contributes to establishing polarizing activity during limb formation [44], epidermal cell growth [45] and hematopoiesis. Deprivation experiments in vivo produces anemia which could be reversed with the administration of vitamin A and thus indicate that this compound was required for normal hematopoieses. More recently, various studies have shown that retinoids play an important role in normal cell growth and differentiation processes for several tissues [46,47,48,49].

Not all the different isoforms of the retinoid receptors are expressed in the hematopoietic system. For example, RARα is expressed constitutively; and during ATRA-induced differentiation, RARβ is expressed. In contrast, whereas RAR often is not expressed [50] . Retinoids (ATRA and 9cRA) both enhance clonal growth of normal human myeloid and erythroid precursors and inhibit their proliferation under different culture upon culture conditions [51,52].

Vitamin D_3 and Retinoids Receptor Expression in Leukemia

Vitamin D_3 Receptors

Examination of a large array of myeloid leukemia cell lines blocked at various stages of maturation showed that they all expressed VDR, albeit at different levels [35]. In addition, lymphocytes of patients with vitamin D resistant rickets type II have various alterations of the VDR [52,53]. Also, fewer receptors have also been detected in the peripheral blood mononuclear cells of patients with X-linked hypophosphatemic rickets [54]. Low levels of VDR expression were detected in low-grade, non-Hodgkin's lymphoma (NHL) tumor samples and in the follicular lymphoma B-cell lines, SU-DHL4 and SU-DHL5 [55].

Retinoic Acid and Retinoid X Receptors

One of the retinoic acid receptors appears to play a major role in the pathology of acute promyelocytic leukemia (APL). This disease is characterized by a reciprocal translocation between the long arms of chromosomes 15 and 17: t(15;17)(q22;q12-21) This translocation, which is diagnostic for APL, is present in 100% of cases [56]. Molecular examinations of the breakpoint revealed it to involve the RARα gene which maps to 17q21.1, distal to the APL breakpoint region, and in the first intron of RARα resulting in a fusion protein. The chimeric fusion protein encoded by the 15q+ derivative retained the DNA- and ligand-binding domains of RARα, whereas the transcription-activating function of the amino-end of RARα will be replaced with PML [56,57]. PML-RARα appears to disrupt transcription at physiological concentrations of ATRA and thus interferes with granulocytic differentiation [58]. The involvement of RARα in the fusion product may explain why pharmacological concentrations of ATRA has the ability to induce differentiation of APL cells into mature granulocytes in the treatment of acute promyelocytic leukemias and lead to a remission in nearly 100% of APL cases but these spectacular results appear to be limited to APL.

Furthermore, an ATRA-resistant HL60 subclone (HL60-R) was shown to be insensitive to retinoid stimulated differentiation because of a mutation in the RARα receptor which resulted in an aberrant ligand binding domain [59]. Introduction of the same mutated RARα gene into a retinoid-sensitive, multi-potent cell line alters its differentiation characteristics implying a significant role for RARα in hematopoieses [60].

The function of the PML protein is beginning to be elucidated. It appears to have some transcriptional activity; and as the chimera with RARα, it is present in large excess, and therefore may exert a dominant negative effect. The chimera disrupts the sub-nuclear organelle known as PML oncogenic domains (PODs). Treatment with ATRA restores wild-type POD structure and reduces levels of PML-RARα (RR 94 96) through a process that requires cyclic AMP (cAMP) [61].

In a minority of cases, translocation occurs at t(11;17)(q23;q21) resulting in a chimeric protein PLZF/RARα [62, 63, 64] or at t(11;17)(q22;21) [65] resulting in another chimeric protein. Each chimera contains proteins (PML, PLZF and NuMA) that although unrelated, contain domains implicated in transcriptional regulation. Of significance, in these three translocations the RAR receptor is targeted underscoring the importance of this receptor in the normal differentiation processes. In the latter two translocations the cells are resistant to differentiation induced by retinoic acid which has been attributed to the different function of the PLZF and NuMA proteins [66].

Inhibition of Proliferation of Leukemia by Vitamin D_3 and Retinoids

Both of these compounds can inhibit the growth in vitro of cancer cells from several different tissues, including glioma, breast, colon, prostate, as well as myeloid leukemia [67, 68, 69, 70, 71, 72]. Retinoids have also been demonstrated to control the growth of various cancers in patients , including those with acute promyelocytic leukemia, head and neck and skin cancer [73, 74, 75].

Vitamin D_3

The role of $1\alpha,25(OH)_2D_3$ in cell differentiation was first described by Abe [76] in the murine leukemia cell metabolite line M1 which was induced to differentiate into more mature cells by $1\alpha,25(OH)_2D_3$. $1\alpha,25(OH)_2D_3$ and related vitamin D_3 compounds have a similar, potent effect on inducing differentiation and inhibiting proliferation of several acute myeloid leukemia cell lines such as HL-60, U937, THP-1, HEL and NB4. In contrast, more immature myeloid leukemia cell lines such as HL-60 blasts, KG1, KG1a and K562 do not respond. HL-60 cells treated with $1\alpha,25(OH)_2D_3$ acquire the morphology and functional characteristics of macrophages. They become adherent to charged surfaces, develop pseudopodia, stain positively for non-specific esterase and reduce nitroblue tetrazolium (NBT) and acquire the ability to phagocytose yeast during incubation with $1\alpha,25(OH)_2D_3$ [73, 77]. In addition, the treated cells acquired the ability to degrade bone marrow matrix in vitro, raising the possibility that the cells may have acquired some osteoclast-like characteristics. The proliferation of HL-60 cells is also inhibited by $1\alpha,25(OH)_2D_3$; in fact, colony formation in soft agar is reduced by 50% (ED_{50}) in the presence of about 10^{-9}M $1\alpha,25(OH)_2D_3$ [78]. Cells of other leukemia cell lines are also inhibited with similar efficiency in their clonal growth, such as U937, HEL, THP-1 and M1 after their exposure to $1\alpha,25(OH)_2D_3$ [78]. Diverse effects of the different cell lines may reflect the higher number of receptors present on the more differentiated cell lines [77].

Also, leukemic cells from AML patients respond to vitamin D_3 compounds when cultured in vitro; however, they are often less sensitive than the cell lines. They are still able to undergo monocytic differentiation as assessed by NBT reduction, morphology, and phagocytic ability. Furthermore, their clonal growth is often inhibited [73, 78]. An antiproliferative effect of $1,25(OH)_2D_3$ was reported by Hickish [55] in the non-Hodgkin lymphoma (NHL) cell lines SU- DHL4 and SU-DHL5; however, this effect was observed only using high concentrations of the compound.

In spite of the promising data obtained from in vitro studies, results of clinical trials with $1\alpha,25(OH)_2D_3$ are limited in scope and thus far have exhibited only mediocre results. For example, the myelodysplastic syndrome (MDS) is associated with anemia, thrombocytopenia and leukopenia and an increased number of myeloid progenitor cells in the bone marrow. Some of these patients develop acute myeloid leukemia. We treated 18 MDS patients with increasing doses of $1\alpha,25(OH)_2D_3$, for up to 12 weeks; and although eight patients displayed limited improvements, the response was not durable and not detectable at the end of the study. Dose-limiting hypercalcemia was a problem [78]. In another shorter study with seven MDS patients; no beneficial effects were observed [79].

All trans and 9-cis Retinoic Acid

Many transformed cell lines, and fresh patient samples may undergo differentiation in the presence of retinoids. The dose of ATRA require to inhibit colony formation of HL-60 in soft agar by 50% (ED_{50}) was 10^{-7} M, with ATRA being considered the most potent modulator of hematopoiesis , binding with high affinity to RAR's and altering gene expression [80, 81, 82, 83]. 9cRA is the ligand for RXR receptors. We have compared both of these natural ligands in AML cell lines and fresh leukemic cells and found that generally 9cRA had a similar activity profile to ATRA being equally potent; but each was equally ineffective at inhibiting growth of chronic myelogenous samples [84]. The myeloblast-promyelocytic cell lines such as HL-60 and NB-4 differentiate along the granulocytic pathway when cultured with retinoids, whereas the monoblastic cell lines such as THP-1 and U-937 mature along the monocytic pathway. Differentiation is generally associated with a decreased proliferative capacity. However, some myeloblastic cell lines (KG1) and fresh leukemia samples are growth inhibited but do not differentiated when exposed to ATRA [85].

The dramatic effects observed in vitro on APL-derived cells lead to APL being the first example of a human cancer to be successfully treated with a differentiating agent (ATRA) [86]. This mode of therapy for APL has recently been extensively reviewed [87]. ATRA induced remissions in about 85% of the patients; but these only last 2-3 months and then retinoid-resistant cells emerged. At this time remission induction is accomplished with ATRA, and then while in remission chemotherapy is administered with very good results.

Although RARα is clearly implicated in the etiology of APL, as it is involved in two separate translocation, the exact mechanism by which ATRA can remediated the effects of this condition is unclear. The PML/RARα oncoprotein is directly down-regulated by ATRA in NB-4 and U937 cells. This down regulation is at the protein level and appears to be posttranscriptional, possibly as a result of ATRA transcriptionally increasing expression of a PML degrading protein. The consequence of this is to change the ratio of PML/RARα to wild-type RARα [88].

Combinations of Vitamin D_3 and Either All trans or 9-cis Retinoic Acid

The toxic hypercalcemic effects mediated by $1\alpha,25(OH)_2D_3$ have lead investigators to examine combinations of this compound with other chemotherapeutic agents that might synergistically promote leukemic cell differentiation yet decrease toxicity. $1\alpha,25(OH)_2D_3$ and retinoids can co- operate together or separately with other differentiating agents such interferons (IFNs). For example, $1\alpha,25(OH)_2D_3$ can potentiate IFN-γ action to induce the expression of CD11b and CD14. Also, the combination of $1\alpha,25(OH)_2D_3$ and either ATRA or 9cRA can potentiate the terminal differentiation process of HL-60 cells down the monocytic/macrophage pathway (~100% induced to express cells CD14+) [18, 89, 90, 91]. Cells cultured in the presence of the combination of $1\alpha,25(OH)_2D_3$ and ATRA develop atypically having a neutrophilic morphology; but in other properties the cells have monocytic features including CD14 expression, the ability to bind to bacterial lipopolysaccharide, and develop sodium-fluoride-inhibited non-specific esterase [91].

In the promyelocytic cell line NB4, carrying the translocation t(15;17) typical of APL, $1\alpha,25(OH)_2D_3$ as single agent was weakly able to induce monocytic differentiation [92]. Bathia [93] showed that the combination of $1\alpha,25(OH)_2D_3$ and TPA resulted in a synergistic response of NB4 cells exhibiting complete differentiation to fully functional macrophages with rapid arrest of cell growth in the first 24 h, with an increasing proportion of cells becoming adherent.

Mechanism of Action of Inhibition

Vitamin D_3

Despite the intense research that has been focused on $1\alpha,25(OH)_2D_3$ since it was first characterized in 1971 [94] the exact mode of action by which it inhibits cancer cells remains largely unknown. This seco-steroid hormone can mediate direct transcriptional and post-transcriptional regulatory events. In normal tissues not directly involved in calcium regulation for example, the well-studied system of keratinocytes exposure to $1\alpha,25(OH)_2D_3$ results in various changes that allow the cell to undergo differentiation, these include increases synthesis of TGF-β1 and β2 [95], decreases EGF receptor expression [96] and dephosphorylation of the retinoblastoma protein [97].

The HL-60 myeloblastic cell line cultured in the presence of $1\alpha,25(OH)_2D_3$ has a 50% decrease of VDR protein levels at about 24 h which returned to normal levels after 72 h; no change of VDR mRNA expression occurred [35]. These data indirectly suggested that a potential site of regulation of VDR expression occurs at the posttranscriptional level. Other non-tissue specific, non-classical effects of $1\alpha,25(OH)_2D_3$ have been associated with cell cycle arrest in G_0/G_1. Many factors can lead to a cell cycle arrest, but CDKIs $p21^{(waf\ 1)}$ and $p27^{(kip1)}$ have been shown to be pivotal in this process. Recently, investigators have shown that $p21^{(waf\ 1)}$ contains a VDRE within its promoter region [24]. Others as well as ourselves have demonstrated both transcriptional and translational upregulation of $p21^{(waf\ 1)}$ and $p27^{(kip1)}$ in the myeloid leukaemia cell lines HL-60 and U-937 after their exposure to $1\alpha,25(OH)_2D_3$ [24, 98, 99]. The marked increase of $p21^{(waf\ 1)}$ protein expression may be due in part to enhanced posttranscriptional stabilization of $p21^{(waf\ 1)}$ mRNA [97]. The up-regulation of $p21^{(waf\ 1)}$ mRNA occurred independently of de novo protein synthesis, further supporting the hypothesis that $p21^{(waf\ 1)}$ is an early response gene of $1,25(OH)_2D_3$/VDR. A transient over expression of p21(waf1) and p27(kip1) in U937 cells promoted the expression of cell surface molecules, CD14 and CD11b, which are specific for monocytes/macrophages. One series of experiments showed that the p21(waf1), p27(kip1), p15 p16, p18 CDKIs were all upregulated in a time-dependent manner after the addition of $1\alpha,25(OH)_2D_3$ [24]. This induction occurred within 4 h of the addition of $1,25(OH)_2D_3$ in the presence of cycloheximide (CHX) suggesting a direct transcriptional activation by VDR. In recent studies the protein expression of different G1-phase regulators has been examined in HL-60 cells exposed to different concentrations of $1,25(OH)_2D_3$. A strong up-regulation of p27(kip1) protein expression was evident after 72 h of exposure to the compound; and it was dependent on the concentration. This up-regulation was also associated with increased levels of D- and E-cyclins, coinciding with the G1 arrest. These results suggested a prominent role for p27(kip1) in mediating the antiproliferative activity of $1,25(OH)_2D_3$ in this cell line [98, 99]. Interestingly, upregulation of p27(kip1) was shown to result from an increase in both the translation rate and half-life of the protein; the mRNA level remained unchanged implying that post-transcriptional effects of $1,25(OH)_2D_3$ are at least as important as the transcriptional ones.

Cells that are arrested in their progression through the cell cycle often exhibit an induction of differentiation or apoptosis. Apoptosis may occur after the process of differentiation as a mechanism of controlling the number of mature cells by inducing cell death within this population. The role of this process in hematopoiesis and leukemia has been extensively reviewed [100]. $1,25(OH)_2D_3$ has a protective effect against apoptosis in HL-60 cells [101]. This effect

lends support to the observation that monocytic differentiation interferes with programs leading to apoptotic death. In other cell types , inhibition of apoptosis correlates with elevated levels of BCL-2; but this does not appear to be the case with myeloid cells. In fact, after culture with $1,25(OH)_2D_3$, a down-regulation of BCL-2 was observed both at the mRNA and protein levels [101]. Previously, studies showed that upregulation of $p21^{(waf\ 1)}$ may play a role in the prevention of differentiating myocytes from undergoing apoptosis [102]. Only a few reports exist of $1\alpha,25(OH)_2D_3$ triggering apoptosis, and these involve the hormonally-dependent breast cancer cell line MCF-7 [103].

Other effects of $1,25(OH)_2D_3$ include upregulation of Jun and Fos proto-oncogenes [104]. Jun-D DNA binding activity is increased during cell cycle arrest in the human chronic myelogenous leukemia cell RWLeu-4 cultured with $1,25(OH)_2D_3$ suggesting that Jun D binding activity may play a role in the regulation of cell proliferation by $1,25(OH)_2D_3$ [105]. Moreover, the binding activity of the proto-oncogene Jun D is enhanced by $1,25(OH)_2D_3$ in these cells during their cell cycle arrest, while it was not decreased in a $1,25(OH)_2D_3$-resistant variant cell line [105].

Other factors appear to be regulated by 1,25(OH)2D3. For example, the levels of expression of c-MYC, c-JUN, c-FMS, p53, N-RAS and protein-kinase C are modulated by $1,25(OH)_2D_3$, but this modulation may not be a direct effect may reflect the entire process of differentiation [106, 107, 108, 109, 110].

Activation of the proto-oncogene c-MYC by retroviral insertion or chromosomal rearrangement is a typical feature of human leukemias. The HL-60 leukemia cell line is characterized by high levels of expression of c-MYC due to gene amplification [111, 112]. Treatment of this cell line with $1,25(OH)_2D_3$ results in a down-regulation of expression of this oncogene related to stimulation of cell differentiation.

The cell lines HL-60 and U937 have been used to attempt to identify early response genes directly regulated by VDR. Bories [113] identified a cDNA coding for fructose 1-6 biphosphatase whose expression is upregulated by $1,25(OH)_2D_3$ in HL-60 cells and peripheral blood monocytes. Interestingly, but of unclear significance, $1,25(OH)_2D_3$ induced differentiation of AML cells from patients resulted in their increased expression of RXRα receptor [114].

All trans and 9-cis Retinoic Acid

The effects of these two ligands are similar to $1,25(OH)_2D_3$ in so far as they mediate a cell cycle arrest and initiate differentiation. The pattern of this process shares certain similarities with $1,25(OH)_2D_3$. A central protein in these processes is the CDKI $p21^{(waf\ 1)}$. Recently, a retinoic acid response element (RARE) has been characterized within the promoter/enhancer region of the gene. It has been shown to be upregulated during monocytic differentiation of U937 cells [25]. The same investigators found distinct regions for $1,25(OH)_2D_3$ and ATRA mediated upregulation. Furthermore the RARE was shown to respond to RAR-RXR heterodimers; a fact which is reflected by differentiation studies undertaken with conformationally restricted retinoids.

Events that follow the induction of $p21^{(waf1)}$ are clearly different depending if HL-60 cells differentiate along either the monocytic lineage in the presence of $1,25(OH)_2D_3$ or the granulocytic lineage in the presence of ATRA. Other specific effects of retinoids that are observed in U937 cells include the increased expression of CD49f, and CD66a. In human promyelocytic cells a rapid induction of CD-38 is observed [115], and stimulation of M-CSF and GM-CSF in AML cells [116].

ATRA directly regulates the transcription of members of the HOX gene family which have a central role in morphogenesis and bone marrow differentiation. Other proteins that are effected include the cell adhesion molecules (e.g., Laminin B), growth factors (e.g., TGF-β) and growth factor receptors (e.g., EGF receptor) [117, 118].

Various groups have looked for early response genes induced by ATRA by differential display and subtractive hybridization techniques. The genes cloned include RIG-E,

which has a suggested role in signal transduction [119], and E3 with an as yet undetermined function [120]. In our own laboratory we have cloned from a promyelocyte-late myeloblast cDNA library, a gene [C/EBP∈] that is a CCAAT/enhancer-binding protein transcription factor. It plays a role in the regulation of a subset of myeloid-specific genes and is upregulated during myeloid differentiation [121]. Prominent expression of C/EBP∈ mRNA occurred in the late myeloblastic and promyelocytic cell lines (NB4, HL60, GFD8), the myelomonoblastic cell lines (U937 and THP-1), the early myeloblast cell lines (ML1, KCL22 and MDS92), and low expression occurred in the T-cell lymphoblastic leukemia cell lines CEM and HSB-2. No C/EBP∈ mRNA was detected in erythroid, B lymphoid or non-hematopoietic cell lines. Most acute myeloid leukemia samples (11 of 12) from patients expressed C/EBP_E. Northern blot, RT-PCR and immunohistochemical staining showed that myeloblasts to metamyelocytes express C/EBP but granulocytes and macrophages express very little of this transcriptional factor analyses.

Synthetic Vitamin D_3 Analogs and Conformationally Restricted Retinoids

Vitamin D_3 Analogs

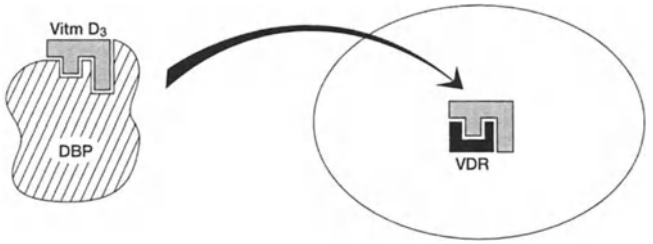
An extensive body of research has examined the effects of novel vitamin D3 analogues on various malignant cell types and highlighted various structural motifs that have increased antiproliferative effects [122, 123, 124]. Structure-function analysis of $1\alpha,25(OH)_2D_3$ has centered on the four main structural motifs of the seco-steroid: the A ring, seco-B ring, C/D ring, and the side-chain. Of the 278 previously investigated analogs, 80% were variants involving structural changes to the side-chain.

The reason for the increased potency of the analogs is becoming clearer and is illustrated in Fig. 1. Vitamin D_3 analogs usually bind less well to the D-binding protein in the blood and therefore are more readily available to enter the cell [as measured by the relative ability of this compound to compete with ^3H-$1,25(OH)_2D_3$ for binding to the vitamin D-binding protein (RCI_{DBP})] [41]. In theory this would facilitate the availability of the unbound compounds for the VDR of the target cells thereby, explaining their potent activity on specific tissues in vivo. In addition, the free compounds might also be cleared faster, explaining in part the lower calcemic activity, or have a different binding affinity for critical VDRE-containing genes such as the VDRE for osteocalcin and the VDREs specific for hematopoiesis [126]. Analogs may also extend the half-life of the VDR [127] or they may induce novel VDR conformations [128], which may in turn allow either more efficient interactions with vitamin D_3 response elements and/or expand the array of vitamin D_3 response elements that can be activated. In addition , metabolic products of analogs may result in potent intermediates in vivo. For example, compared with the parental analogs, the 24-oxo metabolites have the same in vitro anticancer activities but have less effect on serum calcium levels [129].

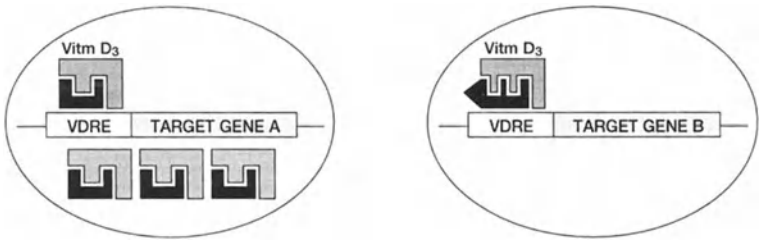
The thrust of vitamin D_3 analog research is to develop compounds with greater antileukemic activity and less hypercalcemic toxicity. To date, more than 50 vitamin D_3 analogs have been identified in vitro which have higher differentiating and antiproliferative activities, but lower abilities to cause hypercalcemia than $1,25(OH)_2D_3$. These analogs could provide a larger therapeutic window for the treatment of hematologic malignancies, retaining the useful properties of $1,25(OH)_2D_3$ [123].

The first attempts using analogs focused on 1α-hydroxy-D_3 (1α-OHD_3), a vitamin D_3 analog that is efficiently converted to $1,25(OH)_2D_3$ in vivo by D_3-25-hydroxylase. This compound was therapeutically more active than $1,25(OH)_2D_3$ [130]. Its conversion to the active form resulted in a more prolonged elevation of plasma levels of $1,25(OH)_2D_3$; and the dose produced insignificant elevation of serum calcium. Also, the administration of 1α-OHD_3 has been shown to produce tumor regression in follicular NHLs in rats [55]. Clinical applications have been limited to studies with MDS patients where the treated group had a greater proportion of patients who did not progress to leukemia as compared to the control group.

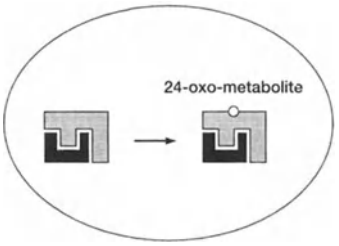
1. Analog binds poorly to D-binding protein and more readily enters cells.



2. Analog induces novel conformation and/or extends half-life of vitamin D receptors.



3. Analog undergoes conversion to potent metabolites.



Summation Of Effects Is To Inhibit Proliferation And Induce Differentiation Of Transformed Cells.

Fig. 1. Mechanism by which vitamin D analogs may have enhanced cellular effects. DBP, D-binding protein; VDR, vitamin D receptor; VDRE, vitamin D response element

Calcipotriol (MC903) has a cyclopropyl group at the end of the side-chain formed by the fusion of the 26th and 27th carbons, a hydroxyl group at carbon 24 and a double bond at carbon 22. This compound was equipotent to $1,25(OH)_2D_3$ at inhibiting the proliferation and inducing the differentiation of the monoblastic cell line U937 [58, 131] and HL-60 myeloblasts [132]. In bone marrow cultures, this compound was able to promote the formation of multinucleated osteoclast-like cells. The effects of this compound on the immune system were very similar to those induced by $1,25(OH)_2D_3$. By interfering with T-helper cell activity, Calcipotriol reduced immunoglobulin production and it blocked the proliferation of thymocytes induced by IL-1 [133, 134]. Follicular NHL B-cell lines SU-DUL4 and SU-DUL5, carrying the t(14;18) characteristic of the disease, were inhibited in there proliferation by MC 903 only at high concentrations of the compound (10^{-7} M) [55]. At the same time, it was 100-fold less active than $1,25(OH)_2D_3$ at inducing hypercalcemia and mobilizing bone calcium in rats [135].

Another promising analog is $1,25(OH)_2$-16ene-D_3 which is over 1000-fold more active than $1,25(OH)_2D_3$ in inhibiting clonal growth of HL-60 cells and 5-fold more po-

tent in inducing their differentiation [126]. This compound has high binding affinity for VDR and has about 50-fold lower affinity for the D-binding proteins present in the serum, therefore increasing the availability of this compound for target tissues. This compound induced hypercalcemia in mice at a comparable rate to that of $1,25(OH)_2D_3$. This analog, as well as other vitamin D compounds, did not inhibit the clonal growth of normal human granulocyte-macrophage committed stem cells [126].

We have shown that $1,25(OH)_2$-16 ene-23 yne-D3 has potent antiproliferative and differentiating effects on leukemic cells in vitro [136]. In preventing HL-60 clonal growth, $1,25\ (OH)_2$-16 ene- 23 yne-D_3 has a potency about 1000-fold more than $1,25(OH)_2D_3$. We had previously shown that this compound potently inhibited the clonal growth of a variety of human leukemic cell lines, had low calcemic potential, and was able to cure leukemia in one third of mice [122]. This compound administered to vitamin D-deficient chicks is about 30 times less effective than $1,25(OH)_2D_3$ in stimulating intestinal calcium adsorption and about 50 times less effective in inducing bone calcium mobilization [58]. A synergistic antineoplastic effect of this compound and ATRA has been shown in HL-60 cells [137]. A HL-60 clone resistant to ATRA (HL-60R) was more than 20-fold more sensitive to inhibition of proliferation by $1,25\ (OH)_2$-16 ene-23 yne-D_3 than was $1,25(OH)_2D_3$. In addition, the induction of differentiation of HL-60R cells by $1,25\ (OH)_2$-16 ene-23 yne-D_3 was much stronger in these cells than in the wild-type HL-60 cells [138].

The $1,25(OH)_2$-20epi-D_3 analog is characterized by an inverted stoichiometry at carbon 20 of the side chain. The monoblastic cell line U937 cultured with this compound showed a strong induction of differentiation [139]. It was also a potent modulator of cytokine-mediated T-lymphocyte activation and exerted calcemic effects comparable to $1,25(OH)_2D_3$ in rats. A recent study by ourselves suggested that $1,25(OH)_2$-20epi-D_3 is the most potent vitamin D3 compound at inhibiting the clonal growth of HL-60 cells and at inducing cell differentiation. In fact, it was about 2600-fold more potent than $1,25(OH)_2D_3$ in preventing the clonal growth of HL-60 cells and about 5000-fold more effective in inhibiting clonal growth of fresh human leukemic myeloid cells [140]. $1,25(OH)_2$-20epi-D_3 exerts its effects by binding directly to VDR as shown by a T-lymphocytic cell line established from a patient with vitamin D-dependent rickets Type II, lacking a functional VDR. Clonal growth was not inhibited after treatment of these cells with high doses of either $1,25(OH)_2$-20epi-D_3 or $1,25(OH)_2D_3$ (10^{-7} M).

The PML-RARα fusion protein can heterodimerize with RXR. In NB4 cells, the RXR is localized to the APL-specific substructures that includes the PML-RARα protein. As mentioned previously, the sequestration of RXRα by PML-RARa is reversed when these cells are treated with ATRA, resulting in RXR returning to a diffuse nuclear pattern [141]. The interaction of PML-RARα with RXR may interfere with other nuclear receptor pathways that involve RXR. One study suggested that, by competing for RXR, PML-RARα can inactivate the vitamin D_3 receptor [142]. The 20- epi-analogs can efficiently enhance homo- and heterodimerization of VDR with VDR and RXR as compared to 1,25D, which predominately forms VDR/RXR heterodimers [19]. The competition between the RAR and VDR for association with RXR may provide an additionally important control step for these ligands [20].

KH1060 is a potent vitamin D_3 20-epi analog with an oxygen placed at carbon 22 and three additional carbons added to the side chain. It is about 14000-fold more potent than $1,25(OH)_2D_3$ in inhibiting the clonal growth of the monoblastic cell line U937. It has also a powerful antiproliferative effect on other leukemic cells [51, 100, 101]. It has the same hypercalcemic activity and the same receptor binding affinity as $1,25(OH)_2D_3$ [142].

We have recently investigated a novel series of vitamin D_3 analogs and found several new structures that are highly effective at inhibiting the clonal growth of HL-60 cells [143]. One particularly potent analog is (22R)-1α,25-$(OH)_2$-16,22,23-triene-D_3 (ED_{50} HL60 2×10^{-11} M). This compound incorporates several of the motifs we had pre-

viously shown to be important for analog potency; a C-16 double-bond and a C-22 or C-23 double or triple bond. The incorporation of two double-bonds on the side-chain appears further to increase activity. The relative competitive index of this compound for VDR (RCI_{VDR}) was 154% as measured by its ability to compete with [^{3}H]-1α,25$(OH)_2D_3$ for binding to the VDR. This is one of the highest RCI_{VDR} reported to date, consistent with formation of a stable complex between the analog and the receptor [125]. The RCI_{DBP} of this compound was 9% of 1α,25$(OH)_2D_3$. Theoretically, this low RCI_{DBP} increases its potency, because it is available to enter the cell rather than remain in the serum bound to DBP. We have also investigated the stereo isomer of this compound (22S)-1α,25-$(OH)_2$-16,22,23-triene-D_3 and found that it was more than a thousand times less effective at inhibiting clonal proliferation of HL-60. This raises some intriguing questions about stereo-selectivity of vitamin D_3 mediated action.

Studies by others as well as ourselves have shown that the lengthening of the side-chain by one or several carbon atoms can slightly increase the activity of the analog [124, 144, 145]. Likewise, replacement of six hydrogens by fluorines at the end of the side-chain can also increase potency of 1α,25$(OH)_2D_3$ analogs [146,147]. Thus, compounds were investigated that encompassed all these modifications, such as, 1,25$(OH)_2$-22E,24Z-diene-24-homo,26,27- F_6-D_3; This analog was very active, approximately 30-fold more potent than 1α,25$(OH)_2D_3$ at inhibiting HL-60 clonal growth. Interestingly orientation of the side chain in the 20-epi-conformation 1α,25-$(OH)_2$- 16,23E-diene-26,27- F_6-20-epi-D_3, was no more potent 1α,25-$(OH)_2$-16,23E-diene-26,27- F_6-D_3 In recent studies with LNCaP human prostate cancer cells, we noted that the structural benefit gained by the 20-epi-conformation is lost by the addition of either the C-26,27 hexafluorines and/or the C-16 ene [148].

This study has helped to decipher structural features that are important to increase the potency of the vitamin D_3 analogs. At least for HL-60 cells, the more potent analogs have a C-16 double bond and either a C-22, -23, or -24 double or triple bond. The most potent analog [(22R)-1α,25- $(OH)_2$-16,22,23-triene-D_3] has the C-16, C-22, and C-23 double bonds incorporated into the parental 1,25$(OH)_2D_3$ structure. This appears more important than lengthening the side-chain, adding fluorides or the 20-epi-conformation. Furthermore, we showed that this active compound markedly inhibited clonal growth of committed myeloid leukemic stem cells from patients. Many of these analogs merit a more thorough evaluation of their antileukemic activities in vivo. In conclusion of this section, new vitamin D analogs have potent antileukemic activity and lower hypercalcemic effects and should be considered for the treatment of hematologic malignancies either alone or in combination with other differentiating agents. Phase I trials are necessary to assess the safety and effectiveness of these treatments.

Conformationally Restricted Retinoids

Conformationally restricted receptor selective retinoids have been used to dissect some of the mechanism of action of the natural ligands. Differentiation and apoptosis are intimately associated processes in the maturation of precursor cells with apoptosis a process for controlling the numbers of mature cells. For example one investigative group provided some evidence that a RAR selective retinoid, such as TTNPB, stimulated differentiation and a RXR selective retinoid, such as AGN191701, initiated apoptosis; and apoptosis (RXR-mediated events) was greatest after differentiation (RAR mediated event) had been initiated [149]. Retinoids induced differentiation leads to a reduction in BCL-2 levels [150] and levels of RXRβ increase [151, 152].

We have shown that synthetic retinoids that are selective for RXR-RXR homodimers (SR11217) are ineffective at inhibiting clonal growth and inducing differentiation of HL-60 cells, whereas retinoids which transactivated both RAR-RXR and RXR-RXR could inhibit clonal growth (SR11256, 9cRA). Furthermore, these compounds in combination with ATRA showed synergistic effects. Thus, we conclude that the RAR-RXR pathway is more important than the RXR-RXR pathway

for inhibition of proliferation and induction of differentiation of AML cells. RARα appears to have a pivotal role as an RARα antagonist could significantly inhibit the cellular response mediated by the combination of AM580 (RARα selective) and an RXR selective ligand. AM580 demonstrated a greater ability than ATRA to induce granulocytic maturation in NB4, but not in HL-60 cells. Furthermore RAR and RARγ selective retinoids were not effective in the same assay systems. These data correlate with the greater ability of AM580 than ATRA to transactivate a RARE containing reporter gene in the presence of PML-RAR [153].

Another synthetic retinoid 4-HPR has potent antiproliferative activity against leukemic cells which occurs in an unique manner. The mechanism of its inhibition requires both RNA transcription and protein synthesis and is regulated by the activity of protein kinase C. This retinoid potently induces apoptosis, although this is not thought to be a direct effect [154].

Antileukemic Effects of the Combinations of Vitamin D_3 Analogs and Conformationally Restricted Retinoids Either Together or with Other Agents

Patients with acute promyelocytic leukemia (APL) relapse after ATRA-treatment because the therapy often fails to eradicate completely the malignant clone and the cells becoming insensitive to the differentiating effects of ATRA. The cause for this insensitivity may be multifactorial including abnormal ligand binding and altered transcriptional activation [155]. Interestingly, we have shown that mutations of the RARα gene are comparatively rare in ATRA-resistant APL, MDS and cell lines [156].

Combining two effective drugs is a mechanism for increasing potency while decreasing toxicity. We have investigated the use of various potent analogs of $1,25(OH)_2D_3$ and 9cis RA. The combination of ATRA (10^{-9} M) and the vitamin D_3 analogs $1,25(OH)_2$-16ene-23yne-D_3 or $1,25(OH)_2$-23yneD_3 (10^{-9} to 10^{-10} M) showed a synergistic effect on the induction of differentiation and the inhibition of proliferation of HL-60 cells [137]. In addition, the inhibition of DNA synthesis of HL-60 cells correlated well with their reduction in a soft-gel clonogenicity assay. A decrease of c-MYC expression was also observed in the presence of ATRA and $1,25(OH)_2$-16ene, 23yne D_3. This downregulation of c-MYC was stronger than that observed using single agents and correlated with the initiation of differentiation [137].

Our data showed that KH 1060 alone was a potent inhibitor of clonal growth of NB4 cells, (ED_{50} 5×10^{-12} M). The combination of KH 1060 and 9-cis-RA synergistically and irreversibly enhanced this effect (ED_{50}, 1×10^{-12} M). Neither 9-cis-RA nor KH 1060 (10^{-6} M, 3 days) were strong inducers of differentiation of NB4 cells. However, 98 % of the cells underwent differentiation to a granulomonocytic phenotype after exposure to a combination of both compounds. Immunohistochemistry and Western blot analysis showed that the bcl-2 protein expression decreased and bax protein increased after incubation with a combination of KH 1060 and 9-cis-RA. After exposure to both analogs, p53 protein strongly decreased, and apoptotic cells increased. Our data demonstrated that nearly all of the NB4 cells can be irreversibly induced to differentiate and some of cells undergo apoptosis when exposed to the combination of KH 1060 and 9-cis-RA. Study of APL blasts from one patient before therapy paralleled our results with NB4.

Other combinations that have been reported include ATRA and interferon which act together, but not alone, to inhibit granulocyte-macrophage colony formation of peripheral blood progenitors from patients with CML [157]. ATRA also potentiated cells to undergo differentiation with hexamethlyene bisacetamide (HMBA), $1,25(OH)_2D_3$, and butyrates [158].

Future Directions

One major focus of research in the field of vitamin D_3 and cancer has been to identify analogs of $1,25(OH)_2D_3$ that have prominent antiproliferative effects against cancer cells without resulting in lethal hypercalcemia when administered in vivo at pharmacologically active doses. The overwhelming majority of analogs examined thus far have been

derived by chemical synthesis. Within a cell, $1,25(OH)_2D_3$ readily undergoes metabolism along several different metabolic pathways as described recently [159] All of these pathways were thought to be catabolic and the intermediate metabolites were believed to have little or no biological activity.

Recently, a major natural intermediate of a potent precursor analog $1\alpha,25(OH)_2$-16-ene-D_3 formed through the C-24 oxidation metabolic pathway has been isolated by using a rat kidney perfusion biotransformation system [129]. This intermediary metabolite, $1\alpha,25(OH)_2$-24-oxo-16- ene-D_3, is significantly resistant to further metabolism and therefore accumulates. This metabolite shares approximately the same level of potency as its parental analog [$1\alpha,25(OH)_2$-16-ene-D_3], in inhibiting proliferation and inducing differentiation of the human leukemic cell line RWLeu-4, and in transactivating a VDRE reporter construct [129]. Most interestingly, the 24-oxo metabolite had reduced calcemic activity as compared to its parental analog [160]. Therefore, the 24-oxo metabolites appear to allow a separation of the anti-proliferative genomic effects of vitamin D_3 compounds from their hypercalcemic side-effects. We have investigated a series of analogs and their 24-oxo metabolites with a variety of cell lines and found with HL-60 that both $1\alpha,25$-$(OH)_2$- 20-epi-D_3 and $1\alpha,25$-$(OH)_2$-24-oxo-20-epi-D_3 were equally able to inhibit clonal growth, arrest the cells in G_0/G_1 of the cell cycle and induce CD11b, but they differed in their effects on the expression of $p21^{(waf\ 1)}$ as the 24-oxo metabolite ($1\alpha,25$-$(OH)_2$-24-oxo-20-epi-D_3) significantly increased expression of this CDKI compared to its precursor analog [161].

Other mechanisms for enhancing steroid hormone action to allow therapeutic effects at lower doses is to recruit additional pathways. For example, HL-60 differentiation can be primed with a pre-treatment with an antiinflammatory drugs such as indomethacin [162]. This compounds inhibit cyclooxygenase activity [163] and also inhibit other enzymic activities that may act to suppress steroid hormone action providing an attractive approach.

The active conformation of steroid hormone receptors is maintained by heterodimerization with other receptors such as RXR [164] and various chaperone and coactivator proteins, the mechanism of which is beginning to be elucidated [165]. One group of these has been identified as the heat shock proteins (HSP). In particular, HSP90 has been shown to be intimately associated with the retinoid receptors and low expression of this protein abolished high affinity hormone binding, and therefore transactivation by the receptor [166, 167]. A synergistic effect of ATRA and $1,25(OH)_2D_3$ to induce monocytic differentiation of U937 promonoblasts was observed [168], and preexposure of these cells to a moderate thermal stress enhanced the $1,25(OH)_2D_3$ and ATRA mediated differentiation, possibly as a result of HSP activity [169]. Possibly synthetic peptides could be designed to mimic HSP activity in vivo and thereby increasing steroid hormone activity.

A large array of orphan receptors has recently been cloned for which the natural ligand has been identified [170]. Many of these appear to interact with other steroid hormone receptors. For example, LXRα has recently been cloned [171] and 9cRA appears to be a natural ligand of it. This receptor is only expressed in certain organs, including the spleen, and in the presence of 9cRA appears to activate biological pathways different from those mediated by RXR/RXR or RAR/RXR. Furthermore within the LXR-RXR heterodimer, the RXR is not a silent, non-ligand activated receptor, as in many other cases, but rather transactivates genes too. This receptor, and other orphan receptors possibly allow for further levels of steroid hormone control of cancer cells and require additional study.

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Growth Factors in the Treatment of Acute Lymphoblastic Leukemia

W. K. Hofmann, O. G. Ottmann and D. Hoelzer

Introduction

Previous studies in lymphomas, solid tumors and following bone marrow transplatation have demonstrated that G-CSF and GM-CSF are able to accelerate neutrophil recovery, reduce the infection rate and allow dose intensification of chemotherapy [1-5]. In ALL patients substantial problems due to cytopenia are presented during induction phase and not only after chemotherapy such as in AML patients. Not only postinduction application of HGF but also parallel administration to chemotherapy because of long induction treatment periods (4-8 weeks) is an approach for the use of G/GM-CSF. Nevertheless, reports of occasional G-CSF and GM-CSF receptor expression by Philadelphia chromosome positive (Ph+) ALL and by ALL coexpressing myeloid markers [6, 7] prompted some theoretical concern that G-CSF could stimulate leukemic cell populations in individual patients (8). To date, several clinical trials in both adult and childhood ALL have examined the feasibility and potential clinical value of HGF in conjunction with induction and early postremission chemotherapy for ALL.

GM-CSF in ALL

There are only a few studies using GM-CSF in ALL-patients. Kantarjian et al. [9] studied 34 refractory adult ALL-patients who received GM-CSF after high-dose cytarabine and mitoxantrone and an identically treated historical control group (n=29). Recovery of granulocyte counts above 500 /µl was observed eight days earlier in the GM-CSF treated patients compared with historical controls (25 days vs. 33 days, $p<0.01$). There was a nonsignificant trend towards lower induction mortality in the GM-CSF group (6% vs. 28%). Neither the incidence of febrile episodes nor of documented infections were reduced with GM-CSF, and complete response rates were identical in both groups. Survival was marginally prolonged in the GM-CSF-treated patients (31 vs. 20 weeks, $p = 0.05$), although this is not clinically relevant.

GM-CSF did not ameliorate the degree or duration of chemotherapy-induced myelosuppression when given concurrently with induction chemotherapy in a randomized, placebo-controlled study in high risk childhood ALL (10). Likewise, GM-CSF did not influence the incidence of fever, number of hospital days, days on i.v. antibiotics or the number and severity of infectious complications.

G-CSF in Adult ALL

The first prospective, randomized study of prophylactic G-CSF after induction therapy fo acute leukemia was conducted by Ohno et al. [11] in relapsed or refractory patients, including 30 patients with ALL. The response

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to therapy was similar in both groups, and importantly, there was no indication of accelerated leukemic regrowth or enhanced relapse rate in response to G-CSF. Overall, G-CSF significantly accelerated neutrophil recovery by approximately 1 week and reduce the frequency of documented infections as compared with the control group.

Administration of G-CSF following intensive early postremission consolidation consisting of high-dose Ara-C, mitoxantrone and vincristine over 3 days was examined by Kantarijan et al. [12] in a nonrandomized study of 14 ALL-patients in first complete remission. The duration of neutropenia < 500/μl in the G-CSF treated patients was significant reduced compared with a historical control group (14 days vs. 18 days). There was a trend towards a lower incidence of febrile episodes, severe infections and early death, although it was not statistically significant due to the small number of patients.

Larson et al. [13] reported a significant reduction in the duration of neutropenia with G-CSF in a randomized, placebo-controlled trial of 184 patients with ALL receiving induction therapy according to CALGB 9111 protocol. Granulocytopenia < 1000/μl was reduced from 21 days with placebo to 15 days with G-CSF in patients < 60 years, and from 29 to 16 days, respectively in the older (> 60 years) patients. Severe infections and death during induction were slightly, though not significantly less frequent in the G-CSF group. Overall, prophylactic administration of G-CSF appeared to benefit the elderly patients in particular. The results are given as a overview in Table 1.

The concomitant administration of G-CSF and induction therapy for adult ALL was first tested in two independent pilot studies by the German multicenter ALL study group (GMALL) [14] and by the Vienna group [15], using a nearly identical chemotherapy protocol. These two trials differed in that G-CSF was given throughout the whole 8 weeks of intensive induction treatment by the Vienna group, that is in parallel to vincristine, daunorubicin, asparaginase, cyclophosphamide, cytarabine, and 6-mercaptopurine. In contrast, the GMALL pilot study restricted G-CSF administration to weeks 5-8 of induction (cyclophosphamide, cytarabine, and 6-mercaptopurine in patients who had achieved a complete or partial remission following the first 4 weeks of induction). Both regimens applied prophylactic cranial irradiation and mediastinal irradiation in patients with T-ALL. These two trials mutually concluded that the administration of G-CSF concomitantly with this specific induction chemotherapy was safe, with a trend towards reduced neutropenia compared with historic controls.

Based on these data, the GMALL in a subsequent randomized open-label study prospectively examined the utility of administering G-CSF simultaneously with chemotherapy, as well as in combination with parallel prophylactic CNS and (in T-lineage ALL) mediastinal irradiation [16]. Seventy-six adult patients with ALL were randomly assigned to receive either G-CSF (n=37) or no growth factor (n=39) parallel to induction chemotherapy and prophylactic cranial irradiation. The median duration of neutropenia

Table 1. Randomized clinical trials with G-CSF or GM-CSF in adult ALL. PR, partial remission; CR1, complete remission 1

Authors	Pts	Chemotherapy	Growth factor	n	ANC < 500 /μl (days)	Infections (%)
Ohno 1993	Refractory	Induction	G-CSF		20	19
			Placebo		28	45
Geissler 1994	De novo	GMALL	G-CSF	12	21	8
		Induction I+II	Control	12	30	42
Larson 1994	de novo	CALGB9111	G-CSF		15	3
		Induction	Placebo		21	66
Ottmann 1995	PR/CR1	GMALL	G-CSF	37	8	43
		Induction II	Control	39	13	56

(<1x109/l) during chemotherapy was 8 days in patients receiving G-CSF and 12.5 days in the control group. In patients with T-ALL, all of whom received additional mediastinal irradiation, a similar reduction from 11.5 to 7 days was observed. Non-viral infections were reduced by 50% (32 episodes in the control arm versus 16 episodes in the G-CSF arm). Chemotherapy was completed significantly earlier with the use of G-CSF (39 versus 44 days, p=0.008), as a consequence of fewer prolonged interruptions of chemotherapy administration. Delays of 2 weeks or more occurred in only 24% of patients receiving G-CSF as opposed to 46% in the control arm. At a median follow-up of 22 months, the probability of disease-free survival was not significantly different between the two groups.

G-CSF in Pediatric ALL

The efficacy and safety of prophylactic G-CSF in ALL is further supported by a randomized, open-label trial in children with high risk ALL treated in the ALL-BFM-90 study [17]. Thirty-four patients were randomized to receive either successive 6-day cycles of multiagent chemotherapy alone or followed by G-CSF starting on day 7. The average incidence per cycle of febrile neutropenia was significantly reduced in the G-CSF group (17% vs 40%; p=0.007). The median total duration over all treatment cycles were 6 days per patient in the G-CSF group as compared with 20 days per patient in the control group (p=0.02).

Despite to this results the largest study at this time involving children with ALL was published by Pui et al. [18] who found that, as compared with a placebo group, children receiving G-CSF after intensive chemotherapy did not have a significantly lower rate of hospitalization for febrile neutropenia (58% vs. 68%), or significant fewer severe infections (5 vs. 6). The G-CSF treatment did not influence the event-free survival at 3 years. However, the children treated with with G-CSF had shorter hospital stays (6 vs 10 days) and fewer documented infections (Table 2).

A recent randomized trial by Mitchell et al. [20] showed similar results to those in the study of Pui but the overall costs were about 20% lower in the G-CSF group. The G-CSF group had a significantly shorter median hospital stay than the placebo group (5 vs. 7 days), fewer median days of antibiotic treatment (5 vs. 6 ays) and higher median neutrophilic counts at discharge (1650 /µl vs. 740/µl).

Different results were obtained in similar trial designed to test the efficacy of G-CSF given concurrently with chemotherapy in children with newly diagnosed, intermediate risk ALL. Thirty-two patients were randomly assigned to a group that received (14 patients) or did not receive (18 patients) G-CSF throughout of the second phase of BFM-type induction therapy. Only 1 of 14 (7%) patients in the G-CSF group and 2 of 18 (11%) patients in the control group completed the course of chemotherapy within the scheduled time. The median lengths of this phase were essentially identical (37 vs. 36 days). The number of days during which patients had granulocyte counts of less than 2000/µl, the number of febrile episodes of unknown origin, the number of bacterial

Table 2. Clinical trials with G-CSF in pediatric ALL. HR, high-risk; CR1, complete remission 1. * over all cycles

Authors	Pts	Chemotherapy	Growth factor	n	ANC <500 /µl (days)	Infections (%)
Calderwood 1994	HR CR1	Consolidation	GM-CSF Placebo	20 20	6 7	81 79
Dibenedetto 1995	HR CR1	Consolidation	G-CSF Control	14 18	16 19	14 11
Welte 1996	HR	BFM90 Consolidation	G-CSF Control	17 17	17* 62*	30 44
Pui 1997		Induction	G-CSF Placebo	73 75	5 13	16 36

and fungal infections and the number of days of hospitalization did not differ in a statistically significant manner between the two groups. In contrast to the results obtained for adults, these data suggest that G-CSF supportive therapy may be unnecessary in children with neutropenia of short duration, for whom the risk of infection is low.

Future Aspects

The majority of studies using G-CSF in ALL obtained a reduced rate of febrile episodes and a trend towards fever infections. This results in a reduction in the duration of hospitalisation. This may be important in the quality of life in ALL-patients, particulary in children, who received intensive treatment for ALL [21, 22]. However, one may argue that this will not outweight the additional costs caused by the treatment with HGF. Thus one of the aim in future studies is to determine the optimal timing of G-CSF post chemotherpy, that is the minimal requirement of G-CSF in days to obtain the above benefits, but with lesser costs. Conventional G-CSF is given from day 1 postchemotherapy up to neutrophilic recovery. The goal is to give it for a shorter duration in a hope to see the same effects. The question is whether a delayed start of G-CSF at a time when early bone marrow progenitor cells start their regeneration after myelosuppression is effective in the same way such as the start of G-CSF administration the day after the end of cytotoxic therapy. A first study of this context was published by Elonen et al. [23]. In a small number of adult ALL-patients who received a treatment with mitoxantrone, etoposide, and intermediate dose cytarabine two schedules for G-CSF application were used: In 19 patients G-CSF was started 2 days after the last dose of chemotherapy. In 16 patients G-CSF was started 9 days after the last dose of cytotoxic treatment. There were no difference between both of the groups with regard to duration of neutropenia (20 vs. 18 days), days with feveral episodes (6 vs. 6 days), days on antibiotics (23 vs. 28) or hospitalization duration (29 vs. 28 days).

Therefore we initiated a randomized, multicentre study to evaluate the efficacy of

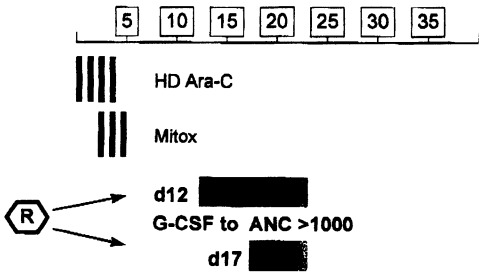

Fig. 1. Treatment schedule of delayed G-CSF after high-dose cytarabine/mitoxantrone treatment in adult ALL

a delayed start of G-CSF after therapy with high-dose cytarabine and mitoxantrone in patients with high-risk ALL who were treated according to GMALL-protocol (Fig. 1). The median onset of granulocytopenia < 500/µl is 7 days, the median duration of ANC~ <500 /µl is 14 days without G-CSF respectively. The aim of the study is to show that there is an improvement of the rate of treatment-associated morbidity and mortality in the G-CSF groups compared with historical controls but without a difference between the two study arms using G-CSF (Fig. 1).

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Control of Leukemia Proliferation in vivo in Patients: In vivo Cytokine Production

H. D. Preisler, X. Z. Gao, H. Chopra, E. Devemy, P. Venugopal, and S. Bi

Abstract. Preisler et al. first provided evidence that cytokine production by AML cells in vitro may not reflect cytokine production in vivo in patients. To address this question we placed bone marrow aspirate cells into GITC immediately after removal from the patient. Parallel marrow aspirates were placed on ice, sent to the laboratory and then placed into GITC after density cut separation or macrophage removal. Multiplex rtPCR was used to detect transcripts for IL1β, TNFα, HSCF, GMCSF, IL6, IL1ra, flt3. Among 13 patients studied, transcripts for IL1β were detected in 12, TNFα in 7, IL1ra in 3 of 9 studied. Among AML which did not produce TNFα, there were 4/4 CRs while there was 6 treatment failures among 6 patients whose cells contained TNFα mRNA with the one "CR" being characterized by persistent dysplasia with leukocytosis upon marrow recovery. Repeat studies during remission induction therapy were performed in 4 patients. HSCF appeared in one patient and was associated with leukemia cell proliferation during treatment. The appearance of IL1ra transcripts was associated with a significant fall in the proliferation rate of the AML cells. As a result of in vitro processing of cells, TNFα transcripts appeared in 4 specimens, HSCF in 4, GMCSF in 4, IL6 in 4. There were 4 CR among 5 pts whose leukemia cells did not produce GMCSF in vitro and no CRs among 4 pts whose AML cells produced GMCSF in vitro. These data demonstrate that cytokine production by AML cells differs among patients and such differences appear to have clinical significance. Further, cytokine production during treatment can change and is associated with clinical consequences. In vitro assessments of cytokine production cannot be used to characterize cytokine production by AML cells in patients but may be predictive of response to therapy.

Introduction

Studies of the proliferation rates of AML cells in vivo in patients [1] led to the surprising observation that long term response to therapy was best in patients with rapidly proliferating disease [2]. This observation seemed paradoxical given the widely accepted fact that rapidly proliferating cells are more sensitive than slowly proliferating cells to cytotoxic therapy. Upon further consideration and additional observations, it became clear that the basis for this relationship is the fact that leukemia cells regrow between courses of therapy and that the more rapidly proliferating the leukemia cells the more rapid their regrowth. Such regrowth offsets at least some of the benefits of the prior course of chemotherapy [3, 4].

Treatment Failure in AML

The treatment problems posed by AML differ depending upon the nature of the dis-

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ease. For patients with standard prognosis disease the problem of extending remission durations is the most important challenge since 75% of these patients will enter remission with currently available therapies. In contrast, for patients with poor prognosis disease [history of toxic exposure, prior myelodysplasia, and/or age over 70] [5] successful remission induction occurs in usually less than 50% of cases. Hence the focus of new therapies should be towards extending remission durations in standard prognosis patients and improving the remission rate in poor prognosis patients.

While there is no doubt that classical drug resistance plays a significant role in treatment failure in all types of AML, treatment failure generally results from the combined effects of both classical and regrowth resistance. This paper will focus on the latter.

Alteration of AML Proliferation in Vivo – Treatment of Standard Prognosis Patients

Given that complete remissions are induced in 85% of patients with standard prognosis AML who survive remission induction therapy and given that upon relapse at least 50% of the relapsed patients can be induced back into remission by the administration of the same chemotherapy, it appears likely that classical drug resistance per se is not the primary cause of treatment failure in these patients. What is meant by this statement is that the leukemia cells of the vast majority of standard prognosis patients are at least moderately sensitive to cytotoxic therapy and that relapse results from the regrowth of these "sensitive" cells. The ease at which one-half of the patients who relapse can be induced back into remission demonstrates that the leukemia cells which regrow are still drug sensitive. The situation is diffent for patients with multiply relapsed disease.

This being the case, then the inhibition of leukemia regrowth between courses of therapy should improve the efficacy of currently available therapy. To this end we evaluated the effects of administration of the combination of 13-*cis* retinoic acid and a-interferon (RA/IFN) on the proliferation rate of AML cells. In vitro studies suggested that the antiproliferative effects of this combination was at least additive in myeloid leukemia (Fig. 1a).

Studies in vivo in patients have demonstrated that this combination of biological agents can slow the proliferative rate of AML cells in some patients [6] and induce apoptosis as well (Fig. 1b,c). These observations led to AML protocol 9102 in which the combination of RA/IFN is administered between courses of remission consolidation therapy and subsequent to the end of treatment. The remission duration curve in Fig. 1d strongly suggest that this regimen is substantially more effective than our predecessor protocol in which the same chemotherapy was administered [7] but without the RA/IFN. On the basis of the initial protocol design an additional 9 patients must be entered onto the protocol and followed for $1^1/_2$ years for the protocol results to be determined.

The Problem of Poor Prognosis AML – The Failure of Postinduction Treatment with RA/IFN

The treatment of poor prognosis AML is much more difficult and complicated than that of standard prognosis disease. In this disease or set of diseases treatment failure among surviving patients can be associated with the regrowth of leukemia or myelodysplastic cells, either at the end of remission induction therapy or as the termination of remission [8, 9]. This reality is a reflection of the fact that the development of secondary AML is the result of the successive overgrowth of increasingly abnormal cell populations (Fig. 2a) and that in the majority of patients all populations of cells are still present in the patient at the time of diagnosis of AML (Fig. 2b). Hence the outcome of remission induction therapy is determined by both the relative drug sensitivity of the populations (which determines the number of cells in each clone which survives remission induction therapy) as well as their relative regrowth rates.

The clone with the greatest proliferation advantage will repopulate the marrow if all cell populations are equally represented at the end of treatment (Fig. 2b). Clearly the

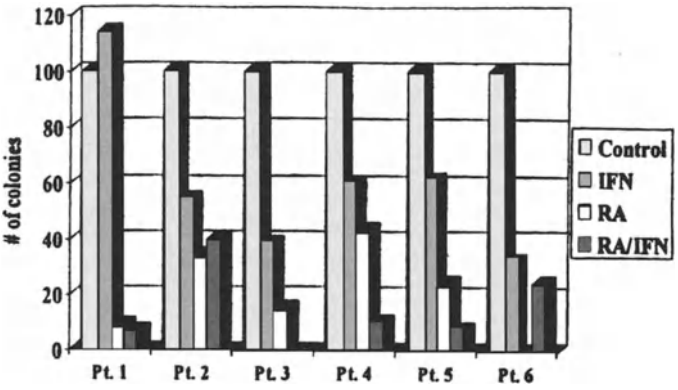

Fig. 1a. Comparison of the effects of RA and IFN alone and in combination on the proliferation of CML cells in vitro

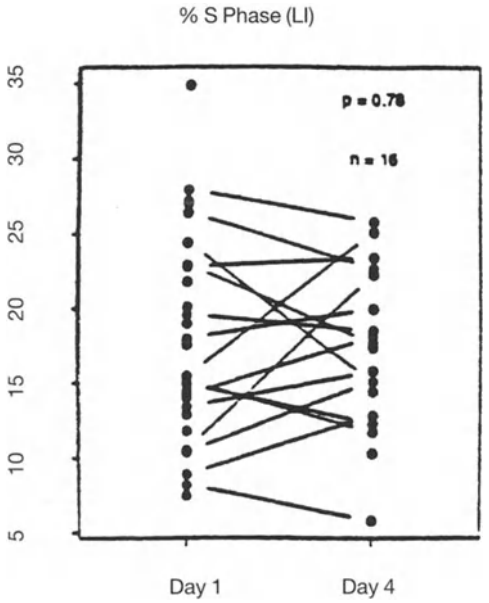

Fig. 1b. Effects of a 3-day administration of the % marrow S phase cells in patients with AML

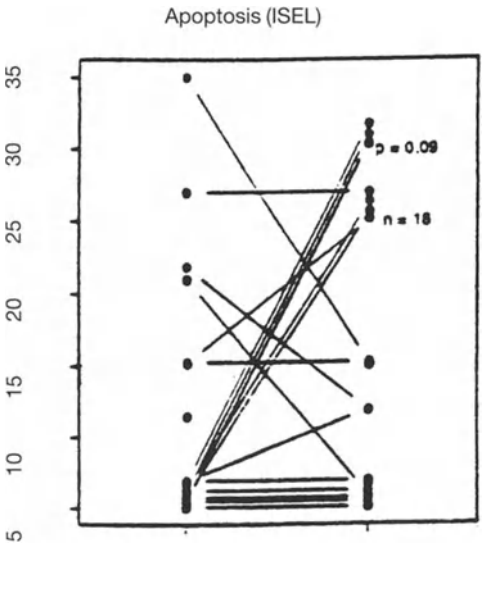

Fig. 1c. Effects of 3 days of RA/IFN on marrow apoptosis in vivo in AML patients

latter is not the case. Since for the development and evolution of the secondary hematologic diseases to occur each succeeding cell population must have a proliferative advantage over its predecessor population, the proliferative advantage must be ordered in the following sequence, AML > MDS > preMDS > normal. Hence the repopulation of the marrow by MDS cells after treatment must indicate that the AML cells were more drug sensitive than the MDS cells. To increase the effectiveness of remission induction therapy in patients, the suppression of the regrowth rates or proliferative advantages of the abnormal cell populations over that of the normal cells must be accomplished.

Our initial attempts to accomplish this goal by the administration of RA/IFN immediately after the end of remission induction therapy was not successful (Table 1). At least one of the reasons for the lack of success of this approach was the increased pro-

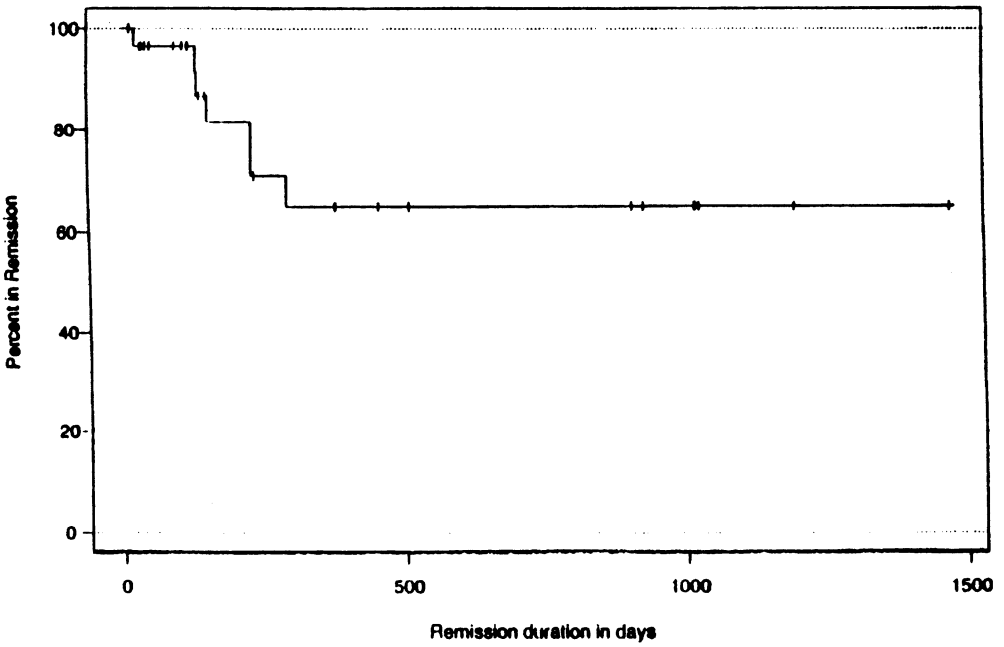

Fig. 1d. Remission duration curve for AML patients who received RA/IFN between courses of consolidation chemotherapy therapy and subsequent to the end of chemotherapy

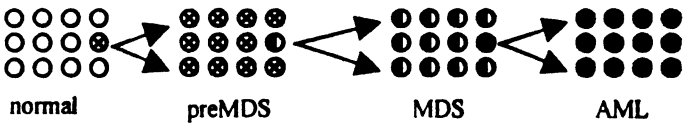

Fig. 2a. The evolution of the secondary hematologicdisordersrepresented as the successive overgrowth of newly emerging cell populations which have a prolife-ration advantage over the population from which they arose

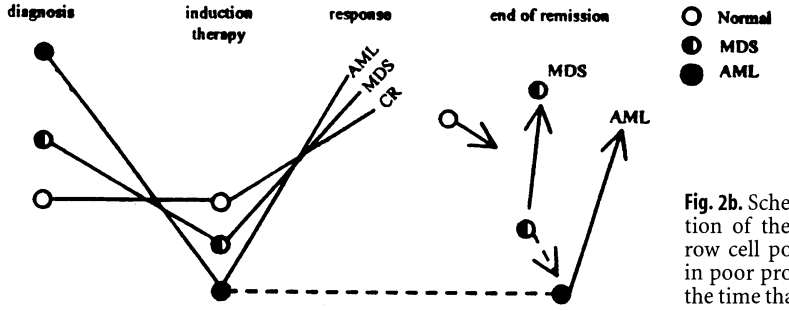

Fig. 2b. Schematic representation of the disordered marrow cell populations present in poor prognosis patients at the time that treatment

portion of deaths during remission induction therapy perhaps a reflection of the failure of these biological agents to suppress the myelodysplastic cell population and perhaps also as a result of the suppressive effects of the combination on the regrowth of normal hemopoietic cells. Studies of the myelodysplastic syndromes suggest that aberrant cytokine production, especially TNFa, may play a significant role in the proliferation advantage of some MDS cell populations [10].

Table 1. Remission induction outcome for patients with poor prognosis AML treated with pulsed HDara C/mitoxantrone therapy

CR	= 11	(includes 2 patients with myelodysplasia in CR marrow)
NCR	= 2	(defined as normal bone marrow but persistent cytopenia)
PR	= 4	
RD	= 4	(documented drug resistant disease)

Died during induction = 6

* Day 1 araC 2 g/m^2 t=0, t=12 h
MTX. - 30 mg/m^2 t=0, t=12 h
Day 4 araC 2 g/m^2 t=0, t=12 h
MTX - 30 mg/m2 t=15 h

Studies of the Biology of AML Cells in Vivo in Patients – Cytokine Production by AML Cells

AML cells do not proliferate autonomously in the sense that their proliferation is dependent upon the presence of cytokines in their environment, whether produced by the AML cells themselves, by other cells in the environment, or added to culture media [11, 12]. Studies by our group [13], as well as by one other group [14], suggested that the in vitro manipulation of AML cells during preparation for study could alter cytokine production. Hence the question was raised as to whether or not information regarding cytokine production by AML cells which is based on in vitro studies provide accurate information.

To address this question we dissolved AML cells in GITC immediately after they were removed from the patient and also after standard in vitro processing. These studies demonstrated that among the cytokines listed in Table 2, AML cells contain transcripts only for IL1β and TNFα. They do not contain either GMCSF or IL6 transcripts (Table 3). The question then arises as to whether cytokine production by AML cells is biologically and clinically significant. As illustrated in Fig. 3a the presence of TNFa transcripts appears to be associated with a reduced likelihood of successful remission induction therapy. Preliminary data suggest that AML cell populations containing TNFα transcripts proliferate at a higher rate than AML cell populations which do not (Fig. 3b) while in vitro studies are compatible with this concept (Fig. 3c).

It had been suggested in the past that the administration of cytotoxic therapy to AML patients was associated with an increase in cytokine production by AML cells and that this increase was associated with an increase in the proliferative rate of the AML cells [15]. We have begun to address this question using the methods which we developed for assessing cell proliferation in vivo and cytokine production. Our initial data are compatible with the concept that cytokine production can change during remission induction therapy and that such changes are associated with a change in the proliferation rates of the AML cells (Fig. 3d). One would expect that an increase in the proliferation rate could be associated with regrowth resistance.

Exploratory studies of cyclin D and cyclin E expression were conducted to determine if the % of cells expressing these genes was related to treatment outcome. Among 22 marrows studied for cyclin D expres-

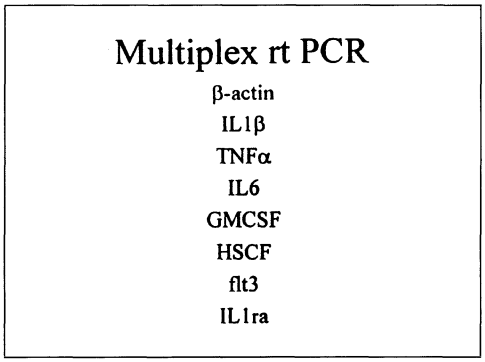

Table 2

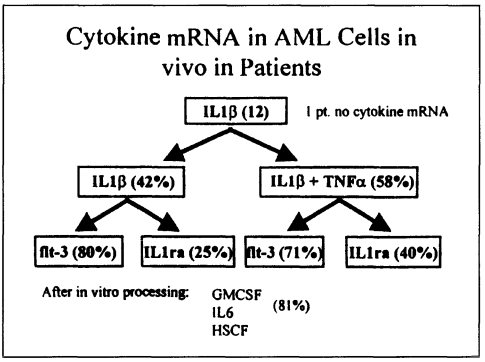

Table 3

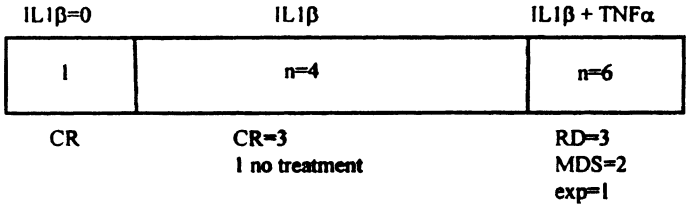

Fig. 3a. Association between TNFα transcripts and remission induction outcome

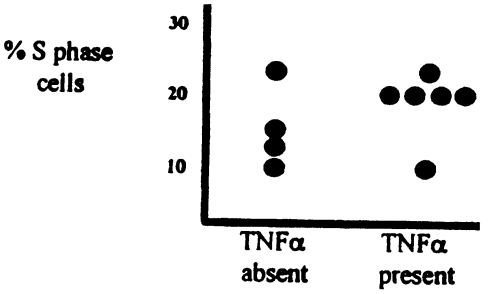

Fig. 3b. Percent S phase leukemia marrow cells in vivo in patients: comparison of AML containing and not containing TNFα transcripts

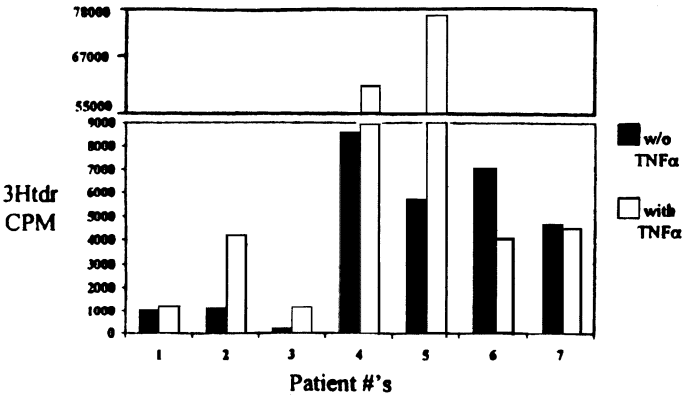

Fig. 3c. Effects of TNFa on DNA synthesis by AML cells in vitro

sion, the average % cells containing the protein was 95±2.5%. With respect to cyclin E, expression was detected in 13.6±13% of cells. Preliminary evaluation failed to detect any relationships between cyclin expression and treatment outcome. In contrast, the % cells containing bcl-2 protein was higher in patients who failed to enter remission than in patients who did enter remission [38% vs. 83%, p=0.0015]. The possible relationship between the expression of this gene and regrowth resistance has already been described [16]. The amount of bcl-2 protein within leukemia cells was also higher in patients who would not enter remission. Division of treatment failures into documented resistant disease and "other" failures [17] demonstrated that both types of failure were associated with high bcl-2 expression.

Insights Obtained from Studies of Cytokine Production by AML Cells in Vivo

The effects of in vitro processing on cytokine gene transcription in AML cells are replicated by the effects of processing in vitro on cytokine production by MDS and chronic myelogenous leukemia cells. Hence at the present time cytokine production by cells can only be accurately performed on cells without in vitro processing. This reality presents obstacles to any studies which are

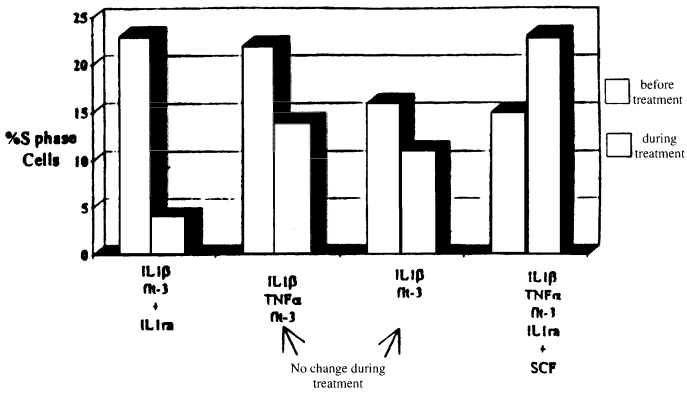

Fig. 3d. Effects of cytotoxic therapy on the %S phase cells in vitro in the marrow and on cytokine transcripts in the same AML cells. Note that in two patients therapy had no effect on cytokine transcripts and in both patients chemotherapy was associated with a modest fall in %S phase cells. In one patient, transcripts for IL1 receptor antagonist appeared and in this patient the % leukemic cells in S phase dramatically fell. In contrast, in one patient transcripts of SCF appeared during treatment and in this patient there was a significant increase in the %S phase AML cells

dependent upon in vitro processing, including cell seperation procedures.

The ability to study cytokine transcripts led to studies directed towards assessing cytokine transcripts present in normal, preMDS (defined as the presence of monoclonal hemopoiesis in patients cured of malignant disease by cytotoxic therapy), MDS, and AML cell populations. Figure 4 compares the cytokine transcripts present in each of these different cell populations. Several interesting observations have been made even at this early date in our studies. With respect to the evolution of the secondary hematologic disorders, the are significant reductions in stem cell factor and IL1 receptor antagonist transcripts during the evolution of these diseases together with what appears to be a gradual fall in flt-3 ligand transcripts.

Several apparent associations are of particular interest:

1. The fall in SCF transcripts which is characteristic of evolution to MDS appears at the same time that the in vitro proliferation defect appears, a defect which is most apparent in the proliferation of erythroid progenitors in vitro. Since the addition of SCF has a profound stimulatory effect on MDS BFUe proliferation in vitro, this observation suggests that the fall in vitro proliferative ability and the fall in SCF production by MDS cells may be causally related.
2. The more modest fall in MDS marrow production of flt-3 ligand parallels the more gradual fall in myeloid colony formation in vitro. A possible causal relationship is suggested by stimulatory the effects of flt-3 ligand on MDS myeloid colony formation in vitro.
3. The continued synthesis of IL1β by cell populations as they evolve to AML together with the fall in IL1 receptor antagonist

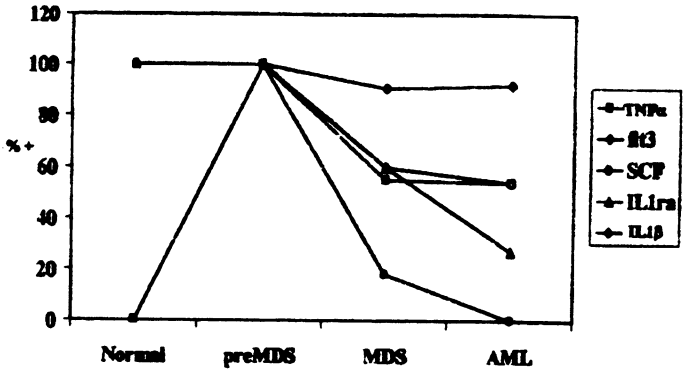

Fig. 4. Changes in cytokine transcript levels during the evolution of the secondary hematologic disorders

transcripts suggests that the loss of feedback control on IL1β production and effects plays a role in the development of the progressive proliferative advantage which characterizes the populations which emerge as the secondary hematologic diseases evolve to more malignant forms.

4 The possible increase in TNFα production with the appearance of preMDS is compatible with our already described relationship between aberrant TNFα production and the genesis of MDS [10].

These data demonstrate that in parallel with the accumulation of genetic abnormalities which characterize the progression of the secondary hematologic disorders, there are parallel progressive changes in cytokine production which contribute to the progressive abnormalities in cell behavior also associated with progression of these disorders.

Potential Therapeutic Approaches to the Treatment of AML

Approaches to the suppression of leukemia regrowth include the direct suppression of the proliferation of these cells, the suppression of the production of cytokines which stimulate leukemia proliferation, and the suppression of the effects of the cytokines on leukemia cells.

With respect standard prognosis disease, while the administration of RA/IFN appears to produce significant prolongation of remission duration, patients still relapse and the therapy produces substantial constitutional symptoms. In some patients, prolonged cytopenia together with the constitutional therapy limits our ability to administer this therapy. Hence one approach to improving remission durations is to develop more effective and less toxic biologic therapies. One interesting aside is that while the therapy was designed on the basis of the apparent ability of RA/IFN to reduce the proliferative rate of AML cells, its apparent beneficial effects may not accrue from this effect since the benefit appears in a higher proportion of patients than the proportion in whom a reduction in proliferation (which occurs during of 3 days' administration of RA/IFN) occurs. It is possible that other effects (induction of apoptosis for example or a direct inhibitory effect on clonogenic AML cells which would not be detected by in vivo assessments of cell proliferation rates), alone or in combination with the effect on proliferation, are responsible for the efficacy of these agents. In addition to these direct effects on AML cells, in some patients the administration of RA/IFN is associated with a reduction in AML production of IL1β but it other cases an increase is seen.

With respect to poor prognosis AML, suppression of the reappearance of MDS cells subsequent to remission induction therapy or during remission by itself would improve treatment outcomes. To this end, the administration of noncytotoxic therapy designed to suppress the regrowth of MDS cells after the end of remission induction therapy might be beneficial. One approach is to administer a combination of pentoxyfillin/ciprofloxacin/dexamethasone which has been shown to produce clinical responses in patients with MDS [18]. Another approach is to administer amifostine after the end of treatment. This agent has been reported to produce clinical responses in MDS patients [19] and to increase the rate of regeneration of normal cells after the end of cytotoxic therapy [20]. Further, as illustrated in Fig. 5a, this agent appears to inhibit the "spontaneous" proliferation of AML cells in vitro. Taken together, the inhibition of MDS and leukemia regrowth, with the facilitation of the regrowth of normal elements, suggests an ideal solution to the problems associated with remission induction therapy in poor prognosis AML. Our current trial in which a single dose of amifostine is administered before remission induction therapy and then three times a week after the end of remission induction therapy is addressing these possibilities.

At the present time we are evaluating the effects of IL4 on AML and CML proliferation in vivo in patients. We became interested in this cytokine because of its reported ability to reduce IL1β production while increasing IL1 receptor antagonist production [21]. As illustrated in Fig. 5b, the agent can reduce leukemia proliferation in patients and can also suppress IL1b production. Trials in CML and AML are being planned.

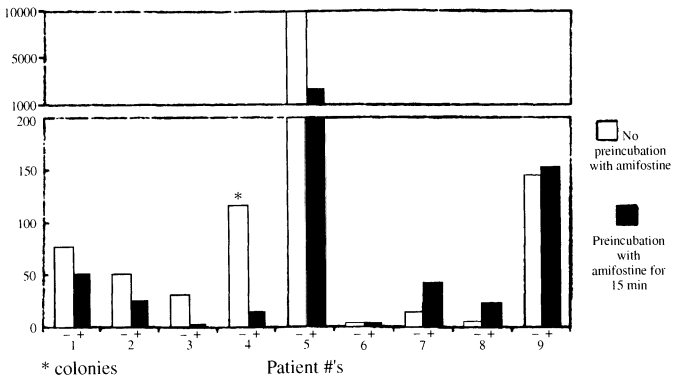

Fig. 5a. Effects of a 15-min preincubation of AML cells with amifostine prior to being plated in methyl cellulose. Cells were cloned in the absence of added cytokines so that the effects of amifostine on "spontaneous" leukemia proliferation could be determined

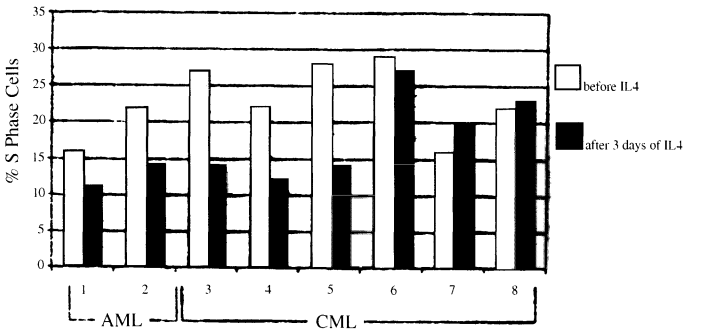

Fig. 5b. Effects of IL4 administered sq for 3 days on the % S phase cells in the marrows of 2 AML patient and 5 CML patients

Two additional biological agents will enter Phase I trials in the Rush Cancer Institute during the 1997/1998. The first is a humanized monoclonal antibody to TNFα. We will first determine if this antibody reduces the proliferative rate of AML cells in vivo in patients and whether or not it is well tolerated. If the answers are yes, we will then conduct a trial in which the antibody is administered immediately after the end of remission induction therapy to, determine if it has the capacity to prevent the regrowth of both MDS and AML cells. The second antibody is an antiIL1b antibody. Interest in this antibody stems from our earlier observations [22], as well as those of others [23], that the addition of the IL1β receptor antagonist prevents the proliferation of AML cells in vitro and from the observations described above that leukemic evolution appears to be associated with unrestrained IL1β stimulation. The possibility that normal hemopoiesis does not require the presence of IL1b makes interference with the effects of this cytokine an attractive possibility.

Finally, both IL1β and TNFα have significant paracrine effects. Hence it is likely that at least part of their biological effects in vivo on MDS and AML cell populations are mediated by other cytokines released in response to stimulation by IL1β and TNFα. Identification of these and their suppression would also be useful in the treatment of these diseases.

Summary

The propensity of leukemia cells [and MDS cells] to regrow between courses of and after the conclusion of cytotoxic therapy continues to present a significant problem. The observations that AML proliferation rates can be reduced by the administration of biological agents offers the possibility for suppression leukemia regrowth. The administration of anticytokines directed against IL1β and TNFα holds out the possibility that the adverse effects of these two cytokines can be suppressed by the administration of anti-

bodies to these two molecules. Interesting pilot studies of IL4 suggest that this agent may also play a role in the suppression of regrowth resistance. Finally, the molecule amifostine appears to have a significant potential for increasing the remission induction rate in poor prognosis AML.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Hematopoietic Growth Factors: Supportive and Priming Effects in AML

T. Büchner, W. Hiddemann, M. Zühlsdorf, B. Wörmann, J. Aswald, S. Aswald, G. Maschmeyer, W.-D. Ludwig, M.-C. Sauerland, and A. Heinecke for the AMLCG

Abstract. Growth factors have been administered after chemotherapy in 12 studies. Eight studies showed benefits in neutropenia and seven studies benefits in infections or survival or remissions. Clinical benefits are mainly found in high-risk patients. There are five negative studies according to major benefits, all five studies using *E. coli* produced GM-CSF. Growth factors before or together with chemotherapy were given in nine studies with trends to more remissions in one and to longer disease-free survival in three studies. Extending the priming strategy to more chemotherapy courses than having been done so far may further improve the antileukemic effect.

For patients with AML hematopoietic growth factors have been administered in order to overcome two limitations of chemotherapy,

1. myelotoxicity and
2. chemoresistance of minimal residual disease.

As a part of supportive care growth factors have been used after chemotherapy in 12 clinical studies [1-12], 8 of them in older-age or otherwise high-risk AML [1, 2, 5, 6, 8-11]. The growth factors used were G-CSF in four [1-4], GM-CSF in seven [5, 6, 8-12] and M-CSF in one [7] of the studies. Beneficial effects could be shown in the duration of neutropenia in eight studies [1-6, 11, 12], frequency of infections or fever in four studies [1, 3, 6, 7], mortality or survival in two studies [5, 6] and remission rate in two studies [2, 4]. The benefits in remissions and survival were found among high-risk patients in three studies [2, 5, 6] and among standard-risk patients in one study [4]. The data from positive studies according to hematological and clinical benefits are listed in Table 1 for G-CSF and in Table 2 for GM-CSF and M-CSF.

One large study in older patients failed to show a clinical benefit possibly due to discontinuation of GM-CSF and placebo in a high-risk group of 34% (GM-CSF) and 31% (placebo) of the patients [10]. One study in younger patients found disadvantages in the remission rate and event-free survival [12], whereas there was no adverse effect of growth factors on therapy resistance, leukemic regrowth or disease-free survival in the other studies. Table 3 shows the negative studies according to major hematological or clinical benefits with growth factors after chemotherapy.

Growth factor priming strategies are based upon their stimulatory effect of AML blasts in vitro [13], their modulation of cellular cytarabine metabolism [14] and enhancement of clonogenic cell kill [15, 16]. Protective effects of growth factors against clonogenic cell kill [17] or apoptosis [18] were also described. There are data from nine clinical studies using growth factors before or concomitantly with chemotherapy [9, 11, 12, 19-24]. Data are listed in Table 4. One study showed a significance [9], two others a

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Table 1. GF supportive use: positive studies I

Study publication	Ref.	Risk	GF	Controls	Benefit Neutropenia	Other
Japan 90	1	Rel./refr. AL	G-CSF	Random	7 d	Infections
France Italy Belgium 95	2	65+ Y	G-CSF	Placebo	6 d	Remissions
Europe Australia 95	3		G-CSF	Placebo	5-7 d	Infections Antibiotics Hospitalisation
Germany Austria Greece 96	4		G-CSF	Placebo	6 d	Remissions

Table 2. GF supportive use: positive studies II

Study publication	Ref.	Risk	GF	Controls	Benefit Neutropenia	Other
Germany 91	5	Relapse or 65+ Y	GM-CSF (yeast)	Historic	7 d	Early death
ECOG 95	6	55-70 Y	GM-CSF (yeast)	Placebo	7 d	Infections Survival
Japan 95	7	Consolidation	M-CSF	Placebo		Infections Antimicrobics Days for 3 courses

Table 3. GF supportive use: negative studies

Study publication	Ref.	Risk	GF	Controls	Modalities	Neutropenia	GF Worse
Houston 90	8	Poor	GM-CSF *(E. coli)*	Historic	120mg/m2		
GOELAM 97	9	55-75 Y	GM-CSF *(E. coli)*	Placebo			
CALGB 95	10	60+ Y	GM-CSF *(E. coli)*	Placebo	Discontinuation in 34% (GM-CSF) and 31% (placebo) pts.	2 d	
EORTC/ HOVON 95	11	60+ Y	GM-CSF *(E. coli)*	Random		faster recovery	Fever Fluid retention
EORTC/ GIMEMA 96	12	10-60 Y	GM-CSF *(E. coli)*	Random		4 d	Remissions Event-free survival

tendency [20, 21] to longer disease-free survival, and two studies showed a trend toward more remissions [24, 25]. A disadvantage in the remission rate and survival was found in one study [12] whereas eight of nine studies did not find evidences for an adverse effect of growth factor priming on the course of the disease [9, 11, 19-24]. In most studies, growth factor priming was only administered in connection with one or two chemotherapy courses. One study giving 4-5 courses found a reduction in relapses during the first 6 months [21] (Fig. 1).

In conclusion, a supportive use of growth factors may have its place in high-risk rather than standard-risk AML. Growth factor priming approaches may not have been adequately investigated so far and an extension of this strategy to more treatment courses now appears more promising.

Table 4. GF priming

Study publication	Ref.	GF	Controls	Modalities	Benefit	GF Worse
Houston 92	19	GM-CSF *(E. coli)*	Historic	20 or 125 μg/m^2 0-8 d before chemoth. 3-4 Courses		Remissions Survival
GOELAM 97	9	GM-CSF *(E. coli)*	Placebo	1-2 Courses	DFS 40% vs. 19%	
South Germany 95	20	GM-CSF *(E. coli)*	Placebo	3 Courses	(DFS ≤ 50 Y, N.S.)	
EORTC/ HOVON 95	11	GM-CSF *(E. coli)*	Random	1 Course		
EORTC/ GIMEMA 96	12	GM-CSF *(E. coli)*	Random	1 Course		
AMLCG 96	21	GM-CSF (Yeast)	Random	4-5 Courses	(DFS < 60 Y, N.S.)	DFS > 60 Y
Buffalo 93	22	G-CSF	Non-random	1 Course		
Houston 94	23	G-CSF	Historic	1 Course		
Japan 94	24	G-CSF	Placebo	1 Course	(Remission, N.S.)	

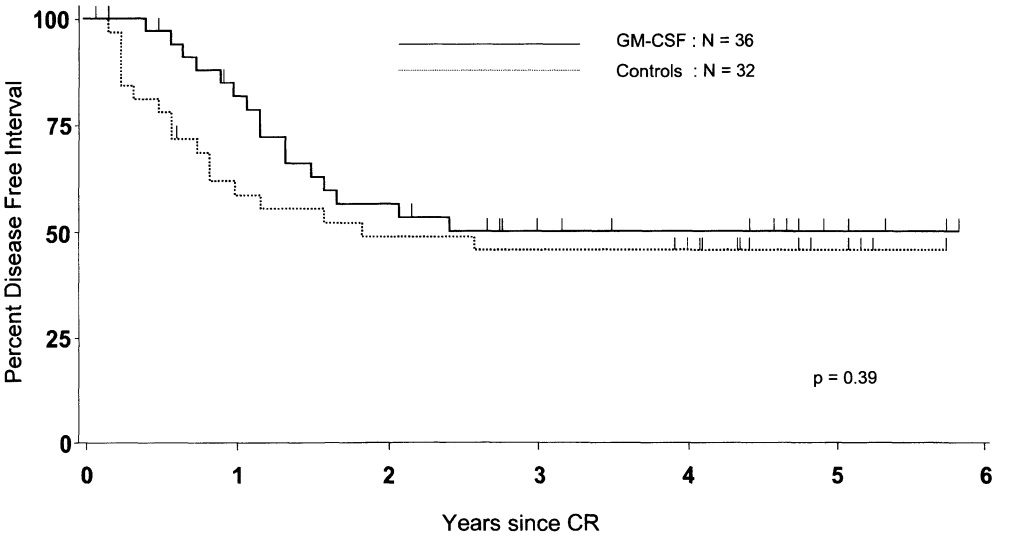

Fig. 1. German AMLCG study on GM-CSF priming in newly diagnosed AML. GM-CSF was given from 24 h before and then during and after chemotherapy in a total of 4-5 chemotherapy courses. *Tick marks* indicate patients without relapse

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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The Role of Growth Factors in the Treatment of AML

H. LINK

Abstract. Infections during neutropenia are major risk factors for morbidity and mortality in chemotherapy of acute myeloid leukemia (AML). Up to 70% of patients suffer from fever during neutropenia. Thirteen comparative studies were analyzed, which were performed aiming at reducing the incidence of infections by stimulation of granulopoiesis with G-CSF or GM-CSF. In three trials the incidence of infections was reduced, whereas the others failed. In the trial of Dombret the rate of complete remissions was significantly increased to 70% with lenograstim as compared with 47% in the placebo group. However, survival was not improved. The concept of sensitizing leukemic cells with G- or GM-CSF before chemotherapy failed in 6 studies. In our placebo-controlled trial with lenograstim (rhu-G-CSF) at a daily dosage of 150 $\mu g/m^2$ following chemotherapy 93 patients received lenograstim and 94 placebo. Within 50 days after the first chemotherapy cycle documented infections occurred in 58/93 (62.4%) patients in the lenograstim group and in 54/94 (57.4%) patients in the placebo group. After two induction cycles, the rate of complete hematological remission was 60.2% with lenograstim and 43.6% with placebo ($p = 0.028$). The number of patients receiving a second chemotherapy cycle was 64/93 (68.8%) with lenograstim and 49/94 (52.1%) with placebo ($p = 0.025$). The duration of remission was similar in both groups. Following the first chemotherapy cycle, neutrophils recovered after a mean (SE) of 12.6 (1.1) days with lenograstim and after 18.2 (1.1) days with placebo ($p < 0.001$). Lenograstim following chemotherapy did not reduce the incidence of fever or documented infections during the first 10 days after chemotherapy, however thereafter fewer patients became infected than with placebo. Conclusion: none of the published studies showed negative effects of G-CSF or GM-CSF on the course of leukemia. G-CSF or GM-CSF can be safely given in AML for accelerating neutrophil recovery Their effect on remission rate and infection has to be determined.

Introduction

Treatment of AML means reducing the malignant cell population with combined cytotoxic therapy, and inducing a hematological complete remission. Once a remission is achieved, further consolidation therapies for prolonging remission are necessary. Preferentially an allogeneic or if no donor is available an autologous bone marrow or stem cell transplantation should be performed. In patients up to fifty years, an event free survival probability of 37% with high dose ARA-C and of 72% with allogeneic BMT can be achieved [1, 2]. All treatments are rather bone marrow toxic in order to maximally reduce the malignant cell clone. This leads to severe and prolonged bone marrow suppression with granulocytes be-

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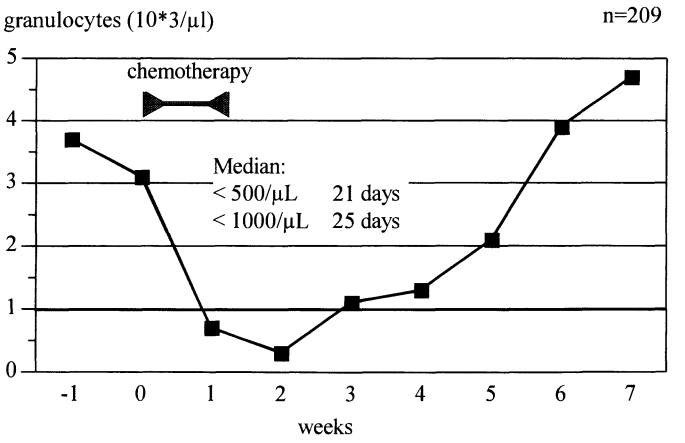

Fig. 1. Duration of granulocytopenia after chemotherapy in AML (AMLGCSF 1-4)

low 500/µl for 20 days and below 1000/µl for 25 days, as analysed in our own study group (see Fig. 1).

The use of the hematopoietic growth factors G-CSF and GM-CSF has become standard after intensive chemotherapy. However, the sensitivity of malignant myeloid cells by expressing specific receptors for these growth hormones has been a major obstacle in using them for prevention of infections after chemotherapy. On the other hand stimulation of clonogenic malignant AML cells with growth factors into cell cycle could render them more susceptible to chemotherapy. Therefore clinical and biological end points have to be considered, if G-CSF or GM-CSF are explored in clinical AML-trials.

First, if G-CSF or GM-CSF are given following chemotherapy, the aim is to reduce the duration of neutropenia and possibly to reduce the incidence and severity of infections. As we know from studies with neutropenic infections after chemotherapy [3], the patients with rising leukocytes respond better with significantly fewer infectious deaths than patients with stable or declining leukocytes, if they had been below 500/µl at the onset of infection (see Fig. 2).

G-CSF (Lenograstim) in Acute Myeloid Leukemia, the Study of the SHG

We have performed a multicenter placebo controlled double blinded study with lenograstim following cytotoxic chemotherapy on newly diagnosed AML, with the aim, to reduce the incidence of infections during neutropenia [4]. By this way outcome should be improved. Furthermore morbidity of chemotherapy induced neutropenia should be reduced. A total of 187 patients was randomised, with a planned interim analysis with 100 patients. The participants of this international study are listed in Table 1.

The objectives of the trial were:

1. The incidence of neutropenic infections during the first 50 days after cytostatic induction chemotherapy.
2. The time to recovery of neutrophils > 500/µl.
3. Influence on rate and duration of hematological remission.
4. The analysis of side effects, especially the assumed stimulation of leukemic blasts.

Table 1. Participants of the lenograstim study in Europe

Cooperative AML study group of the SHG	
G. Ehninger, Dresden	A. Franke, Magdeburg
R. Gäckle, Stuttgart	T. Geer, Schwäbisch Hall
M. Gramatzki, Erlangen	H. Link, Hannover
W. Linkesch, Graz	D. Lutz, Linz (A)
D. Niederwieser, Innsbruck (A)	E. Nikiforakis, Athen (GR)
S. Öhl, Wuppertal	B. Otremba, Oldenburg
E. Pittermann, Wien (A)	H. Schmidt, Tübingen
P. Schönrock-Nabulsi, Hamburg	J. Tischler, Minden
H. Wandt, Nürnberg	J. Weiß, Regensburg
	M. Wilhelm, Würzburg

Supported by S. Badri, Chugai Rhône-Poulenc, Antony (F)

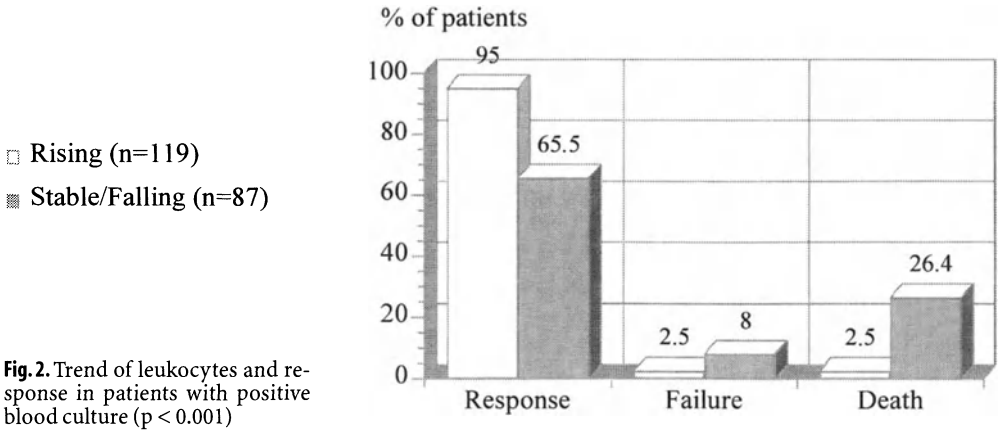

Fig. 2. Trend of leukocytes and response in patients with positive blood culture ($p < 0.001$)

The study drug (lenograstim 150 µg/m^2 or placebo) was given daily as short i.v. infusion, beginning 24 h after the last chemotherapy until 500 neutrophils/µl were reached for 3 consecutive days. The maximal treatment period was 28 days after each chemotherapy cycle. Patients were stratified according to age ≤ and > 60 years and for the different AML-treatment protocols. The study drug was given after each chemotherapy and for a maximum of 4 cycles.

Infections were classified as microbiologically, clinically or clinically and microbiologically documented and as fever of unknown origin [3]. At the final analysis 93 patients had received G-CSF and 94 placebo, their median age was 52 and 54 years respectively. The types of chemotherapy were comparable in both groups.

Documented infection until 50 days after the first induction cycle occured in 58 (62%) of 93 patients with G-CSF and in 54 (57%) 94 patients with placebo. A complete hematological remission after two induction cycles was achieved in 63 % with G-CSF and 45 % with placebo ($p = 0.025$). The median (standard error) time to neutrophil recovery above 500 cells per µl was 12.7 (1.1) days with lenograstim and with placebo 18.2 (1.1) days ($p = 0.001$). There was no difference in adverse events and relapse rate between both groups. Several other comparative trials with G-CSF or GM-CSF have been reported in recent years and are summarized in table 2. When the impact of G-CSF or GM-CSF on the incidence of infections is analysed it is assumed, that this effect is indirect by stimulation of granulopoiesis or additionally monocytopoiesis. Considering the occurence of infections after chemotherapy early during neutropenia, these infections cannot be prevented as granulopoietic regeneration cannot be expected befor day ten if an usual bone marrow toxic chemotherapy regimen is applied. If the incidence of infections is analysed beyond day 10, as in our placebo-controlled study with GM-CSF after autologous bone marrow transplantation [5], then the occurrence of bacterial infections is significantly reduced in parallel to the granulocyte recovery. One study [6] analysed this effect separately for AML patients who had been afebrile for 9 days after the end of chemotherapy. Two (14%) of 15 patients with G-CSF and 7 (29%) of 24 patients with placebo presented infectious episodes. One (7%) patient with G-CSF and 7 (29%) patients with placebo developed documented infections 1 week later (6). The number of patients being too small for a statistical analysis; these data, however, show that infections might be reduced by an accelerated neutrophil recovery. With lenograstim we found a smaller proportion of new infectious episodes after day 10 following chemotherapy (Fig. 3). This effect was also seen after the second and third chemotherapy cycle.

Studies with G-CSF or GM-CSF in AML

Several comparative studies have been performed with either GM-CSF or G-CSF after

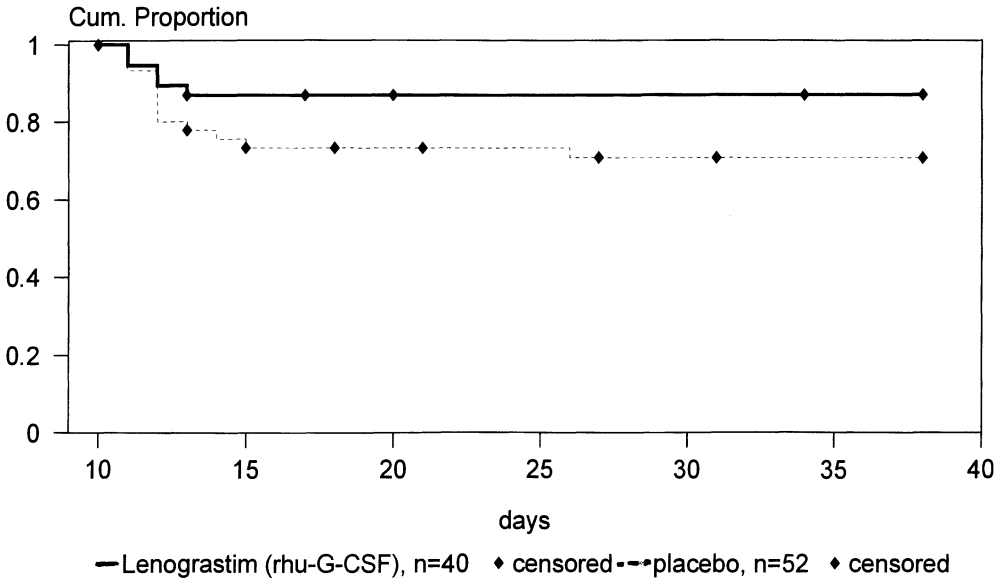

Fig. 3. Incidence of first bacterial infection after chemotherapy

Table 2 a. Effects of G-CSF orGM-CSF in AML therapy, as shown in clinical trials

Study h: historical control p: placebo controlled	G-CSF during therapy	n	Days to neutrophils 500/µl G-CSF, controls		Infection rate	CR-rate % G-CSF, controls		Remission length	Survival
Ohno 1990 (8)	-	61	20	28	↓	57	39	=	=
Ohno 1994 (6) p	+	58	17,	22	=	54	42	=	=
Estey 1994 (9) h	+	197	6[a],	29[a]	=	53	63	=	=
Heil 1995 (10) p	-	521	20	25	↓	69	68	=	=
Dombret 1995 (7) p	-	173	21[a],	27[a]	=	70	47[b]	=	=
Maslak 1996 (11) h	-	26	13	17	=	58	46	=	=
Link 1996 (4) p	-	187	13,	18	=	63	45[c]	=	=

[a] Days to 1000 neutrophils/µl.
[b] $p = 0.002$; [c] $p = 0.012$.

Table 2 b

Study p: placebo-controlled	Stimulation of blasts	n	Days to neutrophils 500/µl GM-CSF, controls		Infection rate	CR-rate GM-CSF, controls		Remission length	Survival
Büchner 1994 (12)	+	94	Shorter with GM-CSF			81	84		
Rowe 1995(13) p	-	124	16	20	↓	61	46[a]	=	↑
Stone 1995 (14) P	-	388	15	17	=	52	54	=	=
Heil 1995 (15) p	+	80	12	11	=	81	79	=	=
Löwenberg 1995 (16)	+	326	Reduced with GM-CSF		=	56	57	=	=
Witz 1995 (17) p	+	244	22	27	=	62	61	Disease free survival ↑	

[a] $p < 0.05$.

AML chemotherapy, see Table 2. In 11 of 13 studies, neutropenia was reduced, however the difference being small in some of them with GM-CSF. The rate of infections was decreased in only three studies. An increased rate of complete remission was reported in two studies and a trend towards a higher remission rate in one trial. Two studies reported an improved survival of the patients. Of note, however, is that in none of these studies was disease progression reported. The higher remission rates in some trials might be due to the fact that therapy could be conducted more consequently with G-CSF than with placebo. In our study significantly more patients received a second chemotherapy cycle for remission induction with G-CSF (68.8%) than with placebo (52.1%), $p = 0.025$. In the study of Dombret [7], a higher response rate with G-CSF was mainly observed in patients with residual marrow blasts by day 8. This was confirmed by our trial, with a complete remission rate of 62% with lenograstim and of 26% with placebo ($p = 0.0009$) in patients with >2% blasts by day 8.

Conclusion

In conclusion, the duration of neutropenia can clearly be shortened, the infection rate and duration of hospitalisation might be reduced. For further reduction of neutropenia and of infections, the addition of autologous peripheral blood progenitor cells might be considered. The improvement of remission rate and survival is only shown by a minority of studies. It might be due to an exacter dosage of drugs and a preciser conduct of the chemotherapy protocol, rather than by biological effcts of the growth factors on the clonogenic malignant cells. However, G-CSF or GM-CSF can be given without the risk of leukemic stimulation. Thus G-CSF can be given safely following the treatment of AML as in the intensive chemotherapy of other malignancies.

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Growth Factors in Elderly Patients with Acute Myeloid Leukemia

P. CHAIBI, H. DOMBRET, and L. DEGOS

Abstract. AML disease in elderly patients is a frequent and severe disease with a high early death rate, low complete remission rate, and short median survival. By reducing the intensity of the induction treatment the complete remission was reduced. The proposal is to give an intensive chemotherapy to elderly patients in order to have a high complete remission rate associated with a drug which potentially minimizes the early mortality. Since the early promising reports, several large prospective, randomized controlled trials have assessed the efficacy of G-CSF in elderly patients. No significant induction of leukemia regrowth was observed in the various trials with either G-CSF or GM-CSF. No clear reduction in chemotherapy related mortality was observed. CSFs increase the complete remission rate after intensive induction treatment in patients with poor prognosis factors mainly patients with persistant blast infiltration in bone marrow after induction treatment. CSFs could also improve the survival time in some cases, mainly in the younger population of elderly persons.

Introduction

Acute myeloid leukemia (AML) in the elderly is a frequent disease. More than 40% of patients with AML are over 65 years old at the time of diagnosis [1, 2]. AML disease in elderly patients often has poor prognostic factors such as involvement of immature progenitor cells [3] or myelodysplastic features or a prior myelodysplastic syndrome [4, 5], and particular chromosomal abnormalities [6, 7, 8] such as monosomy 7 or the loss of the long arm of chromosome 5.

The mortality rate during induction therapy increases with age. For patients over 55 to 60 years of age, the treatment related early death rate is in the range of 25 to 40% [9, 10, 11, 12].

If complete remission is achieved in more than 75% of adults less than 60 years old, only about 45% of elderly patients receiving similar treatment have a complete remission [13, 14, 15]. In the AML 8 trial in UK [13] the complete remission rate is directly related to the age (70% before 60 years old, 52% between 60-69 years old, and 26% after 70 years old).

The median survival, even after complete remission, still remains relatively short between 9 to 12 months, in elderly AML patients [10, 12, 16], although a relatively low median age of enrolled patients ($\leq$ 70 years).

From these poor results, high early death rate, low complete remission rate, short median survival, several approaches were attempted to improve the outcome of the acute myeloid leukemia in elderly patients.

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Is It Possible to Reduce Early Mortality by Reducing the Intensity of the Induction Treatment?

A multicentric randomized trial was conducted comparing low dose Cytosine Arabinoside (LD Ara-C) to conventional chemotherapy (Daunorubicin and Cytosine Arabinoside) [15] in order to reduce the early mortality. The early death rate was 10% in patients treated by LD Ara-C, compared to 32% in patients treated by conventional chemotherapy. The number of transfusions, days of hospitalisation and the antibiotic requirements were reduced in patients treated by LD Ara-C. The median survival was similar in both population. Thus the early mortality rate is reduced while simultaneously the complete remission was also reduced.

Are the Growth Factors Able to Reduce the Early Mortality Rate?

The proposal is to give an intensive chemotherapy to elderly patients in order to have a high complete remission rate, associated with a drug which potentially minimizes the early mortality. Infections cause approximately two third of treatment associated deaths [17, 18, 19]. The use of myeloid CSFs could decrease the time of neutropenia and thus the number of infections. However the potential for in vitro cytokine stimulation of leukemia cells has reduced the enthousiasm in conducting trials with colony stimulating factors.

The first trial was conducted by Buchner et al. [20, 21]. GM-CSF was administered in 30 elderly or relapsed AML patients with an aplastic bone marrow after the completion of chemotherapy. A historical control group of similar patients who had not received GM-CSF was used for comparison. The duration of neutropenia was reduced by 6 to 9 days in patients treated with GM-CSF. The early death rate was significantly reduced (14 vs. 39%) and there was a trend towards more complete remissions in the GM-CSF group (50 vs. 32%). Remission durations were identical in both groups. Two patients experienced marked leukemic regrowth with GM-CSF therapy; however, this was totally reversible in one of them once GM-CSF had been discontinued.

In 1990, Ohno et al. [22] published the results of the first Japanese randomized controlled study of G-CSF administered after the completion of induction chemotherapy in a heterogeneous population of patients with refractory or relapsed acute leukemia. Each patient received an individualized, response-oriented induction course of mitoxantrone, etoposide, and behenoylcytosine arabinoside. Mitoxantrone and occasionally etoposide doses were increased in cases of persistent blast cells in the bone marrow examination on Day 8, Day 10, and sometimes Day 12. Only patients achieving a severe bone marrow hypoplasia after chemotherapy were randomized to receive G-CSF or placebo. Even though patients treated with G-CSF received higher doses of chemotherapy than those treated with placebo, their neutrophil counts recovered significantly earlier to a level higher than $500/mm^3$ or $1000/mm^3$. The duration of neutropenia was decreased by about 1 week. The incidence of documented infections was significantly lower in the G-CSF group. There was no difference between the two treatment groups in terms of leukemia regrowth with G-CSF / placebo therapy. Furthermore, there was a trend towards a higher CR rate in the G-CSF group (50% compared to 36% in the placebo group). Remission durations were similar in both groups.

Since these early promising reports, several large prospective, randomized, controlled trials have assessed the efficacy of CSF (or GM and G-CSF) in elderly patients [23, 24, 25, 26, 27, 28].

No significant induction of leukemia regrowth was observed in the various trials with either G-CSF or GM-CSF.

Some investigators restricted CSF administration to patients with a documented aplastic bone marrow after the induction course, as used in early Japanese and German reports. In contrast, the growth factor was administered not only after the completion of chemotherapy, but also during and occasionally before the chemotherapy in some studies. The effects of CSF in the history of the disease are detailed in Tables 1, 2 and 3.

Table 1. Randomized trails with G-CSF in elderly patients

	G-CSF after the 1st course EACSG (Dombret 1995) Lenograstim	G-CSF after courses 1 and 2 SWOG (Godwin 1995) Filgrastim
Median age	71 (> 65)	66 (> 55)
N	173	193
Randomization	all patients	aplastic patients
Neutropenia	Reduced	Reduced
Survival	Similar	Similar
CR rate	Increased 70 vs. 47%	Similar 42 vs. 49%

Table 2. Randomized trials with GM-CSF in elderly AML patients given after induction chemotherapy

	GM-CSF after the 1st course CALGB (Stone 1995) Molgramostim	GM-CSF after courses 1 and 2 ECOG (Rowe 1995) Sargramostim
Median age	69 (> 60)	64 (55 – 70)
N	388	117
Randomization	All patients	Aplastic patients
Neutropenia	Reduced	Reduced decreased incidence of infections
Survival	Similar	Longer median (overall survival)
CR rate	Similar 51 vs. 54%	Similar 60 vs. 44%

Table 3. Randomized trials with GM-CSF in elderly AML patients given during and after induction chemotherapy

	GM-CSF during and after the 1st course GOELAM (Witz 1995) Molgramostim	GM-CSF during and after courses 1 and 2 EORTC-HOVON (Lowenberg 1995) Molgramostim
Median age	67 (55-75)	68 (> 60)
N	232	316
Randomization	All patients	All patients
Neutropenia	Reduced	Reduced
Survival	Increased (DFS in 55 - 65 y)	Similar
CR rate	Similar 62% vs. 61%	Similar 56% vs. 55%

The period of neutropenia induced by the induction course was consistently reduced after the induction course, sometimes by as much as a week. This effect was not seen after the consolidation courses. No clear reduction in chemotherapy-related mortality was observed.

Are Growth Factors Able to Increase the Complete Remission Rate?

Complete remission rate were similar or higher in the CSF randomized groups than in the control group in all studies. Two studies found a higher CR rate in the CSF group (only one reached a statistical significance). A highly significant increase in the CR rate was observed by Dombret et al. [23] using glycosylated G-CSF. This increase did not result from a reduction in treatment related mortality from infection but rather from a lower incidence of resistant patients. Furthermore, the benefit of G-CSF administration was mainly observed in AML patients with poor prognostic characteristics, such as unfavorable cytogenetics or marrow blasts persisting after the completion of induction chemotherapy (Table 4).

A second trial [26] using GM-CSF after courses 1 and 2 showed an increase (but non significant) of the CR rate (60 vs. 44%).

Analysing the results of the 6 randomized trials, one can conclude that the CR rate is increased if the CSF are given not during the intensive chemotherapy but after the induction treatment, and if the treatment is strong enough to give a CR rate over 60% in patients. The effect seems to be restricted to the population with poor prognosis factors.

This observation of an increase of CR rate suggests that G-CSF may contribute to the antileukemic effect of chemotherapy. The possible mechanisms remain unclear but include a growth advantage for the normal hematopoietic cells, a differentiation effect, and an induction of residual leukemia cell apoptosis, or a cytokine-mediated effect.

Are Growth Factors Able to Increase the Median Survival?

Two trials described an increase in DFS [28] or median overall survival [26]. Both of them used GM-CSF. The growth factor was administered either during and after the induction treatment or only after the induction treatment. Two characteristics have to be pointed out : (1) both trials treated patients from 55 years old, and for one trial [28] the increased DFS was only seen in the cohort 55-65 years ; (2) both trials have a high complete remission rate (over 60%) reflecting the intensity of the induction treatment.

Conclusion

The two principal positive results obtained through the use of granulocytic growth factors in the treatment of AML in the elderly are the safety of their use with respect to the risk of malignant clone stimulation and the reduction in the duration of neutropenia induced by the cytotoxic agents employed for

Table 4. Effect of G-CSF on the complete remission rate in patient with residual bone marrow blast infiltration after chemotherapy. Results of a randomized trial in elderly AML patients [23]

	Complete Remission Rate		
	Lenograstim n = 88	Placebo n = 85	
Residual bone marrow Blasts at day 8 (end of chemotherapy)			
Present	67% (28 / 42)	35% (16 / 46)	P < 0.005
Absent	76% (34 / 45)	63% (24 / 38)	

treatment. It seems that CSFs increase the complete remission rate after intensive induction treatment in patients with poor prognosis factors mainly patients with persistant blast infiltration in bone marrow after induction treatment. CSFs could also improve the survival time in some cases, mainly in the younger population of elderly persons.

One research option could be to define patient subgroups most likely to benefit from the administration of CSFs. In fact, it is possible that the discordant results obtained in published randomized trials are due to differences in the profile of patients treated, and of the type of chemotherapy used for induction treatment.

A second research option could be to test either the combined use of several growth factors or the sequential use of "protecting" factors and CSFs. Such combinations might achieve higher reductions in the duration of chemotherapy-induced neutropenias and consequently an improvement in rates of mortality from infections.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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The Role of Growth Factors in Myelodysplastic Syndromes: Biological and Clinical Factors

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Introduction

The myelodysplastic syndromes (MDS) comprise a heterogeneous group of bone marrow disorders characterized by ineffective and dysplastic hematopoiesis in one or more cell lines. Prognosis in MDS is extremely variable. Evolution to acute nonlymphocytic leukemia (ANLL) occurs in 20–75% of the cases. There is also a high excess mortality rate in these patients compared with that of the general population, especially from infection and/or bleeding, due to cytopenia and defective function of neutrophils.

Treatment

A variety of treatment approaches have been use in MDS. Supportive therapy with antibiotics and/or transfusions of blood cell products has remained the main therapeutic option, due to the advanced age of these patients and their relatively poor responses to chemotherapy. Transfusions are associated not only with increasing inconvenience to the patient, but also with the risk of transfusion reactions, iron overload, and transmission of viral or other infectious agents.

Hormonal Therapy and Differentiation-Inducing Agents

In selected patients corticosteroids, anabolic steroids and differentiation-inducing agents such as *cis*-retinoid acid, vitamin D analogs, and interferon α or γ have been claimed to be active [1].
Although all-*trans*-retinoid acid (ATRA) works remarkably as a differentiation therapy in acute promyelocytic leukaemia, its effect in MDS was modest with respect to neutrophils, hemoglobin and platelets [2]. Previous attempts at treating patients with low dose chemotherapy, such as cytosine arabinoside (Ara-C) as a differentiating and/or cytostatic agent, or 5-azacytidine have resulted almost uniformily in failure to improve long-term survival [3].

Haematopoietic Growth Factors

In theory, the haematopoietic growth factors could exert their action in various ways: decreasing the morbidity and mortality associated with prolonged anaemia, neutropenia and/or thrombocytopenia by stimulation of the proliferation and maturation of residual normal or leukaemic haematopoietic cells into red cells, granulocytes and/or thrombocytes, respectively; they could enhance the impaired functional capacities of granulocytes [4] and may synchronize the leukaemic cells, therapy increasing the effectivity of S-phase-specific agents; they may shorten the aplastic phase after intensive chemotherapy.

Erythropoietin in MDS

Thirty one separate trials, involving 520 MDS patients, have been reported (Table 1)

Table 1. Response rates to colony-stimulating factors in patient with myelodysplastic syndromes[a]

	No. of patients	Increase in neutrophils (%)[b]	Increase in hemoglobin (%)[b]	Increase in platelets (%)[b]	Increase in blasts (%)
G-CSF	73	66 (90)	6 (8)	4 (5)	3 (4)
GM-CSF	263	199 (76)	4 (2)	14 (5)	32 (12)
IL-3	135	49 (36)	6 (4)	29 (21)	11 (8)
IL-6	22	0 (0)	0 (0)	8 (36)	2 (9)
Epo	520	0 (0)	133 (25)	8 (1)	4 (0.7)
IL-3 + GM-CSF	9	7 (77)	0 (0)	3 (33)	1 (11)
Epo + G-CSF	71	62 (87)	18 (25)	1 (1)	6 (8)
Epo + CM-CSF	24	19 (79)	8 (33)	3 (12)	2 (8)
Epo + IL-3	23	16 (70)	4 (17)	5 (21)	0 (0)
Epo + G-CSF + ATRA + α tocopherol	10	9 (90)	4 (40)	2 (20)	NS

[a] Data based on references quoted in the text
[b] Response criteria included an increase in neutrophils (doubling with a minimum of 0.5×10^9/l), or platelets (doubling with a minimum of 50×10^9/l), a rise in hemoglobin by 2 g/dl, or reduction in transfusion requirements by 50%; NS, not stated.

[5]. An increase in hemoglobin level was noticed in 20%, and a reduction of RBC transfusion requirements in 24% of patients. There was considerable variation in rHuEPO dosing, ranging from as low as 30 U/kg three times weekly, up to 100 000 U two times weekly. Erythroid response, if seen, will usually occur within the first 8 weeks of rHuEPO treatment. The current practice of rHuEPO administration is three times per week, subcutaneously, with a starting dose of 150 U/kg, with escalation up to 300 U/kg in patients not initially responding after 4 to 8 weeks of rHuEPO treatment. As expected from the known in vitro actions of erythropoietin, there was virtually no effects on neutrophils and only occasional improvements in platelet counts.

A recently published meta-analysis of 205 patients from 17 studies identified factors associated with response to erythropoietin therapy [6]. These factors included no transfusion need (44% versus 10%), endogenous serum erythropoietin level below 200 U/l, and absence of ringed sideroblasts (21 versus 7.5%). This meta-analysis could identify a group of patients (i.e., patients without transfusion need and MDS other than RARS) with a response rate of $\geq$ 50%, irrespective of their serum level of erythropoietin. At the other extreme, no response was seen in patients with RARS and serum erythropoietin levels $\geq$ 200 U/l.

Granulocyte (G-CSF) and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) in MDS

G-CSF has been administered by both intravenous infusion and subcutaneous injection. Pooled data from five trials of G-CSF that have previously been summarized [7] have shown increased neutrophils in 90% of MDS patients and minimal effect on anaemia and thrombocytopaenia (Table 1), although there have been rare reports of MDS patients who showed trilineage haematologic responses to G-CSF. There was no correlation between the subtype of MDS and response rate. Toxicity during G-CSF therapy was minimal, although sharp decline in platelet counts after starting G-CSF therapy can create serious bleeding problems in the first weeks of therapy. The best dose of G-CSF to be used in MDS is still a matter of debate. A practical approach would be to start with 1 μg/kg day and to adjust the G-CSF dose to obtain neutrophils around 2 500/μl.

Several investigators have reported the results of phase I/II therapeutic trials with GM-CSF [7–9]. These trials have utilized different drug preparations, dosages and schedules of administration of GM-CSF for varied intervals. The combined data from these studies, including 263 MDS patients, showed that GM-CSF was effective in increasing neutrophil counts in 76% of the

patients (Table 1). In addition to the increase of neutrophils, further cell lineages were stimulated, including eosinophils, monocytes, and lymphocytes. The counts generally returned to baseline levels upon discontinuation of the cytokine. The responses in other cell lineages such as platelets and hemoglobin were limited to only a fraction of the patients (2–5%). Adverse effects of GM-CSF which occur in 25% of patients at "conventional dose" (60–250 $\mu g/m^2$), include fever, bone pain, local erythema, phlebitis, decrease in platelet counts, fluid overload, and, rarely an adult respiratory distress syndrome. An interesting approach for long-term GM-CSF therapy seems the administration of "very low dose" of GM-CSF (0.25–0.5 $\mu g/kg$), since recent studies suggest that it might be possible to divorce the toxic effects of GM-CSF from the therapeutic benefits [9].

Two central questions in the use of G-CSF and GM-CSF is whether they produce survival benefit or significant decrease in morbidity from infection, and whether the use of these growth factors causes increased rate of progression to leukaemia. A significant proportion of patients with RAEB and RAEBt receiving GM-CSF (and to a lesser extent G-CSF) do develop transient increases in blast cell counts while undergoing therapy that revert to pretreatment levels when therapy is withdrawn. In contrast, several reports have documented the induction of leukaemic transformation by GM-CSF in CMML [10, 11]. Preliminary results of a randomized, controlled clinical trial comparing GM-CSF for 90 days versus observation, did not show differences in transformation to RAEBt/ANLL in the different treatment arms [12]. As expected, there was a significant increase in WBC in the treatment arm which was associated with fewer major infections during the 90-day treatment period. A multicenter study comparing G-CSF and observation did not show significant differences in the rate of progression to ANLL between treated and untreated patients [13]. However, there was a shorter median survival in the G-CSF treated group of patients with RAEB. Death was due to nonleukaemic, disease-related causes. Decreased survival in treated RAEB patients was probably due to the increased number of high-risk patients included in the G-CSF treated arm and the unusually long survival of RAEB patients in the control arm. Infection rates in the two groups have not yet been reported. No survival benefit has yet been demonstrated for growth factor therapy in MDS. Combined therapy with low-dose Ara-C and GM-CSF or G-CSF, respectively, has not proven to be superior to therapy with low-dose Ara-C alone, with regard to survival, or toxicity, and resulted in CR rate of 15 to 20% and a PR rate of 20% [14, 15].

Interleukin-3 (IL-3) in MDS

One long-term and seven short-term studies have reported the effects of IL-3 therapy in 135 MDS patients (Table 1) [16–19]. There is an overall response rate for neutrophils of approximately 36%, increase in hemoglobin level of 4%, platelets 21%, and a 8% increase in bone marrow blasts. Trilineage response was only seldom seen. These data indicate that IL-3 must be combined with other haematopoietic growth factors to achieve substantial improvement in MDS cytopenias. Adverse effects of IL-3 were significant, especially at higher doses, and consisted of eosinophilia, fever, bone pain, myalgias and headache, necessitating discontinuation of IL-3 in several patients.

Interleukin-6 (IL-6) in MDS

IL-6 has apparent effect on megakaryopoiesis. One short-term study has reported the effects of IL-6 in 22 low-risk MDS patients with $< 5\%$ bone marrow blasts and $< 100\,000/\mu l$ platelets [20]. Eight patients experienced at least a transient improvement in platelet counts. Two of the three patients who received maintenance IL-6 therapy had a persistent increase in platelet counts, during 3 and 12 months of IL-6 therapy, respectively. Moderate to severe toxicity with constitutional symptoms occurred without leukocyte improvement, and worsening anemia developed, but without a significant effect on transfusion requirements.

Combination of Cytokines in MDS

The infrequent multilineage responses with recombinant human growth factors have led to studies involving combinations of growth factors, either simultaneously or sequentially; in order to target proliferation and differentiation of both early and late stages of haematopoietic progenitor cells. However, combination of growth factors, especially that act early in haematopoiesis, must be balanced against potential risk of increased rate of progression to acute leukaemia, and side effects of combinations of growth factors may be additive.

Preliminary trials with the combination of erythropoietin and G-CSF or GM-CSF, respectively, have yield impressive results with erythroid responses of 42% compared to 20–25% with erythropoietin alone (Table 1) [21–23]. Additional studies, however, could not support these synergistic effects of combination therapy, and resulted in erythroid responses comparable to erythropoietin alone [24–26]. Dose schedule, timing and duration of the growth factors may and difference in the study population, at least partly, explain these conflicting results.

Treatment with IL-3 and erythropoietin has so far been dissapointing [27–28]. A worrisome finding was the development of thrombocytopenia in 50% of the patients, probably related to the induction of tumor necrosis factor α (TNFα), a rather potent inhibitor of megakaryopoiesis.

The combination of ATRA with G-CSF, erythropoietin and tocopherol resulted in increased neutrophils in 90%, an erythroid response in 40%, and increased thrombocytes in 30% of the patients, respectively [29]. The induction of TNFα might be responsible for treatment failure.

Sequential therapy with interleukin-3 followed by GM-CSF gave improved absolute neutrophil count in 77% and improved platelets in 33% [30]. However, toxicity was unacceptable.

Administration of growth factors in MDS rarely induces polyclonal haematopoiesis as evaluated by repeated cytogenetic investigations and analysis of restriction fragment length polymorphisms [7, 31].

Intensive Chemotherapy

Therapeutic strategies in MDS have historically been inspired by either "missionary" approaches, i.e., converting malignant cells into normal behaviour, or by "crusader" tactics, i.e., destroying non-compliant elements at the expense of innocent bystanders. At present, long-term benefit, however, can be achieved only by eradication of the abnormal clone and restoration of normal hematopoiesis ("crusader" tactics). This can possibly be accomplished by high-dose chemotherapeutic regimens designed for treatment of ANLL and more durable by allogeneic bone marrow transplantation.

Selection of patients for whom intensive chemotherapy is appropriate is difficult. Patients belonging to the poor-risk MDS category, where the general medical condition allows it, should be considered for ANLL-type chemotherapy in the setting of multicentre, controlled trials. In several clinical trials investigating standard ANLL induction therapy for advanced MDS, complete remission rates have ranged from 13 to 51% [32, 33]. However, a high proportion of individuals showed significant morbidity or suffered a toxic death due to therapy. The duration of response is generally short (3–11 months).

Allogeneic Bone Marrow Transplantation

The use of allogeneic bone marrow transplantation currently offers the only potentially curative treatment. A recent retrospective update by the EBMT demonstrated a disease-free survival for MDS patients without excess of blasts (RA and RARS), with refractory anemia with excess of blasts (RAEB) and with excess of blasts in transformation (RAEBt) and secondary ANLL of respectively 46, 35, 27 and 0% 5 years after the transplantation [34].

However, the use of allogeneic BMT for MDS patients is available only for a minor proportion of patients because of lack of HLA-identical donors and/or because of the high median age of patients with MDS. Transplant-related mortality increase with age, because of a decreased ability to tolerate

the toxicity of the therapy and an increased incidence and severity of both acute and chronic graft-versus-host disease [35].

Alternative Sources of Hemopoietic Stem Cells

Transplants from Unrelated Marrow Donors

The outcome of MDS patients grafted from unrelated marrow donors presently is unsatisfactory with a disease-free survival of 18% at 2 years [36]. However, recent data on unrelated bone marrow transplantations in children are more encouraging. Casper et al. reported that five of nine patients survived 27 to 80 months posttransplant with four of them staying in remission [37].

Autologous BMT

In poor-risk patients not eligible for allogeneic transplantation, the prognosis remains poor. Encouraged by data obtained in ANLL, recent attention has focused on autologous transplantation as a possible alternative. The experience with ABMT in MDS is limited and the literature describes only case-reports [38]. From a large series of 82 adults patients with ANLL 6, patients with a known preceding myelodysplastic state received ABMT in first remission. Three patients relapsed after ABMT, and overall leukemia free survival was worse compared to the group without antecedent hematologic disorder, but two patients were still alive and disease-free after more than 1 year of BMT [39]. One hundred and fourteen recipients of autologous marrow grafts who suffered from MDS or ANLL secondary to MDS have been reported to the EBMT [40]. The overall survival at 2 years of the 79 patients transplanted in first CR was 39%, disease-free survival was 34%, the actuarial relapse rate was 64%. Nineteen patients were transplanted for MDS which had not progressed to ANLL before ABMT. The actuarial disease-free survival at 2 years in these patients was 40% and the relapse rate 58%. Thirty-nine MDS patients had progressed to ANLL before chemotherapy and AMBT. Disease-free survival was 30% and the relapse rate 68%. Twenty-one patients were transplanted for MDS or ANLL which had developed after treatment with chemotherapy for other malignancies. Actuarial disease-free survival of these patients was 36% and the relapse rate 60%. Patients younger than 40 years had a significantly better disease-free survival as compared with patients older than 40 years. The disease-free survival for the transplant-related mortality and death due to failure to engraft did not appear to occur more often than after ABMT for de novo ANLL. However, a substantial proportion of patients are not eligible for ABMT because of delayed hemopoietic recovery after intensive antileukemic chemotherapy and poor marrow harvest.

Autologous Peripheral Blood Progenitor Cell Harvest and Transplantation in MDS: "The Leuven Experience"

Recent studies demonstrate the feasibility of peripheral blood progenitor cell collection (PBPC) and transplantation in a growing number of patients with poor-risk malignant disorders [41, 42]. Some authors reported also a significantly accelerated hemopoietic engraftment and reduced transfusion requirements in a series of peripheral blood progenitor cell transplantations for ANLL as compared to standard autologous bone marrow transplantation [43–45]. We were very interested in the feasibility of PBPC and transplantation in patients with high-risk myelodysplasia who were not eligible for allogeneic bone marrow transplantation because of advanced age or lack of a suitable donor. Our results are summarized in Tables 2 and 3 [46]. All patients received remission-induction chemotherapy containing idarubicine (10 mg/m^2, days 1, 3 and 5, etoposide (100 mg/m^2, days 1–5) and cytosine-arabinoside (100 mg/m^2, days 1–10), and all patients were consolidated with intermediate dose of Ara-C (2 × 500 mg/m^2, days 1–5) and mitoxantrone (8 mg/m^2, days 4–6) (NOVIA). One patient refractory to the induction therapy received high dose of Ara-C (2 × 3 g/m^2, days 1–6), and was subsequently consolidated with high dose Ara-C for 4 days.

Table 2. Patients characteristics and PBPC collection data

Patient	Sex/age (yrs)	Diagnosis	Interval diagnosis to PBPC harvest (days)	Mobilizing agent[b]	No. of PBPC collections	$CD34^+$ cells $\times 10^6$/kg	CFU-GM $\times 10^6$/kg
1[a]	M/48	RAEBt	307	Lenograstim 10 mg/kg/d, days 1–7	4	2.03	46.34
2[a]	F/34	RAEBt	231	Lenograstim 10 mg/kg/d, days 1–6	3	1.02	23.46
3[a]	M/58	RAEBt	344	Lenograstim 10 mg/kg/d, days 1–7	4	1.09	7.23
4	F/53	RAEBt	237	Lenograstim 150 mg/m^2/d, days 1–7	4	0.61	4.34
5	F/30	RAEBt	115	Filgrastim 5 mg/kg/d, days 1–6	2	3.43	65.7
6[a]	F/47	RAEBt	114	Filgrastim 5 mg/kg/d, days 1–6	3	7.16	92.13
7	M/44	sAML	99	NOVIA+Filgastrim, days 20–27	3	0.22	2.23
8	F/33	RAEBt	87	NOVIA+Filgastrim, days 20–27	1	0.05	0.39
9[a]	F/47	RAEBt	63	NOVIA+Filgastrim, days 20–24	2	54.99	461.7
10	M/48	RAEB	104	NOVIA+Filgastrim, days 20–33	5	2.35	19.81
11	F/49	RAEBt	146	Ara-C+Filgastrim, days 20-44	5	0.91	4.18
Median						1.09	19.65
Mean						6.95	66.13

[a] Subsequent autologous PBPC transplantation following BuCy4.
[b] All growth factors were administered subcutaneously.

Table 3. Transplant characteristics of five MDS patients

	Patient 1	Patient 2	Patient 3	Patient 6	Patient 9
Transplant cell dose					
No. $CD34^+$ ($\times 10^9$/l)	1.64	1.02	1.09	4.78	20.35
No. CFU-GM ($\times 10^4$/l)	38.76	23.46	7.23	72.23	164.12
Hemopoietic recovery					
ANC > 0.5 $\times 10^9$/l	11	15	18	14	10
ANC > 1.0 $\times 10^9$/l	12	20	25	16	11
Platelets > 20 $\times 10^9$/l	144	20	NR	47	8
Platelets > 50 $\times 10^9$/l	317	144	NR	89	8
Supportive care					
No. PC transfused	19	2	115	9	2
No. platelets transfused	28	7	104	8	2
Days hospitalization	26	24	35	27	27
Days fever ≥ 38.5 °C	7	0	4	3	5
Days i.v. antibiotics	10	0	14	6	9
Present disease status	CCR	CCR	Death[a]	Death[b]	CCR
Followup postinfusion	832 days	825 days	Died 126 days	Died 242 days	574 days

ANC, absolute neutrophil count; NR, not reached; CCR, continued complete remission;
[a] Untractable hemorrhagic cystitis.
[b] Died, relapse MDS.

PBPC collection was undertaken only in patients in durable complete remission. The first patients (nos. 1–6) were collected in steady state after mobilization with G-CSF. In an effort to optimize the collections, harvest in subsequent patients was attempted at the time of rapid hematological recovery after chemotherapy (NOVIA or high-dose Ara-C). In 7/11 patients (63%) sufficient cell numbers were harvested resulting in a CD34 progenitor cell yield > 1 $\times 10^6$/kg (5 patients after G-CSF mobilization alone and 2 patients after G-CSF + NOVIA). The majority of progenitors harvested were committed progenitors. However, a substantial number of more immature precursors, assumed to be responsible for the long-term sustained engraftment, were also present (data not shown) [46]. Because of our policy at that time, several of these patients underwent

bone marrow harvest before PBPC with inadequate collections in most of them.

Five patients were subsequently transplanted with peripheral blood progenitor cells only and without growth factor support (Table 3). The median time to ANC > 0.5 and 1.0 × 10^9/l was respectively 14 days (range 10–18) and 16 days (range 11–25). Platelets were self-supporting at a level of ≥ 20 × 10^9/l after a median of 41 days (range 8–144). Consistent with the rapid recovery, days of fever, need for parenteral antibiotics, transfusions of red cells and platelets, and total hospitalization duration were restricted.

One patient had a persistent lack of platelet engraftment, and died eventually because of a severe untractable hemorrhagic cystitis. Another patient (patient 6) had a relapse of MDS and died because of bleeding and infection. At present, the other patients are alive and well and in CR, with a median follow-up of 744 days.

The most recent analysis of the ongoing EORTC/EBMT pilot study (protocol no. 06921) compared repopulation data after at least 100 days follow-up for 13 patients receiving ABMT versus 8 patients receiving PBPCT after identical induction therapy (Table 4) [47]. The mean number of CFU-GM reinfused in the ABMT group was 5.0 × 10^4/kg (range 2.3–11.1) versus 51 × 10^4/kg range 7.2–462) in the PBPC group. After BM reinfusion, leukocytes ≥ 0.5 and 1.0 × 10^9/l were reached after a median of respectively 35 and 39 days, versus 11 and 13 days in the PBPCT group. The median duration to reach a platelet count of ≥ 20 × 10^9/l was > 134 days and 5/13 ABMT patients were still platelet transfusion dependant more than 135 days after ABMT. In contrast, the median time to recovery of a platelet count > 20 × 10^9/l was 23 days (range 8–216) in patients receiving PBPC transplantation.

Table 4. Repopulation data after autologous bone marrow transplantation and PBPC transplantation in high-risk MDS patients after identical induction therapy[a]

	ABMT	PBPCT
Number of Patients	13	8
CFU-GM (× 10^4/kg) (range)	5 (2.3–11.1)	51 (7.2–462)
ANC > 0.5 × 10^9/l (days, range)	35 (11–90)	11 (9–14)
Platelets > 20 × 10^9/l (days, range)	> 134	23 (8–216)

[a] De Witte et al. Blood 1995; 86 (suppl. 1):618a.

Feasibility of Harvesting Non-Clonal Benign Hemopoietic Progenitors in MDS

A major concern remains the possible contamination of the peripheral blood progenitor cells by clonal malignant cells. In our patients with chromosomal abnormalities at diagnosis, a normal karyotype and CR state were required at the time of the harvest. In addition, we performed additional clonality studies on the harvest of female patients by using X-chromosome inactivation patterns [48]. We choose to analyse MDS progenitor clonality more directly on cell populations reflecting different stages of hemopoietic development. Highly purified hemopoietic progenitors were obtained from mobilized peripheral blood by CD34 enrichment with immunomagnetic beads followed by FAC-Sorting. Although cells expressing the CD34 glycoprotein at their surface are enriched for hemopoietic progenitors, they still form a heterogeneuous population. For this reason, we sorted different stem cell subfractions, including $CD34^+$ cells with low CD38 coexpression ($CD34^+38^{low}$), being strongly enriched for the most immature and uncommitted progenitors, and $CD34^+$ cells with high levels of CD38 expression ($CD34^+CD38^{high}$), particularly consisting of multilineage committed stem cells. We used polymorphisms in the recently described X-linked HUMARA locus for detection of these X-chromosome inactivation patterns [49]. In four of the patients studied, this assay has shown an unequivocal polyclonal pattern in the mature granulocytic and T-lymphoid cell fractions at the time of PBPC harvesting. This clearly implies the feasibility of restoring polyclonal myeloid hematopoiesis after high-dose chemotherapy. The same polyclonal nature was also found at the level of both immature ($CD34^+38^{low}$, $CD34^+33^{low}$) and more committed ($CD34^+38^{high}$, $CD34^+33^{high}$) stem cells. In one patient (in morphological complete remission after high-dose therapy), al-

lelic ratios of mature and progenitor fractions remained skewed, thus suggesting persistent clonal malignant hemopoiesis. This patient had polyclonal nature of buccal scrapes and T lymphocytes, which makes it very unlikely that skewed patterns could be due to unbalanced lyonization.

Conclusion

Our findings and those of others clearly demonstrate that even though MDS is a clonal disorder of the hemopoietic stem cell, it is possible to harvest non-clonal, putative benign, $CD34^+$ progenitors. In addition, these data indicate that PBPC transplantation is feasible in selected MDS patients and leads to more rapid and complete repopulation when compared to ABMT. This opens new perspectives to deliver high-dose chemotherapy as an alternative treatment option for patients who are to old for or lack an allogeneic marrow donor. However, the benefit of PBPC transplantation compared with high dose consolidation performed after a common induction chemotherapy has to be assessed.

A new EORT/EBMT/GIMEMA/HOVON/SAKK study entitled Autologous peripheral blood stem cell transplantation versus a second intensive consolidation course after a common induction and consolidation course in patients with bad prognosis myelodysplastic syndrome (MDS) and acute myelogenous leukemia secondary to MDS of more than 6 months duration (EORTC no. 06961) will soon be started to further elucidate the role of PBPC transplantation in MDS.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Clinical Trials of Pegylated Recombinant Human Megakaryocyte Growth and Development Factor (PEG-rHuMGDF)

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Abstract. Megakaryocyte Growth and Development Factor, a truncated c-mpl ligand, represents the main cytokine involved in thrombopoiesis. It is involved in all stages of megakaryocyte development from the stem cell. Only 3 years after its discovery, several phase I and phase II clinical trials have been performed in solid tumors and leukemia. In these studies, PEG-rHuMGDF was found to be well tolerated and to have biological efficacy specific for the megakaryocytic lineage. Further studies will be necessary to define its clinical utility for reduction of the duration of thrombocytopenia and platelet transfusion requirements following chemotherapy.

Introduction

Thrombocytopenia is a major dose-limiting side effect of chemotherapy in solid tumors as well as in acute leukemia. Thrombopoietin for a long time hypothesized has been identified as the c-mpl ligand in 1994 [1-3] and has been isolated and cloned [4-5]. It appears to be the main cytokine regulating platelet production, supporting all stages of megakaryocytes development in vitro [2]. Megakaryocyte growth and development factor (MGDF) is a truncated mpl ligand including the 163 N-terminal amino acid domain of thrombopoietin responsible for receptor binding and biological activity [5]. It is known to exert all biological effects of the full molecule, favouring all steps of megakaryocyte development from the immature stem cells [6, 7]. N-terminal conjugation of polyethylene glycol leads to increased stability and in vivo half-life of the molecule, with a 10 times increase in biologic activity [8]. Polyethylene glycol-conjugated recombinant human MGDF (PEG-rHuMGDF) induces thrombocytosis when administered alone [8, 9] and diminishes thrombocytopenia induced by chemotherapy and radiotherapy [9-13] in murine species and non human primates, with few known side effects. Results of recently completed phase I and Phase II clinical studies are summarized here.

Results of Clinical Studies

Phase I Studies in Solid Tumors

Two phase I studies in patients with solid tumors have been recently completed in Australia [14, 15] and the United States [16]. One of these studies included patients with various solid tumors and the other patients with lung cancer. In both studies, patients were randomized to receive escalating doses of placebo or PEG-rHuMGDF (0.03 to 5.0 μg/kg/day) for up to 10 days in the absence of any chemotherapy [14, 16] and up to 16 days after chemotherapy with carboplatin and paclitaxel [15, 16] or until platelet count increased to at least 600 $\times$ 10^9/l. In these two

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studies, a total of 7 patients received placebo and 16 received PEG-rHuMGDF in the absence of any chemotherapy, and 22 received placebo and 69 PEG-rHuMGDF following chemotherapy. In the absence of chemotherapy preceding administration of PEG-rHuMGDF, increases in platelet counts above 600×10^9/l were observed in patients treated with dosages of PEG-rHuMGDF as low as 0.03 and 0.1 μg/l. After chemotherapy, the nadir of the platelet count in the patients given PEG-rHuMGDF was higher than in the patients given placebo at all doses of PEG-rHuMGDF tested [16]. In addition, the nadir and return to baseline platelet count occurred significantly earlier in patients receiving PEG-rHuMGDF than in patients receiving placebo [15, 16]. Intensity of chemotherapy was not sufficient to evaluate potential effect of PEG-rHuMGDF to avoid platelet transfusion since only one patient in the placebo group received platelet transfusion. In both studies, platelet count of patients treated with PEG-rHuMGDF rose progressively over the 20 days of observation, exceeding 600×10^9/l in more than 50% of the patients on day 20 and more than 1000×10^9/l in approximately one third of patients receiving at least 1 μg/kg/day of PEG-rHuMGDF. No significant effect of PEG-rHuMGDF was observed of neutrophil recovery and hematocrit. Among all enrolled patients, the adverse event observed were consistent with the effects of the underlying malignancy or the chemotherapy. At total of 5 patients receiving PEG-rHuMGDF in the two studies developed venous thrombosis, while no patient receiving placebo did. Thrombotic events included two episodes of deep venous thrombosis, one of which was complicated by pulmonary embolism, one asymptomatic and apparently isolated pulmonary embolism in a patient with lung cancer, and two episodes of superficial vein thrombosis. However, these episodes did not appear to be related to the platelet count since no patient with $> 1000 \times 10^9$ platelets/l underwent thrombosis. Normal platelet aggregation and ATP release was observed in all patients studied [15, 17]. It could not be determined whether the observed thrombotic events were related to PEG-rHuMGDF therapy or the predisposing underlying malignancy [15, 16].

Phase II Sstudy in Acute Myelogenous Leukemia

A randomized, placebo controlled study of PEG-rHuMGDF as an adjunct to chemotherapy in adult patients with newly diagnosed AML, excluding M3 (promyelocytic) and M7 (megakaryoblastic) FAB subtypes was recently completed in 10 European centers [18]. Seventy-three patients receiving conventional induction chemotherapy with daunorubicin, 45 mg/m^2/day for 3 days, cytarabine, 200 mg/m^2/day for 7 days and etoposide, 100 mg/m^2/day for 5 days were randomized to receive PEG-rHuMGDF, 2.5 or 5 μg/kg/day or placebo from the day after chemotherapy until recovery of platelet count(50×10^9/l) for a maximum of 21 doses. Seventy of these patients went on to receive at least one dose of blinded study drug, including 24 patients receiving each of the PEG-rHuMGDF daily doses and 22 patients receiving placebo. Median recovery time to an unsupported platelet count of 20×10^9/l was 21 days for all groups and the reduction of platelet transfusions in patients receiving PEG-rHuMGDF was not significant. Overall, a prolonged elevation of platelet count was seen after discontinuing the drug in both groups of patients receiving PEG-rHuMGDF, with 52% of patients reaching platelet counts $> 1000 \times 10^9$/l versus only 9% of patients receiving placebo. The effect of PEG-rHuMGDF appeared to be specific for platelet lineage as no difference in neutrophil count or red blood cell transfusion requirement was observed between the PEG-rHuMGDF and placebo groups. Adverse event profile was similar in the 3 groups of patients. One patient in the placebo group underwent cerebral artery thrombosis when his platelet count was 8×10^9/l while no thrombotic event was observed during induction in the two PEG-rHuMGDF groups. Complete remission rate was 76% in patients who received PEG-rHuMGDF and 86% in patients who received placebo.

Conclusion

In the three clinical studies whose results are available, PEG-rHuMGDF was found to be biologically active, inducing intense stimu-

lation of platelet production, without significant effect on the myeloid and erythroid lineages, as was observed in animal studies [8-13]. Furthermore, PEG-rHuMGDF was well tolerated and adverse events observed in these studies were typical of the underlying disease and chemotherapy. The major increase in platelet count seen in most patients receiving the drug could be explained by drug accumulation, the long plasma half-life [19], the time required for platelet megakaryocyte maturation, and normal platelet survival of 10 days. The absence of demonstrated increase in thrombotic event despite thrombocytosis confirms animal studies showing no in vivo activation of platelets and no increase of platelet adhesion to damaged vessels [9]. The lack of reduction of time to recovery of platelets to 20×10^9/l in the AML study could be related to the delay necessary for PEG-rHuMGDF to increase platelet production from stem cells. Further studies of the activity of PEG-rHuMGDF after myelosuppressive chemotherapy in a variety of tumor settings are ongoing to enable definition of its role in supporting patients undergoing myelosuppressive chemotherapy.

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Increased Numbers of Progenitor Cells in the Apheresis Product of Patients Randomised to Receive Increasing Doses of Stem Cell Factor (r-metHuSCF) Administered in Combination with Chemotherapy and a Standard Dose of Filgrastim (r-metHuG-CSF)

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Abstract. The optimal regimen for mobilisation of peripheral blood progenitor cells remains to be defined. Many clinical studies have shown enhanced mobilisation when using a combination of a chemotherapeutic agent and a hematopoietic growth factor, most notably cyclophosphamide and G-CSF compared with the use of either agent alone. Stem cell factor (SCF), a glycoprotein hematopoietic growth factor, is the ligand for the tyrosine kinase receptor encoded by c-kit. We report here a randomized study in 48 patients with ovarian cancer, the first clinical trial investigating the mobilisation of peripheral blood progenitor cells using SCF in combination with G-CSF following chemotherapy. All patients were treated with cyclophosphamide 3 g/m^2 and were randomized to receive filgrastim 5 μg/kg alone or filgrastim 5 μg/kg plus SCF. The dose of SCF was cohort dependent (5, 10, 15 and 20 μg/kg), with 12 patients in each cohort, nine of whom received SCF plus filgrastim and the remaining three patients receiving filgrastim alone. On recovery from the white blood count nadir following chemotherapy 41 of the 48 patients underwent a single apheresis, and 5 of the 48 patients underwent two aphereses. The apheresis product was divided into 4 aliquots, with one aliquot being reinfused following each cycle of cyclophosphamide (900 mg/m^2) and carboplatin (AUC 7.5 mg/ml/min).

Stem Cell Factor in combination with filgrastim following chemotherapy, enhanced the mobilisation of progenitor cells compared with that produced by filgrastim alone following chemotherapy. This enhancement was dose dependent for CFU-GM, BFU-E, and CD34+ cells in both the peripheral blood and apheresis product. In the apheresis product, 3- to 5-fold increases in median CD34+ and progenitor cell yields were obtained in patients treated with SCF 20 μg/kg plus filgrastim compared with yields obtained in those patients treated with filgrastim alone following chemotherapy. Likewise, in the apheresis product there was a 5-fold increase in the median number of long term culture-initiating cells (LTC-IC) collected in those patients treated with the highest dose of SCF plus filgrastim compared with those patients receiving filgrastim alone.

In addition, peripheral blood values of CFU-GM, BFU-E and CD34+ cells/ml remained above defined threshold levels for a greater period of time with the higher doses of SCF. This has an important clinical implication in that the higher doses of SCF offer a greater 'window of opportunity' in which to perform the apheresis to achieve high yields.

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Introduction

The presence of primitive haemopoietic cells in adult peripheral blood has been recognised for over 30 years. Initial canine and murine studies in the 1960s demonstrated blood cells capable of protecting recipients from lethal doses of whole body irradiation by restoring blood cell formation from circulating donor cells[1-4]. Subsequent studies led to detection and quantitation of progenitor cells by colony assays [5-7] and the effects of growth factors on their numbers in the circulation [8, 9]. More recently, this was followed by investigation of the apheresis product[10-14] as an alternative source to bone marrow cells for transplantation following myeloablation. As a result, not only has intensive investigation into peripheral blood mobilisation regimens been conducted, but quantitative assays for cells capable of reconstituting and sustaining hematopoiesis in transplant recipients been performed [15-19]. LTC-IC are arguably the most primitive human hematopoietic cells detectable by in-vitro functional assays. They are capable of generating myelopoid and erythropoid progenitor cells (CFU-GM and BFU-E) and in murine transplantation protocols the assay detects the marrow repopulating cells [17, 19].

Stem cell factor (SCF), a glycoprotein hematopoietic growth factor, is the ligand for the tyrosine kinase receptor encoded by c-kit [20]. A recent study in primates [21] has demonstrated the ability of SCF alone to mobilise PBPCs. When used in combination with G-CSF, low doses of SCF have been shown to give enhanced mobilisation of PBPCs compared with G-CSF alone. In mice [22], primates [23] and canines [24] the combination of optimal doses of G-CSF with low dose SCF (25 μg/kg/day) resulted in increased numbers of circulating white blood cells (WBC) and both mature and primitive progenitor cells. Transplantation of irradiated mice or canines with these PBPCs resulted in enhanced radioprotection and recovery of platelets and WBC post transplant[22, 24].

Initial clinical studies involving SCF have investigated mobilisation of peripheral blood progenitor cells using the cytokine alone or in combination with G-CSF, but not involving chemotherapy. Early results from these studies have shown increasing mobilisation of progenitor cells (GM-CFC and BFU-E) and CD34+ cells with increasing doses of SCF in combination with filgrastim compared with the administration of filgrastim alone [25]. The optimal regimen for mobilisation of both primitive and more committed progenitor cells remains to be defined. Stem cell factor, when used in a clinical setting in combination with G-CSF following chemotherapy may mobilise more primitive cells as well as more committed cells, compared with mobilisation following chemotherapy and G-CSF alone, therefore increasing the yield of primitive and committed blood cells in the apheresis product. We report here the first study investigating the mobilisation of progenitor cells and frequency of LTC-IC collected in the apheresis product of patients with ovarian cancer who were randomised to receive stem cell factor in combination with G-CSF following chemotherapy versus chemotherapy plus G-CSF alone.

Patients, Materials and Methods

Patients

Previously untreated patients aged between 16 and 65 years with histologically proven epithelial ovarian cancer, International Federation of Gynaecology and Obstetrics (FIGO) stages Ic-IV were entered. All eligible patients were required to have a normal full blood count. Patients were eligible providing they had no history of IgE-mediated hypersensitivities. Three centres entered patients - Cancer Research Campaign (CRC) Departments of Medical Oncology at Christie Hospital, Manchester, Nottingham City Hospital and Newcastle General Hospital. All progenitor cell, CD34+ and CD34+ subset assays were performed in the CRC Department of Experimental Haematology,Paterson Institute for Cancer Research, Christie Hospital, Manchester. Forty eight patients were recruited between September 1994 and November 1995.

Treatment

All patients were treated with cyclophosphamide 3 g/m^2 on day 1, given as a 4-h intravenous infusion, together with mesna 6 g/m^2 as a 12-h intravenous infusion. The patients were randomized to receive filgrastim 5 μg/kg alone or filgrastim 5 μg/kg plus SCF. The dose of SCF was cohort dependent, with twelve patients in each cohort, nine of whom received SCF plus filgrastim (5 μg/kg) and the remaining three patients receiving filgrastim (5 μg/kg) alone. The dose of SCF was 5, 10, 15 and 20 μg/kg in cohorts 1, 2, 3 and 4 respectively. Growth factors were administered daily from day 3 following chemotherapy until the peripheral white blood count was 4×10^9/l, when all patients underwent a single apheresis. This apheresis product was divided into four aliquots and frozen in a controlled rate freezer in the vapour phase of liquid nitrogen (Kryo 10: Planer Biomed Products Ltd. Middlesex, UK) and then transferred to liquid nitrogen and stored at -196 °C. Patients randomized to receive SCF and filgrastim were given cetirizine 10 mg orally 2 h prior to each SCF injection and 2 puffs of salbutamol via a metered dose inhaler with an aerosol spacing device, 30 min prior to this injection, in order to reduce the risk of allergic reaction to SCF.

Patients were planned to receive treatment at 3-weekly intervals and cycles 2 to 5 consisted of cyclophosphamide and carboplatin. Carboplatin dose was prescribed according to the Calvert Formula [26] at an A.U.C. 7.5 mg/ml/min i.e. carboplatin dose = 7.5 (EDTA clearance +25)mg. Carboplatin was reconstituted in 1 l of 5% dextrose and infused over 1 h. Cyclophosphamide 900 mg/m^2 was given immediately after the carboplatin, and infused over 1 h in 1 l of normal saline. Ondansetron and dexamethasone were routinely given as antiemetics. Each cycle of combination chemotherapy was given as an outpatient and was followed 24 h later by reinfusion of one aliquot of apheresis product. Filgrastim 5 μg/kg/day was recommenced the following day and continued until the absolute neutrophil count (ANC) recovery (ANC $\geq 1 \times 10^9$/l for 3 consecutive days or 10×10^9/l for 1 day) was achieved.

Patients were only treated when their WBC was $\geq 3.0 \times 10^9$/l (or ANC $\geq 1 \times 10^9$/l) and platelets $\geq 75 \times 10^9$/l. If the blood count failed to recover by the time of the next planned cycle of treatment the chemotherapy was delayed until recovery had occurred. Treatments were always delivered at full dose and never dose-reduced.

Patients at the Christie Hospital had peripheral blood progenitor cells collected on a Spectra cell separator (Cobe laboratories, Lakewood, CO) using a continuous collection procedure until 2.5 times the patient's blood volume had been processed. Patients at Nottingham and Newcastle had their cells collected on a CS 3000 cell separator (Baxter Fenwal Division, Deerfield, IL). Platelet transfusions were given to maintain a platelet count $\geq 20 \times 10^9$/l and red cell transfusions to maintain a haemoglobin count of ≥ 8 g/dl.

Study Procedures

Full blood counts including manual differential counts were performed on the following days during the mobilisation phase and all subsequent treatment cycles: days 1, 8, 10, 12, 14, 16, 18 and 20. Serum biochemistry and CA 125 levels were measured on day 1 of each cycle of chemotherapy. Mononuclear cell (MNC) counts and progenitor cell assessments (CFU-GM, BFU-E, and CD34+ cell counts) were performed on peripheral blood samples on the same days as the full blood counts during the mobilisation phase and also on the apheresis product.

Results

Forty eight patients were entered into the study, their median age being 49.5 years (range 30-65 years). Five patients were FIGO stage Ic, 8 patients stage II, 28 patients stage III and 7 patients stage IV. The Christie Hospital enrolled 37 patients, Nottingham City Hospital 6 patients and Newcastle General Hospital 5 patients. All patients underwent a single apheresis except for 4 patients enrolled from Newcastle General Hospital, one patient from the Christie Hospital who had

at least two aphereses performed, and the 2 patients described below who were withdrawn from the study.

Peripheral Blood

For CD34+ cells, CFU-GM, and BFU-E mobilised into the blood there was a more rapid rise and a greater maximum value for patients treated with higher doses of SCF with filgrastim compared with the lower doses of SCF plus filgrastim or filgrastim alone. The pattern for MNC was less clear, with similar profiles seen for the SCF 5, 10 and 15 μg/kg treatment groups.

The peripheral blood data were investigated further by examination of:

1. Peak values obtained for CD34+ cells, CFU-GM, BFU-E and MNC for each patient.
2. The number of days these parameters remained above defined threshold values from day 8 to day 18.
3. The area under the cell count versus time curves for each patient between days 8 and 18.

The median peak values (and their ranges) of progenitor and CD34+ cells mobilised into the blood are shown in Table 1. The median peak values for CD34+ cells, CFU-GM and BFU-E increased with increasing SCF dose across the SCF 5, 10, 15 and 20 μg/kg plus filgrastim groups. However, there was little difference between the filgrastim alone group and SCF 5 μg/kg plus filgrastim group. Median peak MNC values were similar in the groups receiving SCF 5, 10 and 15 μg/kg plus filgrastim, however, the group treated with filgrastim plus SCF 20 μg/kg had a higher median peak MNC value. There was approximately a two-fold increase in median peak numbers of MNC, a three-fold increase in median peak numbers of CD34+ cells and BFU-E and a four-fold increase in CFU-GM mobilised in those patients treated with SCF 20 μg/kg plus filgrastim following chemotherapy compared with those treated with filgrastim alone following chemotherapy. Linear regression analysis (on log scale) demonstrated that these increases with SCF dose were statistically significant for all four parameters, both including and excluding the patients receiving filgrastim alone. In addition, analysis of variance confirmed significant differences between the group receiving filgrastim alone and those groups treated with SCF plus filgrastim for all parameters ($p < 0.001$ for CFU-GM, BFU-E and CD34+ cells, $p = 0.04$ for MNC).

The number of days that peripheral blood CD34+ cells, CFU-GM, BFU-E and MNC/ml remained above specified threshold levels is summarized in Table 2. The median number of days above the threshold generally increased with increasing doses of SCF. The greatest number of days above threshold levels was observed for those patients receiving SCF 20 μg/kg plus filgrastim for all parameters. As for the peak data in Table 1, the median number of days above threshold levels

Table 1. Median peak values (range) of progenitor cells mobilised into the blood

	Cyclophosphamide 3 g/m^2 followed by					
	Filgrastim 5 μg/kg n = 12	Filgrastim 5 μg/kg + SCF 5 μg/kg n = 9	Filgrastim 5 μg/kg + SCF 10 μg/kg n = 9	Filgrastim 5 μg/kg + SCF 15 μg/kg n = 9	Filgrastim 5 μg/kg + SCF 20 μg/kg n = 9	p-value[a]
MNC $\times 10^5$/ml	49.6 (18.9-92)	60.7 (31.6-148.8)	69.0 (27-110.8)	62.8 (26.4-171.9)	112.3 (31.9-151.74)	$p < 0.001$
CFU-GM $\times 10^3$/ml	10.8 (1.5-50.9)	10.6 (3.7-29.7)	17.1 (7.6-79.2)	35.0 (15.4-92.8)	46.4 (15.2-118.9)	$p < 0.001$
BFU-E $\times 10^3$/ml	13.4 (1.9-79.1)	9.5 (5.4-32.9)	31.1 (5.8-84.6)	51.3 (25.1-114.1)	42.9 (15.4-127.44)	$p < 0.001$
CD34+ $\times 10^3$/ml	86.8 (18-249.3)	71.2 (45.6-441.4)	118.0 (34.5-361.8)	151.7 (85.6-379.5)	290.5 (126.5-820.6)	$p < 0.001$

[a] Linear regression for SCF dose across all five treatment groups.

Table 2. Median number of days (and their ranges), peripheral blood MNC, CFU-GM, BFU-E and CD34+ cells remained above specified levels, according to treatment group

Number of days for:	Filgrastim 5 μg/kg	Cyclophosphamide 3 g/m² followed by Filgrastim 5 μg/kg + SCF 5 μg/kg	Filgrastim 5 μg/kg + SCF 10 μg/kg	Filgrastim 5 μg/kg + SCF 15 μg/kg	Filgrastim 5 μg/kg + SCF 20 μg/kg	*p*-value[a]
MNC $> 20 \times 10^5$/ml	3.5 (0-5)	6 (1-8)	6 (3-10)	7 (0-9)	8 (7-8)	$p < 0.01$
CFU-GM $> 5 \times 10^3$/ml	5 (0-7)	4 (2-5)	5 (1-8)	8 (5-10)	9 (6-10)	$p < 0.001$
BFU-E $> 5 \times 10^3$/ml	5 (0-7)	5 (2-5)	6 (2-9)	7 (6-10)	9 (6-10)	$p < 0.001$
CD34+ $> 50 \times 10^3$/ml	3.5 (0-5)	3 (0-5)	4 (0-8)	6 (3-9)	7 (6-8)	$p < 0.001$

[a] Linear regression for SCF dose across all five groups.

for the filgrastim plus SCF 5 μg/kg and the filgrastim alone groups were similar. Linear regression analysis across the five treatment groups demonstrated these increases with SCF dose to be significant for all parameters ($p < 0.001$ for CD34+ cells, CFU-GM, BFU-E and $p < 0.01$ for MNC). The linear regression remained significant for CD34+ cells, CFU-GM and BFU-E (all $p < 0.001$) when the patients treated with filgrastim alone were excluded from the analysis. Significant differences between the filgrastim alone group and the combined SCF plus filgrastim groups were found for all four parameters (ANOVA, $p < 0.001$ for CFU-GM, BFU-E and CD34+, $p = 0.02$ for MNC).

The area under the cell count versus time curves (AUC) were calculated for each patient between days 8 and 18 and for each parameter (CD34+ cells, CFU-GM, BFU-E and MNC). There was a progressive increase in AUC as the dose of SCF increased from 5 μg/kg to 20 μg/kg. Again, linear regression analysis (of log transformed AUCs) across the five treatment groups demonstrated the increases with SCF dose to be significant for all parameters (all $p < 0.001$).

Apheresis Product

The results of PBPC and LTC-IC yields obtained from the single apheresis (or first apheresis in the case of the 4 Newcastle patients who underwent more than one procedure), are shown in Table 3. There was an increasing median number of CFU-GM, BFU-E, CD34+ cells, CD34+/33- cells and LTC-IC obtained in the apheresis product with the increasing dose of SCF. There was a 5-fold increase in CFU-GM and BFU-E, a 3-fold increase in CD34+ cells, a 66 fold increase in CD34+/33- cells and a 5-fold increase in LTC-IC in those patients treated with SCF 20 μg/kg plus filgrastim following chemotherapy compared with those treated with filgrastim alone following chemotherapy. These increases were all highly significant using linear regression analysis (on log scale), both including and excluding the group of patients receiving filgrastim alone. There was no evidence for an SCF dose-related increase for MNC, but there was a significant difference between those patients treated with filgrastim alone and the groups treated with SCF plus filgrastim (ANOVA, $p = 0.02$). Analysis of variance also demonstrated significant differences between the group receiving filgrastim alone and those groups receiving SCF plus filgrastim for CFU-GM, BFU-E, CD34+ cells and CD34+/33- cells (all $p < 0.01$).

Toxicities During the Administration of Growth Factors

The majority of patients tolerated the growth factor injections with either mild or no adverse events. The most common side-effect in patients receiving filgrastim alone was musculo-skeletal pain at the time of

Table 3. Median progenitor cell and LTC-IC yields (range), by treatment group, obtained from a single apheresis

	Cyclophosphamide 3 g/m^2 followed by					
	Filgrastim 5 μg/kg n = 12	Filgrastim 5 μg/kg + SCF 5 μg/kg n = 9	Filgrastim 5 μg/kg + SCF 10 μg/kg n = 8[a]	Filgrastim 5 μg/kg + SCF 15 μg/kg n = 9	Filgrastim 5 μg/kg + SCF 20 μg/kg n = 7[a]	p-value[b]
MNC $\times 10^8$/kg	1.7 (0.3-6.0)	3.9 (1.7-7.2)	3.6 (2.1-6.3)	2.5 (1.7-6.7)	3.3 (2.6-5.4)	$p = 0.29$
CFU-GM $\times 10^4$/kg	84 (5-653)	107 (83-138)	151 (119-373)	423 (167-706)	444 (101-930)	$p < 0.001$
BFU-E $\times 10^4$/kg	117 (7-909)	143 (100-308)	235 (134-656)	346 (196-667)	564 (150-871)	$p < 0.001$
CFU-MK $\times 10^4$/kg	3.9 (0-62.2)	6.3 (3.7-14.9)	11.3 (5.6-64.0)	12.2 (2.7-54.2)	4.8 (2.1-34.3)	[c]$p < 0.049$
CD34+ $\times 10^6$/kg	3.2 (1.0-17.0)	4.6 (1.8-25)	6.6 (2.6-15.8)	9.9 (6.3-18.3)	10.1 (5.4-16.2)	$p < 0.001$
LTC-IC/ MNC	1/10916 (2163-46783)	N/A	1/4425 (1919-8768)	1/3150 (1672-4447)	1/2540 (449-3785)	$p < 0.001$
CD34+/33- $\times 10^6$/kg	0.08 (0-8.2)	1.29 (0-7.98)	2.92 (0.27-6.38)	3.91 (0-6.48)	5.55 (0-13.8)	$p < 0.001$

[a] Data unavailable for 1 patient in cohort 2 (due to late arrival of apheresis sample for analysis) and for two patients in cohort 4 (due to failure to obtain venous access in one patient and withdrawal from the study prior to apheresis due to adverse event in the other patient).
[b] Linear regression for SCF dose across all five groups.
[c] Linear regression for SCF dose across four groups, excluding the 20 μg/kg treatment group. N/A - not available

neutrophil recovery from the nadir following cyclophosphamide 3 g/m2. This occurred in 3 out of 12 patients. In the SCF plus filgrastim treated patients musculo-skeletal pain occurred at a similar time of neutrophil recovery as the filgrastim alone treated patients. However, this adverse event occurred more frequently with 17 out of 36 SCF-treated patients affected. The commonest adverse event due to SCF was a local skin reaction at the injection site. These reactions consisted of mild oedema surrounded by a ring of erythema. This type of skin reaction occurred in 27 out of the 36 patients receiving SCF and started approximately 4 h after the SCF injection and lasted for between 24 and 48 hours, after which it resolved completely. No patient suffered any skin reactions distant to the injection site apart from the one patient who developed an anaphylactoid reaction following her first injection of stem cell factor at 20 μg/kg. This patient required intravenous chlorpheniramine, hydrocortisone and intramuscular adrenaline to relieve the urticaria, chest tightness, and stridor resulting from laryngeal oedema. She made a full recovery but as a consequence of this reaction was withdrawn from the study. A second patient in the 20 μg/kg treatment group was withdrawn from the study because it was impossible to obtain adequate venous access in order to perform the apheresis harvest. A third patient was withdrawn prior to receiving her second cycle of chemotherapy after developing acute renal failure secondary to an episode of neutropenic sepsis. However, the patient subsequently made a full recovery.

Delays in Chemotherapy

Only eight of the 45 patients who received dose intensive cycles of carboplatin and cyclophosphamide chemotherapy had any delays in their planned treatment. Five of these eight patients had only one cycle delayed, while the remaining three patients each had two cycles delayed, resulting in a total of 11 cycles delayed out of a possible 134 cycles. All the delays were due to the slow hematopoietic recovery prior to either the penulti-

mate or the final cycle of treatment. Only 2 out of the 12 patients treated with filgrastim alone-mobilised progenitor cells had delays in their planned delivery of chemotherapy compared with 6 patients out of 33 having any delays in the SCF treated groups. Of the 11 cycles delayed 7 were due to inadequate recovery of ANC and 4 due to inadequate recovery of the platelet count.

Discussion

This is the first clinical study reporting on the mobilisation of progenitor cells in patients treated with SCF and filgrastim following chemotherapy. The combination of SCF and filgrastim following cyclophosphamide effectively mobilised progenitor cells. The mobilisation of progenitor cells into the peripheral blood was enhanced by the combination of SCF and filgrastim following chemotherapy compared with the mobilisation produced by filgrastim alone following chemotherapy. This enhancement was dose dependent for the mobilisation of CFU-GM, BFU-E, and CD34+ cells into the peripheral blood and in the apheresis product. Linear regression analysis of peripheral blood data demonstrated a statistically significant increase of these parameters with increasing SCF dose. This was also demonstrated in the yields obtained in the apheresis product, with a 5-fold increase in median numbers of CFU-GM, BFU-E and LTC-IC, a 3-fold increase in median numbers of CD34+ cells and a 66 fold increase in the median number of CD34+/33- cells obtained in patients receiving SCF 20 μg/kg plus filgrastim compared with those receiving filgrastim alone.

We have previously reported the analysis of CD34+ subsets and long term culture-initiating cells (LTC-IC) [27, 28] and demonstrated a highly significant increase in the number of CD34+/33- cells mobilised in the apheresis product as the dose of stem cell factor was increased ($p < 0.001$). However, when analysing the CD34+/38- subsets, virtually no cells of this phenotype were detected in the filgrastim alone, SCF 5 μg/kg, SCF 10 μg/kg and SCF 15 μg/kg treatment groups despite at least 500 000 cells being analysed in each assay. Although, the group receiving SCF 20 μg/kg had a significant mobilisation of CD34+/38- cells, with a median 0.062×10^6 cells/kg (range $0.04\text{-}0.49 \times 10^6$/kg) being collected in the apheresis product. We were also able to demonstrate a highly significant linear trend in median numbers of LTC-IC mobilised with increasing doses of SCF ($p < 0.001$).

The greatly increased number of LTC-IC mobilised by SCF in combination with filgrastim compared with that produced by filgrastim alone following chemotherapy demonstrates that the SCF/filgrastim combination also mobilises more primitive cells in addition to the CFU-GM and BFU-E. As a consequence, there should be excellent capacity of the apheresis product to effect prompt and lasting reconstitution following myeloablative therapy if SCF is part of the patient's mobilisation regimen.

As the dose of SCF is increased not only does the median peak blood value of the particular parameter increase, but the duration that each parameter (CFU-GM, BFU-E and CD34+ cells) remains above an arbitrary threshold value also increased as the dose of SCF was increased. Linear regression analysis across all five treatment groups confirmed these increases to be significant for all parameters with increasing SCF dose (Table 2). This has an important clinical implication in that as the dose of SCF increases there is a greater 'window of opportunity' in which to perform the harvest to achieve high yields.

A further important observation was that as the dose of SCF was increased it tended to reduce the variability of progenitor cell yields between patients within each treatment group, particularly for CD34+ cells, (all patients treated with SCF 15 μg/kg or 20 μg/kg acheived $>5 \times 10^6$/kg CD34+ cells in their apheresis product). A similar observation was described regarding the mobilisation of LTC-IC with increasing doses of SCF[28]. This may have important implications for those patients who mobilise progenitor cells poorly into the peripheral blood, as these patients may well have their progenitor cell / LTC-IC yields increased by the addition of stem cell factor to the mobilisation regimen.

The enhanced yields obtained using the combination of chemotherapy, SCF and fil-

grastim will facilitate investigation of alternative multicyclic dose-intensive regimens, including myeloablative therapies, as SCF in combination with filgrastim following chemotherapy enhances the mobilisation of both committed progenitors and more primitive hematopoietic cells including LTC-IC, CD34+/33- and CD34+/38- cells compared with the mobilisation following the administration of chemotherapy and filgrastim alone.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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The Influence of Surface Receptors for G + GM-CSF, IL-3 and SCF and S-Phase Recruitment of AML Cells on Response to First Phase Chemotherapy

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Abstract. In order to improve the outcome of treatment for AML, several groups have investigated the use of rh cytokines to prime leukaemic cells prior to cell cycle specific chemotherapy. Our group has used immunofluorescent ligand binding assays to determine the proportion of marrow AML cells bearing receptors for G + GM-CSF, IL-3 and SCF and the mean density of these receptors (n=21), prior to incubation in serum free medium ± G+GM-CSF, IL-3, SCF, SCF+G+GM-CSF or SCF+IL-3 for seven days. A significantly greater percentage of cells from AML marrow specimens possessed cytokine receptors compared with normals (n=17). AML marrow cells however, showed no significant difference in mean receptor densities compared with normals except for reduced incidence of IL-3 receptors. Flow cytometric assessment of recruitment into S-phase was undertaken following cytokine priming for seven days (chosen to favour the self renewing compartment of AML cells). The only significant difference in kinetic activity between normal and AML cells was reduced sensitivity to IL-3 in AML. This study sought to determine if these measurements could be used to predict the response to first phase chemotherapy for AML, with response defined as attainment of haematological remission and failure of response as any other outcome. Subjects with complete data sets were used in the predictive analysis (n=13). Results analysed using a multivariate discriminant analysis with quadratic curve fitting demonstrated that the three best discriminators of outcome were

1. recruitment into S-phase by SCF+IL-3 cytokine combination
2. G-CSF receptor density and
3. baseline S-phase activity after seven days incubation in serum free medium.

These factors, taken together, permitted correct prediction of clinical outcome in 11/13 cases. It remains to be seen whether such a predictive model would continue to apply to larger patient cohorts. Despite higher percentages of AML cells bearing similar densities of receptors to normal cells, poor kinetic response to cytokines was frequently observed thus implying possible defective intracellular signalling.

Introduction

Recombinant human cytokines are potent agents whose biological effects include proliferation and differentiation of target cells, enhanced function of cells and prevention of apoptosis. Because of their expense, consensus opinion, both in Europe and the USA has limited the use of cytokines to well defined clinical situations. These are post bone marrow transplantation, to accelerate regeneration of haematopoiesis post chemotherapy and for peripheral blood stem cell harvesting.

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One of the most compelling areas for their use however, is the induction of leukaemia cells into cell cycle prior to and during chemotherapy. Nonetheless, clinical results using cytokines in this fashion have been disappointing and have led investigators to question which patients would benefit from such an approach. What is known, is that G-CSF and GM-CSF can be used to facilitate normal haematopoietic recovery without increasing the incidence of AML relapse, a concept which is embraced by the current AML11 and AML12 Trials in the United Kingdom.

In the Last 5 years, there has been an explosion in our understanding of how cytokines work both at the cell surface and intracellular levels. Recent research has demonstrated mutations in cytokine receptor genes in patients with AML [1, 2]. The work presented here augments previous studies by our group where AML cells, pre-incubated in cytokine cocktails, have been cultured with varying concentrations of Ara-C in vitro and different patterns of clonogenic responsivity observed [3, 4]

Novel aspects of this present work include:

1. Seven day pre-incubation of AML cells with cytokines in order to favour the self renewing compartment [5-7].
2. Fluorescent ligand binding assays to permit the detection of cytokine receptors capable of functional binding combined with an attempt to determine the relative mean receptor density on cells.
3. Comparison of in vitro data with clinical outcome using a multivariate analysis.
4. Parallel in vitro investigations with normal cells.

By adopting such a multifaceted approach it may become possible to identify those patients who will accrue the greatest benefit from chemotherapy combined with S-phase inductive cytokines.

Materials and Methods

Patient Selection and Marrow Preparation

Marrow was aspirated from the posterior iliac crests of 21 patients with AML sixteen of whom were de novo cases, the remaining five having progressed to AML from prodromal myelodysplastia. AML patients were categorised by FAB subtype as : M0: 2, M1: 1, M2: 7, M3: 1, M4: 4, M5: 6. In all cases the blast burden exceeded 80%. AML patients karyotypes were characterised as good (n=5), intermediate (n=5) and poor (n=11) prognostic groups according to recognised criteria [8].

Patients were further categorised according to clinical outcome after one course of inductive chemotherapy (MRC Trial of the time). Complete remission (CR) was defined as attainment of haematological remission and non response (NR) as any other outcome.

Normal marrow samples were obtained from 17 subjects with non-Hodgkins lymphoma undergoing marrow examination for staging purposes whose marrows were used only if subsequently found to be uninvolved and free of reactive features.

Light density marrow cells (LDMCs) were obtained by density gradient centrifugation (SG=1.077) and were depleted of T-cells and monocytes using complement mediated monoclonal antibody cytolysis of CD4, CD8 and CD14 cells. This manoeuvre was performed to remove haematopoietic accessory cells potentially capable of endogenous cytokine production.

Pre-Incubation of Cells with Cytokines

T-cell and monocyte depleted LDMCs were cultured in QBSF 58 serum free medium (SFM) (Sigma) at a concentration of 1.5 $x10^6$/ml. Cultures were maintained for seven days either in SFM alone as a negative control or with the inclusion of G+GM-CSF (100 U/ml each), IL-3 (10 ng/ml), SCF (20 ng/ml) or combinations of these cytokines. SCF was a kind gift of Amgen Ltd. Following liquid culture (pre-incubation), cells were washed and aliquoted for kinetic studies.

Flow Cytometric Studies

The percentage of normal and AML T-cell and monocyte depleted LDMC's in S phase following pre-incubation ± cytokines was

determined by flow cytometric analysis of DNA content using Becton Dickinson's Cycle Test Plus DNA reagent kit as recommended by the manufacturer to obtain, fix and stain nuclei with propidium iodide prior to flow cytometric analysis. DNA histogram analysis was performed using Becton Dickinson's FACScan/cellFIT DNA System software which discriminates G0, S and G2/M phase cells. For all samples 10 000 cells were analysed

The percentage of normal and AML T-cell and monocyte depleted LDMCs bearing surface receptors for G-CSF, GM-CSF, IL-3 and SCF was quantitatively assessed using Fluorokine kits by R&D Systems Europe Ltd. according to the manufacturers instructions. The assay involved incubation of cells with either directly or indirectly fluorochrome conjugated cytokines which when bound to specific surface receptors permitted flow cytometric evaluation. For all samples 10 000 cells were analysed.

In order to obtain a measure of relative mean receptor density per sample, total fluorescence intensity for the entire cell population was measured, expressed in arbitory units, and divided by the percentage of cells bearing that particular receptor.

Statistical Analysis

Significance values were obtained using the Student's T test or the Mann Whitney U test where data was non-parametric.

In order to establish any correlation between in vitro data and clinical outcome, multivariate discriminant analysis with quadratic curve fitting was used. This analysis determined whether individual patients could be correctly assigned to either the complete remission (CR) or no response (NR) clinical outcome groups on the basis of S-phase response to cytokines and/or cell surface receptor levels.

Results

Percentages of normal and AML marrow cells bearing surface receptors for different cytokines are presented in Table 1.

Mean results ± 1SE for normal and AML T-cell and monocyte depleted LDMCs are grouped according to response to first phase chemotherapy. The highlighted sections of Table 1 indicate significantly different values : significantly more AML cells bear receptors for all cytokines than normal LDMCs ($p = < 0.001$ in all cases).

Cytokine Receptor Densities on Normal and AML Marrow cells

Because significantly greater percentages of purified AML cells possessed receptors for all cytokines, compared with normal LDMCs, an attempt was made to further define this difference by looking at mean receptor densities derived from the total fluorescent signal for the whole cell population divided by the percentage of cells positive for the receptor in question.

Mean receptor densities are summarised for normal and AML LDMCs as mean values ±1SE.

No significant difference in densities of surface receptors was apparent on AML cells from responders and non-responders to chemotherapy.

Table 1. Comparison of percentages of normal and AML cells bearing surface receptors for G-CSF, GM-CSF, IL-3 AND SCF.

Receptors	Normals	AML: whole group	AML: CR	AML: NR
G-CSF	15.3±4.1	42±4.2	34.4±.8.9	45.7±4.9
GM-CSF	22.2±4.4	51±4.6	60.4±9.6	46.0±5.5
IL-3	4.5±1.8	31.6±4.1	37.7±10.5	26.1±4.0
SCF	3.1±1.4	33±4.9	46.0±9.5	26.6±5.6
	n=17	n = 21	n = 7	n = 14

Table 2. Comparison of mean G-CSF, GM-CSF, IL-3 and SCF receptor densities on AML and normal cells

	G-CSF	GM-CSF	IL-3	SCF
Normals n=10	1.7±1.0	2.3±1.9	4.5±1.9	4.1±2.3
AML : whole group n=15	0.4±0.1	0.4±0.1	0.5±0.1	0.4±0.1
AML : CR n=5	0.4±0.2	0.4±0.1	0.5±0.3	0.3±0.1
AML : NR n=10	0.4±0.2	0.5±0.2	0.5±0.1	0.5±0.1

The highlighted section of Table 2 indicates the only significant finding that AML cells have lower receptor densities for IL-3 compared with normal cells ($p = 0.022$). Despite the similar trend for other cytokine receptors, no other significant differences in receptor densities were apparent comparing normal and AML cells and comparing responders and non-responders to chemotherapy.

Baseline S-Phase and Recruitment Ratios

In order to examine S-phase recruitment of normal and AML marrow cells by cytokines and their different combinations, a seven day preincubation manoeuvre was employed to favour the self renewing compartment. In AML, this approach would target the AML stem cell which theoretically is responsible for relapse in vivo. Incubation in serum free medium alone for seven days was used as a control for each normal and AML marrow sample. These initial baseline values without cytokines are presented in Table 3.

Table 3. Comparison of baseline S-phase activity of normal and AML cells

	% Cells in S-phase	Ranges
Normals n=14	5.8±0.9	1.1-11.6
AML : whole group n=21	7.3±1.5	0.3-23.8
AML : CR n= 7	5.6±1.3	0.3 - 9.0
AML : NR n=14	5.8±1.2	0.4 - 23.8

Percentages of normal and AML T-cell and monocyte depleted LDMCs in S-phase following seven days incubation in SFM alone are presented as mean values ±1SE.
No significant differences were observed between the AML patient groups and the normal control subjects.

Because of the variable baseline ranges, it was decided to standardise the reporting of cytokine priming results using a "S-phase recruitment ratio" derived by dividing the percentage values following cytokine preincubation by the SFM control result for individual patients. Results for normals subjects and AML patients are presented in Table 4 .

Results are presented as mean values ±1 SE for normal and AML T-cell and monocyte depleted LDMCs following pre-incubation in SFM± cytokines alone or in combination.

Increased S-phase activity in normal cells with IL-3 priming compared to that observed in AMLs from the CR group was the only significant finding (p = 0.042). No significant differences were detected between any of the other patient groups and the normal control subjects in response to any cytokine permutation. There was however a trend towards increased kinetic activity with SCF+G+GM and SCF+IL-3 priming of AML cells comparing responders and non-responders.

Multivariate Discrimant Analysis with Quadratic Curve Fitting

In order to examine whether any of the results presented above could predict clinical outcome retrospectively, multivariate analysis was performed using complete data sets (n=13).

Table 4. Comparison of S-phase recruitment ratios for normal and AML marrow cells

	G+GM-CSF	IL-3	SCF	SCF + G+GM-CSF	SCF + IL-3
Normals n=11	2.0±0.3	2.1±0.3	1.7±0.4	2.5±0.4	2.8±0.5
AML: whole group n=21	2.2±0.5	2.6±0.6	1.6±0.3	3.5±0.3	3.4±0.9
AML: CR n=7	1.4±0.1	1.2±0.2	1.3±0.2	1.5±0.2	1.9±0.2
AML: NR n=14	2.6±0.7	3.3±0.9	1.7±0.4	4.5±1.3	4.2±1.0

Table 5. Predictability of clinical outcome based on multivariate discrimant analysis with quadratic curve fitting

Predictive factors	A	B	C	A+B	A+C	B+C	A+B+C
Probability of correctly predicting outcome	0.587	0.563	0.485	0.618	0.512	0.933	0.996

The % probability results presented are illustrative and demonstrate that the greater the number of predictive parameters used the greater the chance of assigning a patient to the correct group. Factors which had the greatest strength of predicting clinical outcome when applied to the group with complete data sets were (A) S-phase recruitment ratio of AML cells by SCF + IL-3 (B) baseline percentage of AML cells in S-phase and (C) G-CSF receptor density.

Discussion

The concept of inducing proliferation of acute myeloblastic leukaemic cells before and during chemotherapy is an attractive one. Clinical trials incorporating cytotoxic agents with G-CSF or GM-CSF have reported variable outcomes, but encouragingly, there has been no evidence of increased relapse rates due to stimulation of the leukaemic clone [9, 10]. This observation has prompted the increasing use of regimes such as FLAG which incorporate fludarabine, cytosine arabinoside (Ara-C) and G-CSF and the current MRC AML12 trial which uses G-CSF post induction chemotherapy. Implicit in the success of such therapeutic manoeuvres however, must be the presence of functional cell surface receptors on AML cells capable of binding the relevant ligand and intact downstream signalling.

In previous in vitro studies we have noted considerable heterogeneity of response of AML cells to cytokine priming with G+GM-CSF or IL-3 in terms of kinetic response and subsequent sensitivity to Ara-C in clonogenic culture [4]. Only by including SCF in priming cocktails was an element of consistency of AML response observed as SCF synergised with the other cytokines in a proportion of cases [3].

AML is a biologically diverse group of diseases typified by disappointing and unpredictable responses to therapy thus it is hardly surprising that in vitro responses to cytokines should be equally diverse. This observation prompted us to investigate kinetic response to cytokines, pecentages of cells bearing receptors and densities of surface receptors for cytokines on "purified" AML blasts. These analyses were performed in parallel on normal cells in order to delineate "normality" as a comparative measure.

G-CSF and GM-CSF in combination were used as priming agents as they are of particular clinical relevance, however in order to

target more primitive progenitors IL-3 and SCF were included, the latter because of its stimulatory effects at or near the level of the multipotent stem cell [11, 12].

The immunofluorescent ligand binding assays revealed that the percentage of AML cells bearing surface receptors for all these cytokines was significantly greater than on normal cells, with no correlation between these data and clinical outcome for AMLs. Some studies using monoclonal antibodies against receptors report normal numbers of cytokine receptors on "de novo" AML blasts [13, 14]. Our methodology, which only permits detection of receptors functionally capable of binding cytokines, suggests that, at the extra-membrane level at least, AML receptors are functionally intact. Presumably, as the AML cell suspensions used comprise greater than 80% malignant blasts, the increased percentages of cells bearing cytokine receptors, particularly for IL-3 and SCF, reflect the primitive nature of cells under study compared to the relatively later compartments represented by normal LDMCs. Thus, AML cells should be able to respond to more primitive stimulators possibly produced locally by adjacent leukaemic or other cells.

It has been suggested that normal myeloid cell maturation is accompanied by functional changes in the GM-CSF receptor. Normally, both Class I and Class II GM-CSF receptors are present, the former evident on more mature cells (and downregulated by GM-CSF) and both found on more primitive normal cells, the Class II upregulated by ligand. In addition, IL-3 can compete with GM-CSF in binding Class II receptors. In AML, leukaemic blasts have predominantly Class II receptors and this may, in part, explain the increased binding of IL-3 and GM-CSF compared with normal LDMCs [15]. SCF exerts its effect primarily on early pluripotent or multipotent stem cells. Leukaemic progenitors escape normal maturation therefore it is not unreasonable to suppose that a greater proportion of these early malignant cells might bear SCF receptors than a population of normal marrow cells spanning a range of maturation stages.

Regardless of the consistent observation that proportionally more AML cells bear cytokine receptors than normal cells, there was no significant difference noted in mean receptor density with the exception of reduced incidence of IL-3 receptor density on AML cells. The receptor density of GM-CSF receptors on AML blasts has been previously observed to be of a similar to that of normals [16]. Indeed we have observed that GM-CSF, G-CSF, IL-3 and SCF all act optimally at identical concentrations on both normal and AML cells in vitro (unpublished observations).

The higher percentages of AML cells bearing surface receptors and at similar mean densities, except for reduced IL-3, did not translate into increased S-phase activity of AML cells compared to normal cells measured either before or following cytokine priming. Thus, despite the greater potential for S-phase recruitment of AML cells by cytokines capable of binding through functional surface receptors, the kinetic response observed were not proportionally increased. This may imply, that in this cohort of AML patients, defective responses to cytokines do not involve the ligand/receptor interaction but rather a defect further downstream in signal transduction.

Karyotypic abnormalities are well documented in AML and the cytogenetic pattern in individual patients can be an important prognostic predictor. Nonetheless, response to therapy is often unpredictable. We sought to examine whether our in vitro findings might correlate in any way with clinical response to first phase chemotherapy in patients of good, intermediate and severe karyotypic status. Standard induction regimens using current MRC protocols were employed. No correlation was noted between karyotpic grouping and in vitro findings. However, using multivariate analysis, three in vitro variables had particular predictive importance in assigning patients to the correct clinical outcome groups (11/13). These factors were high S-phase recruitment of AML cells by SCF+IL-3, high baseline percentage of AML cells in S-phase and low G-CSF receptor density and were associated with poor clinical outcome. No variable taken alone was predictive. We do not suggest that this multi-faceted analytical approach is a practical way to predict clinical response

to therapy. However, as our repetoire of data increases as more patients are accrued, it may be possible to prospectively identify an in vitro invesigation(s) of predictive value.

These data re-enforce the concept of AML as a biologically complex and unpredictable disease in which flaws in signal tranduction cascades may contribute to the impaired response to cytokines. It may be that the only possibility of a worthwhile predictive model may lie at the molecular level.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Shc Overexpression Induces Selective Hypersensitivity to GM-CSF and Prevents Apoptosis of the GM-CSF-Dependent Acute Myelogenous Leukemia Cell Line GF-D8

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Abstract. Hematopoietic cell self-renewal, proliferation, differentiation, and survival are regulated by a complex network of integrated processes under the control of extra- and intra-cellular mechanisms. Shc is an adaptor molecule implicated in the regulation of cell proliferation. To assess the role Shc may play in regulating hematopoietic cells, we engineered the overexpression of Shc in the recently described GM-CSF-dependent GF-D8 cell line by retroviral gene transfer and analyzed subsequent effects on cell behavior. Early passaged Shc- or mock-transfected GF-D8 clones, cultured in RPMI-1640 with FBS (10%) and GM-CSF (20 ng/ml), were used. Western blot analysis confirmed that the transfected clone (GF-D8/Shc) had a significantly higher expression of Shc as compared to the parental clone (GF-D8), or clones retrovirally transduced with the LXSN vector only (GF-D8/SN). Analysis of nuclear DNA fragmentation by DNA gel electrophoresis revealed that GF-D8/SN cells underwent apoptosis 24 h following GM-CSF deprivation, whereas GF-D8/Shc cells failed to show any evidence of apoptosis up to six days after GM-CSF deprivation. Apoptosis was associated with a progressive decrease of Bcl-2 and increase of CD95 expression. To evaluate the clonogenic response of GF-D8/SN and GF-D8/Shc to growth factors, cells were GM-CSF-starved for 24-48 h and then cultured in methylcellulose with increasing concentrations (0.001 - 50 ng/ml) of different growth factors, including GM-CSF, G-CSF, and MGDF. Cell proliferation was analyzed by assaying colonies (aggregates of $\geq$ 40 cells). As compared to GF-D8/Shc cells, GF-D8/SN cells generated significantly lower numbers of colonies upon stimulation with GM-CSF. Both Shc- or mock-transfected GF-D8 cells failed to give rise to clonal aggregates in response to G-CSF and MGDF. In conclusion, our data demonstrate that Shc overexpression increases GM-CSF sensitivity and prevents apoptosis of GF-D8 cells. These results suggest that:

1. Shc is an important regulator of cell survival and proliferation,
2. our model system might be useful for investigating leukemic hematopoiesis as well as the controlled amplification of genetically modified hematopoietic cells.

Introduction

Hematopoietic cell self-renewal, proliferation, differentiation, and survival are regulated by a complex network of highly integrated processes under the control of regulatory molecules and intracellular mechanisms [1]. Granulocyte-macrophage colony-stimulating factor (GM-CSF) mainly regulates proliferation, differentiation and functional acti-

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vation of normal myeloid cells [2]. In addition, GM-CSF has been shown to be an essential regulator of acute myelogenous leukemia (AML) cell proliferation both in vivo and in vitro [3]. Unlike fresh leukemic cells, most AML cell lines are growth factor independent due to additional genetic abnormalities acquired in vitro which allow their unrestricted proliferation even in the absence of exogenously added growth factors [4]. Recently, a number of AML cell lines have been described which retain their original growth factor dependency for continuous growth [5]. These cell lines including the GM-CSF-dependent GF-D8 cell line [6] represents useful in vitro models for studying signal transduction pathways triggered by growth factors as well as leukemogenic mechanism(s).

All GM-CSF effects are mediated by a heterodimeric receptor comprised of a ligand binding subunit, denoted α, and of a transducing subunit, designed as β [7]. Although GM-CSF does not possess an intrinsic tyrosine kinase domain, several lines of evidence indicate that signaling processes initiated by ligand binding to the receptor induce activation of cellular tyrosine kinases [8]. Signaling from the GM-CSF receptor involve a number of transducing molecules, including Shc, Grb2, Sos1, Ras, Raf-1 [9].

To assess the role Shc may play in regulating hematopoietic cells, we engineered the overexpression of Shc in the GM-CSF-dependent GF-D8 cell line [6] by retroviral gene transfer and analyzed subsequent effects on the behavior of cell proliferation and survival.

Materials and Methods

Cell Line. Early passaged Shc- or mock-transfected GF-D8 cells, maintained in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% FBS (Stem Cell Technologies, Vancouver, Canada) and 20 ng/ml GM-CSF (Sandoz, Basel, Switzerland), were used throughout the study.

Retrovirus Production and Infection. The Shc coding sequence was cloned within the EcoR1 and Hpa1 restriction sites of the LXSN retrovirus plasmide and the GF-D8 cell line transfection was obtained according to protocol previously described [10].

Western Blotting. Ten millions of cells were lysed on ice in 50 mM Tris HCl pH 8, 150 mM NaCl, 1 mM EGTA pH 8, 100 mM NaF pH 8, 10% glycerol, 1.5 mM $MgCl_2$, 1% vol/vol Triton X-100, 1 mM sodium orthovanadate containing freshly added protease inhibitors (1 mM phenylmethyl sulfonhyl fluoride, 10 mg ml-1 leupeptin and 5 mg ml^{-1} aprotinin). Insoluble materials were removed by centrifugation for 20 min at 13,000 rpm at 4 °C and protein concentration determined according to Bradford procedure. Fifty micrograms of total cell lysate was denatured by heating to 100 °C for 3 minutes in SDS gel-loading buffer. Samples were then resolved on 7.5% SDS-PAGE and electrophoretically transferred to gene Screen Plus membrane at 0.3 A overnight at 4 °C. After blocking with 2.5% nonfat dry milk dissolved in PBS-T (NaCl $NaH_2PO_42H_2O$ and Tween-20 0.12%) filters were probed with polyclonal anti-Shc antibody (1:5000) for 2 h at room temperature [11]. After extensive washing in PBS at room temperature, immune complexes were detected with horseradish-peroxidase conjugated species-specific secondary antiserum followed by the enhanced chemiluminescence reaction (ECLTM, Amersham) according to manufacture protocol.

Colony Assay. Colony formation by Shc- or mock-transfected GF-D8 clones was assayed in methylcellulose culture [12]. Briefly, Shc or mock-transfected GF-D8 cells (1 x 10^3/dish) were plated in 35-mm petri dishes in 1-ml aliquots of Iscove's modified Dulbecco's medium (IMDM, Seromed, Berlin, Germany) supplemented with 20% FBS (Stem Cell Technologies, Vancouver, Canada), 2 mM L-glutamine and 1.1% (w/v) methylcellulose (Stem Cell Technologies). Cultures stimulated with increasing concentration of granulocyte-macrophage colony-stimulating factor (GM-CSF, 0.001-50 ng/ml, Sandoz), granulocyte colony-stimulating factor (G-CSF, 0.001-50 ng/ml, Amgen Inc., Thousand Oaks, CA), or megakaryocyte growth and development factor (MGDF, 0.1 - 50 ng/ml, Amgen Inc.) were incubated (37 °C, 5% CO_2) for 10-14 days in a humidified atmosphere. Ag-

gregates of ≥ 40 cells were scored as colonies. Four dishes were set up for each individual data point per experiment.

Immunological Analysis. CD95 and Bcl-2 expression on GF-D8 cells was analyzed by direct immunofluorescence analysis [13]. Cells (0.5×10^6/ml) were stained with fluorescein (Fitc)-conjugated anti-CD95 (clone DX2, Becton-Dickinson, Mountain View, CA, USA) or Fitc-conjugated anti-Bcl-2 (clone 124, Dakopatts, Copenhagen, Denmark). Cells to be analyzed for Bcl-2 expression were fixed with 0.25% paraformaldehyde (15 min, room temperature) and permeabilized with cold 70% methanol (60 min, 4 °C). Control samples labeled with an appropriate isotype-matched mouse IgG for both CD95 and Bcl-2 (IgG1-Fitc) were included in each experiment. Cells were analyzed on a FACSort laser flow cytometry system (Becton Dickinson) equipped with a Macintosh (Apple Computer, Cupertino, CA, USA) Quadra 650 computer using Cell Quest (Becton Dickinson) software.

Nuclear DNA Fragmentation. Nuclear DNA fragmentation was detected by DNA gel electrophoresis [14]. To perform DNA gel electrophoresis, cell samples were collected by centrifugation, washed in PBS, resuspended in 20 µl 10 mM EDTA, 50 mM Tris-HCl (pH 8) containing 0.5% (w/v) sodium lauryl sarkosinate and 0.5 mg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany), and incubated at 50 °C for 1 h. Following addition of 10 µl RNase A (0.5 mg/ml), incubation at 50 °C continued for 1 h. Samples were heated to 70 °C, and 10 µl 10 mM EDTA (pH 8) containing 1% (w/v) agarose, 0.25% (w/v) bromophenol blue and 40% (w/v) sucrose was mixed with each sample before loading into the dry wells of a 2% (w/v) agarose gel containing 0.1 µg/ml ethidium bromide. Electrophoresis was carried out in 2 mM EDTA 800 mM Tris-phosphate (pH 7.8) until the marker dye had migrated 3-4 cm.

Results

As shown in Fig. 1, Western blot analysis confirmed that the transfected clone (GF-D8/Shc) had a significantly higher expression of Shc as compared to the parental clone (GF-D8), or clones retrovirally transduced with the LXSN vector only (GF-D8/SN).

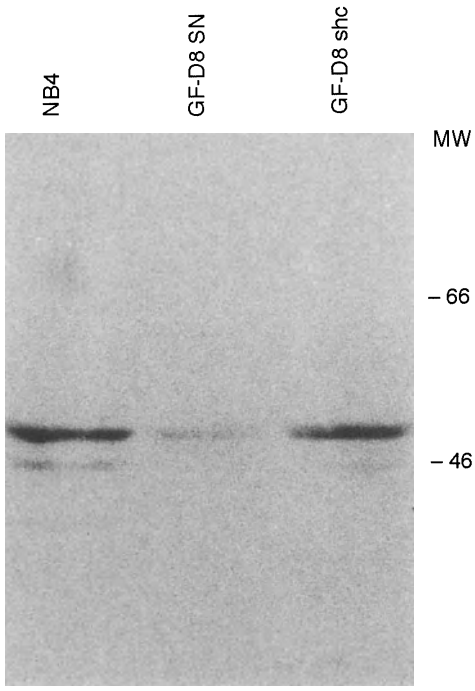

Fig. 1. Western blot analysis showing that the transfected clone (*GF-D8/Shc*) had a significantly higher expression of Shc as compared to clones retrovirally transduced with the LXSN vector only (*GF-D8/SN*)

To evaluate the clonogenic response of GF-D8/SN and GF-D8/Shc to growth factors, cells were GM-CSF-starved for 24-48 h and then cultured in methylcellulose with increasing concentrations of different growth factors, including GM-CSF (0.001-50 ng/ml), G-CSF (0.001-50 ng/ml) and MGDF (0.1-50 ng/ml). Cell proliferation was analyzed by assaying colony formation in semisolid medium. As compared to GF-D8/Shc cells, GF-D8/SN cells generated significantly lower numbers of colonies upon stimulation with GM-CSF (Fig. 2). Both Shc- or mock-transfected GF-D8 cells failed to give rise to clonal aggregates in response to G-CSF or MGDF (data not shown).

Cell survival was analyzed by nuclear DNA fragmentation. This analysis revealed that GF-D8/SN cells underwent apoptosis

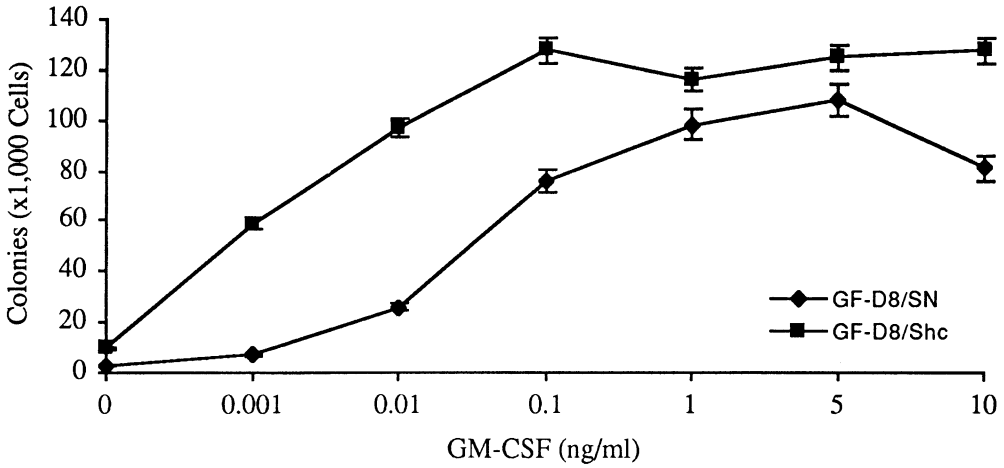

Fig. 2. Colony formation by GF-D8/Shc and GF-D8/SN in response to increasing concentrations of GM-CSF

24 h following GM-CSF deprivation, whereas GF-D8/Shc cells failed to show any evidence of apoptosis up to six days after GM-CSF deprivation (Fig. 3). Both in GF-D8/SN (Fig. 4) and GF-D8/Shc (data not shown), GM-CSF deprivation was associated with a progressive decrease of Bcl-2 and increase of CD95 expression.

Discussion

The Shc gene encodes three widely expressed signaling and transforming proteins of 46, 52 and 66 kDa, containing a C-terminal SH2 domain [15]. Shc proteins are rapidly associated with, and phosphorylated by growth factor receptors with intrinsic tyrosine kinase activity [16]. Shc proteins are involved in mitogenic signal transduction and act by coupling growth factor receptors to the Ras signaling pathway that regulates the proliferation of mammalian cells [17]. Shc proteins are phosphorylated upon activation of GM-CSF receptor and have been shown to be involved in the transmission of GM-CSF signals to Ras [18].

GM-CSF plays a crucial role in the regulation of normal and leukemic myeloid cell proliferation both in vitro and in vivo. The availability of continuous cell lines which have retained the property of growth factor dependency allows useful in vitro models for investigating functional relationships between growth factors and adaptor molecules involved in signal transduction.

We have investigated the effects of Shc overexpression in the recently described GM-CSF-dependent GF-D8 cell line [6] and here demonstrate that Shc overexpression induces hypersensitivity of GF-D8 cells to GM-CSF and prevents apoptosis of these cells following GM-CSF deprivation.

Despite Shc overexpression, GF-D8 cells retained their dependency on GM-CSF, thus suggesting that in this specific model system Shc is devoided of transforming activity. Analysis of GF-D8 proliferative response to GM-CSF, as measured by clonogenic assays, revealed that Shc overexpression was associated with increased number of colonies generated in response to suboptimal (0.001-1 ng/ml) and optimal (5-10 ng/ml) concentrations of GM-CSF. The plating efficiency of GF-D8/Shc cells was increased two- to five-fold by maximal concentrations of GM-CSF (10 ng/ml). In addition, colonies generated by GF-D8/Shc cells were bigger in size than those generated by the parental clone. Taken together, these data implies that overexpression of Shc proteins results in increased recruitment of cells able to proliferate in semi-solid culture, i.e., increased clonogenic activity, and increased proliferative potential of individual clonogenic cells [19]. The number and size of colonies generated by a progenitor cell population stimulated with maximum doses of a given growth factor

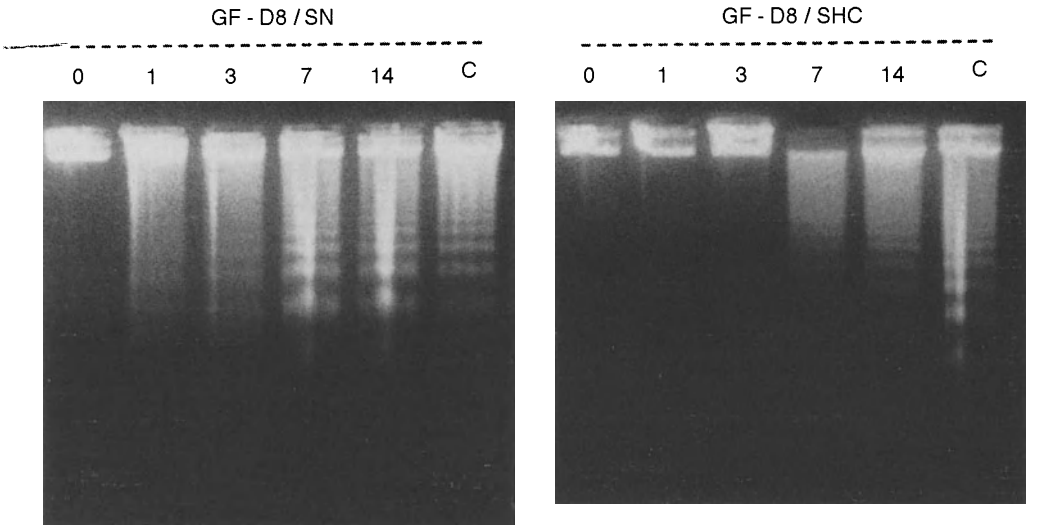

Fig. 3. Following GM-CSF deprivation, GF-D8/SN cells underwent apoptosis within 24 h whereas GF-D8/Shc cells failed to show any evidence of apoptosis up to day +6

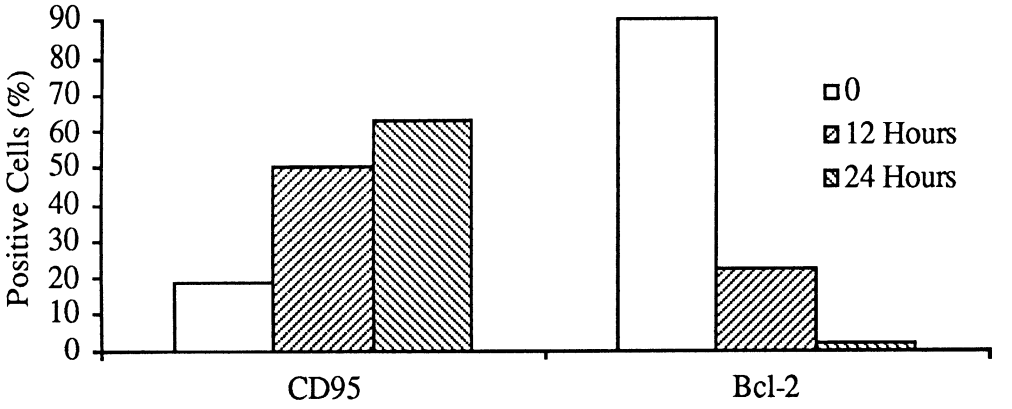

Fig. 4. CD95 and Bcl-2 expression on GF-D8/SN following GM-CSF deprivation for 12 to 24 h

can only be increased by combination of growth factors [20]. Thus, it can be inferred that Shc overexpression in the presence of a single growth factor mimics the functional behavior of progenitor cells stimulated by combination of growth factors.

Homeostasis of multicellular organisms is controlled not only by the proliferation and differentiation of cells but also by cell death [21]. During normal tissue turnover, the death of cells mainly occurs through apoptosis, a process that includes condensation and segmentation of nuclei and fragmentation of chromosomal DNA into nucleosome units [22]. Apoptosis occurs by default when cells fail to receive the extracellular survival signals needed to suppress an intrinsic cell suicide program [23]. The Fas receptor (Fas-R)/Fas ligand (Fas-L) system plays an important role in mediating apoptotic signals both in the lymphoid as well as myeloid system [24]. Following GM-CSF deprivation, GF-D8 cells undergo apoptosis within 24 h. Overexpression of Shc proteins significantly affects this apoptotic process, in that it delays the appearance of DNA fragmentation up to 6 days following GM-CSF deprivation. This finding suggests that Shc overex-

pression activates signal transduction pathways capable of counteracting apoptosis triggered by growth factor deprivation, i.e., Shc overexpression activates survival signals. Changes of CD95 and Bcl-2 expression we detected in GF-D8/SN cells (Fig. 4) and GF-D8/Shc cells (data not shown) following GM-CSF deprivation demonstrate an involvement of the Fas-R/Fas-L system and of the oncogene Bcl-2 in this apoptotic process. Potential relationship among Shc, Fas-R/Fas-L and Bcl-2 pathways remains to be investigated.

In conclusion, our data demonstrate that Shc overexpression increases GM-CSF sensitivity and prevents apoptosis of GF-D8 cells. These results indicate Shc to be an important regulator of cell proliferation and survival, and suggest that our model system might be useful for investigating leukemic and preleukemic hematopoiesis as well as the controlled amplification of genetically modified hematopoietic stem cells.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

ICAM-1 Levels Correlations with Cytokine (IL-1, IL-6 and TNF) Production During the Course of Acute Lymphoblastic Leukemia (ALL) in Children

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Abstract. This study was undertaken to establish the role of ICAM -1 levels and their correlation with cytokines (IL-1, IL-6 and TNF) production in the pathogenesis and in the clinical course of childhood ALL. A total number of 160 children with ALL, 96 boys and 64 girls, aged from 0.5 to 15 years was included into the study. ICAM-1 levels and IL-6 production were studied according to the conventional ELISA Genzyme-test, TNF production was studied in supernatants derived from 24 h PBSC mononuclear culture, according to the method based on growth inhibition of 929 mice fibroblasts sensitive to TNF, IL-1 production after the method based on inhibition of autologous rosette formation by thymocytes of CBA mouse. Thirty seven healthy children served as the control group. A correlation between ICAM-1 and TNF and IL-1 was observed, a decrease after start of chemotherapy of ALL being noted, while a relative increase of IL-6 was observed. It was found that in children with ALL the serum levels of ICAM-1 was higher, during the whole period of therapy while IL-1- production was significantly lower than that observed in the control group of healthy children ($p < 0.005$). After cessation of therapy ICAM-1, IL-1 and TNF production increased, while IL-6 production remained on the same level. During a 10-year period after the end of therapy median values of IL-1 did not reach the values of the control group.

Introduction

Acute lymphoblastic leukemia (ALL) is a malignant process characterized by unrestricted growth and maturation arrest of hematopoietic precursor cells, possibly due to abnormal expression of adhesion molecules and/or impaired cytokines production, or abnormal cytokine receptor expression [1-6]. Adhesion molecules are composed of an increasingly large group of surface membrane molecules, which, stimulated by cytokines, mediate contact between cells on the other parts and other cells or extracellular matrix (ECM) [7]. Their function is that of ensuring tight cell to cell, contact which is important for major biological phenomena such as cell migration during embryogenesis, hematopoiesis, immunopoiesis, inflammation and for the spreading and metastasis of tumor cells [8-11]. Despite the recent progress in our understanding of the role of adhesion molecules and cytokines in the origin of cancer cells, their biological significance for malignant cell proliferation is far from clear. Inadequate cooperation between network cytokines and adhesion molecules expression also plays an important role in the clinical course of the disease [10-14]. We have previously reported the disturbances in cytokine production, in lymphocyte subset numbers and in cytotoxic activity of natural killer cells during the course

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of cytostatic therapy in children with acute lymphoblastic leukemia [15-17]. Therefore, we were interested to learn, if there is a correlation between impaired cytokine production and levels of the soluble form of ICAM -1.

Aim of the Study

This study was undertaken to establish the role of ICAM-1 serum levels and their correlations with the cytokine network (IL-1, IL-6 and TNF) in pathogenesis and the clinical course of childhood acute lymphoblastic leukemia (ALL).

Materials

Between 1988 and 1997, 160 children (96 boys and 64 girls), ranging in age from 0.5 to 15 years (median 9 years) were treated according to the BFM protocol for low and middle risk group and according to the New York protocol for high risk group [15, 16]. All children were observed in the Department of Children Hematology and Oncology, Faculty of Medicine in Wrocaw. Thirty nine healthy children of the same age served as controls for IL-1 level examinations, 20 for TNF production and 22 for ICAM-1 activity. The initial characteristics of the examined children with ALL are presented in Table 1.

Methods

Serum and heparinized vein blood samples were drawn from children at different stages of their disease: at the time of diagnosis, during intensive therapy (induction, consolidation), during maintenance therapy and after completion of the treatment. ICAM-1 in serum was examined. IL-1, IL-6 and TNF in supernatants from 24 h cell culture were checked. Mononuclear cells were isolated on Lymphoprep (Nesco) gradient, then counted, resuspended in Eagle's medium containing 10% FCS and placed in an incubator (37 °C, 5% CO_2) at a concentration of 2×10^6 cells/ml for 24 h. After 24 h of incubation the supernatants were removed.

ICAM-1 and IL-6 Assay [18-20]

For specific measurement of ICAM-1 and IL-6 a commercially available antibody "sandwich" ELISA assay was used (TM ICAM-1, Interleukin-6 Genzyme Elisa kit). First samples, standards and control were incubated with a solid phase monoclonal antibody supplied precoated on microtiter wells and used to capture the ICAM-1, IL-6 present. The solid-phase bound ICAM-1, IL-6 was then incubated with a second antibody, biotinylated mouse anti-human ICAM-1, IL-6 polyclonal. The resulting immune complex was then incubated with adivin-peroxidase conjugate. The substrate

Table 1. Clinical characteristics of children with ALL

		n=150	%
Sex	Boys	96	60.00
	Girls	64	40.00
Age	< 2 years	9	5.63
	2-10 years	139	86.88
	>10 years	12	7.50
Clinical classification	LRG	45	28.13
	MRG	102	63.75
	HRG	13	8.13
FAB classification	L1	129	80.00
	L2	30	18.75
	L3	2	1.25
Immunological classification	pre B	46/63	73.02
	B	2/63	3.17
	T	6/63	9.52
	Non T non B	63/64	15.87

(peroxide) and the chromogen (tetramethylbenzidine) were added. The resulting color development was directly proportional to the amount of ICAM-1, IL-6. Absorbance values were read at 450 nm using an ELISA reader. Accurate sample concentrations of IL-6 were determinated by comparing their respective absorbances with those obtained for the standards plotted on a standard curve.

IL-1 Determination [21]

The method used to determine of IL-1 activity described by Zimecki and Wieczorek was based on inhibition of number of thymocytes forming autologous rosettes [29]. 1.8 ml 10^7 thymocytes of RPMI, were supplemented with 10% FCS and antibiotics, and then incubated with 0.2 ml of supernatant, at various dilutions for 24 h in a CO_2 incubator.

The Rosette Assay. The cells were resuspended in Eagle's medium at a concentration of 3×10^6 cells/ml, supplemented with 10% mouse serum/preabsorbed with syngenic erythrocytes/. To 0.1 ml of the cell suspension 0.1 ml of 12% syngenic erythrocytes was added, mixed and centrifuged for 5 min at 200 g at 4 °C. After 24 h incubation at 4 °C 0.5 ml of Hank's medium 0.1 ml of 0.1% acridine orange solution was added, the cells were gently resuspended and kept in an ice bath. The percentage of autologous rosettes formed by thymocytes from 2-month CBA mice were counted, the number varying from 28-33%. Control samples consisted of referential r-IL-1 dilution. All the results were expressed in units of IL-1. One unit of IL-1 inhibited 50% of rosette formation.

TNF Assay [22,23]

Five $\times 10^4$ mice fibroblasts/200 ml/well were incubated for 4 h at 37 °C. After washing with 100 RPMI 640 + 2% FCS + actynomycin D 1 mg/ml and different dilutions of supernatants (20, 10, 5, 1 and 0.5%) were added. Control samples consisted of referential r-TNF dilution. After further 18 h of incubation (37 °C, 5% CO_2), nonadherent cells were removed by washing mice with PBS. Then crystal violet was used to color the cells. The violet was then extracted and absorbency values were read at 570 nm. Accurate sample concentrations of TNF were determined by comparing their respective absorbance with those obtained for the standards plotted on a standard curve.

Results

The results of our studies on ICAM-1 levels and IL-1 and IL-6 and TNF production at various phases of therapy are shown in Table 2.

At the time of diagnosis before any treatment was started, we found the decrease of median values of ICAM-1, IL-6, parallel with

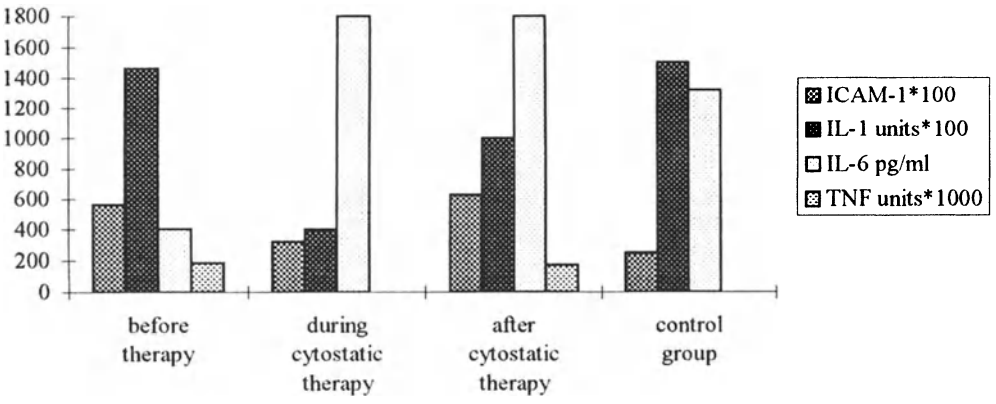

Fig. 1. ICAM-1 serum levels and IL-1, IL-6 and TNF productions in children with ALL during various phases of treatment (median values)

deficient IL-1 production. The values of IL-1 were lower than obtained in the control group consisting of healthy children. The median ICAM-1 and TNF production was higher when compared with children from the control group.

The ICAM-1 serum levels and TNF, IL-1 production decreased after starting chemotherapy and remained low during the whole period of treatment, whereas a relative increase in IL-6 was observed (Fig.1).

After cessation of therapy, an increase in ICAM-1, TNF and IL-1 production was noticed, while IL-6 values remained at the same level. During a period of 5 years after cessation of therapy IL-1 median values did not reach the values of the control group of healthy children, while ICAM-1, IL-6 and TNF production were within normal limits and even above.

Discussion

As an extension of our previous observation [15-17], we hypothesize that impaired IL-1, TNF and IL-6 production observed in children with ALL may influence serum levels of ICAM-1.

Gearing et al. found that ICAM-1 serum levels are higher in HD patients than in normal controls and in patients with more advanced HD [24]. Herman et al. have shown reduced production of IL-1 in patients with malignancies [24, 25]. In the present study we have investigated the ICAM-1 serum levels, IL-1, IL-6 and TNF in children with ALL before, during and after cessation of chemotherapy.

Our findings and literature data suggest that some cancer patients have TNF, IL-1 and IL-6 released by cancer cells themselves, which may be responsible for malignant cell proliferation [26-28]. (IL-6 and TNF for myeloma, plasmocytoma) [29, 30]. IL-1 stimulates malignant cell growth through IL-6 stimulation [31, 32]. Overproduction of IL-1 and TNF could stimulate expression of adhesion molecules on the cancer cell surface and also on the endothelial cell surface, which may lead to easier spreading of neoplasm [33-36].

We conclude that antineoplastic chemo-

Table 2. ICAM-1 serum levels and IL-1, IL-6 and TNF production in children with ALL during various phases of treatment

	Before therapy			During cytostatic therapy			After cytostatic therapy			Control group		
	Median	Mean	Range	Median	Mean	Range	Median	Mean	Range	Median	Mean	Range
ICAM-1 pg/ml	5.619	6.40	3.657-10.89	3.228	3.91	2.39-7.79	6.27	5.61	3.88-9.20	2.35	3.41	1.6-6.9
IL-1 units	14.60	23.27	0-64	4	10.57	0-64	10	19.10	0-72	15	26.21	2-64
IL-6 pg/ml	405	402	0-954	1800	1218	0-1800	1800	1800	0-1800	1320	1230	0-1800
TNF units	0.188	0.798	0-5.3	0	0.43	0-6.2	0.178	0.8	0-3.2	0	0.344	0-1.23

therapy administered to children with ALL may damage the mechanisms in peripheral blood cells, which leads to defective expression of ICAM-1 and production of cytokines TNF and IL-1, which may contribute to an increased risk of life-threatening infection in these patients. Other authors have reported similar results in patients with different types of cancer during chemotherapy [37–39]. Our studies revealed that IL-6 production during the whole period of therapy was higher than from normal healthy controls, which does not correlate with impaired IL-1 activity and TNF production (statistically n.s) and ICAM-1 serum levels. Sudhoff have noted similar results [3].

It is, however, noticeable that after cessation of chemotherapy the level of IL-1 production remains decreased. The reason for this is not clear. The possible role of specific IL-1 inhibitors needs experimental confirmation [40]. After cessation of chemotherapy ICAM-1 serum levels and IL-6 and TNF production were found to be highly elevated. It seems that high IL-6 and TNF activity could be explained by concomitant infection with hepatitis B and C viruses recorded in some of the children [41]. However, the role level of ICAM-1, IL-1, IL-6 and TNF activity after successful chemotherapy needs further studies and observation. Future trials on cytokines and adhesion molecules will better explore the regulatory mechanism of cytokine gene expression, receptor levels and receptor antagonist levels, which may lead to using cytokines and antiadhesive drugs as therapeutic agents not only to reduce hematologic toxicity [42-47].

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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High Serum IGFBP-2 in Acute Lymphoblastic Leukemia may be an Indication for Increased Risk of Relapse

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Abstract. In view of the morbidity after chemotherapy, patients with acute lymphoblastic leukemia (ALL) were treated according to risk-group-directed protocols. Empirical prognostic factors, including biological features of lymphoblasts, are used as factors to identify subgroups of patients needing intensified or less toxic treatment.

The IGF signaling system plays an important role in mitogenic effects during normal and tumor proliferation. IGFs were found in circulation and in extracellular fluids bound to specific IGF binding proteins (IGFBP). IGFBP regulate the bioavailability of IGFs for specific IGF/ IGF-receptor interactions, which are essential for metabolic and mitogenic effects of the IGFs. It has been shown that normal lymphocytes and leukemic cell lines express and secrete IGFBP. A functional type 1 IGF receptor in hematopoetic cells has been demonstrated.

Previously we investigated serum levels of IGFBPs in children suffering from ALL and found elevated concentrations of IGFBP-2 at the time of diagnosis. Out of 53 children, with a follow-up of 2 years or death during that period, 8 developed a relapse (3 patients with HR-ALL, 4 with MR-ALL and one with SR-ALL). The occurrence of relapse was restricted to children with elevated serum IGFBP-2 at diagnosis. We conclude that serum IGFBP-2 at time of diagnosis seems to be an additional parameter to determine clinical outcome in ALL. Further studies are necessary to evaluate the possible use as a risk factor in patients with ALL.

Introduction

A number of factors have contributed to the remarkable advances in the survival rate of children with acute lymphoblastic leukemia (ALL), including randomized controlled clinical studies, introduction of new effective cytostatics and an effective supportive care system [28]. However, endocrine dysfunction and damage of the epipysial growth plates have been reported as late effects of antileukemic treatment during childhood [18]. In view of the morbidity after radiochemothe-rapy [15, 31] current treatment concepts avoided cranial irradiation and modified multiagent regimens. On the basis of empirical prognostic factors patients were treated according to risk-group directed protocols. Various biological features of lymphoblasts are used as factors to identify subgroups of patients who needed intensified or on the other hand less toxic treatment [28].

The IGF axis has been known to be involved in cell proliferation and apoptosis of a variety of normal and malignant cell types [8, 11, 16]. It has been shown, that normal lymphocytes and lymphoblast cell lines express and secrete IGFBP [23, 24]. The presence of a functional type 1 IGF receptor

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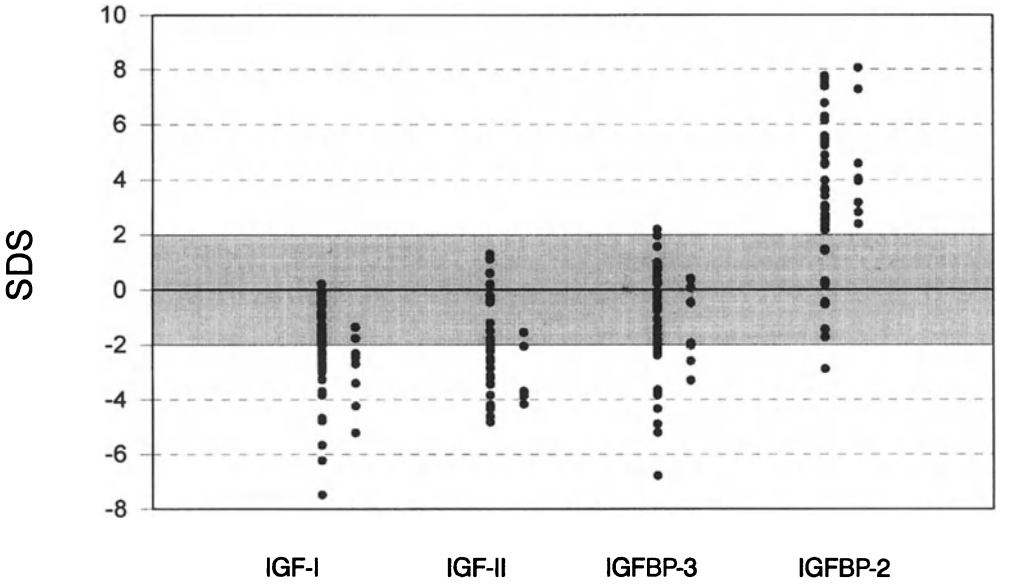

Fig. 1. Serum levels of IGF-I, -II and IGFBP-2 and -3 in patients at diagnosis. Points on the *right side* indicates 8 patients who developed a relapse.

(IGF-1R) was demonstrated in hematopoietic cells [1, 12, 14].

Previously [17, 19, 20, 33] we found altered serum levels of IGF-I, -II and IGFBP-2 and -3 in children with ALL or NHL at the time of diagnosis. In order to evaluate the clinical significance we continued to study these patients after the end of therapy.

Patients and Methods

All blood samples were obtained from patients, who were referred to our oncological unit. Diagnoses were based on the definition of the BFM cooperative study group [32]. Serum was stored at -20 °C until analysis. IGF-I and IGF-II, IGFBP-2 and -3 were determined by radioimmunoassay as described previously [2, 5, 6], except that recombinant hIGFBP-2 (kind gift from Sandoz, Basel, Switzerland) was used for tracer and standard preparation [7]. IGF-I and IGF-II were measured after acid ethanol extraction. Because of the age-dependence of the normal ranges during childhood, the values of IGF-I, IGF-II, IGFBP-2 and IGFBP-3 have been age-adjusted [4] by calculating the standard deviation score (SDS).

Results

Significant decreased serum concentrations of IGF-I, -II and IGFBP-3 and elevated IGFBP-2 were found at diagnosis of ALL (Fig. 1). When patients were grouped according to the age, immunophenotype or ALL risk factors [28, 32] no difference was found with respect to mean IGF-I, IGF-II, IGFBP-2 and IGFBP-3 levels.

Out of 53 children, with a follow-up of 2 years or death during that period, 8 developed a relapse (2 patients with HR-ALL, 5 with MR-ALL and one with SR-ALL). The occurrence of relapse was restricted to children with elevated serum IGFBP-2 at diagnosis. In contrast, at the time of relapse only one patient (one with SR-ALL) had elevated IGFBP-2.

Discussion

Various biological features of lymphoblasts are used to identify subgroups of patients needed intensified or on the other hand less toxic treatment. However, reliable prognostic factors are still missing at least in patients with MR-ALL [28].

Previously we reported data on 28 patients [19], showing that despite the striking changes in the initial serum levels of IGF-I, IGF-II, IGFBP-2 and IGFBP-3 these parameters were not correlated to empirical risk factors, i.e. age at diagnosis, immunophenotype or ALL risk factor [27]. In continuation of this study 53 patients were followed for more than 2 years. A relapse has been diagnosed in 8 patients including 5 children who died later. The occurrence of relapse was restricted to children with elevated serum IGFBP-2 (> 2 SDS) at diagnosis.

IGFBP may potentiate [3, 9] or inhibit [10, 25] actions of IGF. The precise function of IGFBP-2 during postnatal life is not yet known. Elevated serum levels of IGFBP-2 has been described in solid tumors and leukemia [13, 19, 21, 34] and in non-malignant conditions, e.g., after protein restriction or growth hormone deficiency [2, 29]. Interestingly, in tumors of the central nervous system of high grade malignancy a correlation between IGFBP-2 and number of malignant cells in cerebrospinal fluid has been shown [22]. In addition, in Wilms tumor [34] and tumors of the central nervous system [22] high expression of IGFBP-2 in tumor cells was suggested to correlate with tumor proliferation. Binding of IGF to the type 1 IGF receptor has been identified as the limiting signal leading to DNA synthesis and proliferation [26, 30]. Therefore, in view of the decreased IGF-I and IGF-II it might be possible that hematopoietic stem cells secreting IGFBP-2 build up a local reservoir of IGFs.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Thrombopoietin Supports the Continuous Growth of Cytokine-Dependent Human Leukemia Cell Lines

H. G. DREXLER, M. ZABORSKI, and H. QUENTMEIER

Abstract. The search for a factor regulating megakaryocytopoiesis lead to the cloning of thrombopoietin (TPO) that specifically promotes proliferation and differentiation of the megakaryocytic lineage. The availability of recombinant TPO and its imminent clinical use has made a more detailed understanding of its effects on hematopoietic cells more urgent. Normal megakaryocyto- and thrombopoiesis occurs predominantly in the bone marrow, a difficult organ to study in situ, particular in humans due to the low numbers of megakaryocytic progenitors and the difficult isolation as pure populations. We developed an in vitro system which may allow to address questions regarding the biology of TPO. The acute myeloid leukemia (AML)-derived cell lines HU-3, M-07e, M-MOK and TF-1 have absolute dependence on granulocyte-macrophage colony-stimulating factor (GM-CSF). We cultured these cells long term (> 6 months) in the presence of TPO (omitting GM-CSF). TPO alone supported the maintenance and expansion of these sister cell lines, HU-3/TPO, M-07e/TPO, M-MOK/TPO and TF-1/TPO, that displayed longer doubling times, a larger cell size, and a higher percentage of polynucleated giant cells and slightly adherent cells than the corresponding countercultures grown with GM-CSF. In the absence of TPO, the cells died quickly within few days; thus, the TPO-grown cell lines have an absolute dependence on this factor, but could all be switched back to growth with GM-CSF. In comparison with the GM-CSF-treated cells, the receptors for GM-CSF and interleukin-3 (IL-3) were down-regulated and the receptors for stem cell factor (SCF) and TPO were up-regulated in the TPO-exposed cells. A short-term proliferation assay showed a stronger response of the TPO-cell lines to erythropoietin, GM-CSF, IL-3, PIXY-321, SCF and TPO than the GM-CSF-cell lines. Flow cytometric analysis of the GM-CSF- and TPO-cultured lines displayed an up-regulation of the megakaryocytic surface markers CD41, CD42 and CD61 and a down-regulation of the erythroid marker glycophorin A in the latter cell lines, suggesting differentiation along the megakaryocytic lineage. Thus, in long term exposure, TPO appears to have both a proliferative and a differentiative effect on responsive cells. Under serum-deprived culture conditions, TPO acted as a survival factor on the TPO-cell lines. Taken together, these findings indicate that the TPO-dependent cell lines represent important biological reagents for further characterization of the biology of TPO and should provide also a great aid for future in vitro experiments aimed at elucidating megakaryocyto- and thrombopoiesis.

Introduction

Regulation of blood cell production, differentiation, and certain functional responses is mediated in part by specific hematopoie-

DSMZ-German Collection of Microorganisms and Cell Cultures, Department of Human and Animal Cell Cultures, Braunschweig, Germany

tic growth factors. The initial event in growth factor action consists of the factor's binding to its cognate membrane receptor. This binding triggers a series of intracellular mediators involved in the signaling pathways. A novel hematopoietic growth factor, thrombopoietin (TPO), was cloned and shown to be a megakaryocytic lineage-associated growth and differentiation factor. Binding of TPO to its receptor, c-MPL, mediates pleiotropic effects on megakaryocyte development in vitro and in vivo. TPO is clearly the primary regulator of this cell lineage acting at all levels of megakaryocytopoiesis and thrombopoiesis [1].

The availability of TPO will be of considerable clinical importance for the treatment of thrombocytopenias following high-dose chemotherapy, irradiation and other bone marrow failures [2]. While TPO has moved tremendously quickly from theory to clinical trials, more insight into the (patho)physiology of TPO is clearly required. Thus, it is absolutely essential that both in vivo and in vitro TPO research will be performed.

Studies on megakaryocytopoiesis using bone marrow specimens are hampered by the paucity of megakaryocytic progenitors and more committed cells within the total population (requiring sophisticated purification steps) and the heterogeneity of the bystander cells. Continuous cell lines, derived from leukemias involving this cell lineage, represent alternative in vitro models due to their monoclonal homogeneity and unlimited availability. Only few cell lines appear to respond to TPO, in terms of enhanced proliferation or induced differentiation [1, 3]. In order to facilitate future investigations on TPO, we established and characterized cell lines that are absolutely dependent on TPO for growth and survival. These TPO-dependent cell lines provide an experimental setting in which the study of TPO regulation and its effects can be adequately performed.

Materials and Methods

Leukemia Cell Lines

The cell lines were taken from the stock of the cell bank of the DSMZ [4, 5] or were generously provided by the investigators who established the cell lines for research purposes (Table 1). They were grown at 37 °C in a humidified atmosphere of air containing 5% CO_2. The basal growth media (Gibco BRL, Eggenstein, Germany) were supplemented with 5-20% heat-inactivated fetal bovine serum (FBS) (Sigma, Deisenhofen, Germany). For selective experiments, cells were incubated in serum-free medium. The growth factor-dependent cell lines were cultured routinely with recombinant cytokines or with supernatant (10-20% v/v) from the bladder carcinoma cell line 5637. The morphology of the cells was evaluated in May-Grünwald-Giemsa stained cytospins. The

Table 1. Leukemia cell lines used

Cell line	Ref.	Phenotype of cell line[a]	Year	Original disease[b]	Sample site[b]	Age/Sex	Stimulation index (TPO)[c]
HU-3	[6]	Megakaryocytic/erythroid	1991	AML M7	BM	69 F	11.4
M-07e	[7]	Megakaryocytic	1987	AML M7	BM	0.5 F	8.4
MB-02	[8]	Megakaryocytic/erythroid	1986	AML M7	PB	70 M	1.1
M-MOK	[9]	Megakaryocytic	1989	AML M7	BM	1 F	11.0
MUTZ-2	[10]	Myeloid	1993	AML M2	PB	62 M	1.5
MUTZ-3	[10]	Monocytic	1993	AML M4	PB	29 M	2.2
OCI-AML-1	[11]	Myeloid	1987	AML M4	PB	73 F	3.1
OCI-AML-5	[11]	Myeloid	1990	AML M4	PB	77 M	1.5
TF-1	[12]	Erythroid	1987	AML M6	BM	37 M	14.5
UT-7	[13]	Megakaryocytic/pluripotent	1988	AML M7	BM	64 M	1.4

[a] Phenotype based on the expression of immunological surface markers, capability to differentiate upon stimulation, functional features, etc.
[b] According to original publication; BM: bone marrow; PB: peripheral blood.
[c] Stimulation indices (SI) are refered to the untreated control culture; an SI > 2 is considered to be significant.

cell counts and viabilities were examined in standard hematocytometers.

Establishment of TPO-Dependent Cell Lines

The original cultures of these 10 cell lines (Table 1) had all been maintained in 10-20% vol 5637 conditioned medium (CM), the main cytokine ingredients of which are G-CSF (40 ng/ml in 100% 5637 CM) and GM-CSF (2 ng/ml)[14]. The original cells were washed, divided into two aliquots (starting concentration of 0.5×10^6 cells/ml in 1 ml) and incubated in medium containing one of the following cytokines or CM: 5637 CM (10% vol); GM-CSF (5 ng/ml); SCF (50 ng/ml); or TPO (20 ng/ml). During 1 week, cells were weaned of the initially added cytokine by decreasing progressively its concentrations while simultaneously adding and increasing the new cytokine.

Immunofluorescence Analysis of Antigen Cell Surface Expression

Surface expression of receptors (R) was analyzed by indirect immunofluorescence staining and flow cytometry (FACScan; Becton Dickinson, Heidelberg, Germany) applying specific monoclonal antibodies (McAbs): anti-GM-CSFRAAA (CD116; Immunotech, Hamburg, Germany); anti-Kit (CD117; Immunotech); anti-IL-3RAAA (CD123; R&D Systems, Wiesbaden, Germany); and anti-MPL (Genzyme, Rüsselsheim, Germany). Several cell lineage-associated differentiation surface markers were examined with the following McAbs: anti-gpIIb (CD41; Immunotech); anti-gpIb (CD42b; Immunotech); anti-gpIIIa (CD61; Dako, Hamburg, Germany); anti-glycophorin A (VIE-G4; Prof. Knapp, Vienna, Austria); anti-HLA-DR (RFDR-2; Prof. Janossy, London, UK). Non-reactive isotype-specific reagents were applied as controls.

Proliferation Assay

A proliferative response was examined by ^{3}H-thymidine incorporation: cells were seeded in triplicate in 100 µl medium (at 2.5 or 5×10^5 cells/ml) in flat-bottomed 96-well plates; for the last 4 h of the 48-h-incubation period, 1 µCi [methyl-^{3}H]thymidine (Amersham-Buchler, Braunschweig, Germany) was added to each well. Commercially supplied cytokines were used: erythropoietin (EPO; 5 U/ml); G-CSF (10 ng/ml); GM-CSF (10 ng/ml); IL-3 (10 ng/ml); SCF (50 ng/ml) (Boehringer Mannheim, Germany; R&D Systems). TPO (at 100 U/ml) from Zymogenetics (Dr. D. C. Foster, Seattle, WA, USA). PIXY-321 (a GM-CSF/IL-3 fusion protein; at 10 ng/ml) from Immunex (Seattle, WA, USA).

Western Blot Analysis

Two $\times 10^6$ cells were pelleted, resuspended in protease inhibitor buffer and lysed by boiling in SDS-PAGE (polyacrylamide gel) sample buffer. Proteins were separated in an SDS-PAGE by electrophoresis and blotted onto nitrocellulose membranes by electroblotting (Sartorius, Göttingen, Germany). For STAT 3 and STAT 5 detection, the membranes were labelled with the respective murine McAbs (Dianova, Hamburg, Germany) and specific bands were visualized applying a biotin/horseradish peroxidase system (Amersham) in combination with the Renaissance Western Blot Chemoluminescence Reagent protocol (Du Pont NEN, Bad Homburg, Germany).

Results

Establishment of TPO-Dependent Cell Lines

We cultured 10 constitutively growth factor-dependent cell lines (Table 1) in the presence of 20 ng/ml TPO and the respective control cultures in GM-CSF, SCF or 5637 CM for more than 5 weeks (Fig. 1). HU-3, M-07e, M-MOK, MUTZ-3, OCI-AML-1 and TF-1 responded proliferatively in a short-term (48 h) thymidine uptake assay to incubation with TPO (a response with a stimulation index > 2 was considered significant) (Table 1). MB-02 and UT-7 were chosen because they are of megakaryocytic origin and display markers of this cell lineage; myeloid cell

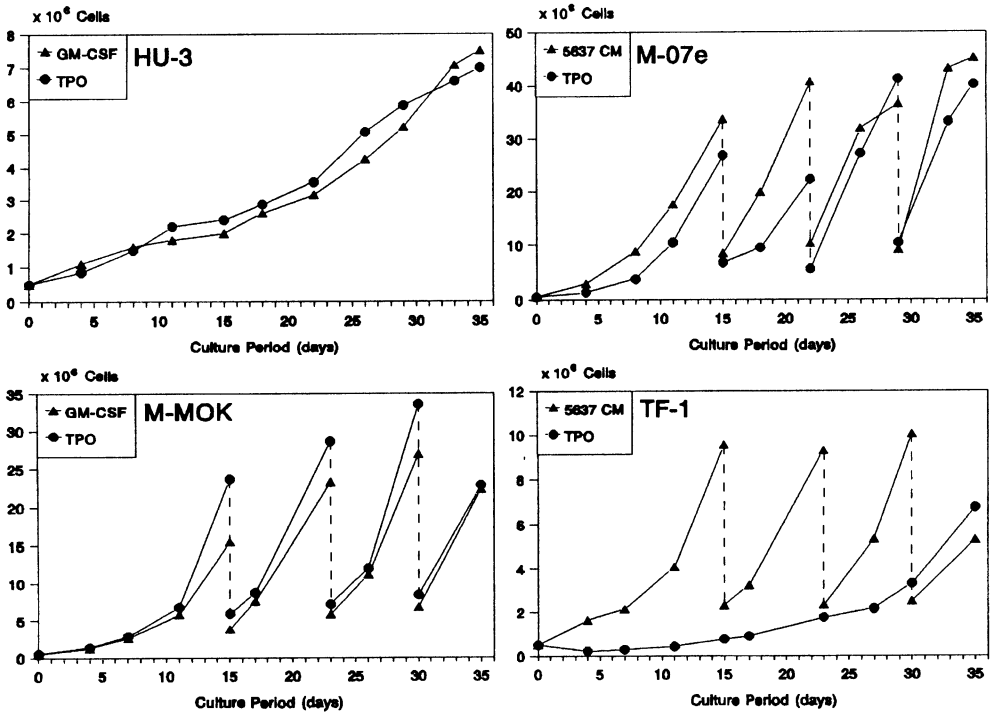

Fig. 1. Establishment of TPO-dependent cell lines. While growth of the GM-CSF- and TPO-treated cultures of HU-3, M-07e, and M-MOK was similar or identical, proliferation of the TF-1/TPO cell line started only very slowly at about day 15-17. At the days indicated, the cultures were split 1:4; spent culture supernatants were exchanged at regular intervals without loss of any cells in order to expand maximally the cell populations

lines MUTZ-2 and OCI-AML-5 were used as controls. MB-02, MUTZ-2, MUTZ-3, OCI-AML-5 and UT-7 clearly did not grow in the continuous presence of TPO while vigorously growing in the presence of GM-CSF, SCF or 5637 CM. HU-3, M-07e and M-MOK grew with TPO as well as they did with GM-CSF or 5737 CM whereas OCI-AML-1 and TF-1 showed a delayed, but still significant proliferation upon exposure to TPO. In the case of OCI-AML-1, it appeared that a cytokine-independent subclone had grown out as cytokine-deprived OCI-AML-1/TPO cells grew as well as their sister cells incubated with either GM-CSF or TPO. All further experiments were performed with the GM-CSF- and TPO-dependent variants of HU-3, M-07e, M-MOK and TF-1. These 4 TPO-dependent cell lines were kept in continuous culture for more than 6 months without loss of their dependency on TPO. We confirmed the dependency of these lines on TPO for cell survival and growth and thus excluded the outgrowth of a cytokine-independent subclone: in the presence of the respective cytokine (GM-CSF or TPO), the HU-3, M-07e, M-MOK and TF-1 cells proliferated normally, but stopped proliferation and eventually died when the cytokines were omitted from the culture medium. When the cells of the 4 TPO-dependent variants were switched back to GM-CSF exposure (without adaption period), the cells grew immediately well or better than with TPO in all four lines; cells continuously incubated with GM-CSF and switched to TPO (in the absence of GM-CSF), did not show immediately vigorous growth. The TPO-variants showed somewhat longer doubling times (Table 2).

Surface Expression of Cytokine Receptors and Differentiation Markers

Using flow cytometry, we examined the GM-CSF- and TPO-treated cell lines for differ-

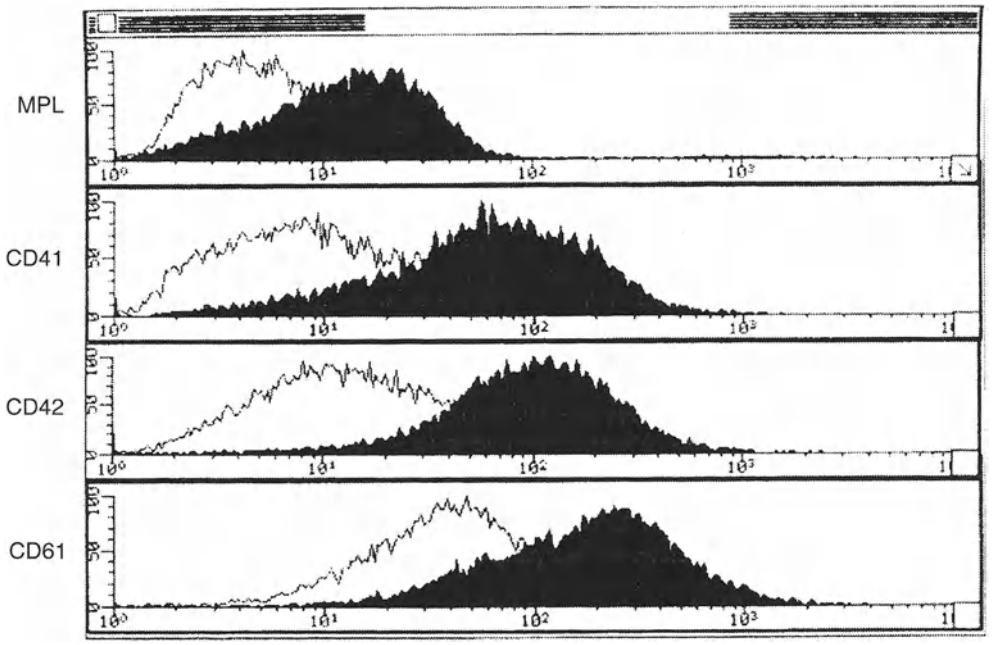

Fig. 2. M-MOK/GM-CSF *(open curves)* and M-MOK/TPO cells *(solid curves)* were analyzed by flow cytometry for marker expression on day 87. Note the upregulation of the expression of all four proteins on the TPO-exposed cells in comparison with the GM-CSF-treated cells

Table 2. Cytokine receptor and surface marker expression by GM-CSF- vs. TPO-dependent cell lines

Cell line	CD 116	CD 123	CD 117	MPL	CD41	CD42	CD61	GlyA	HLA-DR	FSC[a]	Doubling time
HU-3/GM	97[b]	95	85	14	52	16	99	10	70	160[c]	60 ± 11[d]
HU-3/TPO	92	89	99	31	72	21	99	8	47	146	65 ± 15
M-07e/GM	98	89	100	22	50	89	98	45	0	118	46 ± 12
M-07e/TPO	84	64	100	30	56	96	97	43	0	119	59 ± 10
M-MOK/GM	95	75	99	37	60	77	98	18	0	104	45 ± 6
M-MOK/TPO	76	73	99	60	91	99	100	4	0	115	54 ± 11
TF-1/GM	92	63	56	18	97	39	100	18	99	140	39 ± 6
TF-1/TPO	86	36	69	26	98	30	100	6	99	155	74 ± 5

CD116 = GM-CSFRα; CD123 = IL-3Rα; CD117 = c-kit; MPL = TPO-R; CD41 = gpIIb/IIIa; CD42 = gpIb; CD61 = gpIIIa; GlyA = glycophorin A.

[a] Forward scatter indicating the size of the cells.

[b] Percentages of positive cells as detected by flow cytometry. Cells were tested at days 87-108. Similar results were obtained at earlier passages (days 68-83). Differences of 5% or more between the GM-CSF- and TPO-treated cultures were considered as significant.

[c] Mean channel on an arbitrary scale.

[d] Doubling times are given in hours; cells were incubated with 10 ng/ml GM-CSF or 10 ng/ml TPO.

ences in the expression of several cytokine receptors and surface proteins associated with erythroid and megakaryocytic differentiation (Table 2, Fig. 2). We considered a (reproducible) 5% difference in antigen expression to be significant. In comparison with the GM-CSF-dependent variants, the GM-CSFR and IL-3R were down-regulated in 4/4 and in 3/4 TPO-dependent cell lines, respectively; c-kit and MPL were up-regu-

lated in 2/4 and 4/4 cell lines, respectively; levels of the megakaryocytic markers CD41/CD42 and CD61 were increased in 3/4 (percentages of positive cells) and 2/4 cell lines (intensity of expression according to the mean fluorescence channel), respectively, while expression of the erythroid marker glycophorin A was decreased in 2/4 cell lines. M-MOK/TPO and TF-1/TPO cells were significantly larger than their GM-CSF-counterparts in the forward scatter analysis; while the GM-CSF- and TPO-variants of M-07e were of similar size, only the HU-3/GM cells were larger than their TPO-treated sister cells.

Morphological Analysis

Both the GM-CSF- and the TPO-exposed variants of the cell lines HU-3 and M-MOK grew as single cells in suspension with only few cells forming small aggregates. Compared with their GM-CSF-treated sister cultures, significantly higher percentages of the M-07e/TPO and TF-1/TPO cells adhered to the plastic with long cellular extensions. Of note are also the giant, polynucleated cells (1-3%) that were seen in the TF-1/TPO cultures; none of the other cultures, neither the GM-CSF- nor the TPO-treated lines, displayed significant numbers of these giant cells. With regard to the morphological appearance of the May-Grünwald-Giemsa-stained mononucleated cells, there were no significant differences between the respective pairs of GM-CSF- and TPO-incubated cells. The GM-CSF- and the TPO-treated cells displayed very similar or identical morphological features: the surface of the cells was highly deformed with cytoplasmic extrusions, reminiscent of the physiological release of platelets from the periphery of the cell ("membrane blebbing").

Expression of STAT 3 and STAT 5 Molecules

Long-term culture of the 4 cell lines with either GM-CSF or TPO did not alter the pattern of STAT 3 and STAT 5 expression. All variants showed constitutively strong expression of both proteins.

TPO as Survival Factor

We evaluated the ability of TPO to protect the four TPO-dependent cell lines under serum-free conditions. TPO could clearly extend the survival of HU-3/TPO and M-MOK/TPO. TF-1/TPO cells even showed some degree of proliferation in the serum-free medium. We calculated the following half-lives; HU-3/TPO: nil (25 h), 20 ng/ml TPO (73 h), 5 ng/ml GM-CSF (74 h); M-MOK/TPO: nil (42 h), TPO (74 h), GM-CSF (102 h); TF-1/TPO: nil (32 h), GM-CSF (18 h), TPO (growth).

Mitogenic Effects of Several Cytokines

As shown in Table 3, the TPO-treated variants responded proliferatively to the same cytokines as their parental GM-CSF-treated

Table 3. Proliferative response of the GM-CSF- and TPO-dependent cell lines to various cytokines

Cell line	5637 CM	EPO	G-CSF	GM-CSF	IL-3	PIXY-321	SCF	TPO
HU-3/GM	972[a]	639	156	2782	1971	2915	1357	711
HU-3/TPO	1113	954	107	4014	3442	4552	2942	1430
M-07e/GM	554	108	91	1012	881	990	805	411
M-07e/TPO	977	112	102	1916	1507	2289	1371	1404
M-MOK/GM	1272	117	140	2475	2186	3142	5026	1284
M-MOK/TPO	1015	91	99	1940	2026	2604	2465	1624
TF-1/GM	948	450	98	960	908	948	426	312
TF-1/TPO	1094	650	95	1554	1264	1566	598	664

[a] Results are expressed as percentages of the respective untreated control cells. Cells were tested at days 81-110. Similar results were obtained at earlier passages (day 68).

cells. Apart from TPO, 5637 CM, EPO (only on HU-3 and TF-1), GM-CSF, IL-3, PIXY-321 and SCF had mitogenic effects on these cell lines; G-CSF did not stimulate growth in any of these cultures. The TPO-exposed variants of HU-3, M-07e and TF-1 showed higher ^{3}H-thymidine incorporations relative to the respective untreated control cells than the sister cultures grown in GM-CSF. The reverse, a stronger proliferative response of the GM-CSF variant to incubation with 5637 CM, GM-CSF, IL-3, PIXY-321 and SCF was seen for M-MOK. Dose-response curves demonstrated a significantly stronger proliferative stimulus of TPO on all four TPO-cell lines than on the GM-CSF-cell lines.

Discussion

Studies on hematopoietic precursor cells and their immature progeny are hampered by the paucity of these cells in the normal hematopoetic tissues and the necessity for extensive purification procedures. Such studies often take recourse to hematopoietic cell lines established from patients with leukemia which recapitulate many of the developmental steps of normal cells. TPO has a tremendous clinical potential and there is an explosion of research activities in this field. We felt it was important to generate a reproducible, accessible and significant in vitro system that is sufficiently representative to perform the extensive examinations of the biological functions of TPO.

In this study TPO was used to establish TPO-dependent continuous human leukemia-derived cell lines. The four lines derived from constitutively growth factor-dependent cell lines were continuously grown for more than 6 months in TPO-supported liquid culture. The cells are TPO-dependent rather than merely TPO-responsive as omission of TPO in the cell culture completely abrogated cell proliferation. While the swift change from incubation with GM-CSF to TPO was not successful, rather requiring a certain adaptation period, all TPO-treated cell lines could be easily switched back to GM-CSF.

Under serum-free conditions TPO promoted cell viability by repressing apoptosis in the TPO-dependent cultures. Depending on the experimental setting TPO can act as a survival factor or as a proliferation-inducing agent. The survival signal provided by TPO is, however, not specific as also GM-CSF rescued TPO-treated cells from apoptosis upon withdrawal of TPO.

While GM-CSFR and IL-3R were downregulated in the TPO-treated cells, c-kit and MPL were upregulated. The cytokines EPO, GM-CSF, IL-3, PIXY-321, SCF and TPO had stronger growth-stimulating effects on three or all four of the TPO-dependent cell lines than on their GM-CSF-exposed counterparts. The immunophenotypic shift of the TPO-cell lines to a stronger expression of the megakaryocytic surface markers CD41, CD42 and CD61, accompanied by the downregulation of the erythroid lineage-associated surface protein glycophorin A, suggests differentiation along the megakaryocytic cell axis induced by the long-term exposure to TPO.

Komatsu et al. succeeded in isolating a subline designated UT-7/TPO that was absolutely dependent on TPO for growth and survival showing several mature megakaryocytic properties [15]. Recently, we were able to also establish a TPO-dependent UT-7 cell line confirming these results (data not shown). Other studies showed that it is possible to transfer or extend the cytokine-dependency of cell lines from one to another or several factors: an EPO-dependent subline, UT-7/EPO, had been isolated from the parental UT-7/GM cells (grown in GM-CSF); the cell line M-TAT (cultured with GM-CSF as M-TAT/GM-CSF) proliferated similarly for more than 1 year in EPO (M-TAT/EPO) or SCF (M-TAT/SCF) [16, 17].

This switch to incubation of a growth factor-dependent cell line with a different cytokine is not always possible; the fact that a cell line responds proliferatively for a short period of time (24-72 h) to a given cytokine does not automatically imply that these cells can be grown over longer periods of time with this particular factor (in the absence of the "usual diet"). In addition, there is always the possibility that the cells become quickly cytokine-independent, showing vigorous growth, but no longer requiring supplementation of any external growth factor.

In summary, we report here the establishment of four TPO-dependent leukemia cell lines isolated from constitutively growth factor-dependent cell lines. The present data show that the culture systems described herein may be useful to dissect basic mechanisms regulating megakaryocyto- and thrombopoiesis in a simplified and isolated setting.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Generation of Active Oxygen Forms by Neutrophils and Granulocytopoiesis in Patients with Acute Lymphoblastic Leukemia under Therapy with Use of G-CSF Granocyte

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Abstract. It was established that the yield of active oxygen forms (AOF) in neutrophils of children with acute lymphoblastic leukemia (ALL) at the adhesion of cells and at latex action was lower in patients with neutropenia in comparison with normal donors. Simultaneous restoration of granulocytopoiesis and increase in the ability to generate AOF by cells usually occur in patients in the course of therapy with the use of granulocyte colony-stimulating factor (G-CSF). Cessation of G-CSF injection to patients with primary ALL and with relapse results in decrease in oxygen activating ability of cells and increase in the contents of young neutrophil forms in blood. It was found that myeloperoxidase activity of neutrophils in patients with ALL in neutropenia is lower in comparison to normal donors and did not change in the course of therapy with the use G-CSF. It was shown that G-CSF therapy of patients with ALL leads to partial restoration of the activity of redox systems localised in plasma membranes but not inside the cells.

Introduction

Treatment of oncological diseases in children is impossible without employment of complex intensive chemotherapy protocols. Use of such protocols inevitably causes profound myelosuppression [1, 2]. It is shown that neutropenia results in higher risk of exogenous and endogenous infections in the organism. The degree of the risk of infections correlates with the duration and severity of neutropenia [3, 4]. It becomes an obstacle to further administration of intensive chemotherapy cycles and results in poor prognosis.

In the Republic of Belarus the rate of children mortality as a result of infections is 2-3 times higher as compared with the other European countries. Examination of patients in the Belarusian Children's Oncohematological Centre (Minsk, Belarus) has shown that 67.2 % of patients with ALL in complete remission had sepsis [5].

Might be decreased by mortality the use of growth and differentiation hemopoietic factors. The granulocyte colony-stimulating factors (G-CSFs) are very important among these factors. It is known from publications that G-CSFs facilitate: myelopoiesis restoration after transplantation of bone marrow and intensification of chemotherapy schedules due to decrease in severity and duration of neutropenia, reduction of the treatment cost because of decreased consumption of expensive antibiotics, blood preparations and their substitutes and shorter hospitalisation time [6, 7]. In vitro comparison of biological capacities of various commercial recombinant G-CSFs (rG-CSFs) forms has

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shown that the glycosylated form of Granocyte G-CSF (lenograstim) (Chinese hamster ovary cell-derived) has the highest efficiency in comparison with the nonglycosylated form [8, 9].

It should be noted that the functional activity of neutrophils is important for the organism with neutropenia. In order to perform phagocytosis neutrophils require active oxygen forms that are produced under external stimulating effects on the cells [10]. Results of studies on the oxygen - activating function of neutrophils with the use of rG-CSFs are scanty and there are almost no reports about the effects of Granocyte [7, 11, 12].

The goal of our work is to analyse the efficiency of Granocyte G-CSF for stimulation of granulocytopoiesis and to determine the dynamics and mechanisms of changes in the oxygen-activating ability of neutrophils under the action of Granocyte G-CSF in patients with ALL and relapsing of ALL during early neutrophil recovery.

Material and Methods

Neutrophils were obtained from peripheral blood plasma of patients and normal donors by centrifugation in the Ficoll-verografin density gradient [13]. Erythrocyte impurity was deleted by osmotic shock. The cell preparations were washed and resuspended twice in Earl's solution and contained more than 96% of neutrophils.

The generation of AOF by neutrophils was investigated with luminol-dependent chemiluminescence (LDCL) method with the use of a BCL-1 biochemiluminometer (Belarus) [14]. LDCL was observed at adhesion of the cells to the bottom of a cylindrical glass cuvette with the diameter 4 cm. It was analysed after addition of 30 μl of latex (Factory of Bacterial Preparations, Kaunas, Lithuania), 50 μg/ml of phytohemagglutinin (PHA) (Sigma, USA) and 25 μg/ml of arachidonic acid (Sigma, USA). The concentration of neutrophils was 1.6×10^6 cell/ml. Measurements were carried out in Earl's solution (pH = 7.4) at 200 °C. The luminol concentration was 1.25×10^{-5} mol/l.

Myeloperoxidase (MP) activity of cells was studied by chemiluminescence method in system: luminol-hydrogen peroxide-neutrophils. The concentration of luminol was 1.25×10^{-5} mol/l and the concentration of hydrogen peroxide was 2×10^{-5} mol/l. Before investigation, neutrophils were destroyed in three cycles of freezing and melting of cell suspension.

The results of this study were assessed with the Student's test.

We have examined 7 patients with ALL and 6 patients with ALL relapse who survived after chemotherapy in febrile neutropenia condition. Granocyte G-CSF (Chugai-Rhone-Poelenc, Antony, France) was daily injected subcutaneously in a dose of 5 μg/kg. Injections of G-CSF were administered with levels of leukocytes $< 1 \times 10^9$/l and neutrophils $< 0.5 \times 10^9$/l in blood and it the patients' temperature exceeded 38.0 °C. The reason for stopping G-CSF was normalisation of the patients' condition and a peripheral blood count of leukocytes, 2×10^9/l and neutrophils, 0.5×10^9/l. The control group consisted of 10 normal donors at the average age of 12 years. All the patients were hospitalised and treated in the Belarusian Children's Oncohematological Centre (Minsk, Belarus).

Results and Discussion

It is known that neutrophils of normal donors generate AOF at adhesion to glass and under the influence of a number of phagocytosis-stimulating factors [9, 15]. Typical kinetic curves of induced AOF production in neutrophils of normal donors are shown in Fig. 1A. It is evident that for a normal donor the dependence of LDCL intensity on time is a complicated two-staged process of cell adhesion to glass (curve 1).

Addition to the cell suspension of latex particles as a phagocytosis inductor or PHA as a receptor-mediated endocytosis inductor or arachidonic acid stimulating the mechanism of glucose-independent activation of plasma membrane redox systems results in an increase in the yield of AOF formation (curves 2, 3, 4). This process is most pronounced following the addition of arachidonic acid and latex.

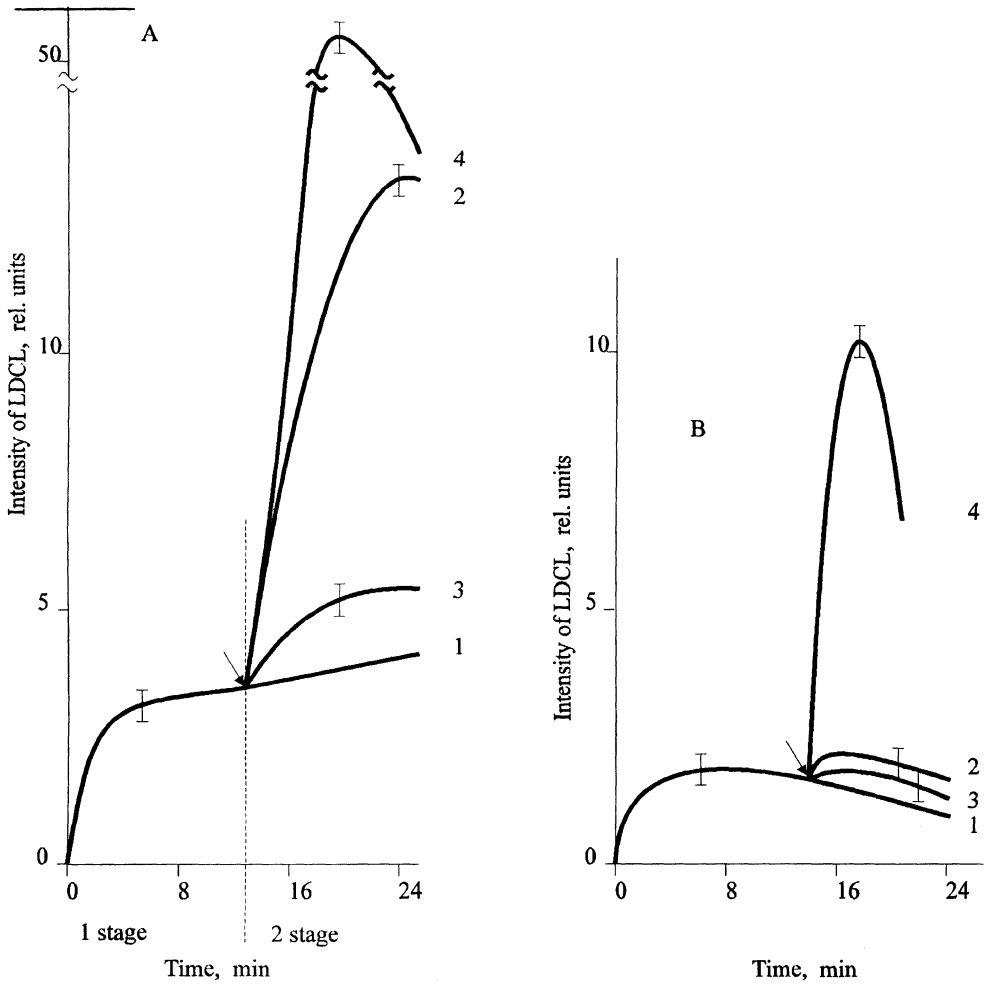

Fig. 1. Typical kinetic curves of neutrophils LDCL intensity of normal donor *(A)* and of a patient with ALL *(B)* at adhesion of the cells to glass *(1)*, at the action of latex *(2)*, PHA *(3)* and arachidonic acid *(4)*

The kinetic curves of the LDCL intensity of a patient with ALL after an intensive chemotherapy course that resulted in neutropenia are shown in Fig. 1B.

Comparison of the curves in Fig. 1 (A and B) shows differences in AOF generation by the cells in the patients and normal donors. For example, for the sick children, the second stage of AOF formation is less expressed in the kinetic curves of the LDCL intensity at contact of the cells with the glass surface. The total yield of AOF induced by adhesion was lower for the patients than it was for the normal donors, which might be due to disturbances of the mechanisms of involvement of the myeloperoxidase system, localised in azurophylic granules of neutrophils, in the processes of AOF generation [16].

In the case of latex particles a low LDCL intensity was observed for cells of the patients with neutropenia in comparison with the normal donors. It is known that with addition of latex AOF generation indicates the level of the phagocytic activity of neutrophils [17]. The present data suggest that the functional activity of neutrophils is significantly reduced in the sick children with neutropenia.

It is known that AOF formation in neutrophils occurs in two ways: with and without participation of glucose [18]. It is shown that arachidonic acid stimulates the glucose-

independent mechanism of oxygen activation in cells [16]. As can be seen in Fig. 1A, the intensity of arachidonic acid-induced LDCL is much lower in the patients in comparison with the normal donors. Decrease in the functional activity of neutrophils is probably caused by inhibition of both glucose-dependent and glucose-independent mechanisms of AOF generation after chemotherapy on the background of agranulocytosis.

It should be noted that under the action of arachidonic acid the AOF yield is much higher in some patients in comparison with the normal donors. Further studies are necessary to find the reason for this phenomenon.

The effect of G-CSF on the change in the total AOF yield under stimulation of neutrophils and the leukopoiesis process for patients with ALL (primary and relapse) is shown in Figs. 2-4. In the examples shown in the figures, the therapy with the use of G-CSF is accompanied by an increase in the number of blood leukocytes, which is caused by stimulation of myelopoiesis. In two of the three presented examples, enhancement of the functional activity of neutrophils determined from AOF formation under the action of latex particles and PHA on neutrophils is observed simultaneously with restoration of granulocytopoiesis (Figs. 2, 3). However, after cancellation of G-CSF injections, the functional activity of neutrophils decreases with a simultaneous increase in their blood concentration.

Results presented in Figs. 2 and 3 are typical of 4 of 7 cases of primary ALL and of 3 of 6 cases of ALL relapse. A constant level or reduction of the oxygen - activating ability of neutrophils was observed in the other patients (an example is shown in Fig. 4). It should be emphasised that heavy complications are characteristic of this group of patients (abscesses of brain, liver, spleen, necrotising ulcerative stomatitis, necrotising glossitis) on the background of staphylococcus and pseudomonas sepsis.

We have found earlier that during Granocyte G-CSF treatment of patients with non-Hodgkin's lymphomas (NHL) and neuroblastomas (NB), leukopoiesis and AOF generation continued to be enhanced even after

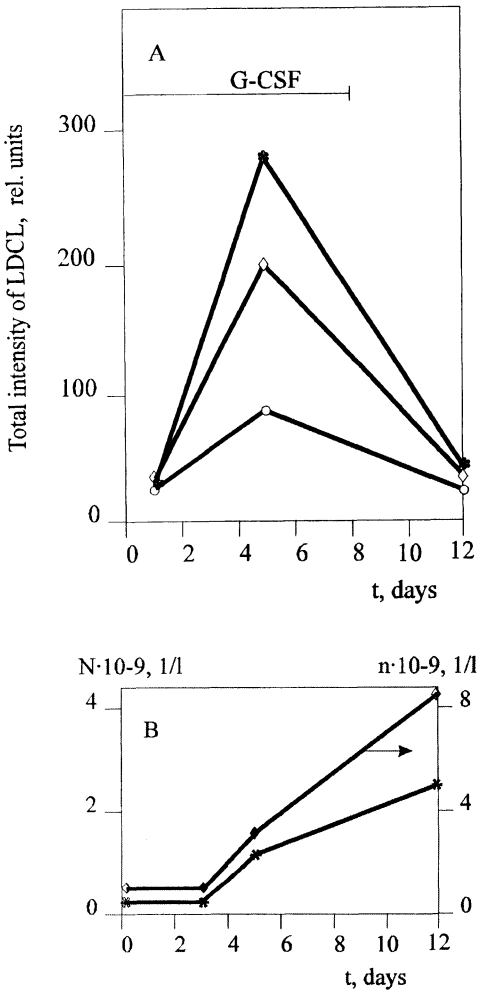

Fig. 2. The effect of G-CSF on the change of the LDCL total intensity of neutrophils *(A)* and on the number of leukocytes *(n)* and neutrophils *(N)* in blood *(B)* of the patient with primary ALL. *t* time after beginning of the treatment with the use of G-CSF; ◊ LDCL intensity at adhesion of the cells to glass; ✻ LDCL intensity at the action of latex; ○ LDCL intensity at the action of PHA

cancellation of G-CSF injections [12]. It is known that unlike NHL and NB, in the case of ALL the hemopoiesis mechanisms are disturbed at the cellular level [19]. These disturbances are accompanied by changes in the production of some cytokines (for example, interleukines) and in transduction of the activating signal during cell growth and differentiation [20]. It can be also suggested that

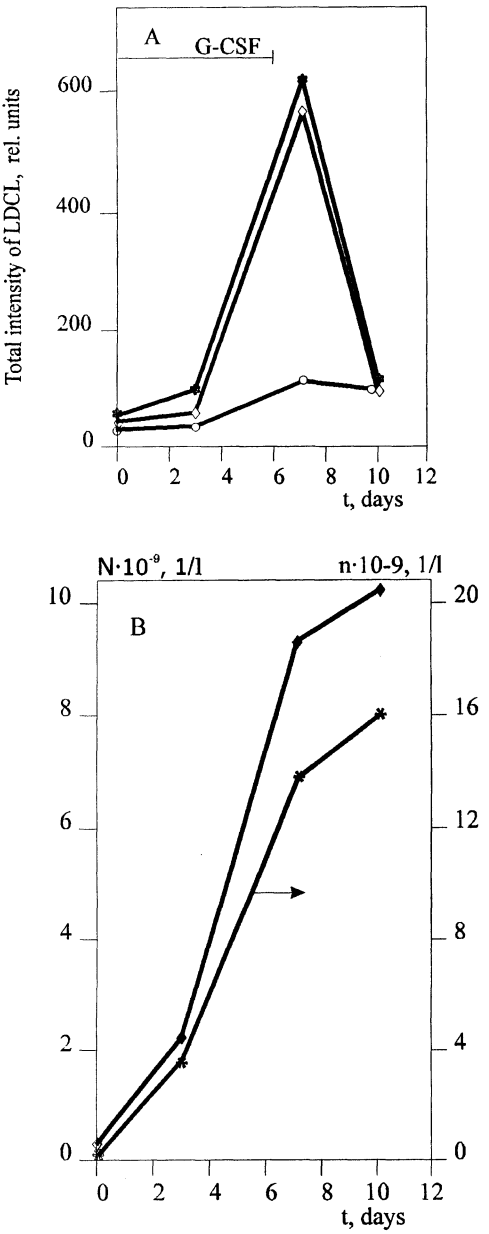

Fig.3. The effect of G-CSF on the change of the LDCL total intensity of neutrophils *(A)* and on the number of leukocytes *(n)* and neutrophils *(N)* in blood *(B)* of the patient with ALL relapse. *t* time after a beginning of the treatment with the use of G-CSF. ◊ LDCL intensity at adhesion of the cells to glass; ✻ LDCL intensity at the action of latex; ○ LDCL intensity at the action of PHA

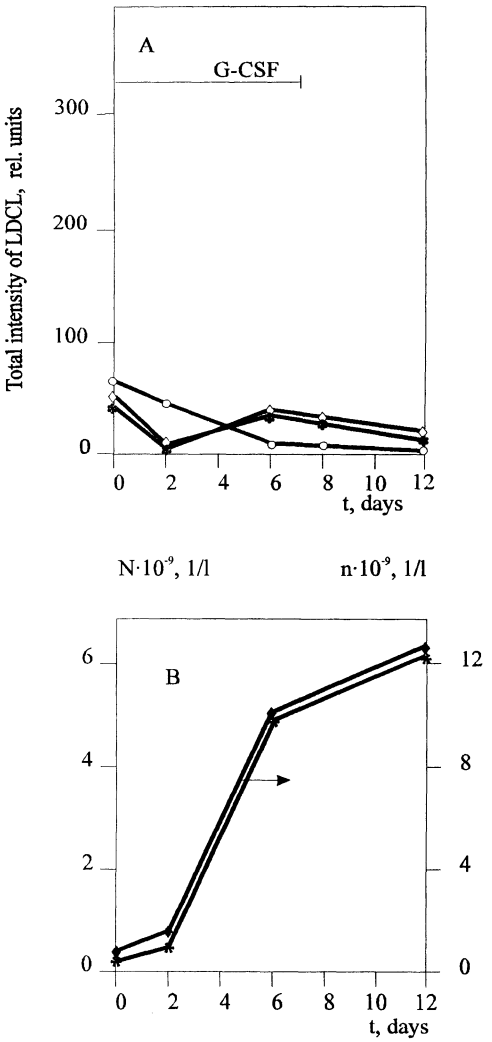

Fig.4. The effect of G-CSF on the change of the LDCL total intensity of neutrophils *(A)* and on the number of leukocytes *(n)* and neutrophils *(N)* in blood *(B)* of the patient with primary ALL. *t* time after a beginning of the treatment with the use of G-CSF. ◊ LDCL intensity at adhesion of the cells to glass; ✻ LDCL intensity at the action of latex; ○ LDCL intensity at the action of PHA

unlike NHL and NB, during ALL therapy the granulocyte colony-stimulating factor is a necessary, but not a sufficient mean for restoration of functional parameters of neutrophils.

In Fig. 5 one can see a plot of the total intensity LDCL over time for normal donors. It

can be seen that the level of AOF induced formation in neutrophils remains constant for 14 days. There results demonstrate that the change in the oxygen-activating function of neutrophils in the patients with ALL is induced by the use of Granocyte G-CSF during treatment and is independent of the individual factors.

As was already noted, children with ALL are characterised by a decreased AOF yield at adhesion of cells to glass in the second stage of the kinetic curve (Fig. 1B), which is ascribed to the intracellular myeloperoxidase system. It can be suggested that either the MP activity of neutrophils decreases, or the redox systems localised in plasma membrane and inside the cell changes in the diseases studied. In Fig. 6 one can see MP activities of neutrophils measured in normal donors and patients with neutropenia before administration of the factor and after its cancellation. As can be seen from Fig. 6, in all the patients examined in this study, the MP activity of neutrophils is much lower in comparison with the normal donors. The increased functional activity of neutrophils determined from their ability to generate AOF upon administration of Granocyte G-CSF is not accompanied by increase in the MP activity of neutrophils. These data indicate that Granocyte G-CSF affects the activity of redox systems localised in the plasma membranes but not inside the cells.

Thus, the following conclusion can be drawn from the present results: in patients with primary ALL and ALL relapse with neutropenia, the functional activity of neutrophils is much lower in comparison with normal donors, which is confirmed by low levels of AOF generation and myeloperoxidase activity of cells. Administration of Granocyte G-CSF stimulates leukopoiesis in all the observed patients with ALL. In about 50% of all cases of primary ALL and relapsing ALL, injection of the factor results in substantial increase in the oxygen-activating ability of neutrophils, which is indicative of an increase in the functional activity of these cells. In the other cases the production of AOF by blood neutrophils either remained unchanged or decreased. After cessation of G-CSF injection, leukopoiesis retarded and the ability of neutrophils to gen-

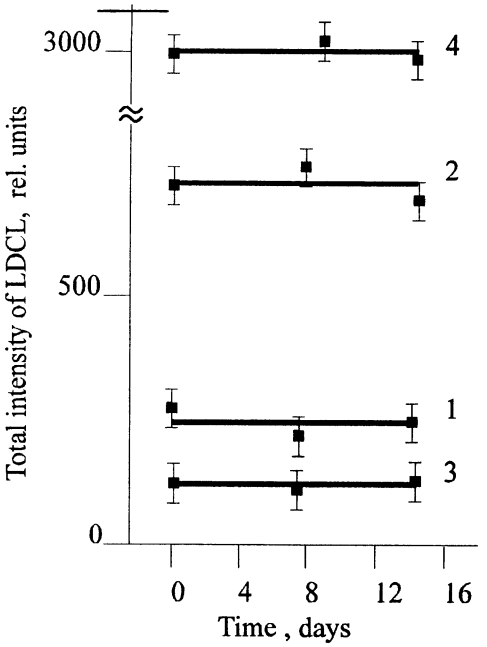

Fig. 5. Dependence of the total LDCL intensity on time for neutrophils of the normal donors at adhesion of the cells to glass *(1)*, at the action of latex *(2)*, PHA *(3)*, arachidonic acid *(4)*

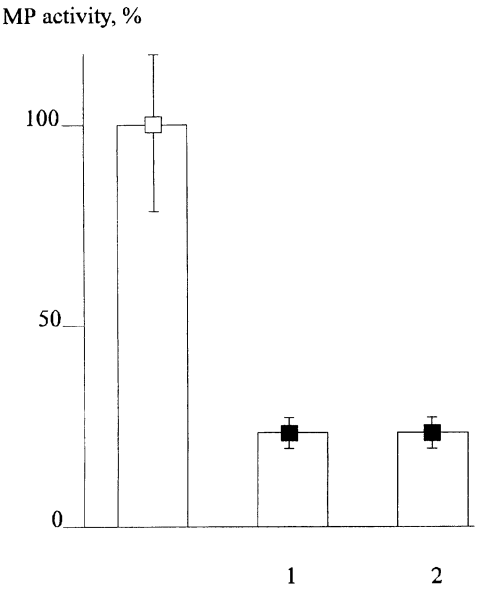

Fig. 6. Myeloperoxidase activity of neutrophils in the normal donors (□) and in the patients (■); *1* at neutropenia before G-CSF administration; *2* after G-CSF therapy at the exit from neutropenia condition

erate AOF decreased in all the patients examined. It is suggested that Granocyte G-CSF is a necessary but not a sufficient means for restoration of the neutrophils functional activity. In the patients' organisms subjected to the action of Granocyte G-CSF, unlike the oxygen-activating ability of neutrophils, the MP activity is not normalised, which testifies to the modifying effect of the factor on the activity of membrane but not intracellular redox systems.

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Drug Resistance

Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Lineage-Tropism of Murine Leukemia Virus Enhancer Activity within the Hematopoietic System

C. BAUM and W. OSTERTAG

Abstract. Tissue-tropism and pathogenicity of murine leukemia retroviruses are strongly influenced by the enhancer assembly located in the U3-region of the long terminal repeat. The polycythemic strain of the murine spleen focus-forming virus (SFFVp) is an acutely transforming retrovirus inducing a bistage erythroleukemia in adult mice. The disease specificity results from the exclusive activity of the env-encoded glycoprotein gp55 which stimulates the cellular receptor for erythropoietin. In contrast to gp55, the enhancer of SFFVp is widely active within the hematopoietic system, including early and late myeloid progenitor cells, rendering it a powerful tool for the design of retroviral gene transfer vectors targeting hematopoietic progenitor and stem cells. We mapped four crucial elements responsible for the high and wide activity of the SFFVp-enhancer. These are the altered upstream control region, the conserved LVb-box, the unique enhancer core, and the E-box overlapping with the distal glucocorticoid-responsive element of the enhancer. These motifs are targets for the ubiquitous transcriptional activator Sp1, Ets-proteins, Polyoma enhancer binding proteins/core binding factors (PEBP/CBF), and basic helix-loop-helix factors, respectively. Stage and lineage-dependent variations of the enhancer activity are presumably highly influenced by the differential developmental distribution and activation of individual members of these complex transcription factor families. Systematically dissecting retroviral enhancer function thus represents a promising system to deepen our knowledge of hematopoietic transcriptional regulation. Furthermore, it might result in the definition of gene-transfer vectors that create a more predictable tropism of transgene expression in defined developmental stages or lineages.

Introduction

Murine leukemia viruses (MLV) belong to the family of simple C-type retroviruses. Replication-competent members encode the *trans*-acting genes *gag-pol* and *env* necessary for virus replication. When injected into newborn mice of permissive strains, these viruses exhibit a slowly transforming potential in hematopoietic progenitor cells. Disease induction occurs after a relatively long latent phase, characterised by progressive replication in susceptible cells. The host range is mainly influenced by the interaction of the viral env-protein with developmentally restricted cellular receptors as well as by the transcriptional potency of the retroviral cis-acting elements residing in the long terminal repeat (LTR) and leader-sequences. Leukemias usually develop on basis of hyperproliferative disorders and involve activation of cellular oncogenes by retroviral insertional mutagenesis [1].

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Some replication defective strains of MLV have been isolated carrying activated oncogenes substituting *gag-pol* or *env*-reading frames. When propagated by replication-competent MLV, these acutely transforming variants induce polyclonal leukemias or sarcomas within a few weeks. In most cases, the acutely transforming oncogene acts dominantly over the cis-active elements of the retrovirus in defining the disease specificity. This indicates that the retroviral oncogenes require a developmentally defined cellular context to exert their pathogenic functions. An example is the polycythemic strain of the spleen focus-forming virus, SFFVp, which transforms erythroid precursor cells. SFFVp encodes the truncated env-protein gp55 able to stimulate the cellular receptor for erythropoietin, preferentially resulting in proliferation rather than differentiation of target cells [2]. In this case, the enhancer in the LTR contributes only to the disease kinetics, but not to its specificity [2]. Similar observations have been made with other acutely transforming MLV [3]. However, at least two cases have been described where the enhancer sequences influence the tissue specificity of acutely transforming MLV. Firstly, the myeloproliferative sarcoma virus (MPSV) carrying the *v-mos* oncogene gains transforming potential in early myeloid cells by virtue of enhancer-alterations [4]. Secondly, the preferential transformation of macrophage progenitors by the malignant histiocytosis sarcoma virus (MHSV) transducing the *ras*-oncogene is strongly influenced by the retroviral enhancer [5], which is highly related to that of SFFVp [6]. In these cases, the transferred oncogenes presumably are more pleiotropic in their transforming ability in vivo. Then, the levels of replication and oncogene-expression determined by the LTR-enhancer can contribute to the disease specificity, which generally reflects the most permissive (but not the only) tissue affected.

When the coding sequences of MLV are replaced with cDNAs encoding diagnostic, protective or corrective entities, replication-incompetent vectors result which are widely used in experimental hematology and gene therapy [7]. MLV-based vectors are produced in safety-modified packaging cell lines releasing replication-incompetent retroviral particles. These particles maintain their ability to stably insert the genetic information of the vector in dividing target cells. As in the case of their leukemogenic ancestors, the phenotypical consequences of the genetic transduction are a function of the expression level and the activity of the encoded entities in the relevant target cell. Striking evidence for the enhancer-dependence of the vector-conferred phenotype was achieved upon transfer and expression of the human multidrug-resistance 1 cDNA, encoding a membrane-located drug efflux pump mediating resistance against a variety of commonly used anticancer drugs [6-8]. Therefore, both the enhancer and the transferred coding sequences are to be considered when employing retroviral vectors in experimental or therapeutic approaches.

On the basis of genetic observations made with slowly or acutely transforming MLV in mice, we have attempted to improve the transcriptional potency of MLV-based vectors in early hematopoietic cells and their progeny [6, 9]. In the absence of a defined in vitro-assay system for hematopoietic stem cells, overall enhancer activities of different strains of MLV were analysed in primary human in vitro clonogenic hematopoietic cells, human and murine cell lines representing distinct stages of hematopoiesis, as well as in embryonic stem (ES) cells, presumably sharing some transcriptional properties with pluripotent, yet uncommitted HSC [6-11]. We observed that the enhancers of SFFVp and MHSV, both related to the family of Friend mink cell focus-forming (FMCF) MLV, are powerful tools for the design of gene transfer vectors mediating high expression levels in myelo-erythroid progenitor and stem cells (6-8). Defining the molecular basis for the activity of these FMCF-type enhancers would be expected to contribute to our understanding of transcriptional control in the hematopoietic system. Furthermore, it might enable to design novel enhancers exhibiting further improved activity in distinct hematopoietic stages or lineages. As indicator systems for these studies, we employed conventional reporter genes like cloramphenicol transferase (CAT), luciferease (luc) or β-

galactosidase (lacZ) as well as dominant selectable markers like the neomycin resistance gene or "cancer drug resistance" genes [6-13].

Material and Methods

Cell Lines, Plasmids, and Transfections

Cell lines used in the assays described in this report were: CCE, murine ES cell line; FDCP-mix 15S, non-leukemic, multipotent myeloerythroid progenitor cells of murine origin; WEHI-3B, murine macrophage progenitor cell line; FDCP-1, murine myeloid progenitor cell line; F4-12-B2, murine erythroleukemia and Friend cell line; K562, human erythroleukemia cell line; CTLL-2; murine cytotoxic T-cell line. Culture conditions have been described [6, 10, 11].

CAT-vectors have been described [12]. Luc-vectors are based on SF-luc [13]; in MP-luc and PC-luc, the enhancer/promoter was derived from MPSV and its derivative PCMV, respectively.

Transfections were performed using an efficient electroporation protocol [14].

Reporter Gene Assays

These were performed as described [6], using the CAT-Elisa (Boehringer Mannheim) to detect CAT-protein, the luciferase reporter gene assay system (Boehringer Mannheim) to detect luc-activity, and the ONPG-assay to detect cotransfected β-galactosidase activity in cell extracts harvested 24-36 hours after electroporation in the presence of 10 μg/assay CAT-plasmid or luc-plasmid plus 10 μg/assay pCMVβ (Stratagene).

Results

The Enhancer of SFFVp Is Strongly Active in Diverse Hematopoietic Progenitor and Embryonic Stem Cells

Previous work of our group has shown that SFFVp contains the most potent retroviral enhancer known so far for gene expression in hematopoietic progenitor cells [6-8].

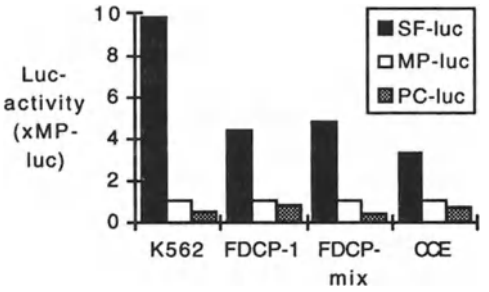

Fig. 1. The enhancer of SFFVp is strongly active in hematopoietic progenitor and in ES cells. Cells were transiently transfected with luciferase (luc) reporter plasmids as described in Material and Methods. In SF-luc, MP-luc, and PC-luc, the luc-gene is under control of the enhancer/promoter of SFFVp, MPSV, and PCMV, respectively. Luc-activity was determined 24-36 h after transfection by electroporation and corrected for cotransfected β-galactosidase activity. Data were normalised with respect to the levels achieved with MP-luc. Standard deviations were below 20%

When compared with the enhancers of MPSV or the MPSV-related PCMV, previously considered to be the strongest enhancers for gene expression in early hematopoietic cells [9, 15], the SFFVp enhancer is up to one order of magnitude more active. This is examplified by the experiments shown in Fig.1. The expression levels obtained in transient transfection of hematopoietic cells correlate well with the relative expression levels of retroviral vectors after stable transduction using various indicator systems ([6, 8, 13] and unpublished data).

Strikingly, the SFFVp-enhancer was also found to be of high activity in transiently transfected ES cells CCE, correlating with an elevated relative transduction frequency as determined with transfer of the neomycin resistance gene (Fig.1, and data not shown). However, the gene expression levels achieved in primitive ES cells were up to two orders of magnitude reduced when compared with more mature hematopoietic progenitor cells (as determined by Northern Blot analysis and quantification of reporter protein expression levels after stable retroviral transduction, data not shown). To better understand the molecular basis for this phenomenon, we initiated a molecular analysis of the SFFVp-enhancer [12].

Four Intriguing Features of the SFFVp-Enhancer

The analysis of the individual cis-acting elements responsible for the strong activity of the SFFVp enhancer was based on a sequence alignment with the related Friend-MLV (F-MLV), the more lymphotropic Moloney-MLV (Mo-MLV), and MPSV [12, 13]. At least four intriguing features of the SFFVp enhancer were noted (Fig. 2).

Firstly, the upstream control region (UCR) located at the 5 basis of the retroviral enhancer contains two highly conserved point mutations creating a GC-box resembling a binding-site for the ubiquitous transcription factor, Sp1 [12] (Fig. 3). In contrast, the UCR of Mo-MLV and F-MLV contains the consensus for a transcriptional repressor of LTR-activity expressed in primitive embryonic cells and known as ELP [16].

Secondly, compared with Mo-MLV, only one out of seven putative recognition sites for Ets-proteins is conserved [12]. This is the LVb-site, located immediately upstream of the enhancer core, and already known to intimately cooperate with the enhancer core in defining the tissue-tropism of replication-competent MLV [18, 19].

Thirdly, the enhancer core itself reveals two point mutations, seemingly diagnostic for FMCF-type enhancers. Compared with Mo-MLV, MPSV and F-MLV, which fulfill the consensus of both Polyoma enhancer binding protein/core binding factor (PEBP/CBF) and CAAT/enhancer binding protein (C/EBP) in the enhancer core, a pyrimidine-switch is found at position 3 of the core, while a purine-switch is noted at position 7. The pyrimidine-switch is indicative for increased affinity to PEBP/CBF, whereas the purine-switch suggests exclusion of C/EBP [12] (Fig. 4).

Last but not least, the E-box overlapping with the distal glucocorticoid-responsive element [22] is the only putative target for bHLH-factors shared with Mo-MLV and MPSV.

Functional Significance of These Four Elements

Using electrophoretic mobility shift and transient transfection assays, we identified transactivators potentially targeting the altered recognition sites found in the UCR and enhancer core [12]. As already suggested from the sequence, Sp1 was able to bind to the UCR of SFFVp, but not to the corresponding sequence of F-MLV or Mo-MLV. Binding of Sp1 correlated with increased ac-

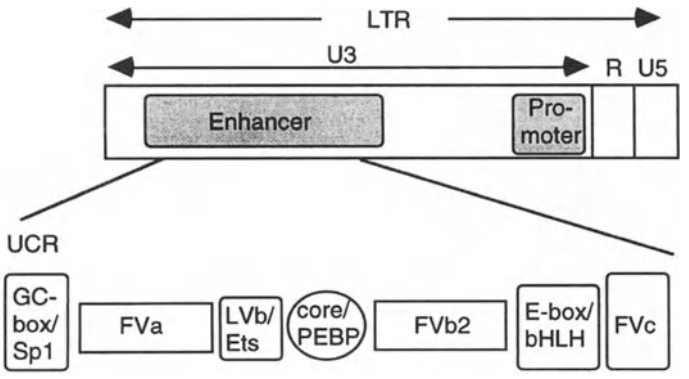

Fig. 2. Simplified representation of the central enhancer region of SFFVp. The order of the hitherto described control elements is shown in the 5' to 3' orientation. For abbreviations of binding sites, refer to the text

```
TCAAGGTCA              ELP
   KGGGCGGRRY          Sp1

TCAAGGTCAGGTA          F-MLV, Mo-MLV
------G-G----          SFFVP, MHSV, FMCF FrNx, FMCF pFMS48
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Fig. 3. Sequence alignment of the UCR of various MLV (adapted from [17]). *Dashes* indicate homology to F-MLV and Mo-MLV. Consensus sequences for ELP and Sp1 are aligned above the MLV-sequences

```
TGYGGTN                 CORE (ref. 21)
TGYGGTY                 PEBP/CBF
 KNNGNAAK               C/EBP

TGTGGTAAG               F-MLV, Mo-MLV, MPSV
--C---G--               SFFVP, MHSV, FMCF1
```

Fig. 4. Sequence alignment of the enhancer core of various MLV (adapted from [12]). *Dashes* indicate homology to F-MLV and Mo-MLV. Consensus sequences for PEBP/CBF and C/EBP are aligned above the MLV-sequences

Table 1. UCR and enhancer core govern the activity of the SFFVp-U3

Construct	Enhancermotif		Activity in cell line				
	UCR	core	CCE	FDCP-mix	WEHI-3B	F4-12-B2	CTLL-2
SSS-CAT	SFFVp	SFFVp	1.0	1.0	1.0	1.0	1.0
SSF-CAT	SFFVp	F-MLV	0.8	0.8	0.8	0.7	0.7
FFS-CAT	F-MLV	SFFVp	0.3	0.5	0.7	0.4	0.5
FFF-CAT	F-MLV	F-MLV	0.3	0.4	0.6	0.4	0.4

CAT-values obtained after transient transfection of the reporter constructs indicated. The activity of construct SSS-CAT in each cell line is set as 1. These constructs contain the complete retroviral enhancer/promoter of SFFVp. Enhancer-motifs in the UCR and core-region were mutated to the situation found in F-MLV using site directed mutagenesis and conventional cloning techniques [12]. Mean values of repetitive experiments, standard deviations were below 20%. Data inferred from [12], with the exception of CTLL-2.

tivity of the SFFVp-type UCR-motif in transfection assays. It was found that the UCR dominates gene expression in ES cells. Moreover, in combination with the altered enhancer core, it accounted for up to half of the transcriptional activity in hematopoietic progenitor cells. Strikingly, the transcriptional dominance of these alterations was observed in a rather lineage-independent manner (Table 1). As far as the ubiquitously expressed Sp1 was concerned, this was not a big surprise. However, the gain of activity obtained with the SFFVp-type enhancer core indicated that the transactivators targeting this sequence would also be widely spread. As already predicted from the sequence alignment (Fig. 4), electrophoretic mobility shift and transfection assays revealed that the SFFVp enhancer core represents an exclusive and high-affinity target for PEBP/CBF, a transcription factor family clearly involved in myeloid and lymphoid differentiation processes [23, 24]. In contrast to Sp1, PEBP/CBF was found to be subject to stage-and lineage dependent posttranscriptional modifications [12, 25]. Interestingly, PEBP/CBF was not detected at significant levels in ES cells. Still, we consistently observed that the SFFVp-type enhancer core was needed to obtain the full activity of the retroviral enhancer in ES cells. One explanation for this phenomenon was the exclusion of inhibitory factors. Indeed, we observed that the core-motif of SFFVp, in contrast to that of Mo-MLV, F-MLV or MPSV was unable to bind C/EBP [12], a member of the bZIP-family of transcription factors [23, 24]. C/EBP was not only detected in all hematopoietic cells analysed, but also in ES cells [12]. Thus, at least in primitive, undifferentiated cells some circumstantial evidence suggested that yet undefined C/EBP family members might represent potential inhibitors of MLV-enhancer activity.

In lymphotropic MLV, the conserved LVb-site and E-box have been shown to be targets for Ets-factors and bHLH-proteins, respectively [19, 22]. A point mutation introduced into the LVb-site and destroying the Ets-consensus also significantly reduced SFFVp-enhancer activity in myeloid and ES cells ([12] and data not shown). PEBP/CBF is known to depend on cooperation with Ets-factors for transactivation of viral and cellular promoters [19, 23, 24]. By cotransfecting the PEBPaB1 cDNA (the mouse homolog of

the oncogene AML1) and the Ets-factor Fli-1 (often found activated in MLV-induced erythroid and myeloid leukemias, [1] in ES cells, it became evident that this cooperation required intact recognition sites in the core and LVb-site of the SFFVp-enhancer. Furthermore, the cooperation of PEBP/CBF and Fli-1 was clearly stimulated in presence of the UCR-located Sp1-site [12].

Finally, more recent data revealed that destroying the single E-box found in the 3'vicinity of the enhancer core significantly reduced activity of the SFFVp-enhancer in multipotent FDCP-mix cells (A. Richters and C.B., unpublished data), similar as previously reported in K562 cells with the homologous site of the more lymphotropic MLV strain, AKV [22]. In AKV, this E-box is activated by the bHLH-factor ALF1 or E2A, predominantly found in lymphoid cells [22]. The corresponding activators recruited in more primitive myelo-erythroid cells and putative interactions with the trias of Sp1, Ets-factors, and PEBP/CBF remain to be defined. Similarly, the identity of the Friend leukemia virus factors a, b, and c (FVa, FVb2, and FVc, [26]) remains as yet unclear. It is quite likely that these factors contribute to the developmental regulation of the SFFVp-enhancer.

Discussion

SFFVp contains the most potent retroviral enhancer configuration known so far for gene expression in myelo-erythroid progenitor cells, and possibly also in ES cells. As outlined above, at least four crucial *cis*-acting elements of this enhancer have been identified thus far that dominate the SFFVp-enhancer both in primitive and more mature stages of hematopoiesis. These are the GC-box in the UCR creating a target for Sp1, the conserved Ets-site LVb intimately cooperating with the enhancer core, the enhancer core itself which is modified by two point mutations to represent an exclusive and high-affinity target for PEBP/CBF, and finally, a conserved E-box consensus for bHLH-factors. Cotransfection experiments in ES cells revealed that transactivation mediated by the interaction of PEBP/CBF with Ets-proteins is supported by the Sp1-site in the UCR. Putative mutual interactions of this trias with bHLH-factors are likely, but remain to be defined.

It must be noted that at this stage we are just beginning to define target sites for complex transcription factor families. This is exemplified by the observation that PEBP/CBF detected in vitro with the enhancer core of SFFVp exhibits striking lineage-dependent variations in its expression pattern [12]. The DNA-binding a-subunit of PEBP/CBF is encoded by at least three distinct genes whose expression and – presumably – function is regulated both at transcriptional and post-transcriptional levels. Likewise, Ets-proteins [29] and bHLH-proteins ([22] and refs. therein) comprise highly complex groups of structurally related, yet functionally distinct factors. Importantly, in most transcription factor families, including the Sp-family, negative regulators have also been discovered [28, 29]. Thus, defining the concrete actors contributing to the developmental control of retroviral enhancers still remains an important challenge.

Several observations indeed reveal that the SFFVp-enhancer is subject to developmentally regulated transcriptional controls. Thus, the SFFVp-enhancer is not as active as that of Mo-MLV or MPSV in lymphoid cells [6], which might be explained by the loss of *Ets*-binding sites and E-boxes. Moreover, while highest activities are observed in actively proliferating, developmentally committed progenitors and precursors of myeloid and erythroid lineages, the enhancer is seemingly suboptimally recruited in more primitive multipotent hematopoietic progenitor and stem cells [13, 27], and definitely up to two orders of magnitude less active in embryonic stem cells (C.B. and M. Hildinger, unpublished data). A fairly simple explanation for the stage-dependence of enhancer activity would be the sequential activation of crucial transcription factors during lineage-commitment and maturation. PEBP/CBF, for instance, is absent or extremely weakly expressed in undifferentiated ES cells, but readily activated upon ES cell differentiation. Although nothing is known about its regulation at the level of the hematopoietic stem cell, from our studies in cell

lines we hypothesize that it is already expressed at very primitive stages of hematopoiesis, i.e., in lineage-negative pluripotent cells (C.B. and K. Itoh, unpublished data). Still, its function might be modulated by posttranscriptional modifications and intimate interactions with other transcription factors, including various *Ets*-proteins. Obviously, lineage-dependent activities of transcription factors are not only defined by their expression pattern, but also by their interaction capacities.

Important and, in the context of MLV-enhancers, widely neglected regulating events might be exerted by the proliferative status of the host cell. Cell cycle-dependent activation and repression of retroviral enhancers has been observed in avian retroviruses [30] and HIV-1 [31], respectively. Such regulations are easily overseen using standard tissue culture conditions. Since actively proliferating progenitor and precursor cells represent the natural habitat of MLV, it is indeed quite likely that their enhancers are responsive to growth-stimuli. Consequently, it can be imagined that the notoriously quiescent status of primitive hematopoietic cells might weaken the strength of retroviral enhancers.

Bearing these caveats in mind, efforts are now needed to concretely define the interplay of hematopoietic transcription factors on the retroviral enhancer. These studies involve a number of key regulators of hematopoietic proliferation and differentiation processes which are frequently affected by translocations contributing to leukemic progression [23, 24]. Therefore, the study of transcriptional control of MLV-enhancers will not only aid in designing further improved gene transfer vectors (e.g., for stem cell protection in cancer-chemotherapy or correction of hematopoietic metabolic disorders), but also contribute to our understanding of developmental controls within the hematopoietic system.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Retrovirus and Adenovirus-Mediated Interferon Gene Transfer into Hematopoietic Stem Cells (CD 34+) of Normal and Chronic Myelogenous Leukemia

N. G. Abraham

Abstract. Gene therapy following gene transfer into hematopoietic cells (CD34+) is now being investigated for several genetic disorders. We have applied a similar approach using interferon gene transfer for treatment of chronic myelogenous leukemia (CML). IFN-α produces a hematologic and cytogenetic response in CML with a survival advantage for cytogenetic responders. Therefore, we examined the effect of transient overexpression of IFN-α using the adenovirus gene transfer approach. The ability of the adenovirus (Adv)-IFN-α gene construct to transfect normal and CML stem cells, CD34+, was examined. The peripheral blood mononuclear fraction from patients with CML treated with G-CSF or GM-CSF/G-CSF was enriched in CD34+ cells. Adv-cytomegalovirus (pCMV) promoter driven IFN-α at multiple doses was assessed to transfect highly purified CD34+ cells in the presence of matrix protein and in co-cultures with the stromal adherent cell layer. The use of cytokines enhances Adv-mediated IFN-α gene transfer into stem cells. Southern blot analysis demonstrated that the Adv-pCMV-IFN-α construct and IFN-α were expressed in cultured CD34+ cells. Transient expression of the IFN-α gene did not suppress proliferation of CD34+ progenitors, CFU-Meg, BFU-E or CFU-CM growth. Reverse transcriptase/polymerase chain reaction (RT/PCR) analysis of RNA from CFU-GM progenitor cells demonstrated transient IFN-α mRNA expression in CD34+ cells. Immunoassay of IFN-α shows that selective expression of IFN-α may be beneficial for CML therapy. We also report on the establishment of novel conditions which permit high efficiency of the retrovirus IFN-α gene transfer into CD34+ cells. Stem cells transfected with retrovirus were infused intravenously to irradiated mice and spleen colony forming units were evaluated for IFN-α marked clones by Southern blot analysis. These studies demonstrate that the retrovirus IFN-α gene can be used to transfect CD34+ cells and possibly for gene therapy of CML.

Introduction

IFN have become important anti-proliferative agents used in cancer therapy. Several clinical trials have demonstrated that administration of IFN-α can be an effective treatment for CML in chronic phases. In those trials, cytogenetic remission can be induced and long-term survival improved compared with other standard therapies [12]. A number of in vitro effects of IFN-α on CML cells have been reported, such as direct inhibition of CML BM progenitor growth [13-14] and restoration of the adherence to stroma [15-17]. In addition, it has been found that IFN-α regulates the paracrine release of growth factors from the stroma by inhibiting the production of stimulating cytokines such as GM-CSF, G-CSF, IL-1b as well as by increasing the production of inhibiting

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cytokines [17-19]. It is thought that inhibitory cytokines and cellular immune surveillance play an important role in the control of expansion of the leukemic clone in CML.

Recently, this laboratory has utilized retrovirus and adenovirus vectors as vehicles for gene transfer into hematopoietic stem cells in vitro and in vivo [31]. Furthermore, we have established novel conditions in which stromal adherence cells permit high efficiency of retrovirus-mediated gene transfer of adenosine deaminase (ADA) into bone marrow hematopoietic progenitor cells [31]. Successful transplantation of the genes were further documented by their appearance in hematopoietic clones of cells from animals many months after reconstitution. Over the past 6 years, investigators have begun to apply retroviral gene transfer technology directed at hematopoietic stem cells in preliminary human clinical trails [32]. Recombinant adenoviruses are the second most commonly used viral vector for gene delivery [33-34], thus minimizing the risk of permanently altering the cellular genotype or of insertional mutagenesis. Adenoviruses are preferentially used when the transient expression of a transgene is sufficient to achieve the therapeutic goals [35].

The current chapter investigates the development of gene transfer vectors and assess the effect of retrovirus and adenovirus-mediated IFN-a gene transfer into CD34+ cells on proliferation and differentiation. Successful gene transfer into CD34+ cells was monitored by Southern blot analysis and Northern blot analysis of genomic DNA and total RNA from the CD34+ cells. Adherent stromal cell layers will be tested to determine their effect or gene transfer efficiency of retrovirus mediated IFN-a gene transfer. The results will allow us to determine the feasibility of developing a gene therapy protocol.

Material and Methods

The replication-deficient adenovirus vector endoding IFN-a (AdCMV- IFN-α) was constructed in our laboratory, as described previously [42]. The recombinant adenoviral vector was constructed by homologous recombination between the E3 deleted adenovirus, dL700 1 and the plasmid containing the human IFN-a. The Adv-CMV-IFN-α plasmid was constructed as follows: Xho1-Xho 1 fragment of the human IFN-a cDNA deleted form the BMGNeo IFN-α plasmid was inserted into the hind III site of the pRC/CMV (invitrogen) to obtain CMV-IFN-α. The virus was replicated and encapsidated into an infectious virus. After 5 days, plaque locations were marked on the plate, and the result in cytopathic effect to the monolayer was observed microscopically until the plaque reached an adequate size (usually 1 week). The plaques were purified, checked for the presence of human IFN-a by polymerase chain reaction (PCR) using IFN-a specific primers, and amplified by propagation in 293 cells.

Bone Marrow Transfection for Retrovirus and Adenovirus

For prestimulation of retrovirus mediated gene transfer a suspension of bone marrow $CD34^+$ cells were incubated over an adherent cell layer (ACL) from 4- to 6-week-old LTBMCs previously irradiated with 15 Gy, or were cultured in gelatin-treated (0.1% solution, 0.5-1 h, Sigma) T-25 flasks, for 2 days at 37 °C in 10 ml of a-MEM with 20% FBS. In some experiments, media was supplemented with rhIL-6(50-100 m/ml; Amgen, Thousand Oaks, CA) and rSCF (50 ng/ml; Amgen). In all cases, cultures of producer cells contained the growth factors. Two different methods of bone marrow transfection were used. One approach utilizes prestimulation with growth factors followed by cell transfer onto a culture of producer cells containing media conditioned by these cells supplemented with the same concentration of growth factors and 4 mg/ml of polybrene; the second approach utilized bone marrow stromal cells for 2 days. Irradiated ACLs were reseeded with bone marrow cells, cultured for 2 days in the absence of growth factors, and then bone marrow cells with the ACL were scraped from flasks, suspended in conditioned media obtained from producer cells. Growth factors were added where indicated, and polybrene was always included

during incubation with producer cells. After 2 days of infection, the hematopoietic cells were extensively washed from producer cells in both protocols, and then the washed cells were used for assays.

$CD34^+$ Cell Separation Techniques

Bone marrow samples were aspirated from the posterior iliac crest of normal volunteers after informed consent was obtained according to the guidelines established by the Human Investigation Committee of New York Medical College. Separation of CD34+ cells from elutriated preparations were then done using McAb cell pro columns (Cell Pro, Seattle, WA). T-cell depletion was achieved by incubation of $CD34^+$ cells with Cdz and CD4 antibodies in the presence of DNAase.

Human IFN-α analysis

The presence of IFN-α in $CD34^+$ cell media was determined using a commercially available quantitative ELISA assay (Endogen Inc., Cambridge, MA). After 24 h incubation, the culture medium was removed and centrifuged at 1,000 pm, 4 °C to remove floating cells. The resultant supernatant was passed through a 0.22 m filter and stored at-70 °C. Human IFN-α levels in the supernatant were determined by radioimmunoassays using anti-human IFN-α nonclonal antibodies following the manufacturer's instructions. $CD34^+$ were placed in fresh IMDM at a concentration of $5x10^6$ cell/ml and assayed for IFN-α after 24 hr. As shown in Table 1, condition medium for non transduces $CD34^+$ produce 29 pg/ml as compared to 360 pg/ml indicating that human IFN-α did not affect the $CD34^+$ cells.

Southern and Northern Blot Analysis of Transfected $CD34^+$ and Colony Assay

Genomic DNA was extracted from $5x10^7$ $CD34^+$ cells and digested with Ban HI, Hind III and Xho I. The digested DNA was electrophoresed on a agarose gel and transferred to nirocellular filters as described [49] Total RNA from $CD34^+$ cells was extracted and the Bgl I fragment of IFN-α cDNA was labeled with [$a^{32}P$] dCTP using a multiprimer DNA-labeling system (15). Methylcellulose technique for multipotent (CFU-GEMM), megakaryoctic (CFU-Meg) and myeloid (CFU-GM), erythroid (BFU-E) colony assays was performed.

Results

A schematic representation of the adenoviral vector containing the IFN-a gene is represented in Fig. 1. We examined the effect of multiple doses of adenovirus containing CMV IFN-α (AdCMV- IFN-α) were used to transfect human $CD34^+$ cells.

In Vitro Expression of IFN-α cDNA Assessment of IFN-α Protein and mRNA

As seen in Table 1, AdCMV-IFN-α caused elevation of IFN-α protein in the condition medium suggesting that the IFN-α gene is expressed into $CD34^+$ cells resulting in the release of IFN-α into the medium (4.1 unit/ml). On the other hand, AdCMV-LACZ on control cells do not express significant levels of IFN-α., the value listed for these two cells represent only about 0.3 unit/ml.

$CD34^+$ cells were plated in Iscoves Modified Medium (IMDM) at $2x10^6$ cells well and incubated with AdCMV-IFN-α 5,10,50,100 pfu/$CD34^+$ cell 5% CO_2 and then washed and used for assessment of mRNA and clonal efficiency. IFN-α mRNA was measured by RT/PCR in $CD34^+$ cells and/or CFU-GM clones.

To evaluate the effect of AdCMV-IFN-α on CFU-GM growth we compared the number of CFU-GM clones in infected and non-infected cells. Results in Fig. 2 shows that there was no significant difference in CFU-GM growth of infected vs control cells for any of the exposure times. Although colony numbers were slightly less for the 24 h cell groups as compared with 4 or 8 h, maximal gene transfer occurred with the 24 h exposure cells. Similar results were obtained for BFU-E and CFU-Meg growth (data not shown). We estimated that the gene transfer

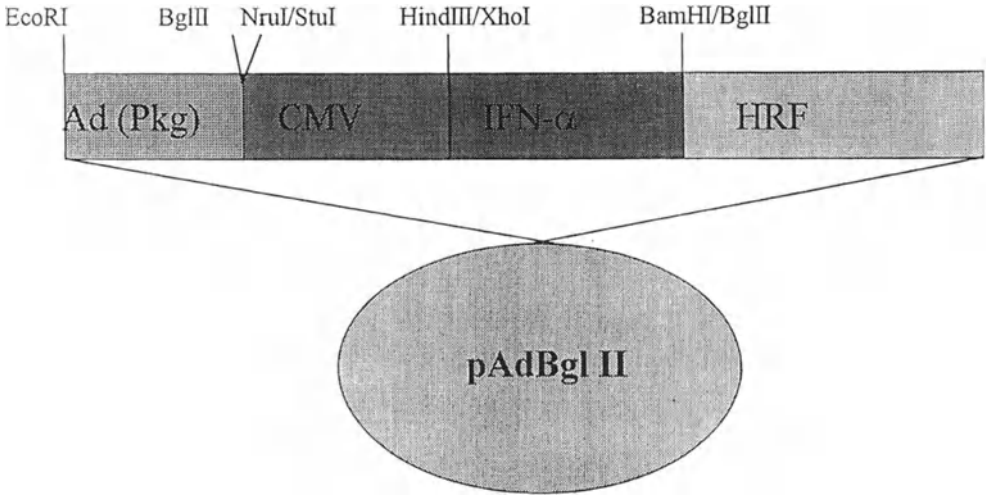

Fig. 1. A schematic diagram of the adenoviral vector containing the IFN-α gene

Table 1. Secretion of IFN-α by Ad CMV- IFN-α transduced human $CD34^+$ cells

Condition	Unit/ml	% Paritive cells
Control $CD34^+$	0.3±0.1	
Transduced $CD34^+$ AdCMV- IFN-α	4.1±0.6	19
Transduced $CD34^+$ AdCMV-LACZ	0.3+0.1	18

Concentration of released IFN-a in $U/10^6$ cell/24 h
Value below assay sensitivity limit were recorded as <0.3 m/control $CD34^+$ cells/24 h

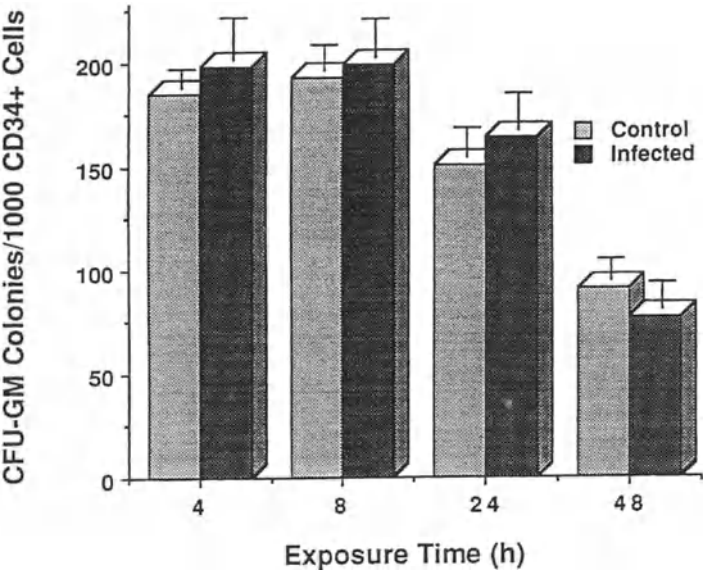

Fig. 2 A, B. *A* Expression of IFN-α in $CD34^+$ cells exposed to different multiplicities of infection (MOI) or plaque forming units (pfu) *B* CFU-GM growth by $CD34^+$ cells exposed to different MOI

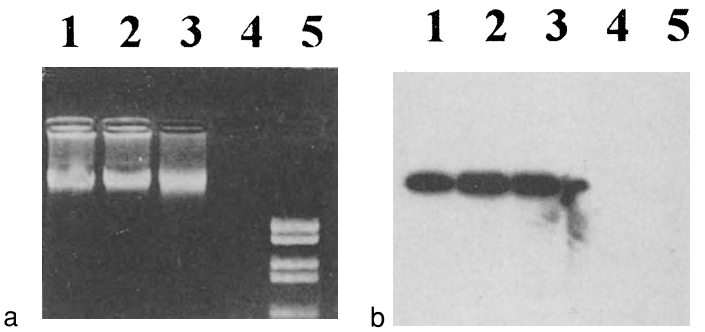

Fig. 3. IFN-α expression in $CD34^+$ cells exposed to adherent stromal cells for different time periods of prestimulation

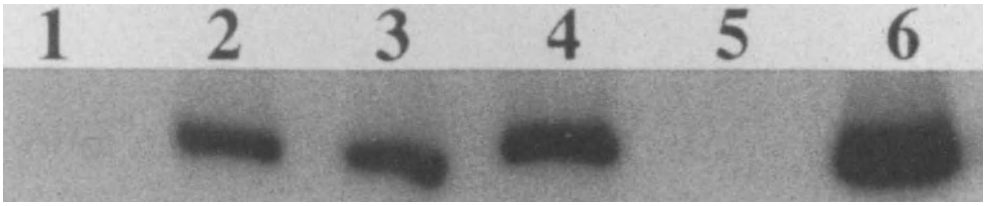

Fig. 4. CFU-GM growth by $CD34^+$ cells exposed to AdCMV for different exposure time periods

efficiency into $CD34^+$ cells was 27-30%. Restriction endonuclease analysis of DNA extracted from CFU-GM clones in CD34+ transfected cells with AdCMV-IFN-a gene was performed. Results are depicted in Fig. 3 where lane 1, represents $CD34^+$ cells after 24 h transfection; lane 2 are CFU-GM after 12 days; lane 3 is a positive control; lane 4 $CD34^+$ not transfected and lane 5 DNA marker. It can be seen that the DNA patterns are similar in lanes 1,2 and 3, indicating adequate transfer to $CD34^+$ cells and its presence in representative colonies (CFU-GM) derived from the $CD34^+$ cells.

Southern blot analysis of PCR product obtained from total RNA isolated from $CD34^+$ cells (and colonies) transfected with AdCMV-IFN-α gene were done. Results are represented in Figure 4 and show that transfected CD34+ cells (lane 2) and clones of CFU-GM (lanes 3,4) and BFU-E (lanes 5,6) (grown for 12-14 days) all show appropriate RNA bands. Lane 1 is a nontransfected control. These results clearly demonstrate that recombinant adenovirus is an effective vehicle for transiently expressing genes in primitive human $CD34^+$ cells, and that the gene is effectively carried to clones of hemopoietic elements (CFU-GM, BFU-E) derived from the $CD34^+$ cells. Although the expression of the gene is transient, we have established optimal conditions for gene transfer thus enabling us to evaluate toxicity and transfer into hemopoietic element.

In the next sets of experiments, we assessed the effect of permanent transfection of IFN-α using the retrovirus stromal adherent cells and growth factors to confirm the presence of stem cells clones expression of IFN-α obtained from mobilized cells by G-CSF. We determined the presence of IFN-α DNA into CFU-S. Southern blot analysis revealed that, in both mice four months after transplantation, there were several different integration sites of IFN-α. All four individual CFU-S studied displayed different clonal pattern. Similarly, different integration sites were also seen in all other CFU-S obtained in other mouse (data not shown).

Discussion

In this report, we used adenovirus as a transient means of gene transfer. Adenovirus (Ad) vectors provide an ideal vehicle for transient expression by which to deliver IFN-α for the purpose of abrogating Ph^+ levels induced in CML cells. Result suggest that transgene of IFN-α can be achieved using

AdCMV vector with efficiency of 45%. Further, there is a direct relationship between increased adenovirus dose and toxicity. At lower concentrations of adenovirus vectors, transduction efficiency is diminished [56]. However, studies with blood cells and hematopoietic progenitors are still limited. The objective of these studies was to define conditions that enhance in vitro adenovirus-mediated gene transfer to hematopoietic cells. Adenovirus IFN-a mediated gene transfer might be of great value to assess the effect of short-term expression of IFN-α on $CD34^+$ cell differentiation. These studies in our laboratory have shown that there is no suppressive effect on $CD34^+$ cell growth after transduction with IFN-α gene. Our result demonstrated that prestimulation of bone marrow cells over an in vitro culture of adherent stromal cell layers (ACL) provides favorable conditions for gene transfer in the absence of growth factors. It appears that utilization of bone morrow microenvironment through the use of an adherent cell results in successful retrovirus IFN-α gene transfer into HSC. These studies demonstrated that an adherent cell layer provides excellent conditions for successful gene transfer. Finally, retrovirus mediated IFN-α gene transfer into mobilized stem cells may be permanently expressed without a cytotoxic effect on $CD34^+$ cell differentiation.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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AML in the Elderly: a Biologically Distinct Disease in which MDR1 Expression and Unfavorable Cytogenetics Contribute to Poor Clinical Response. Studies of the Southwest Oncology Group

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Abstract. Elderly patients with acute myeloid leukemia (AML) respond poorly to chemotherapy compared to younger cohorts. To determine the contribution of biologic factors to this poor response, we studied 211 patients (161 de novo, 50 secondary AML) over 55 years of age (median: 68 years) registered to a single clinical trial for previously untreated AML (SWOG 9031: Phase III randomized trial of standard dose cytosine arabinoside and daunomycin +/- rhG-CSF). Pretreatment blasts were karyotyped, and were analyzed for MDR1 expression and functional efflux by multiparameter flow cytometry. In addition, pre-treatment marrows were analyzed for trilineage dyspoiesis. MDR1 expression was very high (71%) in these cases, distinctly different from a frequency of about 35% detected in a group of younger patients by similar methods. Functional efflux (58% of cases were efflux +) and unfavorable karyotype (e.g. -5/5q-, -7/7q-, complex abn; present in 32% of cases) were also frequent in both de novo and secondary AML cases. Over 80% of evaluable cases showed erythroid or megakaryocyte dyspoiesis while 68% showed myeloid dyspoiesis. Secondary AML, CD34 and MDR1 expression, functional efflux, and unfavorable cytogenetics were all associated with lower complete remission (CR) rates in univariate analysis. Dyspoiesis, while associated with MDR1 expression and unfavorable cytogenetics, was not clearly predictive of CR. In multivariate analysis, three independent predictors of CR were found: secondary AML ($p = 0.0035$), MDR1 expression ($p = 0.0041$) and unfavorable cytogenetics ($p = 0.0031$). Resistant disease was associated with MDR1 expression ($p = 0.0007$) and unfavorable cytogenetics ($p = 0.017$). AML in the elderly is associated with a high frequency of dyspoiesis, unfavorable cytogenetics and MDR1 expression, and thus more closely resembles myelodysplasia related AML than the de novo AML of younger patients. Analysis of biologic disease parameters at AML diagnosis may help identify patients with a high likelihood of achieving CR with current therapies. Biologic studies can identify MDR1+ cases where patients may benefit from therapies which include MDR1 modulators, or non-MDR1 transported drugs. Other biologic factors are also likely important in AML in the elderly, and await further investigation.

Introduction

Elderly patients with acute myeloid leukemia (AML) have a much worse clinical outcome than younger patients with the disease. While 75% of younger patients generally achieve complete remission (CR) on current therapies, and about 25 to 30% survive for over 5 years, the prognosis is much worse in older patients, with less than 50% of

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patients over 55 years achieving CR, and fewer than 10% surviving even 2 years after diagnosis [1-5]. Since the incidence of AML increases with age, and with the aging US population, there is an increasing population of elderly AML patients for whom treatment remains problematic.

Various data indicate AML in the elderly is biologically different from that in younger patients. This difference in leukemia biology between younger and older patients may largely contribute to the much worse clinical response observed in elderly patients. The disease in elderly patients is more often secondary to antecedent myelodysplasia (MDS), or previous leukemogenic therapies; such confirmed secondary AMLs have been reported to be more resistant to therapy than de novo AML [4, 6, 7]. In addition, a subset of de novo AML cases, identifiable by dyspoietic features of residual bone marrow elements, may be biologically more like MDS-related AML; AML cases which have dyspoietic features have been reported to have a poorer response to standard chemotherapy [8-10]. Some reports suggest that this background dysplasia may be more frequent among elderly patients, although the data are conflicting [8-11]. Karyotyping in these elderly patients also shows a higher frequency of MDS-type cytogenetic abnormalities, again suggesting that a subset of de novo disease in these patients is similar to MDS-related disease [12-16].

If, as suggested, AML in the elderly is often similar to MDS-related AML, the frequency of drug resistance mediated by the multidrug resistance protein-1 (MDR1 or p-glycoprotein) may also be high in this patient group, as AML arising from MDS has a high frequency of MDR1 expression [17, 18]. As MDR1 expression has been linked to poorer response in AML, an increased frequency of MDR1 expression would offer a biologic explanation for poor outcome in AML in the elderly [19-25]. This would be of particular interest to clinicians as drug resistance mediated by the MDR1 protein can potentially be blocked by the addition of MDR1 modulators to therapy, and thus may in principle be overcome in vivo [26, 27].

This chapter summarizes studies which we have undertaken in the Southwest Oncology Group (SWOG) to identify biologic factors which might be linked to poor outcome in AML in the elderly, with particular reference to cytogenetics and MDR1 expression [28, 29].

Materials and Methods

Patient Selection

Pre-treatment bone marrow and/or blood specimens were obtained at diagnosis from patients registered to a single Southwest Oncology Group study, SWOG-9031: a randomized, double-blind, placebo-controlled trial of daunomycin (45 mg/m^2 days [1-3]) and standard dose cytosine arabinoside (200 mg/m^2 days [1-7]) with or without rhG-CSF for previously untreated AML patients over 55 years of age (Godwin et al; in preparation, [30]).This trial included patients with de novo and secondary AML apart from those with acute promyelocytic leukemia (FAB M3/M3v).

Morphology Review

The diagnosis of AML in all cases reported here was confirmed by central histopathologic review by the SWOG Leukemia Pathology Committee using standard FAB criteria as modified by SWOG [31, 32]. In addition Wright's stained pretreatment bone marrow and blood smears were examined by two observers (CL and DH) for evidence of dyspoiesis in the background maturing myeloid lineages. If sufficient cells of any lineage were present for evaluation, the degree of dyspoiesis was categorized into absent, mild, moderate, severe or according to criteria previously agreed upon by the examiners, and similar to the criteria of others as described in detail elsewhere (Leith and Head; in preparation, [8, 9, 11, 29, 33]). Briefly, white cell dyspoietic features included Pseudo-Pelger Huet anomaly, nuclear hypersegmentation, megaloblastoid changes and cytoplasmic hypogranulation. Red cells were considered dysplastic when nuclear fragmentation, karyorrhexis, megaloblastoid change, or nuclear budding were observed,

while megakaryocyte abnormalities included mononuclear forms, cells with multiple, distinct small nuclei, or megakaryocytes with bizarre, enlarged nuclei. Severity of dyspoiesis was graded according to the percentage of abnormal cells in any lineage, with severe dyspoiesis correlating with over 50% of the lineage showing dyspoietic changes.

Analysis of MDR1 Expression and Functional Efflux

These assays were performed on pre-treatment bone marrow/peripheral blood samples which had been enriched for leukemic blasts by density gradient separation. MDR1 expression by leukemic blasts was measured using the MDR1-specific antibody MRK16 (Kamiya, Thousand Oaks, CA) in three color flow cytometric assays where blasts were co-stained with MRK16, the hematopoietic stem/progenitor cell antigen CD34, and the pan-myeloid antigen CD33, as previously described in detail [34]. Using this method allowed gating on a phenotypically defined myeloid blast population for MDR1 analysis, as well as correlation of MDR1 and CD34 expression. Appropriately matched isotype controls were used in all assays. Functional activity of the MDR1 protein was assessed by measuring efflux of a fluorescent dye, $DiOC_2$, by leukemic blasts in a single color flow cytometric assay, and the ability of the MDR1 modulator Cyclosporine A to inhibit this efflux [34]. In this assay, leukemic blasts were incubated in media containing $DiOC_2$ for 30 min at 37 °C to allow dye uptake. After the blasts were washed, and an aliquot put on ice to measure baseline dye uptake, leukemic blasts were resuspended in fresh dye-free media with or without the MDR1-modulator cyclosporine A (CsA; 2500 ng/ml; Sandoz Pharmaceuticals, Basel, Switzerland) and incubated for 90 min at 37 °C. Cells were then washed and resuspended in fresh 4 °C medium for immediate flow cytometric analysis. The MDR1(+) DOX cell lines and MDR1(-) 8226/S parental line (kindly provided by W. S. Dalton, University of Arizona) were used as controls in all experiments [35].

Analysis of MDR1 expression and efflux data were performed on a FACScan flow cytometer using Lysis II software (Becton-Dickinson). MRK16 staining of gated leukemic blasts compared to control cells was measured using the Kolmogorov-Smirnov (KS) statistic which measures the difference between the two distribution functions of staining intensity [36]. This method generates a D value ranging from 0 to 1.0, with higher numbers indicating a greater difference, and is useful in identifying small differences in cellular fluorescence and thus low level antigen expression, as often occurs with MDR1 expression in primary patient samples [34, 37]. MRK16 staining intensity was categorized for descriptive purposes as follows: bright ($D \geq 0.25$), moderate ($0.15 \leq D > 0.25$), dim ($0.10 \leq D < 0.15$) and negative ($D < 0.10$). $DiOC_2$ efflux was assessed by analyzing cellular fluorescence of gated leukemic blasts after efflux in the presence/absence of CsA; differences in fluorescence were analyzed with KS statistics and a D value of ≥ 0.25 was used to define a case as efflux (+).

Cytogenetic Analysis

Cytogenetic studies on pre-treatment bone marrow or unstimulated blood samples were performed using standard G-banding with trypsin-Giemsa or trypsin-Wright's staining in SWOG-approved cytogenetics laboratories. Karyotypes were interpreted using ISCN (1995) [38]. Karyotypes were considered normal diploid if no clonal abnormalities were detected in a minimum of 20 metaphases examined and if 2 growth/harvesting methods were used. Each karyotype was independently reviewed by at least three members of the SWOG Cytogenetics Committee.

Statistical Analysis

Demographic and clinical data for patients in this study were collected according to standard procedures of the SWOG. MDR1 expression and efflux were analyzed either as quantitative variables using the KS statis-

tic D, or were categorized as described above. Unweighted least squares (LS) and logistic regression (LR) analyses were performed to identify variables predictive of MDR1 expression or functional efflux [39, 40]. Standard criteria were used to define CR and relapse [41]. Overall survival (OS) was measured from randomization until death from any cause, with observation censored for patients last known alive. RFS was measured from establishment of CR until relapse or death from any cause, with observation censored for patients last known alive without report of relapse. Distributions of OS and RFS were estimated by the method of Kaplan and Meier [42]. Analyses of prognostic factors for treatment outcomes were based on LR models for CR and proportional hazards (PH) regression models for OS and RFS [40, 43]. Prognostic factors considered in the analysis included clinical parameters such as age, white blood cell count, blast count, etc. MDR1 and CD34 expression, functional efflux and cytogenetics [53]. Statistical significance is represented by two-tailed p-values.

Results

Patient and Disease Characteristics

The patient study group comprised all 211 patients registered to SWOG study 9031 who were confirmed to have non-M3 AML by central pathology review. There were 89 females and 122 males who had a median age at study entry of 68 years (range 56 to 88). In 50 patients (24%), the AML was secondary to either antecedent MDS or previous leukemogenic drug/radiation therapy according to their clinical histories. Morphology was examined in all cases, while one or more of the following biologic parameters were also studied in each case: CD34 and MDR1 protein expression on leukemic blasts, functional efflux and cytogenetics. All the above parameters were examined in 130 cases; data was not available for all parameters in the remaining cases either because the specimens contained too few blasts for all analyses, or because the analyses were determined to be inadequate.

Morphologic Features

All subtypes of AML (excluding AML M3, which by protocol was excluded from the study) were represented in this study, with the most frequent sub-types being AML FAB M1 or M2, which were identified in 128 (61%) of the cases. Dyspoietic changes in the background maturing myeloid elements were frequently seen in those AML cases where residual marrow elements were present for assessment. In the myeloid series, 104 of 153 evaluated cases (68%) showed dyspoietic changes including megaloblastoid cells and pseudo Pelger-Huet anomaly. The erythroid series also showed frequent dyspoietic changes, with 125 of 150 cases (83%) showing abnormalities including megaloblastoid changes, broad-based nuclear budding, "cloverleaf" nuclei, multinucleation, and internuclear bridging. In addition, 80 of 91 cases (88%) showed megakaryocyte abnormalities including large, dysplastic forms with bizarre, enlarged nuclei, mononuclear megakaryocytes, and megakaryocytes with multiple small, distinctly separated nuclei.

MDR1 Expression and Functional Efflux

Expression of the MDR1 protein on phenotypically gated leukemic blasts was detected in 135 of 189 cases (71%) including 69 cases (37%) which showed bright staining ($D \geq 0.25$) with the antibody, MRK16. Functional efflux of the dye, $DiOC_2$, was detected in 101 of 175 cases (58%) studied, and was inhibited by cyclosporine A (an MDR1 efflux inhibitor) in each case. As expected, functional efflux was strongly correlated with MDR1 expression: 82 (67%) of 122 MDR1(+) cases were efflux(+) while 32 (67%) of 48 MDR1 (-) cases were efflux(-) ($p < 0.0001$). However, as we and others have previously described, discrepant cases were identified including 16 MDR1(-)/efflux(+) cases and 40 MDR1(+)/efflux(-) cases [34, 44, 45].

Cytogenetics

Complete cytogenetic studies were performed in 164 cases. Ninety cases (55%)

demonstrated abnormalities including 54 (33%) with 2 or more abnormalities. Cytogenetic abnormalities classically associated with MDS as well as AML were particularly common in this group and included 24 cases (15%) with -7/7q-, 21 cases (13%) with -5/5q-, and 25 cases (15%) with trisomy 8. In contrast t(8;21) and inv(16)or t(16;16), all abnormalities classically associated with true de novo AML were only found in a total of only 10 cases (6%). For the purposes of correlation with clinical response, karyotypic abnormalities were grouped into favorable, intermediate, or unfavorable categories based upon published literature [12-16, 46-48]. The unfavorable cytogenetic group included the following: -5/5q-, -7/7q-, inv(3), abnormalities of 11q or 17p, i(17q), del(20q), +13, cases with greater than 3 abnormalities; 52 cases (32%) were classified as unfavorable based on these criteria. In contrast, only 9 patients (5%) were considered to have favorable cytogenetics, as defined by cases with any of the following: t(8;21), inv(16), t(16;16), +14, without additional unfavorable abnormalities. The remaining 103 cases (63%) had other abnormalities or a normal karyotype, and were considered to have intermediate prognosis cytogenetics.

Correlation Between Morphologic and Biologic Features of Disease

Dyspoietic features were marginally associated with biologic disease factors; myeloid cell dyspoiesis was associated both with MDR1 expression ($p = 0.034$) and with unfavorable cytogenetics ($p = 0.029$). In addition to its association with dyspoiesis, MDR1 expression was, as expected, significantly and independently associated with efflux ($p < 0.0001$). In addition, MDR1 was associated with FAB subtype ($p = 0.0037$), mainly because MDR1 expression was much less frequent among FAB M4 and M5 subtypes. Interestingly, there was no independent correlation between MDR1 expression and either secondary disease ($p = 0.76$), or unfavorable cytogenetics ($p = 0.28$).

Unfavorable cytogenetics, in addition to its association with dyspoiesis, were also associated with CD34 expression ($p = 0.0077$) but surprisingly, in view of the frequency of MDS-like cytogenetic features detected, was only marginally associated with secondary AML ($p = 0.046$).

Prognostic Factors for Response to Therapy and for Resistant Disease

Overall 95 (45%) of the 211 elderly patients with AML achieved a complete remission (CR), including 86 patients who achieved CR after one course of induction therapy, and 9 who achieved CR after 2 courses. In univariate analysis, secondary AML was the only clinical parameter strongly predictive for achievement of CR ($p = 0.0005$). Only 24% of patients with secondary AML achieved CR in contrast to 52% of patients with de novo disease. No other clinical parameter including age, white blood count, blast count, or platelet count at presentation, or treatment arm (G-CSF versus placebo) was correlated with CR rate. Among laboratory parameters, CR rate was significantly influenced by CD34 and MDR1 expression, by functional efflux, and by cytogenetics. CR rate significantly decreased as the intensity of either CD34 ($p = 0.0027$) or MDR1 expression ($p = 0.0019$) increased on the leukemic blasts. Among patients with CD34+ blasts, 38% achieved CR in contrast to a CR rate of 59% among the CD34- group, while 34% of patients whose blasts showed moderate or strong MDR1 expression entered CR, in contrast to 45% of patients with dim MDR1 expression, and 67% of patients whose blasts were MDR1-. Similarly, the CR rate decreased with increasing strength of dye efflux ($p = 0.0039$); 35% of patients with efflux+ blasts achieved CR while 58% of patients who were efflux- entered CR. Among patients with unfavorable cytogenetics, only 21% achieved CR in contrast to a 55% CR rate among cases with intermediate or favorable karyotypes ($p < 0.0001$).

In multivariate analysis which took into account clinical as well as laboratory parameters, the CR rate was highly significantly and independently associated with secondary AML ($p = 0.0035$), MDR1 expression ($p = 0.0041$), and unfavorable cytogenetics ($p = 0.0031$). After accounting for these three fac-

tors, there was no significant independent association with any other factor, including CD34 expression ($p = 0.80$) or functional efflux ($p = 0.75$). Only 2 (12%) of 17 patients with secondary AML whose leukemic blasts were MDR1(+) and had an unfavorable karyotype achieved CR. In contrast, 22 of 27 (81%) patients with de novo MDR1(-) AML and favorable/intermediate cytogenetics achieved CR. The CR rate fell among AML patients as MDR1 expression increased, so that patients with de novo disease, and favorable/intermediate cytogenetics, but whose blasts were moderately or strongly MDR1+ had a CR rate of 48% (19/40).

Resistant disease was documented in 73 of 116 patients who failed to achieve CR. Resistant disease was significantly and independently associated with unfavorable cytogenetics ($p = 0.017$) and MDR1 expression ($p = 0.0007$), but not with secondary AML status (p=0.11).

Prognostic Factors for Overall Survival (OS) and Relapse-Free Survival (RFS)

One-hundred and eighty of the 211 patients (85%) have died. The remaining 31 were alive between 18 and 53 months (median 33 months) after registration. Multivariate proportional hazards regression analysis identified three significant independent prognostic factors which predicted for poor OS: unfavorable cytogenetics ($p < 0.0001$), increasing age ($p = 0.014$) and increasing WBC ($p = 0.029$). After accounting for these factors, none of the other variables considered had independent prognostic significance, including secondary AML ($p = 0.29$), MDR1 expression ($p = 0.93$) and efflux ($p = 0.10$).

RFS among the 95 patients who achieved CR was marginally poorer among patients with unfavorable cytogenetics ($p = 0.028$). No other variables were significantly correlated with RFS in multivariate analysis.

Discussion

A major question in the treatment of AML has been whether the poor response rate in elderly patients is due to their inability to tolerate aggressive chemotherapy or to unique features of their disease. The present studies indicate that the poor clinical outcomes of elderly patients when treated with standard AML chemotherapy can, in large part, be attributed to distinct features of the disease found in this patient population. We found that even so-called de novo AML in the elderly has many similarities to AML arising secondary to MDS. Many of these so-called de novo AML cases in elderly people show morphologic evidence of bone marrow dyspoiesis, similar to that found in MDS, as well as a very high frequency of MDR1 expression, similar to the frequency of MDR1 expression found in secondary AML [17, 18, 33]. The frequency of MDR1 expression in the present study is much higher than that seen in a cohort of younger AML patients we have systematically studied by similar methods (Leith et al; in preparation). In addition, the high frequency of MDS-like cytogenetics, and the very low frequency of abnormalities associated with de novo AML in younger patients, point to a strong similarity between many cases of AML in the elderly, and AML arising from MDS [12, 14, 18, 49, 50].

When theses factors were identified and accounted for, it was possible to define a small group of elderly AML patients who had a remarkably good response to therapy. These patients had de novo disease in which the leukemic blasts were MDR1-, and lacked adverse cytogenetics. Such cases, with their high CR rate (of over 80% in this study) appear to resemble the de novo AML cases seen in younger patients with favorable risk features [15, 51]. In contrast, the patients we identified who had both MDR1+ leukemic blasts, and unfavorable cytogenetics, had a very poor response to therapy; such a patient group would clearly benefit from alternative treatment regimens.

In addition, there was a large group of elderly patients whose leukemic blasts expressed MDR1, but whose other prognostic markers were favorable. The CR rate in this group decreased with increasing expression of MDR1. This group in particular may benefit from addition of MDR1 modulators to their therapy.

Analysis of MDR1 expression and function, to identify patients who may benefit

from MDR1 modulators, may thus be a first step towards improving the treatment of elderly patients with AML. However, multidrug resistance is clearly complex in this patient group. The association of RFS and overall survival with cytogenetics, but not with MDR1, indicates that other biologic mechanisms of therapy resistance must operate in these patients. The identification of cases with discrepant MDR1-/efflux+ phenotypes in the laboratory suggests that other drug efflux mechanisms may operate in these patients; these may be contributing factors to therapy resistance. Such resistance mechanisms may include expression of the multidrug resistance protein, MRP, or the lung resistance protein, LRP, although our preliminary identification of some cases which are efflux+ but lack expression of any of these resistance markers, indicates that yet other mechanisms may be involved in this group of elderly AML patients. Our future studies will focus on identifying the efflux mechanisms involved in these unusual cases, and determining if they may further contribute to the unfavorable outcome of elderly patients with AML.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Pharmacological Modulators and Alternate Mechanisms of Multidrug Resistance

A. F. List

P-Glycoprotein Antagonists

The hematologic malignancies, and acute myeloid leukemia (AML) in particular, have emerged as the principal diseases targeted to investigate pharmacological modulation of P-glycoprotein [P-gp] export function. Among the Phase II studies completed using so-called "first generation" P-gp antagonists, two agents – quinine [1] and cyclosporin-A [2, 3] – have shown sufficient activity to enter further testing in Phase III studies (Table 1). Among these, only the GOELAM MAQ2 study is complete [4]. Over 300 patients participated in this trial, which included a heterogeneous group of acute leukemias. Although effective plasma concentrations of quinine were confirmed using an ex vivo model, an improvement in remission rate in the quinine-treated arm was not demonstrable. Overall, 53% of patients receiving mitoxantrone in combination with quinine achieved a complete remission compared to 45% of patients in the control arm. Although this is a comparatively large study, the power to discern a difference in treatment outcome is limited by the failure to stratify patients by P-gp phenotype and the magnitude of disease heterogeneity, the latter of which includes refractory AML, acute lymphoblastic leukemia, blast phase transformation of myeloproliferative disease, and secondary leukemias. Despite this, a retrospective analysis of the data indicates that patients with P-gp overexpression experienced an improved response rate with the addition of quinine compared to P-gp-negative patients (60% versus 35%). Studies testing the bene-

Table 1. Randomized trials investigating Pgp modulation in poor risk acute leukemia

Institution/ investigator	Dates	Modulator	Dx	# Pts	Chemotherapy
GOELAM MAQ2	1992-95	Quinine (30 mg/kg IV)	AL	315	ID-AraC + Mitox
SWOG 9126	1993-	CsA (16 mg/kg IV)	AML (RoR,2°)	220	HiDAC + DNR
HOVON	1995-	CsA (12.5 mg/kg)	AML (Refractory)	NA	Mitox + VP16
MRC	1994-	CsA (2.5-5 mg/kg)	AML (> 60, RoR)	NA	DNR + AraC + 6TG

Note: CsA = Cyclosporin-A; AL = Acute Leukemia (see text); RoR = Relapsed or Refractory; 2° = secondary AML; ID-AraC = Intermediate Dose cytarabine; HiDAC = high-dose cytarabine; Mitox = mitoxantrone; DNR = daunorubicin; 6TG = 6-thioguanine; NA = not available.

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fit of cyclosporin-A (CsA) have targeted poor-risk populations with a high frequency of P-gp expression, including AML in relapse, treatment-induced or secondary AML, patients refractory to primary induction therapy, and/or the elderly. P-gp is believed to contribute to treatment failure in each of these disease categories, and therefore, these studies should prove informative.

Second generation P-gp antagonists are agents selected for clinical development based upon greater potency and selectivity for P-gp, and lower inherent toxicities than the first generation compounds. Among these, the cyclosporine-D analogue, PSC 833, and dexverapamil are the most extensively studied in acute leukemia (Table 2). The latter agent is currently under investigation in only one randomized trial in patients with acute lymphoblastic leukemia (ALL). PSC 833 is a non-immunosuppressive cyclosporine analogue that lacks intrinsic renal toxicity. In both animal models and in vitro, this analogue exhibits up to tenfold greater potency than its parent compound, CsA. In a multicenter Phase I/II trial headed by the Arizona Cancer Center, 44 patients with poor-risk AML received induction therapy with high-dose cytarabine and daunorubicin, the latter administered as a continuous infusion with PSC 833 [5]. Dosages of both the antineoplastic and PSC 833 were escalated in sequence to determine the best tolerated dose for subsequent investigation. Mean steady state blood levels of PSC 833 at the highest dosage administered (10 mg/kg/day) were well tolerated and exceeded by two- to threefold the minimal threshold for P-gp blockade predicted by preclinical models (> 1000 ng/ml). More importantly, patient serum from evaluable patients augmented daunomycin retention in a P-gp-expressing myeloma cell line. The results of this trial indicate that remission rate was not adversely impacted by P-gp overexpression. In addition, sequential comparative studies showed that overexpression of the MDR1 gene message was eliminated from relapsed specimens of remitting patients, whereas overexpression of alternate MDR transporter genes were not affected. These findings support the notion that P-gp export function was successfully and specifically modulated. Based upon these findings, five randomized cooperative group trials were initiated in the past year to test the benefit of MDR-1 modulation with PSC 833 in poor-risk AML (Table 3). Three of

Table 2. Pharmacological modulators of P-gp function in hematologic malignancies

First-generation modulators
Verapamil
Quinine
Cyclosporin-A
Tamoxifen
Second-generation modulators
dex-Verapamil
PSC 833
GG918
LY335979

Table 3. Phase III trials with second-generation P-gp modulators in AML

Institution/ investigator	Date	Modulator dose	Diagnosis	Chemotherapy
French multicenter Trial	1994	Dexverapamil (250 mg q4H po)	ALL (RoR)	HiDAC + VAD
ECOG	1996	PSC 833 10 mg/kg/d CI	relapsed/ refractory	Mitox + VP + AraC (MEC)
CALGB	1996	PSC 833 10 mg/kg/d CI	≥ 60 yrs	DNR + VP + AraC
CALGB	1996	PSC 833 10 mg/kg/d CI	de novo	DNR + VP + AraC
HOVON, MRC	1996	PSC 833 10 mg/kg/d CI	≥ 60 yrs	DNR + AraC
SWOG	1996	PSC 833 10 mg/kg/d CI	≥ 55 yrs	DNR vs. Mitox + AraC vs. Mitox + AraC + PSC

Note: ALL = acute lymphoblastic leukemia; RoR = relapsed or refractory; HiDAC = high-dose cytarabine; VAD = infusional vincristine, doxorubicin and dexamethasone; CI = continuous intravenous infusion; VP = etoposide (VP-16); DNR = daunorubicin.

these trials target elderly patients, whereas only one study is currently planned in de novo AML. Although the frequency of P-gp overexpression is lower in de novo AML (≤ 30%) compared to the elderly population, modulation of P-gp-mediated anthracycline resistance merits investigation in this setting. The relatively high remission rate observed with conventional induction therapy in patients with de novo disease suggests that a limited number of resistance mechanisms may be operative at the time of disease presentation. Knowing that many of these patients are destined for treatment failure, primary treatment may select for emergence of P-gp which thereby contributes to relapse of disease. Indeed, in a recent analysis of 221 AML patients enrolled on EORTC trials, the predictive value of P-gp for treatment failure was greatest in patients with de novo AML with a corresponding predictive value of 90%, compared to 73% and 44% for patients in late or early relapse, respectively [6]. Despite the rising frequency of P-gp expression with disease progression, these observations indicate that other biological features of these leukemias other than P-gp alone contribute to treatment failure in poor-risk groups. Conversely, in vitro co-selection of tumor cell lines with the P-gp antagonist PSC 833 and an anthracycline, suppresses emergence of multidrug resistant clones by tenfold [7]. Knowing this, the greatest potential to impact remission duration and survival may reside in the treatment of de novo AML.

Toxicity and Pharmacokinetic Interactions

Modulation of P-gp-mediated multidrug resistance is considered a target-specific drug interaction that carries with it potential consequences because of expression of the protein in normal tissues. In murine models, administration of vincristine in combination with verapamil at concentrations sufficient to inhibit P-gp function, produces an eightfold increase in antineoplastic concentration in P-gp-expressing tissues such as the small intestines, liver and kidneys, with accompanying excess toxicities to these organs [8]. In addition, these same preclinical models predicted for significant pharmacokinetic interactions between the modulator and anticancer agent [8, 9]. Studies performed using verapamil or CsA in conjunction with anthracyclines or vinca alkaloids have consistently demonstrated delay in clearance of the antineoplastic accompanied by a significant increase in systemic drug exposure [2, 5, 10-14]. Although potentially lethal complications have not been observed in human studies, toxicities to normal tissues with MDR-1 modulation have been reported. Although isolated hyperbilirubinemia occurring in the absence of hepato-cellular injury is observed commonly with the use of CsA, minor elevations occur routinely with the use of other P-gp antagonists, including quinine and PSC 833 [1, 2, 5, 15]. Delayed bilirubin excretion is now recognized to result from direct interaction of these modulators with bilirubin transport proteins [16, 17]. Not surprisingly, major alterations in chemotherapy pharmacokinetics have been reported in clinical trials. Indeed, with each of the more potent P-gp modulators, CsA, dexverapamil and PSC 833, a marked increase in the area under the curve (AUC) has been observed when these agents are combined with either paclitaxel, doxorubicin, daunomycin, or etoposide [2, 5, 10-14]. The magnitude of change in AUC varies widely but on average approximates a two- to threefold increase in systemic drug exposure. An additional concern is the inter-patient variability in pharmacokinetic interactions. In the University of Arizona AML trial, for example [5], PSC 833 increased the mean AUC of daunomycin threefold, however, the magnitude varied from no alteration in AUC to a greater than fourfold increase in drug exposure. Unpredictable pharmacokinetic interactions such as these present a particular challenge for routine chemotherapy dose adjustment when combining anticancer agents with a P-gp modulator. In the absence of limiting nonhematologic toxicity, however, dose modification may not be necessary when the principal intent of treatment is bone marrow ablation. Nonetheless, such pharmacokinetic interactions pose additional challenges to the design and interpretation of randomized trials. An improved outcome with MDR-1 modulation might be ascribed to either increased systemic drug exposure

and/or MDR-1 modulation. In addition, interpretation of these trials must be tempered by the recognition of the number of variables affecting outcome. These include a balanced distribution of functional P-gp-expressing leukemias, achievement of effective blood levels of the chemotherapy modulator, and the presence or absence of non-P-gp mechanisms affecting chemotherapy resistance. In the current SWOG trials, patients are stratified according to P-gp phenotype before assignment to treatment arm. In addition, alternate MDR phenotypes and blood levels of the modulator and antineoplastic are prospectively monitored.

Non-P-GP Multidrug Resistance

Despite successful elimination of MDR1 gene overexpression in phase II studies of P-gp modulators, resistance to salvage therapy indicates emergence or persistence of alternate drug resistance mechanisms. Indeed, multiple cellular mechanisms are now recognized which contribute to an MDR phenotype in vitro. These mechanisms can be distinguished based upon their principal mechanism of action (Table 4). These include genes encoding membrane proteins responsible for intracellular drug entrapment and/or translocation such as P-gp, the MDR-related protein (MRP), lung resistance protein (LRP), P95, and the transporter associated with HLA-processing (TAP); enhanced intracellular drug detoxification by glutathione-conjugation; alterations in nuclear targets such as topoisomerase II and DNA repair enzymes; and lastly, an altered threshold for generation of the apoptosis signal in response to DNA damage. These non-P-gp mechanisms of drug resistance as a group are relatively insensitive to competitive inhibitors of P-gp export function, and therefore if clinically relevant, may limit the benefit of P-gp modulation. Among the non-P-gp transport proteins the MDR-related protein (MRP) [18] and the major vault protein, lung resistance protein (LRP) [19, 20] are the best characterized.

The MRP gene is localized to chromosome 16p13.1, and is a member of the ATP-binding cassette (ABC) super gene family of membrane transporters [21]. The MRP gene encodes a 190-kDa integral membrane glycoprotein localized to plasma membrane and endomembrane structures, that contains two interior nucleotide-binding motifs [22]. Transfection studies have confirmed that MRP overexpression alone is sufficient to confer resistance to a broad profile of amphophilic natural product antineoplastics similar to P-gp, with specific exceptions of paclitaxel and mitoxantrone [23-25]. A major distinction is that MRP is a conjugate carrier that selectively catalyzes the transport of organic anions, or glutathione-, glucuronate-, or sulfate-conjugates of neutral or protonated antineoplastics such as the anthracyclines, vincristine and etoposide [26, 27]. As such, MRP but not P-gp mediated drug transport, is inhibited and chemotherapy sensitivity restored by agents that deplete intracellular glutathione content.

Table 4. Cellular mechanisms contributing to multidrug resistance

Mechanism	Example
1. Transmembrane transport	• P-glycoprotein (MDR-1) • Multidrug resistance related protein (MRP) • P95
2. Intracellular entrapment	• MRP • Lung resistance protein (LRP) • Transporter-associated with Antigen • Processing (TAP)
3. Drug detoxification	• Glutathione (GSTPPP)
4. Altered nuclear target	• Topoisomerase II • DNA repair
5. Altered apoptotic response	• p53 mutations • ↑ bcl-2

MRP is only one member of a recently described family of multispecific organic anion transporter (MOAT) genes that includes the canalicular MOAT (cMOAT) gene overexpressed in the liver and cisplatin resistant cell lines [28]. The latter gene, located on chromosome 10q24, has been implicated in the transport of bilirubin- and cisplatin-conjugates. Mutational inactivation of the cMOAT gene results in a murine model of the Dubin-Johnson syndrome, and has been implicated in the same syndrome in humans. Regulation of MRP gene message in normal blood cells differs from the pattern observed for MDR-1. The MRP gene is constitutively expressed in most, if not all mammalian cells at low levels [29]. Gene knockout studies indicate that such background levels of MRP contribute to a 1.5- to two-fold level of resistance to antineoplastic substrates [30]. Among hematopoietic elements, MRP gene overexpression has been reported only in T-lymphocytes, and unlike P-gp, is not overexpressed in mononuclear cells bearing the progenitor cell antigen CD34 [31]. Preliminary reports examining the frequency of MRP gene overexpression in hematologic malignancies are conflicting. Relative to normal bone marrow, significant overexpression has been observed in the majority of patients with chronic lymphocytic leukemia, unrelated to previous drug exposure, suggesting that native overexpression occurs commonly in this disease [32, 33]. Although the frequency of MRP overexpression in de novo AML is low, recent reports indicate a higher prevalence in clinically resistant and/or secondary AML, and acquisition of this MDR phenotype at relapse in sequentially analyzed specimens [31, 34, 35]. In addition, in patients harboring the favorable cytogenetic abnormality inv(16)p13q22, hemizygous deletion of the MRP gene is associated with a longer interval to treatment failure [36]. Recent reports indicate that overexpression of the MRP gene product precedes upregulation of MDR-1, and it is coordinately regulated with genes encoding glutathione-S transferase and GGG-glutamyl-cysteine synthetase, the rate limiting enzyme in glutathione synthesis [37, 38]. Whether overexpression of the MRP transporter is an important prognostic feature in specific acute leukemia subsets awaits further prospective studies in uniformly treated patient populations. Its low frequency of detection in de novo AML, higher prevalence at relapse, and prognostic importance in AML harboring the chromosome 16 inversion suggest that this drug resistance phenotype may be clinically relevant. However, reports of a high frequency of MRP overexpression in generally responsive acute lymphoblastic leukemia have raised appropriate concern that it lacks universal biological significance [32]. The availability of highly specific monoclonal antibodies to the MRP protein product will further define its biological significance [39]. A recent cytofluorometric analysis of MRP in over 300 de novo AML specimens from patients enrolled on SWOG 8600 indicates a low detection frequency (10%), and absence of a discernible adverse prognostic effect [35].

Overexpression of the gene encoding lung resistance protein, or LRP gene, is associated with an MDR phenotype analogous to that for P-gp. The monoclonal antibody LRP56 recognizes the 110-kDa protein product expressed in MDR tumor cell lines which lack P-gp and MRP overexpression [19, 40]. The amino acid sequence of LRP shows striking identity to the rat major vault protein (MVp)-AAA [20]. Vault proteins are multisubunit protein complexes localized to nuclear membrane pores and cytoplasmic vesicles, implicated in nuclear-cytoplasmic transport. Not surprisingly, transfection of LRP cDNA does not confer chemotherapy resistance in tumor cell lines indicating that expression of this protein alone is insufficient to confer drug resistance. Nevertheless, studies of anthracycline cellular pharmacokinetics support a similar function for the LRP transporter unit, showing rapid drug redistribution from the nucleus to cytoplasmic vesicles in LRP-overexpressing cell lines [41, 42]. More importantly, clinical investigations using the LRP56 antibody have demonstrated prognostic relevance in patients with relapsed or secondary AML, ovarian cancer, childhood ALL, and multiple myeloma [43-46]. Among 87 AML patient specimens analyzed at the University of Arizona, immunocytochemical staining with LRP56

was detected in 33% of cases of de novo AML, 38% of secondary leukemias and 38% of relapsed specimens [44]. Each AML subset was considered poor-risk based upon age or disease stage. LRP overexpression adversely impacted response to chemotherapy resulting in a 35% remission rate compared to 68% in LRP-negative patients ($p = 0.001$). More importantly, when adjusted for P-gp expression, only LRP retained independent prognostic significance in a logistic regression model. Of note, treatment with CsA as a P-gp modulator was associated with emergence of LRP overexpression at the time of treatment failure coincident with loss of MDR-1 expression. This suggests that in poor risk or previously treated leukemias, the use of chemosensitizers that effectively eliminate P-gp-expressing populations may select for this alternate mechanism of multidrug resistance. Nevertheless, these findings indicate that LRP is an important drug resistance phenotype that may contribute to anthracycline resistance in poor-risk AML. Identification of chemotherapy sensitizers which also reverse LRP resistance may therefore hold promise to improve therapeutic outcome. Indeed, recent investigations have identified multifunctional modulators that inhibit both P-gp and LRP function. One such agent, BIBW22, is a dipyridamole analogue with 100-fold greater potency for P-gp inhibition than the parent compound that effectively inhibits LRP function [47]. Clinical development of such multifunctional modulators awaits further investigation.

MDR in drug-selected tumor cell lines may also arise from a decrease in the catalytic activity of, and DNA cleavage by the nuclear enzyme topoisomerase I [48, 49]. The importance of this type of drug resistance in clinical specimens, however, remains unclear because of limitations in development of reliable assays for its application and cell cyclegoverned alterations in enzyme activity [50]. The glutathione-S transferases (GST) are a family of detoxification enzymes responsible for the direct conjugation of glutathione (GSH) to cellular nucleophiles [51]. While enhanced GSH generation and/or conjugation are accepted as important mechanisms of resistance to alkylating agents, direct evidence for a causative role in anthracycline resistance is less convincing. Correlative studies suggest that overexpression of GSTPPP is associated with a higher incidence of treatment failure in de novo AML [52]. However, no correlation was observed with treatment outcome in relapsed patients. Not surprisingly, other investigators have reported a positive correlation between GSTPPP and MDR-1 or MRP overexpression [53, 54]. In AML specimens, GSTPPP gene message remains relatively constant at relapse raising concerns that overexpression may represent a feature of the malignant phenotype. With the exception of MRP, GSH depletion results in minimal change in anthracycline resistance in P-gp overexpressing cell lines, despite concurrent over-expression of the transferase message, implicating P-gp as the dominant mechanism of resistance [55, 56]. More recent investigations suggest that the coordinate regulation of GSTPPP and other transport proteins relates to its role in the conjugation of amphophilic xenobiotics within endosomal structures to afford local protection from lipid peroxidation before export from the cell [37, 38, 57].

Cellular cytotoxicity resulting from DNA damage is dependent upon effective transmission of the growth arrest signal. A variety of antileukemic agents, including the anthracyclines, topoisomerase inhibitors and cytarabine exert their effects by induction of apoptosis or programmed cell death as evidenced by generation of the characteristic DNA fragmentation pattern and/or cytologic changes [58-60]. For this reason, disruption of the apoptotic signal promotes tumor survival and perceived resistance to chemotherapy. The proto-oncogene, bcl-2, is the best characterized of the biological signals responsible for suppression of apoptosis. Indeed, in a cohort of 82 patients with AML, immunodetection of the bcl-2 protein was associated with adverse prognostic features such as an elevated white blood cell count at diagnosis and expression of the stem cell antigen, CD34 [61]. More importantly, overexpression of bcl-2 predicted for an inferior response to induction chemotherapy and shorter overall survival. Overexpression of the bcl-2 protein has also been

reported in lymphoid leukemias, but its prognostic relevance remains under investigation [62].

The bcl-2 gene represents only one of many genes which impact transmission of the apoptotic signal. Several genes, including the tumor suppressor gene, p53, inhibitors of p53 function such as the human homologue of the murine double minute 2 gene (MDM2) and the retinoblastoma gene product (Rb), may be modifiers of the apoptotic response to many DNA damaging agents. Not surprisingly, mutations of the p53 gene and overexpression of MDM2 are demonstrable in both lymphoid and myeloid leukemias and are linked to an inferior treatment outcome [63-65]. Recent reports that P-gp is a lipid translocase with broad specificity that extends to the cell death effector, ceramide, indicates that the MDR1 gene product may limit not only anticancer drug entry, but also translation of the cell death signal [66]. While these observations illustrate the emerging biological complexity of chemotherapy resistance in the acute leukemias, they provide an important framework for development of strategies to enhance the apoptotic response to DNA damage or other stress-related signals.

Conclusions

It is clear from investigations to date that clinical MDR results from the interaction of an ever-increasing number of biologic mechanisms. MDR resulting from overexpression of the MDR-1 gene remains an important biological mechanism of resistance in AML and multiple myeloma that warrants further study. Despite the presence of redundant resistance mechanisms, ongoing clinical trials will provide direction for further therapeutic development. Whether pharmacological modulation of P-gp function offers clinic benefit must await the results of randomized trials which are now in progress. The recent development of more potent and multifunctional modulators indicates that we are only now at the threshold of exploring new, potentially more effective treatment strategies for MDR modulation in hematologic malignancies.

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Acute Leukemias VII
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Some Reflections on P-Glycoprotein Expression in Acute Leukemia

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Abstract. Expression and functional activity of P-gp were measured in 182 acute myelogenous leukemia (AML) patients: 136 patients were treated with the AML-6 protocol (EORTC), containing daunorubicin, vincristine and conventional-dose cytarabine (ara-C), and 21 patients with idarubicin, vepeside and conventional-dose ara-C (ICE-AML-10 protocol/EORTC). Additional 25 patients were treated with a modified dose of idarubicin and ara-C as compared to the ICE protocol, however with the same dose of etopside (ICE-I protocol). P-gp was determined using monoclonal antibody 4E3.16 and functional activity using the rhodamine 123 accumulation test. P-gp positivity was defined as a KS D value ≥ 0.15, P-gp negativity as a KS D value < 0.15. P-gp activity was defined as a ratio of mean rhodamine 123 accumulation with/without verapamil. In AML patients at primary diagnosis and early relapse/refractoriness a significant ($p < 0.05$) difference between P-gp positive and P-gp negative patients was ascertained using the AML-6 protocol; the difference corresponded to the complete remission rate. For ICE and ICE-I-treated AML patients at primary diagnosis this significance could not be shown. Compared to AML patients at primary diagnosis and patients at early relapse or refractoriness, a significantly ($p < 0.05$) increased incidence of non-pumping P-gp and a trend ($p = 0.054$) to a higher percentage of non-P-gp-related mechanisms in AML patients at late relapse was determined. When using the AML-6 protocol, age, activated P-gp and CD34 expression are independent prognostic factors in AML patients. A test system, which determines a functional P-gp overexpression, is a major tool for identifying a poor prognostic group of AML patients. In order to effectively use so-called P-gp modulator substances, the degree of P-gp expression, the activated or non-activated P-gp condition and detection of non-P-gp-related resistance mechanisms are of utmost interest for optimal design and analysis of P-gp modulator trials and for understanding the complexity of chemotherapy-related resistance mechanisms in patients.

Introduction

Chemotherapy resistance is the major reason for treatment failure not only in pretreated acute myelogenous leukemia (AML) patients but also in AML patients at primary diagnosis. One of the most investigated resistance mechanisms in AML patients is the P-glycoprotein (P-gp)-related resistance [10,

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18, 28, 30-32, 39, 40, 45, 47, 50, 52, 53, 55, 58, 69, 72, 73, 76, 77]. This transmembrane 170 kDa protein pumps several cytostatic drugs, such as daunorubicin, doxorubicin, vincristine and etoposide from the inside to the outside of the cell in a way that consumes ATP [12, 13, 19, 23, 25, 27]. It should be emphasized, however, that idarubicin, a new anthracycline, can overcome P-gp-related resistance in vitro and in vivo [6, 7, 38, 51, 54, 68, 70]. A comparison of the results of the numerous above-mentioned studies published in recent years is extremely difficult in the absence of standardized methods. A first step toward a consensus was established at the Memphis Meeting [4]. It is of utmost importance not only to detect P-gp on its phenotype or RNA level in a most specific way, but also to analyze its functional activity. The last-mentioned point should be of special interest with a view to initiating an optimal study design with agents (modulators) that can reverse P-gp-related resistance. Many such trials [3, 5, 9, 14, 16, 20, 34, 41-44, 48, 49, 56, 57, 59, 60, 61, 66, 67, 71] have been initiated in the past. The results, however, were predominantly negative and the P-gp biology not sufficiently analyzed in context with P-gp modulators. Additionally, special attention should be given to the existence and coexistence of alternate pump mechanisms, such as the multidrug resistance-associated protein (MRP) [1, 8, 15, 21, 26, 29, 31, 7, 17, 24, 29, 31, 32, 37, 64, 65, 75] or the lung resistance protein (LRP) [33, 62, 63]. Therefore, the goal of the present work was to characterize the P-gp phenotype by using indirect immunofluorescence with MoAb 4E3.16 und the P-gp pump function with the rhodamine 123 (R123) accumulation test system for diagnosis and treatment regimen.

Patients and Methods

Patients

We studied 182 acute myelogenous leukemia (AML) patients, where 136 patients were treated with the EORTC-AML-6 protocol, including 76 AML patients at primary diagnosis, 31 AML patients at early relapse or refractoriness and 29 AML patients at late relapse. Additionally, 46 AML patients at primary diagnosis were treated with the ICE (AML-10 protocol/EORTC) or ICE-I protocol. According to the FAB classification, 51 were classified as M1, 91 as M2, 24 as M4, two as M4 Eo, seven as M5a / M5b- and seven as M6 patients. Therapy-related leukemia and leukemia evolving from a myelodysplastic syndrome were excluded from this analysis.

Treatment Protocols. AML patients were treated according to the EORTC (European Organization for Research on Treatment of Cancer) AML-6 protocol [3] (daunorubicin 45 mg/m days 1-3; vincristine 1 mg/m, day 2; ara-C at 12-h intervals 50 mg/m^2 and daily continuous infusions 100 mg/m, days 1-7) or according to the ICE scheme (AML-10 protocol/EORTC-GIMEMA) containing idarubicin 10 mg/m on days 1,3 and 5, ara-C 25 mg/m^2 i.v. bolus followed immediately by 100 mg/m^2 given as a continuous daily infusion for 10 days and etoposide 100 mg/m on days 1-5. The ICE-I protocol consists of idarubicin 12 mg/m^2 on days 1,3,5, ara-C 100 mg/m^2 on days 1-7, and etoposide 100 mg/m^2 on days 1-3.

Blast Population Analysis. Morphological and cytochemical analysis of the blast population followed after Ficoll separation. After that, the so-called dominant marker analysis was performed with a leukogate (CD45/CD14) and lymphocytic marker determination by using a FACScan flow cytometer (Becton & Dickinson). In the case of suspected myelocytic leukemia CD34, CD33, CD13, CDw65 and CD15 were used. As a result of this procedure, the dominant immunocytological marker for the blasts was determined and used for further biparametric P-gp analysis and R123 accumulation ratio determination. A specimen was defined as CD34 positive when at least 30% of the leukemic cells showed mAb CD34 binding.

Sample Handling. Fresh bone marrow samples were held at room temperature for no longer than 24 h.

Quality Control. Standardized drug-sensitive and various drug-resistant P-gp-expressing cell lines such as CCRF-CEM (P-gp nega-

tive), CCRF-CEM/VCR100 (low P-gp expression), CCRF-CEM/VCR400 (intermediate P-gp expression), CCRF-CEM/VCR1000 (high P-gp expression) and CCRF-CEM/ACT400 (high P-gp expression) were used.

Monoclonal Antibodies. 4E3.16 is a mouse monoclonal antibody (MoAb) directed toward an extracellular epitope of the human mdr1-encoded P-gp [2]. This MoAb is also an IgG_{2a} class antibody and was originally a gift of Dr. Arceci (Dana-Faber Institute, Boston, MA, USA). It was later purchased from Signet Laboratories,Inc., Dedham, Massachusetts, USA. MoAb 4E3.16 specifically recognizes the human mdr1 P-gp but not the mdr2 product.

P-gp determination in BM: Using MoAb 4E3.16, P-gp was determined by means of the indirect immunocytological method. For 4E3.16 determination, mononuclear cells were concentrated by Ficol separation, washed twice with PBS + 0.05% NaN3 (in order to avoid capping the MoAb) and centrifuged at 200 *g* and 2-8 °C for 5 min. The pellets were resuspended in PBS + 0.05% NaN_3 and 20% FCS at a final concentration of 5 x 10^6 cells/ml. After 15 min on ice, 200 µl of cell suspension were incubated with 5 µl of MoAb 4E3.16. As negative control, a separate tube was filled with the same concentration of a MoAb of the same isotype (IgG_{2a}). Both tubes were incubated on ice for 30-60 min. Samples were centrifuged and washed twice. Pellets were resuspended in 200 µl PBS + 0.05% NaN_3 with 2% FCS and in 10 µl of the 1:10 with PBS-diluted FITC-conjugated secondary antibody (goat anti-mouse IgG_{2a}, Sigma Immuno Chemicals, Deisenhofen, Germany). Both tubes were subsequently incubated on ice in darkness for 30-45 min. After the cells had been washed once, they were resuspended in PBS + 0.05% NaN_3 for flow cytometric analysis. Fluorescence was analyzed with a FACScan flow cytometer (Becton & Dickinson) combined with LYSIS II computer software. P-gp expression was determined as the percentage of P-gp positive blasts. This analysis differentiated between negative (0%), low (1%-9%), moderate (10%-25%) and high (>25%) P-gp expression and correlated with the Kolmogorov Smirnov (KS) statistics [46, 74]. Corresponding to the percentage of P-gp positive blasts when using the KS test a D value was estimated , such as P-gp negative expression ($D<0.05$), low (D between 0.05 and 0.14), moderate (D between 0.15 and 0.24) and bright or high P-gp expression ($D >0.25$). P-gp positive expression was defined as a flow cytometric D value $\geq$ 0.15.

Rhodamine (R-123) Accumulation Test

Isolated mononuclear BM cells ($5x10^5$/ml) were resuspended in RPMI 1640 medium with stable glutamine (Seromed FG 1215). The cell suspension was divided into two tubes. One was preincubated with 10 µM verapamil for 15 min in a 37 °C shaker water bath. After preincubation, rhodamine 123 (Sigma R8004) 200 ng/mL was added to all samples, and the samples were incubated for 60 min at 37 °C. At the end of incubation, accumulation was immediately stopped with 3 ml ice-cold PBS (with or without verapamil). After centrifugation the sediments were resuspended in PBS (with or without verapamil) and cells kept on ice in the dark until flow cytometric analysis. Accumulation of R123 was determined with a FACScan flow cytometer (Becton & Dickinson). The green fluorescence of R123 was collected with a 530-nm band pass filter. Fluorescence intensity of R123 in the leukemic blasts was detected on fluorescence channel one (FL1) of the FACScan using a 1.024-log channel scale. A minimum of 5000 events were collected for each sample. Samples were gated at FSC versus SCC to exclude cell debris and clumps. The analyzed data are described as ratios of mean R123 accumulation with verapamil divided by R123 accumulation without verapamil. A R123 ratio between 0.88 and 1.32 was defined as a high R123 accumulation. In combination with a high P-gp expression (25-98%), however it means a non-functional P-gp. A R123 ratio between 6.2 and 14.28 is defined as a low R123 accumulation in combination with a high P-gp expression value; together with low or non-P-gp expression an indication for other protein pump mechanisms (the last-mentioned not defined in this study) should be considered.

Definitions

- Complete remission was defined as normal cellular (blast count 0-5%) bone marrow without blasts in the peripheral blood and a leukocyte count between 2 and 10 G/l as well as a platelet count > 100 G/l.
- The endpoints fo assessing the prognostic influence of the examined different prognostic factors in AML patients were:
- Clinical drug resistance (survival at day 28 and in five patients at day 32 with > 5% leukemic blasts in the bone marrow) or failure of induction therapy (clinical drug resistance after two therapy cycles). The last definition includes no change (NC) or no response to treatment and progressive disease (PD), i.e. an increase of blast count or more than 25% in the bone marrow after therapy.
- According to the tables, AML patients were treated at refractoriness (NC after at least two conventional induction therapy cycles), early relapse ($\leq$ 6 months) or at late relapse (> 6 months).
- Poor-risk karyotype was defined as t(9;11); Ph^+, t(9;22); t(4;11), t(11q23), -5/del(5q), -7/del(7q), +8 and intermediate- or good-risk karyotype was defined as t(8;21), inv (3), inv (16), t(6;9) t(8;16),), t(21q22) and, of course, normal karyotype according to the classification of Keating et al. (35).

Statistics

The U (WILCOXON) test was used for comparison of various measured values of the patients' (CSS software). The WILCOXON test tests the hypothesis that there are no differences between two paired populations of ordered-metric scores. The test takes into account the magnitude of the differences between two paired variables. *P* values < 0.05 were considered statistically significant. The predictive value for nonresponse in relation to P-gp positivity is the ratio of nonresponding patients to the total number of treated patients with P-gp positivity. Kolmogorov Smirnov (KS) statistics [46, 74] contained in the Lysis II program of the FACScan (Becton & Dickinson) were used, and a D value was generated to define P-gp positivity (D > 0.15). Multivariate analysis was used (CSS software) to calculate the prognostic factors.

Results

Seventy-six adult AML patients at primary diagnosis were treated with the AML-6 protocol; 45 (59%) reached CR. Moreover, 21 AML patients at primary diagnosis were treated with the ICE induction scheme (AML-10 protocol) and 25 with the ICE-I protocol. In the ICE-treated group a CR rate of 69% and in the ICE-I-treated patients a CR rate of 69.5% was observed. Furthermore, 31 AML patients at early relapse or refractoriness (CR rate 29%) as well as 29 AML patients at late relapse (CR rate 41%) were treated with the AML-6 protocol. The median age of these 101 male and 81 female patients was 51 years with a range between 22 and 58 years. Within the FAB classification there was no significant difference in P-gp expression.

P-gp and CD34 Value as Well as R123 Ratio Analysis Before Treatment

Sixty-three (35%) of the 182 AML patients had a D value > 0.15 and therefore ranked as P-gp positive according to the definition criteria. Of the 63, 39 (62%) patients were CD34-positive, and 20 (32%) of the P-gp positive patients showed a non-pumping P-gp. Eleven (55%) of these 20 AML patients were analysed as late relapse patients. This high incidence of non-activated P-gp-overexpression is significantly ($p < 0.05$) increased as compared to AML patients at primary diagnosis and early relapse or refractoriness. No significant difference concerning CD34 expression was seen in AML patients at primary diagnosis, early relapse or refractoriness and late relapse. Nine (45%) of the 20 non-pumping P-gp positive AML patients were CD34 positive. The incidence of CD34 expression between non-pumping and pumping P-gp positive patients was not significant.

Of the 119, 27 (23%) P-gp negative (D value < 0.15) AML patients demonstrated a ten-

dency to a non-P-gp-related mechanism. AML patients at primary diagnosis, early relapse or refractoriness and late relapse showed no significant difference regarding the assumption of a non-P-gp-related mechanism. Only 12 (10%) of 119 P-gp negative AML patients were CD34 positive; compared to the P-gp positive patients the incidence of CD34 expression was significantly ($p < 0.05$) lower. Three (11%) of the 27 P-gp negative AML patients with a non-P-gp-related mechanism showed positive CD34 expression. No significant difference in CD34 expression was demonstrated between P-gp negative patients and patients with a non-Pgp-related mechanism.

AML-6 Treatment

Eighteen (24%) of the 76 AML patients at primary diagnosis were defined as P-gp positive (Table 1). Of the 18 P-gp positive patients 12 showed a bright and six a moderate staining with the MoAb 4E3.16. Fifty-eight (76%) of the 76 patients were P-gp negative (Table 1), whereas in 27 patients a low P-gp expression was detected and in 31 patients a negative P-gp expression was seen. Of the 18 P-gp positive patients, three (17%) showed an R123 ratio between 0.85 and 1.35 (Table 2). This result indicated a non-pumping or non-activated P-gp. Of the 58 P-gp negative patients four (7%) showed an R123 ratio between 6.2 and 14.5. This result must be considered an indication of other, non-P-gp-related pump mechanisms. Two (11%) of the eighteen P-gp positive patients reached CR, with one patient showing bright and one patient a moderate P-gp expression (Table 1). Both patients with CR, however, demonstrated (Table 2) a non-pumping P-gp (R123 ratio between 0.85 and 1.35). Compared to the two P-gp positive responding patients, 43 (74%) P-gp negative patients had CR. The predictive value for non-response was 89%. The CR difference between P-gp positive and negative patients at primary diagnosis was significant ($p < 0.01$).

Twenty-one (68%) of 31 AML patients at early relapse or refractoriness were P-gp positive; 13 of whom had bright and eight moderate 4E3.16 staining (Table 3). Two (9.5%) of the 21 reached CR after the AML-6 protocol and both had a non-pumping P-gp (Table 4). The predictive value for non-responding P-gp positive patients was 93.5%. Seven (70%) of the ten P-gp negative patients achieved CR. Of these responded patients, a non-Pgp-related resistance mechanism must be assumed in two (28.5%), neither of whom reached CR (Table 4). P-gp negative and P-gp positive patients at early relapse/refractoriness treated with the

Table 1. Correlation of 4E3.16 staining as measured by the KS statistic (D value) and the percentage of 4E3.16 positive blasts in 76 AML patients at primary diagnosis treated with the AML-6-protocol in correlation to complete remission

	4E3.16 expression by the percentage of positive blasts							
	Bright (>25%)	CR	Moderate (25%–10%)	CR	Low (9%–1%)	CR	Negative (0%)	CR
4E3.16 expression by KS value (D)	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]
Bright (≥ 0.25)	9	1	2	–	1	–	–	–
Moderate (0.24–0.15)	–	–	4	1	2	–	–	–
Low (0.14–0.05)	–	–	2	1	22	18	3	2
Negative (<.05 or left shift)	–	–	–	–	–	–	31	22

[a] Number of patients with a particular value from the total number of 76 patients studied.
[b] Complete remission is indicated as CR and number (No.) of patients.

Table 2. Correlation of 4E3.16 staining intensity and R-123 accumulation (ratio value) in 76 AML patients at primary diagnosis treated with AML-6-protocol in correlation to complete remission

	4E3.16 expression of gated blasts (KS D value)							
	Bright (≥ 0.25)	CR	Moderate (0.24–0.15)	CR	Low (0.14–0.05)	CR	Negative (<.05 or left shift)	CR
R-123 ratio[a]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]
Ratio 0.85–1.35	2	1	1	1	25	19	29	22
Ratio 6.2–14.5	10	–	6	–	2	2	2	–

[a] R-123 accumulation of gated blasts (ratio).
[b] Number of patients with a particular value from the total number of 76 patients studied.
[c] Complete remission is indicated as CR and number (No.) of patients.

Table 3. Correlation of 4E3.16 staining as measured by the KS statistic (D value) and the percentage of 4E3.16 positive blasts in 31 AML patients at early relapse or refractoriness treated with AML-6-protocol in correlation to complete remission

	4E3.16 expression by the percentage of positive blasts							
	Bright (>25%)	CR	Moderate (25%–10%)	CR	Low (9%–1%)	CR	Negative (0%)	CR
4E3.16 expression by KS value (D)	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]
Bright (≥ 0.25)	9	1	3	–	–	–	–	–
Moderate (0.24–0.15)	–	–	6	1	2	–	–	–
Low (0.14–0.05)	–	–	–	–	5	3	1	1
Negative (<.05 or left shift)	–	–	–	–	–	–	4	3

[a] Number of patients with a particular value from the total number of 31 patients studied.
[b] Complete remission is indicated as CR and number (No.) of patients.

Table 4. Correlation of 4E3.16 staining intensity and R-123 accumulation (ratio value) in 31 AML patients at early relapse treated with AML-6-protocol in correlation to complete remission

	4E3.16 expression of gated blasts (KS D value)							
	Bright (≥ 0.25)	CR	Moderate (0.24–0.15)	CR	Low (0.14–0.05)	CR	Negative (<.05 or left shift)	CR
R-123 ratio[a]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]
Ratio 0.85–1.35	3	1	1	1	4	4	9	3
Ratio 6.2–14.5	10	–	7	–	2	–	4	–

[a] R-123 accumulation of gated blasts (ratio).
[b] Number of patients with a particular value from the total number of 31 patients studied.
[c] Complete remission is indicated as CR and number (No.) of patients.

AML-6 protocol showed a significant difference ($p < 0.05$) in CR rate at early relapse and refractoriness.

In 14 (48%) of 29 AML patients at late relapse a P-gp positivity was analyzed, while in nine a bright and in five a moderate 4E3.16

Table 5. Correlation of 4E3.16 staining as measured by the KS statistic (D value) and the percentage of 4E3.16 positive blasts in 29 AML patients at late relapse treated with AML-6-protocol in correlation to complete remission

3	4E3.16 expression by the percentage of positive blasts							
	Bright (>25%)	CR	Moderate (25%-10%)	CR	Low (9%-1%)	CR	Negative (0%)	CR
4E3.16 expression by KS value (D)	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]
Bright (≥ 0.25)	7	3	2	1	-	-	-	-
Moderate (0.24-0.15)	-	-	4	1	1	-	-	-
Low (0.14-0.05)	-	-	-	-	6	3	1	1
Negative (<.05 or left shift)	-	-	-	-	-	-	8	3

[a] Number of patients with a particular value from the total number of 29 patients studied.
[b] Complete remission is indicated as CR and number (No.) of patients.

Table 6. Correlation of 4E3.16 staining intensity and R-123 accumulation (ratio value) in 29 AML patients at late relapse treated with AML-6-protocol in correlation to complete remission

	4E3.16 expression of gated blasts (KS D value)							
	Bright (≥ 0.25)	CR	Moderate (0.24–0.15)	CR	Low (0.14–0.05)	CR	Negative (<.05 or left shift)	CR
R-123 ratio[a]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]
Ratio 0.85–1.35	7	3	4	1	3	3	3	4
Ratio 6.2–14.5	2	1	1	–	4	2	5	1

[a] R-123 accumulation of gated blasts (ratio).
[b] Number of patientswith a particular value from the total number of 29 patients studied.
[c] Complete remission is indicated as CR and number (No.) of patients.

staining was seen (Table 5). Five (36%) of these patients had CR. Therefore, the predictive value for non-responding P-gp positive patients was 64%. Of the five responding P-gp positive patients, four (80%) had a non-pumping P-gp (Table 6). Fifteen patients were P-gp negative, whereas seven showed a low and eight a negative 4E3.16 staining. Seven (44%) of the 15 negative P-gp patients achieved CR. In nine (60%) of the P-gp negative patients, however, a non-P-gp-related resistance mechanism must be assumed; three of these patients reached CR (Table 6). According to CR rate there was no significant difference between P-gp positive and P-gp negative patients.

The incidence of P-gp positivity in AML patients at primary diagnosis was significantly ($p < 0.05$) lower in comparison with AML patients at early relapse or refractoriness and AML patients at late relapse. Compared to AML patients at primary diagnosis and patients at early relapse or refractoriness, a significantly ($p < 0.05$) increased incidence of non-pumping P-gp and a trend ($p = 0.054$) to a higher percentage of non-P-gp-related mechanisms in AML patients at late relapse was ascertained.

ICE (AML-10 Protocol) or ICE-I Protocol Treatment

Of the 46 AML patients at primary diagnosis treated with the ICE scheme of the AML-10 protocol or with the ICE-I protocol, seven patients showed bright and four patients

Table 7. Correlation of 4E3.16 staining as measured by the KS statistic (D value) and the percentage of 4E3.16 positive blasts in 46 AML patients at primary diagnosis treated with ICE (AML-10-protocol) or ICE-I in correlation to complete remission

	4E3.16 Expression by the percentage of positive blasts							
	Bright (>25%)	CR	Moderate (25%-10%)	CR	Low (9%-1%)	CR	Negative (0%)	CR
4E3.16 Expression by KS Value (D)	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]	No.[a]	No.[b]
Bright (≥ 0.25)	5	3	1	1	1	1	-	-
Moderate (0.24-0.15)	-	-	2	1	2	1	-	-
Low (0.14-0.05)	-	-	-	-	12	10	7	3
Negative (<.05 or left shift)	-	-	-	-	-	-	16	13

[a] Number of patients with a particular value from the total number of 46 patients studied.
[b] Complete remission is indicated as CR and number (No.) of patients.

Table 8. Correlation of 4E3.16 staining intensity and R-123 accumulation (ratio value) in 46 AML patients at primary diagnosis treated with ICE (AML-10-protocol) or IC in relation to complete remission

	4E3.16 Expression of gated blasts (KS D value)							
	Bright (≥ 0.25)	CR	Moderate (0.24–0.15)	CR	Low (0.14–0.05)	CR	Negative (<.05 or left shift)	CR
R-123 ratio[a]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]	No.[b]	No.[c]
Ratio 0.85–1.35	2	2	–	–	19	11	13	11
Ratio 6.2–14.5	5	3	4	2	2	2	3	2

[a] R-123 accumulation of gated blasts (ratio).
[b] Number of patients with a particular value from the total number of 46 patients studied.
[c] Complete remission is indicated as CR and number (No.) of patients.

moderate 4E3.16 staining, which means 24% of the ICE- and ICE-I-treated patients had a P-gp positive value at primary diagnosis (Table 7). Thirty-five (76%) patients were analyzed for the category P-gp negative: 19 had a low and 16 a negative P-gp expression. Two (18%) of the eleven P-gp positive patients had non-pumping P-gp, and in eight (23%) of the 35 P-gp negative patients a non-P-gp-related resistance mechanism was to be considered (Table 8). Of the eleven P-gp positive patients, seven (64%) reached CR, compared to 26 (74%) of the 35 P-gp negative patients who showed CR. Thus, a predictive value of only 36% was determined for non-response in P-gp positive patients. Both non-pumping P-gp positive patients had CR, and of the eight P-gp negative patients with a possible indication of non-P-gp-related resistance 50 % achieved CR. Compared to the P-gp positive AML patients at primary diagnosis who were treated with the AML-6 protocol, the P-gp positive AML patients treated with the ICE or ICE-I protocol showed a significant difference ($p < 0.05$) in CR rate. There was no significant difference between the 21 ICE- and 25 ICE-I-treated P-gp positive patients in relation to CR.

Follow-Up of ICE- and ICE-I-Treated AML Patients

Nine of the 26 P-gp negative AML patients at primary diagnosis (Table 7), who reached CR after ICE (four patients) or ICE-I (five patients), developed an early relapse and

Table 9. Prognostic factors in AML patients according to the AML-6 protocol and the ICE/ICE-I protocol

	CDR/F (P value) according to AML-6 protocol (136 patients)		CDR/F (P value) according to ICE or ICE-1 (46 patients)	
	Univariate	Multivariate	Univariate	Multivariate
Age	0.0027	0.0029	0.0027	0.0025
P-gp expression[a]	0.0063	0.0036	0.045	0.052
R123 Ratio[a]	0.052	0.048	0.050	0.054
CD34 positivity[a]	0.048	0.049	0.038	0.048
Karyotype: (78 patients) [a]				
• poor risk	0.046	0.050	0.048	0.051
• intermediate/good risk	0.051	0.051	0.051	0.050
Blast cell count[a]	NS	NS	NS	NS
WBC	NS	NS	NS	NS
LDH	NS	NS	0.04	NS
FAB subtype[a]	NS	NS	NS	NS

CDR Clinical cytostatic drug resistance (survival at day 28 or 32 with >5% leukemic blasts in bone marrow).
F Failure after induction therapy.
NS Not significant.
WBC White blood cell count.
LDH Lactate dehydrogenase.
[a] Parameters measured in bone marrow.

further three patients after ICE treatment a late relapse. In seven of them a pumping P-gp overexpression was analyzed; five patients had a D value > 0.25 and two a D value between 0.15 and 0.24. One of the three late-relapsed patients showed P-gp positivity (D ≥ 0.25) and non-pumping P-gp.

Univariate and Multivariate Analysis of Prognostic Factors in AML Patients at Primary Diagnosis Treated with the AML-6 Protocol or the ICE/ICE-I Scheme

In 122 AML patients at primary diagnosis, established prognostic factors like age, karyotype, blast cell count, leukocyte count (WBC), lactate dehydrogenase enzyme (LDH) and French-American-British classification (FAB) were weighted with P-gp expression, R123 accumulation ratio and CD34 expression according to clinical cytostatic drug resistance (CDR) and failure (F) by using the AML-6 protocol and ICE/ICE-I treatment. In the multivariate analysis age, P-gp expression, R123 accumulation ratio and CD34 expression as compared to the other parameters (Table 9) are independent prognostic factors in AML patients treated with the AML-6 protocol. In ICE- or ICE-I-treated AML patients an independent prognostic relevance was determined only for the parameters age and CD34 expression, although a trend for P-gp ($p = 0.051$) and R123 accumulation ratio ($p = 0.054$) to influence the treatment response was established in this patient group.

Discussion

The present work analyzes the clinical relevance of P-glycoprotein expression and its function. From our own experience [54, 55] we preferred to use the MoAb 4E3.16 for P-gp analysis in this study. Furthermore, we evaluated P-gp expression by two options, namely percentage P-gp blasts and KS statistics [40], which generates a D value. The reason for the decision to use KS statistic was the fact that this method allows accurate identification of small differences in flouresence between test and control cells [46, 74]. This situation is frequently observed in clinical BM samples. Furthermore, the R123 accumulation test expressed as a ratio (with/without verapamil) was used to detect the activation or pumping function in P-gp positive or P-gp negative blasts. Both methods are necessary to optimally characterize the expression and function of P-gp. As al-

ready known, P-gp transports daunorubicin, vincristine and etoposide. Idarubicin, which is also an anthracycline, can overcome P-gp-related resistance in vitro and in vivo as mentioned in the Introduction section. However, this fact does not mean that idarubicin is not a substrate of P-gp transport, as shown by our group [unpubl.]. A possible explanation for the efficacy of idarubicin in patients with P-gp-related resistance might be that idarubicin is strongly lipophilic [24]. This aspect must be considered for evaluation of the clinical relevance of P-gp expression in leukemic blasts.

The present work showed no significant difference in P-gp expression when using the 4E3.16 MoAb within FAB classifications. This divergence from our former results [55] might be explained by the MoAb C219, which was used in this analysis. The significantly ($p < 0.05$) increased incidence of CD34 expression in P-gp positive AML patients found in this analysis confirms the results of other groups [11, 17, 22, 40, 72].

Only two of the 18 AML patients at primary diagnosis with a positive P-gp expression, who had been treated with the AML-6 protocol, reached CR. Both patients had a non-pumping P-gp (Table 2). A similar situation was found in AML patients at early relapse or refractoriness (Table 4). Compared to AML patients at primary diagnosis and early relapse or refractoriness, a significantly ($p < 0.05$) higher incidence of non-pumping P-gp in AML patients at late relapse (Table 6) was demonstrated and a trend to a higher percentage of non-P-gp-related mechanisms (Table 6) was also shown. An explanation for the significant high incidence of non-functional P-gp in AML patients at late relapse could be the hypothesis that P-gp is not only a marker for so-called P-gp-related resistance, but also a marker for other resistance mechanisms, or a mutant P-gp is the cause. Concerning non-P-gp-related resistance mechanisms two possibilities have to be discussed. The first one is the alternate drug efflux mechanism recently described as multidrug resistance-associated protein (MRP). The 110 kD major vault protein (LRP), which may alter intracellular drug redistribution is a further possible cause of cytostatic drug resistance. It can be assumed, however, that the methods used in this study could not detect the LRP mechanism.

The results of AML patients at primary diagnosis treated with ICE/ICE-I protocols suggest at first view, that it seems to be more natural for an idarubicin-containing protocol to replace future P-gp modulator protocols, but our first follow-up observations indicate that an induction or noneradication of activated P-gp positive blasts after ICE/ICE-I treatment can occur. A very close follow-up is needed to verity these results.

The multivariate analysis underlines the importance of analyzing the P-gp related results concerning the employed protocol in order to examine the clinical relevance of an activated P-gp overxpression in AML patients.

The main conclusion to be drawn from our results is that we should use methods characterizing the quantity and quality of P-gp expression, and that means the compulsory application of tools defining P-gp expression and its function in order to identify a poor prognostic group of AML patients. Particularly the determination of P-gp function must be used to discriminate between pumping and non-pumping P-gp positivity or, in the case of P-gp negativity, to identify non-P-gp-related efflux pump mechanisms. If a non-P-gp-related mechanism is to be assumed, further investigation should follow in order to make a contribution toward recognizing the complexity of drug resistance in patients. Because P-gp modulators such as PSC 833 or cyclosporine A also have a little influence on the MRP too, in clinical trials which aim to prove the efficacy of P-gp modulators we recommend that non- P-gp-related patients be included, but analyzed separately.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

In Acute Myeloid Leukemia only the Coexpression of at Least Two Proteins Including P-Glycoprotein, the Multidrug Resistance-Related Protein MRP, bcl-2, Mutant p53 and Heat-Shock Protein 27 is Predictive for the Response to Induction Chemotherapy

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Abstract

Purpose. Determination of the potential resistance markers P-glycoprotein (Pgp), the multidrug resistance-related protein (MRP), bcl-2, mutant p53 and the heat shock protein 27 (HSP27) in myeloid blasts for patients with de novo acute myeloid leukemia (AML) at the time of diagnosis. Evaluation whether these proteins alone or in combination are predictive for the response to induction chemotherapy with cytosine arabinoside (ara-C) idarubicin (AIDA).

Patients and Methods. The pattern of these patients were determined in 20 non-responders (BP) and compared to 20 patients with complete remission (CR). Pgp and MRP were determined by flow cytometry, bcl-2 and HSP27 by Western blotting and mutant p53 by single-strand conformation polymorphism analysis and luminometric immunoassay. The response to two courses of induction chemotherapy with AIDA (12 mg/m^2/d idarubicin i.v., day 1–3; 100 mg/m^2 ara-C i.v. bolus injection, day 2, immediately followed by 200 mg/m^2/d ara-C 24 h-continuous i.v. infusion, day 2–6) was defined by the criteria of the National Cancer Institute-sponsored workshop.

Results. There was no significant correlation between any of these proteins alone and treatment outcome. In contrast, a significant correlation between the coexpression of at least two of these proteins and response ($p = 0.0298$) was found which turned out to be a significant independent prognostic factor ($p = 0.0329$, relative risk = 1.5) in a multivariate analysis.

Conclusions. We conclude that drug resistance in AML is multifactorial and that the coexpression of different resistance mechanisms may be responsible for the primary drug resistance in de novo AML.

Introduction

Drug resistance is the major limitation in the cytotoxic treatment of AML, which may be present at the time of initial presentation (de novo resistance) or may occur during the course of treatment and relapse (acquired resistance) [1]. Treatment outcome can be affected by a number of host related characteristics such as age, karyotype and immunophenotype or by intrinsic biological mechanisms of resistance [2, 3].

Although several recent studies have indicated the clinical relevance of P-gylcoprotein (Pgp) associated multidrug resistance (MDR) in both newly diagnosed and pretreated AML, the correlation between Pgp gene expression and clinical drug resistance remains controversial and is not yet definitively established [4–9]. It has been suggested that alternative, non-Pgp-mediated mechanisms of drug resistance exist [10]. These

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mechanisms include the (over)expression of other drug transporters such as MRP (multidrug resistance-associated protein) and LRP (lung resistance-related protein) which have been found to correlate with poor response to chemotherapy in drug resistant acute and chronic leukemias [11–13].

More recently, decreased apoptosis including alterations in the tumor suppressor gene p53 and the overexpression of the oncoprotein bcl-2 as well as the expression of heat shock proteins (HSPs) have been associated with drug resistance in hematologic malignancies [14–16]. Whether all these mechanisms also contricute to clinical drug resistance still remains to be determined.

Although drug resistance in AML appears to be multifactorial in the clinical setting, studies on coexpression and the association between the various drug resistance genes and response to chemotherapy are rare [17–19].

Therefore we analyzed the expression of different proteins which possibly contribute to drug resistance such as Pgp, MRP, bcl-2, mutant p53 and HSPs in myeloid blasts of patients with de novo AML before induction chemotherapy. It was the purpose of the study to determine whether these proteins alone or in combination are predictive variables for response to induction chemotherapy with cytosine arabinoside and idarubicin. Thus, the pattern of resistance markers in 20 non-responders were compared to those of 20 complete responders.

Patients and Methods

Patients

Patient characteristics at the time of entry into the study are summarized in Table 1. The data were correlated with the remission status after the first two induction therapies with cytosine arabinoside (ara-C) and idarubicin (AIDA) [20, 21]. The treatment consisted of 12 mg/m^2/d idarubicin i.v., day 1–3; 100 mg/m^2 ara-C i.v. bolus injection, day 2, immediately followed by 200 mg/m^2/d ara-C 24 h-continuous i.v. infusion, day 2–6. The remission status after chemotherapy was evaluated as complete remission (CR) and blast peristence (BP) according to the criteria of the National Cancer Institute-sponsored workshop on definitions of diagnosis and response in AML [22].

Karyotype Analysis

Karyotype analysis was performed on aspirated heparinized bone marrow cells as previously described [23].

Table 1. Patient's characteristics

		CR	BP
Age	(years: median/range)	44 (21–64)	46 (19–68)
Sex	(female/male)	11/9	10/10
FAB			
-M0		–	1
-M1		4	5
-M2		8	5
-M3		–	–
-M4		7	7
-M5		1	3
-M6, M7		–	–
LDH (U/l)	(median/range)	534 (244–1650)	533 (124–6100)
WBC/nl	(median/range)	29 (2–152)	32 (5–160)
Karyotype			
Favorable		–	1
Intermediate		17	11
Unfavorable		2	6
n.d.		1	2

n.d.: not determined; WBC: white blood cells.

Preparation of Myeloid Blasts

Bone marrow aspirated containing more than 80% myeloid blasts were collected from about 100 AML patients at their initial presentation and separated by Ficoll-Hypaque gradient centrifugation (Nycomed, Oslo, Norway). The cellular protein, DNA and viable cells were immediately prepared and stored under the respective conditions as detailed in the method section. According to systemic validation experiments there was no difference in protein expression in all assays used when the results from previously long-term frozen cells or protein were compared to those obtained in fresh material as determined in ten bone marrow samples from normal donors and five AML patients.

When clinical data concerning treatment outcome after two cycles of induction chemotherapy were available, the pattern of resistance markers from the first consecutive 20 non-responders were compared to those of 20 patients with CR.

Flow Cytometry (Pgp and MRP)

Flow cytometry was performed with viable cells which were stored under nitrogen. Detection for Pgp was performed by incubation of myeloid blasts (1×10^6 cells/100 µl) with 1 ml PBS containing 1 µg MRK16 (Syrinx, Frankfurt, Germany) for 60 min at 4 °C, washing and subsequent incubation with 2 µg FITC-conjugated goat antimouse IgG (GAM, Becton Dickinson, Germany) for 60 min at 4 °C. MrP detection: myeloid blasts (1×10^6 cells/100 µl) were washed once with PBS, pelleted, and fixed with 0.4% paraformaldehyde in PBS for 2 min at 4 °C, washed with PBS containing 1% BSA, 0.09% azide, and permeabilized in 500 µl ice-cold methanol/acetone (1:1, v:v). This mixture was then incubated on ice for 15 min and washed twice with PBS plus azide before adding 1 µg MRP antibody (Syrinx, Frankfurt, Germany). After incubation for 15 min at room temperature the cells were centrifuged, resuspended in PBS and further incubated with 2 µg FITC-conjugated goat antimouse IgG (GAM, Becton Dickinson, Heidelberg, Germany) for 15 min at room temperature followed by washing and resuspension in PBS. A mouse antihuman IgG1 for MRP (Coulter, Krefeld, Germany) and IgG2a (MRK16) antibody served as negative controls in all experiments. After appropriate gating, intracellular fluorescence was determined in 15 000 cells per sample using an EPICS XL flow cytometer (Coulter, Krefeld, Germany).

Cell Homogenization and Protein-Isolation

Myeloid blasts (1×10^7) were suspended in 150 µl PBS buffer (NaCl: 148 mM; Na_2HPO_4: 6.4 mM; KCl: 2.7 mM; KH_2PO_4: 1.5 mM, pH 7.2) and disrupted by sonication for 6×10 s (30 W, sonifier B-12; Branson Sonic Power Company, Danbury, CT, U.S.A.). Complete cell breakage was assessed by light microscopy. In order to avoid proteolytic inactivation, 12 mM EDTA, 100 µg/ml PMSF and 1 mM dithiothreitol were added. Disrupted cells were centrifuged at 20 000 g for 30 min at 4 °C (EBA 12 R, Hettich, Tuttlingen, Germany).

Western Blot (bcl-2 and HSP27)

Protein (20–40 µg) was separated on a 12.5% SDS-gel and subsequently transferred to nitrocellulose. Nonspecific binding was blocked by 5% non-fat milk in PBS buffer. Immunoblotting was performed with 5 µg/ml monoclonal mouse anti-human antibody (HSP27: Biomol, Germany; bcl-2: DAKO, Hamburg, Germany) and a subsequent incubation with horseadish peroxidase (HRP) conjugated goat anti-mouse IgG (dilution 1:1 000). HSP27 and bcl-2 were determined by using the ECL system supplied by Amersham (Braunschweig, Germany).

Luminometric Immunoassay (p53)

The sandwich-type luminometric immunoassay (LIA) for quantitative measuring of mutant and wild-type p53 protein in homogenized cells has been described elsewhere in detail [24]. In brief, two monoclonal anti-p53 antibodies named Pab 1801 and

DO1 were used for immobilization and detection of p53, respectively. These antibodies are directed against two different N-terminal constant and denaturation-resistant epitopes. For luminometric detection the DO1 antibody is conjugated with aminobutylethylisoluminol (ABEI). The assay was standardised using pure soluble recombinant wild-type human p53 protein isolated from bacteria. For measurement of luminescence a Berthold luminometer (Wildbad, Germany) and the LIA-mat starter service kit (Byk-Sangtec, Diezenbach, Germany) were used. The concentration of p53 protein was expressed in ng/ml cell lysate protein. The standard curve was calculated with a curve-fitting programme (spline smooth Multicale, Wallace OY, Turku, Finland), the p53 content (ng/ml) was determined per RLU (relative light unit). The detection limit (zero standard + 3 standard deviations) was approximated to be 0.01 ng/ml.

Evaluation of p53 Gene Mutations

1. Amplification of p53 exons 5–6, 7 and 8–9 by polymerase chain reaction (PCR): Isolation of genomic DNA using phenol/chloroform extraction and amplification of p53 exons 5–6, 7, 8–9 by PCR was performed as previously described [25].
2. Single-strand conformation polymorphism (SSCP): PCR products (8 μl) were mixed with 3 μl sample buffer containing 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene xyanol, 3% Ficoll and formamide. This mixture was heated at 95 °C for 2 min, chilled on ice and loaded on a 6% neutral polyarcylamide gel [MDE (mutation detection enhancement) gel solution] containing 90 mM Tris-borate and 2 mM EDTA. After electrophoresis at 550 V for 16 h, DNA was visualized by silver staining. p53 mutations were detected due to atypical DNA banding pattern using PCR products from healthy donors as reference.

Statistical Analysis

Correlation between the expression and co-expression of gene products and treatment outcome in terms of response was assessed by the chi-squared test. Multivariate analysis was performed with the simultaneous regression method by means of the "stepwise proportional hazards general linear model procedure". One-way-analysis of variance (One-way Anova) followed by Bonferroni's post test for multiple comparisons were used to compare p53 amounts in normal donors and AML patients.

Results

Patients

We studied 40 AML patients (20 non-responders and 20 CR-patients) with de novo AML before induction chemotherapy. Patients' characteristics are given in Table 1. The karyotype could be determined in 37 patients. In the group of responders 16 patients had an intermediate karyotype [normal (n = 14); +7, $11q^-$, $1q^+$ (n = 1 each)] and in two patients the karyotype was characterized as unfavorable [t(8;21), $9q^-$], (+8, $9q^-$] (n = 1 each). One favorable karyotype (inv16) and 11 intermediate karyotype [normal (n = 11)] were found in 12 BP patients and six patients had an unfavorable karyotype [multiple abnormalities (n = 4), −7, t(10;11) (n = 1 each)].

Expression of Different Proteins

The expression of the various proteins studied is shown in Table 2. The amount of Pgp or MRP measured by flow cytometry higher than 15% was determined as positive. With this cut-off point, the application of curve fitting and substraction analysis revealed Pgp expression in 8/20 patients with CR (40%) and in 13/20 patients with BP (65%). MRP expression was detected in 4/20 patients with CR (20%) and in 9/20 patients with BP (45%).

bcl-2 Expression. When patients were stratified for basic bcl-2 expression as found in normal donors and overexpression of bcl-2, 10/20 patients with CR (50%) versus 12/20 with BP (60%) were found to have an overexpression of bcl-2.

Table 2. Expression of Pgp, MRP, bcl-2, mutant p53 and HSP27 in AML patients (n = 40)

	Number of patients	Number of patients (%) with protein expression for				
De novo AML	40	PgP	MRP	bcl-2	mu p53	HSP28
- with complete response (CR)	20	8 (40)	4 (20)	10 (50)	2 (10)	6 (30)
- with blast persistence (BP)	20	13 (65)	9 (45)	12 (60)	1 (5)	10 (50)

Evaluation of Mutant and Wild-Type p53 Protein. AML patients were first stratified with regard to the absence and presence of p53 mutations as analyzed by single-strand conformation polymorphism (SSCP). Mutations could only be detected in 2/20 patients with CR (10%) (one patient with a mutation in exon 7, one patient with a mutation in exon 8/9) and 1/20 patients having a BP (5%) (mutation in exon 7).

Figure 1 gives a more detailed analysis of p53 expression. AML patients were stratified with regard to treatment response (CR+, BP+, n =20 each, respectively) after two cycles of induction chemotherapy and the presence (M+; n = 3) or absence (M-; n = 37) of p53 mutations at the time of diagnosis. The concentration of p53 in three patients with a mutation in exon 7 (p53: 20.5 and 22 ng/ml) and exon 8/9 (p53: 3.5 ng/ml) was 2 to 20-fold higher than in 37 patients without any mutation detected (p53: 0.125–1.7 ng/ml).

Furthermore, one-way-analysis of variance (One-way Anova) followed by Bonferroni's post test for multiple comparisons were used to compare 37 AML patients without p53 mutations with p53 values established for bone marrow and peripheral cells or tissue from normal donors (p53: 0.18 ± 0.14 ng/ml, range: 0.01–0.38 ng/ml, as determined by Borg et al., 1995 and our group) [34]. The test revealed significant differences in p53 concentrations when values for donors were compared with AML CR+/M− ($p < 0.001$) or AML BP+/M− ($p = < 0.001$). No differences were obtained when both AML groups were compared with each other.

HSP27 expression: HSP27 is not detected in bone marrow cells from normal blood donors as studied by Western blot analysis. Six out of 20 patients with CR (30%) and 10/20 patients with BP (50%) were shown to be positive for HSP27.

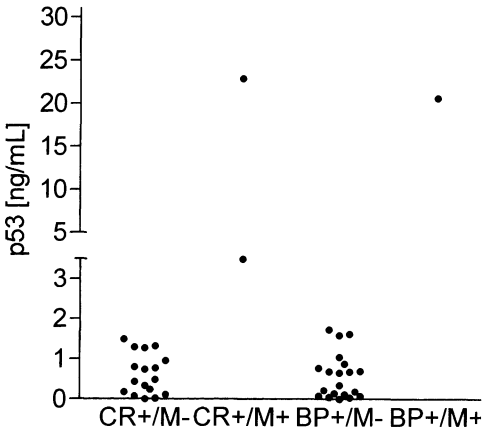

Fig. 1. Results from 40 myeloid blast lysates from untreated AML patients were stratified with regard to treatment response (CR+: complete remission, n = 20; BP+: blast persistance, n = 20) after the first two cycles of induction chemotherapy and the presence (M+; n = 3) or absence (M-; n = 27) of p53 mutations at the time of diagnosis. One-way-analysis of variance (One-way Anova) followed by Bonferroni's post test for multiple comparisons revealed significant differences when basal values in cells and tissue from normal donors (p53: 0.18 ± 0.14 ng/ml, range: 0.01–0.38) were compared with CR+/M- ($p < 0.001$) or BP+M- ($p < 0.001$). No differences were obtained when both AML groups were compared

Univariate Analysis for Prognostic Variables

For the following analysis, all parameters, including patients' charcteristics and the expression of different proteins, were evaluated comparing patients who achieved a complete remission with patients who had a blast persistence. The predictive values of the respective variables prognostic for response to induction chemotherapy are listed in Table 3. There was no significant association between any of the factors studies and response to induction therapy. However, when patients were analyzed for the expression of at least two different proteins and treatment outcome, coexpression was found

Table 3. Relationship between prognostic variables, protein expression and treatment outcome

	Univariate analysis	Multivariate analysis
PgP	0.1028	0.8514
MRP	0.1007	0.4141
bcl-2	0.3798	0.4084
Mutant p53	0.8231	0.3644
HSP-27	0.2507	0.8068
Coexpression ≥ 2	0.0298	0.0329
Age	0.0778	0.0626
Sex	0.5814	0.7009
FAB	0.5012	0.4615
WBC	0.0796	0.0583
% Blasts in bone marrow	0.5957	0.5714
LDH	0.6109	0.6347
Karyotype	0.9923	0.7653

to be a significant prognostic factor (p = 0.0298).

Coexpression of Pgp, MRP, bcl-e, Mutant p53 and HSP27

The different patterns of coexpression of the respective proteins in AML patients according to the remission status is detailed in Table 4. As indicated in section A, 17/20 patients with a BP (85%) showed a multifactorial resistance resulting in the coexpression of four proteins (n = 2), three proteins (n = 6), and two proteins (n = 9), respectively. In contrast, only 8/20 patients in CR (40%) displayed a coexpression of three or four different proteins (n = 3 each) and two proteins (n = 2), respectively. Interestingly, relapses occurred in five out of eight patients coexpression two proteins or more within 6 months in contrast to only two out of 11 patients without coexpression.

When AML patients were analyzed according to the coexpression of these proteins in a multivariate model including host-related characteristics, the coexpression of at least two of these proteins turned out to be a significant independent prognostic factor for treatment failure (p = 0.0329, relative risk = 1.5) (Table 3).

Discussion

The clinical resistance of malignant cells against cytotoxic drugs is supposed to be simultaneously caused by multiple mecha-

Table 4. Coexpression of different proteins in AML according to response

No. of coexpressed proteins	PgP	MRP	bcl-2	mu p53	HSP-27	n
A) Blast persistence (BP), coexpressions in 17/20 patients (85%)						
4	+	+	+	–	+	2
3	+	+	+	–	–	2
3	+	–	+	–	+	2
3	–	–	+	+	+	1
3	+	+	–	–	+	1
2	+	–	+	–	–	4
2	+	–	–	–	+	2
2	–	+	–	–	+	2
2	–	+	+	–	–	1
B) Complete remission (CR), coexpressions in 8/20 patients (40%)						
4	+	+	+	–	+	1
4	+	+	+	+	–	1
4	+	–	+	+	+	1
3	+	–	+	–	+	1
3	+	+	+	–	–	2
2	–	–	+	–	+	1
2	+	–	+	–	–	1

nisms in the majority of cases. Up to now, a number of different gene products including Pgp, MRP, LRP, bcl-2, p53, HSPs, topoisomerase II and glutathione-S-transferase (GST) have been indicted to be causative in AML. The contribution of these proteins to drug resistance still has to be investigated in more detail and, furthermore, their coexpression and possible interaction has to be elucidated.

Here we demonstrate by the analysis of various gene products possibily responsible for drug resistance, such as Pgp, MRP, bcl-2, p53 and HSP27, that no single gene product was of significant prognostic value with respect to treatment outcome in AML when all responders were compared to nonresponders. In contrast, the coexpression of at least two of these gene products was significantly predictive for the clinical outcome of these patients. Thus, our results indicate that drug resistance is multifactorial in AML.

Univariate Analysis

Although Pgp overexpression remains a good predictive factor for clinical resistance against daunorobicin-containing chemotherapy of patients with AML [4, 5, 6, 9] a variety of studies reported a number of patients with AML refractory to chemotherapy not expressing the MDR_1 gene. Furthermore, a consensus conclusion on the prognostic significance of Pgp is hampered by the fact that the methods for its determination as well as sample preparation and fixation are not standardized [26]. Although a conclusive evaluation is still pending, the results of a number of studies demonstrated a prognostic significance for the drug transporter MRP in AML as well as a variety of other human malignancies [13, 27, 28]. In our study, when analyzed a single factors, neither Pgp nor MRP was predictive for treatment outcome in AML patients treated with an induction chemotherapy regimen containing the anthracycline idarubicin instead of daunorubicin. Induction chemotherapy with two AIDA courses has produced significantly higher CR rates in the range of 70 to 80% as compared to the induction chemotherapy with daunorubicin and ara-C in three randomized studies [29–31] and we have achieved a CR rate of about 70% on the basis of 100 patients which is in agreement with the results preliminary reported in a smaller group of patients [21]. The higher efficacy of idarubicin in comparison to daunorubicin has been attributed to the finding that it is a less suitable substrate for Pgp [32]. However, its major metabolite idarubicinol which in contrast to daunorubicinol has about the same cytotoxicity as its mother compound [33] turned out to be a Pgp-dependent anthracycline [34]. Furthermore, clinical outcome in patients with AML treated with the AIDA regimen was significantly dependent on the cellular uptake of idarubicinol in myeloid blasts in vivo [35].

The expression of gene products involved in the inhibition of apoptosis such as p53 and bcl-2 have become more and more important in the discussion of treatment failure in AML. Variable levels of bcl-2 were found in AML which were significantly higher in the FAB subtypes M1 and M2 as in the other ones [36]. Additionally, the poor prognosis previously associated with AML blasts expressing the CD34 antigen, may be in part related to high expression of bcl-2 [37]. Furthermore, the expression of bcl-2 was shown to be predictive for poor response to intensive chemotherapy and shorter survival in AML [38]. In our study overexpression of bcl-2 alone was not predictive for treatment failure and did not correlate with other patient characteristics.

The low incidence of p53 mutations in three out of 40 patients (8%) is in agreement with findings obtained by other groups who demonstrated that mutations within the highly conserved portions of the p53 coding region infrequently occur in most types of AML [39]. Wattel and coworkers reported that p53 mutations, although infrequent, are a strong prognostic indicator for response to chemotherapy and survival in AML, myelodysplastic syndrome and chronic lymphocytic leukemia [15]. Although the number of patients with p53 mutations in our study was small, a poor treatment outcome could be documented in one nonresponder and one patient with a CR of only three months. Furthermore, elevated concentrations of p53 without detectable mutations were found in half of the patients, which is in agreement

with the findings by Zhang and coworkers [40]. These findings could either correspond to an overexpression of a nonmutant p53 or to p53 mutations in a minor subclone of the malignant cells undetectable by SSCP. Furthermore, they can be related to the presence of a mutation outside the exons investigated, although such mutations appear to be very rare.

According to clinical investigations in breast cancer, HSPs may resemble a separate mechanism of resistance [41]. Increased levels of HSPs were found in a number of malignancies including leukemia [14, 42, 43]. Our results did not show a correlation between the expression of HSP27 and treatment outcome. Furthermore, no increased amounts of HSP60 and HSP70 could be detected in the blast samples as compared to normal blood donors (data not shown).

Multivariate Analysis

When patients were analyzed according to the coexpression of Pgp, MRP, bcl-2, p53 and HSP27, there was a significant correlation between the coexpression of at least two of these proteins and response which turned out to be a significant independent prognostic factor for treatment failure according to multivariate analysis.

The fact that different mechanisms can be simultaneously coexpressed has been recently confirmed for various tumor entities including leukemia [44, 45]. For instance, the functional MDR phenotype associated with the simultaneous overexpression of Pgp and MRP [4, 19, 46, 47] as well as their coexpression together with the expression of HSP27 [48] has been shown to predict clinical response in AML. Additionally, the coexpression of Pgp and GST has been suggested to be responsible for drug resistance in acute leukemia [27]. In this context it should be emphasized that most of the studies published so far semiquantitatively analyzed the gene expression at the mRNA level by cDNA-PCR which reveals the relative activity of genes, not of gene products. Thus, in this study for the first time the simultaneous expression of five mechanisms of drug resistance is investigated in myeloid blasts on the protein level with respect to clinical response after induction chemotherapy.

In conclusion, future investigations will have to consider a variety of gene products as well as their coexpression to better characterize clinical drug resistance. Furthermore, it will be of great importance to quantify the expression of these proteins simultaneously to define their prognostic significance for the establishment of individual treatment intensity and to possibly contricute to the development of strategies for overcoming clinical drug resistance.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Human P-Glycoprotein/Mdr1-Gene Product: Surprising Potential for Nuclear Localization and Coupling to Intracellular Growth Factor Signalling

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Abstract. The human p-glycoprotein (P-gp)/mdr1-gene product is an integral plasma membrane protein whose overexpression is associated with chemotherapeutic drug resistance. In the present study, we scanned the amino acid sequence of p-gp for clues which may point to additional functions of this important protein. This bioinformatics-based approach yielded two new and surprising results. P-gp contains structural information compatible with a nuclear translocation of this protein and with a complex formation of P-gp with the insulin-degrading enzyme (IDE). These interesting data suggest that P-gp may also directly act at the level of the cell nucleus, possibly influencing nuclear events such as cell cycle progression, and additionally affect the intracellular turnover of growth factors such as insulin. As such, these are the first hints towards a common mechanism of P-gp-dependent and growth factor-mediated drug resistance of cancer cells.

Introduction

The premise underlying our present study was that there is a shortcut for most signals influencing the fate of the cancer cell. This relay propels extracellular or membrane-bound molecules directly into the nuclear compartment, the site of gene regulation. One recently elucidated and potentially major example for this concept is the nucleocrine pathway [1]. The overexpression of P-gp, an integral plasma membrane protein whose overexpression correlates with chemotherapeutic drug resistance, has recently been shown to reliably predict treatment failure in patients with acute leukemia [2]. The specific aim of the present investigation was to assess whether P-gp may also employ a fast track to the genes, thereby rendering cancer cells more resistant towards various treatments.

Materials and Methods

P-gp was first visually inspected for the presence of nuclear localization signals, as defined previously [3]. In order to find novel interaction partners for p-gp, we retrieved the human p-gp cDNA from the NCBI data base and applied the complementary peptide strategy [4] to it using the DNA Strider software. The resulting complementary peptide sequences were then checked for homologies to known proteins by employing the BLAST search procedure contained in the OWL data base navigator.

KK NFFKLNNKSE KDKKE.

Fig. 1. Proposed bipartite nuclear localization signal (NLS) in the human p-glycoprotein, residues 13-29 (basic residues such as K located in the two clusters of the bipartite NLS are underlinded)

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A H_2N- GAGL-COOH p-gp_{62-65}
HOOC- ASSK -NH_2 IDE_{87-84}
Potential contact paint #1 between p-gp and IDE

B H_2N- RGWK -COOH p-$gp_{210-213}$
HOOC- PPPL -NH_2 IDE_{27-24}
Potential contact point #2 between p-gp and IDE

Fig. 2

Results and Discussion

We have now found that human P-gp harbors a bipartite nuclear localization signal (Fig. 1).

By employing the complementary peptide strategy we have further identified potential P-gp binding sites for intracellular proteins, in particular for IDE (Figs. 2a and b).

IDE is a large cytosolic protein which causes insulin degradation [5]. IDE-like proteins have been implicated in membrane fusion, muscle differentiation and intracellular turnover of proteins suggesting similar involvements of IDE itself [5]. Interestingly, the predicted P-gp binding site #2 is in the immediate vicinity of P-gp transmembrane region 3 to which rhodamine 123 and other P-gp modulators bind [6]. This suggests that these modulators may disrupt the physical association of P-gp with IDE through sterical displacement such that IDE is then free to bind and degrade intracellular insulin. The putative intracellular interaction between P-gp and IDE may be of paramount importance in P-gp action given that growth factors have previously been shown to mediate drug resistance. Based on our present findings, it is now tempting to speculate that intracellular/nuclear P-gp may block IDE action, thus extending the intracellular lifespan and facilitating the nuclear translocation of insulin - a scenario reminiscent of the effect of agents which block IDE function [7]. The net result of our model would be an enhancement of the growth-promoting actions of insulin. Moreover, P-gp may also directly participate in gene regulation by interacting with intranuclear proteins. Taken together, our data are intriguing first hints towards a unifying view of P-gp-dependent and growth factor-mediated drug resistance of cancer cells.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Treatment of Poor Prognosis Acute Myelogenous Leukemia with PSC-833 and Mitoxantrone, Etoposide, Cytarabine

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Introduction

A major reason for failure of conventional treatment in acute myeloid leukemia (AML) is primary or secondary resistance of the leukemic cells to anti-neoplastic drugs. One of these mechanisms is characterized by the multi-drug resistance (MDR) phenotype that results from overexpression of the MDR-1 gene product, p-glycoprotein (P-gp) [1, 2]. P-gp has been linked to a number of adverse prognostic variables including age, CD34 phenotype, cytogenetic pattern or leukemia arising secondary to cytotoxic therapy or antecedent hematological disorders [3, 4, 5, 6]. The MDR phenotype is characterized by lower intracellular drug accumulation [7, 8] and reduced sensitivity to the anthracyclines [5, 6, 9]. Several agents capable of reversing drug resistance in vitro have been used clinically [10, 11, 12]. We report here the preliminary results of a multicenter phase I-II trial evaluating PSC-833, a non-nephrotoxic, non-immuno-suppressive analogue of cyclosporin and an inhibitor of the MDR-1 pump, in combination with Mitoxantrone (M), Etoposide (E), Cytarabine (C) (termed PSC-MEC) for treating a poor prognosis subgroup of patients with AML.

Patients

This is a preliminary report of an ongoing open phase I-II multicenter trial initiated in June, 1995. The protocol was approved by the Institutional Review Boards and Human Subjects Committees of all the participating institutes and is the pilot study for a planned Eastern Cooperative Oncology Group (ECOG) randomized phase III trial. Eligibility criteria included patients between the ages of 18-70 with a diagnosis of AML by the FAB criteria with the following characteristics:

1. relapse (6 months after first complete remission (CR)
2. refractory to chemotherapy, either initial induction or first reduction;
3. relapse after autologous or allogeneic bone marrow transplant;
4. second or greater relapse;
5. secondary AML or AML evolving from myelodysplastic syndromes (MDS) or myeloproliferative disorders (MPD) (not CML).

Patients were also required to have an ejection fraction > 50%, ECOG performance status of 0-2, normal hepatic/renal function and no evidence of active infection. The main exclusion criteria were presence of central nervous system (CNS) leukemia and M3 leukemia.

Stanford University Med Ctr, Stanford, CA, Moffitt Cancer Ctr, Tampa, FL, Northwestern Med Ctr, Chicago, IL Rochester Med Ctr, Rochester, NY, Montefiore Med Ctr, Bronx, NY and Sandoz Pharmaceuticals, E. Hanover, NJ, USA

Chemotherapy Treatment

PSC-833 (Valspadar, provided by Sandoz / Novartis Pharmaceutical Corporation, East Hanover, NJ) was administered as a loading dose at 2 mg/kg over 4 h, pretreatment with a concomitant continuous infusion at 10 mg/kg/IV for 120 hours. Doses for chemotherapy (PSC-MEC) in Cohort I were M 5 mg/m^2/day, E 50 mg/m^2/day and C 1 G/m^2/day all given IV for 5 days. In cohort II the dose of M was reduced to 4 mg/m^2 and E to 40 mg/m^2. Growth factor support with GM-CSF or GCSF was initiated daily once marrow hypoplasia was documented, 8-10 after post-induction treatment initiation. If residual leukemia was noted, the induction course was repeated for 5 days if the blastic cellularity was $\geq$ 10% and for 3 days if it was 5-10%. A maximum of two induction cycles were permitted to achieve bone marrow hypoplasia. If the disease progressed thereafter the patient was taken off the study and considered to have failed to respond. Patients who entered a CR were scheduled to receive consolidation treatment with PSC-MEC. Toxicity was assessed as per the Leukemia Common Toxicity Criteria.

Measurement of Response

Complete remission (CR) required adequate marrow cellularity, with < 5% blastic cellularity documentation at pre-consolidation evaluation, no peripheral blasts, ANC (1500 mm^3 platelets $\geq$ 100 000 and no evidence of extramedullary leukemia. Partial remission (PR) required all criteria as in CR except that the bone marrow may contain 5-25% blasts, or the bone marrow has < 5% blasts, but the peripheral counts do not meet CR criteria. Failure consisted of refractory leukemia at time of the pre-consolidation marrow or death during/following induction therapy (pre-consolidation).

MDR-1 Analysis of Blasts

Leukemic blasts from bone marrow or peripheral blood were evaluated. Immunophenotype analysis utilized the 4E3.16 anti-P-glycoprotein antibody [13]. For MDR function the rhodamine-123 efflux assay was used and modulation of rhodamine efflux by PSC-833 was evaluated.

Pharmacokinetic Evaluation

Patients had venous blood drawn prior to the start of PSC-833 infusion with subsequent levels evaluated on day 3 and day 5 just prior to the mitroxantrone dose.

Results

Twenty five patients were entered into the trial and all patients are evaluable for response, with the following pretreatment characteristics: early relapse (n = 8), $\geq$ (second relapse (n = 5), secondary/post MDS-MPD (n = 6), refractory AML (n = 4) and relapse-post BMT (n = 2). The median age was 54 yrs (27-70 yrs) with 15 males and 10 females. Of the 19 patients with de novo AML all had first CR durations of < 12 months and 14 had first CR durations of < 6 months. Twelve patients had 1-4 prior salvage treatments with only 4 patients responding to these therapies. Six of the 19 patients had unfavorable cytogenetics. In the group with an antecedent hematologic disorder, 5 of 6 had unfavorable cytogenetics.

Chemotherapy Effects

Cohort I consisted of the first 6 patients. All 6 achieved marrow hypoplasia, however there were 3/6 early deaths (i.e., within 30 days) due to severe mucositis and infection, with prolonged marrow hypoplasia. Therefore for Cohort II, the doses of M and E were reduced 20% as described in the Methods section. Nineteen patients received Cohort II doses, which were well tolerated. Overall, 22 of 25 patients achieved adequate hypoplasia (one bone marrow was not evaluated). Fifteen patients required one cycle and 10 patients required two cycles of chemotherapy.

Responses

Overall 7 CRs and 3 PRs were achieved, and 15 patients failed therapy. The responses/failures were evenly distributed between the diagnostic subgroups and within both cohorts. Three of 10 patients > 60 years achieved remissions (2 CRs, 1 PR). Five of 7 patients with CR remain alive with a median survival of 7 months (2.5-11 months); their median remission duration was 3-8 months (0.6-10 month). The median survival of the PR group is 6.3 months (0.6-7.6 months). During the hypoplastic period, 8 deaths were noted: 7 were secondary to infection (Cohort I -3 /6 patients, Cohort II - 4 /19 patients) and 1 was due to an arrhythmia. Six patients failed due to leukemic regrowth resistance and one patient had refractory leukemia.

Toxicity

Grade 3 or 4 mucositis was reported in 3 of 6 patients in Cohort I and 3 of19 patients in Cohort II. Other significant toxicity included 1 patient with mental status changes and 1 patient with neuropathy. No significant ataxia occured. 19 patients had transient hyperbilirubinemia > 1.5 mg %, most likely related to PSC administration. Eight deaths were noted as described above.

Results of MDR-1 Analysis

P-gp positive blast cells evaluated by using the monoclonal antibody 4E3.16 [13] ranged from 1-77% (median 35%). Of 15 patients evaluated, 11 demonstrated rhodamine-123 efflux, which was inhibitable by PSC-833 in 7 of these 11 patients. The level of 4E3.16 positively correlated with the inhibitability of rhodamine-123 efflux by PSC-833, rather than efflux activity per se.

Pharmacokinetics

Target levels (>1000 ng/ml) of PSC-833, which are capable of in vitro MDR-1 modulation, were achieved in all 10 patients evaluated over 15 cycles and were maintained for the 5 days of treatment.

Discussion

Among the best understood mechanisms of drug resistance is mediated by the multidrug transporter P-pg and encoded by the MDR-1 gene [14, 15, 16].Cells which express MDR-1 are cross resistant to several important antileukemic drugs including daunomycin, mitroxantrone and etoposide. Cyclosporine in high doses is one agent which has been used in clinical trials attempting to reverse MDR [11]. PSC-833 is two- to tenfold more potent than cyclosporine in modulating MDR in vitro and in animal models [17]. Because we anticipated substantial pharmacokinetic interaction between PSC-833 and the MDR-related agents used for leukemia treatment, we initially used significant dose reductions of etoposide and mitroxantrone in this trial compared to the doses used with these agents without PSC [18] and with cyclosporin plus MEC (E491) [19].

In spite of this reduction in the M and E doses the initial 6 patients treated (cohort I) had significant toxicity with Grade 3 mucositis in 3 patients, 3 early deaths and prolonged neutropenia in 5 patients. This required a further 20% dose reduction in mitroxantrone and etoposide for cohort II doses of PSC-MEC. This latter regimen has been capable of achieving adequate marrow hypoplasia and was relatively well tolerated, even in elderly patients.This dosing schedule is being confirmed for the remainder of this study. Adequate blood levels of PSC were achieved. Detailed analysis of pharmacokinetic interactions with M and E are still pending.

The MD Anderson group [20] has recently proposed placing poor risk AML patients into four categories which correlated response to treatment with presenting clinical features [20]. Patients were categorized as being in one of four prognostic risk groups, based on their background treatment history. Group 1 patients had expected CR rates of $\approx$ 70% (These were patients with an initial CR duration in excess of 2 years who were receiving their first salvage attempt). Group 2

had expected CR rates ≈ 40% (patients with an initial CR duration of 1 to 2 years who were receiving their first salvage attempt),- Group 3 expected CR rates were ≈ 10-20% (patients with a first CR lasting less than 1 year , or with no initial CR , who were receiving their initial salvage attempt) and Group 4 had expected CR rates of <1% (patients with an initial CR under 1 year, or with no initial CR , who were receiving a second or subsequent salvage regimen, having not responded to a first salvage attempt).

In the above context, categorizing relapsed patients into prognostic risk groups, most of our patients (19 of 25 individuals were in the very poor prognostic risk groups 3 and 4).

Our study demonstrated encouraging anti leukemic effects, even in these patients with poor prognosis, with 7 CRs and 3 PRs among the 25 patients.

Although MDR -1 is expressed in a minority of patients with AML at presentation, studies have reported 60-80% expression of this gene product in a relatively increased % of blasts from AML of patients whose disease was refractory or relapsed after chemotherapy [5, 6, 7, 9, 16, 21] In our study, a substantial number of patients (11 of 15 individuals) demonstrated increased levels of Pgp by flow cytometry, median 35% (1-77%) and the level of expression positively correlated with the inhibitability of rhodamine-123 efflux by PSC-833 (11/15 = 64%), rather than efflux activity per se. The number of patients evaluated for MDR-1 expression' are too small for clinical correlation.

Adequate blood levels of PSC were achieved and the drug doses were well tolerated. Detailed analysis of pharmacokinetic interactions are still pending but suggest that the current doses in Cohort II produce adequate marrow hypoplasia without undue toxicity.

The predictive value of Pgp as an independent marker for treatment failure in AML remains controversial as some studies show good correlation [5, 22] while others fail to do so [12, 19]. Evidence indicates that mechanisms other that Pgp contribute to the MDR resistance phenotype in human malignancies [23, 24, 25, 26]. A recent preliminary report of a randomized study using quinine as an MDR modulator plus intensive chemotherapy for AML post MDS and high risk MDS indicated that quinine increased the CR rate and survival in Pgp positive patients but not in the Pgp negative patients [27]. Another recently reported investigation [28] using PSC plus chemotherapy for relapsed/refractory AML has also shown encouraging results. Phase III randomized studies will be required to compare this regimen to regimens lacking MDR modulating therapy for determining the relative contribution of PSC-833 to clinical response in this setting. Such a study is currently being coordinated through ECOG.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Approaches to Overcome Multidrug Resistance: PSC and CdA/ara-C Combination Chemotherapy

G. JULIUSSON

Abstract. Multidrug resistance (MDR) is a potential major problem in acute myeloid leukemia (AML). Approaches to overcome MDR include pharmacological reversal of P-glycoprotein-mediated resistance, and use of effective drugs not affected by MDR, such as nucleoside analogues. Drugs that confer reversal of MDR in vitro include verapamil and cyclosporin A. We have analysed concentrations of anthracyclins and their metabolites within leukemic cells in vivo during continuous infusion of doxorubicin, daunorubicin, and idarubicin, respectively, and documented an elevated steady state level of active metabolites during concomitant treatment with verapamil, and cyclosporina A (CyA).

In a Scandinavian phase I/II study, the CyA analogue PSC833 was given together with daunorubicin and cytosin arabinoside (ara-C) to patients with refractory or relapsing AML to assess a feasible dose level of PSC and daunorubicin. The overall CR rate in 41 patients during the dose finding part of the study was 27%. Patient recruitment into the phase II part continues using a PSC dose of 10 mg/kg/day on days 1-4, and daunorubicin 45 mg/sqm/day on days 1-3, with ara-C 1 g/sqm/2h q 12 h, days 1-4.

2-Chlorodeoxyadenosine (Cladribine, CdA) is a purine analogue with a documented single-drug activity in de novo and relapsed pediatric AML. In analogy with the studies on fludarabine/ara-C combinations, we evaluated ara-CTP concentrations in AML cells during ara-C treatment with or without CdA pretreatment. Priming with CdA gave a 36% median increase in ara-CTP concentrations. Thirty patients (AML n = 21, ALL n = 4, MDS n = 1, MDS-AML n = 4; previously untreated n = 20, previously treated n = 10, 4 of them with relapse following stem cell transplants), aged 20-76 years (median 47 yrs), were given a combination of CdA, ara-C and idarubicin. The overall CR rate from one course was 73%, and among previously untreated patients 85%. Toxicity was limited, and recovery from thrombocytopenia (> 50) and neutropenia (> 0.5) was prompt and occurred on day 21 and 23 from start of treatment (median), respectively. The projected 3-year survival of the 20 previously untreated cases is 70% (with 4 autotransplants, and 6 allogeneic stem cell transplants during follow-up). CR rate and overall survival in refractory patients were not as encouraging.

Introduction

MDR is a well-known clinical problem in relapsing AML, and overexpression of the transmembrane multidrug transporting P-glycoprotein (Pgp) is common (reviews in [1, 2]). However, in primary AML, Pgp expression is less common, but the frequency is increased with poor-prognosis features, such as poor-risk cytogenetics, high age, and prior hematologic disorder. Pgp-expression

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is also linked with the expression of the stem cell marker CD34, perhaps indicating a physiological role of MDR in hematopoietic stem cells. Thus, MDR is likely to be an important issue also in de novo AML, since relapse may well be caused by the emergence of MDR-positive subclones present already at diagnosis. Additional non-Pgp-related proteins resulting in MDR are also reported, such as the multidrug resistance-related protein (MRP) and lung resistance protein (LRP).

Approaches to overcome MDR may include a pharmacological reversal of MDR, and the use of effective chemotherapeutic agents not affected by MDR. In the present report the first approach will be elucidated by

1. the influence of MDR-reversing drugs on intracellular pharmacokinetics of anthracyclins and their active metabolites in leukemia cells in vivo, and
2. preliminary data from a Scandinavian dose-finding study using cyclosporin-analogue PSC833 in the treatment of refractory or relapsing AML.

The second approach will be exemplified by phase I and phase II studies of combining 2-chlorodeoxyadenosine (cladribine, CdA) with cytosin arabinoside (ara-C) in the treatment of myeloid and lymphoid leukemias.

Several Chemically Different Drugs are Shown to be Affected by MDR In Vitro

These usually have a natural origin, and may be described as small nitrogen-containing hydrophobic molecules. Common chemotherapeutic drugs are included in this group, such as the anthracyclins (doxorubicin, daunorubicin, idarubicin) and similar compounds (amsacrine, mitoxantrone), the vinca alkaloids (vincristine, vinblastine), the epipodophyllotoxins (etoposide, teniposide), and taxol. In contrast, alkylating agents, cisplatin, and antimetabolites are regarded as unaffected by Pgp.

Likewise, several chemically different drugs, preferrably lipophilic molecules, have the ability to block Pgp-mediated MDR. This group includes the calcium-channel blockers (verapamil, nifedipine), calmodulin inhibitors (prochlorphenazine, fluphenazine), antibiotics (erythromycin, tetracyclines), and immunosuppressive drugs (cyclosporin A, FK506). Many of these drugs have been used clinically with the aim to reverse MDR. A significant problem however, is other pharmacological effects since high plasma concentrations usually are required for MDR reversal. For example, patients had to be monitored in coronary care units during chemotherapy plus high-dose verapamil. Thus, analogues with the same (or improved) MDR modulating capacity but less other effects have been developed, such as dexverapamil, and the cyclosporin analogue PSC833.

Another problem when designing clinical studies with MDR modulators is that they may alter Pgp expression in biliary canaliculi and renal tubuli, which blocks chemotherapy drug elimination and increases plasma concentration, leading to an increased toxicity unless dose is adjusted. Since normal hematopoietic CD34+ stem cells express Pgp, a potential problem is that MDR modulators may aggravate toxicity of chemotherapy to normal stem cells.

CdA/ara-C Drug Combination

Cytosin arabinoside is an anti-metabolite that is the backbone of treatment for AML. The nucleoside is incorporated in the leukemic cells, and through the action of the enzyme deoxycytidine kinase (dCK) the nucleoside is phosphorylated to the active nucleotide, the triphosphorylated ara-CTP. In studies by Gandhi et al. [3], it was shown that the purine analogue fludarabine, if given before ara-C, leads to an increased area-under-the curve (AUC) for ara-CTP in leukemic cells, indicating a synergistic action based on pharmacological interaction. Subsequently, clinical studies have evaluated the effects of the drug combination fludarabine + ara-C with or without G-CSF in AML.

Fludarabine has a cytotoxic effect against AML cells, however, to be effective highly toxic dose levels have to be used [4]. In clini-

cally used doses, fludarabine is ineffective by itself in AML. In contrast, another purine analogue, CdA, has a documented single-drug activity in relapsed [5] and de novo [6,7] pediatric AML, in doses that may be given without significant toxicity (except for short-term cytopenia), i.e., 8.9 mg/sqm for 5 days. Of interest is that this CdA dose was sufficient for eradication of CNS leukemia [6], a finding that was predicted from our previous evaluation of CdA drug levels in the cerebrospinal fluid [8].

The structural similarity between fludarabin and CdA, also including mechanism of action, raised the hypothesis that CdA might also be capable of augumenting the ara-CTP concentration in leukemic cells, and provided us with the basis for testing the CdA + ara-C combination.

Materials, Methods and Results

Anthracyclin Drug Concentrations in Leukemic Cells in Vivo

Treatment with MDR modulating drugs may increase the concentration of anthracyclins and their metabolites in leukemic cells in vivo. We treated a patient with an aggressive leukemic relapse of centroblastic/centrocytic lymphoma following an autologous bone marrow transplantation. Treatment was given with CVAD, including a continuous infusion of doxorubicin 9 mg/sqm/day over 4 days. During the doxorubicin infusion of the first course a 6-hour infusion of verapamil was added. During the second course, oral verapamil was given together with three injections of cyclosporin A. As shown in Fig. 1 (from Tidefelt et al. Eur J Haematol 1994; 52: 276, reprinted with the permission of the publisher), there was a steep increase in the doxorubicin concentration during infusion of MDR modulators as compared to the previous steady state level [9].

We have also performed similar studies in vivo analysing daunorubicin and idarubicin with metabolites in leukemic cells during continuous infusions with and without CyA 10 mg/kg/day, or PSC833, 10 mg/kg/day through continuous infusions.

Scandinavian Phase I/II Study of PSC833 in Refractory AML

A clinical study with the cyclosporin A-analogue PSC833 is currently ongoing. In the first part, feasible doses of PSC and daunorubin were to be established. We treated 41 patients with AML that was resistant to two or more regimes, or with relapse within 9 months, or with second or subsequent relapse, or with relapse following autologous or allogeneic bone marrow transplantation, or with secondary leukemia [10].

In the dose-finding study, all patients received ara-C, 1 g/sqm iv during 2 h twice daily for 4 days. Daunorubicin was given by bolus injection on days 1-3 in the doses of 25.3 or 33.75 or 45 mg/sqm in different patient cohorts. PSC was given in a loading dose of 2 mg/kg in 2 h day 1, followed by a continuous infusion of 2 or 4 or 6 or 8 or 10 mg/kg/day on days 1-4. Dose-limiting toxicity (DLT) was defined, including neutropenia more than 28 days (subsequently prolonged to 42 days), hepatic toxicity more than grade III, or other toxicity (except alopecia) more than grade II. Five patients had DLT: two died in aplasia, two had prolonged neutropenia, and one developed hepatic failure. At the PSC dose of 10 mg/kg the mean plasma level during infusion was about 3500 ng/ml. The CR rate was 5/20 (25%) among primary refractory patients, 3/14 in those with early relapse, 2/3 in second or subsequent relapse, and 1/3 in relapse following BMT. Overall CR rate was 11/41 (27%), and 4 patients have continuing CR after 8-23 months [10].

The highest dose level with daunorubicin 45 mg/sqm and PSC 10 mg/sqm was found to be feasible, and the study continues as a phase II trial on this dose level.

CdA/ara-C Combination

Initially, we treated nine patients with advanced myeloid leukemia. There were 4 patients with relapsed/refractory AML, and five with chronic myeloid leukemia in blastic crisis. CdA 8.9 mg/sqm was given by 2-h infusions for 1 up to 5 days, with or without ara-C 1 g/sqm/2 h twice daily. Blood leukemia cells were analysed for CdA and ara-C

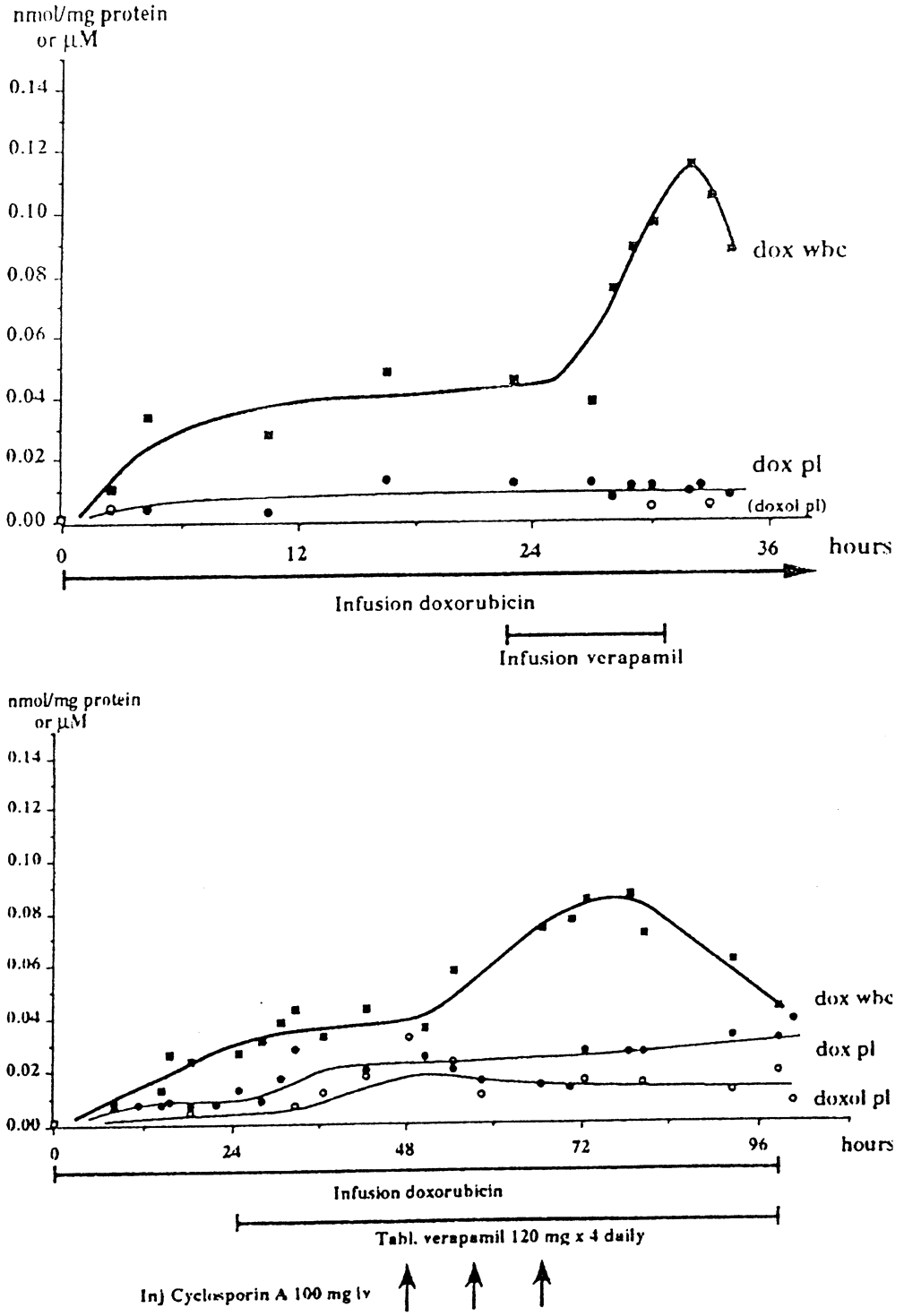

Fig. 1. Intracellular concentrations of doxorubicin during continuous infusion of doxorubicin during two separate courses given to a patient with a leukemic relapse of lymphoma. Verapamil and Cyclosporin A were added as shown

nucleotides by HPLC. There was a very rapid elimination of circulating leukemic cells in all patients with limited toxicity. However, no durable responses were seen. As previously presented [11], CdA nucleotides in leukemic cells had an AUC of 150 ± 83 μM·h (n = 8), and the half-life was 9.0 h ± 3.6 h. The median AUC of ara-CTP was 751 μM·h following the first dose (n = 5), and we found a median increase in ara-CTP-concentration of 36% when the ara-C infusion was preceeded by CdA. The median ara-CTP half-lives were 2.7 h and 4.1 h, respectively [11]. Subsequently, we proceeded with a phase II design. Patients with myeloid or lymphoid leukemias, either newly diagnosed or previously treated, were given CdA + ara-C + idarubicin (CCI regime). Cytosin arabinoside was given in a 1 g/sqm/2 h twice daily for 4 days schedule. CdA was given either as 5 mg/sqm/1 h every 12 h for 4 days preceeding the ara-C doses, or 8.9 mg/sqm/2 h preceeding one of the two daily doses of ara C. Idarubicin was given at a dose of 10 mg/sqm/1 h infusion/day for 2 consecutive days. We included 21 patients with AML (15 newly diagnosed, one of them with AML secondary to chemotherapy for non-Hodgkin's lymphoma), 4 patients with ALL (2 newly diagnosed), 4 patients with AML preceeded by myelodysplasia (MDS) (3 previously untreated), and 1 pretreated patient with MDS type 5. Four patients (1 ALL, 3 AML) had relapsed from bone marrow transplantation (1 allogeneic, 3 autologous). The ages ranged from 20 to 76 years (median 47 yrs).

The outcome of the first CCI course is shown in Table 1. Complete remissions were seen in one patient with a Philadelphia-positive ALL who failed two courses of induction combination chemotherapy, in one patient with AML relapsing following autologous transplantation, as well as in one elderly patient with MDS-AML refractory to two courses of TAD. Recovery from cytopenia was prompt in all responding patients. Untransfused platelet counts of more than 50 x 10^9/l were found on day 21 from start of CCI treatment (median, range 16-32), and neutrophil counts of more than 0.5 x 10^9/l were found on day 23 (range 17-43) (Fig. 2). Previously treated patients and patients with MDS-AML had a somewhat slower platelet recovery than newly diagnosed patients (24 vs. 21 days) or patients with AML or ALL (26 vs. 20 days); as regards neutrophils there was no difference. There was a median of 6 days with fever of more than 38 °C (range 0-14), and 17 days of intravenous antibiotics (range 6-31). Nausea and vomiting was minimal. However, fever during chemotherapy infusion without documented infection was common, and extensive skin rashes were frequently seen during the week following chemotherapy. Ten patients received a second CCI-course, in some cases with abbreviated doses. Platelet recovery to more than 50 x 10^9/l was delayed until day 35 (range 26-40) following the second course; however, bleeding was not a problem, and there was no increased need for platelet transfusions (3 infusions of pooled platelets per course) since the time to recovery of platelet counts to more than 20 was unchanged. Thirteen patients had their remissions consolidated by stem cell transplantations (5 autologous, 8 allogeneic). Autologous stem cell harvest was achieved using G-CSF following consolidation with daunorubicin 45 mg/sqm for 2 days and cytosin arabinoside 200 mg/sqm/day as continous infusion for 5 days. One to three stem cell harvests were required to achieve 2x 10^6 CD34+ cells/kg.

Table 1. Results of CCI therapy

		CR rate
ALL	Untreated	2/2
	Relapse/refractory	1/2
AML	Untreated	12/15 (80%)
	Relapse/refractory	2/6
MDS-AML	Untreated	3/3
	Refractory	1/1
MDS	Pretreated	0/1 (partial response)
Total		22/30 (73%)
Total untreated		17/20 (85%)
Total pretreated		5/10 (50%)

Overall survival for AML-patients according to previous therapy is shown in Fig. 3. Overall survival for all patients, with those who subsequently underwent stem cell transplant censored at time of transplant, is shown in Fig. 4.

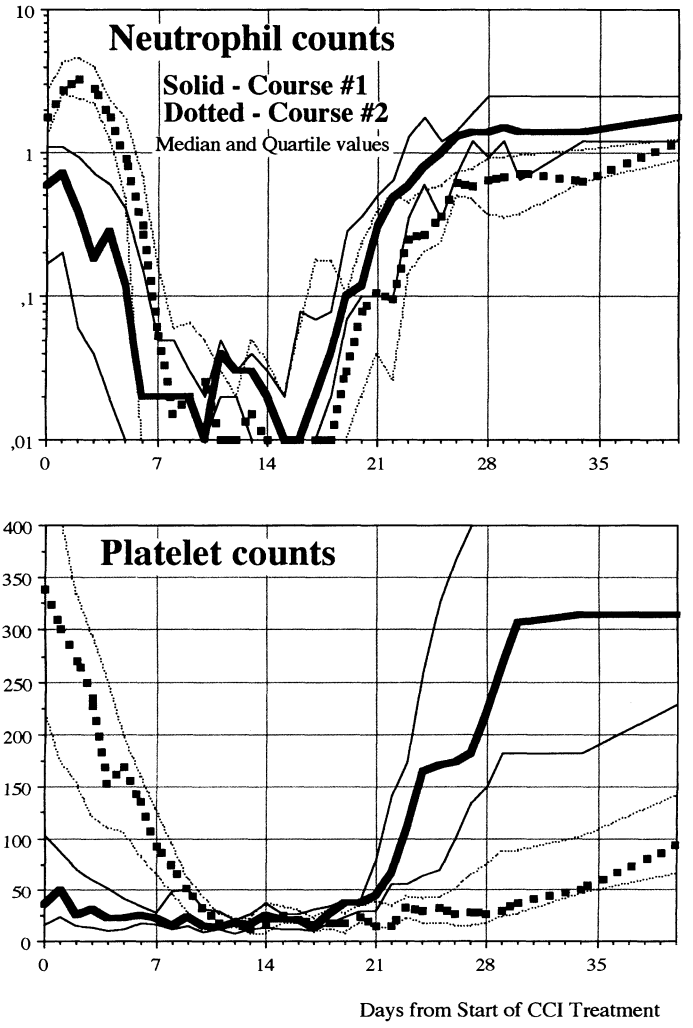

Fig. 2. Median and quartile values of neutrophil and platelet counts (x 10^9/l) during course #1 and course #2 of CCI (CdA + ara-C + idarubicin) chemotherapy to patients with acute lymphoid and myeloid leukemias

Discussion

MDR-modulating drugs have previously shown to increase concentrations of chemotherapeutic drugs within tumor cells in vitro, and also induce increased cell kill in vitro. We here present data showing that increased concentrations of clinically important anthracyclins through treatment with different MDR modulating drugs can be achieved also in vivo during clinical conditions. This is a prerequisite for further clinical studies. In a Scandinavian phase I dose escalating study, it was shown that it was feasible to give daunorubicin 45 mg/sqm/day for 3 days, PSC833 10 mg/kg/day for 4 days with intermediate dose cytosin arabinoside for 4 days to adult patients with refractory/relapsing acute myeloid leukemia. Complete responses were seen during the dose escalation phase. The study now proceeds with a phase II trial on the established dose level.

Our clinical study on the in vivo pharmacokinetic interaction between CdA and ara-C showed that CdA potentiates metabolism of ara-C in leukemia cells similar to fludarabine. There was a median increase of 36% in ara-CTP concentration when ara-C was preceeded by a bolus infusion of CdA [11]. This finding was subsequently confirmed and documented also when CdA was given through a continuous infusion [12]. How-

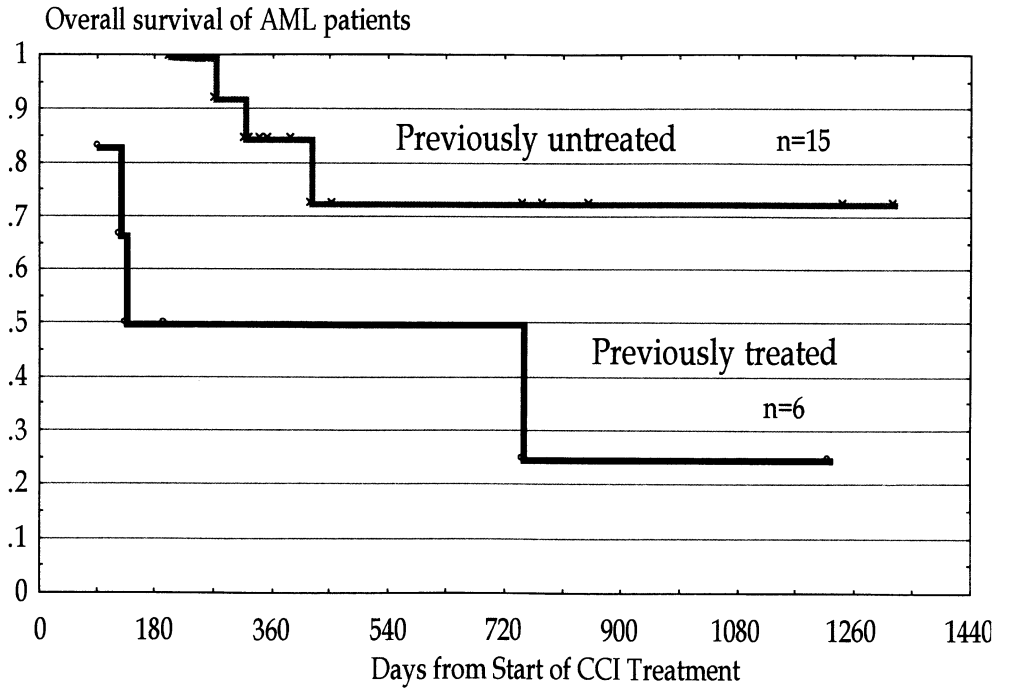

Fig. 3. Overall survival of AML patients from start of CCI chemotherapy (n = 21)

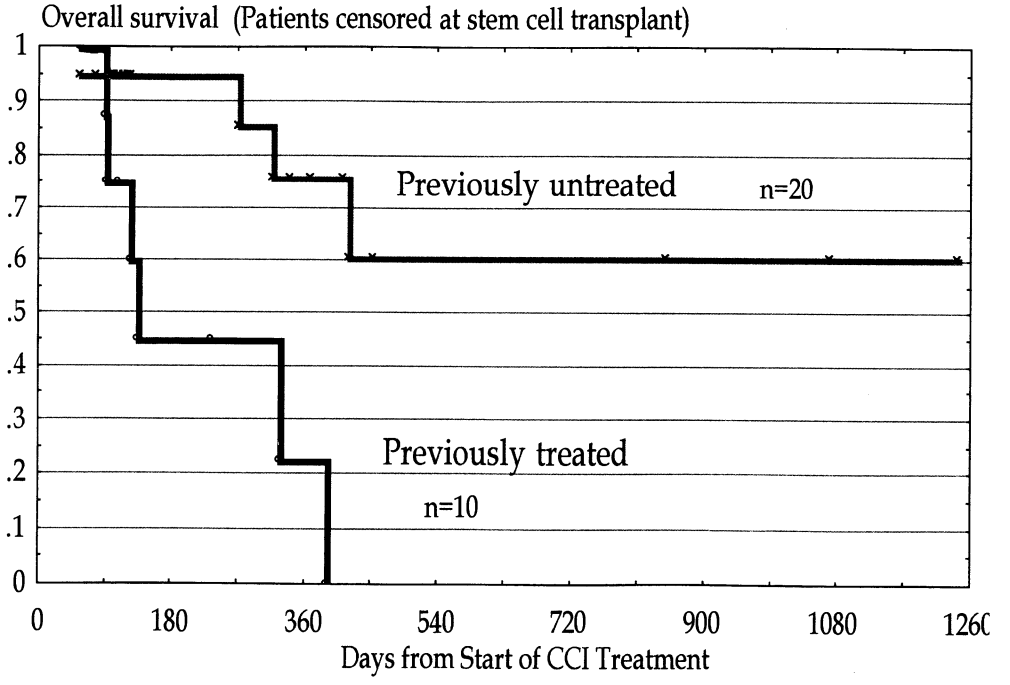

Fig. 4. Overall survival of all patients from start of CCI chemotherapy (n = 30). Patients subsequently entering autologous or allogeneic stem cell transplant were censored at transplant

ever, a major synergistic effect between these two drugs might well be on the level of nucleic acids rather than solely through a pharmacokinetic interaction. The response rate in our study was encouraging, although we could not state for sure that similar results would not have been achieved also through an ara-C + idarubicin combination without CdA. However, the idarubicin dose was fairly modest, leading to a low level of nausea and vomiting. Furthermore, the recovery from cytopenia was prompt and rapid. Many patients had a clear-cut thrombocytosis following the first course, and a reactive increase of blasts was a common bone marrow finding early in the recovery, necessitating a second marrow aspirate to assess complete remission. This was in many cases also documented by remission cytogenetics and flow cytometry.

However, the therapeutic results in refractory cases and leukemia with poor-risk cytogenetics were less encouraging, similar to what previous investigators have found [7, 13, 14].

If the response rate of the CCI combination is unrelated to MDR expression is not known. However, CD34 is known to correlate with Pgp expression, and we did not see an adverse effect on prognosis by the CD34 marker.

In conclusion, in vivo data of MDR modulators indicate that a beneficial clinical effect might be expected. Such data however has to come from large-scale clinical studies. The CCI regimen has been a feasible way to induce rapid complete remissions, with quick recovery from cytopenia and limited toxicity, primarily in standard-risk acute leukemia and MDS-AML. Occasionally, patients refractory to standard chemotherapy protocol achieved complete remissions. We therefore find that this drug combination is worth further clinical testing.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Zidovudine Induces Resistance to Antineoplastic Agents and Alterations in Apoptosis in T-Cell Lymphoma Cells

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Abstract. Prolonged treatment of leukemic cell lines with antiretroviral agents such as nucleoside analogues may result in the development of cell resistance against antiviral agents. We tested whether prolonged treatment of H9 cells (T-cell lymphoma cell line) with 3′-azido-3′-deoxythymidine (AZT) interferes with sensitivity to antitumor agents commonly used for treatment of AIDS-associated malignancies. H9 cells grown for more than 2 years in medium containing 250 μM of AZT were at least 100-fold less sensitive to cytotoxic effects of AZT than parental H9 cell line. These cells designated $H9^rAZT^{250}$ were 5- to 20-fold less sensitive to toxic effects of antitumor agents including cisplatin (CP), vincristine (VIN), doxorubicin (DOX) and etoposide (VP-16). The resistance to these drugs was associated with inhibition of apoptosis as demonstrated by the terminal deoxynucleotidyl transferase-mediated nicked-end labelling (TUNEL) assay and DNA fragmentation assay. The expression of genes involved in regulation of apoptosis such bcl-2 was enhanced in $H9^rAZT^{250}$ cells. The results demonstrate that prolonged treatment of tumor cells with AZT may result in the development of resistance to antineoplastic agents due to the inhibition of apoptosis.

Introduction

Infection with human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immune deficiency syndrome (AIDS), predisposes to neoplastic conditions. As patients survive longer, due to improved prophylactics and management of underlying opportunistic infections, the number of HIV positive patients presenting malignancies will continue to increase. The tumours which occur with an increased frequency in HIV-infected individuals include non-Hodgkin lymphoma, Kaposi's sarcoma and also intraepithelial cervical neoplasma and anal neoplasia and possibly seminoma [1, 2]. Cytotoxic therapy used in patients with AIDS may be influenced by different factors which are not found in patients without HIV infection. For example, most of HIV-infected individuals with malignant disease undergo long-term therapy with antiretroviral agents [3]. Thus impact of antiretroviral therapy on effects of antitumor agents in malignant cells should be studied.

Various 2′,3′-dideoxynucleoside analogs have been reported to inhibit selectively the replication of HIV-1 [4, 5]. These compounds act as chain terminators of the HIV-1 reverse transcriptase reaction following intracellular phosphorylation by cellular ki-

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nases to their triphosphates [6]. Selective antiviral activity of nucleoside analogues is gained from the relative inability of human cellular DNA polymerases to use their triphosphates as a substrate. However, some incorporation of residues of nucleoside analogues into human cellular DNA does occur which accounts for the cytotoxicity of the drug [7]. It is a common feature of eucariotic cells to develop different mechanisms to escape deterious effects of cytotoxic drugs. Tumour cell populations in patients who undergo antineoplastic chemotherapy develop readily multidrug resistant mechanisms which account for resistance to different chemical drugs [8]. It is possible that nucleoside analogues used for treatment of HIV infection induce resistant mechanisms in tumour cells in AIDS patients.

In the present study we observed whether long-term incubation of human lymphoid H9 cells with anti-HIV agent zidovudine (3′-azido-3′-deoxythymidine, AZT) influences their sensitivity to antitumor therapy. We also studied whether long-term treatment of H9 cells with zidovudine influences expression of genes involved in regulation of cell sensitivity to toxic agents.

Material and Methods

Cells

$H9^rAZT^{250}$, $H9^rDDC^{500}$ and $H9^rARA\text{-}C25^{250}$ resistant sublines were established by cultivation of parental H9 cells (T-lymphoma) in medium containing increasing concentrations of drugs. The cells grow now over a period of more than two years in medium containing 250 μM AZT, 500 μM DDC and 250 μM ARA-C respectively. All cells lines were routinely cultivated in IMDM supplemented with 10% foetal bovine serum, in 5% CO_2.

Antitumoral Agents

All the drugs were obtained from Sigma (Deisenhofen, Germany). Stock solutions of doxorubicin and etoposide (VP-16) were dissolved in dimethyl sulfoxide, vincristine sulfate and cisplatin in culture medium. Prepared solutions of these drugs were stored at -20 °C.

Cytotoxicity Assay

The cytotoxic effects of drugs were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrasolium (MTT) method, as previously described [9]. Briefly, 10^5/ml cells were seeded in a 96-well plate containing different concentrations of testing agents in IMDM-medium, supplemented with 10% FCS. After 5 days incubation 2% MTT solution was added to each well and viability of the cells was measured with determining the absorbency by multiwell ELISA reader.

Measurement of Apoptosis

Morphological features of the cells and DNA-fragmentation were investigated with Hoechst-33258 staining [10]. To quantify the amount of apoptosis, TUNEL-(terminal deoxynucleotidyl transferase (TdT^2) mediated UTP-fluorescent nicked-end labelling) method was used [11]. DNA-strand breaks were also quantified by ELISA DNA-fragmentation assay, based on binding of specific biotin labelled antibodies to DNA-histon complexes. The expression of gene bcl-2 has been detected with the fluorescein-conjugated monoclonal antibody (Hölzel Diagnostica) directed against Bcl-2 protein [12]. Flow cytometry for quantification of DNA-strand breaks and Bcl-2 was performed on a FACscan flow cytometer (Becton Dickinson). The data from 1×10^4 cells were analyzed using program Lysys II.

Results

The all cell sublines proliferated to a similar extend as parental H9 cells. The cytotoxicity of doxorubicin, etoposide, vincristine, cisplatin in H9 parental cell line and $H9^rAZT^{250}$, $H9^rDDC500$, $H9^rARA\text{-}C^{250}$ resistant cell lines is demonstrated in Fig. 1. The $H9^rAZT^{250}$ cells showed 5- to 20-fold lower sensitivity to cytotoxic effects of all drugs, when compared with H9 parental cell line. Both $H9^rDDC^{500}$ and $H9^rARA\text{-}C^{250}$ sublines

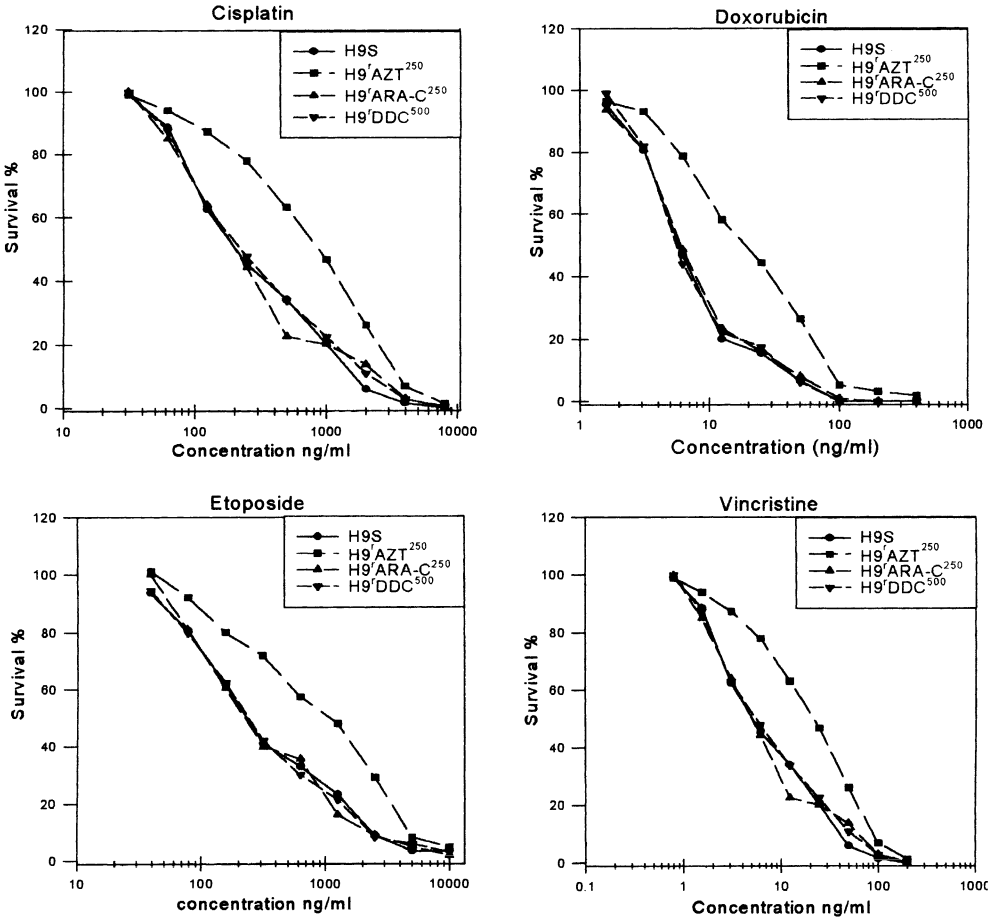

Fig. 1. MTT values of H9 parental, H9^{r}AZT^{250}, H9^{r}ARA-C^{250} and H9^{r}DDC^{500} cell lines

showed similar sensitivity to these agents as parental H9 cells.

H9 parental cells showed the morphological features associated with apoptosis induced by cisplatin while H9^{r}AZT^{250} were not significantly influenced by this drug. Figure 2 shows chromatin abnormalities (condensation and fragmentation), cell shrinkage, which were significantly more expressed in H9 parental cells.

Amount of DNA-breaks, induced by cisplatin was up to 3-fold greater in H9 parental than in H9^{r}AZT^{250} cultures (Fig. 3). Bcl-2 expression was significantly greater in H9^{r}AZT^{250} than in H9 parental cells (Fig. 4). As measured by FACs, the mean values were 47.13 $\pm$ 3.2 and 102 $\pm$ 6.7 for H9 parental and H9^{r}AZT^{250} cells, respectively.

Discussion

In the present study we demonstrated that prolonged treatment of human lymphoid cell line H9 with AZT resulted in resistance to cytotoxic effects of antitumor agents commonly used for treatment of AIDS-related lymphomas and other malignancies. Moreover, we demonstrated that prolonged incubation of H9 cells with AZT was associated with increased expression of apoptosis preventing bcl-2 gene. To our knowledge this is the first report dealing with overexpression of bcl-2 protein following AZT treatment. Interestingly, prolonged cultivation in the presence of other nucleoside analogues such as ARA-C or DDC did not induce resistance to apoptosis in H9 cells.

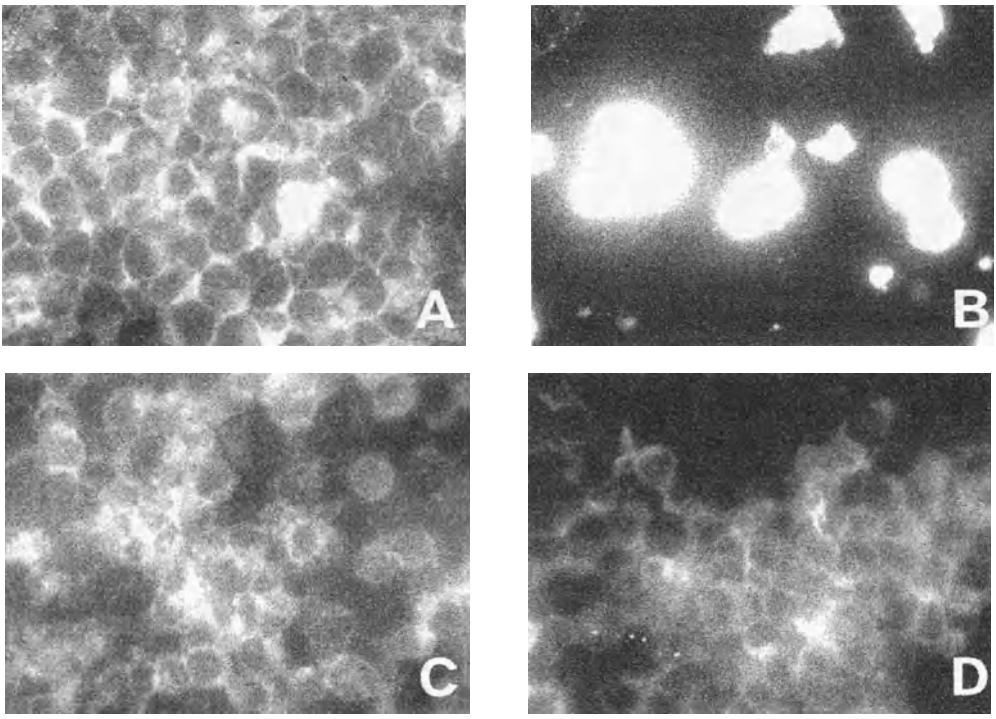

Fig. 2. H9 parental *(a, b)* and H9^{r}AZT^{250} *(c, d)* before *(left)* and after incubation with cisplatin *(right)*. Hoechst 33258 staining

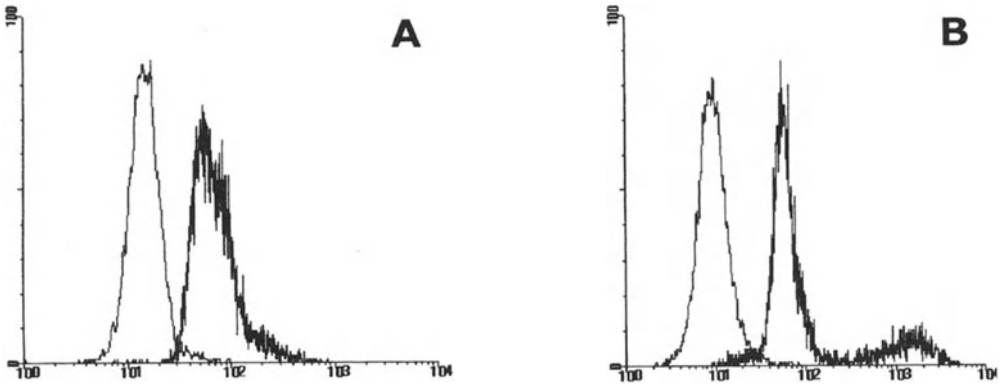

Fig. 3. Induction of DNA breaks by cisplatin in H9^{r}AZT^{250} *(A)* and H9 parental *(B)* cells, measured by TUNEL-assay

A variety of antitumor agents exert their effects due to inducing of apoptosis [13]. The loss of ability of tumor cells to undergo apoptosis is frequently associated with a treatment failure and/or cancer progression [14]. Bcl-2 is an important protein exerting a protective effect on cell death [15]. It may function as a "life-saving" protein to determine the thresholds of apoptosis in different kinds of cells [16, 17]. Moreover, the cells with overproduction of bcl-2 protein become resistant to wide variety of chemotherapeutics, radiation and immune effector mechanisms which result in cell distribution

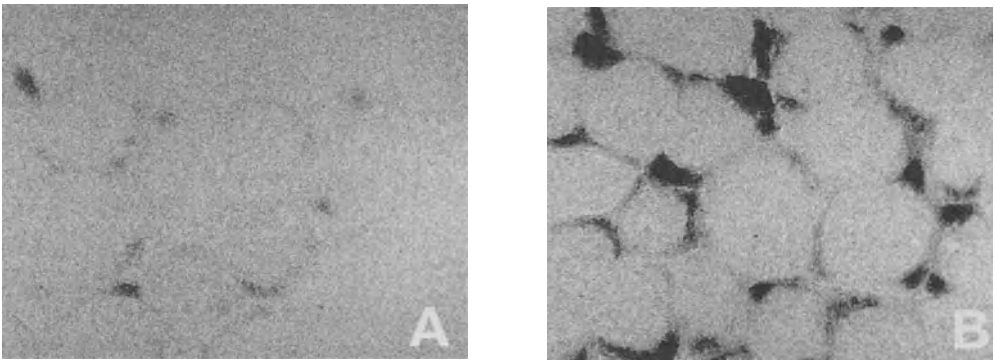

Fig. 4. Bcl-2 expression in H9 parental *(A)* and $H9^{r}AZT^{250}$ *(B)* cell lines

[18]. It is possible that bcl-2 overexpression accounts for resistance to apoptosis in H9 cells. On the other hand, expression of genes such as p 53 or c-myc could be envolved in the regulation of cell death in H9 cells.

In conclusion, our results suggest that AZT treatment of lymphoma cells may lead in resistance to antitumor agents. Increased expression of bcl-2 gene could account not only for resistance to antineoplastic drugs but also contribute to increased malignity by disturbing pathways controlling programmed cell death.

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Bcl-2 expression selectively prolongs survival of growth factor deprived hemopoetic cell lines. J Immunol 144: 3602-3614
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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Idarubicin Activity Against Multidrug-Resistant (mdr-1+) Cells is Increased by Cyclosporin A

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Abstract. Multidrug resistance related to functional overexpression of P-170 glycoprotein (mdr-1 gene) is often responsible for treatment failure in acute leukaemia. Attempts to restore drug sensitivity with revertants and less vulnerable drugs are underway. We compared the ability of cyclosporin A to modulate mdr-1 resistance of T-lymphoblastic CEM cells to daunorubicin and idarubicin. To obtain clinically useful informations, experimental conditions reproduced partially in vivo pharmacology (drug peak plasma levels, alcohol metabolites, exposure times) of a single intravenous bolus with daunorubicin 45 mg/m^2 or idarubicin 10-12 mg/m^2, plus cyclosporin A 16 mg/kg/d given as continuous infusion (List schedule). Study methods were cytofluorimetry for detection of anthracycline early uptake, retention and pro-apoptotic effects (binding to fluoresceinated annexin V) at the single cell level, and the standard MTT growth inhibition assay for cytotoxicity. The results showed greater drug uptake/retention and apoptotic rates with idarubicin than with daunorubicin, with a further increase by cyclosporin A. MTT results were in favour of idarubicin with or without cyclosporin A, and greatly influenced by cyclosporin A itself. Altogether, study results in mdr-1+ cells with idarubicin/idarubicinol at 100/20 ng/ml, corresponding to levels achievable in vivo with a single idarubicin dose $\geq$ 12 mg/m^2, were in the range of those obtained with standard-dose daunorubicin in mdr-1-cells. These findings underscore the potential usefulness of an idarubicin plus cyclosporin A combination in mdr-1+ leukaemias, and prompt further studies on associations with other modulators of P-170 functional activity.

Introduction

Daunorubicin (DNR) and idarubicin (IDA) act primarily as topoisomerase-II inhibitors [1]. Blocking of this nuclear enzyme, that regulates DNA topology during cell cycle, switches on programmed cell death or apoptosis [2]. Following rapid intravenous administration of DNR 45 mg/m^2 and IDA 10-12 mg/m^2, the mean peak plasma levels of both drugs reach approximately 200 ng/ml and 50 ng/ml, respectively. Corresponding intracellular levels are about tenfold higher, owing to active cell uptake and intracellular transport. Rapid liver metabolism releases alcohol metabolites daunorubicinol (DNRol) and idarubicinol (IDAol), at low concentration but long half-life, particularly IDAol (> 50 h) [3-5]. Interestingly IDAol is cytotoxic but DNRol is not or only minimally so. Among known resistance mechanisms to anthracyclines that correlate with treatment failure, the most studied is multi-drug resistance by functional overexpression of a 170 Da membrane P-glycoprotein (P-170 type MDR) [6]. P-170

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substrates are actively extruded from P-170+ cells and cannot exert their cytotoxic, pro-apoptotic effect. Although the superiority of IDA over DNR against P-170+ leukaemia was proved by more than one in vitro study, these experiments were carried out with very high drug concentrations [7, 8], very long drug incubation times [9, 10], or without the cytotoxic alcohol metabolites [7, 9]. Although these studies were informative as regards gross pharmacologic differences, they neither attempted to reproduce in vivo pharmacology of anthracyclines nor unequivocally demonstrated IDA superiority over DNR in P-170+ leukaemias. We undertook a comparative in vitro study using a P-170+ reference cell line and setting experimental conditions as close as possible to known clinical pharmacology of both IDA and DNR. The assessment of effects by the mdr-1 inhibitor cyclosporin A (CsA) was an integral part of this study.

Materials and Methods

CEM Cell Lines and Drugs

Human T-lymphoblastic CEM and vinblastine-resistant P-170-positive CEM-VBL cell lines (courtesy of Dr. L. Capolongo, Centro Ricerche Pharmacia, Nerviano, Italy) were grown in cell culture medium supplemented with 10% fetal calf serum, L-glutamine, penicillin and streptomycin in humidified 5% CO_2 at 37 °C. Cell concentration was maintained at 2-3 $\times$ 10^5 cell/ml. DNR, DNRol, IDA and IDAol were obtained from Pharmacia-Farmitalia Group (courtesy of Dr M. Grandi, Milan, Italy). 10 μg/ml stock solutions were stored at -80 °C. Working solutions were prepared by dilution in cell culture medium; DNR: 200, 400 ng/ml; DNRol 50, 100 ng/ml; IDA 50, 100 ng/ml; IDAol 10, 20 ng/ml. These concentrations correspond to peak (drug) and steady state (metabolite) plasma levels obtained in vivo with an intravenous bolus administration of DNR 45 mg/m^2 (230 ng/ml, DNRol 50 ng/ml) and IDA 10-15 mg/m^2 (50-100 ng/ml, IDAol 10-20 ng/ml), respectively [3-5]. Cells were incubated with drugs and metabolites for 30 min 12 h, respectively, to mimic the short and long plasma half lives of these compounds at the concentrations considered. Combined drug plus metabolite experiments were performed using the lower or higher concentrations of such, e.g. DNR 200 ng/ml plus DNRol 50 ng/ml etc., and incubating cells with the drug first and then, after two washings in medium, with the drug metabolite, for the reported times. P-170 blocking agent CsA was obtained from Sandoz Company (Bern, Switzerland) as vials for intravenous use and diluted in absolute ethanol and cell medium to a cellular ethanol < 0.1%. CsA was added 90′ before anthracyclines and maintained until end of exposure to drug/metabolite at a final concentration of 1.5 μg/ml, that is moderately toxic to patients and apparently able to affect P-170-mediated drug efflux in vivo [11].

Cytofluorimetry and Cytotoxicity Assays

Purified, fluorescein isothiocyanate (FITC)-labelled annexin V was used for the cytofluorimetric determination of apoptotic cells (Bender MedSystems, distributed by Prodotti Gianni, Milan, Italy) [12]. FITC-annexin V 2.5 μg/ml was added to cellular suspensions according to the manufacturer's technical recommendations. FITC-annexin V, and anthracycline ± CsA incorporation/efflux studies were performed according to published methods [12, 13] on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) equipped with an argon excitation laser light (488 nm wavelength) and 530/30 nm (FL1) and 585/42 nm (FL2) band pass filters. Cellular fluorescence of 1 $\times$ 10^6 cell/ml was determined using FL1 (FITC-annexin V) and FL2 (ANT) filters with use of the appropriate negative controls and fluorescence compensation. Results of drug/metabolite incorporation studies were expressed as fluorescence index (FI), corresponding to percent cellular fluorescence x mean fluorescence intensity of samples. All values were subtracted of negative control experiments. Cell death rates by anthracyclines ± CSA were measured by the standard MTT test [14]. Cells at

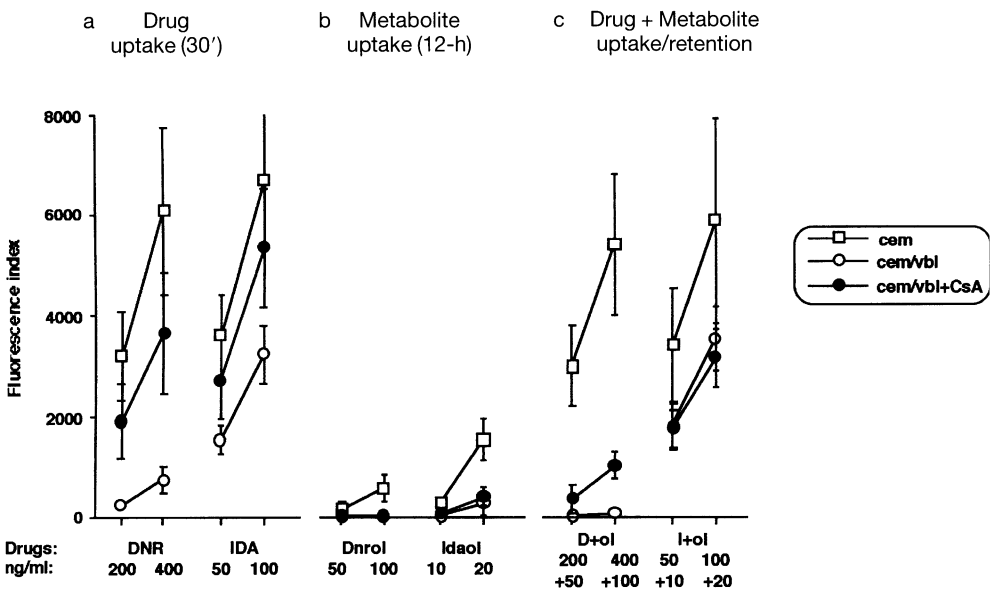

Fig. 1 a, b, c. FI from different drug concentrations ± CsA. *a* DNR and IDA early uptake (after 30 min incubation). *b* DNRol and IDAol uptake (after 12 h incubation); *c* DNR/DNRol and IDA/IDAol uptake/retention

a concentration of 2×10^5 cell/ml were dispensed in 96-well tissue culture plates, and drugs were added at the concentrations shown above. Plates were incubated for 96 h at 37 °C in humidified 5% CO_2. Ten µl of MTT 5 mg/ml in phosphate-buffered saline were added to each well and the plate was incubated for 4 h. Formazan crystals were dissolved with 0.1 ml of 0.04 N HCl-isopropanol. Optical density (OD) of wells was measured after 15 min a Multiscan MCC340 microplate reader (Labsystem, Helsinki, Finland) at 540 nm and 620 nm. The cytotoxicity index (CI %) was calculated by the equation (1-OD treated well/OD untreated well) x 100.

Statistics

Results are means and SD of three different experiments, each performed in duplicate. Comparisons were by means of Student's test. Reference values for each test were obtained in CEM or CEM-VBL cells with the less active drug (DNR/DNRol), at lower concentration (200/50 ng/ml) and without CsA. Comparative results were also expressed as fold-increments from baselines.

Results

Drug Uptake and Retention Studies

Cellular drug uptake after 30 min incubation and without CsA was higher for IDA than for DNR, in a fashion that was proportional to drug concentration and, notably, more evident in CEM-VBL than CEM cells (Fig. 1a).

The figure documents how IDA enters mdr-1+ cells at a much greater rate than DNR, even at the fourfold lower concentrations corresponding to the therapeutic ranges achievable in patients. By adding CsA, FI of both drugs increased significantly in CEM-VBL cells, up to the levels of DNR in CEM cells, with a marginal advantage for IDA. Higher drug concentrations were associated with increased uptake rates, which in turn were better with IDA than DNR and with CsA than without (Fig. 1a). Numerical details of tests performed with lower DNR and IDA concentrations are given in Table 1.

Figure 1b shows results of intracellular DNRol and IDAol accumulation after 12-h incubation with low concentrations of both compounds. Only low IDAol FI levels were detectable in CEM-VBL cells, and apparently there was no effect by CsA. Lastly, Figure 1c shows uptake and retention study results of

Table 1. Comparative DNR and IDA uptake by CEM-VBL cells, ± CsA

Cells	Drug(s)	DNR/IDA ng/ml	FI (± SD)	Fold variation[a]	*p* value[a]
CEM	DNR	200	3197 ± 870		
CEM-VBL	DNR	200	262 ± 73	-12.2	0.004
CEM-VBL	DNR + CsA	200	1914 ± 747	-1.67/+7.3	ns/0.019
CEM-VBL	IDA	50	1554 ± 295	-2/+5.93/-1.23	0.036/0.002/ns
CEM-VBL	IDA + CsA	50	2728 ± 767	-1.17/+10.4/+1.42	ns/0.005/ns

[a] Compared with CEM/DNR (first figure), CEM-VBL/DNR (second figure) and CEM-VBL/DNR+CsA (third figure) ns denotes nonsignificant *p* value ≥ 0.05.

Table 2. Comparative uptake/retention by CEM-VBL cells of varying concentrations of IDA/IDAol and DNR/DNRol, ± CsA

Cells	Drugs[a]	D/ol, I/ol ng/ml	FI (± SD)	Fold variation[b]	*p* value[b]
Lower drug concentration:					
CEM	D/ol	200/50	2987 ± 804		
CEM-VBL	D/ol	200/50	36 ± 23	-82.9	0.003
CEM-VBL	D/ol + CsA	200/50	355 ± 280	-8.4/+9.86	0.006/ns
CEM-VBL	I/ol	50/10	1818 ± 465	-1.64/+50.5/+5.12	ns/0.003/0.01
CEM-VBL	I/ol + CsA	50/10	1762 ± 368	-1.69/+48.9/+4.96	ns/0.001/0.006
Higher drug concentration:					
CEM	D/ol	400/100	5409 ± 1411		
CEM-VBL	D/ol	400/100	88 ± 73	-61.5	0.003
CEM-VBL	D/ol + CsA	400/100	1043 ± 245	-5.2/+11.8	0.006/0.003
CEM-VBL	I/ol	100/20	3538 ± 657	-1.5/+40.2/+3.39	ns/0.001/0.004
CEM-VBL	I/ol + CsA	100/20	3136 ± 569	-1.7/+35.6/+3	ns/0.001/0.004

[a] D/ol, DNR+DNRol; I/ol, IDA+IDAol.
[b] Compared with D/ol in CEM cells (first figure), D/ol in CEM-VBL cells (second figure) and D/ol+CsA in CLM-VBL cells (third figure).
ns denotes nonsignificant *p* value ≥ 0.05.

drug (30 min incubation) plus metabolite (12-h incubation) combinations, which is quite similar to what happens in vivo as previously described. In CEM-VBL cells, FI with IDA/IDAol was distinctly higher than with DNR/DNRol, at any concentration, but, contrary to early drug uptake studies (Fig. 1a and Table 1) and differing from the latter drug type, the result was not modifiable by CsA. This means that, with IDA, incremental effects by CsA were maximal during early cell uptake, after which time long-term retention was independent of CsA co-administration (and P-170 mechanism), while the continuous DNR extrusion was still liable to the modulatory effects of CsA. Numerical data of drug plus metabolite experiments are given in Table 2. Most notably, FI values of IDA/IDAol 100/20 ng/ml in CEM-VBL cells were as high as with DNR/DNRol 200/50 ng/ml in CEM cells.

Apoptosis and MTT Cytotoxicity Assay

The results of drug-induced apoptosis were evaluated after 36 h from end of incubation with drugs. In previous tests (data not shown), we found that annexin V binding by cells exposed to increasing concentrations of anthracyclines was detectable after 12 h and peaked after 24-48 h. Results of the assays carried out with drug plus metabolite combinations are presented in Fig. 2a and Table 3.

Apoptotic cell rates were higher with IDA/IDAol and with CsA. The IDA/IDAol 100/20 ng/ml plus CsA combination gave, in CEM-VBL cells, results similar to that by DNR/DNRol 200/50 ng/ml in CEM cells, thus reproducing strictly the observations made in the drug uptake and retention cytofluorimetric study.

The MTT assay evaluating cytotoxic effects by anthracyclines and performed with-

Table 3. Comparative apoptosis of CEM-VBL cells by varying concentrations of IDA/IDAol and DNR/DNRol, ± CsA

Cells	Drugs[a]	D/ol, I/ol ng/ml	Annexin V positive (%)	Fold variation[b]	*p* value[b]
Lower drug concentration:					
CEM	D/ol	200/50	29 ± 9		
CEM-VBL	D/ol	200/50	0	-29	0.005
CEM-VBL	D/ol + CsA	200/50	0	-29	0.005/ns
CEM-VBL	I/ol	50/10	6 ± 3	-3.83/+6/+6	0.014/0.022/0.022
CEM-VBL	I/ol + CsA	50/10	13 ± 10	-2.23/+13/+13	ns/0.035/0.035
Higher drug concentration:					
CEM	D/ol	400/100	67 ± 11		
CEM-VBL	D/ol	400/100	0	-67	0.0001
CEM-VBL	D/ol + CsA	400/100	3 ± 5	-22.3/+3	0.001/ns
CEM-VBL	I/ol	100/20	14 ± 2	-4.8/+14/+4.6	0.001/0.0001/0.02
CEM-VBL	I/ol + CsA	100/20	35 ± 7	-1.9/+35/+11.7	0.014/0.001/0.003

[a] D/ol, DNR+DNRol; I/ol, IDA+IDAol.
[b] Compared with D/ol in CEM cells (first figure), D/ol in CEM-VBL cells (second figure) and D/ol+CsA in CEM-VBL cells (third figure).
ns denotes nonsignificant *p* value ≥ 0.05.

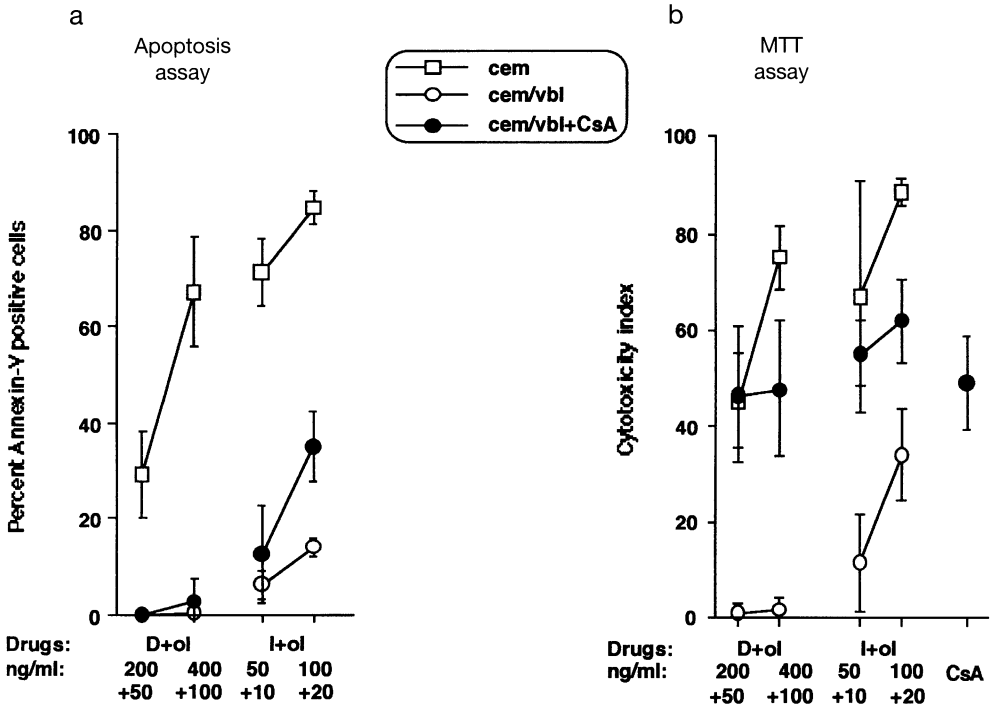

Fig. 2 a, b

out CsA confirmed the greater cytotoxicity of IDA/IDAol towards CEM-VBL cells, particularly at higher drug concentrations (Fig. 2b). A notable finding was that CsA alone exerted a significant inhibitory effect on cell growth. CsA accounted approximately for > 95% of cell growth inhibition in DNR/DNRol tests and for > 75% in IDA/IDAol tests (Fig. 2b and Table 4).

Table 4. Comparative cytotoxicity on CEM-VBL cells by varying concentrations of IDA/IDAol and DNR/DNRol, ± CsA

Cells	Drugs[a]	D/ol, I/ol ng/ml	Cytotoxicity index (%)	Fold variation[b]	*p* value[b]
CEM-VBL	CsA		49 ± 10		
Lower drug concentration:					
CEM	D/ol	200/50	45 ± 10		
CEM-VBL	D/ol	200/50	1 ± 2	-450.0001	
CEM-VBLD/ol + CsA		200/50	47 ± 14	+1.04/+47	ns/0.0001
CEM-VBL	I/ol	50/10	12 ± 10	-3.7/+12/-3.9	0.0001/0.05/0.002
CEM-VBL	I/ol + CsA	50/10	55 ± 7	+1.2/+55/+1.2	ns/0.0001/ns
Higher drug concentration:					
CEM	D/ol	400/100	75 ± 5		
CEM-VBL	D/ol	400/100	2 ± 3	-37.5	0.0001
CEM-VBL	D/ol + CsA	400/100	48 ± 14	-1.6/+24	0.004/0.0001
CEM-VBL	I/ol	100/20	34 ± 10	-2.2/+17/-1.4	0.0001/0.0001/ns
CEM-VBL	I/ol + CsA	100/20	62 ± 9	-1.2/+31/+1.3	0.019/0.0001/ns

[a] D/ol, DNR+DNRol; I/ol, IDA+IDAol.
[b] Compared with D/ol in CEM cells (first figure), D/ol in CEM-VBL cells (second figure) and D/ol+CsA in CEM-VBL cells (third figure).
ns denotes nonsignificant *p* value ≥ 0.05.

Discussion

In this chapter we evaluated comparatively the in vitro effects of therapeutic dosages of DNR and IDA on a T-lymphoblastic cell line exhibiting the mdr-1 multidrug resistance mechanism. Our observations validate and extend prior studies indicating an advantage for IDA. Berman and McBride [7] and Ross and others [8] performed a similar study using drug concentrations of 1000 ng/ml, that cannot be achieved in vivo. Damiani and others [10] and Kuffel and others [9] tested the cytotoxic effects of lower IDA concentrations, including those we considered here, but after very long incubation times of 3-7 days that are again beyond feasibility in patients. Moreover in two of these studies the activity of the cytotoxic IDA alcohol metabolite was not tested [7, 10]. Instead, we used combinations of drug plus metabolite at the concentrations previously ascertained in plasma of leukaemia or cancer patients receiving intravenous DNR 45 mg/m^2 and IDA 10-15 mg/m^2, respectively [3, 5]. Pharmacokinetic data from these studies were used to select the appropriate incubation times, which were rather short (30 min) for parent drugs, the peak plasma level of which is subject to a rapid fall, and longer (12 h) for their metabolites with prolonged half-life. The decision of adding CsA 1500 ng/ml fixed-dose was developed along the same lines of evidence, because similar or even higher CsA levels were achieved in patients with refractory acute leukaemia tolerating intravenous CsA 6 mg/kg as a bolus followed by 16/mg/kg/d as continuous infusion for four consecutive days [11]. The ultimate rational behind our study was that, since IDA was reported less vulnerable to mdr-1-mediated transport than other anthracyclines, it would perhaps represent the anthracycline of choice for in vivo attempts to overcome mdr-1 overexpression with CsA or other P-170 downregulator. Favourable results with IDA and the novel and more potent P-170 inhibitor PSC 833 were preliminarily reported [15].

The data collected fit the widely accepted view that IDA enters mdr-1+ leukaemic cells at a greater rate than DNR, due to a greater lypophilicity and relative P-170 invulnerability. Additionally, it appears from our comparative analysis of cellular FI that it is only in the early phase of drug-cell interaction that IDA uptake may benefit from P-170 downregulation by CsA. The data obtained with DNR were different, showing long-term modulability by CsA which correspond to a cellular pattern of continuous drug extrusion. This difference could reveal advanta-

geous to patients, because CsA could be given for a shorter period thereby causing less toxic side effects. We are currently studying in greater detail CsA-IDA interectations, to confirm whether and when exposure to CsA could be stopped after IDA.

In the second part of our study we assessed pro-apoptotic effects by anthracycline drugs and their metabolites by means of a highly sensitive and specific cytofluorimetry technique revealing early apoptosis-related structural changes in cell membranes [12]. As expected, apoptosis rates correlated well with drug uptake/accumulation, varying significantly in function of cell type (mdr-1- vs mdr-1+), drug type (DNR/DNRol vs IDA/IDAol), concentration (low vs high) and CsA (- vs +). In keeping with cellular drug accumulation study results, pro-apoptotic effects by IDA/IDAol 100/20 ng/ml +CsA in mdr-1+ cells were as high as with standard DNR/DNRol concentrations in mdr-1-cells, whereas the activity of DNR/DNRol was negligible even in the presence of CsA.

Lastly, examining DNR and IDA effects by the conventional MTT assay for in vitro drug cytotoxicity, we obtained results consistent with FI and apoptosis assays and in favour of the higher IDA concentration. The marked cytotoxicity from CsA was an unexpected finding. Because CsA is primarily an inhibitor of T lymphocyte proliferation, it could, by analogy, suppress the growth of a T-cell related malignancy. However a recent report from St. Jude's Hospital (Memphis, TN, USA) documented cytotoxicity (increased apoptosis after 96 h by CsA against several T and B lineage leukaemic cell lines including multidrug resistant lines [6]. The fact that we did not observe any significant degree of apoptosis by CsA alone after 36 h may simply indicate that CsA-dependent cytotoxicity may become detectable later, namely in the MTT assay at 96 hours. Because of this, and of the presence of an independent mdr-1 downregulator in intravenous CsA preparations, cremophor EL [17], intravenous CsA stands out as a downregulator of (mdr-1+) leukaemic cell proliferation by at least three distinct and perhaps synergistic mechanisms: direct cytotoxicity unrelated to P-170, inhibition of P-170 function and enhancement of cytotoxicity by P-170 substrates, and concurrent inhibition of P-170 by CsA oily vehicle cremophor EL. The effects of IDA plus new powerful mdr-1 negative regulators, such as PSC-883 [15], and the spectrum of their activities in comparison with the complex properties of CsA, need to be almost entirely worked out.

If these findings are put in relation to the management of patients with mdr-1+ leukaemias, the conclusions are that IDA should be preferred to DNR in clinical studies with CsA or other P-170 modulator, and that chosen IDA dosages should warrant an intracellular IDA/IDAol accumulation as high as that we found relatively effective in the in vitro study (corresponding to 100/20 ng/ml in the medium or, alternatively, in patient plasma). Available in vivo pharmacokinetic data show this to be possible with a single IDA dose ≥ 12 mg/m^2.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Protection of Marrow from Methotrexate Toxicity by Gene Transfer of Mutant Forms of Dihydrofolate Reductase into Hematopoietic Progenitor Cells

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Abstract. A potential clinical use of gene transfer technology would be to introduce via a retroviral vector, cDNAs encoding mutant forms of dihydrofolate reductase (DHFR) into hematopoietic progenitor cells, thus allowing high doses of methotrexate MTX to be safely administered. Mice bearing a chemotherapy sensitive breast cancer tumor, treated with high dose cyclophosphamide treatment and transplantated with bone marrow cells transfected with a mutant DHFR cDNA, tolerate high doses of MTX post transplant, leading to cure.

Additional studies are described that indicate that the use of a mutated nerve growth factor receptor (mNGFR) engineered into a vector also containing the S 31 mutant DHFR cDNA, allows identification of $CD34^+$ hematopoietic progenitor cells that are expressing this surface protein.

Introduction

Several clinical studies are planned or are in progress: [1] to introduce suicide genes into tumor cells (e.g., HSV thymidine kinase, bacterial cytosine deaminase), then treat patients with non-toxic prodrugs that are activated by tumor cells selectively; [2] to introduce cytokine genes into the tumor cells, with the aim to stimulate the immune response; [3] to introduce normal tumor suppressor genes to replace defective genes; [4] to introduce anti-sense sequences that would decrease expression of oncogenes; or [5] to introduce drug resistance genes into bone marrow progenitor cells. The rationale for this last approach is that the drugs used in cancer chemotherapy usually have a steep dose-response, curve and increasing doses to even 2-3 fold may increase anti-tumor response significantly [3]. The two drug resistance genes tested extensively for this purpose are mutant dihydrofolate reductase (mDHFR) [4-8] genes and the multi-drug resistance (MDR-1) gene [9-19]. Other cDNAs that impart a resistance phenotype to transfected cells are also under investigation, including glutathione transferase (resistance to alkylating agents), [20] 0^6alkyl transferase (nitrosoureas), [21-23] and aldehyde dehydrogenase (cyclophosphamide) [24-25].

Another important use of drug resistance genes is as selectable markers. Introduction of a second, non-selectable gene in the retroviral vector may allow it to be co-expressed when cells are treated with a selecting agent. Variant forms of DHFR cDNAs are particularly attractive for this purpose, as they are relatively small and thus other genes can be accommodated in retroviral vectors. MTX, the selecting agent, has enjoyed wide use as an anticancer agent, and low doses have been safely employed to treat non-neoplastic diseases, e.g., rheumatoid arthritis, graft versus host disease, and psoriasis [26].

DHFR catalyzes the formation of tetrahydrofolate, a folate coenzyme that mediates the transfer of one carbon units in purine

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and pyrimidine biosynthesis [26]. This enzyme is a target for MTX, a widely used chemotherapeutic agent. Certain mutations or an increase in the activity of this enzyme result in resistance to MTX. We recently discovered that a mutation in codon 31 (phe ® ser) decreases markedly the binding of MTX to DHFR, yet is functional as an enzyme, and thus may serve as a dominant selectable marker [7]. This mDHFR, introduced into hematopoietic stem cells via a retroviral vector protected these cells from MTX toxicity [8]. More recently, we have generated "double" mutations in codons 22 and 31 respectively. These double mutants provide even higher levels of resistance to MTX than other single DHFR mutations [27].

Retroviral vectors have been extensively studied, and efficiency, safety, and feasibility for clinical use have been tested [28]. Retrovirus vectors are the most efficient vectors when target cells are hematopoietic cells. Since the development of the newer generations of virus packaging cell lines, which require more than three recombination events to produce replication competent retrovirus particles, generation of helper viruses has not been observed [29, 30]. Also, to date, a large number of patients have been treated with gene therapy protocols using retroviral vectors and no obvious side effects or secondary cancers have been reported.

In this paper we review studies with mutant forms of DHFR (mDHFR) cDNA introduced into hematopoietic cells to provide protection against MTX toxicity. In a model system, using mice bearing a transplanted breast cancer, we show that post transplant doses of MTX can improve curability of this tumor in animals with marrow transplanted after infection with a mDHFR retroviral construct.

Materials and Methods

CBA/J male mice, 7-11 weeks of age are used as marrow donors. Bone marrow is harvested 4 days after treatment of 5FU (150 mg/kg), and mononuclear cells are recovered after Ficoll separation. Bone marrow cells are infected by co-culture with virus producer cells irradiated with 1500 cGy. The infections are carried out in IMDM media containing 20% FCS, with either 10% WEHI-B conditioned media or with a combination of Kit-Ligand, IL-3 and IL-6, in the presence of 8 μg/ml of polybrene [18] or 4 mg/ml of protamine.

CFU-GM assays are carried out as follows: 10^5 marrow cells are plated on a grid petri dish (10 × 35 mm), in 1-2 ml of media containing 1% α-methylcellulose, 20% FCS treated with thymidine phosphorylase, 10% WEHI-B media and 1×10^{-7} M MTX. CFU-GM colonies are scored after 12-14 days.

Detection of Transduced Genes. DNA-PCR and RT-PCR analysis for the altered DHFR gene and the NEO gene are performed on peripheral blood nucleated cells isolated by Ficoll-Hypaque separation. Two months after transplant, these mice are used as donors and additional mice are transplanted after irradiation and on day 12, mice are sacrificed to analyze CFU-S and bone marrow cells for NEO by Southern blot analysis and PCR amplification. DNA sequencing is performed for the DHFR gene to confirm the presence of the mutated gene in CFU-S, marrow and peripheral blood nucleated cells [7].

PCR Analysis of the Altered DHFR cDNA. High molecular weight DNA is extracted from bone marrow or CFU-S. Using this DNA as a template, asymmetric PCR amplification is performed with GTNC-1 and DHFR250 oligos at the ratio of 50:1. Direct sequencing is performed with an internal primer, DHFR151.

The sequences of primers used are:

DHFR151: 5'-CCAGATTCTGTT-TACCTTCTAC-3'

GTNC-I 5'-CCTCGGCCTCTGAGC-TAT-3'

DHFR250: 5'GAG GTT CCT TGA GTT CTC TGC-3'

GTHinDIII-1: 5'TTCAGTATAAGCTTAT-CCGCGTGCTGTCATGGTT-GGTTCGCT-3'

RT-PCR to detect the expression of a mDHFR DHFR cDNA is carried out as follows: Total cellular RNA is extracted and re-

versely transcribed. A PCR is performed with following primers:

GTHinDIII-1 and DHFR250. Product size 260 bp.

Results

Generation of New DHFR Mutants

We have created several new mutations of DHFR by site-directed mutagenesis [27]. Mutagenesis is performed using a polymerase chain reaction technique involving two separate sets of primers; one set flanking the entire cDNA of DHFR and the second set being complimentary to each other and containing the desired mutations. The primers used for mutagenesis are as follows:

To introduce a F22 mutation:
5-CCAGGGTTTGTCCCCGTT-3
3-GGTCCCAAACAGGGGCAA-5

To introduce a Y22 mutation:
5-TGGCCAGGGTACGTCCCCGTTCTC-3
3-ACCGGTCCCATGCAGGGGCAAGAA-3.

Double mutants, F22S31 and Y22S31 were created with these primers by using S 31 mutated DHFR cDNA as a PCR template. The PCR products were digested with NcoI/HinDIII sites and subcloned into a pKT7 bacterial expression vector containing the same restriction sites. The resulting ligation mixtures were used to transform *E. coli* BL21 to express the enzymes to be characterized. Also, the complete cDNAs of the different DHFR mutant species were sequenced and no other mutations were found. These DHFR mutant enzymes were characterized (Table 1). The double mutant Y22S31 is potentially 200-fold more effective than the previously used ser 31 mutant for generation of high level resistance as estimated by $K_i \times K_{cat}/K_m$.

The Mouse as a Model to Test Gene Transfer in Vivo

Experiments are in progress to determine if whole body irradiation is required for engraftment of transduced marrow in mice, and to test the value of MTX as a selectable agent. Using DC/SVhDHFRS31, transduced bone marrow was transplanted to unirradiated recipient mice. With low dose MTX selection, we were successful in obtaining PCR signals of mDHFR and Neo from spleen colonies, although the percentage of PCR positive colonies was lower than those of transplanted irradiated mice. These preliminary experiments show that with MTX selection, transplanted bone marrow can engraft and effective chimerism can be induced without prior irradiation. Additional studies are planned using the optimal mDHFR construct to test the feasibility of such an approach.

Mice Bearing a Transplanted Mammary Carcinoma Tolerate Curative Doses of MTX After Transplantation with a Retroviral Construct Containing a Mutated Dihydrofolate Reductase cDNA

In these experiments (repeated × 2), mice were inoculated subcutaneously with 5×10^6 E0771 mammary adenocarcinoma cells, a tumor moderately sensitive to both cyclo-

Table 1. Enzyme kinetic parameters and binding affinity of MTX for recombinant wild-type and mutant variants of human DHFR

Enzyme	K_i (MTX) (nM)	K_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	$K_i.K_{cat}/K_m$ ($10^3 \times s^{-1}$)	Fold increase
wt	0.0012	159	0.19	1
Ser 31	0.24	16	3.8	20
Phe22	0.11	46	4.9	26
Gly31	0.35	30	10	53
Tyr22	1.98	10	20	100
PHE22-Ser 31	25.6	3.6	90.0	478
Phe22-Gly31	29.4	3.3	96.5	508
Tyr22-Ser 31	41.6	3.8	160	842
Tyr22-Gly31	150	1.4	216	1140

phosphamide (CTX) and MTX. Eight days later, when the tumors had grown to 0.5-0.7 cm in volume they were treated with CTX, 200 mg/kg i.p.,q.d.x3 (LD_{100}), followed 24 h later by transplantation with marrow from donor mice that had been transduced by a retroviral construct containing a mutant DHFR cDNA, or marrow not transduced with a retroviral construct. Following the transplant, treatment with low dose MTX (5 mg/kg, i.p., b.i.w x 2 weeks) was followed one week later by a single i.p. dose of MTX (600 mg/kg). A control group that did not receive a transplant died of CTX toxicity. A second control group transplanted with normal marrow, but not treated with MTX post transplant, died of tumor regrowth. In contrast, the animals in the experimental group were able to tolerate the post transplant dose of MTX and 5 of 10 animals had complete and lasting tumor regression (Fig. 1). Integration of a marker NEO gene and the mDHFR gene in DNA from marrow cells was demonstrated by PCR. These results support the premise that transfection of bone marrow cells with a cDNA imparting drug resistance may allow dose intensification and improve curability for certain tumors.

Increasing MTX Resistance with Co-Expression of Genes Increasing Purine or Thymidine Salvage

DHFR is required for thymidylate and purine synthesis. Therefore, resistance to MTX may be potentiated by genes which allow increased salvage of thymidine or purines when co-expressed with a mutated DHFR. We recently constructed two vectors which express HSV-tk or XGPRT as well as Serine 31 DHFR (DC/SV6s31-tk and DC/SV6s31-GPT). HSV-tk or XGPRT alleviate MTX toxicity partially, as they rescue thymidine and purine synthesis pathways, respectively. Thus, both vectors augment MTX resistance conferred by S 31 DHFR. In particular, DC/SV6S31-tk infected 3T3 cells are very resistant to short term exposure to MTX, and the ED_{90} of these cells is more than 30-fold higher than that of DC/SV6S31 infected 3T3 cells (Fig. 2). Also, this vector renders infected cells sensitive to ganciclovir as this anti-viral is an excellent substrate for the HSV-tk enzyme. This suicide function imparted by HSV-tk can be very useful as a backup to eradicate contaminating tumor cells in the peripheral stem cell/bone marrow preparation, if necessary, by treatment with ganciclovir.

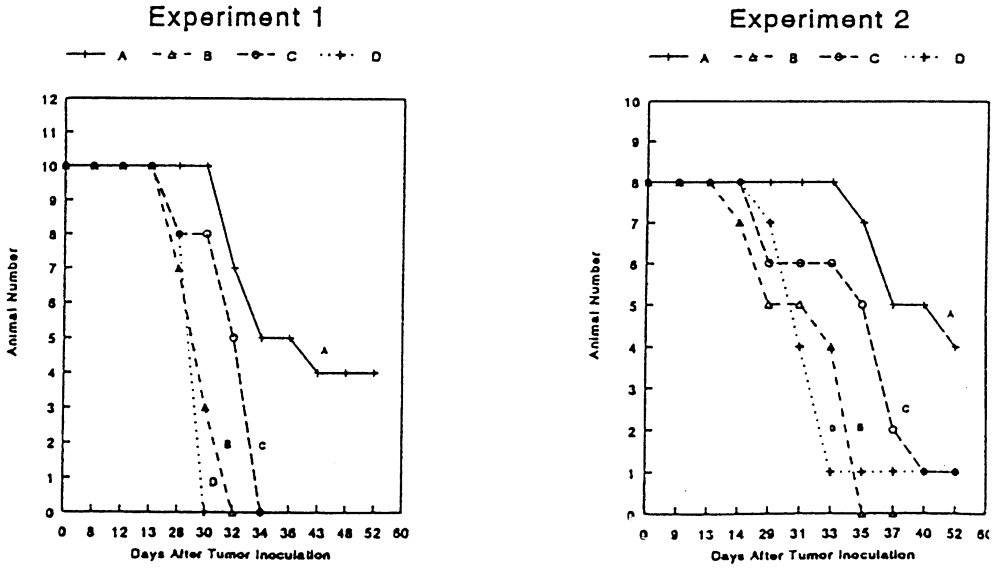

Fig. 1. Results of two different experiments using groups of mice bearing the E0771 mammary carcinoma. After treatment with CTX as described in the text, the animals were randomized into groups and treated as follows: A, +—+, experimental group; B, △—△, control group, not receiving a marrow transplant; C, O—O, a control group receiving a transplant with normal marrow, but not receiving MTX post transplant; D +- - -+, a control group receiving a transplant with a retroviral construct not containing mutant DHFR (mock)

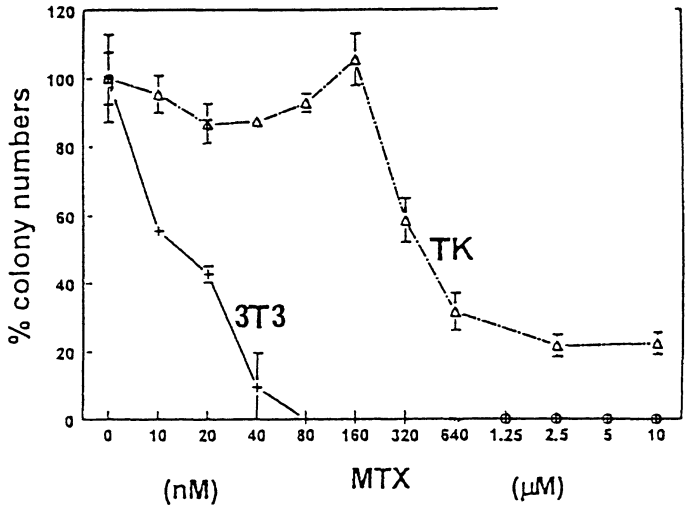

Fig. 2. Cytotoxicity assays of 3T3 cells, and cells transfected with mDHFR/Neo(DC/SV6531) and mDHFR/HSV-tk(DC/SV6531-tk) retroviral constructs

Utilization of a Retroviral Vector Encoding a Cell Surface Marker as a Means of Evaluating Transduction and Expression Efficiency as well as Providing a Mechanism for Enrichment of Transduced Cells

We have developed a system to rapidly and reliably detect the successful transduction of $CD34^+$ cells utilizing a retroviral vector based on the MFG retroviral backbone encoding a mutated Nerve Growth Factor Receptor (p75) (NGFR) expressed on the cell surface. This mutated receptor does not bind Nerve Growth Factor. Using a tagged antibody against this vector-encoded molecule, we can readily determine levels of gene transfer and expression in a transduced cell population and cells expressing the protein can be isolated by FACS or by immunomagnetic bead separation.

In multiple experiments with a high titer amphotropic retroviral vector, we performed $CD34^+$ cell separation from chemotherapy/cytokine mobilized peripheral blood mononuclear cells, followed by 72-96 h of cytokine prestimulation (with IL1, IL3 and KL), 24 h of co-culture transduction, and a 72 h period of proliferation to allow for protein expression. Over this time period, we observed a 12.1- to 22.4-fold expansion of cell numbers, which is comparable to our previously reported experiments, and was similar regardless of whether cells were co-cultured on retroviral producer cells or packaging cells without virus [8]. At the end of this time period, when this cell population was stained with an anti-NGFR antibody (and secondarily FITC-labeled), significant levels of gene transfer and subsequent cell surface protein expression were detected by flow cytometric analysis (Fig. 3). Cells were also co-stained with a PE labeled anti-CD45 antibody to confirm that they were of hematopoietic origin (rather than contaminating low levels of retroviral producer cells from the co-culture); 3.4 to 15.3% of the cell population transduced in this initial system demonstrated vector-derived NGFR protein on their cell surface (as well as CD45 expression). Background detection with this method, on cells cocultured on packaging cells without vector, is minimal (less than 1%).

Furthermore, 11.5 to 14.3% of the cell population in these experiments (with IL1, IL3 and KL) are CD34+ at the end of this culture period. Co-staining with a PE-labeled anti-CD34 antibody reveals that 4.9 to 11.5% of these CD34+ cells have also been successfully transduced and are expressing the surface protein (Fig. 3). Similar rates of transduction and expression were observed with the cytokine combination of IL3, KL and IL6 (3.8 to 13.4% of cells co-expressed CD45 and NGFR). Our data suggests that a progenitor cell population had been successfully transduced and was expressing vector-derived protein following this initial protocol.

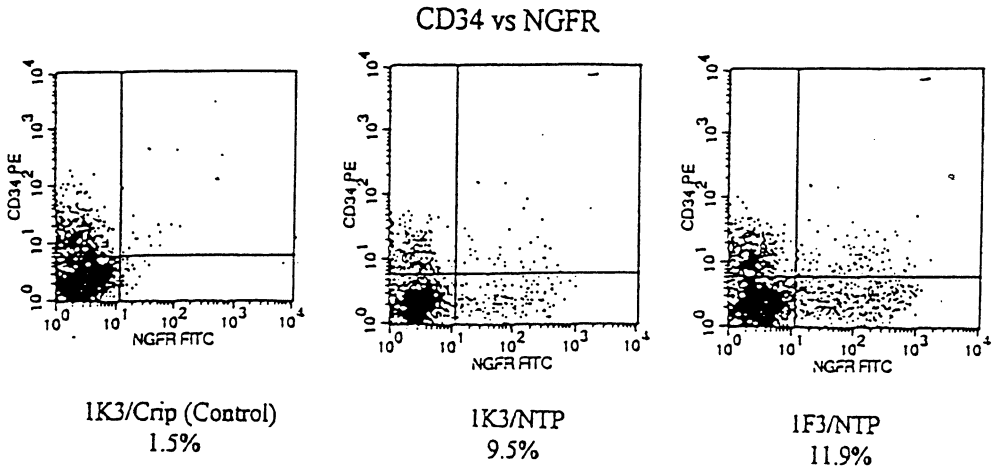

Fig. 3. Comparison of 1K3 vs. 1F3 prestimulation NTP (amphotropic) vector. 1K3: IL-1, KL and IL-3, IF3: IL-1, FLT ligand and IL-3

Discussion

Work from this laboratory and other laboratories have identified several mutant forms of DHFR that maintain catalytic activity and yet bind MTX poorly [27, 31, 32]. In particular, substitutions at the 22 position (phe or tyr for leu), or the position 31 of DHFR (gly or ser for phe) give rise to mutants with desirable properties for gene transfer studies as described in this chapter Recently, we have described several "double mutants", e.g., mutations in both codons 22 and 31 that may even be more useful for generating high levels of resistance to inhibitors of this enzyme [27].

Introduction of a second cDNA into retroviral vectors affecting a metabolic pathway (either purine salvage or thymidine salvage) may also increase the level of resistance produced to MTX and related DHFR inhibitors. Of particular interest is the DHFR and the HSV-tk construct; not only does it produce a high level of resistance to MTX, but the HSV-tk gene imparts a suicide function if tumor cells are inadvertently transfected with this retroviral construct.

The studies with the breast cancer model demonstrate nicely the potential of this approach for the treatment of human malignancies. In patients with breast cancer who are transplant candidates (responding advanced disease, or patients with more than 10 positive axillary nodes), peripheral blood stem cell transplants have been used following intensive chemotherapy regimens in an attempt to obtain long term regressions and even cure. However results to date for patients with advanced disease show complete regressions lasting 2 years or more in only 20% of individuals, indicating that there is considerable room for improvement [33, 34]. Introduction of drug resistant genes into PBSC may allow treatment and even dose intensification post transplant, perhaps providing the additional cell kill necessary for complete tumor eradication.

Encouraged by these results and our studies showing that transduced cells may be selected by the use of antibodies to a co-expressed membrane protein (mNGFR), we are planning to initiate a clinical trial in patients with breast cancer in the near future.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

In Vitro Drug Resistance in Childhood Acute Myeloid Leukemia: a Preliminary Analysis

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Abstract. Cellular drug resistance is an important determinant of the clinical outcome with chemotherapy, but has not been well studied in childhood acute myeloid leukemia (AML). We therefore initiated a cooperative study on drug resistance, using the colorimetric methyl-thiazol-tetrazolium (MTT) assay in Amsterdam, of childhood AML patient samples treated according to the German AML-BFM protocols. This report concerns a preliminary analysis of the results on 55 successfully tested AML samples obtained at initial diagnosis. In 8 cases the MTT assay failed, so the technical success-rate of the assay was 87% (55/63). The group of patients with a prognostically favorable FAB type (M1 and M2 with auer rods, M3, M4 with eosinophils) was not more drug sensitive in vitro compared to the complementary group. FAB M5 samples were more sensitive to vincristine and l-aspagarinase than the other FAB types. The initial response to induction chemotherapy (cytarabine, etoposide, and daunorubicin or idarubicin) as determined by the percentage of bone marrow blasts at day 15 of treatment was related to in vitro drug resistance. The group of patients with more than 5% blasts had AML cells that were more resistant to daunorubicin (3-fold, p=0.03), borderline significantly more resistant to idarubicin (2.4-fold, p=0.06) and etoposide (3.3-fold, p=0.07), and not significantly more resistant to cytarabine (1.6-fold, p=0.12) than the patients with less than 5% blasts.

In conclusion, the MTT assay may identify subgroups of AML patients that are particularly sensitive or resistant to certain drugs. In addition, response to induction chemotherapy may be predicted by cellular drug resistance testing. However, more patients and longer follow-up are necessary to allow definite conclusions.

Introduction

Cellular drug resistance, together with clinical pharmacokinetics and the regrowth potential of residual cells, determines the clinical outcome after chemotherapy [1, 2]. However, there is a lack of knowledge on cellular drug resistance in childhood acute myeloid leukemia (AML). Yet, treatment results in childhood AML need further improvement [3]. We therefore initiated a cooperative study on drug resistance in childhood AML. Samples of both initially diagnosed and relapsed patients treated according to the German AML-BFM treatment protocols are sent to our research laboratory of pediatric oncology in Amsterdam. There, the colorimetric methyl-thiazol-tetrazolium (MTT) assay is performed, among other techniques. The MTT assay is a reliable short-term total cell kill drug resistance assay [4]. In childhood acute lymphoblastic leukemia (ALL), we and others showed that the results of the MTT assay correlate well with clinical and cell bio-

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logical features [2, 5-7] and to the long-term clinical outcome [2, 8, 9]. In childhood AML, several groups reported significant correlations between in vitro drug resistance and clinical outcome in small patient-groups [2, 10-13], including our own group [14].

In this chapter, we will report the preliminary results on childhood AML samples obtained at initial diagnosis, with emphasis on the correlation of cellular drug resistance with FAB type and initial response to induction chemotherapy. Previous clinical studies showed that FAB type, together with information on the presence of auer rods and eosinophils, has prognostic significance [3].

Materials and Methods

Patients

By the end of 1996, samples of 100 AML patients had been sent to Amsterdam (research laboratory of pediatric oncology, Free University Hospital) for cellular drug resistance testing. In 79 patients, samples had been obtained at initial diagnosis. Sixteen samples were not tested, mainly because of too few blasts, rarely because of a delay in the transport of the sample to Amsterdam. Out of the 63 remaining samples, 55 (87%) were successfully tested. According to FAB type, it concerned 8 M1, 13 M2, 2 M3, 15 M4, 7 M5 and 2 M7 cases. In 8 cases FAB type was not (yet) known. According to previous AML-BFM studies, the group of FAB type M1 and M2 with auer rods, M3, and M4 with eosinophils have a more favorable prognosis than the complementary group.

Most patients were treated according to German AML-BFM protocol 1993, some according to protocol 1987. Induction chemotherapy consisted of cytarabine, etoposide, and either daunorubicin or idarubicin. At day 15 of induction, bone marrow aspiration was done to determine the response by the percentage of leukemic blasts.

MTT Assay

Leukemic cells were isolated from bone marrow or blood by gradient centrifugation and incubated with or without drugs in 96-well microculture plates in culture medium for four days (4). Six wells contain culture medium only to blank the reader. After 4 days, MTT is added. Only living cells can reduce MTT into formazan crystals. These crystals are solubilised using acidified isopropanol, and the optical density is measured spectrophotometrically. Each drug is tested at 6 concentrations in duplicate. The results are corrected for the control cell survival (mean survival of 6 wells with leukemic cells in medium only), which is set at 100%. The dose-response curves are used to calculate LC50 values, the drug concentration lethal to 50% of the cells. A large number of drugs is tested, including more typical AML drugs such as cytarabine (ARA), daunorubicin (DNR), idarubicin (IDR), etoposide (VP16), and thioguanine (6TG), but also more typical ALL drugs such as vincristine, l-asparaginase, and prednisolone, and newer agents such as 2-chloro-deoxyadenosine and 5-azacytidine.

Results

FAB Type and Drug Resistance

A prognostically favorable FAB type (up to 25 samples successfully tested) was associated with a remarkable resistance to cytarabine and etoposide compared to a prognostically unfavorable FAB type (up to 19 samples successfully tested). This is shown in Table 1.

Table 1. For five drugs important in current AML treatment, the median LC50 value of the patients with a clinically prognostically favorable FAB type is divided by the median LC50 value of the other group. A ratio above 1 means that the group of patients with a favorable FAB type is more resistant to that drug

Drug	Resistance ratio of clinically low versus high risk FAB type	P-value
Cytarabine	1.7	0.01
Etoposide	3.9	0.03
Daunorubicin	1.8	0.28
Idarubicin	1.4	0.29
Thioguanine	0.7	0.72

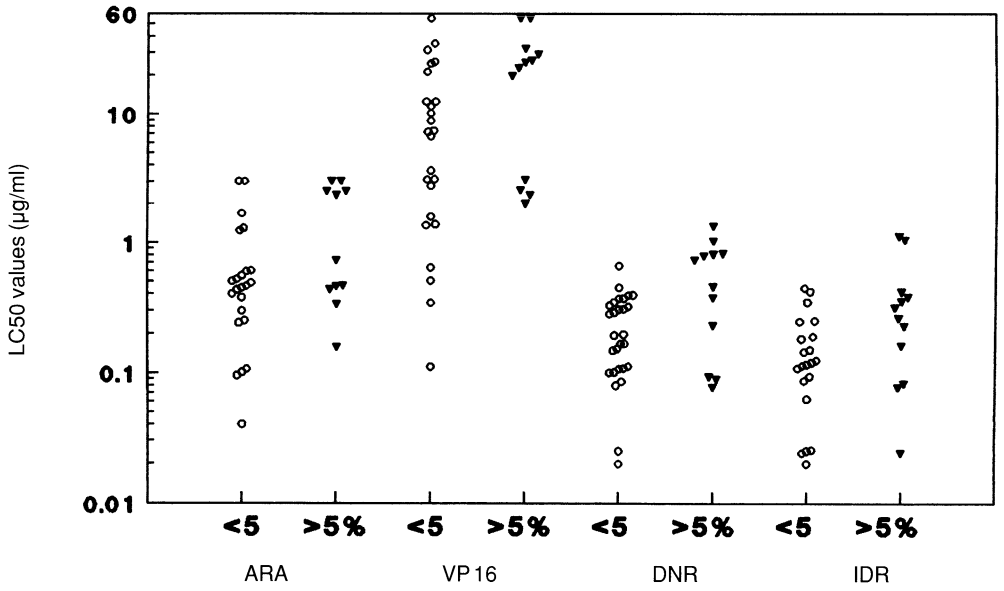

Fig. 1. Relation between in vitro resistance to induction drugs (*ARA* cytarabine, *VP16* etoposide, *DNR* daunorubicin, *IDR* idarubicin) and initial response to induction treatment, defined as good (<5% blasts in bone marrow at day 15) or poor (>5%)

The drug resistance profiles of all separate FAB types were compared, which was hampered by the small numbers tested sofar. However, the group of 7 FAB M5 samples was relatively in vitro sensitive to many drugs, including vincristine (median 8.5-fold), l-asparaginase (median 3.5-fold) and 2-chloro-deoxyadenosine (median 3.6-fold), compared to the group of patients with other FAB types.

Response to Induction Chemotherapy and Drug Resistance

Patients with >5% BM blasts at day 15 of induction treatment (n=12) had more resistant AML cells in vitro than patients with < 5% blasts (n=28). This was significant for daunorubicin and a borderline significance for etoposide and idarubicin, as shown in Table 2. Individual LC50 values are shown in Fig. 1.

Two out of three patients who did not achieve complete remission (CR) because of resistant disease, had AML cells resistant in vitro to etoposide and anthracyclines, as shown by the LC50 values in Table 3. The follow-up on the patients is too short for a meaningful analysis of the relation between the occurrence of a relapse and in vitro cellular drug resistance.

Table 2. For the induction drugs, the median LC50 value of the patients with more than 5% blasts in their bone marrow at day 15 of treatment was divided by the median LC50 value of the other group. A ratio above 1 means that the group of patients with a less favorable response to induction chemotherapy is more resistant to that drug

Drug	Resistance ratio of group with >5% versus <5% BM blasts at day 15	P-value
Cytarabine	1.6	0.12
Etoposide	3.3	0.07
Daunorubicin	3.0	0.03
Idarubicin	2.4	0.06

Discussion

Knowledge on cellular drug resistance in childhood AML is limited. An ongoing cooperative study has been initiated, and prelim-

Table 3. LC50 values in μg/ml for three patients who did not achieve complete remission in relation to the median LC50 value of each drug for the group of patients who did enter complete remission (CR)

Drug	Patient 1	Patient 2	Patient 3	CR patients
Cytarabine	Not tested	0.46	0.72	0.55
Etoposide	Not tested	31.7	25.0	10.8
Daunorubicin	0.28	1.33	0.45	0.30
Idarubicin	0.15	0.38	1.12	0.19

inary results on the AML samples obtained at initial diagnosis are presented here.

Remarkably, patients with a prognostically favorable FAB type had AML cells that were relatively in vitro drug resistant. Obviously, the favorable prognosis can not be explained by differences in cellular drug resistance, at least not as detected by the MTT assay. The MTT assay is most suited to look at the total cell population, and may not identify a subpopulation of, e.g., 5 or 10% highly resistant cells. However, such a subpopulation may be responsible for a relapse. Alternatively, regrowth resistance may play a role [15]. Of interest, FAB M5 AML cells were relatively in vitro sensitive to drugs typically used in ALL, especially vincristine and l-asparaginase. For vincristine, this is in agreement with the fact that FAB M5 cells have relatively low myeloperoxydase activity, while it has been shown that myeloperoxydase activity is associated with resistance to vincristine [16, 17]. In general, AML samples were significantly more resistant to vincristine than ALL cells [18]. For l-asparaginase, our observation is in agreement with the results of Dübbers et al. [19], who showed that AML cells of patients classified as FAB M5 have low asparagine synthetase activity, that would render cells sensitive to l-asparaginase treatment. The relatively poor prognosis of AML type FAB M5 might be improved by a more intensive use of these ALL drugs, vincrisine and l-asparaginase. FAB M5 cells were also relatively sensitive to 2-chloro-deoxyadenosine, a promising new agent in the treatment of AML. It has been reported that for children with AML the clinical response to a monotherapy with 2-chloro-deoxyadenosine was highest among AML FAB M5 patients [20].

Patients with a relatively poor response to induction chemotherapy as determined by the bone marrow at day 15 had AML cells that were more in vitro resistant than those of the relatively good responders. This is in agreement with several studies showing that initial treatment response correlates well with in vitro cellular drug resistance measurements in childhood AML [2, 10-14]. This study will be continued and longer follow-up will have to show whether long-term clinical outcome can also be predicted by the MTT assay.

In conclusion, it is feasible to perform a study on childhood AML samples send from German centers to Amsterdam. The MTT assay may identify subgroups of AML patients (e.g., FAB M5) that are particularly sensitive or resistant to certain drugs, which information would be very useful for rational improvements of current treatment protocols. In addition, response to induction chemotherapy may be predicted by cellular drug resistance testing. However, more patients and longer follow-up are necessary to allow definite conclusions.

Acknowledgments. We thank all clinicians cooperating in the German AML-BFM trials for sending their AML samples to Amsterdam, and kindly ask them to continue doing so.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Correlation of in Vitro Drug Sensitivity with Clinical Outcome in Adult AML

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Abstract. Advances in chemotherapy and supportive care have significantly improved the prognosis of patients with AML, but 5-35% of them fail to respond to induction therapy and up to 90% of patients who achieve complete remission relapse within 2 years. Drug resistance is presumed to be the major cause of these chemotherapy failures. Several studies have reported the clinical relevance of both long-term clonogenic and various short-term non-clonogenic in vitro drug resistance assays. In particular, the MTT assay is a rapid, simple automated test based on the reduction by living cells of dimethylthiazol-diphenyl-tetrazolium bromide to a coloured formazan product. After 2 days of drug exposure, in vitro responses of leukemic samples can be measured by spectrophotometry.

We evaluated in vitro chemosensitivity with the MTT assay on blast cells from adult patients with AML at onset of disease. 62 patients with AML diagnosed according to the FAB criteria (2 M0, 3 M1, 32 M2, 7 M4, 12 M5, 5 M6, 1 M7) were included in our study. The drugs tested were ARA-C, MITOX, DNR, IDA and VP-16; each drug was tested in different concentrations in triplicate. We also evaluated the cytotoxicity of ARA-C plus VP-16 or DNR or IDA or MITOX, in different concentrations. Leukemic cell survival (LCS) was calculated by the following equation: LCS = OD treated wells/OD control wells x 100. Sensitivity was defined as less than 50% LCS at the highest concentration of the drug.

We found in vitro sensitivity in 79% of samples tested with IDA, in 73% with DNR, in 62% with MITOX, in 47% with VP-16 and in 44% with ARA-C. The combination of ARA-C plus VP-16 showed a significantly higher cytotoxicity than ARA-C or VP-16 alone, whereas the cytotoxicity of DNR, IDA and MITOX was not increased by ARA-C. The MTT test correlated with the in vivo response in 62% of patients; in particular, patients with correlation between the MTT test and in vivo response were significantly younger than patients without correlation.

Introduction

Despite treatment improvements in adult acute myeloid leukemia (AML), 5-35% of patients fail to respond to induction therapy and up to 90% of patients who achieve complete remission relapse within 2 years [1, 2].

As the first cause of these poor results seems to be drug resistance of all leukemic cells or only a subset of them with high clonogenic activity, many in vitro drug resistance assays have been developed aiming to predict clinical response to induction therapy and to select drugs for individualizing therapy.

Several studies have reported that the results of in vitro drug sensitivity assays could be associated with clinical outcome: in particular, the long-term clonogenic assay was first described in AML to have a good clini-

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cal correlation, as regards response to induction treatment and duration of complete remission.

If the chemosensitivity of clonogenic cells does not differ significantly from that of the predominant non-clonogenic leukemic clone, non-clonogenic short-term assays may also be able to predict clinical response and long-term outcome [4].

The differentiating staining cytotoxicity (DiSC) and fluorimetric microculture cytotoxicity assays (FMCA) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) test are a better choice for selecting drugs to use for induction therapy because they provide results in only 48 h compared to the 14 days of the clonogenic test [5-7].

Among them, the MTT assay is particularly interesting as in addition to being simple to perform, it is automated and reproducible, being based on the reduction by living cells of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, to a formazan precipitate measurable by spectrophotometry at 540 nm [8-11].

We have already reported the feasibility and the ability of the MTT assay to evaluate in vitro chemosensitivity in AML patients; in this study we confirm these results and compare in vitro sensitivity to drugs used for induction therapy with clinical response in 62 AML patients [12].

Materials and Methods

Cells

A total of 62 leukemic cell samples was obtained from the peripheral blood and/or bone marrow of patients with newly diagnosed adult acute myeloid leukemia. The mononuclear cells were isolated on a Ficoll density gradient, washed twice and resuspended in Iscove's Modified Dulbecco Medium containing 20% heat-inactivated fetal calf serum, 1.250 µl/ml gentamycin and 40.3 µ l/ml of a 7.5% sodium bicarbonate solution. Viability was determined by the Trypan-Blue exclusion test and stained smears indicated that there were more than 90% leukemic cells in all cases. There was no difference in drug resistance between bone marrow and peripheral blood samples.

Treatment

Patients under 60 years were treated according to the GIMEMA-EORTC protocol including DNR 50 mg/m^2 or MITOX 12 mg/m^2 or IDA 10 mg/m^2 (3 days) plus ARA-C 100 mg/m^2 (10 days) plus VP-16 100 mg/m^2 (5 days). Patients over 60 years were treated according GIMEMA protocol including MITOX 8 mg/m^2 or IDA 10 mg/m^2 (3 days) plus ARA-C 100 mg/m^2 (5 days) plus VP-16 100 mg/m^2 (3 days).

Drug Exposure

The drugs tested were Cytosine-Arabinoside (ARA-C), Mitoxantrone (MITOX), Daunorubicin (DNR), Idarubicin (IDA), Etoposide (VP-16). Each drug was tested in three concentrations in triplicate. The concentration ranges (0.001-10 µM for DNR, IDA and MITOX, 0.01-100 µM for VP-16, 0.1-1000 µM for ARA-C) cover clinically achievable concentrations in the serum of patients. Aliquots (90 µl) of cell suspension (3-4 $\times$ 10^6 cells/ml) were added to 10 µl of the various drug solutions in a 96-well round bottomed microculture plate. Three wells containing cells and 10 µl of medium were used to determine control cell survival. The plate was incubated in a humidified atmosphere for 2 days at 37 °C in 5% CO_2. Cells were continuously exposed to the drug throughout this period.

We also evaluated the cytotoxicity of ARA-C plus VP-16 or DNR or IDA or MITOX in different concentrations.

MTT assay

Ten µl of MTT solution (5 mg/ml) were added to each well and, after gently shaking for 1 min, the plate was incubated for a further 5 h. MTT was reduced to a coloured formazan and then the formazan crystals were dissolved with 100 µl of DMSO. The optical density (OD) of the wells, linearly related to

the cell number, was measured with a microplate reader (Titertek Multiskan) at 540 nm. Leukemic cell survival (LCS) was calculated by the following equation: LCS = (OD treated wells / OD control wells) x 100. Sensitivity was defined as less than 50% leukemic cell survival at the highest concentration of the drug.

CD34 and Lymphoid-Associated Antigen Expression

A leukemic cell suspension of 100 ml was incubated with 10 ml of conjugated monoclonal antibody (CD34, CD2, CD4, CD7, CD10, CD19) for 20 min at 4 °C in the dark; an isotype matched monoclonal antibody was used as negative control. After washing, cells were processed with a FACScan flowcytometer (Becton Dickinson); samples were considered positive when more than 20% of the cells expressed the antigen.

MDR Expression

MDR expression was analyzed using the MDR specific antibodies C219 and MRK-16 with the techniques described in detail elsewhere; samples were processed on a FACScan and considered positive when containing more than 10% MDR+ cells.

Marrow Leukemic Index

The marrow leukemic index (MLI) calculated on bone marrow aspirates, representing the residual (%) of initial leukemic cell mass (% blasts × cellularity), was calculated according to the following formula:

$$\text{MLI} = \frac{\text{Blasts (\%)} \times \text{cellularity (after the first course of therapy)}}{\text{Blasts (\%)} \times \text{cellularity (pretherapy)}} \times 100.$$

Clinical Correlation

We considered the in vitro results to be correlated with clinical response when patients achieved complete remission and at least one of the drugs used in vivo was in the range of the in vitro sensitivity or when patients had resistant disease and all the drugs used in vivo were in the range of in vitro resistance. The in vitro results were judged not to be correlated with clinical response when the patient had resistant disease whereas at least one drug used in vivo was in the range of in vitro sensitivity or when patients achieved complete remission whereas all the drugs used in vivo were in the range of in vitro resistance.

Statistics

Differences in clinical and laboratory features among patients with or without correlation between clinical response and MTT test were analyzed using the Wilcoxon test. Statistical analysis of the in vitro-in vivo relationship was performed with Chi Square contingency table analysis.

Results

Patients and Drugs

62 patients with AML at onset were included in our study: 30 males and 32 females with a median age of 58 years (range 16-78); white blood cell counts ranged from 0.5 to 207x106/ml (median 21.4); there were 2 M0, 3 M1, 32 M2, 7 M4, 12 M5, 5 M6 and 1 M7 cases according to the FAB criteria [13].

The characteristics of the patients, divided into two groups according to the correlation of the MTT results with clinical response, are reported in Table 1; statistical analysis indicated that patients with correlation were significantly younger ($p < 0.01$). There were no differences between the two groups as regards FAB type, Marrow Leukemic Index, CD34, lymphoid-associated antigens and MDR expression.

In vivo, 32 patients (52%) responded to chemotherapy and 30 failed to respond while in vitro, 47 samples (76%) were found to be sensitive to at least one of the drugs used in vivo and 15 were resistant to all tested drugs. In detail, we found in vitro sensitivity in 45/47 (79%) samples tested with Idarubicin, in 41/56 (73%) with Daunorubicin,

Table 1. Characteristics of patients

	S/S R/R	S/R R/S	
Total cases	37	25	
M/F	19/18	13/12	
Median age (range)	51 (16-78)	61 (19-72)	$p < 0.01$
CD34+	6/36	15/24	
LDH	704 (143-1411)	550 (285-2057)	
MLI	2.45 (0-712)	24.1 (0.5-184.6)	
Ly+	15/37	10/25	
C219+	17/20	11/16	
MRK-16+	9/11	11/12	

Abbreviations: S (Sensitive), R (Resistant): in vitro/in vivo

in 38/61 (62%) with Mitoxantrone, in 29/61 (47%) with Etoposide and in 25/57 (44%) with Cytarabine. All the drugs showed concentration-dependent cytotoxicity.

Clinical Correlation

The clinical correlation could be evaluated in all patients because at least 3 of the 5 drugs tested in vitro were then used in vivo.

The MTT test correlated with the in vivo response in 37 of 62 patients (60%): 27 in vitro sensitive patients achieved complete remission and 10 in vitro resistant patients had resistant disease. Among the patients with no correlation, 20 in vitro sensitive patients had resistant disease and 5 in vitro resistant patients achieved complete remission. The 5 patients who were resistant in vitro but responded in vivo to chemotherapy showed significantly lower values of Marrow Leukemic Index (MLI) compared to the 20 in vitro sensitive patients who failed to respond ($p < 0.05$), but had a very low disease free-survival (DFS) (Table 2).

When we analyzed patients according to age we found that the MTT test correlated with in vivo response in 73% (25/34) of patients under 60 years of age but only in 43% (12/28) of patients over 60 (Table 3) (Fig. 1).

Table 2. Characteristics of patients with no correlation

	S/R	R/S	
Total cases	20	5	
M/F	11/9	2/3	
Median age (range)	61 (19-72)	63 (43-71)	
CD34+	14/19	1/5	
LDH	467 (285-2057)	1097 (457-812)	
MLI	45.1 (0.6-184.6)	1.1 (0.5-2.5)	$p < 0.05$
Ly+	8/12	2/5	
C219+	9/13	2/3	
MRK-16+	10/10	1/2	

Abbreviations: S (Sensitive), R (Resistant): in vitro/in vivo.

Table 3. Clinical response and in vitro results according to age

	Total cases		< 60 years		> 60 years	
	S	R	S	R	S	R
CR	27	5	21	2	6	3
NR	20	10	7	4	13	6

Abbreviations: CR: complete remission; NR: non responder; S: in vitro sensitive; R: in vitro resistant.

Table 4. Clinical response and in vitro results according to each single drug

	ARA-C		VP-16		DNR		IDA		MITOX	
	S	R	S	R	S	R	S	R	S	R
CR	18	12	15	11	6	1	14	5	9	2
NR	7	19	4	18	3	1	11	5	3	1
p	<0.05		<0.02		n.v.		n.s.		n.v.	

Abbreviations: CR: complete remission; NR: non responder; S: in vitro sensitive; R: in vitro resistant.

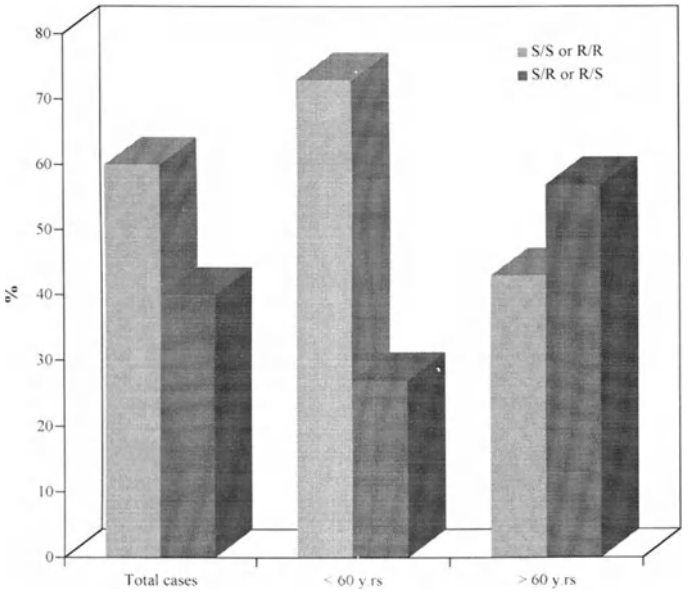

Fig. 1. Clinical correlation according to age

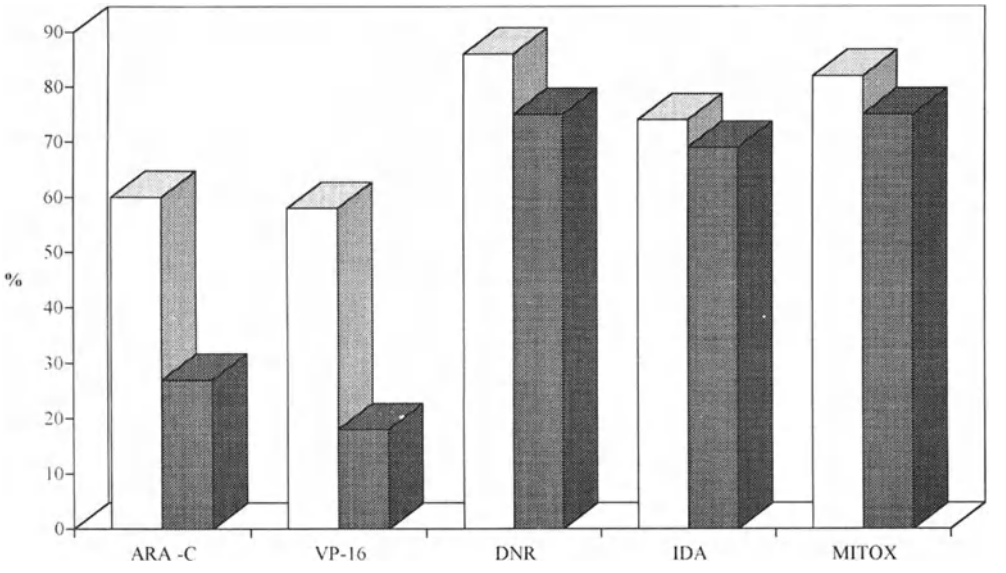

Fig. 2. Percentage of in vitro sensitivity in patients who achieved complete remission *(blank blocks)* and in patients who had resistant disease *(filled blocks)*

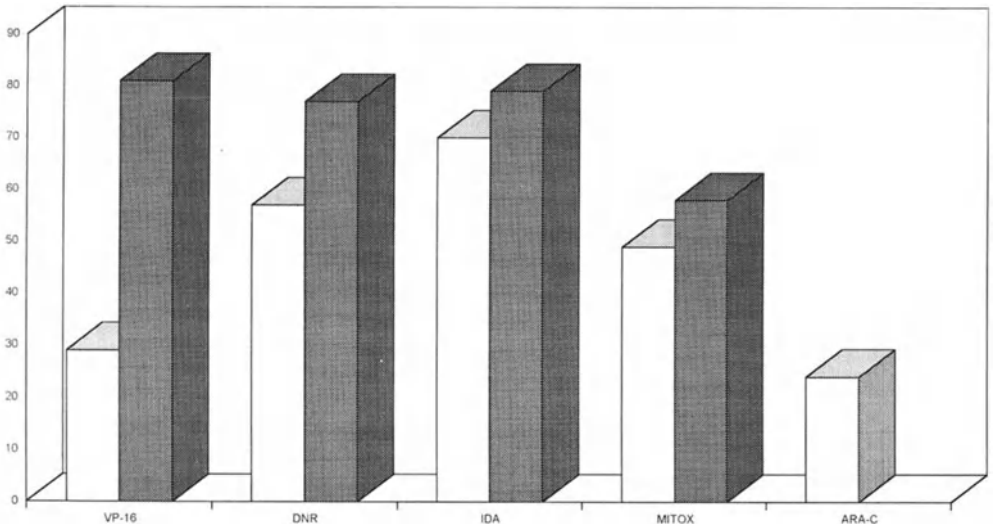

Fig. 3. Percentage of patients sensitive in vitro to VP-16, DNR, IDA, Mitox, ARA-C alone *(blank blocks)* or associated with ARA-C *(filled blocks)*

As regards correlation of in vitro results for single drugs, only for Cytarabine and Etoposide was there a significantly higher frequency of sensitivity in vitro in patients that achieved complete remission compared to patients that failed to respond ($p < 0.05$ and $p < 0.02$ respectively) (Table 4, and Fig. 2); moreover, the age factor did not influence these results. Only in 19/35 (54%) of patients treated with Idarubicin was there correlation with clinical response; for Mitoxantrone and Daunorubicin it was not possible to analyze the results statistically because of the low number of assessable patients, but correlation was found in 10/15 (67%) of patients treated with Mitoxantrone and in 7/11 (64%) treated with Daunorubicin.

The combination of Cytarabine plus Etoposide showed a significantly higher cytotoxicity than ARA-C or VP-16 alone, while the cytotoxicity of Daunorubicin, Idarubicin and Mitoxantrone was not increased by Cytarabine (Fig. 3).

Discussion

Advances in chemotherapy and supportive care have significantly improved the prognosis of patients with AML, but 5-35% of them fail to respond to induction therapy and 50-90% of patients who achieve complete remission relapse within 2 years. Drug resistance is presumed to be the first cause of failure in the treatment of adult AML, while CD34 positivity has been associated with the expression of P-glycoprotein. Other Authors have also reported that CD34 positivity is associated with in vitro DNR resistance [14, 15].

In the present study we found no correlation between the FAB subtype, MLI, CD34, lymphoid associated antigens or MDR expression and in vitro chemosensitivity. Instead, there was a strong correlation between the age of the patients and the results of clinical correlation. In particular, analyzing patients according to age we found that the MTT test correlated with in vivo response in 73% of patients under 60 years of age versus 43% of patients over 60.

The lack of correlation in elderly patients could be attributable in part to less aggressive chemotherapy, while the percentage of in vitro chemosensitivity did not change with the age of patients. These results suggest that the MTT assay may be useful in evaluating chemosensitivity and in selecting drugs for induction therapy only in younger AML patients.

Idarubicin, Daunorubicin and mitoxantrone showed the highest overall cytotoxic-

ity while the chemosensitivity of etoposide and cytarabine was significantly correlated with clinical response, regardless of the patient's age.

The increased cytotoxicity showed by the combination of cytarabine and etoposide seems to justify the clinical use of these drugs despite the low cytotoxicity they demonstrated when assayed alone.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Drug Resistance Testing of Acute Myelogenous Leukemia in Adults – Initial Results with the MTT Assay

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Abstract. Drug resistance is a major cause of treatment failure. Drug sensitivity in vitro can serve as prognostic parameter in leukemias. While a considerable amount of data is available in children, data of short-term assays in adult leukemia are still limited.

We therefore studied adult patients with newly diagnosed AML. MTT tests were performed according to Pieters et al. More than 14 cytostatic drugs were tested in a wide range of concentrations over 4 days and compared with controls. If necessary, blasts were enriched > 80% by negative selection with dynabeads. Percent S-phase was measured at begin of assay and after 4 days. Up to now, samples from 40 adult AML patients were prospectively tested in vitro at diagnosis. Technical success rate was about 90% and IC50 values as a measure for drug resistance were highly reproducible. Large differences in drug resistance between patient samples were observed (over 1000 fold on average).

There was a correlation of in vitro sensitivity between the topoisomerase II related drugs mitoxantrone, daunorubicin, amsacrine and etoposide. Also we found a correlation of in vitro drug resistance with cytogenetic risk for drugs like ara-C and mitoxantrone, whereas other clinical prognostic parameters like patient age, initial WBC, FAB subtype and % S-phase do not show a correlation up to now. Correlations with outcome, and comparison of MTT test with SIA assay will be performed. Tests like these may aid in the rational choice of drugs in patients with poor prognosis or relapse and with conditioning regimens, and thus improve therapy outcome.

Introduction

Drug sensitivity in vitro can serve as a valuable prognostic parameter in leukemias, as has been shown in prospective studies especially in children with ALL [1]. In adults there are relative few data regarding short-term assays in AML [2-8], but there is also emerging evidence of the prognostic relevance of in vitro testing in adult AML [9, 10]. A major aim of our study is to evaluate the MTT in vitro test for AML in adults. In particular, we want to investigate its feasibility, reproducibility, interindividual variability and prognostic significance – like correlations with known prognostic factors, initial clinical response and ultimately disease free survival.

Materials and Methods

Up to now, samples from 40 adult patients with AML at diagnosis have been prospectively tested in vitro, including two patients with secondary leukemia and two patients at relapse. Median age (range) was 51 years (17-72) and WBC 66 $x10^9/l$ (7-359); there were three M0, eleven M1, twelve M2, two M3, six M4, five M5 and one M6 according to

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FAB classification. Patients were treated according to SHG (Süddeutsche Hämoblastosegruppe) cooperative study AML 91/96 or EORTC APL 93 protocol.

MTT tests are performed according to Pieters et al. [11]: MNC are obtained from peripheral blood or bone marrow after density centrifugation. Each drug and concentration is tested on microtiter plates in duplicate with 8-16 x 10^4 cells per well over 4 days and compared with controls without drug on day 4 (= 100% cell survival). More than 14 cytostatic drugs are tested over a wide range of concentrations. Drugs are stored in dilutions at -20 (C for < 3 months prior to testing. Less than 107 blasts are needed to test 3 cytostatic drugs. If necessary, RBC lysis is performed and blasts are enriched > 80% by negative selection with dynabeads (12). Blasts are then incubated for 4 days at 37 (C with 5% CO_2 in RPMI-1640 DM with 15% FCS and addition of ITS (insulin, transferrin, selenite).

MTT – a tetrazolium dye – is added after 4 days, and cells are incubated for another 6 hours. Living cells metabolize the dye and form coloured crystals, which are dissolved by the addition of acidic isopropanol. Absorption is measured by ELISA reader at 570 nm. Samples are considered evaluable if the drug-free control wells contain > 80% leukemic cells before and > 70% leukemic cells after 4 days of culture, and if control optical density (OD) on day 4 exceeds 0.15. Data is obtained by Mikrowin software (from Mikrotek), and concentration-survival data are analyzed by sigmoid regression using the 4-parameter function with Sigma-Plot (from Jandel Scientific). IC_{50} and IC_{90} values are calculated – the concentration at which 50 or 90% of cells are killed – and serve as a measure for the degree of cytostatic drug resistance. Percent S-phase of leukemic blasts is determined by methods described elsewhere [13].

Results

Median cell viability of control (range) at start of assay was 97% (88-99) and after 4 days 80% (47-97); optical density of controls

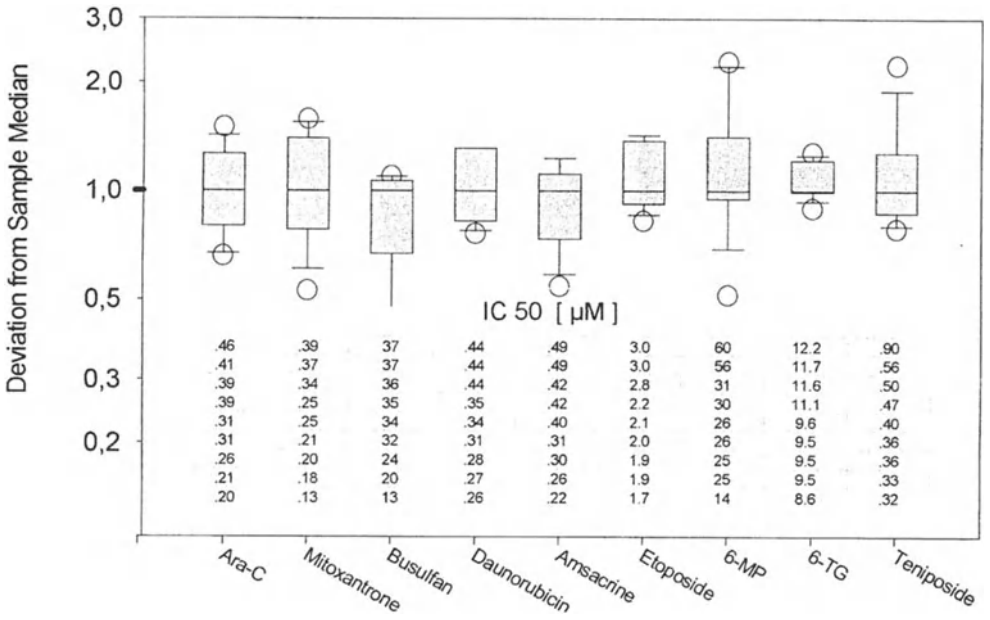

Fig. 1. Assay reproducibility from day to day. Frozen samples from a CML patient in blast crisis with 86% blasts were tested on different days by different people. Shown are results and interassay variability for n=9 experiments, also the 10, 25, 75 and 90% percentiles and individual values outside this range. Measured IC_{50} values are sorted from high to low for each drug tested

Table 1. IC_{50} values – the concentration at which 50% of leukemic blasts are killed – and median relative resistance of adult AML compared to children (n=40). Relative resistance is calculated by dividing IC_{50} values of adults by median IC_{50} values reported for children with AML where available. If relative resistance is > 1, then AML blasts from adults are more resistant than those from children and vice versa. # Concentration of L-asparaginase in IU/ml

Drug	IC_{50} [μM] Median	Range	Relative Resistance
Ara-C	3.3	(0.3–1478)	1.5
Mitoxantrone	0.57	(0.07–15)	1.8
Busulfan	235	(17–>1000)	
Daunorubicin	0.46	(0.1–7.1)	1.3
Amsacrine	1.4	(0.01–>30)	0.9
Etoposide	9.7	(0.6–208)	0.9
Prednisolone	172	(0.1–>3000)	0.4
Dexamethasone	> 3000	(0.01–>3000)	
L-Asparaginase	0.54 #	(0.01–>30)	0.5
6-MP	927	(8.6–>3000)	1.5
6-TG	37	(7.4–185)	0.7
Teniposide	2.3	(0.1–121)	3.6
Idarubicin	0.36	(0.03–2.8)	
Vincristine	10	(0.01–>100)	3.4

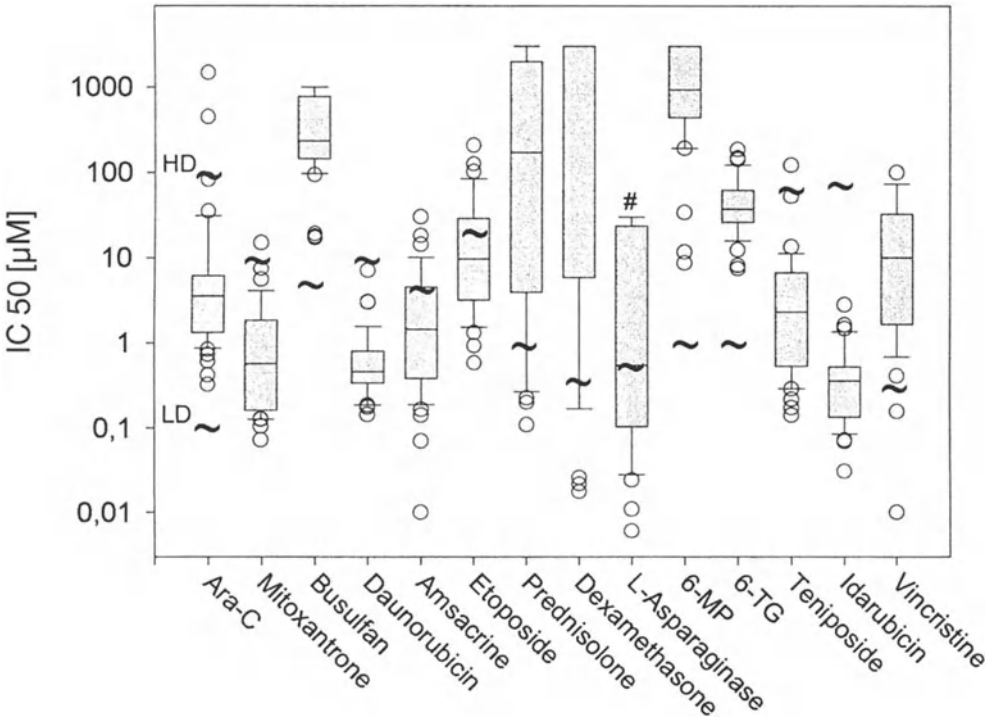

Fig. 2. IC_{50} values as measure of cytostatic drug resistance of adult AML in vitro (n=40). Shown are the 10, 25, 75 and 90% percentiles and individual values outside this range. For orientation, population peak levels from the literature of cytostatic drugs at usual dosages are inserted (~).
HD = high dose, *LD* = low dose. However, in the clinical evaluation other factors also have to be taken into account: e.g. IC_{90}, pharmacokinetics and variability, infusion duration, drug stability, protein binding, metabolism and mechanism of action. # Concentration of L-asparaginase in IU/ml

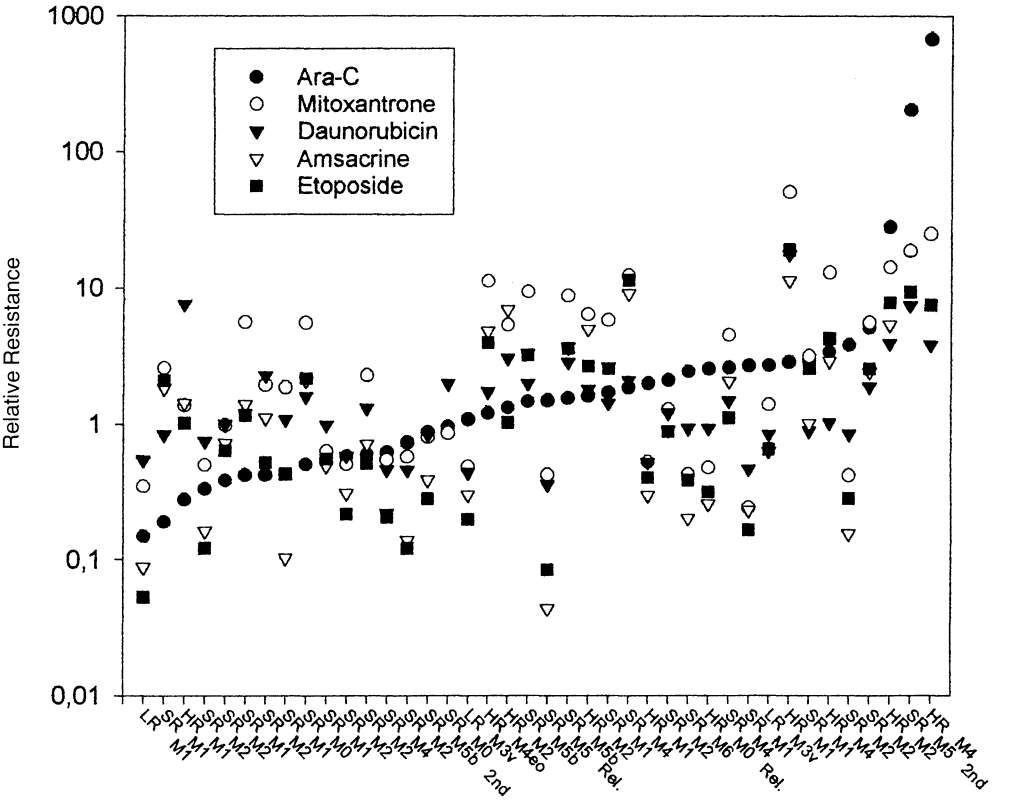

Fig. 3. AML in vitro drug resistance versus cytogenetic risk and FAB classification (n=40). Patients are sorted by increasing ara-C resistance of their blasts in vitro. Relative blast resistance for each drug was calculated dividing IC_{50} values of adult patients by median IC_{50} values reported for children with AML. LR (n=3), SR (n=27) and HR (n=10) - cytogenetic risk groups; stratification according to SHG (Süddeutsche Hämoblastosegruppe) cooperative study AML 96: *LR* = low risk: t(8;21) without other abnormalities except -Y (M3 is being included here). *SR* = standard risk: all patients with no LR or HR abnormalities. *HR* = high risk: -5/del(5q), -7/del(7q), other monosomies, inv(3q), abnl 12p, abnl 11q, + 11, +13, +21, +22, t(6;9), t(9;22), t(9;11), t(3;3), multiple aberrations or documented MDS. *M1–M6* subtype according to FAB classification. *Rel.* = relapse, *2nd* = secondary leukemia. Note the similar behaviour of in vitro resistance for mitoxantrone, daunorubicin, amsacrine and etoposide in comparison to ara-C

after 4 days was 0.40 (0.13-0.94). Technical success rate was about 90% in fresh samples even if sent by overnight mail, so that in vitro drug resistance testing could be successfully performed in most patients. Percent S-phase at begin of assay was 3% (0.1-15) and after 4 days 9% (2-34). In vitro results were highly reproducible. Interday and interperson variablility of measured IC50 values was small (Fig. 1). Median CV in % (range) of the IC50 for n=9 experiments and several drugs tested was 28% [13-47].

Leukemic blasts from 40 adults with AML were tested in vitro. Interindividual differences in cytostatic drug resistance were large, as can be seen from the range of measured IC_{50} values (Fig. 2). IC_{90} values could be generated for all drugs, except busulfan, prednisolone, dexamethasone, L-asparaginase and 6-MP, and parallelled IC_{50} values (data not shown). For better comparison IC_{50} values of adults were divided by median IC_{50} values reported for a larger cohort of children with AML (14). Median IC_{50} values and median relative drug resistance of adult AML compared to children are shown in Table 1.

Large differences between individual drugs were observed, with individual resistance profiles for each patient. Figure 3 shows data from drugs commonly used in AML protocols, and patients are sorted by

relative in vitro resistance to ara-C, a drug that has been shown to be of prognostic relevance in adult AML [9]. Patients resistant to one drug are not necessarily so to another. However, in vitro resistance to mitoxantrone, unlike ara-C, correlated with resistance to daunorubicin, amsacrine and etoposide (Fig. 3). If patients with IC_{50} values above the median are classified resistant then 7 of 10 patients with high risk cytogenetics were in vitro resistant, both to ara-C and mitoxantrone. 5 of 10 patients with high risk cytogenetics in their leukemic blasts were in vitro resistant to both drugs.

So far no correlation could be found between in vitro drug sensitivity and other clinical risk factors like patient age, initial WBC, FAB classification and % S-phase (data not shown). Other risk factors like immunophenotype, prior MDS and relapse are being evaluated; further samples need to be tested before firm conclusions can be drawn.

Discussion

Ninety percent of AML samples can be assayed by the MTT test; this is a high technical success rate compared to data published for ALL. Only 10-20 million leukemic cells are needed to test six cytostatic drugs, which equals on average 1 ml of bone marrow or a few mls of peripheral blood. Reproducibility of the in vitro data in our hands was remarkable for biological assays with an average CV of $\approx$ 25 % between days and technicians.

Large interindividual differences exist in drug sensitivity between AML patient samples; IC50 values varied by a factor of $\approx$ 1000 (25-300 000 depending on the drug). It is likely that these differences somehow translate into different clinical response and therapy outcome. The in vitro sensitivity obtained in adults with AML is somewhat higher than that reported for children [14]. In vitro results in part reflect clinical experience in AML and pharmacological knowledge. The poor response to steroids in adult AML, for instance, is mirrored in vitro; also the better response of AML to 6-TG than 6-MP.

Relative drug resistance of patient blasts in comparison to a cohort of patients (individual IC_{50} /median IC_{50}) is meaningful and perhaps can serve as an important biological parameter. Interestingly, resistance to mitoxantrone correlated with resistance to daunorubicin, amsacrine and etoposide – all drugs which act via topoisomerase II – whereas there was no correlation between mitoxantrone and the antimetabolite ara-C. A similar observation was made by Klumper et al. [9].

The differences in prognosis between cytogenetic risk groups might be explained, at least in part, by the differences of in vitro sensitivity to cytostatic drugs. Correlations between in vitro results and initial clinical response or survival data are still too early to allow a conclusion regarding the prognostic value of MTT in vitro testing in adults with AML from our data; further patient accrual and longer follow-up are required.

Comparison of MTT test with SIA assay [15] – a stromal cell based, flow cytometric assay – will be performed. Short term assays like these could provide valuable information for risk group stratification or aid in the rational and individualized selection of drugs, and thus perhaps improve therapy outcome. In vitro testing is also used in our laboratory to examine new drugs, modulators and drug combinations.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Predictive Value of Pretherapeutic In-Vitro Chemosensitivity Testing in Adult AML

P. Staib, B. Lathan, K. Mickel, E. Janz, T. Schinköthe, D. Voliotis, and V. Diehl

Abstract. Individual prognosis in adult AML may still not be determined by known prognostic factors, e.g. age, cytogenetics. Short term in-vitro drug sensitivity tests such as the differential staining cytotoxicity (DISC) assay were developed to determine individual treatment outcome. We prospectively correlated DISC assay results and treatment outcome in patients (pts.) with AML using a new method of analysis. Pts. were treated according to the TAD-HAM regimen (thioguanine, Ara-C, daunorubicin-high dose Ara-C, mitoxantrone) in de-novo AML or the Ida-FLAG regimen (idarubicin, fludarabine, Ara-C, G-CSF) in relapsed or MDS-AML. The DISC assay was performed as described by Weisenthal et al. [1]. All drugs used for therapy were pretherapeutically tested at 5 different concentrations in primary cell cultures in triplicate samples of each patient. Assay results measured in % tumor cell survival (TCS) were transformed into a mathematical equation, which described the dose response relation. The area under the curve (AUC) and the calculated TCS at the middle test-concentration were then transformed into an index called chemosensitivity index C_i. If C_i was > 0.5 probability of clinical response to that drug was defined to be high, and if C_i was < 0.5 to be low. Remission duration and survival were estimated according to the Kaplan-Meier method. In 65 pts. clinical outcome was evaluable; since 5 assays failed, 60 pts. were eligible for correlation; 43 received the TAD-HAM regimen, 17 the Ida-FLAG regimen. 53 pts. reached CR or total blast clearance in control BM aspirates and were found with $C_i > 0.5$ of at least one drug used for therapy (TP=true positive correlation). 6/7 pts. were nonresponders and identified with $C_i < 0.5$ of all drugs given for treatment (TN=true negative). Only 1 pt. with $C_i < 0.5$ came into CR (FN=false negative). TP=53, TN=6, FN=1. Thus, overall predictive accuracy was 98%. Mean survival of the group with $C_i > 0.5$ was 672 days, median is not yet reached. Mean survival of the group with $C_i < 0.5$ was 86 days, median 92 days ($p<0.01$, logrank). Mean remission duration (n=42; $C_i > 0.5$) was 449 days, median 275 days. In conclusion, in-vitro chemosensitivity tests may provide a valuable tool for prediction of individual treatment outcome in pts. with AML. Prospective clinical trials using the DISC assay for treatment stratification and assay directed therapy strategies would be justified.

Introduction

Several prognostic factors have been identified for treatment outcome in adult acute myeloid leukemia (AML), e.g., age, cytogenetic abnormalities, initial white blood count, LDH, FAB-subtype etc. However, individual prognosis and response to treatment may still not be determined by these factors. Consequently, during more than 40 years of research many in- vitro drug sensitivity tests

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have been developed in order to predict individual treatment outcome in AML and numerous other neoplastic diseases. It could be shown that the differential staining cytotoxicity assay (DISC-assay) [1, 2], and also metabolic function assays such as the MTT-assay [3] are the short term in-vitro drug sensitivity tests of choice in acute leukemias. The accuracy of an in-vitro assay should be assessed not only by correlation of assay results with response rates but also with remission duration and survival [4]. The majority of published data consists of retrospective rather than of prospective clinical correlations [4]. We, therefore, conducted a prospective study to correlate response to therapy, remission duration and overall survival with its prediction by the DISC-assay in adult patients with AML using a new method of analysis.

Material and Methods

Patients and Treatment

At our institution patients with de-novo AML were included into the German prospective randomized multicenter trial of the AMLCG (AMLCG-´92 trial) [5]. Induction therapy consisted of the TAD-HAM double induction regimen containing thioguanine, Ara-C, daunorubicin-high dose Ara-C, mitoxantrone. Patients suffering from relapsed AML or AML with a history of preexisting myelodysplastic syndrorme (MDS-AML) were treated with the Ida-FLAG regimen consisting of idarubicin, fludarabine, Ara-C and G-CSF [6, 7].

DISC Assay

The DISC-assay was performed according to the methods described by Weisenthal et al. with minor modifications [1]. Briefly, bone marrow or peripheral blood specimens from each patient with AML were collected into heparin prior to the administration of chemotherapy. Leukemic blast cells were isolated by Ficoll density gradient centrifugation, washed and suspended in RPMI 1640 culture medium supplemented with fetal calf serum (FCS). All drugs used for treatment were tested at five concentrations in triplicate. The middle test concentration of each drug was chosen within the range of clinically relevant steady state plasma levels [8]. Drug (20 µl) was added to 90 000 cells in 180 µl medium; phosphate-buffered saline (PBS) in medium served as a control. After 94 h of incubation (37 °C, humidified 5% CO_2), 50,000 fixed duck erythrocytes (DRBCs) were added to each tube in 10 µl PBS containing 2% fast-green and 1% nigrosin. The cells were transferred to collagen surfaced microscope slides by cytocentrifugation, air-dried and counterstained with May-Grünwald-Giemsa stain. Subsequent evaluation of slides by light microscopy facilitated the determination of drug efficacy at each concentration compared with controls. Assay results are expressed as percent tumor cell survival (% TCS).

Data analysis

TCS-data were transformed into the following mathematical equation by logarithm and linear regression for description of the dose response curve:

$$TCS\ (conc) = e^{a+b*conc}$$

(conc = drug test concentration; e = exponential e; TCS = percent tumor cell survival; a = y-intercept; b = gradient; * = multiplication).

A typical dose response curve for Ara-C and prednisone in a patient with AML is illustrated in figure 1a and 1b:

The area under the curve (AUC) as a measure for the in-vitro dose-response relation and the calculated TCS at the middle test concentration (TCSm) were transformed into an index called chemosenisitivity Index C_i:

$$C_i = e^{-2.354*AUC*TCS_m}$$

(-2.354 is a constant derived after correlation of Ci data of 20 AML patients with clinical response to induction therapy).

For practical reasons the cut-off point for the prediction of resistance or response was adjusted at 0.5. If C_i was > 0.5 the probability of clinical response to that drug was defined to be high, and if C_i was < 0.5 probability of clinical response to be low. The highest scoring C_i of those drugs clinically used was

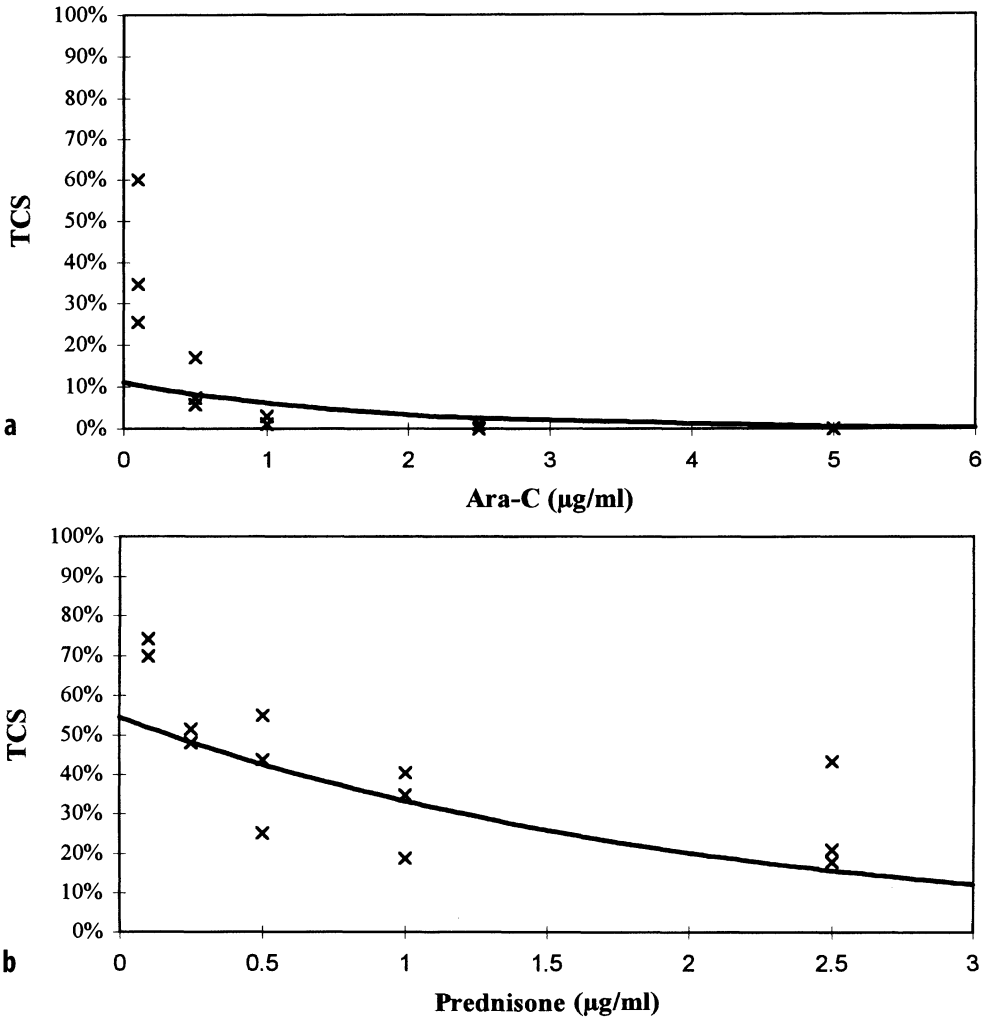

Fig. 1a, b. Computerized dose response curve (—) for Ara-C *(a)* and prednisone *(b)* derived from DISC-assay results (x) in a patient with AML. *TCS* = tumor cell survival

called C_i-max. Correlation of C_i-max with patient response was assessed according to the definitions given in Table 1:

Remision duration and survival curves were estimated according to the method described by Kaplan and Meier. Differences in survival between patients with C_i-max < or > 0.5 was assessed by the logrank test.

Table 1. Definition of correlation of chemosensitivity index C_i with clinical response

C_i-max	Response	No response
> 0.5	True positive (TP)	False positive (FP)
< 0.5	False negative (FN)	True negative (TN)

Results

In 65 patients clinical outcome was evaluable; since five assays failed, 60 of 65 patients were eligible for correlation analysis. 43 patients received the TAD-HAM regimen and 17 the Ida-FLAG regimen for induction therapy. Results of correlation of C_i with clinical response are summarized in Table 2:

Table 2. Correlation of C_i-max with clinical response

C_i-max	Response	Non-response
> 0.5	53 (TP)	0 (FP)
< 0.5	1 (FN)	6 (TN)

Results describing the accuracy of the DISC-assay in predicting treatment outcome are listed in table 3:

Correlation of C_i-max with survival and remission duration is shown in figures 2 and 3. Mean survival of the group with C_i-max > 0.5 was 672 days, median is not yet reached. Mean survival of the group with C_i-max < 0.5 was 86 days, median survival 92 days. The difference in survival between the two groups was statistically highly significant ($p < 0.01$, logrank). Mean remission duration was 449 days, median 275 days.

In patients with C_i-max < 0.5 predominantly unfavourable karyotypes were found except for one case with a t(8;21) translocation (Table 4).

Table 3. Accuracy of DISC-assay results

Parameter	Definiton	Value (%)
(+) Predictive accuracy	TP/(TP+FP)	100
(-) Predictive accuracy	TN/(TN+FN)	86
Overall pred. accuracy	(TP+TN)/ (TP+TN+FP+FN)	98
Sensitivity	TP/(TP+FN)	98
Specifity	TN/(TN+FP)	100

Table 4. Cytogenetics in patients with C_i-max < 0.5

Karyotype (C_i max < 0.5)	n
-5	2
-7	1
Complex aberration	1
t(8;21), X0	1
n.d.	2

Discussion

In-vitro drug resistance assays have been developed with the ultimate aim of predicting clinical drug resistance and to tailor

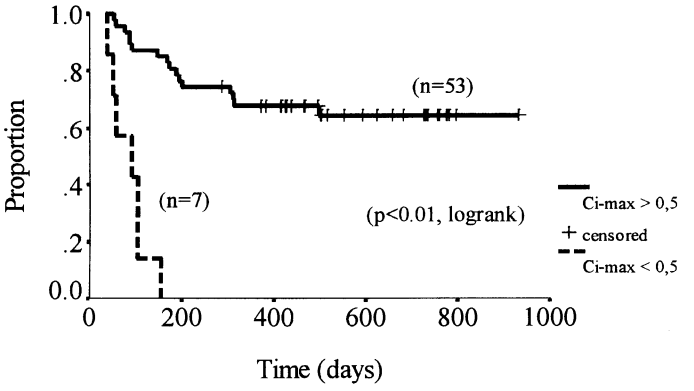

Fig. 2 Overall survival dependent on C_i-max > 0.5 or < 0.5

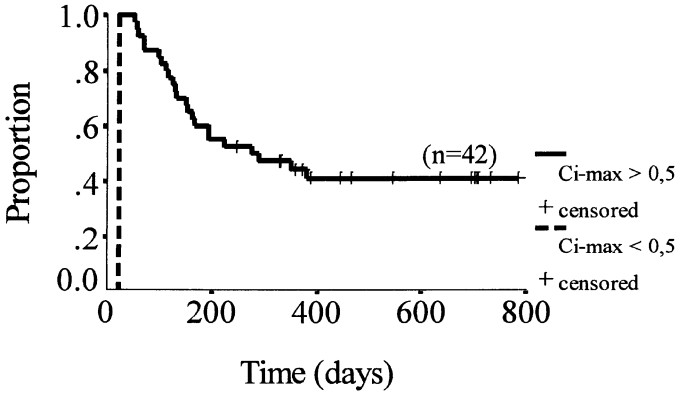

Fig. 3 Remission duration dependent on C_i-max > 0.5 or < 0.5

chemotherapy in patients with resistant disease or poor prognosis. The vast majority of both clonogenic and non-clonogenic in-vitro chemosensitivity assays were able to predict the short term clinical outcome in terms of CR [2, 4, 9], but few have reported the long-term clinical outcome in AML [8, 10, 11] or ALL [12]. For the assessment of the accuracy of an in-vitro chemosensitivity assay it is necessary to compare the test results not only with short term outcome (clinical correlation) but also with patient survival [4]. Clinical correlations are usually obtained by categorising into sensitive or resistant both test results and patient response and comparing the two. The definition of patient response is usually easy (response = CR or PR) whilst test results are not so easily categorized as the test results will usually make up a continuum from very sensitive through very resistant [4]. Defining a cut-off point between sensitive and resistant in vitro can in many cases only be done by comparison with the clinical data. In many studies the percentage of surviving tumor cells (e.g., 30%) at a particular drug concentration or the inhibitory drug concentration to achieve a certain percentage of killed tumor cells (e.g., IC90) were used for cut-off points [2,3,9]. In contrast to these conventional, rigid cut-off point systems our newly developed chemosensitivity index Ci takes into account the total dose response relation by using the AUC.

The application of the C_i method for analysing DISC-assay results in this series of AML patients demonstrated a precise prediction of clinical response to induction chemotherapy with an overall predictive accuracy of 98%. Furthermore, we found that patients with a C_i-max over 0.5 had a significant longer survival than patients with a C_i-max below 0.5. It is important to notice that we could identify 6 patients with primary resistant disease within four days. Interestingly, the majority of these patients had prognostic unfavourable cytogenetic abnormalities. Only one patient found with C_i-max below 0.5 achieved a CR which lasted 22 weeks; this patient had the prognostic favourable translocation t(8;21), but died of septicemia after consolidation therapy without any signs of relapse. A possible reason for this false negative correlation may be a reduced assay quality since viability of the control cells was less than 50%.

Our data confirm that the DISC-assay is one of the most suitable in-vitro drug sensitivity tests for acute myeloid leukemias. Together with the chemosensitivity index C_i analysing method the DISC-assay provides a valuable tool for the prediction of individual treatment outcome in adult AML in terms of clinical response and also survival. The next stage must be to extend these observations to further prospective clinical trials where

1. patients are randomised to receive best available therapy or assay directed therapy or
2. treatment is stratified according to risk groups identified by assay results.

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Functional Studies of Daunorubicin Transport in Human Leukemic Cells

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Abstract. We have compared daunorubicin accumulation and efflux in human leukemic cell lines (K562 and K562/Vcr a mdr1-gene-expressing multidrug resistant subline) and in leukemic cells from patients with acute myeloid leukemia.

The accumulation and efflux were measured by flow cytometry after incubation with daunorubicin for 1 h followed by washing and reincubation in a drug-free medium. The efflux rate was determined by the decline in cellular drug concentration at 12 time points within 60 min.

By incubating K562/Vcr cells with a 6-fold higher daunorubicin concentration, similar intracellular concentration could be achieved as in the parental K562 line. On washing and reincubation in drug-free medium the efflux of daunorubicin was more rapid from the resistant cells only during the first minutes.

The metabolic inhibitors sodium azide (10 mM) and iodoacetate (1 mM) had little or no effect on daunorubicin accumulation in K562/Vcr cells when used separately but when used in combination cellular daunorubicin accumulation increased about 6-fold. This was higher than the increase, about 4-fold, caused by cyclosporin A (3 μM). However, the efflux curves were parallel.

Large differences were found in the accumulation of daunorubicin in the leukemic cells isolated from patients with AML. Cyclosporin A increased the accumulation in 11 of 12 patients, median increase 15%, (range 1-29). The combination of iodoacetate and sodium azide was more effective in that respect, with median increase 43%, (range 18-99). However, the modulating agents had no effect on daunorubicin efflux from the patients' leukemic cells.

The results indicate that cyclosporin A and metabolic inhibitors influence other processes than daunorubicin efflux across the plasma membrane.

Introduction

Several mechanisms for drug resistance have been identified in cell lines in which resistance has been induced by incubation with successively increased concentrations of an antineoplastic drug ([1] review). Most interest has been focused on multidrug resistance, characterized by cross resistance to a wide range of anticancer drugs of natural origin but with different chemical structures and mechanisms of action. Classical multidrug resistance is caused by an increased P-glycoprotein (P-gp)-mediated drug efflux from the tumor cells which can be blocked by various modulators [2]. P-gp is encoded by the mdr1 gene on chromosome 7 [3]. A number of non-toxic substances are known to inhibit the function of P-gp and these are of potential interest for use in patients resistant to conventional treatment.

Depts of [1] Clinical Pharmacology, [2] Hematology and Infectious Diseases, [3] Pathology; Hematopathology laboratory, Karolinska Hospital, Stockholm, Sweden

Expression of Pgp in acute myelocytic leukemia (AML) has been associated to a poor treatment response [4-8]. Many techniques are available for assessment of P-gp expression and there is no consensus on which technique is the most relevant [9]. Furthermore additional membrane proteins have been identified which can affect drug efflux or intracellular drug distribution [10, 11].

The aim of the present study was to establish a functional assay of autofluorescent daunorubicin (Dnr) transport in leukemic cells. The intention was to develop a technique to be able to study the rate of drug transport and not just the intracellular concentration at a certain time point. Here we report a comparison between accumulation and efflux properties of Dnr sensitive and resistant human leukemic cell lines and of leukemic cells isolated from patients.

Material and Methods

Cell Lines

The human leukemic cell line K562 [12] and K562/Vcr, a multidrug-resistant subline were used. K562/Vcr cells were developed by continuous exposure to increasing concentrations of vincristine and were grown in medium containing 150 nM vincristine. The mdr1 mRNA level in these cells is about 200 transcripts per cell as determined by a quantitative RNase protection assay [13]. In K562 no mdr1 mRNA was detected.

Patients

Leukemic cells from 12 patients with AML were studied (Table 1). The diagnosis was based on morphology and immunophenotyping and classification was made according to FAB criteria [14]. Eight patients had de novo AML and 4 AML secondary to MDS. Five samples were taken at diagnosis, 3 at relapse and 4 were taken from patients with progressive disease resistant to cytostatic drugs. The study was approved by the local ethics committee.

Mononuclear cells were isolated from peripheral blood on Ficoll-Paque (Pharmacia, Uppsala, Sweden) by centrifugation at 500 g for 20 min. The cell samples were analyzed on the same day or frozen in a programmed freezer in 50% human AB serum, 40% phosphate-buffered saline (PBS) and 10% dimethylsulphoxide, stored for 24 h in -70 °C and thereafter in liquid nitrogen. For analysis frozen cells were rapidly thawed with a few drops of newborn calf serum (NSC) in a waterbath (37 °C), diluted in 10 ml NCS, centrifuged, washed in medium RPMI 1640 with 20 mM HEPES (Gibco, Life Technologies Ltd, Paisley, Scotland) supplemented with 10% NSC and 1% L-glutamine and resuspended in medium. Cells were adjusted to a concen-

Table 1. Patient characteristics

Pt. no.	Sex/ Age	AML FAB type	WBC $\times 10^9$/L	WBC % leukemic cells	Stage of disease	mdr1 mRNA transcripts per cell
1	M81	M2	17	73	progress	1.2
2	M65	M2	92	88	progress	0.2
3	F/40	M2	37	88	progress	nd
4	F/76	M2	16	66	diagnosis	1.1
5	M/61	M5a	11	80	relapse	0.6
6	F/78	M5	17	92	diagnosis	1.4
7	F/43	M2	25	85	relapse	< 0.15
8	M/68	M2	20	80	relapse	< 0.15
9	M/60	M2	35	88	diagnosis	nd
10	F/29	M5a	33	73	diagnosis	< 0.15
11	M/55	M5a	24	81	diagnosis	nd
12	F/83	M0	110	70	progress	nd

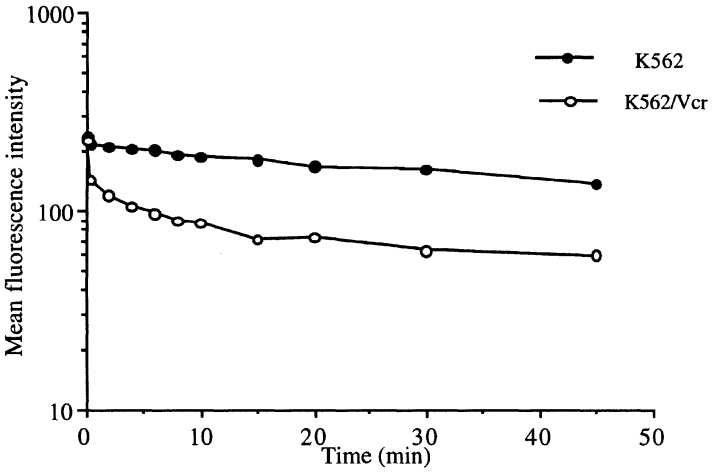

Fig. 1. Accumulation and efflux of daunorubicin in K562 and K562/Vcr cells, incubated with 0.5 and 3 μM, respectively

tration of 5×10^5/ml. Viability was determined using trypan blue exclusion and was always above 85%.

Flow Cytometry Assessment of Daunorubicin Accumulation and Efflux

Cells were incubated for 60 min at 37 °C with Dnr (1 μM for patient cells) alone, or with addition of cyclosporin A (CyA, 3 μM) or the metabolic inhibitors, sodiumazide (S, 10 mM) and iodoacetate (I, 1 mM). The cells were then centrifuged at +4 °C and resuspended in Dnr-free medium with or without CyA or S/I. Dnr efflux was followed for 60 min by assessment of mean cellular fluorescence at frequent intervals (12 times) using a FACScan flow cytometer (Becton Dickinson) with an argon laser (excitation at 488 nm) and Lysys II software. Forward and side scatter signals were collected using linear scales and fluorescence-2 using logarithmic scale. The flow cytometer settings were standardized by fluorescent microspheres (Standard-Brite). Gating on forward scatter was done to exclude dead cells, damaged red blood cells and debris. Blasts were gated based on forward scatter-side scatter properties according to results from immunophenotyping with monoclonal antibodies.

At least 10 000 events were counted. The intracellular Dnr concentration was expressed by the mean cellular fluorescence intensity. Efflux curves were plotted assuming a one-compartment kinetic model (Ae^{-kt}) and the efflux rate (k-value) was calculated from the mean cellular fluorescence values.

Results

Daunorubicin Accumulation and Efflux in K562 and K562/Vcr Cell Lines

Incubation with 0.5 μM Dnr for K562 cells and 3 μM Dnr for K562 resistant cells gave similar intracellular drug concentrations (Fig. 1). After centrifugation and reincubation in a Dnr-free medium the initial Dnr efflux was much faster in the resistant cells but only during the first 10 minutes.

Since the P-gp is believed to serve as an ATP-dependant efflux pump we studied the effect of metabolic inhibitors on Dnr accumulation and efflux in K562/Vcr cells. Sodiumazide (10 mM), an inhibitor of oxidative phosphorylation had no effect on Dnr accumulation and efflux whereas iodoacetate (1 mM), an inhibitor of glycolysis increased drug accumulation about 2-fold (Fig. 2). The combination of the two inhibitors had a much larger effect with a 6-fold increase in drug accumulation. As shown in Fig. 3, the effect of both the metabolic inhibitors on Dnr accumulation was higher than that of CyA (3 μM) (Fig. 3). However, the drug efflux curves showed a high degree of parallelity.

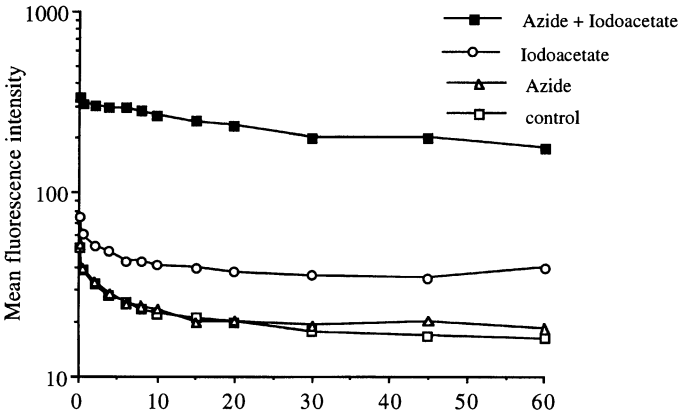

Fig. 2. Accumulation and efflux of Dnr in K562/Vcr cells incubated for 60 min with 1 μM Dnr, efflux in Dnr-free medium. Addition during incubation and efflux of 10 mM sodiumazide, 1 mM iodoacetate, both metabolic inhibitors, and no additive (control)

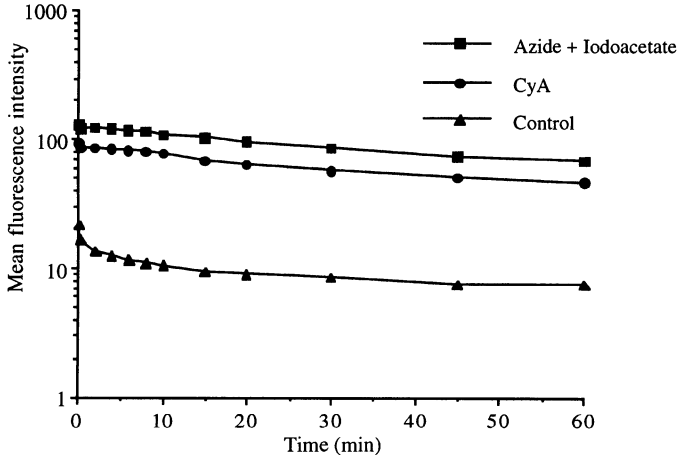

Fig. 3. Dnr efflux from K562/Vcr. Cells were incubated with 1 μM Dnr with 10 mM sodiumazide and 1 mM iodoacetate or with 3 μM cyclosporin A. After 60 min cells were washed and reincubated in Dnr-free medium but with metabolic inhibitors or CyA

Daunorubicin Accumulation in Leukemic Cells from Patients with AML

The accumulation of Dnr in leukemic cells showed a large variability (Table 2). In all but one patient the addition of cyclosporin A or of metabolic inhibitors increased drug accumulation. The highest observed increase was 99% (median 43%, range 18-99) with metabolic inhibitors and 29% (median 15, range 1-29) with CyA, which is much less than in the K562/Vcr cell line, where the increase was 6-fold for metabolic inhibitors and around 4-fold for cyclosporin A. In one patient (no. 11) neither metabolic inhibitors nor cyclosporin A increased Dnr accumulation, however, cells from this patient had by far the highest Dnr accumulation. There was no correlation between Dnr accumulation and the effect of modulators (not shown).

Table 2. Daunorubicin accumulation in leukemic cells from patients with AML. Cells were incubated for 1 h with 1 μM Dnr in the presence or absence of the metabolic inhibitors (10 mM sodiumazide + 1 mM iodoacetate) or CyA (3 μM)

Pt. no.	Dnr accum MFI	% Increase met inhibitors	CyA
1	56	27	11
2	55	58	29
3	67	42	13
4	34	21	26
5	52	54	15
6	75	63	23
7	71	99	15
8	39	26	5
9	47	43	15
10	72	18	1
11	213	−4	−10
12	57	61	25

Daunorubicin Efflux from Leukemic Cells from Patients with AML

Dnr efflux was studied in cells loaded for 1 h in the presence of CyA, washed and reincubated in Dnr-free medium with or without the metabolic inhibitors or CyA. The mean efflux after 30 min was 14% (not shown). In the presence of CyA the mean efflux was 12% and in the presence of metabolic inhibitors it was 14% (not shown).

Discussion

It is generally accepted that P-gp-mediated active drug efflux from resistant cells is the mechanism behind classical multidrug resistance [1]. Previous results have indicated that P-gp expression is associated with poor treatment outcome in AML [4-8]. Consequently clinical trials are being conducted to investigate the possibility to inhibit drug efflux by resistance reversing agents like PSC 388. However, discrepancies have also been found between the ability of AML blast cells to accumulate daunorubicin and P-gp antigen expression [15].

Evidence for active efflux of anthracyclines across the plasma membrane was also found in non resistant cells and a "pump and leak" hypothesis was proposed as a mechanism for cellular anthracycline accumulation [16].

In the present study we have used flow cytometry to characterize cellular daunorubicin accumulation and efflux and to compare these processes in human leukemic cell lines and in cells isolated from patients with AML.

The results in cell lines are compatible with the current hypotheses, although drug efflux was higher in the presence of metabolic inhibitors or CyA only during the first 10 min.

The results in leukemic cells isolated from patients are more difficult to interpret in the current framework. The lower effects of metabolic inhibitors and CyA on Dnr accumulation on cells from patients as compared to the resistant cell line are in accordance with a much lower mdr1 expression in the patients' cells than in the resistant cell line. The Dnr efflux was slow which can also be understood in view of the difference in mdr1 expression but no increase in Dnr efflux could be observed by metabolic inhibitors or cyclosporin A. The results indicate that other processes than drug efflux can be affected by resistance modulators, e.g. cellular storage capacity.

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Pharmacokinetics

Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Pharmacokinetics and Pharmacodynamics of Asparaginase

J. Boos

Abstract. L-asparaginase (Asp.) from different biological sources *(Escherichia coli, Erwinia chrysanthemi)* is prescribed to completely deplete asparagine in blood. Faced with increasing reports of treatment complications a program to monitor enzyme activity and asparagine levels in serum was started. Based on the monitoring data, dose optimisation of the Asparaginase medac product (produced by Kyowa Hakko) was then established.

Part I: Trough levels of asp.activity and asparagine were measured in 49 children who received different *E. coli* preparations (Asp. medac, Crasnitin) for induction treatment (8 applications, 10000 U/m^2 every 3rd day) of childhood acute lymphatic leukemia and non-Hodgkin's lymphoma, and in 52 children on reinduction (Asp. medac, Crasnitin, and, in the event of allergic reactions, Erwinase). During induction, both E. coli preparations led to the desired reduction in asparagine, however, asp. activity with Asp. medac was significantly higher than with Crasnitin (median trough levels 430 vs. 74 U/l). Under reinduction (trough levels: Asp. medac 528 U/l, Crasnitin 49 U/l, Erwinase < 20 U/l) complete asparagine depletion was recorded in more than 90% of Asp. medac samples, 64% of Crasnitin samples and a mere 26% of Erwinase samples.

Part II: Following pharmacokinetic simulation based on the monitoring data, 11 children received induction therapy with only 5000 U/m Asp. medac. The median trough level was still 265 U/l. In a subsequent group of 15 children on 2500 U/m^2 Asp. medac a median trough level of 102 U/l was measured. In both groups, asparagine was depleted in plasma and cerebrospinal fluid.

Moreover, treatment related changes in a broad panel of coagulation parameters were significantly less distinct in children receiving 5000 or 2500 U/m^2.

Conclusions: The monitored asp. activity covaries with pharmacodynamic parameters (amino acids, coagulation parameters) which may serve as surrogates of effect and toxicity. Different asparaginase preparations are not readily interchangeable. When Crasnitin is replaced by Asp. medac during induction therapy, comparable enzyme activities will be reached with only $^1/_2$ to $^1/_4$ of the asp. dose. When substitution of an alternate source of asparaginase is necessary, monitoring of the activity is advisable. Erwinia based treatment is expected to require schedules with much higher dosages.

Introduction

The enzyme L-asparaginase is an important element of well-proven therapeutic efficacy in the treatment of acute lymphoblastic leukemia in children and adults [1].

The mechanism of action has been related to the observation that the growth of lymphoblastic blast cells requires extracellular asparagine, since the malignant cells lack

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sufficient activity of the enzyme asparagine synthetase [2, 3]. The enzyme catalyses the hydrolysis of L-asparagine to aspartic acid and ammonia. Hence, pharmacologically, the objective of asparaginase treatment is to deplete the patient's blood of asparagine. Glutamine is another substrate of asparaginase, and changes in glutamine metabolism have been discussed to contribute to asparaginase-associated side effects such as hepatotoxicity, hyperglycemia and haemorrhagic as well as thrombotic events [4, 5, 6]. In addition, allergic events have been a common clinical problem.

Biologically, the enzyme L-asparaginase is derived from either *Escherichia coli (E.coli)* (Asparaginase medac, Crasnitin) or Erwinia chrysantemi (Erwinase), which translates into different chemical and immunological properties of the commercially available preparations [3].

Erwinia asparaginase is considered to induce minor toxicity [7]. In many institutions, including our own, it is frequently used in the event of allergic reactions to *E. coli* asparaginase. Erwinia asparaginase is known to have a shorter half-life compared to *E. coli* asparaginase [8]. In clinical usage, however, those preparations are usually interchanged without adjusting the dosage or the application interval. While the recommendations of the ALL/NHL-BFM 90 treatment protocol were based on the *E. coli* asparaginase preparation Crasnitin (Bayer company), the available alternatives were equally applied at a dose of 10000 U/m^2 every third day. As of 1990, the supply from Bayer was no longer sufficient to satisfy the demand, and substitution of other products became necessary.

Increasing reports of hemorrhagic and thrombotic events prompted us to establish a monitoring program in our own institution, using asparaginase activity as the primary parameter. Additional pharmacodynamic parameters were the asparagine levels, which represent the main biochemical correlate of the desired therapeutic effect, and the aspartic acid concentration as well as the levels of glutamine and glutamic acid, which are considered potential indicators of toxic side effects.

Patients

Part I. Trough levels were measured on a routine basis in 49 children who received different *E. coli* preparations (Asp. medac, Crasnitin) for induction treatment of acute lympho-blastic leukemia and non-Hodgkin's lymphoma. They had 8 applications, 10000 U/m^2 every 3rd day on days 8,11,15, and 18, along with prednisone, vincristine, and daunorubicin on days 8, 15, 22 and 29 (see Fig. 1).

On reinduction, another 52 children were also included into the monitoring program. They received the *E. coli* preparations Asparaginase medac or Crasnitin and, in the event of allergic reactions, Erwinase. The asparaginase dosage for reinduction was 10000 U/m^2, given as 1-hour infusion, applied 4 times at 3- or 4-day intervals (days 8,11,15 and 18 along with daily dexamethasone and weekly vincristine and doxorubicin) (see Fig.1).

Part II. The first step of dose reduction in protocol I included 11 children (2-14 years) on a dose of 5000 U/m^2 Asparaginase medac. Nine of the 11 patients were maintained on the same dose and monitored during protocol II. In a second step, 15 children (6 months - 13 years) were treated with 2500 U/m^2 of the medac product in protocol I. The children of this group received 10000 U/m^2 during reinduction.

Erwinase only was applied when allergic reactions to the *E. coli* preparation were observed.

Methods

Sample Collection. Parents and patients had consented to the procedure and serum samples were withdrawn 1-2 h prior to each asparaginase infusion on occasion of the usual pretreatment laboratory tests. The regular interval to collection of the samples was thus 3 days from the previous application, except on day 15 of reinduction, when the interval was 4 days. Additional samples were available from blood sampling for clinical reasons, e.g., coagulation analysis, obtained at variable intervals from the last application.

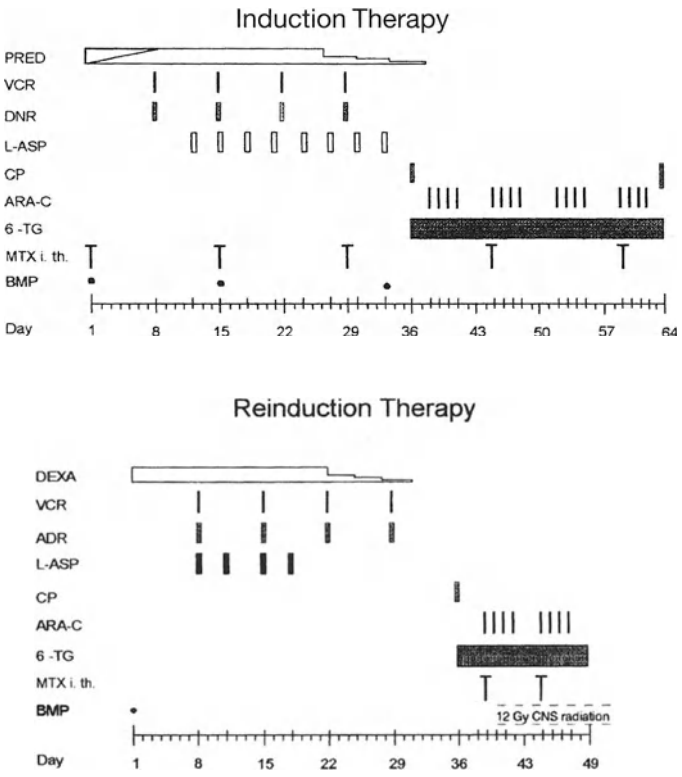

Fig. 1. Treatment flow sheet for induction and reinduction therapy according to the ALL- and NHL-BFM-90 trial. (*BMP* Bone marrow puncture, *L-Asp* Asparaginase

The samples were immediately centrifuged and divided in half. After extraction of the cellular components one part was immediately deproteinized and deep frozen within 15-20 min after withdrawal for storage until amino acid analysis. The other part was immediately frozen and used to determine the asparaginase activity. No asparaginase inhibitor was added, as those available proved highly unstable in our tests and inhibitory activity was only seen at concentrations high enough to produce multiple major interference peaks in the HPLC chromatograms during the analytical procedure.

L-asparaginase activity was quantified by photometrical determination of the ammonia release following incubation with an asparagine buffer solution for 45 min and addition of Nesslers solution. For details of the asparaginase assay and the in vitro comparison of different asparaginase preparations see [9].

Amino acid levels in the serum were measured using an RP-HPLC technique following precolumn derivation with o-phthaldialdehyde (OPA) and fluorescence detection. For details see [10].

Definitions. Trough levels are concentrations determined before application of asparaginase on the third day after the previous infusion (see Fig. 1). This was termed day 3 (defining the day of application as day 0). Under physiological conditions, asparagine levels are approx. 40-80 μM. The pretreatment asparagine values were within the normal range: children on Asparaginase medac (median 61 μM) were comparable to children on Crasnitin (median 58 μM). The depletion of asparagine was graded: complete depletion: ≤ 0.1, almost complete depletion: $> 0.1 - \leq 0.5$ μM, moderate reduction: $> 0.5 - \leq 1.0$ μM, slight reduction: $> 1.0 \leq 40$ μM, no reduction: > 40 μM.

Statistics. Mann-Whitney rank sum test (comparison of two groups) and Kruskal-Wallis One Way Analysis of Variance on Ranks (comparison of more than two groups) using Dunn's Method in addition.

Results

Part I. During induction, both of the *E. coli* preparations led to the desired reduction in asparagine. All samples were almost completely or completely depleted by both *E. coli* asparaginase products (see Table 1).

Asp. medac, however, showed significantly ($P < 0.01$) higher asparaginase activity than Crasnitin. The median trough levels were 430 U/l in children on Asparaginase medac and 74 U/l in children on Crasnitin (see Fig. 2, groups on 10000 U/m^2).

Changes in amino acids other than asparagine included a significant increase over pretreatment levels of aspartic acid in all treatment groups. The increased levels, however, remained within the range of normal values. Glutamine levels were markedly reduced by 75% one day after application of Asparaginase medac, in some patients the reduction lasted until day 3. Glutamic acid was enhanced in this group (for details see [10]).

Under reinduction, the trough levels were 528 U/l in Asp. medac treated patients and 49 U/l in children on Crasnitin. After application of Erwinase the trough levels were under the limit of detection (< 20 U/l). Complete asparagine depletion was recorded in more than 90% of Asp. medac samples, 64% of Crasnitin samples, and a mere 26% of Erwinase samples [10]. The ability of Crasnitin to reduce the serum levels of asparagine to levels < 0.5 μM was significantly lower during second exposure. While 100% of the samples in the induction therapy were completely or almost completely depleted, 29% of the samples showed levels higher than 1 μM during reinduction. Those children on Crasnitin exhibited a loss of pharmacological activity without any clinically apparent allergic reaction, a phenomenon which is comparable to observations of other groups, the so-called silent inactivation [8].

Part II. Following the pharmacokinetic simulation of each asparaginase product based on those data obtained in the monitoring series (using topfit 2.0 Pharmacokinetic and Pharmacodynamik Data Analysis System), an Asparaginase medac dosage of 2000-2500 U/m^2 every 3rd day, was calculated to produce a sufficient trough level of about 100 U/m^2.

In a first step, 10 children of our study received induction therapy with only 5000 U/m^2 Asp. medac, i.e., 50% of the dosage prescribed by the treatment protocol. The median trough level was 265 U/l in this group and this was comparable to the one which had been simulated (about 250 U/m) (see Fig. 2). Each sample was successfully depleted of asparagine.

Subsequently, another group of 15 children on only 2500 U/m Asp. medac showed a median trough level of 102 U/l.

In both of these groups we recorded complete asparagine depletion in the plasma and, most important, in 100% of the cerebrospinal fluid samples in protocol I, 2 to 3 days after application.

Discussion

The drug monitoring program showed significantly different trough activities in children on different asparaginase products. To find an approximately 6-fold biological activity in plasma with the product Asparaginase medac compared to Crasnitin was a

Table 1. Distribution of asparagine levels in serum under treatment according to protocol I of the ALL/NHL BFM 90 trial. Evaluation restricted to trough levels on the third day after asparaginase application [10]

	Asparaginase medac			Crasnitin		
L-asparagine (μM)	% of samples	n	pat	[%]	n	pat
≤ 0.1	88	148	37	66	33	9
> 0.1 - ≤ 0.5	12	20	13	34	17	9
> 0.5 - ≤ 1	–	–	–	–	–	–
> 1	<1	1	1			
total:	100	169	51	100	50	18

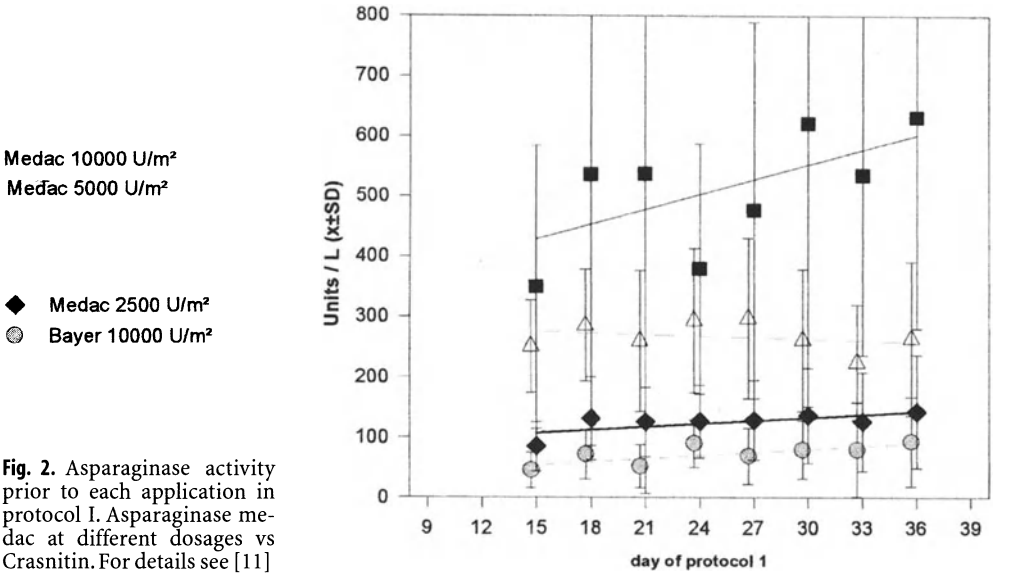

Fig. 2. Asparaginase activity prior to each application in protocol I. Asparaginase medac at different dosages vs Crasnitin. For details see [11]

surprise. Both products are *E. coli* asparaginase preparations. Nevertheless, biological differences between *E. coli* strains and mutants have been described. The pharmacokinetic difference resulted not only in different trough levels but also in higher C_{max} and a longer half-life of asparaginase medac [10]. The significantly higher plasma activities of the medac product were correlated with more intense changes in coagulation parameters compared to Crasnitin [12].

The subsequent dose reduction of the product asparaginase medac was targeted to obtain serum trough activities of about 100 U/l. This level had been postulated as therapeutic in the 1980 [13] and the children on Crasnitin, the agent used at the outset of the empirical development of the BFM-trial protocols, had shown levels in the same order of magnitude.

A significant improvement of the coagulation parameters was observed when the dosage was reduced to 5000 U/m^2. Further reduction to 2500 U/m^2, however, was accompanied by only a slight additional positive effect [11, 14]. The mean serum trough activities of the children on 2500 U/m^2 fitted the target concentration of >100 U/l perfectly. The ranges of the trough asparagine activity, however, showed wide interindividual variability and some children repeatedly had trough levels of 50 U/l or less. Serum as well as cerebro-spinal fluid were completely depleted even in these samples. Drug monitoring, however, is currently unavailable for the German multicenter trial and the group, therefore, decided to define 5000 U/m^2 as the standard dose of Asparaginase medac. In our own institution, however, the clinical pharmacology workgroup currently investigates the feasibility of individual drug targeting.

Erwinase has been discussed to lack antigenic cross reactivity with *E. coli* products. In this study, however, all children on Erwinase without exception had previously shown an allergic response to an *E. coli* preparation. Hence, in addition to the well-known shorter half-life of Erwinia asparaginase [8], antigenic cross-reactivity still has to be taken into consideration.

There are several reports on comparably low trough levels in blood or cerebro-spinal fluid indicating reduced activity or silent inactivation of Erwinia asparaginase on this 3-day schedule [15, 16, 17]. Based on these data, our workgroup recently initiated a dose optimisation study with increasing Erwinase dosages and shorter application intervals. The study is ongoing.

Our data indicated the necessity to specify the optimal dosage and schedule for every

commercially available asparaginase product separately. Especially, dosage and scheduling for the Erwinase preparation has to be defined when it is to be substituted for the *E. coli* asparaginase application on an 3-day schedule.

We have shown that the asparaginase serum activity covaried with changes of representative coagulation parameters [12] and thus had good reason to avoid high trough activities. The treatment rationale defining the minimum trough activity, however, was based on the empirical treatment protocol (developed on Crasnitin) and on data published by Riccardi and coworkers [13].

The most important question, however, about the clinical relevance of the pharmacokinetical differences between the asparaginase products monitored could not be answered by these data. A randomised clinical trial has recently been presented which compared identical dosages of Asparaginase medac and Erwinase [18]. The 4-year event free survival was significantly lower in children on Erwinase compared to children on E.coli asparaginase (62% versus 75%). These results indicate a significant impact on treatment outcome of the higher treatment intensity obtained with the *E. coli* preparation. We may, therefore, conclude with respect to asparaginase, that parameters of pharmacokinetics, pharmacodynamics, toxicity and clinical response are closely linked.

Summary

Asparaginase is an important drug in the treatment of acute lymphoblastic leukaemia. Different asparaginase preparations are not readily interchangeable. Changes regarding the asparaginase preparations, the application schedules or the asparaginase dosage require careful observation and possibly pharmacokinetic drug monitoring.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Asparagine Synthetase in Pediatric Acute Leukemias: AML-M5 Subtype Shows Lowest Activity

A. Dübbers, P. Schulze-Westhoff, E. Kurzknabe, U. Creutzig, J. Ritter, and J. Boos

Abstract

Objective. The enzyme L-asparaginase is used to achieve maximum reduction of L-asparagine in blood and CSF. Lack of sufficient cellular activity of asparagine synthetase in blast cells compared to normal tissues is thought to be the basis of the antileukemic effect in ALL. While L-Asparaginase is routinely used in acute lymphoblastic leukemia its role and value in the treatment of acute myeloblastic leukemia is still being discussed. As the drug causes a number of relevant side effects, measuring the asparagine synthetase activity of individual patients' blast cells might help to restrict the use to patients with low expression and to exclude others with high synthetase capacity from this treatment. We, therefore, established asparagine synthetase monitoring.

Methods. Peripheral or bone marrow blast cells were separated by Ficoll gradient centrifugation. Intracellular proteins (6000 g-supernatant) were incubated in excess of required substrates and the synthetased asparagine levels then measured after 0, 40, 80, 100, and 140 min (HPLC). The interassay coefficient of variation was < 20% and showed good reproducibility (activity: nM asparagine / mg protein / h).

Results. The observed activity (nM/mg protein/h) was significantly lower in AML-M5 than in the other myeloid leukemias ($p < 0.005$, Mann Whitney rank sum test). There was no significant difference between myeloblastic and lymphoblastic leukemia.

	n	Median (range)	Mean±standard dev.
ALL	18	10 (3.4-45)	14±11
AML (not M5)	18	12.6 (0.8-45.5)	16±12.8
AML-M5	9	3.8 (0.96-7.9)	3.9±2.2

Conclusion. As AML and ALL both showed comparable expression of asparagine synthetase, the possible value of L-asparaginase in the treatment of AML should be reconsidered. As a consequence we have initiated a multicenter trial to test the clinical activity in patients with relapsed AML-M5 and to determine the relationship between the response to L-asparaginase treatment and cellular asparagine synthetase activity, mRNA expression (d'Incalci, Milano) and in vitro asparaginase sensitivity (Pieters, Amsterdam).

Introduction

The L-asparaginase therapy of acute lymphoblastic leukemia is based on the fact that lymphoblastic cells require external asparagine for growth as they lack sufficient activity of asparagine synthetase [1-3]. The bio-

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synthesis of asparagine in normal and malignant tissues by cellular asparagine synthetase interferes with the effectiveness of L-asparaginase in the treatment of lymphoblastic leukemia [4, 5], because asparagine synthetase catalyses the formation of L-asparagine, AMP, PP_i and L-glutamate from L-aspartate, ATP and L-glutamine in the presence of Mg^{2+} and Cl^-. L-glutamine, the amide nitrogen donor, may be displaced by NH_4^+ [6]. Side effects of and resistance against L-asparaginase are thought to be affected by cellular asparagine synthetase activity [5, 7].

In order to distinguish patients with low expression in blast cells from those with high asparagine synthetase capacity we compared the ability of peripheral or bone marrow blasts to synthesize asparagine prior to treatment with L-asparaginase.

Patients and Methods

Patients

From June 1993 to March 1996, 27 patients with newly diagnosed AML, aged 4 months to 15 years (median 9.5 years, mean 8.9), and 18 patients with newly diagnosed ALL, aged 4 months to 15 years (median 8 years, mean 7.8), underwent asparagine synthetase monitoring at the time of diagnosis.

Sample Collection

Samples were obtained from routine diagnostic peripheral venous or bone marrow punctures. Blast cells were separated by Ficoll gradient. Trypan blue solution was used for cell enumeration. Cell pellets were frozen at -80 °C until further sample preparation and analysis. The analysis was performed within 1 day to 2 years (median and mean 12 months). The asparagine synthetase activity in peripheral or bone marrow blast cells frozen at -80 °C was shown to be stable for at least 11 months. The enzyme activity in HL-60 cells (human promyelocytic leukemia cell line) was stable for at least 4 months, the coefficient of variation was < 20%.

Materials

L-Glutamine, ATP, mercaptoethanol, boric acid and trypan blue solution were obtained from Sigma Chemical Co., St. Louis USA. L-aspartic acid, potassium chloride, Mg-acetate H_2O, ammonium chloride, sodium chloride, potassium hydroxide and O-phthaldialdehyde were supplied by Merck, Darmstadt Germany, absolute ethanol by Carl Roth GmbH & Co, Karlsruhe Germany, disodium hydrogen phosphate 2 H_2O and potassium dihydrogen phosphate anhydrous by Serva, Heidelberg Germany, Lymphoprep by Nycomed Pharma AS, Oslo Norway, and high purity water (HPLC grade) was obtained from Milli-Q UF Millipore, Eschborn Germany. Glacid acetic acid and methanol were purchased from Baker Chemicals, Heidelberg Germany. All agents used were of analytical grade.

Protein Concentration

The protein concentration in each preparation was determined using the Bio-Rad DC protein assay (Bio-Rad Co., Richmond USA) based on the method of Lowry et al [8] with bovine serum albumin as the standard.

Sample Preparation

One hundred µl of aqua dest. was added to 10 million frozen cells followed by disrupting in three cycles of freezing, thawing and sonication at 0 °C (Ultrasound bath Senorex TK 30 Bandelin Electronic, Berlin, Germany). Homogenates were centrifuged at 6000 g, 4° C, for 5 min (Megafuge 1.0 R Heraeus, Osterode, Germany). The supernatant was divided in two equal parts, one for protein assay and one for enzyme assay, and stored at -20 °C until incubation.

Enzyme Assay

The asparagine synthetase activity was measured according to the - slightly modified - method described by Hongo and Sato [9]. 500 ml of the reaction mixture contain-

ing 1.5 mmol/l L-aspartic acid, 20 mmol/l L-glutamine, 10 mmol/l ATP, 50 mmol/l magnesium acetate, 20 mmol/l KCL, and 20 mmol/l ammonium chloride in phosphate buffered saline (PBS) titrated with KOH (pH 7.7) were added to 100 µl of the cell lysate. The vortexed mixture was incubated at 37 °C for 0, 40, 80, 100, and 140 min (Thermomixer 5436 Eppendorf, Hamburg, Germany). The reaction was stopped by heating in boiling water for 90 s and the mixture was subsequently centrifuged for 5 min (Biofuge A Heraeus, Osterode, Germany). Asparagine was determined by RP-HPLC based on the method described by Unnithan et al. [10]. The specific activity of the enzyme was expressed as nmol asparagine / mg protein / h.

High-Performance Liquid Chromatography Method

A high-performance liquid chromatography (HPLC) system from Pharmacia/LKB (Bromma, Sweden) was used in combination with fluorescence detection (Shimazdu RF 535, Shimazdu Duisburg, Germany) at λ_{ex} 340 nm and λ_{em} 450 nm after precolumn derivation with ophthaldialdehyde (OPA) [11]. Continuous on-line quantifikation of the HPLC results was obtained with a Nelson interphase and the Nelson Analytical Software. Separation was done on an ET 250/8/4 Nucleosil 120-5 C18 column (Machery & Nagel, Düren, Germany). A high pressure gradient was formed between two helium degassed solvents: solvent A = 0.2 M potassium hydroxid buffer pH 6 filtered through 0.45 mm filter (Millipore), solvent B = methanol (HPLC grade) (min : s-min : s/% methanol: 0:00 - 7:00 / 18% - 7:05 - 13:00 / 21% - 13:05 - 17:00 / 18% - 17:05 - 25:00 / 75% - 25:05 - 30:00 / 18%). The injection volume was 20 µl of the supernatant. At a flow rate of 1.3 ml/min the retention time for asparagine was 15 min and the time for analysis 32 min. The detection limit of the method was 0,1 µM. Daily calibration curves from 0.1-75 µM showed linearity > 0.99 for asparagine. The interassay coefficient of variation of the asparagine synthetase assay was < 20%. The intraassay coefficient of variation was < 5%.

Results

The results are summarized in Tables 1 and 2. It can be seen that the asparagine synthetase activity in lymphoblastic leukemias including c-ALL, pre pre B-ALL, and T-ALL ranged widely from very low to high activity. Identical results were obtained in myeloblastic leukemias including M0-M4 and M7, but not in M5 (Fig. 1). Blasts of monocytic leukemias, as compared with the other myeloid and lymphatic blasts, showed the lowest enzyme activity prior to treatment (Fig. 1); the difference was highly significant. Surprisingly, there was no significant difference of the synthesized asparagine levels between acute myeloblastic and acute lymphoblastic

Table 1. Characteristics of patients and asparagine synthetase activity in blast cells at the time of diagnosis, mean ± SD and median (range)

Diagnosis	No. of Patients	Age (years) Median (range)	Mean	Activity (nM Asn/mg protein/h) Median (range)	Mean±SD
ALL	18	8 (0.3-14.7)	7.8	10 (3.4-45)	14±11
AML (not M5)	18	11.4 (2.1-15.4)	10.6	12.6 (0.8-45.5)	16±12.8
AML-M5	9	5.7 (0.3-14.3)	3.9	3.8 (0.96-7.9)	3.9±2.2

Table 2. Asparagine synthetase activity at the time of diagnosis in T-ALL compared to other ALL, mean ± SD and median (range)

Diagnosis	No. of Patients	Activity (nM Asn/mg protein/h) Median (range)	Mean±SD
ALL (not T-ALL)	13	11.7 (3.4-45)	15.5±12
T-ALL	5	8.6 (4.1-22.9)	10±7.5

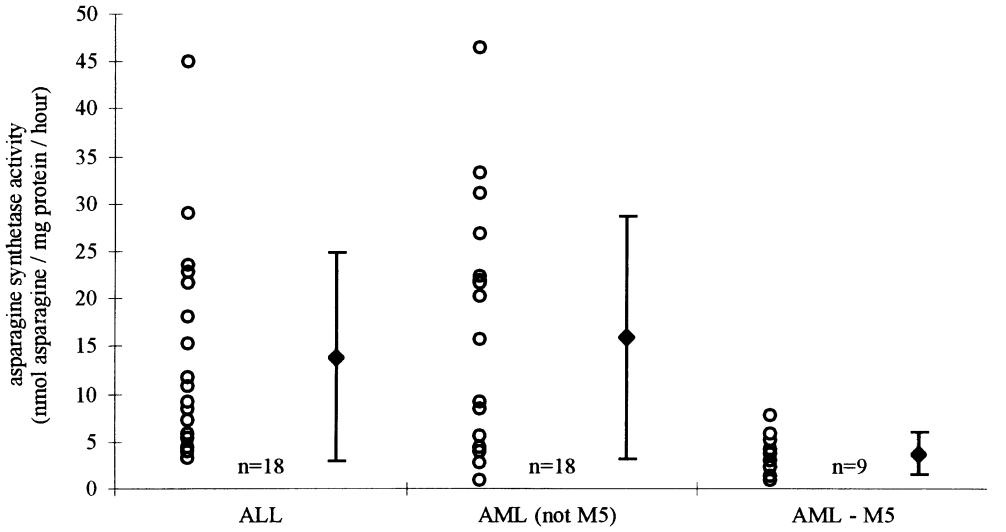

Fig. 1. Asparagine synthetase activity in ALL, AML (not M5) and AML-M5 blast cells at the time of diagnosis, mean ± standard deviation. Activity is significantly lower in AML-M5 compared to other myeloid leukemias ($p < 0.005$, Mann Whitney rank sum test) and to lymphatic leukemias ($p < 0.001$, Mann Whitney rank sum test), no significant difference between myeloblastic and lymphoblastic leukemias ($p > 0.025$ Mann Whitney rank sum test).

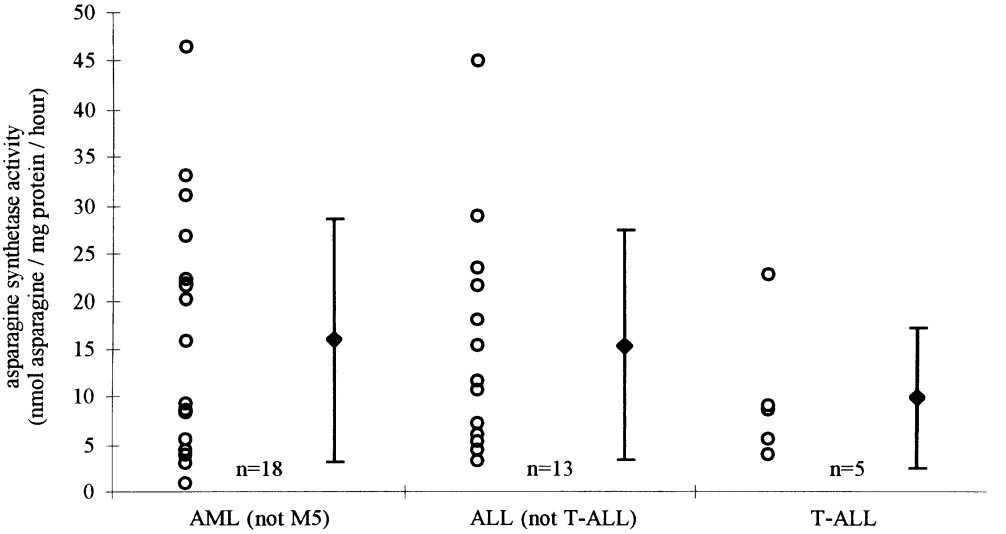

Fig. 2. Asparagine synthetase activity in T-ALL, ALL (not T-ALL) and AML (not M5) blast cells at the time of diagnosis, mean ± standard deviation. There is no significant difference between T-ALL and other lymphoblastic leukemias ($p > 0.025$ Mann Whitney rank sum test).

leukemias (Fig. 1), and between T-ALL and other ALL blasts (Fig. 2).

Discussion

Several earlier in vitro studies documented that T-lymphoblastoid cell lines are sensitive to L-asparaginase, whereas B-lymphoblastoid and myeloid cell lines are resistant to this agent [12, 13]. Papers concerning the asparagine synthetase activity in human leukemia blast cells are rare and showed only a few patients who underwent asparagine synthetase monitoring [5, 14]. Kiriyama et al. correlated the sensitivity to L-aspa-

Table 3. Sample characteristics

Diagnosis	Peripheral blast cells No. of samples	Bone marrow blast cells No. of samples	Not specified No. of samples
ALL (not T-ALL)	5	7	1
T-ALL	2	2	1
AML (not M5)	7	10	1
AML-M5	4	5	0

raginase with asparagine synthetase activity [13]. The sensitive, namely T-lymphoblastoid, cell lines had an enzyme activity of 41.1 ± 25.3 and 40.2 ± 26.1 (mean ± SD) pM/min/mg protein or 2.4 ± 1.5 and 2.4 ± 1.6 expressed as nM/mg protein/h. The resistant, i.e., promyelocytic, B-lymphoblastoid and non-B non-T lymphoid, cell lines had activities of 179.8 to 374.3 (mean) pM/min/mg protein or 10.8-22.5 expressed as nM/mg protein/h. Our results differed markedly from the cited study in that we showed the AML-M5 subtype to represent the lowest activity, whereas all other AML and ALL blast cells did not differ significantly. Not even the results of T-ALL blasts were as low as expected. The range of activity found in our ALL and AML blast cells covered the enzyme activity of sensitive and resistant cell lines described by Kiriyama.

The cytotoxic effect of L-asparaginase has been reported to correlate inversely with the asparagine synthetase activity [5]. Hence, it seems to be useful to distinguish those patients with low asparagine synthetase expression from patients with high synthetase capacity, in order to restrict the therapy to the first group. As it has been found that asparagine synthetase activity could increase with L-asparaginase therapy [5] and decrease with higher tumor differentiation [15], it would be important to study the change in enzyme activity during and after treatment and in case of relapse.

As a consequence, we initiated a multicenter trial to test the clinical activity in patients with relapsed AML-M5 and to determine the relationship between the response to L-asparaginase treatment and cellular asparagine synthetase activity, mRNA expression (d'Incalci, Milano) and in vitro asparaginase sensitivity (Pieters, Amsterdam) [16].

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Pharmacokinetic Basis for an Oral Chemotherapy with Idarubicin and Etoposide: Dose-Dependent Biological Effects of Topoisomerase-II-Inhibitors

F. GIESELER

Abstract. In the treatment of hematological malignancies with idarubicin or etoposide two different pharmacological phases can be discriminated. Shortly after i.v. application, a peak with high plasma concentration can be found which lasts shorter than 3 h. As well after i.v. application as after oral application, a long phase with 10-100 fold lower concentration (through level) can be found which lasts for hours or even days. The cellular reaction upon a short exposure of topoisomerase II inhibitors at high concentration is completely different than the cellular reaction upon a long exposure of topoisomerase II inhibitors at very low concentrations. Especially the epipodophyllotoxines have a high capacity to induce cellular differentiation at these conditions which is not the case with anthracyclines. For the induction of apoptosis, a exposure-time which is longer than the cell-cycle-time of the leukemic cells seems to be much more important than especially high concentrations of topoisomerase II inhibitors.

Introduction

The inhibition of topo(isomerase) II is a very potent principle of chemotherapy. Since a few years etoposide and idarubicin are available in an oral form which allows completely oral chemotherapies of hematological malignancies. In this chapter the pharmacological differences of the i.v. and oral applications are reviewed and it is shown that these result in specific biological effects which highly depend upon drug concentration.

Idarubicin

Chemical and Biological Characteristics

A main structural component of anthracyclines is the chinone-ringsystem (Fig. 1). This part of the molecule is able to intercalate into the DNA-double strand. The biochemical characteristics of each anthracycline are determined by the side-chains. Important elements are the doublebound oxigen and the sugar with NH_2-group. The sugar is responsible for the inhibition of DNA-processing enzymes after intercalation of the drug. Interaction with the oxigen seems to be an important mechanism for the generation of free oxigen-radicals. The chemical difference between daunorubicin and idarubicin is the loss of the methoxy-group in the D4-position which results in important biochemical and pharmacological changes.

First of all, idarubicin has a higher acid-stability which allows oral application, hence it is the first anthracycline which can be orally applied. The lipophilicity is higher, with a distribution coefficient of 27.7 as compared to 15.3 of daunorubicin [1,2]. This results in a higher and faster cellular accumulation, 1.8 times higher than daunorubicin [8]. Idarubi-

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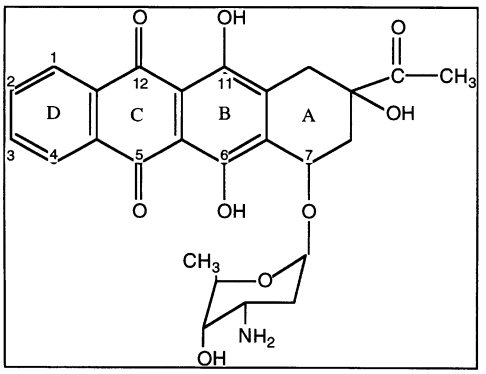

Fig. 1. Chemical structure of idarubicin (4-demethoxy-daunorubicin)

cin is also about 4 times more cytotoxic which is due to it's higher DNA binding rate and it's active metabolite. Interestingly, the difference in cytotoxicity is higher in myeloid cells than in lymphatic cells.

All anthracyclines are metabolized in the same way. The urine excretion rate is only 1-5%, depending on the way of application. The major way of metabolization is hepatic via aldoketo-reductase [4]. For pharmacodynamic studies it is important to take idarubicinol into consideration, because in contrast to daunorubicinol, this metabolite has the same cytotoxicity as idarubicin. Idarubicinol has even some characteristics which are superior to idarubicin. It has a higher polarity which results in cellular retardation. The metabolite is hold longer inside the cells which has been called metabolic retention [5]. Taken together, the cellular concentration of idarubicin and idarubicinol is about 300 times higher than the plasma concentration. The intracellular DNA binding rate of idarubicin is about 20% higher than the one of daunorubicin and the one of idarubicinol is still 15% higher [3]. Both drugs are found in low concentrations in the liquor cerebrospinalis, yet the clinical relevance of this finding is unknown. The binding to plasmaproteins is very high which must be taken into account with co-medication.

Cellular Effects of Anthracyclines

Antracylines exhibit a number of different cellular effects, two of them seem to be important for the clinician. Anthracyclines inhibit the topo II alpha. The topoisomerases control the three-dimensional structure of the DNA, thus being life-important for every cell. Anthracyclines inhibit their DNA-interaction after the topo II has introduced a double-strand-break and is covalently bound to the DNA. This "cleavable complex" activates a cellular program which results in apototic cell death.

The second important effect is the generation of free oxigen radicals. The amount of free oxigen radicals is directly proportional to the number of intracellular anthracycline-molecules. It is believed that the oxigen radicals are responsible for the most serious side effect in the treatment with anthracyclines, that is cardiotoxicity. The reason is that myocardial cells have only limited capacity to eliminate oxigen radicals and once this capacity is exceeded DNA damage occurs. This is the reason why cardiotoxicity is closely linked to high plasma peak levels, whereas cytotoxicity is linked to a high area-under-the-time-curve (AUC) [6].

Differences Between i.v. and Oral Applications of Anthracyclines

In Fig. 2, two characteristic time-plasma-curves of i.v.- and orally-applicated idarubicin are shown [7]. As mentioned before, not only idarubicin but also idarubicinol must be taken into account and for the clinical effect, idarubicinol might even be more important than idarubicin. After i.v. application of 13 mg/m^2 idarubicin within 15 min it is not surprising to find a high plasma peak which is up to 300 ng/ml plasma. The plasma decay follows a three-compartment model with a high decay rate in the first hours and a longer terminal half-life which is about 20 h for idarubicin. In contrast, the terminal half-life for idarubicinol is about 60 h, which results in a higher AUC. The plasma-time-curve for oral application is different. Please note the different scales for the y-axis. In this case, the hepatic metabolization capacity is high enough to metabolize all idarubicin very fast. Within a short period of time, the plasma concentration of idarubicinol is higher than the one of idarubicin. Taken to-

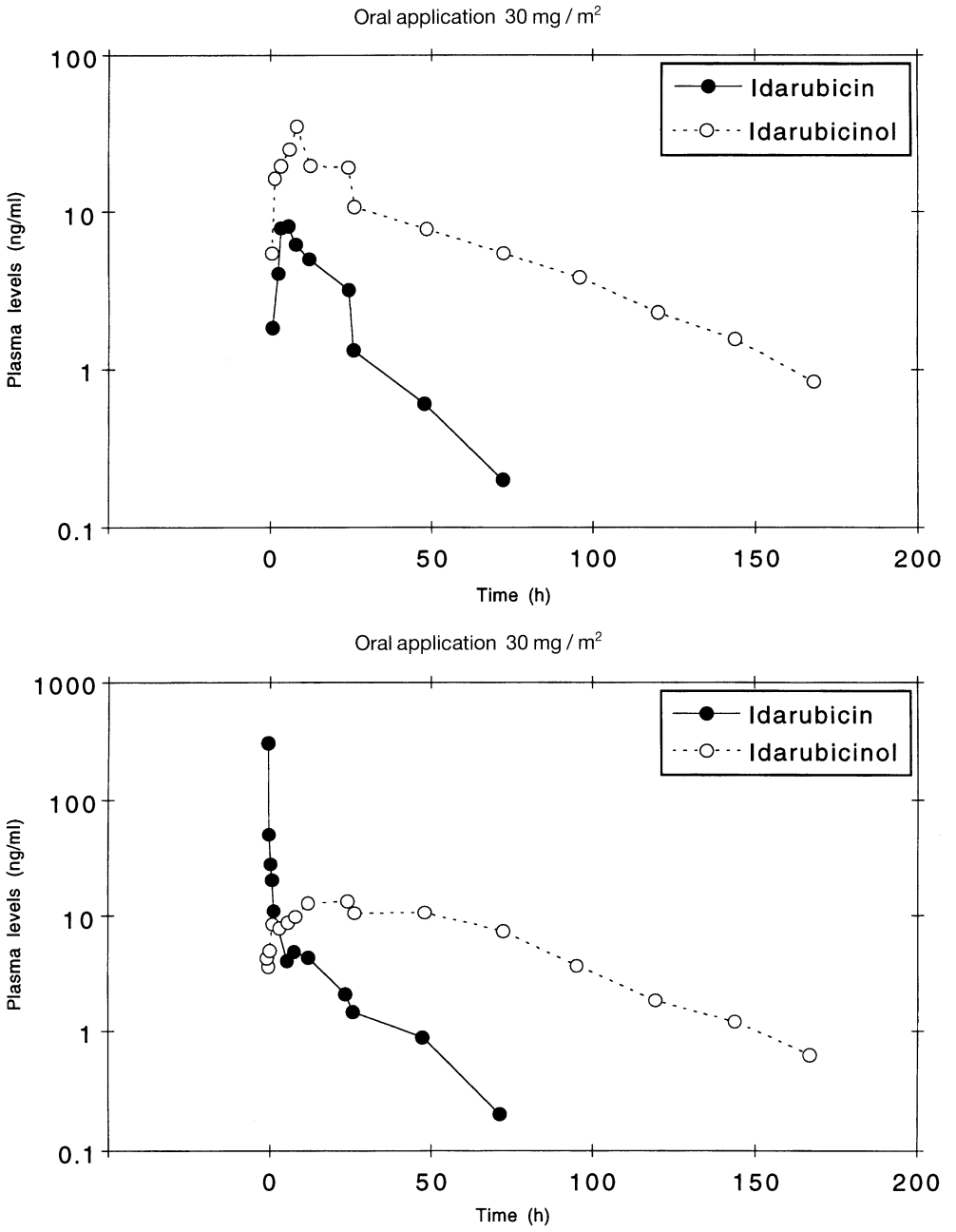

Fig. 2. Pharmacokinetic of idarubicin and idarubicinol after oral and i.v. application [2]

gether both drugs, the resulting AUC after oral application is higher than after i.v. application and the plasma peak level is lower.

In Table 1, the pharmacological data of four cross-over studies are shown [8-11]. The used dosages are different. As mentioned before, the terminal half-life of idarubicin is substantially shorter than the one of idarubicinol. In the very right column the important information that the AUC-ratio

Table 1. Pharmacokinetic parameters of idarubicin in four crossover studies

Reference	No. of courses	Dose (mg/m^2)	ida $t_{1/2}$ (h)	idaOL $t_{1/2}$ (h)	AUC ratio idaOL/ida
Smith 1987					
i.v.	5	15	34.7	52.1	2.30
oral	5	15	20.4	40.4	5.26
Zanette 1990					
i.v.	9	8.5-15.5	12.6	71.6	5.11
oral	9	25-45	9.7	57.8	14.4
Stewart 1991					
i.v.	13	15	15.5	51.4	3.00
oral	13	45	11.7	32.7	5.68
Camaggi 1992					
i.v.	21	12	16.2	48.7	4.85
oral	21	30-35	16.9	47.2	8.85

of idarubicinol to idarubicin is substantially higher after oral application in all four studies is shown. Taken together, after oral idarubicin a lower plasma peak, a higher AUC-ratio idarubicinol/idarubicin which results in longer terminal half-life and metabolic retention can be found. One important flaw must be considered: the uncertain bioavailability which is between 28 and 49% [12]. This means that the haematological parameters of every orally treated patient must be monitored to eventually adjust the dosage.

Etoposide

Chemical and Biochemical Characteristics of Etoposide

Extracts from Podophyllum peltatum were already used by American Indians two centuries ago. Etoposide is a beta-D-glycoside of podophyllotoxin, its structure is shown in Fig. 3. For maximal antitumor activity, stereochemical restraints on the C-ring are needed. Substitution of the 4-hydroxyl function at the E-ring results in considerable loss of antineoplastic activity [13]. Approximately 30-40% of an i.v. etoposide dose is excreted in the urine as unchanged drug, there is a close relation between etoposide plasma-clearance and creatinine clearance. Direct biliary excretion seems to be the minor route of drug elimination. Several etoposide metabolites such as the hydroxy acid metabolite or etoposide-aglycone have been confirmed and postulated. Formation of these metabolite could have potential importance, while the hydroxy acid metabolite is considerably less cytotoxic, the aglycone retains cytotoxicity [14].

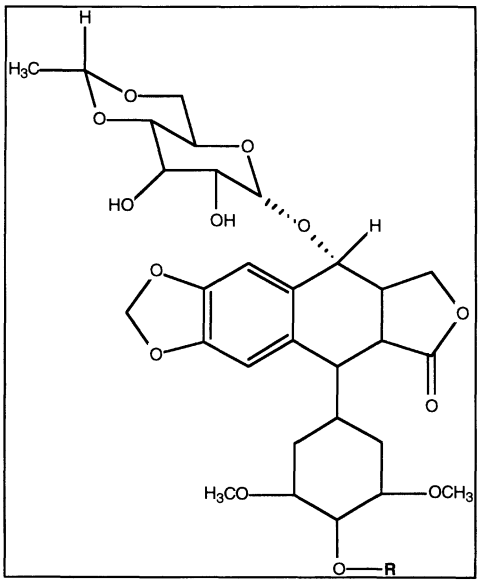

Fig. 3. Chemical structure of etoposide (R = H) and etoposide phosphate (R = H_2PO_4)

Cellular Effects of Etoposide

In contrast to anthracyclines which are DNA-intercalators, epipodophyllotoxines bind directly to the topo II alpha. This alters the enzyme's three-dimensional structure

and inhibits it's religation activity, thus resulting in DNA double-strand breaks. This mechanism is somewhat more specific and leads to more defined DNS-breakes. One problem arising from this is secondary hematological malignancies arising after chemotherapy with etoposide. The cumulative risk estimate of 12% (95% confidence interval (CI): 3%-46%) at 5 years for patients with Hodgkin disease and 18% (95% CI: 5%-49%) at 3 years for patients with small cell lung cancer. Early after initial treatment these patients might present balanced translocations 11q23 and 21q22 [15].

Differences Between i.v. and Oral Application of Etoposide

Etoposide is poorly and eratically absorbed from the gastrointestinal tract, most studies have shown a 30 to 76% bioavailability with a wide interpatient variability. To bypass these problems, the water-soluble analog etoposide-phosphate was synthesised (Fig. 3). This prodrug is converted to etoposide within minutes in the plasma and its bioavailability is 68.0% ± 17.9% (SD) [16]. The plasma pharmacokinetic parameters after i.v. administration and oral administration are shown in Table 2 and typical plasma-time-curves are shown in Fig. 4 [16].

Dose Dependent Biological Effects of Topo-II-Inhibitors

In a typical plasma-concentration-curve two major phases can be discriminated: high plasma drug concentration after i.v. application which lasts not longer than 3 h and a long terminal half-life which is basically the same after i.v. or oral application. The through-level drug concentrations are 10-100-fold below the peak. To compare the biological effects of topo II inhibitors we incubated human myeloid leukemia HL-60 cells for different times and with different concentrations with idarubicin (Fig. 5) and with etoposide (Fig. 6). The cells have been incubated with the indicated drug concentration for different times (0.5-48 h), then the cells were washed and reincubated in medium without drugs. The medium had been changed every day and the viable cells determined by Trypan-blue exclusion.

The doubling time of HL-60 cells is about 28 h. It seems that the presence of the drug for this time is more important than a higher drug concentration. Interestingly, at very low concentrations of etoposide we found quite a high viability, but the cells were quiescent and died after about 10 days in culture. Performing a nitro-blue-tetrazolium assay (NBT test) which indicates granulocytic differentiation showed that up to 53% of the cells were differentiated (Table 3) [17]. The concentrations used in these experiments are in the range of plasma levels reached after oral application.

Discussion

The cellular effects of topo II inhibitors highly depend upon the concentration in which they are used. At high drug concentrations which are reached shortly after i.v. application the outcome of treatment depends highly upon the reaction of the cell to the molecular effects of drug treatment. In most cases, the cells do not die immediately,

Table 2. Plasma pharmacokinetic after 1 h i.v. administration of etoposide, or after oral administration of the prodrug etoposide phosphate in cancer patients (mean ± SD). (After Chabot 1996)

Drug (route)	Dose (mg/m^2)	C_{max} (μg/ml)	AUC (μg h/ml)	Terminal $t_{1/2}$ (h)
E (i.v.)	80	15 ± 3	88 ± 22	8.1 ± 4.1
EP (p.o.)	50[a]	4 ± 0.4	30 ± 3	11.9 ± 7.8
EP (p.o.)	100[a]	8 ± 4	68 ± 19	7.8 ± 3.5
EP (p.o.)	125[a]	10 ± 4	91 ± 30	11.4 ± 6.4
EP (p.o.)	150[a]	14 ± 4	127 ± 38	8.5 ± 3.3

Abbreviations: E, etoposide; EP, etoposide phosphate. [a]EP dose is expressed in E equivalent.

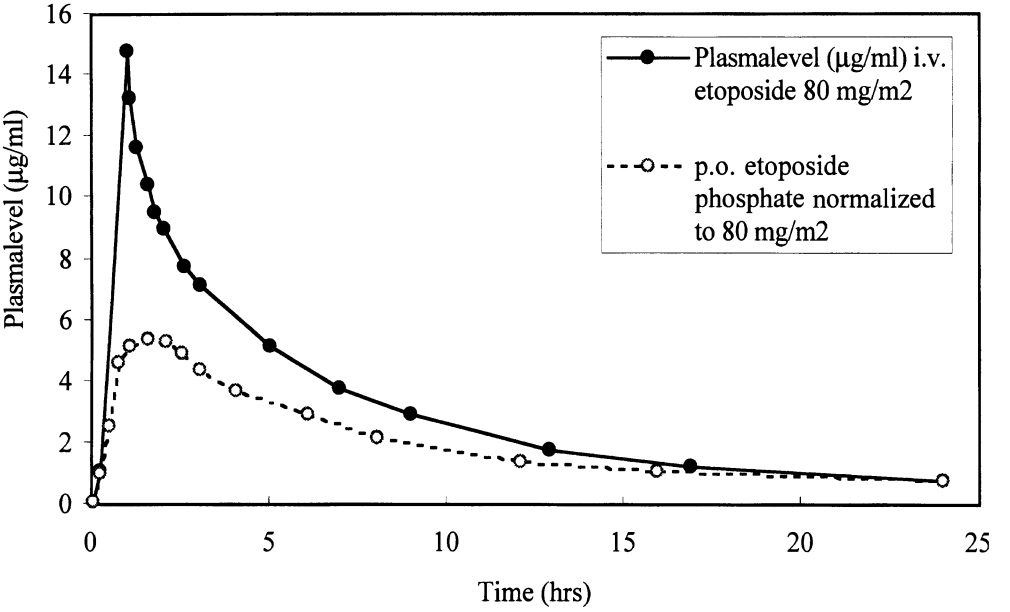

Fig. 4. Pharmacokinetic of etoposide i.v. and etoposide phosphate p.o. [16]

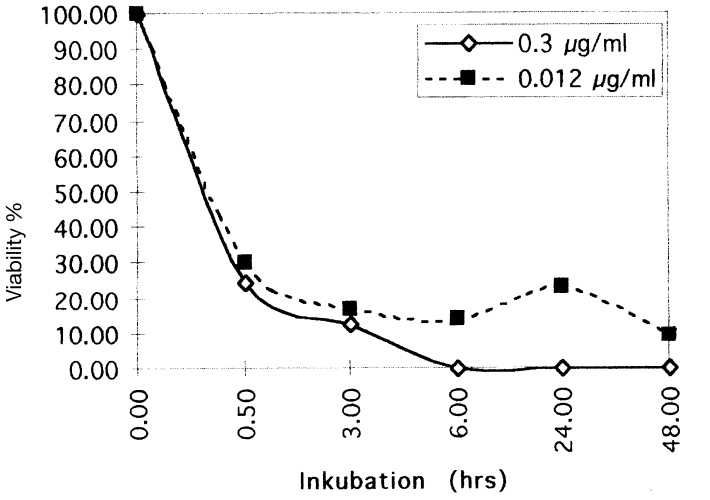

Fig. 5. Viability (%) of HL-60 cells after different times of incubation with idarubicin. Viability of control-cells was 80-90%

but after the initiation of a frustrated rescue attempt. In the treatment of sensitive cells with lethal doses of topo II inhibiting drugs, two stops can be distinguished. With a few exceptions the drugs stabilize a topo II-DNA complex at different stages of the enzymes' action cycle after the induction of DNA strand breaks by the enzyme. Obviously, this genotoxicity is a prerequisite for the initiation of cellular reactions which depend upon the expression of specific genes, such as p53, c-myc or bcl2 and on the effect of growth factors.

Drug concentrations 10-100-fold below the peak level result after oral therapy and can be found in the plasma for many hours or even several days. At these concentrations the cellular reaction on treatment is different and depends upon the mode of drug-action (e.g., DNA intercalation site, inhibition

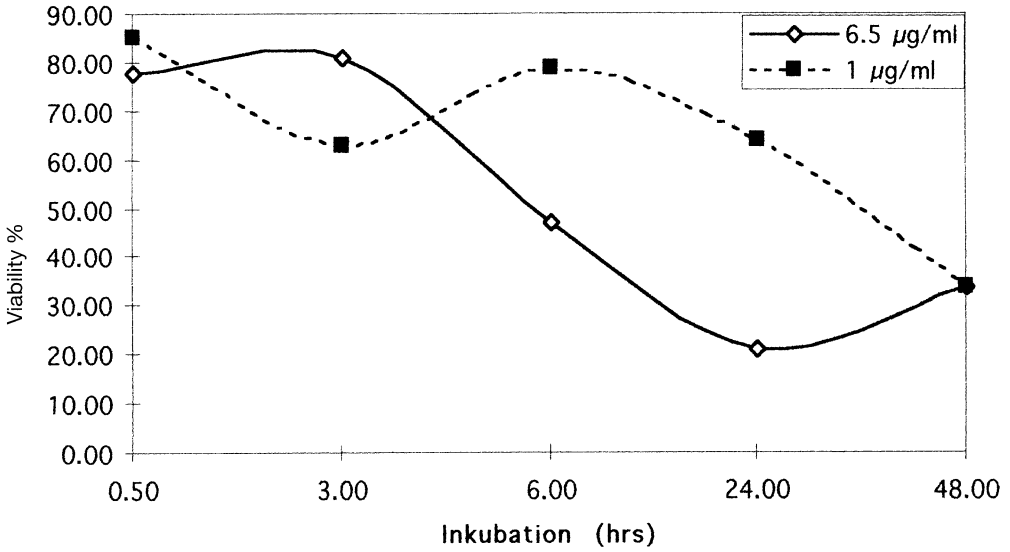

Fig. 6. Viability (%) of HL-60 cells after different times of incubation with etoposide. Viability of control was 80-90%

Table 3. Differentiation inducing capacity of topo II-inhibiting drugs

Induction[a]	Drug[b]	Mode of action
No (0-8%)	Novobiocin, coumermycin	ATPase site binder
Low (18-22%)	Anthracyclines, mAMSA	DNA-intercalator
High (26-53%)	Epipodophyllo-toxines	Non-intercalator

[a] Number of differentiated cells in percent from all living cells.
[b] Optimal concentration for induction 10-100 times below plasma peak concentration of drug.

of DNA binding of topo II, inhibition of isoenzymes, etc.). It might result in specific cellular reactions such as inhibition or induction of cellular differentiation or proliferation. At these concentrations, the cells are still able to compensate for the functional loss of the enzymes as apoptosis in not induced.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Involvement of ICE-Like Proteases in Gemcitabine-Induced Programmed Cell Death

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Abstract. Activation of ICE-like-protease cascade is a crucial step in Fas/APO-1 (CD95) induced apoptosis in different cell lines. Here we determined the role of interleukin-1 ß-converting enzyme (ICE) and CPP32/YAMA in gemcitabine induced apoptosis in leukemic cell lines. Gemcitabine (dFdc) is a purin analog, that induces typical features of apoptosis in several leukemic lines.

After starting gemcitabine incubation CCRF-CEM cells exhibited an 9-fold increase in CPP32-activity and an 3.5-fold increase in ICE-activity. In HL-60 CPP32 activity increased 15-fold and ICE an 2-fold after gemcitabine. Preincubation with ICE- and CPP-inhibitory peptides mainly prevented enzyme activation. However, survival was not affected by inhibiting ICE and CPP32. We therefore concluded that either CPP32 and ICE-function is not essential for gemcitabine induced apoptosis or other members of the ICE-family res. other proteases take over proteolytic function.

Introduction

Proteolytic cleavage of functionally essential cellular key enzymes play a central role in the biological processing of apoptosis [1]. The involvement of sequential activation of interleukin-1 ß-converting enzyme (ICE)-like proteases in FAS induced apoptosis has been demonstrated for several CD95-positive cell lines [2-6]. ICE proteases apparently play also a crucial role in staurosporine-induced programmed cell death (PCD) [7]. ICE-like proteases are cysteine proteases with a high affinity to aspartic acid [8, 9]. Several ICE homologues have been identified.

With regard to cleavage function during the course of apoptosis, interleukin-1 ß-converting enzyme itself and CPP32/Yama are by now the best characterised ICE-like proteases.

ICE is known to cleave pro-IL-1ß and pro-CPP32/Yama [10]. CPP32 has been shown to cleave lamins, globular actin, the nuclear mitotic apparatus protein (NuMA), the 70-kDa protein component of the U1-ribonucleoprotein (U1-70 kDa), poly(ADP-ribose)polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) [10-12]. At least PARP and DNA-PK are believed to play an important role in regulating the cell cycle and repair in response to DNA damage and repair.

We hypothesised that cytotoxic drug induced apoptosis also involve activation of ICE-like proteases. Therefore we applied Gemcitabine (2′,2′-difluorodeoxycytidine, dFdc), a nucleoside analog to induce DNA

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fragmentation leucemic cells [13]. As target cell lines CCRF-CEM (T-ALL) and HL-60 (AML) were used.

Material and Methods

Materials

Gemcitabine (Lilly Deutschland GmbH, Bad Homburg) was provided as pure substance and dissolved in sterile distilled water before each experiment in order to obtain constant quality of preparations.

CPP32 inhibitor tetrapeptide DEVD-cho (N-acetyl-Asp-Glu-Val-Asp-CHO) and ICE inhibitor YVAD-cho (N-acetyl-Tyr-Val-Ala-Asp-CHO) were purchased from Bachem Feinchemikalien, Bubendorf, Switzerland. Fluorogenic substrates DEVD-AFC ((N-acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin) and YVAD-AFC (N-acetyl-Tyr-Val-Ala-Asp-7-amino-4-trifluoromethyl coumarin) were purchased from Biomol Feinchemikalien, Hamburg, Germany. All peptides were stored as stock solutions in DMF.

Cell Lines and Culture Conditions

CCRF-CEM cells and HL-60 were grown in RPMI 1640 containing 10% FCS, 10 mM HEPES, pH 7.3 and 2 mM l-glutamine. For experiments cell lines were washed twice in RPMI 1640 and then cultured in medium with addition of gemcitabine and/or peptides.

Assay of Cell Viability

Cell viability was assessed morphologically by staining nuclei of cells with Hoechst 33342 and PI.

Determination of Apoptosis

For analysis of DNA-fragmentation, cell pellets were treated with lysis buffer at 37 °C overnight. Protein was then precipitated with NaCl and centrifuged at 3300 g. DNA in the supernatant was precipitated overnight with ethanol at -20 °C. After centrifugation at 3300 g, the pellet was dissolved in Tris-HCl/EDTA and incubated with RNase. Subsequently electrophoresis on a 1.4% agarose gel was performed followed by ethidium bromide staining.

Western Blot Aanalysis of CPP32 and ICE

For Western blotting, proteins obtained from whole-cell lysates were separated by 12% SDS-PAGE and transferred to ECL membranes (Amersham, Braunschweig, Germany). Detection of CPP32 was performed using a monoclonal mouse-anti-CPP32 antibody (Clone 19, Transduction Laboratories, Lexington, USA) For 45 kDa ICE precursor a rabbit polyclonal antibody was applied (Clone A19, Santa Cruz Biotechnology, Santa Cruz, California). For detection ECL-system was used (Amersham). Equal loading was proven by actin control staining.

Assay of Enzyme Activity

Treated cells (3×10^6) were collected, washed three times with PBS and suspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 10 mM EGTA. After the addition of 10 μM digitonin, cells were incubated at 37 °C for 10 min. Lysates were centrifuged at 15000 rpm for 3 min and cleared supernatants were collected. Protein concentrations were adjusted to 30 μg/ml. Aliquots (2 ml) were incubated with 10 μl of DEVD-AFC (50 μM) at 37 °C for 15 min or YVAD-AFC (50 mM) at 37 °C for 30 min. The release of 7-amino-4-trifluoromethyl coumarin was monitored by a spectrofluorometer (Hitachi F-2000) using an excitation wavelength of 400 nm and an emission wavelength of 505 nm. Activity of controls were taken as 100%.

Results

ID50 (48 h) for gemcitabine was 0.0075 μmol/l in HL-60 and 0.02 μmol/l in CCRF-CEM. Drug incubation was done perma-

nently with gemcitabine at a concentration of 0.01 µmol/l i.e., without intercurrent drug removal by washing. Gemcitabine induced cell death was in both lines associated with typical oligonucleosomal DNA-fragmentation.

Fluorimetrical monitoring for specific cleavage of AFC-conjugated tetrapeptides was performed for interleukin-1 ß-converting enzyme (YVAD-AFC) and CPP32 (DEVD-AFC) separately. Highest levels of proteolytic activity were measured 8 h after starting gemcitabine (0.01 µmol/l) treatment in HL-60 and CCRF-CEM.

In HL-60 CPP32 activity after gemcitabine reached 8 h after starting treatment 1480% of activity in untreated control. ICE activity increased up to 220%. In CCRF-CEM CPP32 activation peaked at 950% and ICE at 370% of untreated control (Fig. 1).

In parallel protein levels of CPP32 and ICE-precursor were measured using ECL-western blots. After gemcitabine incubation levels of inactive 32 kDa CPP32 precursor as well as inactive 45 kDa ICE precursordecreased because of activation by specific cleavage.

ICE res. CPP32 enzyme activities were inhibited by pretreatment and additional permanent treatment with aldehyd-conjugated synthetic tetrapeptides. Therefore we used YVAD-CHO for ICE- and DEVD-CHO for CPP32-inhibition. Pretreatment was started 30 min before drug treatment. Peptide concentrations were 50 µMol for YVAD-CHO and 50 µM for DEVD-CHO, which were the highest non-cytotoxic concentrations.

Increase of enzyme activity could be mainly neutralised by peptide pretreatment. In HL-60 CPP 32 increase was cut from 1480 to 270%, ICE from 220 to 110%. Corresponding movements were 950 to 250% and 370 to 160% in CCRF-CEM (Fig. 1).

With regard to survival after gemcitabine treatment there was no significant difference between cells pretreated with ICE- or/and CPP32 inhibitors and non-pretreated cells (Table 1).

Table 1. Vitality after gemcitabine treatment with and without pretreatment

Vitality (%)	HL-60	CCRF-CEM
control	98	99
Gemcitabine 0.01 mmol/l	38	55
Gemcitabine 0.01 mmol/l + YVAD-Cho	36	54
Gemcitabine 0,01 mmol/l + DEVD-Cho	42	58
Gemcitabine 0.01 mmol/l +YVAD-Cho + DEVD-Cho	33	53

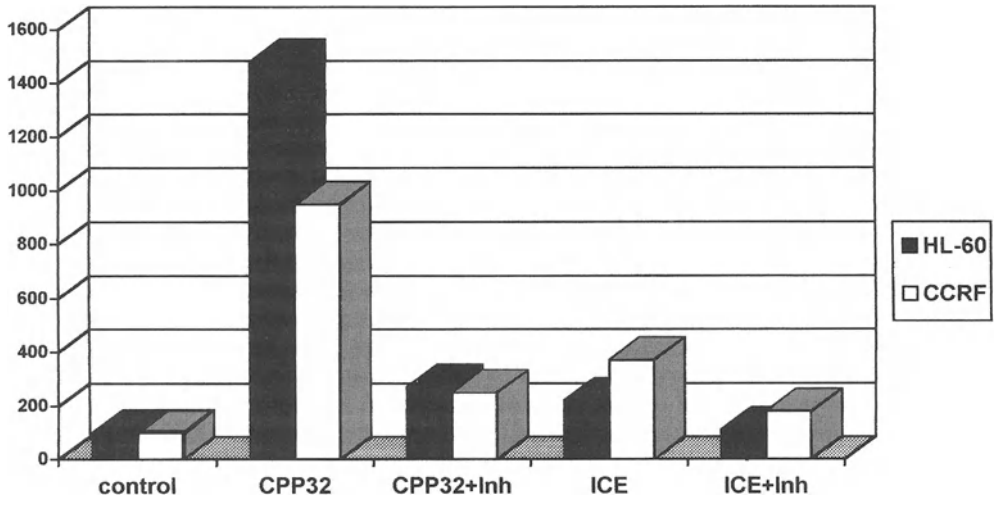

Fig. 1. Proteolytic activity of CPP32 and ICE in HL-60 and CCRF-CEM after incubation with Gemcitabine (8 h). Pretreatment with specific tetra-peptide enzyme inhibitors. Controls are untreated. *+Inh* plus inhibitory peptide

Discussion

It is now clear that members of the ICE-like protease family play key biological roles in apoptosis induced by different stimuli. Best examined induction mechanism involving ICE-like proteases is Fas/APO-1-mediated apoptosis. In HeLa and Hep G2 cells CPP activity increases dramatically shortly after exposition to Fas agonistic antibody [4]. Selective inhibitors of CPP32 prevent Fas mediated apoptosis while ICE-inhibitors are much less effective [4, 5]. Recent works demonstrated the involvement of the Fas/APO-1 receptor/ligand system and subsequently also the ICE-proteases in drug-induced apoptosis in leukemia cells [14]. But there are only few reports on the effect of cytotoxic drugs on the ICE-proteases. Jacobsen et al. demonstrated that staurosporine induced apoptosis in GM701 cells can be blocked by the use of specific CPP32-inhibitors but not by ICE-inhibitors [7].

We decided to determined the role of ICE and CPP32 in gemcitabine induced apoptosis. Gemcitabine (2′, 2′-difluorodeoxycytidine, dFdc) was chosen because of its ability to induce typical morphological and biochemical patterns of apoptosis in leukemic cell lines.

ICE-like proteasese are cysteine proteases with a high affinity to aspartic acid [8, 9]. Several ICE-homologues have been identified (e.g., ICE, CPP32/Yama, ICH-1, Mch2). In order to survey the growing number of ICE-members recently even an own ICE-nomenclature has been created. The family name caspases was proposed. C stands for the cysteine protease mechanism, aspase refers to their ability to cleave after aspartic acid. By now caspases were devided into 10 subgroubs [15].

With regard to cleavage function during the course of apoptosis, interleukin-1 ß-converting enzyme (caspase-1) itself and CPP32/Yama (caspase-3) are well characterised. We therefor decided to concentrate our investigations on CPP32 and ICE.

In HL-60 as well as in CCRF-CEM we observed a marked increase in CPP32 proteolytic activity after gemcitabine exposure whereas ICE activity rised only slightly.

This corresponds well with the data known from Fas and staurosporine induced apoptosis [4, 5, 7]. The increase of both activities could be suppressed in part but not totally using CPP32 and ICE inhibitors. In parallel to activity increases protein concentration of inactive CPP32 and ICE-precursors decreased as expected after (auto)-activation by cleavage.

After all, surprisingly, inhibition of CPP32 and/or ICE had no significant effect on cell survival after gemcitabine. Although CPP32 and ICE-activity increase could be abrogated at least in part, there was no protective effect on gemcitabine induced toxicity in both cell lines. These results are standing in direct contrast – as mentioned above – to what has been reported about Fas and staurosporine induced apoptosis.

A potential explanation for our results could be that depending on the mechanism of apoptosis-induction different components of the large number of ICE-like-family members are activated. Another possibility would be that activation of the ICE-cascade is an arbitrary mechanism in the course of gemcitabine-induced programmed cell death. Then gemcitabine would intiate alternative mechanism to kill leukemic cells via apoptosis.

In order to answer these questions further investigations are necessary. Monitoring of other ICE-like components has to be done. Also interesting is to study the involvement of other PCD-relevant systems like PARP and mitochondrial function.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Effect of Combined Treatment with 4-Hydroperoxycyclophosphamide and Fludarabine on Cytotoxicity and Repair of Damaged DNA

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Abstract. For treatment with cyclophosphamide (CP) rapid repair of CP-induced DNA interstrand cross-links (ICLs) seems to be an important problem in the lack of activity. This implies the idea of combined treatment of CP with nucleoside analogs such as fludarabine or gemcitabine, which should be able to inhibit repair of damaged DNA. Using multiple drug effect analysis we investigated the interaction between the cyclophosphamide prodrug 4-HC and the nucleoside of fludarabine, F-ara-A. Simultaneous exposure to the drug combination 4-HC/F-ara-A for 4 h resulted in synergistic reduction of clonogenicity in the lymphoblastic cell line CCRF-CEM. This finding was in agreement with repair experiments of 4-HC induced ICLs. After 4 h exposure to 10 μM 4-HC alone and washing into drug-free media, more than 80% of the ICLs were repaired after 6 h. In contrast, the retention of ICLs was increased in cells incubated with 4-HC in combination with F-ara-A, such that most of ICLs remained after 6 h. These results suggest a possible role of nucleoside analogs to suppress the repair of damaged DNA. This may be a mechanistic basis for the synergistic cytotoxicity of combined treatment with alkylating agents and nucleoside analogs.

Introduction

High dose combination chemotherapy remains the principal approach to achieving maximal responses during clinical treatment of leukemias and solid tumors in comparison to the observation that single agent treatment in the past was unable to eradicate most haematologic malignancies. Drug selection for combination chemotherapy regimes is generally based on the knowledge of the single agent activity against the malignancies, the known dose-limiting toxicities, and cross-resistance data. However, to develop novel strategies in the treatment of leukemia and solid tumors it is necessary to investigate the mechanism of interaction between different cytostatic drugs. This rationale is illustrated by the combination of DNA damaging agents, such as cyclophosphamide (CP), with fludarabine or gemcitabine, nucleoside analogs that inhibit DNA replication and repair.

CP is a commonly used antineoplastic agent that shows activity against a variety of neoplastic diseases and is also used as an immunosuppressive agent in bone marrow transplantation [1-3]. The DNA alkylating agent 4-hydroperoxycyclophosphamide (4-HC) is an active derivative of CP, which is metabolized to the same active alkylating end products as CP, but does not require P450 liver enzymes. 4-HC is used for in vitro studies and for ex vivo purging of dormant leukemic cells from peripherial stem cells of AML patients prior autologous transplantation [4]. CP and 4-HC are each metabolized in two different competing

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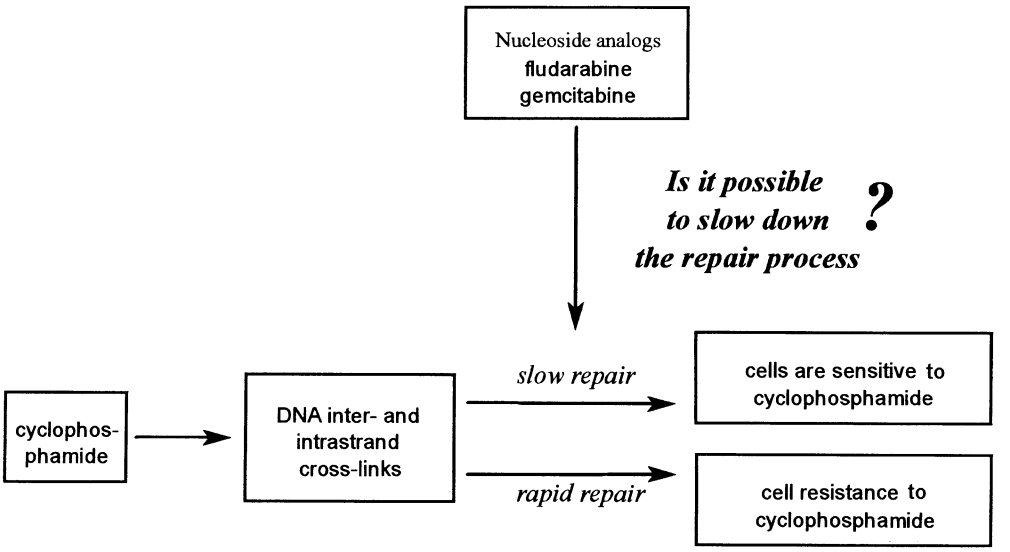

Fig. 1. Mechanism of interaction between cyclophosphamide and the nucleoside analogs fludarabine and gemcitabine

pathways to phosphoramide mustard and acrolein and on the other hand to nornitrogen mustard [5, 6]. The cytotoxic action is thought to result mainly from phosphoramide mustard-induced DNA interstrand and intrastrand cross-links. Acrolein is reported to bind to proteins, to form DNA mono adducts, to induce DNA single and double-strand breaks. In contrast, the action of nornitrogen mustard results in DNA protein cross-linking [5-8]. Some studies about alkylating agents showed that cell resistance is correlated with rapid repair of DNA interstrand cross-links, increased aldehyde dehydrogenase activity or elevated levels of glutathione [9-16]. Overcoming cell resistance to alkylating agents implies the idea of combined treatment of these cytostatic drugs with nucleoside analogs, which should be able to slow down the repair process of damaged DNA (Fig.1). The nucleoside analog fludarabine is an effective drug in the treatment of leukemia [17, 18]. The activity of this drug is dependent on the phosphorylation of the prodrug nucleoside, F-ara-A, to the active metabolite F-ara-ATP, which inhibits DNA, and RNA synthesis, and leads to cell apoptosis [19, 20]. Especially the inhibition of key enzymes which are involved in DNA metabolism, e.g. ribonucleotide reductase [19, 21], DNA primase [22], the polymerase ε-associated 3 to 5 exonuclease [23] and DNA ligase [19] are associated with the possibility that these nucleoside analogs are effective inhibitors of DNA repair.

To develop better strategies in the treatment of leukemia it was the aim of the present study to investigate whether a synergistic effect can be obtained in CCRF-CEM cells incubated with 4-HC and F-ara-A. In addition we studied the role of F-ara-A on the repair of 4-HC-induced DNA interstrand cross-links.

Materials and Methods

Chemicals

F-ara-A was a gift from Dr. V. L. Narayanan of the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD). 4-HC was obtained as a gift from Dr. Michael Colvin, John Hopkins University School of Medicine (Baltimore, MD). Immediately before use, the drug was dissolved in Millipore-pure and filtered water. Proteinase K and Rnase (DNase free) were from Boehringer Mannheim (Indianapolis, Ind.). All other reagents were of analytical grade and commercially available.

Cell Lines

The human T-lymphoblastic cell line CCRF-CEM was obtained from the American Type Culture Collection (Rockville, MD). The cells were kept in exponential growth at 37 °C and maintained in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco). Under these conditions, the population doubling time was between 20 and 24 h.

Clonogenic Assay and Analysis of Drug Interactions

The determination of the cytotoxicity was performed using a clonogenic assay with the following modifications. Plastic dishes (60 mm) were filled with 4 ml bottom layer supplemented with RPMI 1640, 20% FBS, 1% penicillin/streptomycin and 0.7% low melting agarose (Gibco) and 1 ml upper layer including cells in RPMI, 20% FBS, 1% antibiotics and 0.35% low melting agarose. Cells were incubated without antibiotics with various concentrations of 4-HC (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 μM) and/or F-ara-A (0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10 μM) for 4 h and washed twice with drug free medium. Because of the instability of 4-HC ($t_{1/2}$ in tissue culture is about 2 h), no experiments were done with 24 h incubations. Depending on the drug concentration, 200-1000 cells were plated in quadruplicate and incubated for 11 days. At the end of the incubation period, the clones were stained with p-iodonitrotetrazolium violet (Sigma) and colonies of more than 100 cells were counted with an inverted microscope. To analyze drug interaction, cells were incubated with these different concentrations of 4-HC and F-ara-A as single agents, and simultaneously in combination at a fixed concentration ratio of 1:1. The fraction unaffected (Fu) and fraction effected ($F_a = 1-F_u$) were calculated by dividing the number of colonies in drug-treated plates by the number of colonies in the control plates. Dose response interactions (antagonism, additivity, and synergism) between 4-HC and F-ara-A were analyzed by the method of Chou and Talalay, calculated using a computerized program of Chou and Chou [25, 26]. The results were plotted as combination index ,CI, versus fraction affected, Fa. According to this method synergism is indicated by a CI less than 1, additivity by a CI equal to 1, and antagonism by a CI greater than 1. Linear correlation coefficients, r, were generated for each curve of drug interaction to determine precision, which in all experiments was > 0.84.

Isolation of Genomic DNA for the Determination of DNA Interstrand Cross-Links

4-HC-induced DNA interstrand cross-links were determined after cells were incubated for 4 h with 10 μM 4-HC alone or in combination with 10 μM F-ara-A. At the end of the drug exposure, cells were washed twice and incubated in drug-free media for up to 6 h. In part of the cells, ICLs were measured at the end of the 4 h drug exposure. At these times, 5×10^6 - 2×10^7 cells were lysed with 150 μg/ml RNAse in digestion buffer containing 0.5% sodium dodecyl sulfate, 10 mM Tris-HCl buffer, pH 8, and 1 mM EDTA for 1 h at 37 °C and for additional 2 h at 50 °C with 0.5 mg/ml proteinase K. At the end of the incubation, genomic DNA was isolated in three steps by extraction first in an equal volume of phenol, second in equal volumes of phenol/chloroform/isoamylalcohol (25:24:1) and finally chloroform/isoamylalcohol (24:1). The DNA was precipitated with two volumes of cold 100% ethanol and half a volume of cold 5 M ammonium acetate. All steps of the DNA extraction procedure were performed in low light. Finally, the DNA was washed twice with 70% ethanol, air dried and resuspended in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8). The purified DNA was kept in the dark at 4 °C until determination of the DNA interstrand cross-links. Under these conditions the interstrand cross-links were stable for up to 1 month.

Quantitation of DNA Interstrand Cross-Links

For quantitation of the DNA cross-links an ethidium bromide fluorescence (EBF) assay was used as described previously [27, 28]. Briefly, from each sample 10 μg of the purified DNA (as described above) were trans-

ferred in duplicate in two brosilicate tubes. After adding 400 µl of assay buffer, containing 20 mM potassium phosphate and 2 mM EDTA, pH 12.0, one of the tubes was heated in a water bath at 100 °C for 10 min and then rapidly cooled in a 27 °C water bath. Meanwhile, the other tube was kept at 27 °C. Finally 2.5 ml assay buffer containing ethidium bromide (1 µg/ml) was added to each tube and the fluorescence was measured in 1 cm^2 cuvettes at room temperature in a Perkin Elmer LS-50 fluorescence spectrophotometer (Norwalk, CT) with an excitation wavelength of 305 nm and an emission wavelength of 590 nm. In each assay, salmon sperm DNA (Sigma) with a known number of interstrand cross-links, formed by treatment with CDDP at 37 °C for 4 h [28], was incubated as a reference. The relative cross-link index ,CLI, was calculated as follows:

$CLI = (N_d - N_c)/ N_c$

N_d = number of cross-links per DNA molecule in the drug treated sample

N_c = number of cross-links per DNA molecule in the drug free control

$N = -\ln(1- (H/NH))$

H = fluorescence value of the heated sample

NH = fluorescence value of the non heated sample.

Thereby 1-(H/NH) is the fraction of fluorescence representing the amount of non-cross-linked DNA.

Results

Cytotoxicity and Interaction Between 4-HC and F-ara-A

A highly synergistic effect was obtained for a combined treatment of CCRF-CEM cells with 4-HC and F-ara-A in a concentration ratio 1:1 at concentrations higher than 1.8 µM. At lower concentrations this was converted to antagonism. The concentrations at which synergy was observed are clinically relevant for high dose chemotherapy. The mean single-agent IC50 values (the concentration of drug that inhibits cell growth by 50% in comparison to the control without drug) was 4.7 µM for F-ara-A and 5.5 µM for 4-HC after 4 h incubation. Control cells had a colony forming efficiency of 29±7%.

Effects of F-ara-A on the Repair of 4-HC-Induced DNA Interstrand Cross-Links

DNA-DNA interstrand cross-link formation (ICL) was maximal after a 4-h incubation with 4-HC. In CCRF-CEM cells, the ICL index was 0.35±0.16 after a 4-h incubation with 10 µM 4-HC alone and 0.38±0.15 for the combined treatment with 10 µM 4-HC and 10 µM F-ara-A. More than 80% of the DNA interstrand cross-links were repaired during the first 6 h, if cells were incubated with 4-HC, only. After this time, the cross-link index signal decreased below the limit of detection, 0.1. The elimination half-life for ICLs was 3.2-0.6 h. In contrast, the retention of ICLs was increased in cells incubated with the combination, such that most of the ICLs remained after 6 h.

Discussion

In this study, treatment of CCRF-CEM cells in vitro with 4-HC in combination with the nucleoside analog, F-ara-A, produce synergistic cytotoxicity. The interaction effect was synergistic at drug concentrations that are clinically relevant.

To investigate the mechanism of interaction further, we studied drug-induced DNA damage. Previous studies showed that phosphoramide mustard, one active metabolite of 4-HC and CP, caused mainly DNA-DNA inter- and intrastrand cross-links. These studies were focused on 4-HC treatment as a single drug and it´s role in damage of DNA [6, 16, 29-33]. In our experiments we demonstrated, in the interaction between 4-HC and F-ara-A, the role of F-ara-A was to slow down the repair of 4-HC-induced DNA interstrand cross-links. In CCRF-CEM cells, F-ara-A was able to inhibit the repair of damaged DNA. Similar observations were described by Yang et al. for the drug combination of cisplatin and F-ara-A [28]. Furthermore, our results are in accordance with

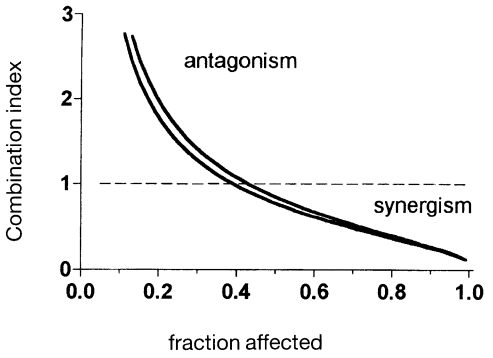

Fig. 2. Interaction between 4-HC and F-ara-A: CCRF-CEM cells were incubated for 4 h with 4-HC (0.05-50 μM) and F-ara-A (0.05-50 μM) alone or in the fixed ratio 1:1 and clonogenicity was determined after 11 days. Combination Index plots were generated by computerized median-effect analysis using the mutually non-exclusive isobologram equation *(upper curves)* and the mutually exclusive isobologram equation *(lower curves)*. The values of Combination Index more than 1 represents antagonism and less than 1 synergism

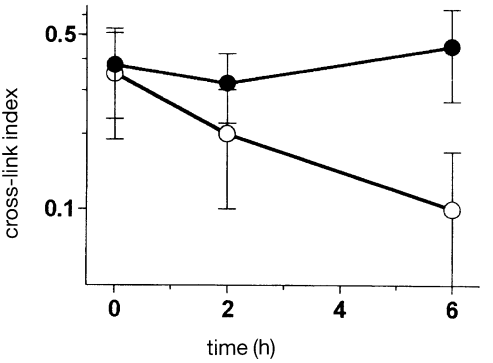

Fig. 3. Influence of F-ara-A on the repair of 4-HC induced inter-strand cross-links: CCRF-CEM cells were incubated with 10 μM 4-HC alone *(open symbols)* or in combination with 10 μM F-ara-A *(closed symbols)* for 4 h and after removing in drug free medium for up to 6 h without drugs. DNA interstrand cross-link index was determined by the EBF-assay. Time point 0 represents the ICL formation after the 4-h incubation

the first clinical results for treatment of chronic lymphocytic leukemia with cyclophosphamide and fludarabine at M.D. Anderson Cancer Center. This drug combination showed a higher response rate in previously treated patients resistant to fludarabine alone and also to alkylating agents [34].

Data in the literature about a correlation between cell toxicity and formation and repair of 4-HC-induced cross-links are conflicting [11, 12, 29]. Inhibition of the repair of DNA-DNA interstrand cross-links by nucleoside analogs seemed to be only one of several events, which contributes to the synergism between 4-HC and nucleoside analogs. Other reasons are described: First, 4-HC is also known to induce DNA protein cross-links, intrastrand cross-links, which may contribute to single- and double strand breaks [6, 30-32]. Second, the removal of ICLs does not mean that the lesion at the DNA is entirely repaired. Complete cross-link repair requires subsequent removal of the adducted base in both opposite strands, as well as repair patch synthesis and ligation in both strands [8]. The study of Wang et al. showed that repair of ICLs and DNA-protein cross-links might be an early event, which produce secondarily single strand breakage [6]. This could be dependent on the concentration of 4-HC, and might be an explanation that in our study ICLs could be detected reproducibly by an ethidium bromide assay for concentrations between 1 and 20 μM 4-HC. Third, the repair mechanism of damaged DNA is a multifactorial process with reversion, excision and tolerance of DNA lesions [13]. Although the excision repair process seemed to be most important for the repair of DNA cross-links, other repair mechanism like recombinational repair, mismatch repair, and a mechanism of DNA repair similar to the SOS repair in prokaryotic cells may also play an important role in the repair cycle [13, 14, 35, 36].

Summarizing, our studies indicate that combined treatment with cyclophosphamide and the nucleoside analog fludarabine might help to overcome cell resistance to cyclophosphamide due to rapid repair of adducted DNA. In a second sense, the initiation of repair processes that include DNA resynthesis steps permit the incorporation of fludarabine into DNA. Thus, complementary mechanism of actions of the combined treatment with 4-HC and F-ara-A point at a hopeful new aspect in the treatment of relapsed CLL. Further investigations should be focused on the understanding the ability of alkylating agents to initiate different DNA

repair mechanisms and the inhibition of these processes by nucleoside analogs. Finally, a better understanding about the expression of specific genes involved in the repair of damaged DNA may provide a pharmacodynamic basis for the design of schedules of drug administration in the clinic.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Enhanced Effects of Adriamycin by Combination with a New Ribonucleotide Reductase Inhibitor, Trimidox, in Murine Leukemia

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Abstract. Ribonucleotide reductase is the rate limiting enzyme of de novo DNA synthesis; its activity is significantly increased in tumor cells related to the proliferation rate of the tumor cell. Therefore the enzyme is considered to be an excellent target for cancer chemotherapy. In the present study we tested the in vitro and in vivo antitumor effects of a drug combination using trimidox (3,4,5-trihydroxybenzohydroxamidoxime), a novel inhibitor of ribonucleotide reductase with adriamycin, a widely used anticancer drug. This combination was selected because adriamycin generates free radicals, which are responsible for cardiotoxic side effects of adriamycin treatment, and because trimidox has been shown to be a good free radical scavenger. The in vitro cytotoxic effect of the drug combination was examined in L 1210 mouse leukemia cells employing an MTT chemosensitivity assay. Simultaneous in vitro incubation of these cells yielded antagonistic cytotoxic effects compared to either drug alone. These effects were not caused by the involvement of p-glycoprotein mediated drug efflux. However, when the effect of trimidox and adriamycin in combination was examined in L 1210 leukemia bearing mice, antitumor effects of adriamycin could be enhanced by the presence of trimidox. Animals were treated on day two after tumor cell injection with 5 mg/kg adriamycin and received 250 mg/kg trimidox on days 2,3 and 4. Mice treated with adriamycin or trimidox alone yielded a 41 and 38% increase in life span, respectively. However, animals, which were treated with both drugs, showed a 89% increase of their life span. Our data indicate, that in vitro results of drug combinations should be interpreted with extreme caution and suggest that the in vivo combination of adriamycin together with trimidox might be beneficial for the treatment of malignancies.

Introduction

Ribonucleotide reductase (RR) is the rate limiting enzyme of de novo DNA synthesis. The enzyme was shown to be significantly increased in activity linked with malignant transformation and proliferation [1, 2]. It was therefore considered to be a good target for cancer chemotherapy [3, 4]. Although various inhibitors of RR have been synthesized, presently only hydroxyurea, a relatively weak inhibitor of the enzyme, is clinically used for the treatment of malignancies.

A newer group of inhibitors of RR are polyhydroxy-substituted benzoic acid derivatives [1, 5-8]. Among these compounds, trimidox (3,4,5-trihydroxybenzohydroxamidoxime) was one of the more effective enzyme inhibitors and demonstrated excellent anticancer activity in animal tumor models. It inhibited the growth of various tumor cell

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lines more effectively than hydroxyurea and demonstrated in vivo activity in L1210 leukemia bearing mice superior to hydroxyurea [9, 10]. Trimidox was also shown to exhibit iron binding capacity and to be a free radical scavenger [11, 12].

Adriamycin is a potent anticancer drug with various mechanisms of action ascribed to its effectiveness. It intercalates with the cellular DNA, generates free radicals and causes cell membrane damage [13, 14]. Adriamycin is clinically used in a broad range of tumors, such as breast, bladder, endometrium, lung, stomach or thyroid carcinomas. It is also used in the treatment of sarcomas of the bone and soft tissue, pediatric solid tumors or in the treatment of haematological malignancies [13]. However, its action is compromised by the formation of free radicals, in particular hydroxyl radicals, which are responsible for toxicity, particularly the anthracyclin-induced cardiotoxicity [15].

The aim of the present study was to investigate the combination effects of adriamycin with trimidox in vitro and in L1210 leukemia bearing mice. As both agents have different modes of action and trimidox was shown to scavenge free radicals, we expected trimidox to be capable of protecting against the toxicity caused by adriamycin [11, 16]. In addition, didox (3,4-dihydroxybenzohydroxamic acid), a first generation polyhydroxy-substituted benzohydroxamate improved the antitumor effect of adriamycin in leukemia bearing mice [16]; therefore we now tested trimidox, the more effective RR inhibitor regarding its combination effect with adriamycin. In vitro cytotoxicity against L 1210 mouse leukemia cells was examined using a MTT (3-[4,5-dimethylthiazo-2-yl]-2,5-diphenyl tetrazoliumbromide chemosensitivity assay. Also the combination effect of trimidox together with adriamycin was examined in L 1210 leukemia bearing animals.

Material and Methods

Cell Lines

The L 1210 mouse leukemia cell line was purchased from the American Type Culture Collection (Rockville, MD, USA).

Cell Culture

The cell line was kept in RPMI 1640 medium supplemented with 10% heat inactivated FCS (Grand Island Biological Co., Grand Island, NY), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 : 95% air.

MTT Chemosensitivity Assay

Cells were incubated at a concentration of 10 000- 20 000 cells /well in 96-well microtiter plates in supplemented RPMI 1640 medium. Cells were incubated simultaneously with various concentrations of adriamycin and trimidox for 4 days. Then the reduction of the tetrazolium compound (3-[4,5-Dimethylthiazo-2-yl])-2,5-diphenyl tetrazoliumbromide (MTT) was assayed using an assay kit (Promega) according to the supplier's manual. The absorbance was recorded using a Microplate Autoreader (BIO-TEK Instruments) [9].

Animals

Inbred male mice of the DBA/2J strain (Velaz, Prague, Czech Republic) weighing 18-20 g were caged in experimental groups under standard conditions.

L1210 in Vivo Leukemia and its Treatment

L 1210 leukemia cells (10^5 per mouse) were inoculated into the peritoneal cavity. Six or more animals were treated per group. The animals were observed daily for the development of ascites and toxic or leukemia-related death.

Adriamycin Transport Study by Rhodamine Incorporation into L 1210 Cells

Cells (5×10^5/ml)l were incubated for 60 min at 37 °C in media containing 500 ng/ml rhodamine 123 (R123) as previously described by Feller and coworkers [17]. Verapamil (10 µM)

or trimidox (1 nM, 1 μM and 1 mM) were used as modulators and added to the cell suspension 30 min prior to incubation with rhodamine. After incubation with R123, cells were washed two times in ice cold PBS. Non specific binding of R123 was then measured after incubation of the cells at 4 °C for 5 min. Accumulation of R123 was determined using a FACStar flow cytometer (Becton-Dickinson). The excitation wavelength was 488 nm and fluorescence was monitored through a 530 nm band-path filter. Data were accumulated and evaluated using Consort30 and PC-LYSYS software packages (Becton-Dickinson).

Statistical Calculations

The calculations of dose response curves and determinations of synergism or antagonism were performed using a software designed by Chou and Talalay (Biosoft, Ferguson, MO, USA) [18]. Comparison of in vivo results were calculated using an unpaired t-test.

Results

In Vitro Cytotoxcicity of Adriamycin and Trimidox in L 1210 Cells

L 1210 cells were simultaneously exposed to various concentrations of adriamycin and trimidox; cytotoxicity was then determined by an MTT assay as described in the materials and methods. When cells were incubated with various concentrations of adriamycin for 4 days, the 50% growth inhibitory concentration (IC_{50}) was 0.06 μM. Under the same conditions, trimidox yielded an IC_{50} of 53 μM. When both drugs were used together, antagonistic results were observed as shown in Table 1. Combination indices were all >1, indicating, according to Chou and Talalay's calculation parameters, antagonistic growth inhibitory effects [18].

Table 1. Cytotoxic effects of adriamycin and trimidox on L 1210 leukemia cells

Agent/ combination	Conc. (μM)	Absorbance (% of control)	Comb. index[a]
Adriamycin (A)	0.03	78.4	
	0.04	68.7	
	0.05	52.1	
	0.1	27.1	
	0.2	9.4	
Trimidox (B)	20	97.3	
	30	91.7	
	40	74	
	80	4.9	
Adriamycin + Trimidox	0.03 20	90.3	2.406[b]
Adriamycin + Trimidox	0.04 20	81.1	2.095[b]
Adriamycin + Trimidox	0.05 20	61.7	1.592[b]
Adriamycin + Trimidox	0.03 30	71.9	1.649[b]
Adriamycin + Trimidox	0.04 30	56.1	1.472[b]
Adriamycin + Trimidox	0.05 30	46.1	1.422[b]
Adriamycin + Trimidox	0.03 40	33.4	1.110[b]
Adriamycin + Trimidox	0.04 40	29.7	1.161[b]
Adriamycin + Trimidox	0.05 40	19.9	1.059[b]

Data are means of three or more determinations; S.D. values were within 5%.
[a] Combination index was calculated according to Chou and Talalay.
[b] Antagonism: combination index >1.

Effect of Trimidox on Rhodamin Efflux in L 1210 Cells

As the in vitro combination of trimidox with adriamycin yielded antagonistic growth inhibitory effects, we investigated whether P-glycoprotein mediated drug efflux might be responsible for this antagonism. Cells were incubated with 10 μM verapamil or 1 mM, 1 μM and 1 nM trimidox for 30 min prior to rhodamine incubation. Then rhodamine accumulation was measured using FACS analysis. However, neither verapamil nor trimidox altered the rhodamine accumulation in comparison to previously untreated L 1210 cells (data not shown).

Antitumor Activity of Adriamycin with Trimidox

Murine leukemia L 1210 cells were injected intraperitoneally to DBA/2J mice. In the first experiment undertaken, adriamycin

treatment occured only on day 1 with a single dose of 5 mg/kg. The adriamycin treatment produced one cure out of the six animals tested. Treatment with 100 mg/kg trimidox alone also started in this initial experiment on day 1 and was repeated on days 2 and 3. This trimidox treatment caused a 23% increase of life span. When animals were treated with both adriamycin and trimidox on the schedule described above, three out of seven animals were cured (data not shown). Therefore we repeated this experiment delaying the treatment until day 2 after tumor cell injection. Animals were treated once on day 2 with 5 mg/kg adriamycin and with 250 mg/kg trimidox on days 2,3, and 4 singly and in combination. As shown in Fig. 1, adriamycin alone caused a 41% increase of life span when compared with untreated control animals. Mice treated with trimidox alone showed a 38% increase of life span, however, the tumor-bearing animals which were treated with both drugs had an 89% increase of their life span when compared with untreated controls. This value is significantly different from the increases in life span which were caused by either drug alone.

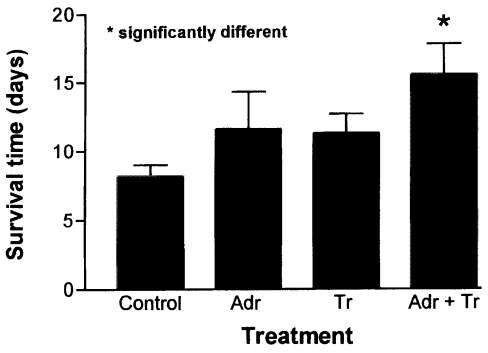

Fig. 1. Mean values ± standard deviation of medium survival times are plotted (6 animals per group). Adriamycin treated animals received 5 mg/kg adriamycin on day 2 after tumor cell injection. Trimidox (250 mg/kg) was administered on days 2,3 and 4. Animals treated with both drugs together showed an 89% increase in life span, when compared with untreated control animals. This is significantly different ($p < 0.02$) from the medium survival times of animals which were treated with either drug alone

Discussion

Ribonucleotide reductase (RR) is the rate limiting enzyme of de novo dNTP synthesis. The enzyme activity is significantly increased with malignant transformation and proliferation rate [1, 2]. Therefore RR is considered to be a good target of cancer chemotherapy [3, 4]. Van't Riet and coworker have synthesized a number of polyhydroxy-substituted benzoic acid derivatives as inhibitors of the enzyme [7, 8, 19]. Didox (3,4-dihydroxybenzohydroxamic acid), one of these compounds, proved to be an effective antitumor agent in vitro, in animals, and was used in clinical phase I and II studies [20-22]. Trimidox (3,4,5-trihydroxybenzamidoxime) and amidox (3,4-dihydroxybenzamidoxime) are second generation compounds, which were also shown to inhibit tumor cell growth with IC_{50}s significantly lower than those of didox or hydroxyurea, the first clinically used inhibitor of RR [9, 10, 23]. Didox and trimidox were shown to form complexes with iron and to be excellent free radical scavengers [11, 12, 19]. Didox also was shown to potentiate the antitumor effects of adriamycin, a widely used anticancer drug [16]. As adriamycin generates free radicals which can cause severe cardiotoxicity, didox was used in this combination to scavenge these free radicals, thus inhibiting anthracycline induced toxic side effects. Indeed, we could show that didox in combination with adriamycin significantly prolonged the life span of L 1210 bearing mice compared to either drug alone [16]. As trimidox acts also as a free radical scavenger and could even prevent ischemia reperfusion injury in the isolated rabbit heart [24], we now combined trimidox with adriamycin in order to potentiate the cytotoxic effects caused by adriamycin and to eliminate the side effects of adriamycin generated free radicals [11, 19, 24]. The combination was first tested in vitro employing a MTT chemosensitivity assay. Unexpectedly, in L 1210 mouse leukemia cells, simultaneous incubation of the cells with trimidox and adriamycin caused antagonistic cytotoxic effects. We initially believed that this antagonism could have been caused by mechanisms involving multidrug resistance, however the adriamycin trans-

port experiment indicates the in vitro modulation of adriamycin toxicity by trimidox is not due to P-glycoprotein mediated drug efflux. In addition, incubation of the L 1210 cells with verapamil, an inhibitor of P-glycoprotein did not increase the intracellular rhodamine accumulation. This observation underlines that MDR is not responsible for the observed in vitro combination effects of trimidox with adriamycin. In vivo, however the combination of adriamycin and trimidox was significantly synergistic prolonging the life span of L 1210 leukemia bearing mice compared to either drug alone. This can be explained by the different modes of action of trimidox and adriamycin. Trimidox may enhance the effectiveness of adriamycin by potentiating the DNA damaging effect of adriamycin by depleting the deoxynucleotide endogenous pools and thereby inhibiting DNA repair. Moreover, trimidox might have scavenged the cardiotoxic hydroxyl free radicals, which were generated by adriamycin. Elimination of the cardiotoxic side effects of adriamycin could have contributed to the significant increase in survival time of those animals which were treated with both agents. Also, our results indicate that in vitro cytotoxicity results have to be interpreted with caution. These results illustrate the danger of relying only on in vitro results. In particular when effects, like free radical scavenging effects, are involved in the action of drugs, data obtained from intact organisms rather than from cells in culture are usually more informative. Nevertheless, our results suggest the in vivo combination of adriamycin with trimidox to be an additional promising option for the treatment of malignancies.

Acknowledgments. This investigation was supported by the Jubiläumsfonds der österreichischen Nationalbank Grant No. 5267 to T.S. and by a grant of the Slovak Grant Agency VEGA grant Nos. 95/5305/353 and 1331/97 to L.N.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Pharmacological Aspects of the Oral Application of Etoposide

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Abstract. The oral application of etoposide is attractive for clinical use, but there are doubts about its reliability as regards the interpatient-variability. This study takes account of the duration of exposure and of the AUC and demonstrates that specific target concentration ranges can be obtained by oral etoposide therapy showing an interpatient-variability comparable to the one resulting from intravenous therapy.

Introduction

There is a need for effective oral chemotherapy regimens for outpatient treatment, but the oral use of etoposide is considered to be less reliable than its i.v. application. The anti-tumour activity of etoposide has been associated with the maintenance of low levels (0.5-2 μg/ml and 0.5-3 μg/ml), whereas levels >2 μg/ml and >3 μg/ml correlate with neutropenia [1, 2, 3]. Therefore it seems interesting to compare the duration of etoposide levels within the limits of 0.5 to 2 μg/ml and its interpatient variability resulting from oral and i.v. therapy. Additionally, we focussed on this target concentration range when comparing the area under the concentration-time-curve (AUC∞) and its variability (coefficient of variability, CV).

Material and Methods

Thirteen patients received etoposide orally (37-149 mg/m^2, median 88 mg/m^2), 15 children received short-time infusions (67-200 mg/m , median 150 mg/m^2) within different treatment schedules. A mean of 8 (oral) and 5 (i.v.) samples was taken per 24 h and measured using HPLC. As within this dosing range the kinetics of etoposide are linear [4], it was possible to normalise the data to the dose of 100 mg/m to ensure comparability.

Results

Duration of the Exposure to Various Etoposide Concentration Ranges

Oral application of 100 mg/m etoposide results in a significantly longer exposure to the concentration range of 0.5-2 μg/ml in comparison to i.v. therapy. The duration of higher serum levels of >2 μg/ml etoposide can be reduced by oral application.

The interpatient-variability resulting from oral treatment compared to i.v. therapy is higher only for the time of exposure to the higher serum levels above 2 μg/ml. There is no significant difference concerning lower etoposide concentrations above 0.5 μg/ml. With regard to the duration of levels between 0.5 and 2 μg/ml, the variability is even lower after oral application.

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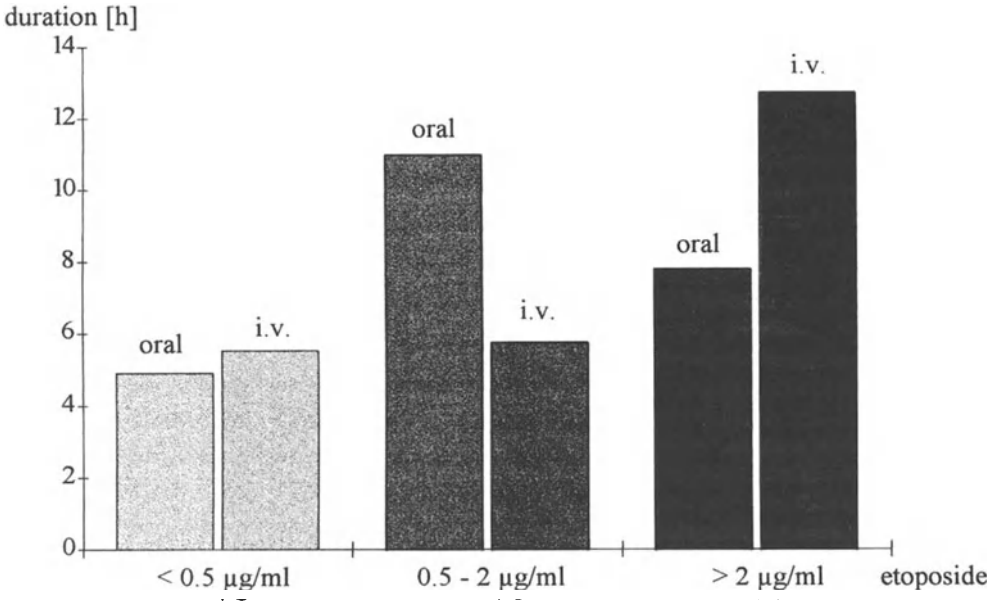

Fig. 1. Time of exposure [h] to various etoposide concentration ranges following the application of 100 mg/m^2 etoposide intravenously and orally; n=13 (oral), n=15 (i.v.)

Table 1. Time of exposure [h] to various etoposide concentration ranges and interpatient-variability (CV in brackets) after intravenous and oral therapy respectively; dose normalised to 100 mg/m^2; *p*-values for comparison of exposure resulting from t-test and Mann-Whitney Rank-Sum Test

Etoposide 100 mg/m^2	Exposure (h) to <0.5 µg/ml	Exposure (h) to >0.5 µg/ml	Exposure (h) to >2 µg/ml	Exposure (h) to 0.5-2 µg/ml
i.v.	5.5 (73%)	18.5 (22%)	12.7 (31%)	5.8 (36%)
Oral	4.9 (105%)	19.1 (27%)	7.9 (42%)	11.2 (24%)
p =	0.519	0.519	0.002	<0.0001

Contribution of Various Concentration Ranges to the Total AUC∞

The differentiation of the total AUC∞ shows that the percentage within the limits of 0.5-2 µg/ml is significantly higher following oral application of etoposide (38% vs. 30%). Peak levels higher than 2 µg/ml contribute to only 28% of the AUC in the case of oral therapy in contrast to 56% after i.v. treatment.

The interpatient-variability concerning the extent which the etoposide levels between 0.5 and 2 µg/ml contribute to the total AUC∞ after oral treatment is comparable to the one after i.v. application (17% each).

Discussion

There is no doubt about the advantage of oral therapy regimens concerning the benefits for the patient. The contribution of outpatient treatment and oral application of chemotherapy to an improvement of the quality of life should not be underestimated. Economic considerations, too, support the use of outpatient therapy with the option of considerable cost savings [5]. The discussion about the validity of the schedule-dependency of etoposide for different tumour types is still going on [6]. In a trial of the German Society for Pediatric Oncology / Hematology for the treatment of neuroblastoma continuous infusion schedules producing low etoposide steady-state levels

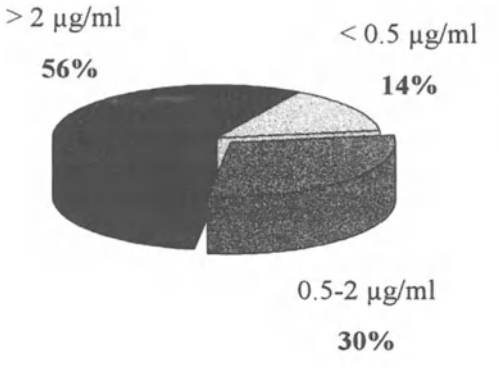

Fig. 2 a. Distribution of AUC (%) after *i.v.* application of 100 mg/m^2 etoposide (n = 9)

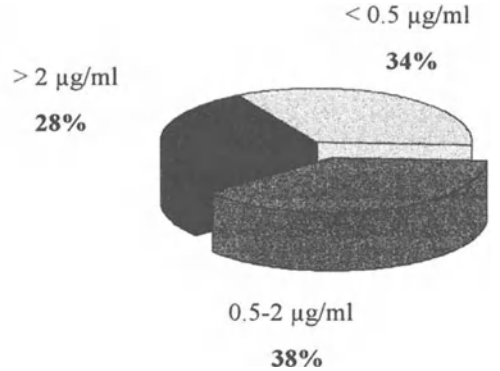

Fig. 2 b. Distribution of AUC (%) after *oral* application of 100 mg/m^2 etoposide (n = 9)

Table 2. Contribution of various etoposide concentration ranges to the total AUC∞ (%) and interpatient-variability (CV *in brackets*) after i.v. and oral therapy respectively; dose normalised to 100 mg/m ; *p*-values for comparison of percentage resulting from t-test and Mann-Whitney Rank Sum Test

Etoposide 100 mg/m^2	Total AUC∞ (µg × h /ml)	% of AUC∞ (>0.5 µg/ml)	% of AUC∞ (>2 µg/ml)	% of AUC∞ (0.5-2 µg/ml)
i.v.	80 (27%)	86 (3%)	56 (12%)	30 (17%)
Oral	42 (40%)	66 (17%)	28 (61%)	39 (17%)
p =	0.0002	<0.0001	<0.0001	0.0009

were used (7). It could be demonstrated that lower etoposide levels correlated with milder bone marrow toxicity. Our data suggest that oral etoposide is just as reliable as its respective i.v. form as regards the achievement of therapeutic target concentration ranges. The specific target concentrations for the therapy of different tumour types remain to be identified by clinical studies.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Pharmacokinetics and Metabolism of Low-Dose ATRA in Children – First Observations

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Abstract

Objective. As the treatment of childhood acute promyelocytic leukemia (APL) with 45 mg/m^2/day all-trans-retinoic acid (ATRA) was associated with rather dramatic side effects in virtually all children, the AML BFM-study group decided to reduce the dosage to 25 mg/m^2/day. Therefore, we started to examine pharmacokinetic and metabolism of ATRA in children and adults with APL.

Patients. Up to now we determined ATRA and its active metabolites in plasma of six children, who received 17-45 mg/m^2/day, and two adults, who received 45 mg/m^2/day.

Methods. Plasma samples were obtained frequently over a period of 8 h on day one and in three children after 4 weeks of ATRA therapy. ATRA and metabolites were measured in plasma by normal phase high-performance liquid chromatography with UV-detection at 350 nm.

Results. In children the time, until absorption of ATRA was observed, varied from 0.75-3 h. Maximal plasma levels were reached between 2-3.5 h and ranged from 3.8-86.23 ng/ml. For low-dose ATRA the elimination half-life and AUC were calculated to be 1.0±0.3 h and 88.2±54.2 ng/ml × h (mean ± standard deviation). Similar metabolic patterns were observed in children and adults. However, isomerisation was observed to a lesser extent in children receiving low-dose ATRA compared to adults treated with 45 mg/m^2/day. ATRA is known to induce its own metabolism. In two children the C_{max} and AUC values decreased over time. However, in the third patient C_{max} on day 28 was about four times higher compared to day one. The elimination half-life was reduced on day 28, as in the other patients indicating an induction in ATRA catabolism. This patient received ATRA for second remission induction at a reduced dosage due to severe side effects during first remission induction with ATRA.

Conclusion. Besides the interpatient variability of ATRA with respect to AUC and C_{max} levels we also observed great intrapatient variations in ATRA bioavailiability, which might be related to the incidence of side effects in children. Whether ATRA bioavailiability is influenced by chemotherapy warrants further investigations in this ongoing trial.

Introduction

All-*trans*-retinoic acid (ATRA) induced complete remissions in a high portion of patients with acute promyelocytic leukemia (APL) by overcoming the differentiation block and inducing differentiation of the leu-

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kemic cells along the granulocytic pathway [1]. However, remissions were short and patients were at risk of developing the ATRA-syndrom, a life-threatening side effect, which was preceded by an increase in peripheral white blood cell count. To overcome ATRA-resistance and to avoid the ATRA-syndrom combination with cytoreductive chemotherapy proofed to be effective [2].

Though 60 mg/m^2 were determined the maximal tolerated daily dose in children [3], the introduction of ATRA at a dose of 45 mg/m^2 combined with induction chemotherapy for treatment of APL was associated with rather dramatic side effects in virtually all children, which necessitated dose reduction or discontinuation of ATRA therapy.

Since in adults complete remissions were also obtained with 25 mg/m^2 ATRA [4], the AML BFM-study group decided to reduce the dose to 25 mg/m^2 (low-dose ATRA).

All-trans-retinoic acid as well as 13-*cis*- and 9-*cis*-retinoic acid are important physiological regulators, which are generated in vivo by oxidation of all-*trans*-retinol (vitamin A). Retinoic acids take influence on gene transcription by interaction with nuclear receptors, the retinoic acid receptors. Retinoic acid effects are controlled by isomerisation and oxidation. Isomerisation results in retinoic acids with different effects due to different receptor affinities. As little is known about ATRA metabolism and pharmacokinetics of low-dose ATRA in children and about the influence of chemotherapy on pharmacokinetic and metabolism of ATRA, we started to determine ATRA and metabolites in children and adults, who received ATRA along with chemotherapy for treatment of APL.

Patients and Methods

Patients

Up to now six children (four male and two female) were treated with ATRA according to the AML-BFM 93 protocol. The age ranged from 9 to 18 years with a median of 14 years.

Except for one patient the t(15/17) translocation, which is characteristic for APL, had been detected in blasts of all patients. Because of severe coagulation disorders and unplain morphology this patient was first supposed to suffer from AML-M3 and, therefore, received ATRA treatment at the beginning.

In addition, plasma levels of two adults receiving 45 mg/m^2 ATRA for APL treatment according to the protocol of the Adult AML Cooperative group have been examined.

ATRA capsules were self-administered with meal. In five out of six children the daily dose was applied every 12 hours as recommended by the Childhood AML BFM-study group, while one child received ATRA once a day like the adult patients.

Informed consent was obtained from all patients and parents prior to the pharmacokinetic studies.

Pharmacokinetic Studies

Citrated-blood sampes were collected prior to drug administration and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6 and 8 h. Samples were stored at 4 °C prior to centrifugation. After centrifugation (3500 rpm for 4 min at 4 °C) the plasma layer was removed and stored at –80 °C.

Evaluation of pharmacokinetics and metabolism were performed on the first day and, when possible, repeated after four weeks of ATRA therapy.

Pharmacokinetic parameters were determined by non-compartmental and compartmental analysis using TOPFIT 2.0.

HPLC Analysis

Since retinoic acids and vitamin A are light-sensitive, blood samples were transported wrapped in aluminium foil and all handling with plasma samples and reference compounds were performed under dim yellow light. Plasma samples were extracted and assayed for retinoic acids and metabolites by HPLC as previously described [5]. In brief, retinoids were extracted from 500 µl plasma by liquid-liquid extraction. Separation was performed on a silica gel adsorption column using a binary multistep gradient. Retinoids were detected at 350 nm. This method al-

lowed the separation of all-trans-retinoic acid, 13-*cis*-retinoic acid, 9-*cis*-retinoic acid, their 4-oxo-metabolites and all-trans-retinol. The limit of detection was 0.5 ng/ml for retinoic acids and 10 ng/ml for all-*trans*-retinol, which allowed determination of endogenous retinoic acid concentrations in human plasma.

Results

Pharmacokinetics of ATRA

Non-Compartmental Analysis

The model independent pharmacokinetic parameters for each child are summarized in Table 1.

In children there were great interpatient variabilities with respect to peak plasma concentrations, area under the curve values and elimination half-lives. Patient No. 5 only showed a marginal increase in ATRA plasma levels, which might also be contributed to endogeneous vitamin A turnover. In adults peak plasma levels were determined 61.0 and 109.9 ng/ml. The elimination half-lives (t1/2: 0.7 h and 1.5 h) were in the range of those calculated for children.

In children the time till adsorption of ATRA [plasma concentration significantly above the physiological range (Table 2)] was observed ranged from 0.75 to 3 h. Peak plasma concentrations were obtained within 2 to 3.5 h and occured later on the first day of ATRA therapy compared to day 28 during induction chemotherapy.

In all patients examined on day 28 of ATRA therapy the elimination half-life decreased over time. In two of three patients ATRA peak plasma levels and AUC values were also decreased by 40 to 70% on day 28 compared to day 1. This was in accordance with previous studies, which observed decreased peak plasma levels and AUC values in nearly all patients examined during chronic ATRA administration. In patient No. 4 the peak plasma concentration was raised about four times and the AUC value was increased about 70% compared to the first day of ATRA therapy, which had not been reported from previous studies [3, 6, 7, 8].

Compartmental Analysis

After neglecting of endogenous and slowly increasing ATRA concentrations by defining lag-times between 0.5 and 3 h the plasma concentration-time curves of ATRA in chil-

Table 1. Individual pharmacokinetic parameters determined by model independent analysis

No. of patients	Dose (mg/m^2)	C_{max} (ng/ml)		t_{max} (h)		AUC (ng/ml/h)		t1/2 (h)	
		day 1	day 28	day 1	day 28	day 1	day 28	day 1	day 28
1	12.8	68.2	20.7	3	2	207.5	61.0	1.4	1.2
2	13	26.4	13.1	3.5	2	65.9	39.6	1.2	0.9
3	12.5	44.2	-	2	-	97.1	-	0.9	-
4	17.9	20.5	86.2	3	2	72.8	126.7	0.7	0.4
5	15.4	3.8	-	2	-	26.3	-	1.3	-
6	20.7	54.8	-	2.5	-	99.2	-	0.6	-

Table 2. Pretreatment retinoic acid concentrations (Mean ± SD) in plasma of children with APL compared to endogenous retinoic acid concentration in plasma of healthy children

	Plasma concentration (ng/ml)	
	Children with APL (n = 5)	Healthy children (n = 27)[a]
13-*cis*-retinoic acid	1.26 ± 0.60	0.97 ± 0.31
13-*cis*-4-oxo-retinoic acid	0.74 ± 1.01	2.22 ± 1.82
all-*trans*-retinoic acid	1.02 ± 1.00	1.07 ± 1.82
all-*trans*-4-oxo-retinoic acid	-	-

[a] The data from healthy children were derived from [9].

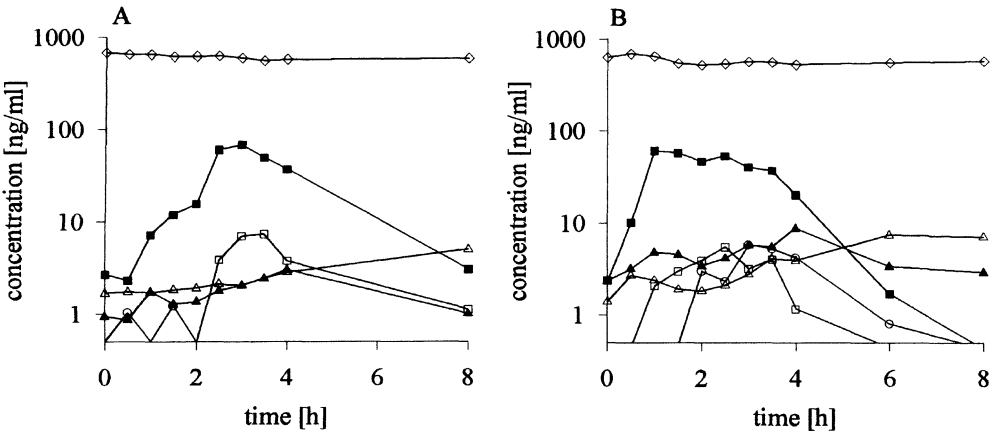

Fig. 1. Plasma concentration-time curves of ATRA and metabolites on day 1 after ingestion of *A* 12.8 mg/m^2 ATRA in a child – *B* 47.6 mg/m^2 ATRA in an adult, -▲- 13-*cis*-retinoic acid; -⊖- 9-*cis*-retinoic acid; -■- all-*trans*-retinoic acid (ATRA); -⊟- all-*trans*-4-oxo-retinoic acid; -△- 13-*cis*-4-oxo-retinoic acid; -◇- all-*trans*-retinol (Vitamin A)

dren fitted to a one compartment model, which well described the terminal adsorption and the elimination phase ($r^2 > 0.96$). Secondary peaks were observed in patient No. 4 and in patient No. 5 on day one of ATRA therapy, which made model dependent pharmacokinetic analysis impossible.

In children with respect to AUC values and elimiation half-lives the results from one-compartimental analysis were in good correlation with the results from non-compartimental analysis ($p < 0.05$, Pearson Correlation).

Metabolism

Plasma concentration-time curves of ATRA and metabolites determined in two patients after ingestion of different dosages are depicted in Fig. 1. So far in adults and children the same metabolic pathways were observed. All-*trans*-4-oxo-retinoic acid was the main metabolite of ATRA detected in children and adults. Peak plasma concentrations of this metabolite occured concomitantly with ATRA and accounted for up to 10% of the parent drug in children as well as adults (Fig. 1). As previously reported for adults there was no increase in all-*trans*-4-oxo-retinoic acid concentrations in children on day 28 of ATRA-therapy [8]. 13-*cis*-retinoic acid plasma levels increased about 1.5 to 7 times compared to pretreatment levels in adults and children. In addition, together with secondary ATRA peak plasma levels the 13-*cis*-retinoic acid concentration increased in plasma. 9-*cis*-retinoic acid was detected more often in adults, treated with 45mg/m^2 as a single dose, compared to children, treated with 17 to 45 mg/m^2 divided in two doses (Fig. 1). After ingestion of ATRA 13-*cis*-4-oxo-retinoic acid increased in plasma, however, in contrast to all-*trans*-4-oxo-retinoic acid the increase of 13-*cis*-4-oxo-retinoic acid was delayed (Fig. 1). So far, no 9-*cis*-4-oxo-retinoic acid had been detected in children and adults during ATRA therapy.

Endogenous Retinoid Concentrations

Up to now there seemed to be no difference between the endogenous retinoic acid concentrations determined in children with APL and those determined in healthy children [9] (Table 2).

Vitamin A levels were in the normal reange 100-1000 ng/ml [10]. The application of ATRA had no influence on all-trans-retinol levels during the 8 hours examined. However, comparing the all-trans-retinol levels, determined on day one with those determined on day 28 during induction chemotherapy, they clearly decreased in the patients examined (Table 3).

Table 3. All-*trans*-retinol plasma levels (mean ± standard deviation) determined in children on day 1 and on day 28 during ATRA and induction chemotherapy

No. of patients	all-*trans*-retinol concentration (ng/ml)	
	Day 1	Day 28
1	619 ± 38.9	482 ± 32.6
2	389 ± 47.7	288 ± 26.3
4	238 ± 21.4	120 ± 9.2

Discussion

On account of the sensivity and the numerous metabolites determined by the employed method several aspects of ATRA pharmacology had been examined in the present study.

So far the most striking observation was the intrapatient variability in ATRA bioavailiability, which resulted in one patient in a fourfold increase in the ATRA peak plasma level together with an increase of the AUC value of about 70% on day 28 of ATRA-therapy. This patient received ATRA for second remission induction at a reduced dosage due to severe side effects during first remission induction with ATRA.

Chronic administration of ATRA had been reported to result in decreased peak plasma levels and AUC values in nearly all patients examined [3, 6-8], which had been caused by increased catabolism rather than by reduced bioavailibilty, because it could be reverted by coadministration of cytochrome P450 inhibitors, like ketoconazol [7]. ATRA is a potent regulator of a number of physiological processes. Autoinduction of metabolizing enzymes is, therefore, an important regulatory mechanism to overcome increased exposure to this potent drug.

Variations in ATRA bioavailiability as observed in our study, might explain for the increased incidence of side effects observed by the Childhood BFM-study group. Whether bioavailiability of ATRA is influenced by the applied chemotherapy warrants further investigation in this ongoing trial.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

In Vitro Monitoring of Asparaginase: Unphysiological Alteration of the Culture Medium

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Abstract

Purpose. Drug sensitivity testing, in this instance of asparaginase (ASNase), is a major tool in developing target therapy concepts. It is generally assumed that the in vitro drug concentration and the culture medium do not change within the incubation time. We propose unphysiological alteration of the culture medium since enzyme products may accumulate whereas substrates may decline. ASNase catabolizes asparagine to aspartic acid. Besides, the inherent glutaminase activity, which is of minor importance in vivo, results in the desamination of glutamine to glutamic acid and ammonia. We addressed the question of a dose-response relationship between the ASNase activity and related substrates and products in vitro.

Methods. At various concentrations of ASNase, the enzyme activity, asparagine, aspartic acid, glutamine, and glutamic acid were measured by HPLC for 24 h, and the ammonia release was photometrically determined (n=4). The area under data of the concentration versus time curve (AUD) of substrates and products of ASNase (0-24 h) was related to rising levels of ASNase.

Results. Without ASNase, all parameters remained constant. At a concentration of 2 IU/l ASNase the medium appeared asparagine-depleted within 4 h. At concentrations of 200 IU/l or more of ASNase glutamic acid and ammonia increased.

Conclusion. The dose-response relationship between ASNase and the related substrates and products is more pronounced than the ASNase-associated changes in the asparagine level. In vitro changes in the amino acid pattern are not representative of the in vivo situation. The contribution to cytotoxicity of various changes in vitro remains unclear. An evaluation of the mechanism of ASNase cytotoxicity in vitro based on a specified amino acid composition of the medium is needed. Current results of drug sensitivity testing involving ASNase should be interpreted with caution since a number of artificial changes occur in the cell culture system.

Introduction

Drug sensitivity testing of established cell lines and of patient samples is a major tool in the preclinical testing of chemotherapeutics. Target therapy concepts have been developed from results of drug resistance testing using various in vitro assay systems like MTT, DiSC or clonogenic assays [1, 2, 3]. The ability to predict the sensitivity of an individual's leukemia cells to the cytotoxic effect of, e.g., asparaginase (ASNase) by in vitro assays is a fascinating concept and approach to identify those children who might benefit from such therapy.

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Regarding in vitro assay systems, it is generally assumed that the drug concentration and the composition of the culture medium do not alter significantly within the incubation time.

When testing active enzymes like ASNase, however, one should be aware that enzyme products may accumulate because they are not metabolized in vitro. The depletion of enzyme substrates may be more pronounced in vitro than in vivo, as the lack is not counterbalanced by biosynthesis, e.g., of the liver.

ASNase catabolizes asparagine to aspartic acid. The lack of externally available asparagine causes apoptotic death, especially in malignant lymphoblastic cells. Besides, the inherent glutaminase activity results in desamination of glutamine to glutamic acid and ammonia. This phenomenon is of minor importance in vivo.

In order to clarify the extent of changes in the amino acid pattern of the culture medium, we investigated the dose-response relationship between the ASNase activity and changes in related substrates and products in vitro.

Material and Methods

Drugs and Reagents

ASNase pharmaceutically graded as *E. coli* enzyme (Medac GmbH, Hamburg, Germany), RPMI-1640 medium and fetal calf serum were obtained from Gibco, BRL (Maryland, USA), HPLC reagents O-phthaldialdehyde, eluant buffers, regenerant buffers, sulfosalicylic acid, and ammonium sulfate as reference substance were purchased from Merck (Darmstadt, Germany), acetic acid and ethanol from Baker Chemicals (Heidelberg, Germany), and amino acid standards from Sigma Chemical Co. (St. Louis, USA).

Analytical Methods

ASNase was measured by Photometer Novaspec II (Pharmacia LKB, Sweden). Concentrations of asparagine, aspartic acid, glutamine and glutamic acid were determined by RP-HPLC-System (Bromma LKB, Sweden). The ammonia release was measured photometrically. Further details of the ASNase assay were published elsewhere [4].

Incubation Methods

RPMI 1640 medium containing 2 mM glutamine and 9% FCS was incubated (at 37 °C in 100% humidity, 24 hours) with various concentrations of ASNase (0, 2, 200, 2000 and 10,000 U/l, n=4). The enzyme activity, asparagine, aspartic acid, glutamine, glutamic acid, and ammonia were determined at 1, 4 and 24 h of incubation.

Pharmacokinetics

Pharmacokinetics, i.e. the area under data of the concentration versus time curve (0-24 h), were calculated by TOPFIT version 2.0, a pharmacokinetic and pharmacodynamic data analysis system for the PC. [5] The AUD of ASNase substrates and products was related to rising levels of the enzyme.

Results

Effects After Incubation Without and with Low Concentrations of ASNase(0-2 IU/l)

Without the addition of ASNase, the amino acid profile remained stable throughout the incubation time. At a low concentration of 2 IU/l ASNase, which is ineffective in vivo, the medium appeared asparagine depleted within 4 h. Correspondingly higher levels of aspartic acid were detectable, but the effect on the glutamine-glutamic acid system was still weak. At that time the rise in the concentration of ammonia was also still limited.

Effects After Incubation with Higher Concentrations of ASNase (200-10 000 IU/ml)

At higher concentrations of ASNase (> 200 IU/l) the depletion from asparagine was complete within 1 h. Glutamine was desaminated to glutamic acid and ammonia (see

Table 1. Concentrations [μM/L] of substrates (light columns) and products (dark columns) of ASNase at different time points related to different concentrations of ASNase (n=4)

Time (h)	ASNase IU/l	Asparagine μM/l	Aspartic acid μM/l	Glutamine μM/l	Glutamic acid μM/l	Ammonia μM/l
0	0	294	145	1466	183	484
1	0	335	154	1494	194	452
	2	273	198	1511	199	517
	200	0.44	446	1393	222	769
	2000	< 0.1[a]	483	1251	491	1086
	10000	< 0.1[a]	492	458	1226	2086
4	0	304	149	1498	191	525
	2	89	380	1554	200	780
	200	< 0.1[a]	476	1370	419	1019
	2000	< 0.1[a]	483	384	1285	2171
	10000	< 0.1[a]	557	2.25	1475	2437
24	0	286	162	1414	194	699
	2	0.24	414	1295	189	975
	200	< 0.1	463	452	1265	1983
	2000	< 0.1	434	2.2	1569	2570
	10000	< 0.1	444	< 0.1[a]	1579	2486

[a] Under limit of detection (< 0.1 μM/l).

Table 1). At 200-2000 IU/l ASNase glutamic acid and ammonia increased exponentially, whereas glutamine decreased and asparagine and aspartic acid remained constant.

After a 4 h incubation time with 10000 IU/l of ASNase the medium was nearly glutamine depleted, with the level of glutamic acid rising high ($AUD_{0\text{-}24\,h} > 30$ mM × h/l) and a subsequent dramatic increase of ammonia ($AUD_{0\text{-}24\,h} > 50$ mM × h/l).

By contrast, the in vitro effect of ASNase on the concentration-time curve of asparagine appeared to be nearly independent of the applied concentrations of ASNase.

Discussion

Dose-Response Relationship

In vitro changes in the amino acid pattern of the medium exceed the asparagine depletion. The dose-response relationship between the ASNase activity and related substrates and products is more pronounced than the ASNase-associated changes in the asparagine level. High and possibly toxic levels of ASNase products like glutamic acid and ammonia are even reached at low concentrations of ASNase. Our observation that the asparagine in the culture medium was already markedly diminished by adding low concentrations of ASNase is consistent with the findings of other investigators [6].

Impact on in Vitro Assay Systems

Drug sensitivity assays of children's leukemic cells and normal lymphocytes from peripheral blood using the MTT assay usually involve ASNase concentrations of 3-10000 U/l. The LC_{50} for peripheral leukemic cells was reported to be about 200 U/l, and about 360 U/l for bone marrow cells [7]. According to our results, the area under data curves (0-24 h) of ammonia and glutamic acid at this dosage show an exponential increase. The level of aspartic acid rises continuously up to 200 U/l ASNase and after complete asparagine depletion reaches a plateau due to the unavailability of asparagine.

Such effects depend on the amino acid composition of the medium. The level of products like glutamic acid and ammonia is directly dependent on the content of glutamine in the medium.

The increase of ammonia corresponds to the equimolar conversion of glutamine and asparagine, even at higher ASNase concentrations and after 24 h of incubation time (Fig. 3).

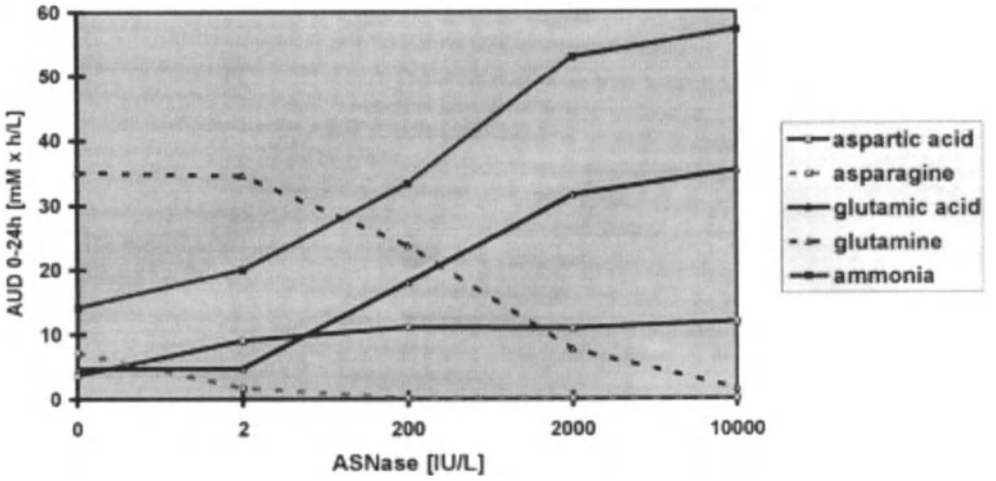

Fig. 1. AUDs (area under the data of the concentrations-time curve, 0-24 h) of the ASNase substrates asparagine and glutamine and products aspartic acid, glutamic acid, and ammonia (mM × h/l) after incubation with rising levels of ASNase (0-10 000 IU/l). AUD was calculated by using the linear trapezoidal rule

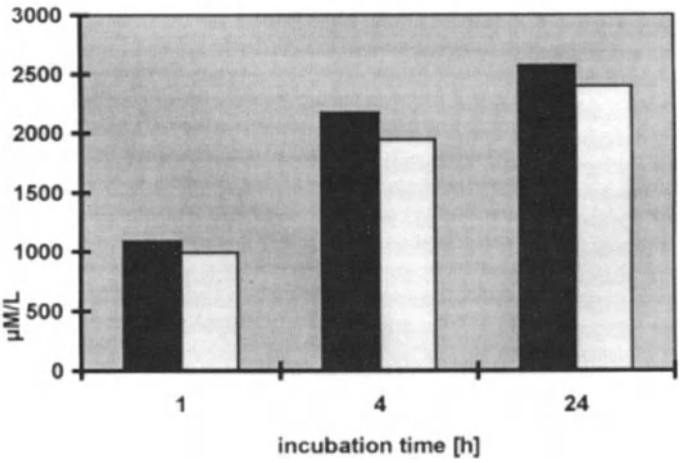

Fig. 2. Comparison of the ASNase product ammonia *(dark columns)* and the amount of equimolar conversion of the ASNase substrates glutamine + asparagine *(light columns)* at 2000 IU/l ASNase within the incubation time (n=4). The rising levels of ammonia are almost entirely explained by the desamination activity of ASNase

In Vitro-in Vivo Comparison

Continued high levels of glutamic acid are of minor impact in vivo [4]. The products glutamic acid and ammonia are detoxified in vivo.

In vitro a lack of substrates, namely glutamine, occurs. The extent of the glutamine decrease probably exceeds the decrease seen in patients, regardless of the ASNase dose, as the degradation of glutamine is largely counterbalanced by the glutamine synthetase of the liver [8]. In vitro changes in the amino acid pattern are not representative of the in vivo situation. Hence, the contribution to cytotoxicity of various changes in vitro remains unclear.

Additional Cytotoxic Effect of Unphysiological Alterations in Vitro?

Asselin and coworkers developed techniques to measure the in vitro and in vivo cell killing ability of ASNase and have recently suggested that the in vitro sensitivity of a patient's lymphoblasts to ASNase may correlate with the patient's short term outcome [6].

A dose-related response, however, was not confirmed by clinical trials. Between 1 and 100 IU/l ASNase the in vitro killing of leukemic cells was independent of the concentration. At 1000 IU/l (and complete asparagine depletion) there was a slight increase in cytotoxic activity measured by the MTT-assay, which suggests that there may be an additional in vitro mechanism of cell killing. Neither glutamine nor glutamic acid, aspartic acid or ammonia were determined in these studies. The additional cytotoxic effect may be due to the unphysiological pattern of substrates and products of ASNase.

Conclusion

The phenomenon of asparagine depletion is of minor importance whereas changes in the media due to different ASNase concentrations are dramatic. Asparagine depletion seems to be only one parameter of ASNase toxicity in vitro. The pharmacological effect of ASNase in vitro may be influenced by various factors due to the unphysiological alterations in the culture medium.

Hence, the most important question arising from our results concerns the significance of such unphysiological alterations of the culture medium in respect to the cytotoxic effect of ASNase in vitro.

The evaluation of the mechanism of ASNase cytotoxicity in vitro based on a specified amino acid composition of the medium is needed. Reducing the available substrates for ASNase may cause a reduction of potentially cytotoxic products. A better understanding of the role of asparagine depletion as well as the pharmacological effect of ASNase in vivo is essential. At this time, results of in vitro cytotoxicity assays involving ASNase may be helpful in defining the biological responsiveness to ASNase, but should be interpreted with caution and should not immediately lead to clinical decisions.

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Pharmacokinetics and Mechanisms of Action of Cytosine Arabinoside

Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Modulation of Ara-C Metabolism to Improve AML Response

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Abstract. Cytarabine (ara-C) is an effective agent for therapy of acute myelogenous leukemia (AML). Ara-C is phosphorylated to its triphosphate (ara-CTP) which acts as a cytotoxic metabolite. Strong correlations have been observed between ara-CTP accumulation and elimination, and response to ara-C therapy for patients with acute myelogenous leukemia (AML), suggesting a need to increase intracellular levels of ara-CTP. Previously we have demonstrated that administration of fludarabine prior to intermittent infusion of ara-C augments the levels of ara-CTP in the circulating blasts. As an alternative schedule, ara-C could be administered as a continuous infusion (CI) with fludarabine as a modulator. To model C.I. ara-C during in vitro studies, K562 human leukemia cells were incubated with 1 μM ara-C for 4 hours to reach steady-state levels of ara-CTP (ara-CTP_{ss}). At this time, the cultures were split in two and in one 15 μM fludarabine was added. The analysis of ara-CTP accumulation in these cells demonstrated that the ara-CTPss were augmented 2-3-fold by incubating cells with fludarabine. Similar in vitro studies in primary leukemia cells from patients with AML confirmed the effectiveness of such a combination. The clinical protocol designed based on these results stipulated a 1.5 g/m^2/d C.I. of ara-C. At 5 h, when the ara-CTP reached a steady-state, fludarabine (30 mg/m^2) was infused. To achieve a high concentration of plasma fludarabine, the infusion duration was reduced to 5 min instead of the standard 30 min administration. Subsequently, to further increase plasma fludarabine and cellular fludarabine triphosphate, the infusion duration of fludarabine was shortened to 1 min. This strategy was followed by increasing the dose of fludarabine to 50 mg/m^2. Of a total of 15 evaluable patients, 10 who achieved ≥10 μM intracellular fludarabine triphosphate increased ara-CTP_{ss} by a median of 45%. In contrast, only 1 of the 5 patients who failed to accumulate 10 μM fludarabine triphosphate in blasts was able to augment ara-CTP_{ss} ($p = < 0.004$, Fisher exact test). Of the 24 evaluable patients with relapsed AML, 8 achieved complete remission. Based on the prognostic criteria, the 33% overall response rate represents an improvement in the clinical outcome in these patients. In conclusion, these studies demonstrate that fludarabine successfully modulates ara-CTP accumulation in circulating blasts during CI or intermittent infusion of ara-C, and this improves clinical responses.

Introduction

Ara-CTP as a Cytotoxic Metabolite

The clinical efficacy of cytarabine (ara–C) in the therapy of patients with acute myelogenous leukemia (AML) was first demonstrat-

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ed more than 25 years ago [1]. Although many drugs and biologicals have been evaluated since that time, it remains one of the most effective chemotherapeutic agents in use against hematologic malignancies [2–5]. For this reason, novel strategies are still sought to optimize the use of cytarabine, particularly in combinations with other effective agents. For ara-C to exert its cytotoxicity, it must first be phosphorylated intracellularly by the rate-limiting enzyme dCyd kinase and subsequently to its 5′-triphosphate, ara-CTP [6, 7]. Ara-CTP then competes with dCTP for incorporation into DNA [8]. Numerous in vitro studies have demonstrated that the amount of ara-CMP (ara-C monophosphate) incorporated into cellular DNA is a predictor of loss of clonogenicity in human leukemia cells [9-11]. Although technically possible [12], it is extremely difficult to reliably and reproducibly measure the incorporation of ara-CMP into the DNA of circulating human leukemic myeloblasts during therapy. Thus, alternative predictors of the clinical cytotoxic effect of ara-C have been sought.

In the in vitro setting, the major determinant of ara-CMP incorporation into DNA was found to be the product of the intracellular ara-CTP concentration and time [11]. This observation has been confirmed in patients in vivo where statistically significant correlations have been demonstrated between the intracellular pharmacokinetics of ara-CTP and clinical response to single-agent high-dose ara-C therapy given either on an intermittent schedule [13, 14] or by continuous infusion [15]. Similarly, very low incorporation of ara-C into DNA in vitro was predictive of an adverse outcome with subsequent ara-C-based therapy in vivo [16].

Biochemical Modulation of Intermittent Infusion of ara-C

Based on the studies demonstrating the importance of ara-CTP formation and retention on treatment outcome [13-15], our laboratory [17, 18], and others [19] have investigated potential means to favorably modulate ara-CTP metabolism. One of the most promising methods of biochemical modulation identified was the capacity of pretreatment with 2-fluoro-arabinosyladenine (F-ara-A) to enhance the rate of ara–CTP accumulation in a human leukemia cell line [20] when ara-C concentration in the medium was 10 μM, the level at which the rate of accumulation of ara-CTP is saturated. Mechanistic studies suggested that the major mechanism by which F-ara-A augments the intracellular anabolism of ara-C to ara-CTP is through an enhanced rate of ara-CTP accumulation [20, 21] due to both a direct stimulatory effect of F-ara-A triphosphate (TP) on dCyd kinase and indirectly through reduced concentrations of dCTP, which feed-back inhibits the activity of dCyd kinase. Subsequent studies extended this observation during investigations in circulating human leukemia cells obtained from patients and incubated in vitro with F-ara-A followed by ara-C [22]. Similarly the leukemic lymphocytes obtained from patients with chronic lymphocytic leukemia (CLL) receiving fludarabine therapy achieved higher levels of ara-CTP when incubated in vitro with ara-C compared to cells obtained from same patients prior to therapy. Clinical trials with the fludarabine and intermediate-dose ara-C combination have demonstrated the effectiveness of this strategy in modulating ara-CTP accumulation during therapy in circulating AML blasts (21), chronic lymphocytic leukemia lymphocytes [23], or leukemic lymphoblasts [24] where F-ara-A is administered as a more soluble form, fludarabine (the monophosphate of F-ara-A).

These studies of ara-C modulation by F-ara-A or fludarabine were carried out at concentrations of ara-C (10 μM or above) that saturate the rate of ara-CTP formation in human leukemia cells in vitro and during therapy [25]. This concentration of ara-C can easily be attained or exceeded in vivo with intermittent high-dose or intermediate dose ara-C therapy [25, 26]. However serum concentrations of ara-C are generally less than 10 μM when maximally tolerated continuous infusion schedules are administered [27]. Continuous infusion schedules of single-agent ara-C have been extensively used for treatment of acute leukemias [28-30] and

have demonstrated clinical efficacy in previously untreated AML [31]. Thus the present study aimed to explore the capacity of F-ara-A to modulate the metabolism of ara-C at concentrations below 10 μM initially in a cell line model system, followed by in vitro incubations in leukemia blasts obtained from patients with AML. The results provided a rational basis for the clinical application of bolus fludarabine together with continuous infusion ara-C in the treatment of patients with relapsed and refractory AML.

Results and Discussion

Continuous Infusion of ara-C as a Single Agent

As indicated by experimental [32] and clinical [17, 33, 34] studies, the extent and duration of inhibition of DNA synthesis in leukemia cells during therapy is critically dependent upon the cellular concentration of ara-CTP. Cellular levels of ara-CTP fluctuate as much as 100-fold during intermittent infusions of high-dose and intermediate-dose ara-C because of large changes in plasma ara-C inherent to these infusion schedules. Leukemia cells that are unable to retain inhibitory ara-CTP concentrations recover DNA synthesis, which is associated with the failure to respond [33, 34]. Our initial approach to maintaining high levels of ara-CTP in the circulating blasts during intermittent infusion was to add a biochemical modulator of ara-C metabolism to increase the peak ara-CTP levels. By virtue of the increased accumulation of ara-CTP, the critical inhibitory concentration of the triphosphate was maintained longer in the cells, although it was eliminated at the same rate as single agent ara-C [21]. As an alternative strategy, ara-C could be administered as relatively high-dose continuous infusions. Our studies demonstrated that ara-CTP_{ss} in leukemia blasts is a function of ara-C concentrations in plasma, and that the ara-CTP_{ss} concentration is proportional to the ara-C dose rate within the dose range of 500 to 3000 mg/m^2/day (27). After a minimum of 5 years of follow-up after therapy, treatment of previously untreated adult AML with continuous infusion high-dose ara-C (1500 mg/m^2/day × 4 days) has demonstrated that long-term survival after single agent ara-C administered in this fashion is equivalent to that of patients treated with our best previous therapies, namely lower infusion rates of ara-C in combination with either anthracyclines or amsacrine [31]. Furthermore, there was a strong correlation between remission induction and steady-state concentrations of ara-CTP in leukemia blasts [15]. In contrast to intermittent infusions of ara-C at intermediate (10 to 15 μM) or high doses (30-100 μM) which saturate the rate of ara-CTP accumulation [25, 35], high-dose continuous infusion (CI) ara-C achieves only 1 to 3 μM ara-C in plasma [27], and thereby signals the opportunity to use a modulation strategy to increase steady-state ara-CTP levels.

K562 as a Model System for Combination of CI ara-C with Fludarabine

Using K562 as a model system for AML, we planned to modulate ara-CTP accumulation, when the steady-state ara-C is 1 μM. With this approach, we hypothesize that due to modulation, the level of ara-CTP in the leukemia cells would be high compared to single agent ara-C, and that these levels would be maintained due to continuous exposure to ara-C [36]. Hence, it could be expected that these maintained ara-CTP intracellular levels would increase the extent and duration of DNA synthesis inhibition in the leukemia cells.

Our initial experiments in K562 cells suggested that 50 μM intracellular fludarabine triphosphate (physiologically achieved in circulating leukemia blasts during fludarabine therapy, [21]), increased ara-CTP accumulation by 2-fold when ara-C was given at different doses (Fig. 1). Therefore, it appears that increse in cellular ara-CTP could be achieved at different concentrations of ara-C, for example at 10 μM ara-C (which would represent plasma concentration at intermediate dose ara-C), metabolism is modulated to same extent as 1 or 3 μM exogenous ara-C (which would represent concentrations achieved with high-dose continuous infu-

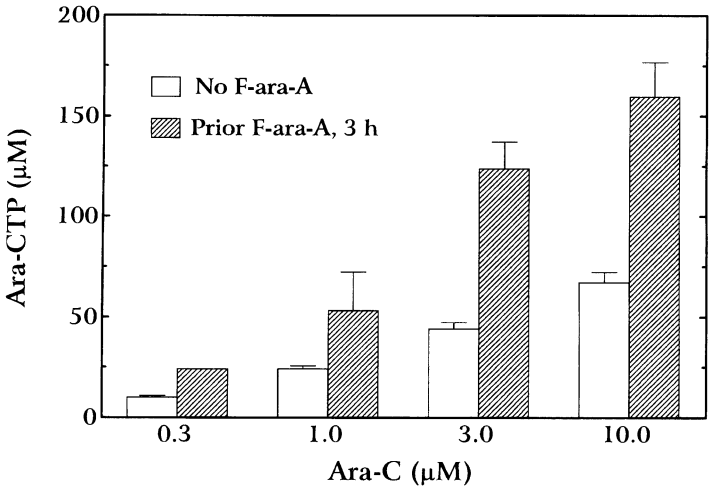

Fig. 1. Influence of F-ara-A preincubations on ara-CTP accumulation at different concentrations of ara-C in K562 cells. Parallel cultures of cells were incubated with no drug or 30 μM F-ara-A for 3 h, washed and then incubated with indicated concentrations of [3H]ara-C for an additional 3 h. Nucleotides were extracted and separated by HPLC. Data points are mean ± SEM of duplicate determinations from 3 to 6 experiments [36]

sion of ara-C). Hence, these data suggested that fludarabine may be combined with continuous infusion schedule of ara-C to achieve higher steady-state levels of ara-CTP. To mimic a clinical trial combining CI ara-C with fludarabine, we used K562 cells and incubated with 1 μM ara-C for 10 h. At 3 h, the culture was divided into 2; one received 30 μM F-ara-A while other was left without any new additions. The culture containing F-ara-A accumulated ara-CTP at twice the rate as that with ara-C alone. This resulted in increased intracellular ara-CTP concentrations which were maintained during the duration of ara-C incubation (Fig. 2). Additional in vitro studies are proposed herein to establish a comprehensive background for the design of a clinical trial.

In Vitro Combination of Fludarabine and CI ara-C in Primary AML Cells

As described earlier, the studies using the K562 cell line suggested that fludarabine infusion modulates the metabolism of ara-CTP when ara-C is present at low concentration (1 μM) for long durations, thus mimicking a continuous infusion schedule. These studies were extended to AML blasts to demonstrate the role of fludarabine on ara-CTP pharmacokinetics. Peripheral leukemia blasts from patients with AML or chronic myelogenous leukemia in blast crisis (CML-BC) were isolated and incubated with 1 μM ara-C for 10 h. At 5 h, cultures were divided into two; one was left with ara-C alone while 15 μM F-ara-A was added to the second culture. The duration of incubation, and concentration of ara-C were selected to represent CI ara-C. To maintain the steady-state levels of ara-C at 1 μM, tetrahydrouridine at 50 μM was added to the cultures to inhibit deamination. The pharmacokinetic studies, as illustrated in Fig. 3, demonstrated that ara-CTP accumulation reached a steady-state level by 4 h, and remained at this level throughout the incubation time with ara-C. In contrast, the blasts co-incubated with F-ara-A, accumulated ara-CTP at a 5-fold increased rate which in many cases did not reach a steady-state level during the 5 h incubation time. Similar results were obtained in leukemia blasts from 6 patients. These results demonstrated that biochemical modulation of ara-CTP accumulation was feasible in leukemia blasts during continuous infusion of ara-C. The caveat for the study design was the concentration of F-ara-A (15 μM). From our previous studies in AML patients, we know that peak plasma F-ara-A levels during standard dose fludarabine (30 mg/m^2 over 30 min) are 3 μM [36]. Moreover, the competing concentration of ara-C in plasma [20], and the affinity of deoxycytidine kinase for these substrates would result in a low rate of phosphorylation of F-ara-A [37]. To ameliorate this problem, higher plasma F-ara-A is needed, which may be

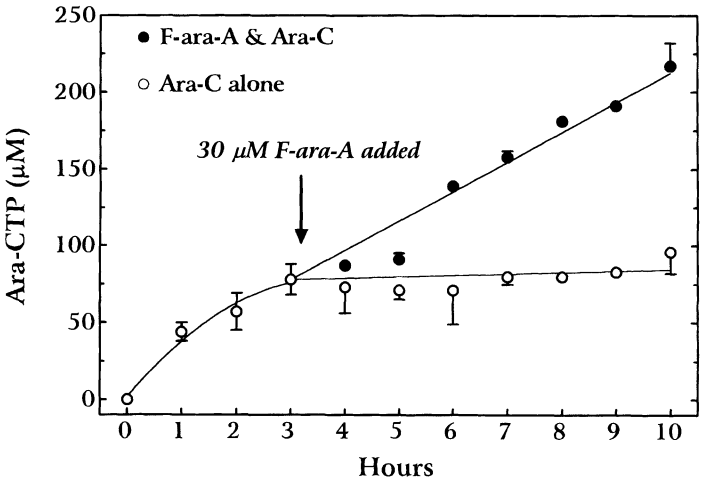

Fig. 2. Influence of F-ara-A addition on ara-CTPss accumulation in K562 cells. Cells were continuously exposed to [^{3}H]ara-C. Samples were taken every hour up to 3 h, and then cultures were split into 2. F-ara-A at 30 μM was added in 1 culture. Cells were then taken hourly from each culture and ara-CTP accumulation was quantitated. Data points are mean + SD of 2 separate determinations in 2 experiments. For some data points, because of very low deviation from mean value, error bars fit within the symbol and therefore are not shown [36]

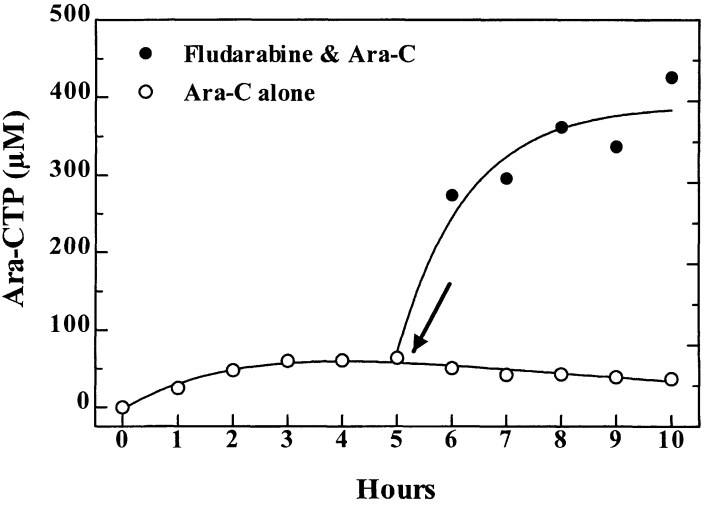

Fig. 3. Influence of F-ara-A addition on ara-CTPss accumulation in leukemic blasts obtained from patient with AML. Cells were continuously exposed to 1 μM [^{3}H]ara-C. Samples were taken every hour for 3 h, and then cultures were split into 2 portions. F-ara-A at 15 μM was added in one culture. Cells were then taken every h up to 10 h from each culture and ara-CTP accumulation was quantitated

achieved by reducing the infusion duration of fludarabine.

Fludarabine and CI ara-C, During Therapy

Based on the results obtained during in vitro experiments, a protocol was designed to administer ara-C at 1.5 g/m^2/d by CI. At 5 h, after ara-CTP had reached a steady-state level, fludarabine (30 mg/m^2) was infused. To achieve high plasma levels of F-ara-A, infusion duration of fludarabine was reduced to 5-min instead of a standard 30 min administration. Pharmacokinetic studies in the first ten patients demonstrated that peripheral leukemia blasts from 6 patients increased ara-CTP$_{ss}$. The circulating leukemia blasts from these patients accumulated 10 μM or higher cellular F-ara-ATP suggesting that this concentration is needed to modulate ara-CTP accumulation. Because the median F-ara-ATP peak concentration was 10 μM in these patients, it would be expected that half of the patients will not benefit from fludarabine's effect on ara-CTP metabolism. Indeed, 4 patients whose blasts accumulated less than 10 μM F-ara-ATP had similar ara-CTP$_{ss}$ before and after fludarabine infusion. To achieve higher F-ara-ATP during this regimen, the infusion duration of fludarabine was shortened to 1 min in 3 patients. This

strategy was followed by increasing the dose of fludarabine to 50 mg/m^2. Cellular pharmacology of F-ara-ATP in these patients suggested an advantage of shorter duration and higher dose of fludarabine infusion; the median was 12 μM with 30 mg/m^2 over a 1-min infusion and 20 μM when 50 mg/m^2 was infused over 1 min. Assuming 10 μM as the cut-point for the effectiveness of F-ara-ATP modulation, of the 15 evaluable patients all 10 who achieved 10 μM F-ara-ATP or more increased cellular ara-CTP$_{ss}$ by more than 20% (median 45%). In contrast, only 1 of the 5 patients who failed to accumulate 10 μM F-ara-ATP in blasts was able to increase ara-CTP$_{ss}$ by 20% ($p = <0.004$, Fisher exact test). Thus, we conclude that the pharmacokinetics during this regimen illustrated that ara-CTP$_{ss}$ in leukemia blasts achieved during CI ara-C administration is increased by fludarabine infusion, and this augmentation is dependent on the intracellular F-ara-ATP concentration.

Conclusion

Our previous and present investigations have demonstrated that fludarabine could be used as a modulator of intracellular ara-CTP pharmacokinetics in human leukemia cells when ara-C is given as either intermittent infusions at intermediate doses or as a continuous infusion. Hence, the biochemical modulation of ara-CTP accumulation by fludarabine is not dependent on the dose of ara-C, rather on the accumulation of fludarabine triphosphate which mediates this change in ara-CTP metabolism. Because it has been previously established that clinical responses to ara-C therapy are related to ara-CTP accumulation and retention, the combination of fludarabine with ara-C (with or without growth factor, [39]) is expected to improve clinical responses for patients with AML [40, 41].

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Response of AML Blasts to Ara-C: Role of BCL-2 in the Regulation of Drug Sensitivity

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Abstract. The sensitivity of AML blast stem cells exposed to ara-C in culture is altered by such growth regulators as retinoic acid (ATRA), hydrocortisone (HC) and growth factors (regulated drug sensitivity or RDS). ATRA and G-CSF usually sensitize cells to drug, while GM-CSF and HC are protective. We have shown that the lethal effects of ara-C are mediated in part by the production of reactive oxygen intermediates(ROI). We have proposed that bcl-2 acts after the formation of ROI to influence whether cells recover or go to apoptosis; and that regulators influence the ara-C sensitivity by changing the activity of bcl-2. ATRA decreases bcl-2 translation as seen by northern blot and decreases the stability of bcl-2 protein, as determined from the half-life of protein labelled with S^{35}-methionine. HC has no effect on transcription but increases protein stability. These findings are compatible with the opposite effects of ATRA and HC on ara-C sensitivity. Growth factors do not change either bcl-2 transcription or the sensitivity of the protein. Factor responsive blasts were grown in G-CSF; the G-CSF/GM-CSF fusion protein pIXY was added for 24 h, and then the cells returned to G-CSF. Exposure to pIXY doubled bcl-2 protein synthesis as measured by incorporation of S^{35}-methionine (1 h pulse exposure). We conclude that multiple mechanisms exist for the regulation of bcl-2, including translational and post-translational mechanisms, such as modifications of synthesis and protein stability.

Introduction

The treatment of acute myeloblastic leukemia (AML) is a paradox. Most patients respond to treatment initially, evidence that the disease is chemosensitive. Most patients subsequently relapse, evidence of chemoresistance. Tissue culture of leukemic cells provides a way to look for a resolution of the paradox; in culture the responses of the cells can be examined without the complications and ethical restraints that limit clinical trials. Further, cell cultures may be manipulated in experiments designed to discover the mechanisms of both cell kill and cell survival. Mechanistic studies of shown clearly that drug-injured cells usually die by apoptosis [1]. A complex series of molecular controls govern physiological cell death; these regulators also influence response to drugs. The opportunity exists to manipulate cell response to encourage cell kill and avoid resistance.

It is convenient to consider two categories of the events that lead from drug-cell contact to either death or recovery: first, proximal events are those that begin with drug entry into the cell and end with damage to the cellular target, usually DNA. Second, distal events are those that influence the fate of the damaged cells, so that they either survive or enter apoptosis. Distal mechanisms have only recently been subjected to extensive study, a new interest based on improved basic knowledge of mechanisms of apoptosis.

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In this paper, work will be described in which AML blast stem cells in culture were used to search for distal mechanisms that might explain how sensitivity to ara-C is altered by growth regulators. The report is a continuation of the studies summarized in the proceedings of the Leukemia VI conference [2].

Distal Mechanisms

Injured cells initiate the processes that may lead to apoptosis. Often damage in DNA is followed by changes in cellular redox, with a shift towards oxidizing conditions, then lipid peroxidation in membranes and the release of intracellular ionized calcium $[Ca^{2+}]_i$. This sequence favors the endonuclease activity that cleaves DNA in the final stage of apoptosis. Accompanying these changes in the redox environment, a number of proteins act either to rescue cells or to facilitate apoptosis. Many of the proteins are enzymes; some are DNA repair enzymes; others are proteases that are required for enzyme activation or inhibition; others are kinases or phosphatases, part of signalling pathways or required for cell cycle regulation. Negative oncogenes are also activated; their protein products alter the cell cycle and may be required for apoptosis or protect against it. The bcl-2 family proteins include members that are protective and others that promote death. The mechanisms of action of these proteins remains obscure; they appear to act in concert with a number of other mechanisms, including those required for repair and cell cycle regulation. Bcl-2 protein also has important anti-oxidant properties. None of these protein-mediated activities works alone or as a single pathway; rather it appears that an interacting network of protein-mediated events determine whether injured cells die or enter apoptosis. A brief description of some of the major mechanisms will be provided, with particular emphasis on the bcl-2 family.

Oxidative Stress Following Injury

Production of excess ROI is frequently a consequence of drug-induced DNA damage. Although ara-C is known to induce DNA breaks [3], we were unaware of evidence of oxidative stress contributing to ara-C toxicity. To test for ROI production after ara-C treatment, we used N-acety-cysteine (NAC) as a radical scavenger. We found NAC to be highly protective if given before drug but to be ineffective if given with or after ara-C [4]. This result provided indirect evidence that ROI are produced in cells treated with ara-C and may contribute to cytotoxicity. The well established series of proximal events that lead to ara-C incorporation and termination of chain elongation [5, 6] continue to be required since dCK negative mutants are resistant to ara-C [7]. It is probable that the generation of ROI follows injury DNA and may be a response to it.

To look at the contribution of ROI to cytotoxicity, free radicals were introduced into blast cells by adding H_2O_2 to the cultures; dose response curves were measured for cells of the AML lines, using the clonogenic assay as an endpoint. Variation in H_2O_2 sensitivity among cell lines was found; there was a correlation between sensitivity to ara-C and to H_2O_2, a finding consistent with a role for ROI in ara-C killing.

To seek more direct evidence for the participation of ROI in ara-C killing, we used flow cytometry to measure the molecules that are important in maintaining cellular redox state. A major contributor is glutathione (GSH) which protects against ROI by binding free radicals or by reducing endogenously produced H_2O_2. In this balanced, reduced state the cell is protected against oxidative damage; membrane integrity and enzyme function are maintained. When membranes are damaged by ROI (lipid peroxidation) calcium is released from stores and calcium-dependent enzymes are activated, including the endonucleases required for apoptosis [8-10].

We asked if these events occured after ara-C damage. We used vital fluorescent dyes, quantitated by flow cytometry, to assess ROI, GSH and intracellular ionized calcium ($[Ca^{2+}]_i$) in ara-C treated cells. Regularly, we observed a sequence of events that began with the emergence a population with elevated ROI and GSH. Then cells were seen with elevated ROI but little GSH; with time,

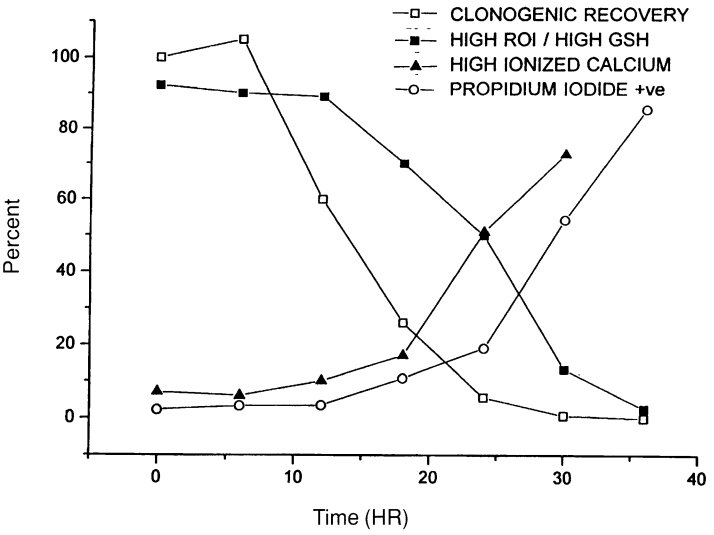

Fig. 1. Time course of changes in OCI/AML-2 cells, cultured in the presence of ara-C at a concentration of 7×10^{-6} M. Clonogenic cell recovery was measured by plating in methylcellulose cultures. The other parameters were measured by flow cytometry [68]

$[Ca^{2+}]_i$ increased in these cells, which then lost membrane integrity and viability as measured by failure to exclude propidium iodide and loss of colony-forming ability. Figure 1 shows the results of a kinetic experiment in which OCI/AML-2 cells were exposed to a 90% lethal concentration of ara-C and then assayed at intervals for clonogenic cell survival, GSH, ROI and capacity to exclude propidium iodide. The findings further support a role for oxidative stress in ara-C toxicity. The development of this aspect of the work is described in an accompanying paper (Hedley et al.) in the Symposium Proceedings.

Repair Enzymes and Proteases

A variety of enzymatic mechanisms are used in repair of damaged DNA [11-14]. DNA repair may also require poly(ADP-ribose) polymerase (PARP); this DNA-binding nuclear enzyme converts NAD to nicotinamide, catalyzing the ADP-ribosylaton of nuclear polypeptides. These polymers remain bound to PARP and modify its structure; the modification alters binding to DNA and may facilitate access of DNA repair enzymes to single strand breaks. A role for PARP in distal mechanisms is postulated because it is enzymatically cleaved early after exposure of AML cells to DNA, with resulting reduction in catalytic and DNA binding activity [15]. The enzyme responsible for PARP cleavage is YAMA/CPP32b, the mammalian homolog of the sed-3, one of the genes required for apoptosis during embryogenesis of *C. elegans.* This is a member of the ICE (Interleukin-2b converting enzyme)/sed family of serine proteases that have regulatory functions in apoptosis [16, 17]. Protease-mediated cleavage of PARP is an early response to treatment of AML cells with many DNA-damaging chemotherapeutic drugs [15].

Signalling by the MAP Kinase Family

The binding of a regulator to its receptor initiates a signalling cascade; information is passed from protein to protein by enzymes with tyrosine of serine/threonine specificity. Receptor-ligand binding plays a key role in the regulation of the growth of normal and leukemic cells. Binding initiates signalling by the mitogen activated protein (MAP) kinase family of extracellular signal regulated kinases (ERKs); these are serine-threonine kinases that ultimately act in the nucleus on the AP-1 transcription factor (jun/fos dimers) [18]. The usual observed effects are either increased cell growth or differentiation. Analogous and parallel to the ERKs is a cascade of MAP kinases that is activated by

many cellular stresses, including chemotherapy [19, 20]. This stress-activated protein kinase (SAPK) is the final stage in a cascade that leads to cell death by apoptosis. Thus, cells possess at least two similar signalling systems, using similar mechanisms, but leading to either survival (ERK) or death (SAPK)[21].

Negatively Acting Oncogenes

Cells frequently respond to damage by arresting in the G1 phase of the cycle. Prominent mediators of G1 arrest are the negative oncogenes, p53 and RB [22-25]. Up-regulation of p53 is usual in cells damaged by irradiation and chemotherapy [26, 27]. Cells may emerge from arrest as survivors, or may enter apoptosis; each of these fates may be p53 dependant. The amount of the protein present may alter the dose response to either irradiation or drugs [26,27].

The Bcl-2 Family

Bcl-2 was first identified from chromosomal breakpoint analysis of a t(14;18), it is developmentally regulated, with expression predominantly in immature lymphocytes; expression is also seen in normal bone marrow, brain, intestine and skin [28-30] Bcl-2 protein is localized on mitochondrial, endoplasmic reticulum and nuclear outer membranes [31-33]. The protein is found in many cancers, including lymphomas and leukemias.Bcl-2 is the first of a family of genes that function to either prevent or enhance apoptosis [34, 35]; it is homologous to the *C. elegans* survival gene ced-9 [36]. The family members share extensive homology in two highly conserved region, BH1 and BH2. Members of the bcl-2 family fall into two functional groups, those that protect against apoptosis, and those that promote cell death. In addition to bcl-2, the protective members include of bcl-xl [37, 38] which acts like bcl-2. In normal hemopoiesis, bcl-x but not bcl-2 is expressed in the most primitive precursors [39]. A third protective protein is bag-1, which has minimal homology to the bcl-2 family but protects against apoptosis [40]. Other weakly protective family members are inducible; MCL1 responds to phorbol esters; it was isolated from a leukemic cell line and may play a role in differentiation [41]. Bfl-1, the human equivalent of the murine GM-CSF responsive gene A1, was isolated from fetal liver and is expressed predominantly in normal marrow [40]. The most prominent member of the opposite, death promoting, class of family members, is Bax discovered by its capacity to dimerize with bcl-2 [42, 43]. Bcl-xs is an alternatively spliced form of Bcl-x that has death promoting activity [37, 38]. Bad was isolated by a yeast 2-hybrid screen; it forms heterodimers with bcl-2 and both forms of bcl-x, but not with bax, although, like bax, it promotes apoptosis [44]. Bak was isolated on the basis of homology with BH1 and BH2. It is widely distributed, and, like bax, promotes apoptosis [45-47]. Human forms are described for all of the above proteins except Bad and Bag.

A major characteristic of the bcl-2 family is capacity to form homo or heterodimers. Both bcl-2 and bcl-x form heterodimers with bax; it is proposed that the level of bax homodimers determines the extent of its death promoting ability [43]. However, it is of note that bax transgenic mice are viable, suggesting that high levels of bax alone do not produce death [48]. Bad may promote apoptosis indirectly by displacing bax from bcl-2/bax dimers [44], providing evidence for the regulatory importance of dimerization.

The Response to Drugs of AML Blast Cells in Culture

The relevance of distal mechanisms to chemotherapy is obvious from the brief outline of the network given above. It is also evident that in a network characterized by a balance between positive (survival) and negative (death) probabilities, a single paradigm is unlikely to be satisfactory. Significant differences are to be expected based on such variable as tumor, species and growth conditions. For the investigator, it is necessary to chose a system and a problem; only when these are dissected can it be asked if the results have general significance. The

blasts cells as they respond to drugs in culture provide an experimental model that is easily perturbed. The role of distal mechanisms in the outcome of changes in the culture environment was explored, using drug treatment as a way of introducing damage.

AML Blasts in Cell Culture

Large numbers of AML blasts can safely be obtained from the peripheral blood of patients; populations containing >95% blasts can readily be purified, using ficoll hypaque separation and T cell depletion [49]. The blasts cells will proliferate in suspension culture, providing suitable growth factors are included in the medium; the specific growth factor requirements of the cultures vary greatly from patient to patient, although most respond to G-CSF, GM-CSF, the ligand for C-KIT [50, 51]. The blast population contains a subpopulation of cells that are capable of colony-formation in cultures made viscid by agar or methylcellulose [52, 53]; replating experiments show that blast colonies contain new colony-forming cells at a low plating efficiency, evidence that clonogenic blast cells have the stem cell property of self-renewal [54]. Suspension cultures and clonogenic assays are complementary methods in the study of AML. Large cell numbers can be manipulated in suspension; clonogenic assays can be used to monitor the results of the manipulation. Such experiments support an hierarchical model of the cellular organization of blast cells. Clonogenic stem cells are considered to be derived from pluripotent leukemic transformants; these then maintain an independent lineage of stem cells, consisting largely of their terminally-dividing or proliferatively inert descendants [55].

Distal Events in Drug-Treated Suspension Cultures

Together the suspension culture and clonogenic assay can be used to model events in vivo [56]. For example, cells in suspension can be exposed to drugs or regulators for varying times and concentrations and then plated in suspension to measure clonogenic cell survival [57]. This design is readily adapted to the study of distal events in blasts; the cells are treated with drug in suspension and then assayed for proteins that regulate apoptosis and for clonogenic survival. The measurement of blast stem cells insures that molecular assays performed on bulk populations in suspension also occur in the crucial stem cell subpopulation. In a paper in these proceeding Hedley et al. describe experiments of similar design, where changes in oxidative stress were measured along with survival of blasts exposed to drug in culture.

Regulated Drug Sensitivity (RDS) in Culture

The importance of distal mechanisms was re-enforced as we began to investigate how regulators alter the sensitivities of blasts to cytosine arabinboside (ara-C) and daunorubicin (DNR). Using the cell culture assays we have explored the capacity of growth factors, all trans retinoic acid (ATRA) and hydrocortisone (HC) to alter the sensitivities of blast cells to chemotherapeutic agents. Regularly, blasts are found to be more ara-C sensitive in G-CSF than in IL-3 or GM-CSF [58-60]. ATRA affects blast sensitivity to ara-C or daunorubicin (DNR); the response may be either an increase in sensitivity or protection, depending on schedule; sensitization is the usual effect when ATRA is given after drugs [61-63]. Hydrocortisone (HC), another ligand for a member of the intracellular steroid receptor family, has little effect on blasts in culture except at very high doses. However HC protects cells against the lethal effects of ara-C or DNR [62, 64].

Our unsuccessful search for proximal mechanism was reviewed in the 1995 Acute Leukemia Symposium [2]. Examination of distal events after drug injury proved more revealing. We used a flow cytometric method to measure nicks in DNA [3, 65]. We followed the kinetics of cells with nicked DNA after ara-C [66] or DNR [62], and determined how these changes were affected by ATRA or HC. The data were consistent with the hypothesis that HC permits damaged cells to persist and eventually undergo repair, while cells treated with ATRA have has-

tened cell death; these findings support our view that these regulators of drug sensitivity affect distal mechanisms of drug sensitivity. The findings confirmed results of others showing apoptosis after exposure to ara-C [67].

A Distal Mechanism Based on Bcl-2

The nicked DNA assay provided general evidence that distal mechanisms might explain regulated drug sensitivity. In a search for a specific mechanism, we examined the effects of regulators on bcl-2. We found that ATRA, but not the other RDS regulators, decreases bcl-2 translation, as seen in northern blots. Bcl-2 is able to protect against a wide variety of damaging agents including chemotherapeutic drugs that produce DNA breaks and oxidative stress. The evidence that cells respond to ara-C damage by producing ROI was reviewed earlier. If ara-C-induced ROI are part of the mechanism of RDS, then ROI toxicity from another source such as H_2O_2, should be regulated. We found that ATRA given after H_2O_2 increased sensitivity while HC given before H_2O_2 was protective; this is the same pattern of regulation seen with ara-C [4].

These results led to the hypothesis that bcl-2 is an important part of the mechanism by with ATRA sensitizes cells to ara-C. ROI are produced as a response to incorporation of ara-C into DNA and contribute to the cytotoxic action of the drug; levels of bcl-2 protein modulate the toxic effects of ROI. We postulated that regulators affect drug sensitivity by altering the activity of bcl-2 [4]. The hypothesis rested on largely on correlative data; our data did not address the issue of protection against ara-C damage by hydrocortisone, or the opposite effects of G-CSF and GM-CSF on the drug response. We sought to remedy these defects.

Tests of the Hypothesis

Earlier we described the direct evidence for the generation of ROI in ara-C treated blast cells [68]. These findings are essential of the model. Further support for the model came when bcl-2 cDNA was transfected into blasts to see if increased bcl-2 protein would protect call against injury. Next, studies of the bcl-2 protein itself, using western blots, immunoprecipitation and pulse labelling with 35S-methionine, provided evidence for post-translational changes consistent with a role for bcl-2 in RDS. These experiments supported post-translational mechanisms by which not only ATRA, but also hydrocortisone and growth factors, could alter bcl-2 activity and through it, change ara-C sensitivity.

bcl-2 Transfectants

If, as postulated in the model, bcl-2 protein protects against ROI generated in ara-C treated blasts, an increase in the amount of bcl-2 protein should lead to decreased sensitivity to both H_2O_2 and ara-C. We transfected cDNA with a CMV promoter into OCI/AML-2 and OCI/AML-5 in the sense and anti-sense orientations. As expected, western blots showed increased bcl-2 protein in the sense but not in the anti-sense transfectants. Concurrent dose response curves for controls (transfected with vector only) and transfectants were obtained for both H_2O_2 and ara-C. Typical results for OCI/AML-5 and its transfectants are shown in Fig. 2. It is evident that two independently-obtained sense transfectants (5sa and 5sb) were less sensitive to both agents than the vector-only controls (5neo). Although anti-sense transfectants (5as) were slightly more sensitive to ara-C than controls, the difference did not reach statistical significance. Thus, cells with increased bcl-2 protein were protected against both exogenous ROI (H_2O_2-treated cells) and ROI generated after exposure to ara-C, compared to controls transfected with vector only, or transfectants with bcl-2 in the anti-sense orientation [69].

The bcl-2 cDNA was transfected with a CMV promoter; its transcription, therefore, should not be regulated in the same way as endogenous bcl-2 with its own regulatory machinery. We prepared ara-C dose response curves for vector-only controls and bcl-2 sense transfectants, with and without

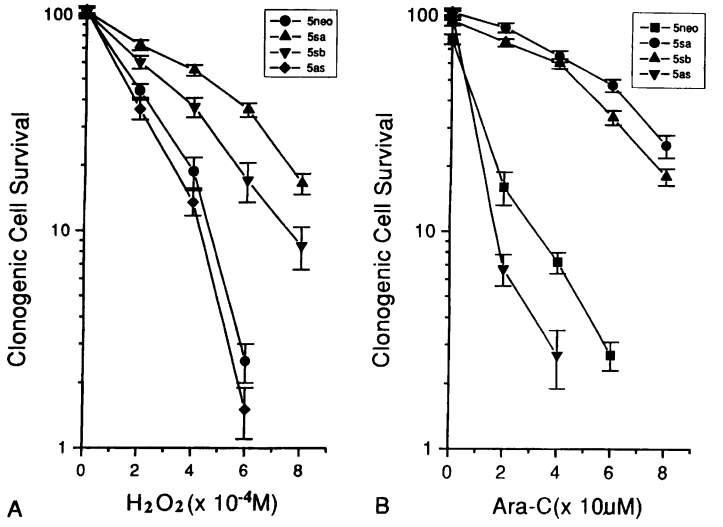

Fig. 2. Survival curves demonstrating the effects of transfected bcl-2 genes of sensitivity of clonogenic blast cells to ara-C or H_2O_2. The H_2O_2 dose response curves are shown in the *left panel,* the ara-C curves in the *right panel.* For both agents the sense transfectants were less sensitive than controls; the antisense transfectants showed increased sensitivity that did not reach significance [69]

ATRA after drug. As expected, the vector only cells were sensitized to ara-C by ATRA. The transfectants were more resistant to ara-C than parental cells; when treated with ATRA, the transfectants showed much less sensitization than parental cells. We consider that this result is consistent with the response of endogenous bcl-2 as compared to transfected bcl-2 with a CMV promoter [69].

Post-Translational Modifications of bcl-2 Protein

Nuclear run on experiments were used to show that ATRA was downregulating the translation of bcl-2 message; the nuclear run-on data, however, did not provide a complete explanation [4]. We decided to look for post-translational modifications of bcl-2 protein. We used the technique of metabolic labelling. The cells were incubated in medium without methionine for 30 m, then pulsed for 20 m with 35S-methionine; then the cells were either collected immediately or returned to growth medium for periods of time as indicated by the design. When harvested the cells were lysed, bcl-2 immunoprecipitated and electropheresed on PAGE gels. Gels were dried, the radioactivity in bcl-2 measured using a phosphoimaging screen. The outcome of the experiment was a measurement of initial labelling and the half-life of the protein.

Bcl-2 Protein Stability

We exposed OCI/AML-2 cells to either ATRA or hydrocortisone for 24 h, and then compared the decay of labelled bcl-2 protein to protein from controls. The densitometer readings from the phospho-images are plotted in Fig. 3. The control protein had a half-life of 20 h; in contrast, protein from ATRA treated cells was less stable (half-life 12 h) while that from HC exposed cells was more stable, with a half-life of 43 h. Similar results were obtained with OCI/AML-5 cells, although the control protein was so stable that neither this protein or that from HC-treated cells reached a half-life value in the 72 h duration of the experiment [70].

The opposite changes in bcl-2 half-life after ATRA or HC are consistent with the effects of these regulators on ara-C sensitivity; a more stable protein might be more effective in protecting cells, as is seen after HC, while the less stable protein found in ATRA-treated cells is consistent with its sensitizing action. Thus, the bcl-2 hypothesis for the mechanism of RDS can now be extended to the protective effect of HC.

Growth Factors and bcl-2 Synthesis

Growth factors, like HC, change drug sensitivity but do not alter transcription of bcl-2, as seen in northern blots. To test for growth-factor induced post-translational changes, we choose two cell lines with different factor

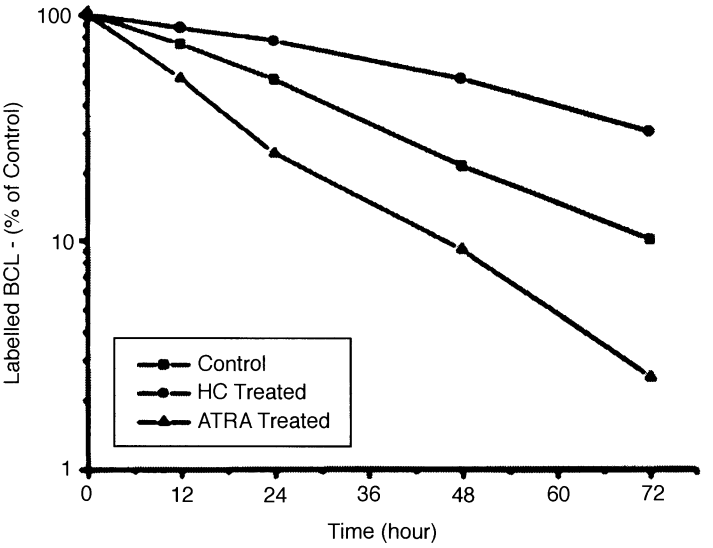

Fig. 3. Changes in expression of bcl-2 in OCI/AML-5 cells following exposure to ATRA (10^{-7}M) or HC (10^{-5}M). The densitometer readings for metabolically-labelled bcl-2 protein are shown, as percentage of control [70]

responses. The major response of OCI/AML-5 cells is to GM-CSF, with a minor response to G-CSF, and clear synergism when both are included in the cultures. OCI/AML-10 grows best in G-CSF; GM-CSF is weakly mitogenic, but again synergism with G-CSF is seen. For each cell line we compared cells growing in G-CSF, GM-CSF and both factors together. Comparison of cells in G-CSF and GM-CSF, or both together showed no difference in protein stability. A difference was found, however, when western analysis was used to look at quantities of bcl-2 protein in cells grown in different conditions. For both OCI/AML-5 and OCI/AML-10 cells, there was a 30-40% increase in bcl-2 protein after 72 h in GM-CSF or G-CSF + GM-CSF, compared to cells in G-CSF alone. The finding was consistent even though the mitogenic responses to growth factors of the two cell lines were different.

With these experiments, we could only conclude that GM-CSF increased the amount of bcl-2 protein in AML blast cells. A different experimental system was required to support a post-transciptional mechanism for the growth factor effect. The cell line OCI/AML-1 had desirable properties for our purpose. Bcl-2 expression is low in these cells, detectable in RNA only by PCR, and the protein gives a weak protein band in western blots. OCI/AML-1 cells readily show regulated sensitivity to ara-C by growth factors. They are very sensitive to the drug in G-CSF but much less sensitive in G-CSF + GM-CSF[58]. Preliminary experiments showed that bcl-2 protein was increased when GM-CSF was added to AML-1 cells growing in G-CSF; the increase was more marked when the cells were cultured in G-CSF with the addition of the GM-CSF/IL-3 fusion protein pIXY [71].

We used kinetic experiments to ask if the increased amount of bcl-2 protein in cultures with G-CSF + pIXY compared to G-CSF alone could be explained by a change in synthesis. Western blots were used to measure the mount of protein; metabolic labelling provided a measure of synthesis. OCI/AML-1 cells were maintained in G-CSF for 24 h; then pIXY was added to one set of cultures, the other remaining as controls. After a second 24 h period the cells were washed and recultured in G-CSF alone. At intervals during the 72 h of the experiment, cells were harvested for western blots. Other cultures were pulsed with 35S-methionine for 60 minutes, immunoprecipitated and incorporation of label into bcl-2 protein determined. The data are shown in Fig. 4, as kinetic plots of the densitometer reading of the western blots (amount of protein) and the phosphoimages (incorporation of label). The densitometer readings for both were normalized to the lowest value, each shown in the Figure at 1. The data in the figure show

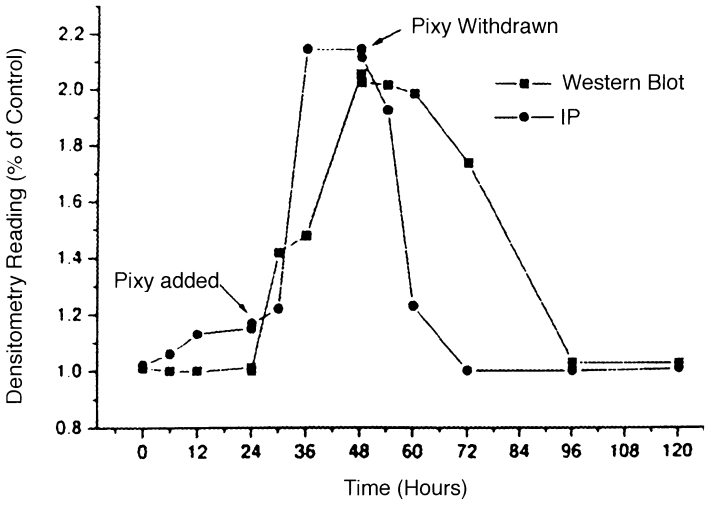

Fig. 4. Kinetics of OCI/AML-1 cells grown in G-CSF, transferred after 24 h to medium containing both G-CSF and pIXY; after a day, they were returned to G-CSF alone. At intervals cultures were collected and either assayed for bcl-2 protein by Western blot, or cultured in methionine negative medium and pulsed for 1 h with 35S-methionine. Immunoprecipitated bcl-2 was tested for incorporation of radioisotope. The densitometry readings were normalized against 1 for the lowest value, for both Western blots and radioautographs [72]

that bcl-2 synthesis had more than doubled by 12 h after the addition of pIXY and fell almost as rapidly when pIXY was withdrawn. The changes in bcl-2 protein were almost as large; the protein level peaked after 24 h, and fell more slowly than synthesis. We concluded that growth factors acted after transcription to alter the rate of synthesis of bcl-2 protein. The increased protein synthesis seen in cultures with G-CSF + pIXY compared to G-CSF alone, is consistent with the protection afforded against ara-C toxicity by IL-3 and GM-CSF [72].

Discussion

We can now propose a more detailed model of regulated drug sensitivity. Bcl-2 remains at the center; drug sensitivity decreases as bcl-2 becomes more active. Growth regulators effect bcl-2 activity in at least three ways. First, ATRA acts in part at the level of translation, as it down-regulates bcl-2 mRNA. Second, growth factors do not change mRNA levels, but rather work at the post-translational level to change the rate of protein synthesis. Third, ATRA and hydrocortisone, both ligands for receptors of the steroid superfamily, change the stability of bcl-2 protein; since such a change may well be the consequnce of a structural alteration in the protein, the effect may be considered post-transcriptional. in each instance the change in bcl-2 in consistent with the known effect of the regulator on ara-C sensitivity. ATRA down-regulates transcription and reduces protein stability, as might be expected from the observed decrease in sensitivity seen when blasts are exposed to ATRA after drug. Hydrocortisone stabilizes bcl-2 protein and also protects blast cells against the toxic effects of ara-C. GM-CSF increases bcl-2 synthesis, as might be expected from its capacity to decrease cell kill after ara-C. How bcl-2 modifies response to injury is unknown. It is plausible to think that the protein acts, in part, to maintain the cellular redox environment in a reducing state.

While recent work has amplified and extending ideas about RDS, an unresolved problem remains. Bcl-2 protein is very stable, even in cells that have been exposed to ATRA. Yet changes in drug sensitivity occur in 24 h. It seems unlikely that bcl-2 protein as measured in western blots or by immunoprecipitation can be the direct mediator that explains RDS. The studies with ATRA and HC, however, show that the protein can be modified. It seems possible that small isoforms may exist that are the truly active agents. Experiments using two dimensional gels are being conducted to test for this possibility.

Finally, bcl-2 and its family are put one part of the interacting molecular events that

happen in injured cells. These are an information system, where modifications of proteins carry the message. These modifications include phosphorylation, enzymatic cleavage and dimerization. Bray has suggested that the protein-based information is fundamentally similar to the language used by computers[73]. It is striking that the multiple and interactive protein network yields a binary results - cells either recover or die. An attractive possibility is that a stochastic process is responsible for the choice of outcome. From this point of view, the complex and redundant protein-based information network may serve not to determine outcome, but rather to set the probability of life or death.

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Acute Leukemias VII
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AraC Metabolism in Fresh Leukemic Blasts/ Normal Bone Marrow/ Hematopoetic Stem Cells and its Impact on the Lipid Composition of Leukemic Cells (HL60)

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Abstract. Metabolites of tritium labeled AraC (AraCMP, -CDP, CTP, -CDP-choline) were determined by HPLC analysis. No significant differences in the formation of these metabolites could be found between fresh leukemic blasts, mononuclear cells from normal bone marrow and CD34/ 38 positive hematopoetic stem cells. AraCTP half-life, which has previously been shown to correlate with clinical parameters like CR duration, was longest in leukemic blasts. The intracellular pool of AraCTP precursors (AraCMP, -CDP) was similar and of low abundance (13-20% of AraCTP at all tested AraC concentrations) in all investigated cell types and therefore did not explain the differences in AraCTP half-life.

AraCDP-choline formation seemed to be saturated at higher AraC incubation levels than AraCTP and might therefore have a significance for AraC pharmacodynamics of high dose AraC regimens. Since this metabolite is structurally similar to CDP-choline – a precursor of phospholipid metabolism – the effect of AraC on cellular lipid metabolism of HL60 cells was investigated: AraC induced a significant reduction (30%) in cellular phosphatidylcholine (PC) content. Antagonism of these alterations by lysoPC resulted in almost complete reversal of the AraC induced loss of viability (tested by trypan blue exclusion). The AraC induced loss of viability at high AraC concentrations might be caused partially by the destabilization of cell membranes, which has also been observed after PC alterations from other causes.

Introduction

The pyrimidine analogue AraC (Ellison et al. 1968) depends on its intracellular activation (via phosphorylation) or inactivation (via deamination) for its cytotoxic activity (Rustum et al. 1987). S-phase dependent cytotoxic activity due to incorporation of the nucleoside into the DNA and interference with enzymes of DNA metabolism (DNA polymerase α and β, DNA ligase) is mediated by the triphosphate AraCTP (Kufe et al. 1980, 1984). Since this drug has demonstrated significant clinical activity especially in acute myeloid leukemias the intracellular formation of AraCTP has been investigated extensively in leukemic blasts. On the other hand, the distinct hematotoxicity after AraC treatment is believed to be mediated by the same pharmaocological mechanisms in mononuclear cells of normal bone marrow or more precisely in hematopoetic stem cells as the physiological counterpart of leukemic blasts. Nevertheless information on the differential AraC metabolism, i.e., qualitative or quantitative differences in the formation and degradation of AraC metabolites between leukemic cells and normal cells, are not available.

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The first part of this study was initiated to investigate the formation of araC-mono, -di and -triphosphate, as the main mediator of cytotoxicity and its precursors, in fresh leukemic blasts, fresh normal bone marrow and in hematopoetic stem cells. Since the half-life of AraCTP in leukemic blasts has been shown to correlate with such clinical parameters as the duration of complete remissions (Rustum et al. 1987) this pharmacokinetic variable was also to be assessed in the aforementioned cell types.

The pharmcodynamic mechanisms of AraC treatment during the highly efficient high dose therapies (Hiddemann et al. 1993) have so far remained partly unlucidated. The observation of a rapid blast lysis during such treatments (Daley et al. 1990, Strum et al. 1994) has evoked interest in the potential interference of AraC metabolites with the cellular lipid metabolism with subsequent disturbances of cellular membranes. The metabolite AraCDP-choline is of particular interest because of its structural similarity to CDP-choline – a precursor of phosphatidylcholine (the most abundant phospholipid in cellular membranes).

The second part of this investigation was therefore focused on the formation of AraCDP-choline in the three cell types (Kucera et al. 1992) and on the changes in lipid composition of leukemic cells during and following AraC exposure.

Materials and Methods

Materials

AraC was obtained from Upjohn (USA), tritium labeled AraC (3H-AraC, 20 Ci/mMol) and L-lyso-3-phosphatidylcholine, 1-(1-14C)palmitoyl (56mCi/mMol) was from Amersham (Braunschweig, Germany). Nucleotide and lipid standards, lysophosphatidylcholine and trypan blue were from Sigma (Munich, Germany). All solvents (HPLC quality) were purchased from JT Baker (Deventer, The Netherlands) or Merck (Darmstadt, Germany). Merck also provided thin-layer chromatography plates. Cell culture medium (RPMI1640) and fetal calf serum were from Gibco Life Technologies (Eggenstein, Germany). For HPLC analysis a type 501 pump by Waters (Erkrath, Germany), a 250/ $^1/_4$ /4.6-120-5 µ C18 reversed phase column by Macherey and Nagel (Düren, Germany) and a Ramona 92 scintillation counter by Raytest (Groß-Gerau, Germany) were used.

Sample Acquisition

Fresh cell samples were obtained by bone marrow aspiration from the posterior iliac crest. Mononuclear cells (leukemic blasts and normal bone marrow cells respectively) were isolated by Ficoll hypaque centrifugation, washed 3 times and then diluted for the respective experiments.

In order to ensure a sufficient amount of CD34/ 38 positive hematopoetic stem cells these cells were isolated from samples obtained by stem cell harvesting after chemotherapy plus G-CSF application. The samples underwent Ficoll centrifugation; CD34/ 38 positive cells were then isolated via cell sorting.

Cultured cell lines (HL60, Raji) were kept in RPMI1640 medium which was supplemented with 20 mM HEPES, 100 µg/ml streptomycin, 10 mM L-glutamin and 10% FCS. Cultures were kept at 37 °C, 5% CO_2 and 95% humidity. The medium was exchanged every 3 days; cells in the exponential growth phase were used for the experiments.

Viability Changes

Cells were incubated at a concentration of 0.2×10^6 cells/ml and exposed to various concentrations of AraC. Cell counts were obtained using a Neubauer cell counting chamber. Cell viability was assessed by the trypan blue exclusion method.

Detection and Quantification of AraC Metabolites

Detection and quantification of tritium labeled AraC metabolites was performed by a previously described high performace liquid chromatography assay (Braess et al.

1996). Briefly this method was based on ion-pairing using a C-18 reversed phase as the stationary phase. Using on-line solid scintillation detection of tritium labeled substances all 9 known metabolites of AraC (AraC, AraCMP, AraCDP, AraCTP, AraCDP-Cholin, AraU, AraUMP, AraUDP and AraUTP) could be detected. The limit of detection ranged from 40 to 200 pg (absolute) for AraC and AraUTP respectively. Due to the detection limits quantification of the respective substances was possible for 7 out of 9 metabolites. For AraUDP and AraUTP only a limit of detection could be defined; exact quantitation was not possible due to the small amounts found persistently during the validation of the assay.

Lipid Changes

Lipid extraction was performed using chloroform/ methanol/ water (volumes 8/10/4). After drying the lipid extract under nitrogen (60 °Celsius) it was redisolved in chloroform/water (volumes 1/1) and applied to high performance thin layer chromatography plates. For the analysis of phospholipids plates were developed in chloroform/ methanol/acetic acid/ water (volumes 60/50/5/2) - for neutral lipids hexane/ isopropanolol was used (volumes 5/7). After drying, the plates were immersed in an 10% copper sulphate solution and made acidic with 8% phosphoric acid. After charring the charred spots were quantitated using a personal densitometer (Molecular Dynamics, Krefeld, Germany).

Results

Metabolite Formation

Cells were incubated with AraC at a concentration of 1 and 5 µg/ml for 5 h. For leukemic blasts and normal mononuclear cells a concentration of 0.5×10^6 cells/ ml was used. In the case of CD34/38 positive stem cells various concentrations ranging from 0.14 to 0.5 $\times 10^6$ were used depending on the yield of the respective sample acquisition. The pharmacologically relevant metabolites, AraC, AraCMP, AraCDP, AraCTP, and also AraCDP-Choline were detectable in all cell types. As shown in Table 1 AraCTP was the most abundant metabolite followed by AraCDP-Choline and AraCDP. There was a significant variability between different samples of the same cell type. These variances were most pronounced in leukemic blasts perhaps reflecting the greater biological het-

Table 1. Formation of AraC metabolites in various cell types at two dosages (5 hours) mean and standard deviation (italics)

Cell type	n	AraC incubation µg/ml	AraC	AraU	AraCMP	AraCDP	AraCTP	AraCDPChol
HL60	2	1	10.4	5.0	1.7	7.0	69.9	10.8
			3.3	*1.4*	*1.3*	*2.6*	*16.6*	*5.2*
	2	5	18.9	9.5	4.7	15.2	120.1	20.4
			6.5	*3.4*	*2.3*	*5.5*	*22.6*	*4.5*
RAJI	2	1	64.0	12.9	12.7	63.5	330.7	86.1
			8.1	*4.0*	*6.5*	*8.9*	*39.9*	*13.6*
	2	5	104.6	25.2	23.8	75.8	438.3	158.0
			13.3	*3.4*	*6.4*	*12.2*	*39.9*	*51.8*
AML blasts	9	1	5.8	2.9	2.7	7.5	99.0	12.9
			2.4	*2.5*	*1.7*	*7.3*	*103.3*	*9.6*
	9	5	24.0	7.7	6.7	13.0	187.8	30.5
			15.0	*6.8*	*3.8*	*12.1*	*173.9*	*21.5*
Normal bone	4	1	3.4	1.6	1.3	3.3	30.4	4.0
			1.0	*0.4*	*0.5*	*1.3*	*13.5*	*1.7*
marrow	4	5	22.8	5.0	3.2	5.3	50.6	9.4
			4.9	*1.2*	*1.2*	*2.3*	*31.1*	*4.6*
CD34/38+	4	1	7.1	1.5	1.6	2.3	16.0	4.2
			7.7	*2.1*	*1.5*	*3.6*	*24.5*	*3.3*

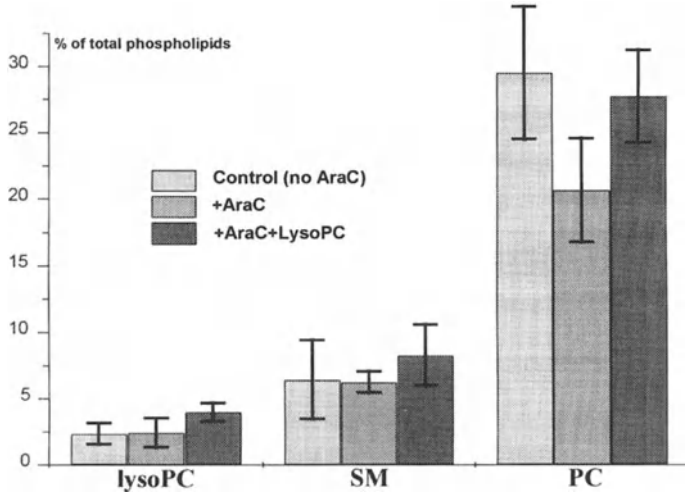

Fig. 1. Effects of AraC and lysoPC on phospholipid content in HL60 cells (AraC 12.5 µg/ml for 5 h, lysoPC 10 µg/ml for 20 h)

erogeneity of this cell type. No significant differences in the absolute formation of AraC metabolites were found between the groups.

The relation of the not completely phosphorylated AraC anabolites (the sum of AraCMP and AraCDP) to AraCTP did not change during dose escalation. In contrast the relative abundance of AraCDP-Choline in relation to AraCTP was increased at higher AraC concentration, perhaps reflecting a saturation of AraCDP-Choline formation at higher concentrations of AraC than is the case for AraCTP (Plunkett et al. 1987).

AraCTP Half-Life

AraCTP half-lives were shortest in CD34/ 38 positive stem cells (median 1.9 h) and longest in leukemic blasts (3.3 h) with mononuclear cells of normal bone marrow featuring an intermediate $t_{1/2}$ of 2.9 h. The differences between the various cell types were not yet statistically significant. There was considerable variability in different samples of the same cell type. Again these variances were most pronounced in leukemic blasts (variation coefficient 0.61) with some cells featuring AraCTP half-lives of up to 7.7 h.

Cell Viability and Lipid Changes During AraC Exposure

Cultured leukemic cells (HL60 cells) were exposed to 12.5 µg/ml AraC for 48 h. A significant loss of viability (60-70%) was consistently observed as demonstrated in Fig. 1. Figure 2 demonstrates that the cellular lipid composition featured significant decreases (-20 to 30%) in the contents of phophatidylcholine (PC) – the most abundant phospholipid of cellular membranes. The amount of other phospholipids as lysophosphatidylcholine or sphingomyeline remained unchanged. Neutral lipids (diglycerides, triglycerides) did not show any significant alterations (data not shown). In order to investigate the pathogenetic relevance of these lipid changes cells were exposed first to AraC and subsequently to lysophasphatidylcholine (lysoPC), a precursor of PC that readily incorporates into cellular membranes and via acylation is turned into PC. This treatment resulted in almost complete reversal of the observed changes in lipid metabolism as shown in Fig. 1.

The reversal of cellular lipid changes following AraC treatment was accompanied by a significant reduction of AraC induced loss of viability. This effect was even more pronounced when cells were again exposed to lysoPC after 24 h. No influence of lysoPC on the uptake of AraC into the cells or on its metabolism was observed.

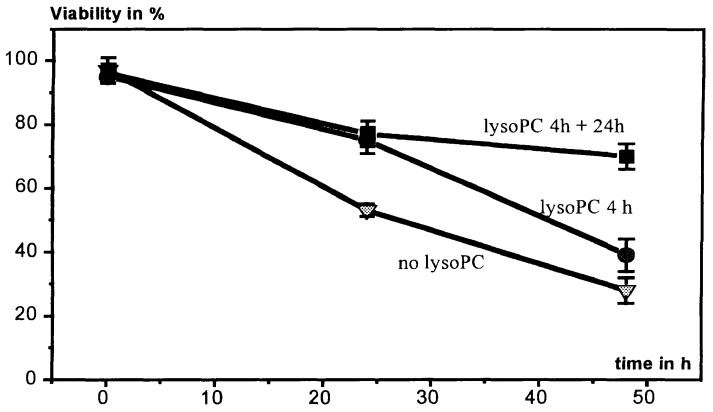

Fig. 2. Viability loss following AraC exposure (12.5 µg/ml) without lysoPC, lysoPC during the first 24 h, lysoPC during the first 48 h

Table 2. AraCTP half-lives (median, range, standard deviation) in various cell types

Cell type	n	Median $t_{1/2}$ in h	Range
Normal bone marrow	9	2.9	2.3 - 8.6
SD		1.4	
AML blasts	9	3.3	1.6 - 7.7
SD		2.0	
CD34/38+	4	1.9	1.1 - 2.7
SD		0.8	

Discussion

In the present study the formation of various metabolites of AraC was investigated in malignant and normal cells. No significant difference, either qualitatively or quantitatively, could be found in the abundance of either AraCMP, -CDP, -CTP or AraCDP-cholin in leukemic blasts, mononuclear cells of normal bone marrow and CD34/38 positive hematopoetic stem cells. Especially no significant differences could be found between mononuclear cells from normal bone marrow and CD34/38 positive hematopoetic stem cells. This observation suggests that for any further investigation into the metabolism of AraC in non malignant hematological cells these experiments can be performed on unsorted mononuclear cells as a realistic surrogate for the hematopoetic stem cell. This statement has to be put into the context that the present investigation employed slightly more differentiated stem cells than the true omnipotent – solely CD34 positive – stem cells. Even though unlikely it is therefore impossible to rule out that differences in AraC metabolism might exist between the cells analysed in this investigation (CD34/38 positive stem cells) and its even more primitive predecessors.

During dose escalation AraCTP formation was saturated at the highest dose level (5 µg/ml AraC) which is in concordance with data by other authors. In all cell types the added amounts of AraC mono- and di-phosphate amounted to 13-20% of the amount of AraCTP on a molar basis at the respective point in time. This ratio did not change significantly during dose escalation. Especially there was no tendency towards increased amounts of not completely phosphorylated AraC metabolites at dose levels that exceeded the one needed for the saturation of AraCTP formation. This finding has to be put into a context with the result that - after termination of AraC exposure - leukemic cells tended to retain AraCTP longer than their physiological counterparts. Even though the $t_{1/2}$ of AraCTP in leukemic blasts has been shown to be a pharmacokinetic parameter with significant correlations to parameters of clinical outcome like rate and duration of complete remissions (Rustum et al. 1987) the pharmacological or metabolic basis responsible for differences in AraCTP retention have so far not been elucidated.

Since AraC is rapidly eliminated in vivo only the already phosphorylated compounds like AraCMP and AraCDP – unable to leave the cell – are available for further

synthesis of AraCTP after the end of the AraC exposure. Because no differences could be found in the amount of AraCMP / AraCDP between leukemic and normal cells it is unlikely that the prolonged synthesis of AraCTP from these two precursors contributed significantly to the longer half-life of AraCTP in leukemic blasts. It is therefore tempting to speculate that the activity of AraCTP degradating enzymes (phosphatase activity potentially mediated by alkaline phosphatase and 5′-nucleotidase) is responsible for the clinically relevant differences in AraCTP $t_{1/2}$ (Hiddemann et al. 1992).

Unlike AraCMP / AraCDP there was a trend towards increasing amounts of AraCDP-choline even at AraC concentrations that were sufficient to saturate AraCTP formation. This finding might be due to a saturation curve of AraCDP-choline formation that is shifted to the right compared to AraCTP. The potential relevance of this finding is due to the observation that a superior antileukemic activity of high dose AraC regimens were observed, during which AraC plasma levels significantly exceed the concentrations needed for the saturation of AraCTP formation. Since AraCDP-choline tends to increase even after AraCTP formation has plateaued AraCDP-choline mediated mechanisms of action might be of relevance at these high dosages.

The second part of this investigation was therefore focused on a potential pharmcodynamic action of AraCDP-choline: a disturbance of phospholipid metabolism could result from AraCDP-choline mediated inhibition of phosphatidylcholine (PC) synthesis (the most abundant phospholipid in cellular membranes) due to its structural similarity to CDP-choline, a physiological precursor of PC synthesis. In fact during exposure of leukemic cells (HL60) to AraC cellular PC content was significantly reduced. In contrast other phospholipids [lysophosphatidyl choline (lysoPC), sphingomyeline (SM)] and neutral lipids remained unchanged. The cytotoxic relevance of these changes were determined by antagonizing the AraC induced PC decreases by adding another precursor of PC synthesis. LysoPC readily incorporates into cellular membranes and is formed into PC via acylation – a CDP-choline independent mechanism. In fact the addition of lysoPC to AraC exposed cells resulted in a complete reversal of the observed lipid changes. This effect was not due to altered AraC uptake into cells or changes in AraC metabolism since these variables remained unchanged during lysoPC exposure.

The reversal of AraC induced lipid changes was accompanied by a significant reduction of the AraC induced loss of viability (tested by trypan blue exclusion). This was most pronounced when lysoPC was applied not only during the first 24 h after AraC exposure but also during the following 24 h, suggesting a prolonged inhibitory effect of AraC on PC synthesis.

The most likely – lipid dependent – mechanism for the observed loss of cell viability is the destabilization of cellular membranes which has also been observed after PC alterations from other causes. Aside from these structural changes caused by the interference of AraC with the phospholipid metabolism functional changes, i.e., alterations in phospholipid dependent signal transduction pathways, are another potential mechanism of AraC induced cytotoxicity. Especially activation of protein kinase C (PKC) following AraC exposure (Emoto et al. 1996; Brach et al. 1992) and consequent activation of downstream signal transduction pathways might be the result of AraC induced rises in DAG (diacylglycerol) – a second messenger known to activate certain isoenzymes of PKC – from the inhibition of PC synthesis (Nishizuka 1992).

In conclusion these investigations suggest that a differential metabolism of AraC – either qualitatively or quantitatively – is unable to explain the relative selectivity of this antimetabolite for leukemic cells as compared to normal cells. Instead enzymatic activity responsible for the degradation of AraCTP might constitute a factor that differentiates susceptible cells from more resistant counterparts.

At high AraC concentrations the pharmacodynamics in HL60 cells seem to involve perturbations of the cellular lipid metabolism by reduction of PC content resulting in a higher susceptibility to membrane lysis. So far the exact mechanisms how AraC – most

likely via its metabolite AraCDP-choline – influences the phospholipid metabolism remains to be established.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Interaction of 1-β-D-Arabinofuranosylcytosine with Lipid Metabolism

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Abstract. We have studied the effects of 1-β-D-arabinofuranosylcytosine (ara-C) and other deoxycytidine analogs on lipid metabolism and cell death. The hypothesis is that ara-C affects lipid metabolism and causes cellular differentiation and/or apoptosis. Ara-CDP-choline is an ara-C metabolite that has been identified in ara-C-treated cells. Two possible pathways for ara-CDP-choline formation are through the catalytic activity of CTP:phosphocholine cytidylyltransferase or CDP-choline: 1,2-diacylglycerol cholinephosphotransferase, two enzymes in the CDP-choline pathway for phosphatidylcholine biosynthesis. The ara-C metabolite, ara-CMP, is a substrate for cholinephosphotransferase, K_m = 1.4 mM. However, ara-CTP is not a substrate for the cytidylyltransferase in the same biosynthetic pathway. In vitro, ara-CMP reverses the activity of cholinephosphotransferase catalyzing the conversion of ara-CMP and phosphatidylcholine to ara-CDP-choline and diglyceride. Ara-CDP-choline has no known function while diglyceride activates protein kinase C leading to downstream signaling events. These downstream events include the phosphorylation and activation of deoxycytidine kinase, the rate limiting step in the conversion of ara-C from a prodrug to its active form, ara-CTP. The activation of protein kinase C also induces cellular differentiation. Furthermore, ara-C increases the intracellular levels of ceramide through the action of a neutral sphingomyelinase. The increase in ceramide is an intracellular signal for the cell to undergo differentiation and apoptosis. Ara-C-induced diglyceride and ceramide signaling pathways are complementary or converging with respect to cellular differentiation and possibly antagonistic with respect to apoptosis. In conclusion, the effects of ara-C on lipid metabolism appear to be through two mechanisms;

1. the activation of PKC and subsequent cellular differentiation and
2. the formation of ceramide and subsequent initiation of apoptosis.

Chemotherapy regimens in the future should capitalize on the signaling mechanisms involved in ara-C cytotoxicity to improve the efficacy of this drug and other deoxycytidine antimetabolites.

Introduction

Although ara-C is an effective and widely used agent for the treatment of AML, full elucidation of its cellular mechanism(s) of action has not been realized. Ara-CTP is a competitive inhibitor of DNA polymerase that competes with dCTP for incorporation into DNA [1]. Ara-CTP incorporation into DNA results in the termination of DNA elongation [2], and the extent to which ara-C is incorporated into DNA has been correlated

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with decreased cell survival [1, 3]. Several other cellular effects of ara-C have been noted including: impaired replicon initiation [4], fragmentation of nascent DNA [5], and induction of double replication of some DNA fragments [6]. Moreover, ara-C has been associated with the initiation of apoptosis [7-11]. Based on the available evidence, we believe that there are a number of mechanisms involved in the cytotoxicity of ara-C including DNA effects as well as effects that ara-C may have on phospholipid metabolism.

Ara-CDP-choline is a metabolite of ara-C that has been identified in cultured cells, ascites cells and leukemic blast cells isolated from AML patients [12-15]. Lauzon et al. [12,13] also identified ara-CDP-ethanolamine in cells treated with ara-C. Ara-CDP-choline has no known cellular function yet it has a relatively long half-life [14,15]. The biosynthetic pathway for phospholipid biosynthesis is dependent upon cytidine coenzymes such as CDP-choline [16] and dCTP [17]. In addition, ara-C stimulates the formation of the sphingolipid, ceramide. Therefore, it is hypothesized that the ara-C metabolite, ara-CDP-choline, and ceramide play a role in phospholipid [18] and sphingolipid metabolism [19].

Differentiation and programmed cell death or apoptosis are two terminal processes in cellular growth. Differentiation of leukemia cells has been identified in cells treated with ara-C [20-23]. This differentiation is mediated through an as yet unknown mechanism possibly involving protein kinase C (PKC). PKC activity is increased in cells treated with ara-C [24]. Furthermore, recent evidence has determined that apoptosis is activated in cells treated with ara-C. Thus, several signaling pathways within the cell are stimulated through the action of ara-C and are affected by lipid mediators.

Material and Methods

Ara-C (Upjohn) was dissolved in sterile deionized water. TPA was purchased from LC Services and stored in DMSO (experimental DMSO concentrations never exceeded 0.03%). ET-18-OCH_3 (Medmark Pharma, GmbH, Grunwald, Germany), and C_2-ceramide (Matreya) were stored at -20 °C. Vehicle controls were included and were consistently found to be without effect on the parameters studied. *E. coli* diglyceride (DG) kinase was purchased from Calbiochem. CTP, dCTP, ara-CTP, CMP and ara-CMP were obtained from Sigma Chemical Co. (St. Louis, MO), [5-^{3}H]CMP (25 Ci/mmol), [5,6-^{3}H]ara-CMP ammonium salt (10 Ci/mmol), [2-^{14}C]-2'-deoxycytidine, (56 Ci/mmol), and [5-^{3}H]ara-C (25 Ci/mmol) were from Moravek Biochemicals Inc. (Brea, CA). [Methyl-^{3}H]choline chloride (88 Ci/mmol) was from New England Nuclear (Boston, MA). Purified PKCα was obtained from Gibco BRL Life Technologies (Gaithersburg, MD). [γ-^{32}P]ATP ammonium salt (4000 Ci/mmol) was from ICN Biomedicals (Irving, CA). All other chemicals were reagent grade or better and were obtained from either Sigma or Fisher.

L5178Y murine leukemia cells were grown in Fischer's medium supplemented with L-glutamine, penicillin and streptomycin (Gibco, Grand Island, NY) and 10% horse serum. HL-60 cells were grown in RPMI 1640 supplemented with L-glutamine, penicillin and streptomycin and 10% fetal bovine serum. The cells were routinely passaged three times weekly. The cultures were grown at 37 °C in an atmosphere of 5% CO_2: 95% air. Exponentially growing cells were used for all experiments.

CTP:Phosphocholine Cytidylyltransferase Activity

Cytidylyltransferase was prepared from L5178Y murine leukemia cells as previously described [18]. Enzyme activity was monitored according to the method of Weinhold et al. [25] as modified in [18] with CTP, ara-CTP, or dCTP and [methyl-^{3}H]phosphocholine (as prepared elsewhere [26]) as substrates. The reaction mixture contained 1 mM [methyl-^{3}H]phosphocholine, 6 mM magnesium acetate, 75 mM Tris-succinate, pH 6.5, total volume was 100 µl. The enzymatic reaction was stopped by boiling, and the reaction product, x-CDP-[methyl-^{3}H]choline (x-CDP-choline refers to either CDP-choline, ara-CDP-choline or dCDP-

choline) was separated from the [methyl-^{3}H]phosphocholine substrate by ion-exchange chromatography on minicolumns made up of 1 ml bed volume of DEAE-Sephadex A-25 according to the method of Yang et al. [14]. The columns were eluted with 4 ml of H_2O followed by 4 ml of 0.12 M TEA formate, pH 6.5. [Methyl-^{3}H]phosphocholine eluted from the DEAE-sephadex with the H_2O wash, and the x-CDP-[methyl-^{3}H]choline eluted from the column with 0.12 M TEA formate. Data are reported as nmoles of x-CDP-choline produced/min/mg of protein.

CDP-Choline:1,2-Diacylglycerol Cholinephosphotransferase Activity

Microsomes containing cholinephosphotransferase activity were prepared from sonicated L5178Y murine leukemia cells as described [18]. Cholinephosphotransferase activity was monitored in the reverse reaction by a modification of the method of Goracci et al. [27] using [^{3}H]CMP (10000 dpm/assay) or [^{3}H]ara-CMP (10000 dpm/assay) as substrates. The reaction mixture contained 15 mM NaF, 10 mM $MgCl_2$, 0.1 M Tris, pH 7.4, 0.5 to 2 mM CMP and/or ara-CMP, and 50 µl microsomes (0.2-0.3 mg of protein per assay) as obtained above in a total reaction volume of 100 µl. Two mM AMP was added to each reaction to inhibit 5'-nucleotidase. Each assay also contained 10 nmol of exogenous phosphatidylcholine (Sigma Type XI-E from egg yolk) that was co-sonicated with the microsome preparation (50 µl) prior to addition to the reaction. Blanks contained all but the microsome preparation. The reaction was initiated by the addition of the microsome preparation and was stopped after 20 min by boiling for 2 min. X-CDP-choline was separated from xCMP by the method of Yang et al. [14] on minicolumns of 4 ml bed volume DEAE-sephadex A-25 ion-exchange resin eluted with 8 ml H_2O followed by 8 ml 0.12 M TEA formate, pH 6.5 (x-CDP-choline fraction) and 8 ml 0.22 M TEA- formate, pH 6.5 (x-CMP fraction). Data are reported as nmoles of x-CDP-choline produced/min/mg of protein.

Measurement of Diglyceride and Ceramide Concentrations by DG Kinase

Ara-C (10 µM final) was added to HL-60 cells (2.5-3.0 3 105 cells/ml in 2.5 ml/assay) for a specified period of time. Cell numbers and viability were determined using a hemacytometer and the Trypan blue exclusion assay. Incubations were terminated by centrifugation of equal cell numbers of each sample in glass 100 3 15 mm tubes. Dead cell debris was removed by aspirating the supernatants and the cell pellets were resuspended in 2.0 ml of methanol. Total lipids were extracted using the method described by Bligh and Dyer [28]. To determine diglyceride and ceramide amounts, a modified DG kinase assay was performed [19, 29, 30]. The reaction products, [32P]phosphatidic acid and ceramide-1-[32P]phosphate were isolated by organic extraction and resolved by thin-layer chromatography. DG and ceramide mass amounts (pmol/106 cells) were determined by liquid scintillation counting and based on standard curves and number of cells used.

Measurement of Diradylglycerol Subclasses by HPLC

L5178Y cells (5×10^5 cells/ml) were incubated at 37 °C with increasing concentrations of ara-C for 24 h. Cells (5×10^8 total cells/condition) were harvested by centrifugation and aspiration of media supernatants. Lipids were extracted by the method of Bligh and Dyer [28]. Phospholipid content was measured by the method of Rouser et al. [31]. In preparation for HPLC analysis, diglycerides from the samples were separated from other lipids by TLC, the lipids migrating with an R_f value similar to an authentic diglyceride standard were recovered from the silica gel and derivatized with benzoylchloride in pyridine [32]. The derivatized diglyceride subclasses were separated by HPLC on a normal phase silica column as described earlier [32]. Quantitation of the subclasses was made based on areas under the curves of known amounts of benzoylated diglyceride standards also subjected to HPLC.

Deoxycytidine Kinase Activity

L5178Y cells (3×10^5/ml) were treated with 50 nM TPA for various periods of time. At the indicated time an aliquot (1 ml) of the cell suspension was removed, the cells were disrupted by sonication, and dCyd kinase activity was measured using [^{14}C]dCyd as a substrate according to the method of Cheng et al. [33].

PKC Phosphorylation of MARCKS

Phosphorylation of MARCKS (myristylated alanine-rich C-kinase substrates, p80) proteins were monitored as a measure of endogenous PKC activity in response to TPA or ara-C according to the method of Chepenik and Haystead [34]. L5178Y cells were labeled with [^{32}P] for 60 min. The cells were washed twice with phosphate buffered saline, pH 7.2 (PBS) and treated with TPA or ara-C for up to 120 min. Following the incubation period the cells were precipitated, resuspended in SDS-PAGE sample buffer. The samples were boiled, centrifuged and the resulting supernatant containing the heat stable MARCKS proteins was loaded onto a 10% SDS-PAGE gel. The proteins were separated by electrophoresis, the gel was dried and radiolabeled proteins were visualized by autoradiography. The proteins migrating at 80 kDa were excised from the gel and quantified by scintillation counting.

DNA Fragmentation

DNA fragmentation was analyzed by conventional agarose gel electrophoresis as reported previously (10). HL-60 cells ($0.5\text{-}1 \times 10^6$) were treated with the indicated agents. Following treatment, the cells were washed with serum-free ice-cold PBS. The cell pellet was lysed in 50 µl lysis buffer (10 mM Tris-HCl, pH 7.4, 20 mM EDTA, and 100 mM NaCl) containing 200 ng/ml RNAse A. After 1 h at 37 °C the lysate was diluted to 300 µl in the same buffer but containing 225 ng/µl Proteinase K and 0.5% SDS and incubated at 50 °C for 16 h. Total genomic DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The DNA was resuspended in 10 mM Tris-HCl, pH 8, 20 mM EDTA. Equal DNA was loaded into wells of a 2% agarose gel and DNA fragments were resolved by electrophoresis at 115 volts for 90 min. A 200-bp DNA ladder, for molecular size reference was run in parallel. DNA was visualized by ethidium bromide staining and exposure to UV light.

Results

Ara-CDP-Choline Formation

Since ara-CDP-choline had been identified as an ara-C metabolite, it was of interest to determine the pathway leading to the formation of this metabolite. Possible mechanisms responsible for the formation of ara-CDP-choline include CTP:phosphocholine cytidylyltransferase and CDP-choline:1,2-diacylglycerol cholinephosphotransferase, two enzymes in the biosynthetic pathway for phosphatidylcholine biosynthesis. To determine which enzyme was responsible for the formation of ara-CDP-choline, cytidylyltransferase and cholinephosphotransferase were partially purified from mouse leukemia L5178Y cells and the enzymatic activities of these enzymes were measured using the appropriate substrates.

In the cytidylyltransferase assay system, cytidylyltransferase showed an activity of 1.05 and 0.56 nmol/min/mg when CTP or dCTP were used as substrates, respectively (Table I). However, ara-CTP was not a substrate for this reaction. In addition, when

Table 1. CTP:phosphocholine cytidylyltransferase activity

Substrate	nmol/min/mg
2 mM CTP	1.05
2 mM ara-CTP	n.d.
2 mM dCTP	0.56
2 mM ara-CTP+2 mM CTP	0.65
4 mM CTP	1.02

n.d. not detected.

Cytidylyltransferase activity was measured using [^{3}H]phosphocholine plus the indicated substrate as described in Materials and Methods (data from [18]).

equal molar amounts of CTP and ara-CTP were included, ara-CTP inhibited the reaction by approximately 60% suggesting a substrate dilution effect since a K_i value of 6 mM was determined for ara-CTP inhibiting the conversion of CTP + phosphocholine to CDP-choline via cytidylyltransferase [18].

The other possible source of the ara-CDP-choline is through reversal of the cholinephosphotransferase. This enzymatic activity has previously been reported in the literature [27,35,36]. Using [^{3}H]CMP or [^{3}H]ara-CMP and a preparation of cholinephosphotransferase from mouse leukemia L5178Y cell microsomes it was determined that ara-CMP was, in fact, a substrate for the cholinephosphotransferase (Table 2).

Table 2. CDP:1,2-diacylglycerol cholinephosphotransferase activity

Substrate	V_{max} (nmol/min/mg)	K_m (mM)	K_i (mM)
CMP	0.78	0.34	
ara-CMP	0.22	1.4	3.0

Reverse CDP:1,2-diacylglycerol cholinephosphotransferase activity was measured using either [^{3}H]CMP or [^{3}H]ara-CMP as substrate and formation of radiolabeled CDP-choline or ara-CDP-choline was measured as described in Materials and Methods. V_{max} and K_m values were calculated from double reciprocal plots (data from [18]).

Diglyceride and Ceramide Formation

Since it was determined that ara-CDP-choline can be formed from the reverse activity of cholinephosphotransferase, the measurement of diglyceride formation was undertaken to confirm the previous results. HL-60 cells incubated with 10 μM ara-C for increasing time exhibited a 2-fold increase in diglyceride content by 12 h. This increase persisted for at least 24 h (Fig. 1). Diglyceride levels in control cells were not changed during this time period. Ceramide levels, measured simultaneously with the diglyceride levels reported above, indicated that the intracellular concentration of ceramide increased 3-fold during the same time period (Fig. 2) and these levels of ceramide persisted for at least 24 h. As with the diglyceride levels in the control cells, ceramide levels were unchanged under control conditions.

The subclasses of diglycerides induced by ara-C were of interest since different diglycerides have distinct intracellular responses [37,38]. In order to determine the species of diglyceride that ara-C induces, diglyceride subclasses were determined using HPLC. In comparing alkenylacyl-, alkylacyl- and diacylglycerols from L5178Y cells treated with 0, 10 and 100 μM ara-C for 24 h, there was little increase in either alkenylacyl- or alkylacyl-linked diglycerides. The greatest increase was observed in the diacylglycerol subclass following the 24 h exposure of the cells to ara-C (Fig. 2). Therefore, it can be inferred that the increase in diglycerides observed in Fig. 1 was due mainly to an increase in the diacylglycerol subclass.

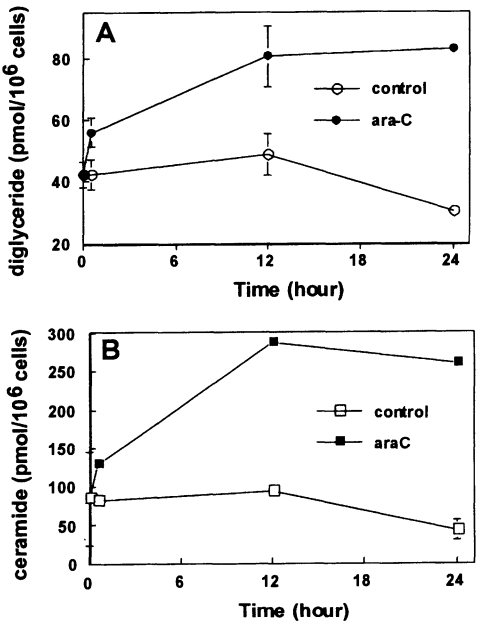

Fig. 1. Diglyceride and ceramide levels in ara-C-treated HL-60 cells. HL-60 cells were stimulated with *(solid symbols)* or without *(open symbols)* 10 μM ara-C over the period indicated. At the times indicated, amounts of diglyceride and ceramide from 1×10^6 cells were determined by the DG kinase assay as described in Materials and Methods. The results are depicted as filled and open circles diglyceride (pmols/10^6 cells ± s.d., n = 3) *(A)*, filled and open squares ceramide (pmols/10^6 cells ± s.d., n = 3) *(B)*. The data are representative of three separate determinations

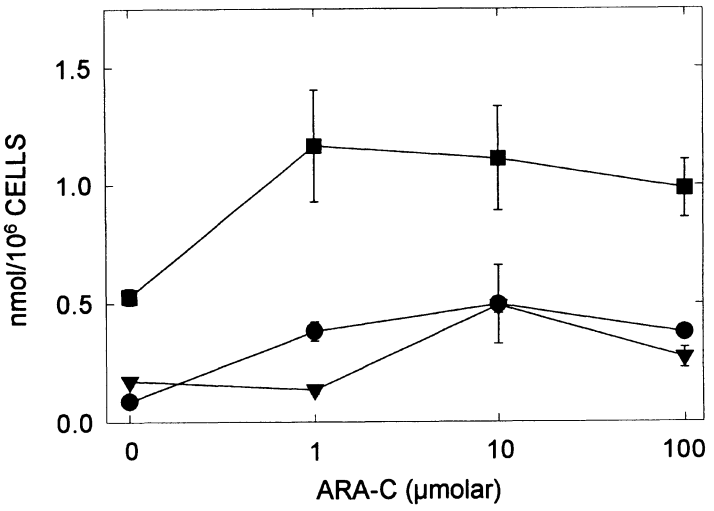

Fig. 2. Diglyceride subclasses in ara-C-treated L5178Y cells. L5178Y cells were exposed to 0, 10 or 100 μM ara-C for 24 h. The resulting diglycerides were extracted, chromatographed on TLC, derivatized and quantified by HPLC as described in Materials and Methods. Points are the mean ± the range of a representative experiment. Filled squares are diacylglycerol, filled circles are alkenylacylglycerol and filled triangles are alkylacylglycerol

PKC Activation

Activation of PKC following treatment with ara-C has been reported earlier [24]. We have further characterized this PKC activation and below are experiments aimed at understanding the effects of altered PKC activity in ara-C treated cells.

In L5178Y cells treated with 10 nM TPA, a 20% increase in deoxycytidine kinase activity was observed within the first 10 min of treatment. The deoxycytidine kinase activity quickly reached a maximum and returned to an activity level below that of the initial level after 2 h (Fig. 3).

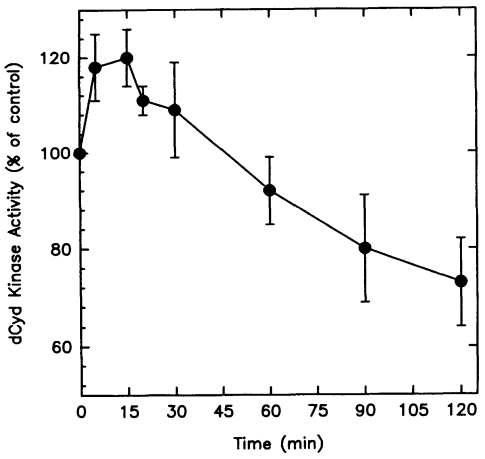

Fig. 3. In situ activation of dCyd kinase activity with TPA. L5178Y cells were treated as described in Material and Methods. *Points* are plotted as a percent of control and represent the mean ± s.e. of 3 experiments done in duplicate

Because of the initial rise in dCyd kinase activity, we used purified PKC and deoxycytidine kinase in vitro experiments to determine if dCyd kinase was a substrate of PKC and what effects phosphorylation by PKC had on dCyd kinase activity. In vitro incubation of dCyd kinase with PKC and [γ-^{32}P]-ATP lead to a rapid and sustained increase in the amount of [^{32}P] associated with dCyd kinase [39]. This result suggests that specific phosphorylation of dCyd kinase was occurring. This phosphorylation was readily observable on SDS-polyacrylamide gel electrophoresis of the deoxycytidine kinase following the incubation with PKC and the radiolabeled ATP. Furthermore, phosphorylation of the deoxycytidine kinase by PKC resulted in an increase in the deoxycytidine kinase V_{max} from 52 to 104 nmol/min/mg of protein and an increase in the K_m from 2 to 6.9 μM [39].

To observe ara-C-activation of PKC in vivo we used a method described by Chepenik and Haystead [34]. This method makes use of the specific heat stable PKC substrate, MARCKS that has been previously characterized by others [40, 41]. Increase in PKC specific phosphorylation of MARCKS in response to ara-C treatment is shown in Fig. 4. Clearly, ara-C treatment of the L5178Y cells caused a 3-fold increase in the phosphorylation of the PKC specific substrate as a func-

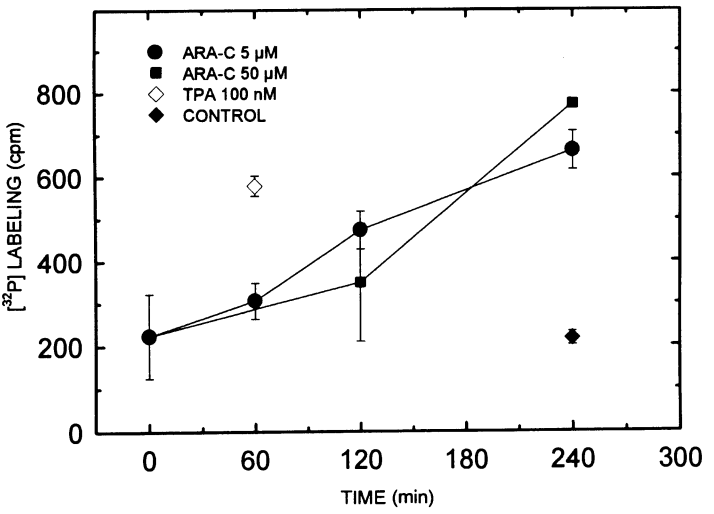

Fig. 4. PKC phosphorylation of MARCKS in $[^{32}P]_i$-labeled L5178Y cells. L5178Y cells were prelabeled with $[^{32}P]$ as described in Materials and Methods and treated with either TPA or ara-C at the indicated dose. MARCKS proteins were isolated as described and quantified following excision from the SDS-PAGE gel by scintillation counting

tion of time. TPA, a known stimulator of PKC activity, also increases phosphorylation of the MARCKS protein. There is no increased phosphorylation of the PKC substrate in the absence of ara-C or TPA. Neither ara-C nor ara-CMP are able to activate purified PKC in vitro (personal communication L. Ballas); therefore, ara-C, indirectly, must activate PKC.

Differentiation and Apoptosis

Low doses of ara-C can induce cellular differentiation in vitro. This may be related to the formation of diglycerides and ceramides following ara-C treatment. This is significant since these two lipid metabolites have been shown to accumulate in cells upon treatment with ara-C and induce monocytic differentiation [42-48]. Furthermore, PKC has been identified as a differentiating agent in hematopoietic cells [49-51]. Therefore, TPA, was used to stimulate PKC in L5178Y cells followed by the addition of ara-C, and clonogenic efficiency was measured (Fig. 5).

At a dose of 0.5 μM ara-C there is little decrease in cloning efficiency with the addition of TPA. However, at 1 and 10 μM ara-C, the addition of TPA produced an additive decrease in cloning efficiency. This decrease in clonogenic survival may be due to increased differentiation of cells as a result of PKC activation.

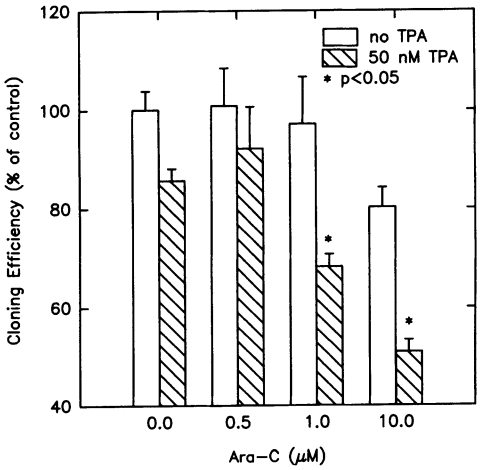

Fig. 5. The effect of TPA on ara-C cytotoxicity. L5178Y cells were treated with or without 50 nM TPA for 5 min, followed by the addition of ara-C (0, 0.5, 1, 10, 100 μM). The cells were allowed to incubate for 3 h, then were washed with drug-free medium. Clonogenic assay on soft agar was completed and cloning efficiency reported as a percent of control

HL-60 cells undergo classic DNA fragmentation representative of apoptosis. This is illustrated by laddering the DNA on an agarose gel in a time and dose-dependent manner in response to ara-C. Figure 6 illustrates the laddering pattern of fragmented DNA following 1 μM ara-C treatment for 4 h (lane 7) and the inhibition of ara-C-induced DNA fragmentation by TPA (lanes 8-11). TPA by

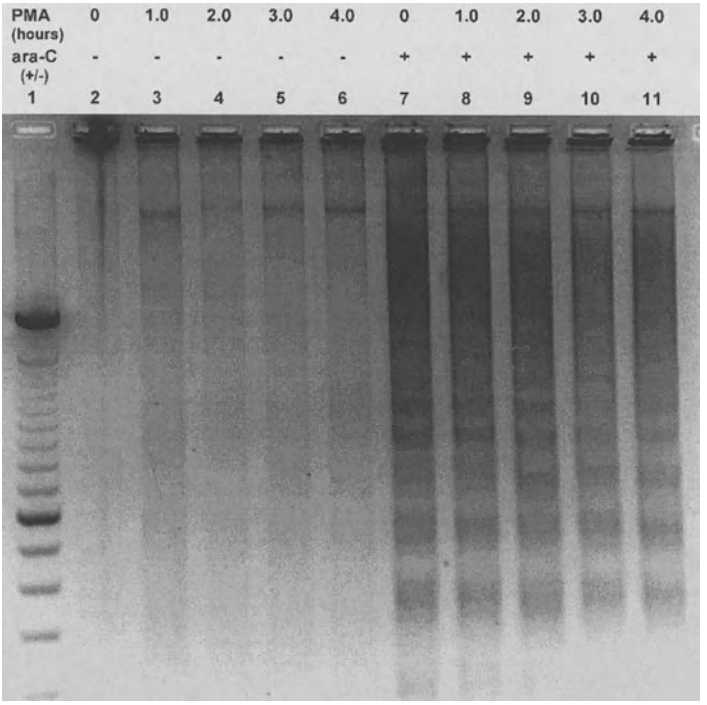

Fig. 6. Agarose gel electrophoresis of ara-C-treated HL-60 DNA in the presence of TPA. HL-60 cells were treated with or without 1 μM ara-C for 4 h. Where indicated, the cells were also treated with 50 nM TPA alone or in combination with ara-C for the indicated time. Following the incubation period, the cellular DNA was extracted as described in Materials and Methods and resolved by electrophoresis on a 2% agarose gel. The DNA was stained with ethidium bromide and visualized by UV light

itself does not induce DNA fragmentation under the conditions used (lanes 3-6). TPA (50 nM) inhibits the degree of internucleosomal DNA fragmentation in a time dependent manner.

Discussion

AML is a chemotherapy-sensitive neoplasm, but drug resistance remains a major obstacle to cure. At the onset of therapy approximately 10% of patients are drug resistant, and approximately 65% achieve complete remission. Of the patients achieving complete remission, 80% will relapse at some point and eventually die with drug-resistant AML [52]. Clearly, new therapies are necessary for the treatment of this disease. Laboratory experiments have linked the cytotoxic effects of ara-C with its degree of incorporation into DNA [1, 2] and clinical pharmacological data are consistent with these observations [53]. However, recent evidence suggests that additional mechanisms are involved with ara-C cytotoxicity. The goal of this report is to describe additional mechanisms of ara-C action that may be exploited to optimize drug efficacy and to provide a rationale for ara-C combination chemotherapy.

With the advent of high dose ara-C chemotherapy, patients who were refractory to conventional doses of ara-C, exhibited a renewed response to therapy. Clinical observations of these patients receiving high dose ara-C revealed a unique phenomenon in that these patients exhibit a rapid lysis of leukemic blasts (36-48 h) whereas patients treated with standard dose ara-C have a lowering of their WBC count over the course of a week [54]. Therefore, it is hypothesized that ara-C affects cells in ways that are inconsistent with DNA alterations since the cell cycle time of circulating blast cells is 76 h [55]. Moreover, ara-C has cytotoxic effects on postmitotic neurons, that are not presumably synthesizing DNA [56].

Early studies indicated that an increase in the turnover or synthesis of phosphatidylcholine followed the treatment of cultured cells with ara-C [57, 58]. Since phospholipid biosynthesis is dependent on cytidine containing coenzymes, it can be inferred that

ara-C plays a role in lipid metabolism. It has been suggested that there are different pools of deoxyribonucleosides [59]. One pool is for the synthesis of DNA and another pool for the metabolism of phospholipids. Furthermore, it has been suggested that ara-CDP-choline and dCDP-choline are synthesized from separate pools in lymphoma cells [60]. In the presence of unlabeled ara-C, synthesis of dCDP-choline was not inhibited when [^{3}H]dCyd was used as a precursor. Furthermore, ara-C enhances the labeling of dCDP-choline in lymphoma cells.

From Tables 1 and 2 it can be seen that the ara-C metabolite, ara-CDP-choline, is derived from the reverse activity of cholinephosphotransferase. This ara-C metabolite has been identified since 1978 [12, 13]. However, to date it has no known cellular function. It was originally presumed that ara-CDP-choline was derived from ara-CTP through the catalytic activity of cytidylyltransferase. However, ara-CTP was not utilized as a substrate by cytidylyltransferase whereas CTP and dCTP were catalyzed to CTP- and dCTP-choline (Table 1). The only other pathway available for the synthesis of ara-CDP-choline was through the reverse activity of cholinephosphotransferase. Table 2 shows evidence that ara-CMP is the precursor for ara-CDP-choline. As stated earlier, ara-CDP-choline has no known cellular function at the present time, nevertheless, the other product of this reaction, diglyceride, does have known cellular signaling functions.

Diglyceride formation in ara-C treated cells was observed with two different experimental methods (Fig. 1 and 2). Figure 1 not only shows that diglyceride levels are elevated in ara-C-treated cells, but that ceramide levels are also elevated. The increased diglyceride can activate PKC since increased PKC activity has been observed in ara-C treated cells [24]. The ceramide that is produced following ara-C treatment is a result of neutral sphingomyelinase activity [19]. Figure 2 indicates that the primary diglyceride produced in ara-C-treated cells is of the diacylglycerol subclass. While the alkenylacyl- and alkylacylglycerol subclasses are only formed in small amounts in response to ara-C treatment. These ether-linked diglycerides have been shown to have inhibitory effects on diglyceride-induced PKC activity [38].

Since increased PKC activity has been measured in ara-C treated cells, we were interested in downstream effects of ara-C metabolism. Treatment of L5178Y cells with TPA increased the activity of dCyd kinase, the rate limiting enzyme in ara-C anabolism at early time periods. After 15 min the dCyd kinase activity began to decrease and went to a level 75% of the initial activity by 2 h. This decrease in activity may have been due to competing phosphorylation/dephosphorylation reactions. Nevertheless, these data suggest that PKC activity increases ara-C anabolism at early time points.

Ara-C-induced activation of PKC activity has been observed previously [24]. Downstream effects of this increased PKC activity, appear to be the in vitro phosphorylation and activation of dCyd, reduction in clonogenicity (Fig. 5) and reduced internucleosomal DNA fragmentation (Fig. 6). Because TPA has been shown to be a cellular differentiating agent, it is tempting to assume that the increased cytotoxicity of the ara-C/TPA combination is due to increased differentiation of the treated cells. Clearly, from Fig. 6, it can be seen that the addition of TPA does not increase the degree of DNA fragmentation resulting from ara-C treatment. In fact, TPA inhibits the DNA fragmentation observed with ara-C. Furthermore, inhibition of PKC activity by ET-18-OCH3 in combination with ara-C increases the DNA fragmentation observed with apoptosis (S. P. Whitman, Ph.D. dissertation). We have observed phenomenon similar to that reported here with other deoxycytidine analogs such as 2,2-difluorodeoxycytidine. Therefore generalizations to other deoxycytidine analogs can be made.

In summary, we suggest that ara-C plays a multifunctional role in cytotoxicity related to lipid metabolism. Ara-C induces the formation of two lipid second messengers, diglyceride and ceramide. The diglyceride activates PKC leading to cellular differentiation. Activated PKC can also inhibit apoptosis. The mechanism for this inhibited apoptosis is currently under investigation and is thought to involve Bcl-2 (S. P. Whitman, Ph.D. dissertation). Early activation of PKC

also increases the amount of ara-C metabolites formed due to an increase in the activity of dCyd kinase. Furthermore, inhibition of PKC in conjunction with ara-C treatment results in increased apoptosis as measured by DNA fragmentation. The other second messenger identified in ara-C treated cells is ceramide. This important lipid second messenger is known to stimulate cells to undergo apoptosis as well as affect cellular differentiation. Therefore, in designing clinical strategies for the treatment of AML one must consider the potential interactions of combination chemotherapies. Combination chemotherapies should take advantage of the known signaling pathways in order to optimize therapeutic efficacy.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Cellular Metabolism and T-ALL Specificity of Arabinosylguanine: a Review

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Abstract. A metabolic disorder resulting from deficiency of a purine nucleoside catabolizing enzyme first focused attention on the specific sensitivity of immature T-cells to deoxyguanosine nucleotides. Recognition that T-cell malignancies might have similar sensitivities coupled with the clinical success of other arabinosyl nucleosides subsequently led to the preclinical evaluation of arabinosylguanine for this purpose. Strong correlations between the specificity of arabinosylguanine for immature T-cells was explained by the favorable anabolism of the drug in cell lines and primary human leukemia cells in vitro. Development of a prodrug of ara-G, compound 506U, has now permitted clinical trials. Preliminary reports indicate that the laboratory studies were predictive of the spectrum of clinical activity; a high initial response rate was seen in T-cell malignancies. Pharmacology studies in circulating leukemia cells during therapy demonstrated that the peak accumulation of the active metabolite, arabinosylguanine triphosphate, was strongly correlated with clinical response. Strategies to maximize accumulation of the triphosphate in non-T-cell malignancies are now being designed.

Introduction

In the early 1960s, arabinosyl nucleosides of cytosine [1] and adenine [2] had been shown to have impressive cytotoxicity in bacterial and mammalian cell systems. Hindsight assures that these initial findings were soon to form the basis for the clinical development of arabinosylcytosine, and eventually of fludarabine.

Synthesis of Arabinosylguanine

Synthetic chemistry efforts clearly reflected the expectation that arabinosyl nucleosides would be capable of exerting antimetabolic activity regardless of the nucleic acid base. It was this rationale that stimulated Reist and Goodman [3] to devise procedures for the chemical synthesis of 9-β-D-arabinosylguanine (ara-G). Deoxyguanosine is the natural nucleoside with which ara-G would compete for transport into the cell, for subsequent metabolism, and ultimately for incorporation into DNA (Fig. 1). Subsequently, ara-G has been synthesized enzymatically from two different routes. Pursuing the metabolism and biological activities of a different arabinosyl nucleoside, arabinofuranosyl-2,6,-diaminopurine, Elion et al. [4] discovered that this compound was deaminated by adenosine deaminase to generate ara-G. Inhibition of adenosine deaminase decreased ara-G levels. This same group also demonstrated the feasibility of enzymatic interconversion of arabinosyluracil with purine bases by uridine phosphorylase to produce arabinosylpurine nucleosides such as ara-G [5]. These enzymatic approaches assure the

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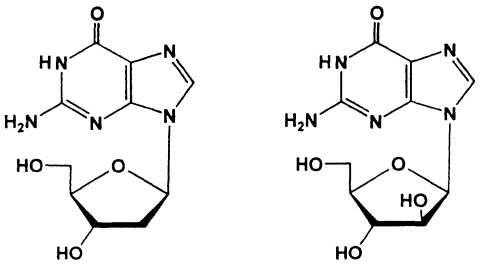


Fig. 1. Structures of deoxyguanosine and arabinosylguanine

exclusion of the α-anomer, which plagues most chemical syntheses.

Biological Activity of ara-G

The first studies of the biological activity of ara-G were reported by Brink and LePage [6], who demonstrated a partial inhibition precursor incorporation into the DNA, but not into the RNA or protein of TA3 tumors borne in the peritoneal cavity of mice that were injected with 0.5 mg of ara-G. There was, however, no evidence of therapeutic efficacy against either L1210 or TA3 ascites tumors when mice were treated i.p. with 10 mg/kg twice a day for 6 days. An interesting sidelight of these studies was the finding in parallel studies that radioactively labelled arabinosyladenine was in part converted to ara-G nucleotides by these tumors. Incubation of ara-G for 3 days with mouse L cells (fibroblasts) or a line of human cells (Detroit 98 cells) in culture indicated it was more toxic than the prodrug arabinosyl-2,6-diaminopurine, but less potent than arabinosyladenine [4]. Thus, the initial in vitro studies and experimental chemotherapy trials generated little enthusiasm for the clinical development of ara-G.

Rationale for T-cell Specificity

A Metabolic Disease Identifies a Target

Clinical interest in guanine nucleoside analogs was subsequently activated with the discovery of genetic diseases and preclinical data which indicated that perturbations of dGTP metabolism and dGTP analogs have specificity for normal T-cell development and T-cell malignancies. Giblett [7] observed that genetic deficiency of purine nucleoside phosphorylase (PNP) results in a profound T-cell lymphopenia, but only minor effects on other organ systems. Pharmacology studies demonstrated that cytotoxicity was associated with elevated plasma deoxyguanosine [8] and the pronounced intracellular accumulation of dGTP in erythrocytes [9]. Models of this metabolic disease demonstrated that immature T lymphocytes and T-lymphoblastoid cells were selectively sensitive to treatment with deoxyguanosine, whereas lymphocytes of B cell lineage did not accumulate high levels of dGTP and were much less sensitive to deoxyguanosine [10-12]. The imbalance of dNTP pools associated with dGTP accumulation was thought to cause toxicity by upsetting the regulation of ribonucleotide reductase and other effects that impair DNA synthesis [13].

Therapeutic Implications

These findings immediately suggested that inhibitors of purine nucleoside phosphorylase might be effective in the treatment of T-cell malignant lymphoproliferative diseases, or to affect specific T-cell functions such as tissue rejection in organ or marrow transplantations. However, at the time, the potential usefulness of known inhibitors was limited by their relatively low potency against the enzyme [14, 15]. As an alternative, the direct use of deoxyguanosine as a therapeutic agent was also limited because of its poor solubility, and because the high levels of PNP present in RBCs would likely cleave the nucleoside [16].

T-cell Specificity of ara-G

These limitations on therapeutic options caused investigators to seek out and evaluate analogs of deoxyguanosine; ara-G was the primary choice. Although earlier studies had suggested that ara-G was cleaved by purine nucleoside phosphorylase in mouse

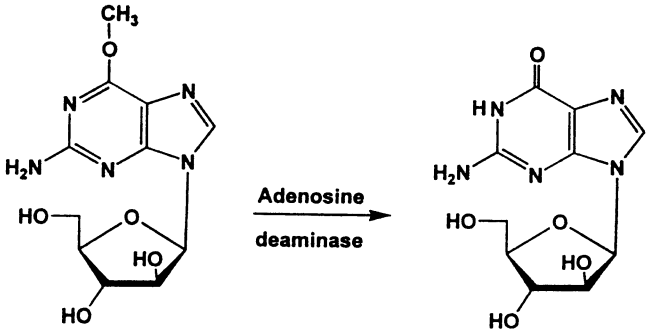
and human RBCs [4], Cohen et al. [17] subsequently indicated that the V_{max} of this reaction was < 1% of that of deoxyguanosine. Several laboratories have demonstrated that it is selectively toxic to T-lymphoblasts relative to B-lymphoblasts or null cells [17-20], suggesting that immature T-lymphoblasts possess biochemical properties which render them highly sensitive to ara-G, and that these properties are not present in mature T-cells. Consistent with this conclusion is the finding that normal T-lymphocytes and leukemic CD4+ and CD8+ cells accumulate lower levels of dATP and dGTP from the respective nucleosides than do cultured T-lymphoblasts [21]. T-cell selectivity may be explained in part by the high specific activity of enzymes which phosphorylate deoxyguanosine or ara-G and the relatively low activity of nucleotideases [17, 20]. The accumulation of ara-G triphosphate (ara-GTP) is greater in T-cell lines than B-cell lines [17, 19]. As with other arabinosyl nucleotides, ara-GTP competes with the corresponding deoxynucleotide (dGTP) for incorporation into DNA [22] and serves as an effective DNA chain terminator when evaluated by primer extension assays [23]. Incubation of freshly isolated leukemia cells from patients with T-acute lymphocytic leukemia, non-T, non-B-cell acute lymphocytic leukemia, AML, and CLL demonstrated a greater ability of T-cell disease to accumulate ara-GTP [24]. Interestingly, cells from the same patients did not differ in their ability to accumulate ara-CTP, and the ability to accumulate ara-GTP was not predictive of ara-CTP accumulation in any cell type. This suggests that factors in addition to or other than phosphorylation, such as nucleotide degradation, may regulate differential accumulation of nucleotide analogs [19, 24].

506U: A Prodrug of ara-G

Despite this compelling evidence for its T-cell specificity and the pressing need for active agents in these diseases, ara-G had not been evaluated in clinical trials, probably due as much to the lack of a pharmaceutical sponsor as the low solubility of the compound. The recent development of the more soluble 6-methoxy-prodrug (2-amino-9-β-D-arabinofuranosyl-6-methoxy-9H-purine, Compound 506U) of ara-G by Burroughs Wellcome Co. has now made such trials possible [25].

Generation of ara-G from 506U

Compound 506U is a poor substrate for direct phosphorylation, which prohibits further anabolism of the parent drug [25]. Rather, ara-G is liberated when adenosine deaminase demethoxylates compound 506U (Fig. 2). The majority of this most likely occurs in the circulation by the relatively high levels of adenosine deaminase in RBCs, although large body organs such as spleen and especially the thymus are known to be rich in this enzyme. Entry of 506U into the RBCs is likely to be facilitated by the equilibrative



2-Amino-6-methoxypurine arabinoside (506U)

Arabinosylguanine (ara-G)

Fig. 2. Demethoxylation of compound 506U by adenosine deaminase to generate ara-G

nucleoside transporter which is inhibited by nitrobenzylthioinosine, the sole nucleoside transport system in these cells. Because RBCs have a relatively low capacity to phosphorylate ara-G, the analog is probably free to exit the cell by as yet undefined mechanisms and to accumulate in the blood. The generation of ara-G in the plasma of monkeys infused with 506U was rapid [25]. The $t_{1/2}$ of 506U was 11 min, reflecting the metabolic conversion to ara-G by adenosine deaminase and a smaller element of renal clearance. In contrast, the elimination half-life of ara-G was 1.7 h.

Cellular Metabolism of ara-G

The generation of ara-G and its cellular metabolism are summarized in Fig. 3. Although no studies have been reported on the mechanisms of cellular permeation of 506U, ara-G is transported into a T-lymphoblastoid cells via at least two systems; the facilitative nucleoside transport system that is inhibited by nitrobenzylthioinosine, and to a lesser extent by a second facilitative transporter that is not sensitive to this inhibitor [26]. Despite the relatively low affinity of these systems, they have a generally high capacity that is not likely limit cellular metabolism of ara-G. Rather, the phosphorylation of ara-G to the corresponding monophosphate appears to be the rate limiting step in triphosphate formation. This phosphorylation is conducted by both high affinity, low specific activity mitochondrial deoxyguanosine kinase [27, 28] and high specific activity, low affinity cytosolic deoxycytidine kinase [29]. The relative importance of each of these enzymes for ara-G phosphorylation in cells remains to be determined. Subsequent phosphorylation steps, first to the diphosphate, presumably by dGMP kinase, and then by nucleoside diphosphate kinase are required to generate the triphosphate ara-GTP. This is the proximal active metabolite which competes with dGTP for incorporation into DNA, after which the analog is resistant to further deoxynucleotide addition in DNA replication and repair reactions [22-25].

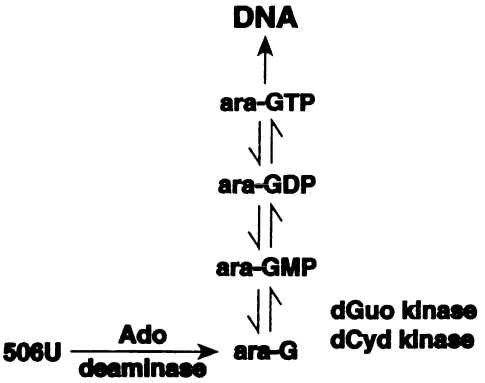


Fig. 3. Pathway for generation and metabolism of ara-G

Clinical Evaluation of Compound 506U

A single phase I multi-institutional clinical trial and pharmacology study of compound 506U has been reported [30-34]. Initial results of the first phase I trial have demonstrated responders, predominantly in the T-cell malignancies [30-32]. Importantly, correlations between clinical response and the ability of tumor cells to accumulate and retain ara-GTP during therapy. Both the responses seen and the laboratory correlates are likely to accelerate accrual and provide further opportunity for investigations of the relationship between response and the cellular pharmacology of ara-G nucleotides.

Phase I Trial of Compound 506U in Hematologic Malignancies

Compound 506U was administered intravenously daily for 5 consecutive days to both adult and pediatric patients. The doses ranged between 10 and 75 mg/kg daily dose. As of the last report [32], 70 patients (45 adults and 25 children) with relapsed or refractory hematologic malignancies had been entered. Responses were scored three weeks after completion of the first course. Of the 25 patients evaluable with T-cell ALL, 11 achieved complete remissions and 8 attained partial responses. One complete remission and seven partial remissions were reported of the 12 patients with T-cell lymphomas entered, and 4 of the 6 patients with

T-CLL achieved partial remissions. The 3 of the 12 patients with B-cell disease attained partial responses, whereas none of the 15 patients with non-lymphocytic disease responded. Thus, the results of the in vitro studies appeared to have been predictive of the responses of the T-cell malignancies.

Plasma and Cellular Pharmacology

During this trial, it was possible to conduct studies of 506U pharmacology in the plasma of many of the patients [34]. A recent report of these investigations indicated a linear proportionality between the 506U dose and the concentration of the drug in plasma at the end of the infusion [34]. Elimination was rapid, with a $t_{1/2}$ of 17 min. This reflected the appearance of ara-G in plasma, which generally reached a peak at the end of the 506U infusion. The elimination kinetics were linear with a similar half-life in children (2.6 h) and adults (3.4 h). No correlations could be found in plasma pharmacokinetics with regard to clinical response.

Cellular Pharmacokinetics of ara-GTP

Pharmacokinetic studies in the circulating leukemia cells of 17 patients demonstrated that the major intracellular metabolite was ara-GTP [34]. Although there was an indication of a dose response (20, 40, and 60 mg/kg), the circulating T-lymphoblasts from patients with T-ALL accumulated significantly greater peak ara-GTP concentrations (median 140 μM, n = 6) compared to other diagnoses (median 50 μM) which included AML, T-CLL, B-ALL, and normal mononuclear cells. Elimination of ara-GTP was generally slow in all diagnoses (median 20 h, range 9 to > 24 h, n = 9), and particularly so in the three T-ALL patients (each was > 24 h). Overall, there was a strong correlation between peak ara-GTP concentrations in leukemia cells and clinical response. Patients who achieved complete (n = 4) or partial remission (n = 2) accumulated significantly greater ara-GTP peak cellular concentrations (median 157 μM) compared to the 8 patients (median 43 μM) who failed to respond ($p = 0.002$). These results indicate that the cellular pharmacokinetics of ara-GTP are related to clinical responses to therapy with compound 506U.

Indications for Future Directions

Because of these findings, strategies to increase ara-GTP levels in leukemia cells of patients treated with compound 506U are being evaluated. A preliminary report has recently Investigated the effectiveness of three feasible clinical trial designs to accomplish this goal [35]. First, incubation of primary human leukemia cells in vitro with 100 to 300 μM ara-G produced a linear increase of ara-GTP accumulation. This response, which is unlike that of either ara-C [36] or gemcitabine [37] which become saturated at 1 to 20 μM, suggests that dosing approaches to achieve greater plasma ara-G such, as increasing the 506U dose rate, may result in more ara-GTP accumulation. Second, the ara-GTP accumulation rate was generally linear for more than 4 ht. This suggests that an extended infusion duration of 506U may maintain plasma ara-G levels that would support continued ara-GTP accumulation. Finally, as indicated above, the rate-limiting step in the accumulation of ara-GTP is phosphorylation by either deoxycytidine kinase or deoxyguanosine kinase. Because the activity of each of these enzymes is regulated in part by deoxynucleotides, a decrease of cellular deoxynucleotides may result in kinase activation, and ultimately greater ara-GTP accumulation. Fludarabine was used to inhibit ribonucleotide reductase and subsequently the deoxynucleotides of the cells were decreased, as has been detailed for combinations with ara-C [38]. Subsequent incubation of the cells with ara-G in a doubling of the rate of ara-GTP accumulation in the leukemia cells of patients with non-T-cell disease. Taken together, these approaches represent new possibilities for achieving greater ara-GTP accumulation in non-T-cell leukemias.

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Acute Leukemias VII
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Modulation of Ara-C Cytotoxicity by Coadministration with Antisignalling Drugs in HL60 and Ara-C-Resistant HL60/Ara-C Cells

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Abstract. Ara-C (cytosine arabinoside) is one of the most effective drugs in the treatment of acute myeloid leukemia (AML). Ara-C is phosphorylated to Ara-CTP (cytosine arabinoside triphosphate) which inhibits DNA polymerase and induces DNA strand breaks after incorporation into DNA. There is increasing evidence that ara-C also affects some cellular signal transduction pathways; it induces transcription/expression of c-*fos*, c-*jun*, NF kappaB, protein kinase C δ, mitogen-activated protein kinase and cyclin E and downregulates cyclin-dependent-kinase-2 and retinoblastoma protein phosphorylation. These events may contribute to ara-C cytotoxicity. The antisignalling drugs all-trans retinoic acid (ATRA), staurosporine, quercetin and bryostatin-1 have recently been shown to enhance ara-C cytotoxicity.

We chose 7 different antisignalling agents [ATRA and the kinase inhibitors quercetin, genistein, CGP 52411, tyrphostin A48, nordihydroguaiaretic acid (NDGA) and staurosporine] and compared their ara-C sensitising potencies with those of hydroxyurea (HU), arabinosyl-2-fluoroadenine (F-Ara-A) and 2-chlorodeoxyadenosine (2-CdA), agents currently in use as "second line treatment" in resistant and relapsed AML. Cytotoxicity was assessed by the tetrazolium (MTT) assay in HL60 cells and a newly derived ara-C-resistant subline (HL60/ara-C) in at least three separate experiments. Supraadditive cytotoxicity was found for combinations containing ATRA, CGP 52411, tyrphostin A48 and HU in both cell lines, for 2-CdA, staurosporine and NDGA in HL60, and for F-Ara-A in HL60/ara-C cells. Quercetin and genistein did not sensitise cells against ara-C.

To elucidate the mechanism of sensitisation in HL60 cells we studied the influence of the modulators of ara-C cytotoxicity on cellular markers of apoptosis. After 4h-coincubation we measured cell size (volume), DNA loss by flow cytometry (sub-G1 peak) and DNA fragmentation by conventional gel electrophoresis. Tyrphostin A48, NDGA, ATRA and HU increased ara-C-induced apoptosis, whereas staurosporine did not affect it. CGP 52411 decreased the effect of ara-C on apoptotic indicators after 4h-, but no longer after 12h-coincubation.

The results suggest that antisignalling drugs such as ATRA, CGP 52411, tyrphostin A48, staurosporine and NDGA may be efficacious alternatives to the already clinically applied ara-C modulators. Among the clinically used ara-C modulators, HU sensitised more potently against ara-C than F-Ara-A and 2-CdA.

Introduction

Ara-C (1-β-D-arabinofuranosylcytosine, cytarabine) plays a central role in the treat-

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ment of leukemias, particularly in the treatment of acute myeloid leukemias (AML). In the cell ara-C is phosphorylated to the toxic metabolite ara-CTP via a sequence of kinases. The first phosphorylation step from ara-C to ara-CMP via the deoxycytidinkinase (dCK) is rate-limiting. Incorporation of ara-CTP into DNA and induction of DNA strand breaks are supposed to be the major mechanisms of ara-C action. Various clinical studies showed a correlation between ara-CTP levels and cytotoxicity resp. ara-CTP retention and clinical outcome [1, 2, 3].

Ara-C resistance is a major cause of AML treatment failure. It can involve each of the steps of the toxification pathway to ara-CTP and might be overcome by combination of ara-C with other drugs [4]. Pretreatment of cells with the ribonucleotide reductase inhibitors hydroxyurea (HU), 2-chlorodeoxyadenosine (2-CdA) and arabinosyl-2-fluoroadenine (F-Ara-A) can increase cellular ara-CTP levels by 200 to 300%. Combinations of ara-C with F-Ara-A and 2-CdA, respectively, are already under phase I and II clinical evaluation [5,6]. However a comparison of ara-C sensitising potencies between ribonucleotide reductase inhibitors under identical conditions has not been published. Other drugs which have been shown to sensitise cells against ara-C are the differentiation inducing agent all-trans retinoic acid (ATRA) [7, 8], quercetin [9], staurosporine [10] and bryostatin-1 [11, 12]. These findings should be interpreted in the light of the observations that ara-C affects a variety of signal transduction pathways such as the transcription factors NFkappa B and AP-1 [13, 14], c-*fos* and c-*jun* oncogene transcription [15] and elements of signal transduction pathways germane to apoptosis, such as MAPKinase [16], PKC [17], cdc2 [18], cyclin E and retinoblastoma protein phosphorylation [19, 20]. The role of these events in the overall mechanisms of ara-C cytotoxicity is unclear. In the light of the fact that ara-C sensitisation by staurosporine was not concomitant with augmented ara-CTP levels [10], staurosporine and perhaps other anti-signalling drugs may modulate ara-C cytotoxicity via cellular changes which are primarily unrelated to DNA synthesis.

We studied the effects of the ribonucleotide reductase inhibitors 2-CdA, F-Ara-A and HU as sensitisers of cells against ara-C and compared their ara-C sensitising potencies with those of ATRA, quercetin and staurosporine. Furthermore we studied the ara-C-sensitising properties of some hitherto untested agents: CGP 52411, tyrphostin A48, genistein and nordihydroguaiaretic acid (NDGA), because they are, like quercetin and staurosporine, protein tyrosine kinase (PTK) inhibitors. CGP 52411 [21] and tyrphostin A48 [22] specifically inhibit the EGF receptor linked PTK (EGFR-PTK), quercetin, genistein [23] and staurosporine [24] are non-specific kinase inhibitors, and NDGA is a specific PDGF receptor linked PTK (PDGFR-PTK) inhibitor [25]. We also explored the possibility that induction of apoptosis might be involved in the mechanism of sensitisation against ara-C in HL60 cells.

Overall the work attempted to compare known and novel agents as ara-C sensitising drugs with the ultimate rationale to increase the drug armamentarium in the therapy of leukemias.

Materials and Methods

Drugs

Ara-C, HU, 2-CdA, F-Ara-A, ATRA, staurosporine, genistein, quercetin were purchased from Sigma Chemical Co. (Dorset, UK), nordihydroguaiaretic acid (NDGA) from Fluka Chemie AG (Buch, CH) and tyrphostin A48 from Calbiochem (Nottingham, UK). CGP 52411 was a kind gift from Ciba-Geigy (Basel, CH). Ara-C, HU, 2-CdA and F-Ara-A were dissolved in phosphate-buffered saline, the other drugs in DMSO resp. ethanol. Stock solutions were stored at -20 °C. Concentrations of DMSO and ethanol did not affect cell growth and viability. Incubates with 2-CdA, ATRA, tyrphostin A48, genistein and quercetin were protected from light.

Cell Lines

HL60 promyelocytic leukemia cells [26] were obtained from the European Collection

of Cell Cultures (Salisbury, UK) and tested negative for mycoplasmal infection. An ara-C resistant HL60 cell line was isolated using a protocol described by Bhalla et al. (1984) [27]; ara-C resistance was 1000-fold in comparison to the maternal HL60 cell line. Cells were maintained in RPMI-1640 medium (with phenol-red) (Gibco, UK) supplemented with 10% heat-inactivated fetal calf serum, glutamine (2 nM) and incubates for the MTT assay with penicillin (100 U/ml) and streptomycin (50 μg/ml). Cultures were grown under 5% CO_2 in a humidified atmosphere at 37 °C.

MTT Assay

The effect of drug combinations on HL60 cell growth and viability was determined using the 3-(4,5 dimethylthiazol-2-yl-)5 diphenyltetrazolium bromide (MTT) assay [28, 29]. Briefly, cells (1×10^6) were seeded in 96 microculture round bottom well plates (Nunc, Naperville, USA) and incubated with drugs for 24 h. Cells were centrifuged (800 *g*, 5min), washed twice in PBS and resuspended in a solution of MTT (5 mg/ml RPMI). After incubation for 4 h, cells were washed (PBS) and the formazan chrystals were dissolved in DMSO. Optical density was measured at 540 nm using a platereader (Labsystems iEMS Reader MF). Experiments were carried out in octuplets at least three times each. Potential chemical interactions between drugs and MTT were outruled in incubates with non-viable cells [30]. G2 arrest as a cause of falsely-high MTT reduction [31] did not play a role, since ara-C cytotoxicity is mainly S phase specific [32] and since the modulators were merely used at non- or low-toxic concentrations. Cytotoxicity by modulators alone did not exceeded 22%. Modulators were tested in combination with two ara-C concentrations in each cell line. Comparing the cytotoxicities of the single drugs with the cytotoxicity of the drug combination the term "sensitisation" refers to significant supraadditive cytotoxicity of the combination treatment ($p < 0.05$).

In HL60 cells we applied the following drug concentrations: ara-C 0.1 and 10 μM, 2-CdA 0.05 μM, F-Ara-A 1 μM, HU 100 μM, ATRA 0.1 to 10 μM, quercetin 50 μM, staurosporine 0.01 μM, CGP 52411 10 μM, tyrphostin A48 50 μM, genistein 10 μM and NDGA 10 μM. Because of less drug sensitivity we increased in HL60/ara-C cells the concentrations of ara-C (100 and 2500 μM), 2-CdA (100 μM), F-Ara-A (100 μM) and genistein (50 μM). The 50% inhibitory concentration (IC_{50}) of ara-C in HL60 cells was 8.7±0.39 μM, in HL60/ara-C cells 6399 ± 211 μM.

Marker of Apoptosis

Gel electrophoresis and DNA fragmentation: After drug exposure 1×10^6 cells/lane were analysed for oligonucleosomal fragmentation on 1.8% agarose gels as previously described [33]. Electrophoresis was at 20 V for 1 h in the presence of 2% SDS and proteinase K (1.25 mg/ml) to ensure cell lysis, and then for 3-4 h at 100 V.

Flow Cytometry. In order to quantitate hypodiploid cells (sub-G1 peak) the DNA histogram was measured essentially as described by Ormerod et al. (1990) [34]. Following drug exposure, cells were centrifuged (10 min, 90 g, 4 °C), washed once in PBS and fixed by exposure to 70% ethanol overnight at 4 °C. Subsequently, cells were washed and resuspended in a solution containing RNaseA (0.05 mg/ml) and propidium iodide (PI, 25 μg/ml). The DNA histogram of the PI-stained nuclei was measured on a FACScan (Becton Dickinson) (486 nm, flow rate 200 cells/s). Clumped cells were excluded by bivariate histogram of the peak and the integrated area of the red fluorescent signal.

Cell Shrinkage. Cell size was analysed with a Casy 1 (Schaerfe system, Reutlingen, Germany) cell counting and sizing system using a 150 μm aperture for routine analysis. Mean cell volume of untreated HL60 cells was 1070 fl.

Statistical Analysis

Differences in cytotoxicity and apoptosis between experimental conditions were eval-

uated using Student's t-test (paired observations).

Results

Modulation of Cytotoxicity

Table 1 shows that the ribonucleotide reductase inhibitors sensitised cells against ara-C. HU was the most potent of these agents; it caused sensitisation in both cell lines. 2-CdA was effective against the low concentration of ara-C in HL60 cells and F-Ara-A against the high concentration of ara-C in HL60/ara-C cells.

Of the antisignalling drugs ATRA, staurosporine, CGP 52411, tyrphostin A48 and NDGA sensitised cells against ara-C. ATRA was efficacious even at 0.1 μM, which is 100-fold below the concentration at which it causes cytotoxicity on its own. ATRA as well as the specific EGFR-PTK inhibitors CGP 52411 (Fig. 1) and tyrphostin A 48 sensitised HL60 and HL60/ara-C cells towards ara-C; staurosporine and NDGA were active merely in HL60 cells. In contrast, quercetin and genistein did not induce ara-C sensitisation in either cell line.

Effect on ara-C-Induced Apoptosis

We investigated if the observed sensitisation of HL60 cells to ara-C by HU, the kinase inhibitors and ATRA was the consequence of increased susceptibility of cells to ara-C-induced apoptosis. Incubation of HL60 cells with ara-C alone for 4 h induced apoptosis as adjudged by occurence of sub-G1 peak in the flow cytogram, decrease in cell size and DNA fragmentation. Table 2 and Fig. 2 show that tyrphostin A48 (50 μM), ATRA (50 μM), NDGA (10 μM) and HU (100 μM) augmented ara-C-induced apoptosis, whereas staurosporine (0.05 μM) and CGP 52411 (10 μM) did not. Paradoxically CGP 52411 inhibited

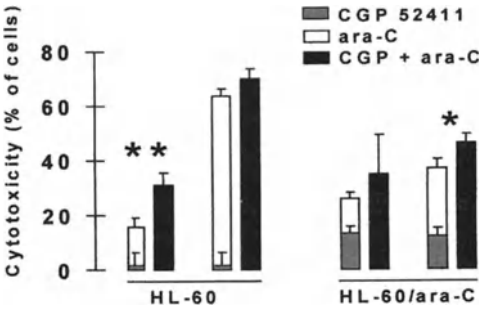

Fig. 1. Modulation of ara-C cytotoxicity by CGP 52411 assessed by the MTT assay in HL60 and HL60/ara-C cells. Of the pairs of bars, the *left one* shows the cytotoxicities of the single drugs and the *right one* the cytotoxicity of the drug combination. In HL60 cells ara-C concentrations of 0.1 and 10 μM, in HL60/ara-C cells of 100 and 2500 μM were used. *Bars* and *error bars* represent the mean±SD of at least 3 independent experiments each conducted in octuplets. *Stars* indicate that the difference between the sum of the single drug cytotoxicities and the cytotoxicity observed for the combination was significant (* $p < 0.05$, ** $p < 0.01$)

Table 1. Modulation of ara-C cytotoxicity by modulators assessed by the MTT assay in HL60 and ara-C resistant HL60/ara-C cells

	HL60		HL60/ara-C	
	ara-C 0.1 μM	ara-C 10 μM	ara-C 100 μM	ara-C 2500 μM
HU	+*	±	±	+**
2-CdA	+**	±	±	-*
F-Ara-A	±	±	±	+*
ATRA	±	+**	+*	+*
CGP 52411	+*	±	±	+*
Tyrphostin A48	+*	±	±	+*
Staurosporine	+*	+*	±	±
NDGA	+*	±	±	±
Quercetin	±	±	±	±
Genistein	±	±	±	±

Cytotoxicity of the drug combination was subadditive (-), additive (±) or supraadditive (+). Stars indicate that the difference between the sum of the single drug cytotoxicities and the cytotoxicity observed for the combination was significant (* $p < 0.05$, ** $p < 0.01$).

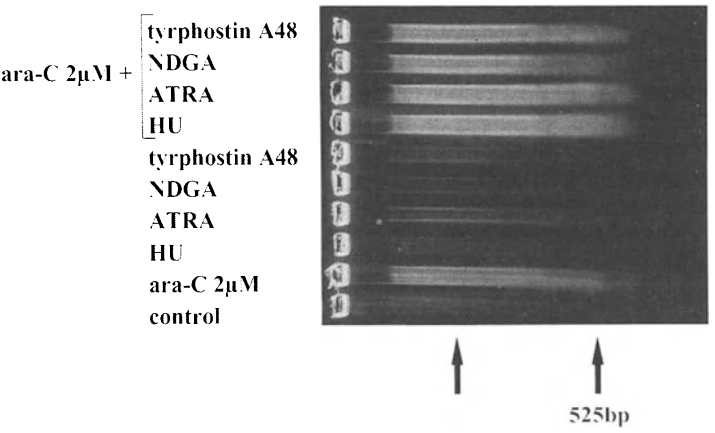

Fig. 2. DNA fragmentation after 4-h exposure of HL60 cells to ara-C alone and to combinations of ara-C with tyrphostin A48, NDGA, ATRA and HU, respectively

Table 2. Modulation of ara-C-induced apoptosis by modifiers of ara-C cytotoxicity in HL60 cells. Incubation time was 4 h

	sub-G1 peak	cell size
CGP 52411	–*	0
Staurosporine	0	0
Tyrphostin A48	+	–
ATRA	+*	–*
NDGA	+*	–*
HU	+**	–**

Markers of enhanced apoptosis are increase of sub-G1 peak (+) and reduction in cell size (–). No change in ara-C-induced markers of apoptosis is characterised by "0", decrease of sub-G1 peak by "–". Stars indicate that the degree of apoptotic changes caused by the combination of ara-C with modifiers was significantly greater than that seen with ara-C alone ($*p < 0.05$, $**p < 0.01$).

ara-C-induced apoptosis after 4-h coincubation, albeit not after 12 h. Induction of apoptosis by etoposide was not inhibited by CGP 52411 (data not shown).

Discussion

Increased ara-C cytotoxicity by combination with other drugs is a major goal in the development of better treatments for AML. We tested a series of ribonucleotide reductase inhibitors and novel antisignalling drugs for their ara-C sensitising potencies.

Comparison of the ara-C sensitising potencies between the ribonucleotide reductase inhibitors characterised HU as a more efficacious modifier of ara-C cytotoxicity than 2-CdA and F-Ara-A with sensitisation against ara-C even in the ara-C-resistant HL60/ara-C cells. Sensitisation against ara-C by HU in ara-C resistant HL60 cells has been described previously [35]. Recently combinations of ara-C with the novel ribonucleotide reductase inhibitors F-Ara-A and 2-CdA, respectively, have entered clinical phase I and II studies [5,6]. In the light of our results and the relatively low rate of toxic side effects of HU [36] its combination with ara-C might be reconsidered in AML treatment.

Our results show that in HL60 and HL60/ara-C cells ribonucleotide reductase inhibitors are not superior to certain antisignalling drugs. Cells were efficiently sensitised against ara-C by the PTK inhibitors CGP 52411, tyrphostin A48, NDGA and staurosporine. It can be suggested that these and perhaps some other antisignalling drugs may be usefully employed as modulators of ara-C cytotoxicity. The specific EGFR-PTK inhibitors CGP 52411 and tyrphostin A48 sensitised cells against ara-C. However, HL60 cells have been shown to lack EGF receptors [37], which renders it unlikely that inhibition of this enzyme is crucial for ara-C sensitisation. It is conceivable that inhibition of PTKs other than EGFR-PTK plays a mechanistic role in sensitisation against ara-C. All of these notions warrant further investigation in vitro and in vivo.

The differentiation inducing agent ATRA sensitised at several therapeutical concentrations [38] against ara-C. This finding is

consistent with the strategy of coadministration of ara-C and ATRA in the treatment of acute promyelocytic leukemia. Early clinical trials on this combination are promising [39, 40]. As described above ATRA increased ara-C-induced apoptosis in HL60 cells which is compatible with recent reports that ATRA affects apoptosis by down-regulation of bcl-2, an inhibitor of apoptosis [41, 42].

The observed modulation of ara-C sensitivity by staurosporine and ATRA is in accordance with previous reports [11, 7, 8], yet the lack of effectiveness of quercetin is inconsistent with results reported by Teofili et al. (1993) [9]. These authors evaluated quercetin-ara-C interaction by the "isobole method" [43] and delineated synergy and potentiation of ara-C cytotoxicity by quercetin. The discrepancy might be the corollary of differences in experimental design and evaluation of results.

Sensitisation of HL60 cells against ara-C by tyrphostin A48, NDGA, ATRA and HU can be partly explained by enhancement of ara-C-induced apoptosis. CGP 52411 exerted a biphasic effect on ara-C-induced apoptosis with inhibition after 4 h, but not after 12 h coincubation. Staurosporine did not affect ara-C-induced apoptosis neither after 4 h nor after 12 h coincubation. This contrasts with previous reports [11, 44] according to which ara-C-induced apoptosis in HL60 cells was augmented after preincubation with staurosporine for 1 h or 3 h and subsequent exposure to ara-C for 6 or 3 h. It is likely that the ability of staurosporine to modulate ara-C-induced apoptosis is subject to subtle experimental differences.

The plethora of mechanisms by which agents modulate ara-C-mediated cytotoxicity remains to be fully unravelled. Combinations of ara-C and antisignalling drugs might present a novel approach to enlarge the therapeutical armamentarium and to improve prognosis in AML.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Cytotoxic Activity and Pharmacology of an Ara-CMP Prodrug in T-leukemic Sublines Resistant to Ara-C

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Abstract. Treatment with Ara-C in patients with acute leukemia may result in development of resistant cell populations. For the study of these cellular resistance mechanisms we have used T-leukemic C8166 sublines resistant to Ara-C, established by incubation with increasing concentrations of Ara-C. C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250} were grown for more than two years in medium containing 5 μM or 250 μM Ara-C, respectively. Cytotoxic measurements showed, that cytotoxicity of Ara-C is strongly diminished in Ara-C resistant cell sublines in comparison to parental cells. Pharmacokinetic studies demonstrated that the ability of resistant cells to accumulate Ara-C to its triphosphate is abolished. Ara-CTP levels in Ara-C resistant sublines, after incubation with a biolabile bis(tBuSATE)Ara-CMP prodrug are higher than in parental cells. However, Ara-CMP prodrug is able to bypass only partly these cellular resistance mechanisms, as demonstrated by cytotoxic assay. These results showed that C8166 cells developed resistance to Ara-C probably due to deoxycytidine kinase deficiency, but other mechanisms may be also involved. C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250} are novel T-leukemic cell sublines which may be valuable models for the investigation of Ara-C resistance.

Introduction

1-β-D-Arabinofuranosylcytosine (Cytarabine, Ara-C) is an effective drug in the treatment against acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) [1]. However, long-term chemotherapy may induce cellular resistance mechanisms [2]. Intracellular phosphorylation of Ara-C to its triphosphate derivative is necessary for the competition of Ara-CTP with the natural dCTP. Both derivatives, dCTP and Ara-CTP, compete for the binding on DNA-polymerase and incorporation into cellular DNA [3], which induces inhibition of DNA-polymerase or leads to the inhibition of DNA-strang elongation. It has been shown, that this process correlates with cytotoxicity in vitro [4]. Different cellular resistance mechanisms have been proposed to be responsible for the diminished efficacy of Ara-C in vitro and in vivo. For example activity of deoxycytidine kinase (dCK), which phosphorylates Ara-C to Ara-CMP could be supressed [5, 6]. Furthermore increased activation of cytidine deam-

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inase or decreased uptake of the substance may induce a diminished intracellular Ara-CTP level which results in a diminished antileukemic efficacy of Ara-C [7].

The use of monophosphate prodrugs constitutes an alternative strategy for overcoming cellular resistance, which is due to a diminished intracellular monophosphorylation of the parent nucleoside analogs. These substances were able to bypass cellular nucleoside kinase deficiency in different cell culture systems [8]. In this regard, bis(SATE)phosphotriester derivatives of nucleoside analogs, which represent lipophilic nucleoside analog monophosphates with neutral biolabile substituents, have been developed recently [9, 10, 11, 12].

In this study we observed cellular resistance mechanisms which can be induced in the T-leukemic C8166 cell line by long-term Ara-C exposure. Two different Ara-C resistant sublines of C8166 parental cell line with a different grade of resistance against Ara-C have been established (C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250}). Cytotoxicity of Ara-C in resistant cells was compared with that of bis(tBuSATE)Ara-CMP, an Ara-CMP prodrug, which was constructed for the intracellular delivery of Ara-CMP. For investigating the ability of Ara-CMP prodrug to overcome cellular resistance mechanisms we measured the accumulation of Ara-CTP in sensitive and resistant cell lines after incubation with bis(tBuSATE)Ara-CMP.

Material and Methods

Selection of Ara-C-Resistant Cell Lines

For the selection of Ara-C-resistant cell sublines, C8166 (MRC, AIDS Reagent Project, Hertfordshire) parental cell line was exposed to increasing concentrations of Ara-C over a period of 6-17 months. The cells grow now for more than 2 years in medium with 5 μM or 250 μM Ara-C, respectively. The resistant sublines which were used in this study were designated as C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250}. Both drug-sensitive and resistant cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS.

Antitumoral agents

Ara-C and Ara-CTP was obtained from Sigma (Deisenhofen, Germany). The synthesis of bis(tBuSATE)Ara-CMP, a bis(*S*-acyl-2-thioethyl)phosphotriester derivative of Ara-C (Fig.1) was carried out according to an already published general procedure [10] and will be reported elsewhere. This compound was characterized on the basis of its physical and spectroscopic properties. Its purity was ascertained by high-pressure liquid chromatography. Stock solutions of the drugs were prepared by dissolving in dimethyl sulfoxide at concentration of 250 mM for Ara-C and Ara-CTP and 10 mM for bis(tBuSATE)Ara-CMP. Solutions were stored at room temperature.

Cytotoxicity Assay

To determine the inhibitory effect of Ara-C and bis(tBuSATE)Ara-CMP in C8166, C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250} cells, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) method [13] was used. The procedure was performed as described previously [14]. Briefly, cells were

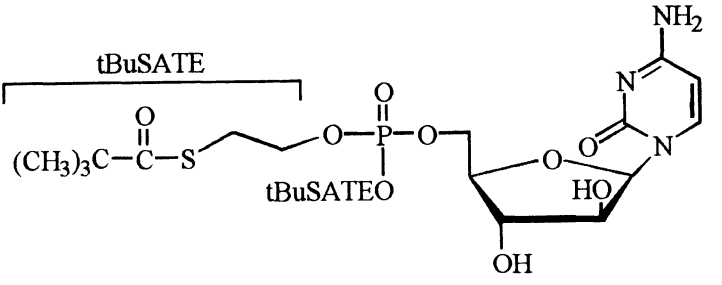

Fig.1. Structure of the studied bis(SATE)phosphotriester derivative of Ara-C

seeded in a 96-microtitre plate, at a concentration of 105 cells per ml IMDM-medium supplemented with 10% heat inactivated FBS. Substances in different concentrations were added and the cells were incubated 5 days (37 °C, 5% CO_2). The viability of the cells was measured by adding MTT solution to each well and determining the absorbance by using a multiwell ELISA reader. The cytotoxic activity of the drugs were expressed as CC_{50}, representing a concentration reducing the cell viability by 50%.

Cell Extraction

Parental and Ara-C resistant cells (10^6/ml) were incubated 4 h with or without 10 μM Ara-C or bis(tBuSATE)Ara-CMP. We used 10 μM Ara-C for the pharmacokinetic measurements, because the formation of Ara-CTP is saturated at this concentration [15]. The plateau of Ara-CTP accumulation differs in different cell lines but after 4 h incubation with Ara-C, the Ara-CTP level remains to be constant up to 11 h [15]. After incubation cells were washed for two times with cold PBS. Cell viability and cell counts were determined with the trypan blue exclusion method. Cells were extracted by adding 300 μl of HPLC-buffer (pH 2.35) and centrifuged at 1000 g for 10 min at 4 °C [15].

Ion Pair Chromatography

Ara-CTP levels were measured as described previously [15]. Aliquots of the nucleotide extracts were injected into a Milton Roy liquid chromatograph equipped with the automatically Waters injector Wisp 712. The nucleotides were analyzed by reversed-phase HPLC on a ODS2 C-18 column (4.6 × 25 cm, 5 μM) with isocratic elution of 0.1 M KH_2PO_4, 5 mM tetrabutylammonium phosphate and 0.5% acetonitrile (pH 2.6, flow rate 0.8 ml/min during 18 min, 1.8 ml/min 18-25 min) and detected by UV-spectroscopy (280 nm). The retention times for the nucleoside triphosphates were: CTP 12 min, Ara-CTP 14 min, dCTP 16 min, ATP 21 min. The amounts of Ara-CTP were measured with the external standard method. A computer evaluation program (727 software, LDC) was used to compare the integrated peak areas of the standards with those of the samples in order to calculate the concentrations in the samples.

Results

The cytotoxicity of Ara-C and bis(tBuSATE)Ara-CMP in C8166 and Ara-C resistant cell sublines is shown in Table 1. Both C8166^{r}Ara-C^5 and C8166^{r}Ara-C^{250} cells were resistant to cytotoxic effects of Ara-C. CC_{50} values were about 10^4-fold higher for C8166^{r}Ara-C^5 cells and at least 10^5-fold higher for C8166^{r}Ara-C^{250} cells than for parental cells (Table 1).

It was shown that different bis(SATE)-phosphotriester derivatives were able to bypass the first enzymatic catalyzed phosphorylating step [9, 10, 11, 12, 16]. In our Ara-C resistant cell systems, the bis(tBuSATE)Ara-CMP was able to overcome partly the resistance to Ara-C. Interestingly, cytotoxicity of Ara-CMP prodrug depends on the degree of Ara-C resistance. Cytotoxic effects of this Ara-CMP prodrug is lower in the cell subline resistant against greater concentration of Ara-C, than for the cell subline resistant against lower Ara-C concentration.

Pharmacokinetic studies of Ara-CTP level in parental and Ara-C resistant cell sublines

Table 1. Cytotoxic effects of Ara-C and bis(tBuSATE)phosphotriester derivative of Ara-C in parental and Ara-C resistant cells

Drug	CC_{50} [μM]		
	C8166	C8166^{r}Ara-C^5	C8166^{r}Ara-C^{250}
Ara-C	0.04 + 0.002[a]	367.7 + 18.2	> 2000
bis(tBuSATE)AraCMP	0.3 + 0.05	5.2 + 0.3	22.5 + 1.3

[a] Values represent mean value + SD from at least five independent experiments.

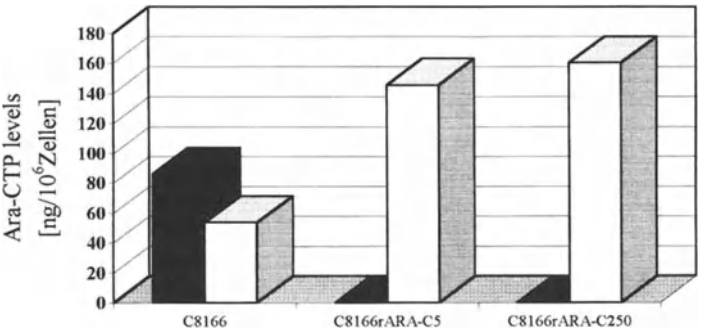

Fig. 2. Ara-CTP levels in parental and Ara-C resistant cell sublines after incubation with 10 μM Ara-C (■) or bis(tBuSATE)Ara-CMP (□). Values are means from three independent experiments. Standard deviation did not exceed 15%

demonstrated that the SATE Ara-CMP prodrug bypasses the dCK-dependent enzymatic pathway (Fig. 2). Ara-CTP was not measurable in C8166^{r}Ara-C^{5} or C8166^{r}Ara-C^{250} resistant cell sublines after incubation with 10 μM Ara-C. Contrasting this, incubation of the resistant cells with bis(tBuSATE)Ara-CMP resulted in accumulation of great amounts of Ara-CTP (C8166^{r}Ara-C^{5}: 145.4 ng Ara-CTP/10^{6}cells; C8166^{r}Ara-C^{250}: 160.2 ng Ara-CTP/10^{6} cells). Surprisingly, the SATE monophosphate prodrug was converted to Ara-CTP in Ara-C resistant cells up to two fold greater extent than in parental cells (Fig. 2).

Discussion

In the present study we showed that bis(tBuSATE)Ara-CMP, an Ara-CMP prodrug, is able to overcome partly the Ara-C resistance mechanisms in C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250} cells. Therefore, our results lead to the assumption that the resistance mechanisms is due to a deficiency of deoxycytidine kinase activity and/or increased activity of deoxycytidine deaminase. These mechanisms were already described in different cell lines and leukemic cells in patients, who were treated with Ara-C [17, 18].

Although high amounts of Ara-CTP accumulated in the resistant cells incubated with bis(tBuSATE)Ara-CMP, the drug only partially overcome resistance mechanisms. These results suggest that cellular mechanisms other than altered deoxycytidine kinase or deoxycytidine deaminase activity may contribute to Ara-C resistance in the Ara-C resistant cell sublines. For example, an increase in activation of unspecific phosphorylases in the resistant cell sublines may occur [19]. An other explanation for this phenomenon may be a decreased Ara-CTP incorporation into the cellular DNA in Ara-C resistant cells [20, 21]. Despite different sensitivity to toxic effects of Ara-C, both resistant cell sublines formed similar amounts of Ara-CTP when incubated with bis(tBuSATE)Ara-CMP. This showed that different resistance mechanisms to Ara-C may develop in resistant cells depending on the grade of resistance.

In conclusion C8166^{r}Ara-C^{5} and C8166^{r}Ara-C^{250} cell lines provide a good instrument to study the mechanisms of cellular resistance to Ara-C as well as to test treatment strategies to overcome resistance mechanisms.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Retinoids Modulate Ara-CTP Pharmacology in the HL-60 Acute Leukemia Cell Line

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Abstract

Purpose. To determine the ara-C-modulating efficacy of retinoids in leukemic blast cells.

All-*trans* retinoic acid (ATRA) has been shown to increase the sensitivity of AML blast cells to cytosine-arabinoside (ara-C). Ara-CTP accumulation in leukemic blast cells is considered to be the main determinant of ara-C toxicity in vitro and in vivo. To test the hypothesis that retinoids exert their ara-C sensitizing effect by enhancing the intracellular ara-CTP accumulation we studied the effect of the retinoids ATRA and 13-*cis* retinoic acid (13-*cis* RA) on the intracellular ara-CTP-formation of HL60 cells in vitro after 24 and 72 hours of preincubation in retinoid containing medium and 1 hour exposure to ara-C.

Results. Cells pretreated with 0.01-10 μM ATRA and 13-*cis* RA equally exhibited a significantly decreased amount of intracellular ara-CTP in a preincubation time-dependent manner. The same pretreatment did not show any effect on ara-C metabolism of K562 cells. Cytotoxicity testing by MTT assay confirmed the synergism of ATRA and ara-C in HL60 cells. These observations are in contrast to results from our studies with other known ara-C-modulating agents such as fludarabine (F-ara-A) and hydroxyurea which effected a parallel increase in ara-C-cytotoxicity and intracellular ara-CTP-formation. The mechanisms by which retinoids exert their inhibiting effect on ara-CTP-formation remain unclear. The differentiation-inducing properties of retinoids might be responsible for changes in ara-C-uptake and phosphorylation.

Conclusion. Retinoids decrease the rate of ara-CTP accumulation in HL60 cells. However, this effect is not mediating resistance to ara-C. In contrast, considerable synergism with ara-C could be demonstrated. Thus, ara-C and ATRA seem to interact by other mechanisms than those identified for other known ara-C modulators. Intracellular ara-CTP-concentration can not exclusively be considered as the main determinant of ara-C toxicity in vitro.

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Introduction

Retinoic acids (RA) are vitamin A derivatives which play an important role in embryogenesis and differentiation of normal tissues. Experimental data indicate that RA also induce cellular differentiation in several hematological precursor cell lines and in blast cells from patients with acute promyelocytic leukemia (APL) [1, 2, 3]. Today, RA are applied in the treatment of adult and

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pediatric APL [4]. In contrast to other anticancer agents, the RA isomer ATRA induces remission in patients with APL without causing bone marrow hypoplasia. Another isomer 13-*cis*-retinoic acid (13-*cis* RA) has successfully been used in the treatment of myelodysplastic syndrome (MDS) [5].

Recently RA has been shown to sensitize AML blasts to 1-ß-D-arabinofuranosylcytosine (ara-C), one of the most effective agents in pediatric and adult AML treatment [6, 7]. According to preliminary clinical results regimens containing ATRA and ara-C induced remission in AML subtypes other than APL as well [8, 9]. The mechanism by which RA affect ara-C-mediated cytotoxicity is not understood yet.

Strategies to optimize ara-C treatment by coadministration of ara-C modulators, such as fludarabine, hydroxyurea and cladribine, have focussed on the enhancement of intracellular accumulation of the active metabolite ara-CTP which is supposed to correlate with ara-C cytotoxicity [10-17]. In order to further clarify the mechanisms of interaction between RA and ara-C we investigated the effect of the retinoids ATRA and 13-*cis* RA on cellular ara-C phosphorylation in the RA-responsive acute myelogenous HL-60 cell line.

Methods

Drugs

Ara-C, all-*trans*-retinoic acid, 13-*cis*-retinoic acid, natural nucleotides and ara-CTP were obtained from Sigma Chemical Co (St Louis, MO). All other chemicals were of the highest purity available.

Stock solutions were prepared in steril water (ara-C) and 100% ethanol (retinoic acids), respectively. Stocks were aliquoted and stored at -20 °C. Directly before use, they were diluted into RPMI medium to achieve the desired concentrations. The final concentration of ethanol in the culture was shown not to affect cell growth. All the procedures involving retinoic acids were performed in subdued light; retinoic acid containing tubes and tissue culture flasks were light-protected by a cover of aluminium foil.

Cell Line

The human acute myeloid leukemic cell line HL-60 [18, 19], and the K-562 chronic erythroleukemia cell line [20] were obtained from the European Collection of Cell Cultures, Salisbury, UK, mycoplasma-screened and used throughout the study.

The cells were maintained in suspension culture at exponential growth phase in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY), supplemented with 10% heat-inactivated fetal calf serum (GIBCO), under standard conditions (37 °C, humidified atmosphere, 5% CO_2). Cells were replaced by cells from the initial charge every six weeks to prevent the effect of spontaneous HL-60 cell differentiation on our experiments. Proliferation of cells was determined using a hemocytometer after staining with trypan blue dye exclusion.

Cellular Pharmacology

Exponentially growing cells (0.3-0.5 $\times$ 10^6/ml) were incubated with various concentrations of ATRA or 13-*cis* RA for a defined preincubation time under the above described conditions. Cells were washed and resuspended in RPMI 1640 medium (+10% FCS), followed by incubation in ara-C containing medium in a shaking water bath at 37 °C. The incubation time and the final ara-C concentration were initially determined to be optimal to achieve an ara-CTP accumulation below the saturation level in K-562 and HL-60 cells. The cells were then washed twice in ice-cold phosphate-buffered saline (pH 7.2) and counted immediately prior to extraction.

Nucleotides were extracted using tetrabutyl ammonium hydrogen sulfate as described elsewhere [21]. Ara-CTP was separated from other nucleotide triphosphates by an isocratic ion pair high-performance liquid chromatography (HPLC) method using a reversed phase C_{18} column (NOVA-PAK, Waters-FRG) and 0.09 M phosphate buffer at pH 6 containing 0.35% tetrahydrofuran and 0.01 M tetrabutyl ammonium hydrogen phosphate [21]. Quantification was done by UV detection at 270 nm; the limit of detec-

tion was 25 ng/ml ara-CTP. Anthranilic acid was used as an internal standard. Ara-CTP levels were expressed as picogram (pg) ara-CTP per 10^7 cells [21].

Chemosensitivity Testing

The effect of drug combinations on HL 60 cell growth and viability was determined using the 3-(4,5 dimethylthiazol-2-yl-)5 diphenyltetrazolium bromide (MTT) assay [22]. Briefly, HL60 cells (10^6) were seeded in 96 multi-well plates (Nunc, Naperville, USA) and incubated with drugs for 24 h. Cells were centrifuged (800 *g*, 5min), washed twice in PBS and resuspended in a solution of MTT (5 mg/ml RPMI). After incubation for 4 h, cells were washed (PBS) and the formazan crystals were dissolved in DMSO. Optical density was measured at 540 nM using a platereader (Labsystems iEMS Reader MF). Experiments in octuplets were carried out three or more times. Potential chemical interactions between drugs and MTT were checked in incubates with non-viable cells [22].

In HL60 cells, the 50% inhibitory concentration (IC_{50}) for ara-C was 5-10 μM, for ATRA 10-100 μM.

Statistical Analysis

Intracellular ara-CTP-concentrations and proliferation kinetics with and without preincubation with retinoic acids were compared using the Kruskal-Wallis One Way Analysis of Variance on Ranks. To isolate the groups which differed from the others we used the Dunn's All Pairwise Multiple Comparison Procedure. Differences in cytotoxicity between single and combined drugs were evaluated using the Student paired t-test (paired observations).

Results

Ara-CTP Accumulation in HL-60 and in K-562 Cells

The mean intracellular ara-CTP concentration after incubation with 10 μM ara-C for 1 h was 387.71 ± 110.99 pM/10^7 cells for HL-60 cells (n = 60) and 292.74 ± 100.38 pM/10^7 cells for K-562 (n = 17). Following incubation with increasing ara-C concentrations for 1 hour, both cell lines showed a similar pattern of ara-CTP accumulation characterized by a maximum at ara-C 10 μM. Varying incubation times in the presence of 10 μM ara-C (30 min to 24 h), maximum intracellular ara-CTP levels were achieved after 2 h in both cell lines.

Effect of Retinoids on Cellular Accumulation of ara-CTP in HL-60 Cells

HL-60 cells were preincubated with ATRA for 24 or 72 h and then uniformly exposed to 10 μM ara-C for 1 h. Following 24 h of preincubation with 1 μM ATRA, the intracellular ara-CTP accumulation in HL-60 cells decreased to median values of 63±12% ($p < 0.05$) compared to unpretreated controls. Lower ATRA concentrations of 0.1 μM and 0.01 μM affected a minor decrease of ara-CTP levels to 73±11% and 76±10%, respectively ($p < 0.05$). Longer preincubation with ATRA 1 μM for 72 h further decreased ara-CTP to 41±4% (Fig. 1). Similarly, a highly significant decrease in ara-CTP accumulation to 62±8% after 24 h and 38±8% after 72 h was achieved by using the ATRA isomere 13-*cis* RA at a concentration of 1 μM (Fig. 1).

Effect of Retinoic Acids on Cellular Accumulation of ara-CTP in K-562 Cells

In K-562 cells, which are known not to undergo RA-induced differentiation, preincubation with neither of the isomers ATRA and 13-cis RA resulted in significant decreases of ara-CTP accumulation (Fig. 2). After 72 h of preincubation we even found a slight increase which, however, was not statistically significant.

Effect of Retinoic Acids on ara-C cytotoxicity in HL-60 Cells (Fig.3)

Coincubation of ATRA (0.1, 1, 10 μM) with ara-C (0.1, 10 μM) resulted in supraadditive

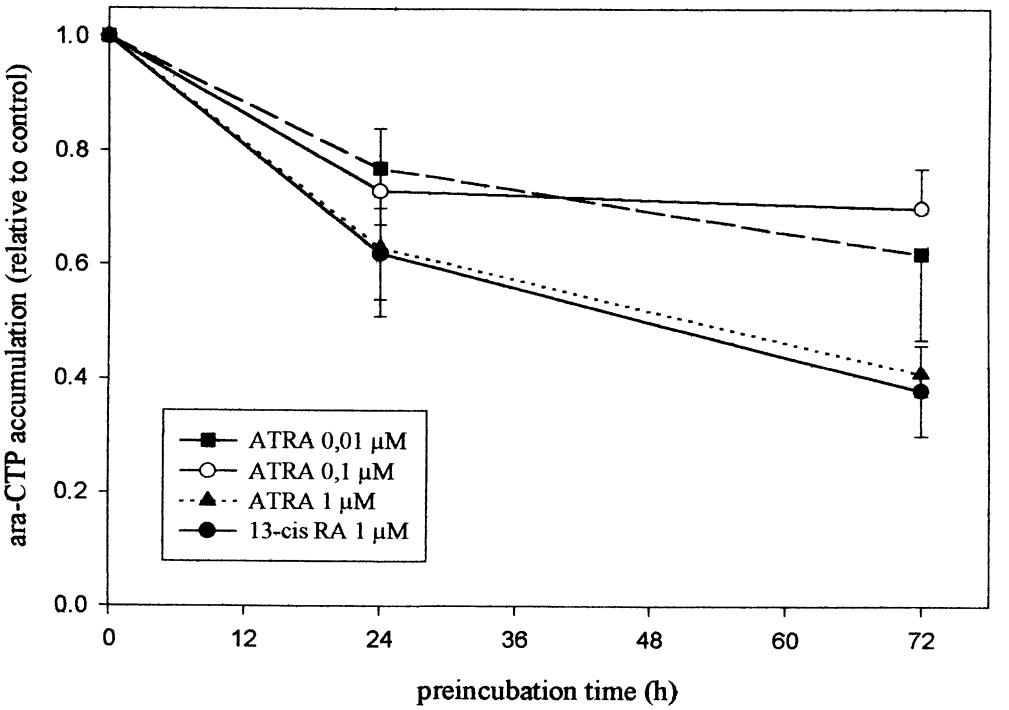

Fig. 1. Effect of 24 and 72 h preincubation with ATRA on ara-CTP accumulation in HL-60 cells as compared to unpretreated controls. Each point represents 2-3 independent experiments in triplets. Standard deviation indicated by error bars never exceeded 20% of mean. Decreases of ara-CTP values were significant after 24 h-preincubation with ATRA 0.1 and 1 µM and after 72 h-preincubation with ATRA 0.01 and 1 µM

increases of cytotoxicity. e.g. while ara-C 10 µM arrested cell growth by 52%, the combination with ATRA 0.1 µM (no cytotoxicity itself) reduced viability by 72%. This decrease in cell viability was highly significant ($p < 0.01$). Plasma peak levels under ATRA treatment are around 1 µM. So therapeutical ATRA concentrations are capable of sensitizing against ara-C in HL60 cells.

Discussion

Preincubation with the retinoids ATRA and 13-*cis* RA at therapeutical concentrations [33] significantly decreased cellular ara-CTP accumulation in HL-60 cells. However, ara-C-induced cytotoxicity was significantly enhanced, as it has already been described for various AML cell lines and fresh leukemic blast cells [6,7].

The finding of RA-induced ara-CTP decrease in spite of increased cytotoxicity is in contrast to a number of studies which consistently showed a positive correlation between accumulation and retention of ara-CTP and cell kill in vitro and in vivo [23-31]. The ribonucleotide reductase inhibitors fludarabine, hydroxyurea and 2-chlorodeoxyadenosine increase cytotoxicity and ara-CTP levels by uncoupling the feedback inhibition of deoxycytidinetriphosphate (dCTP) on the rate-limiting enzyme of ara-C phosphorylation, deoxycytidine kinase, mainly by reduction of cellular dCTP-pools [10]. Preincubation with these ara-C modifiers resulted in 2-4-fold ara-CTP concentrations in HL-60 cells [32] and other myeloid leukemia cell lines [10-17], including the K-562 cell line. K-562 cells have been shown to be unresponsive to the differentiation-inducing and growth-inhibiting effects of RA [1]. We found that ara-CTP levels were not decreased by preincubation with ATRA in K-562 cells. So, the RA-induced downregulation of ara-

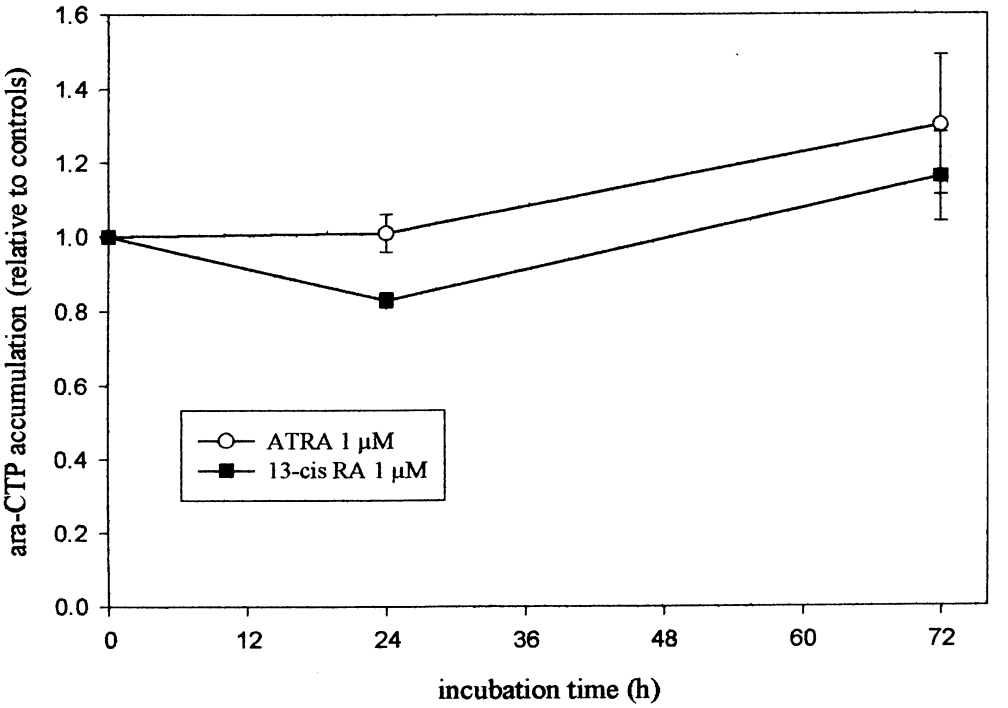

Fig. 2. Effect of preincubation with ATRA 1 μM and 13-*cis* RA 1 mM on ara-CTP accumulation in K-562 cells as compared to unpretreated controls. Each *point* represents 2-3 independent experiments in triplets. Standard deviations indicated by *error bars* never exceeded 20% of mean. There were no statistical significant deviations from control

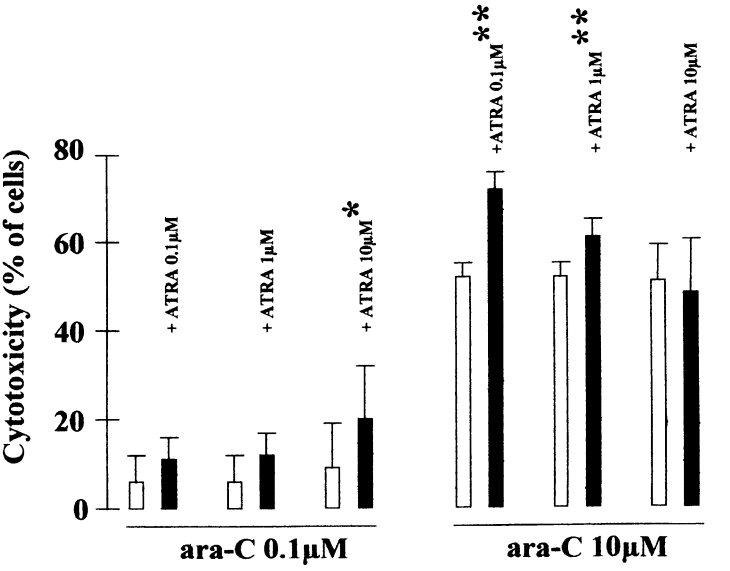

Fig. 3. Modulation of ara-C cytotoxicity by combination with ATRA (0.1, 1, 10 μM) in HL60 cells assessed with the MTT assay. The *bars* are presented in pairs. The *left bars* show the cytotoxicity of ara-C alone, the *right bars* the cytotoxicity of ara-C with ATRA. ATRA alone caused no cytotoxicity. *Bars* and *error bars* represent the mean and standard deviation of at least 3 independent experiments in octuplets. *Stars* indicate significant difference to control (* $p < 0.05$, ** $p < 0.01$)

CTP accumulation might be a specific phenomenon of cells responsive to RA, such as HL60 cells.

The two retinoic acid isomers ATRA and 13-cis RA, which differ in their receptor binding affinity profile, displayed comparable effects on cell kill and ara-CTP levels. In vitro studies comparing the differentiation inducing potencies of the isomers have not been unanimous [1,34,35]. Considering that in vitro studies showed spontaneous nonenzymatic isomerization resulting in an isomer equilibrium independent of the initial isomer, isomer concentration and cell line used [36] in vitro comparisons between different RA isomers should be regarded with precaution.

The mechanism of ara-CTP decrease by RA in leukemic blast cells has not been elucidated yet. It might well be that ATRA modulates the amount of intracellular nucleotides and the expression of proteins involved in the phosphorylation of ara-C via interaction with retinoic acid receptor (RAR, RXR) transcription factors [33].

The way of ara-C sensitization by retinoids seems to be independent of ara-CTP levels. This indicates that incorporation of ara-CTP into DNA followed by termination of chain elongation is not the only mechanism of ara-C mediated cell kill. Apoptosis-related events have been described to be involved as well [38, 40, 41]. Hu et al. proposed that downregulation of the proto-oncogene bcl-2, which is known to prolong cell survival by suppressing the onset of apoptosis [37], may in part be responsible for the ara-C-sensitization by RA in vitro [38, 39].

In summary we found that sensitization against ara-C cytotoxicity by RA is independent of ara-CTP enhancement. This demonstrates that intracellular ara-CTP-concentrations can not always be applied as indicators of cytotoxicity. Furthermore, in the light of the low myelotoxicity profile of ATRA its combination with ara-C should be further exploited in the treatment of AML.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Induction of Apoptosis in Vitro by ARA-C, VP-16, MITOX, DNR, IDA and FLU in Myeloid Leukemic Cells

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Abstract. A number of anticancer drugs such as Cytosine Arabinoside (ARA-C), Etoposide (VP-16), Mitoxantrone (MITOX), Daunorubicin (DNR), Cisplatin and Amsacrine have been shown to induce programmed cell death (apoptosis) in leukemic cells. Apoptosis is morphologically characterized by early cell shrinkage, chromatin condensation, nuclear fragmentation, cell surface blebbing and membrane-bound apoptotic bodies. We investigated the in vitro induction of apoptosis by ARA-C, FLU (Fludarabine), VP-16, MITOX, DNR and IDA (Idarubicin) in the blast cells of 23 adult patients with untreated acute myeloid leukemia (2 M0, 2 M1, 10 M2, 2 M4, 4 M5, 2 M6 and 1 M7). Mononuclear cells from the bone marrow and/or peripheral blood were isolated. Cell cultures were exposed to different concentrations of ARA-C, VP-16, FLU, IDA, DNR and MITOX at 37 °C for 24 h. Samples were analyzed using DNA gel electrophoresis and flow-cytometry after staining with propidium iodide. We also examined the morphology of 200 leukemic cells under light microscopy. The three methods gave comparable results. The most active drug in inducing internucleosomal fragmentation of DNA was FLU (82%); VP-16 and ARA-C induced apoptosis in a smaller number of cases (64 and 55%, respectively). MITOX, DNR and IDA- treated cells did not show fragmentation of DNA. Our results confirm the ability of some drugs to induce apoptosis in vitro in leukemic cells and demonstrate a good correlation between the detection of apoptosis by DNA electrophoresis, flow cytometry and light microscopy.

Introduction

Apoptosis or programmed cell death (PCD) is an active energy-dependent process of cellular self-destruction. Apoptosis is morphologically characterized by early cell shrinkage, chromatin condensation, nuclear fragmentation, cell surface blebbing and membrane-bound apoptotic bodies; in contrast, necrosis is characterized by cell swelling, rupture of the plasma membrane and leakage of the cellular contents into the extracellular environment [1]. These features enable us to detect apoptotic cells on an MGG stained cytospin under light microscopy.

Biochemical studies of nuclear damage have shown that the cellular DNA is cleaved into multimers of about 180-200 base pairs which may be visualized as a distinct ladder of bands following agarose gel electrophoresis [2].

Furthermore, apoptotic cells can be identified in flow cytometry by their diminished stainability with several DNA-specific fluorochromes due to degradation and subsequent leakage of DNA from the cell; apoptotic cells appear as an apparently hypodiploid population [3].

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Since apoptosis is an active process, it can also be modulated and its modulation could provide new tools in the treatment of malignant disease [4]. Recently, some authors have reported that Daunorubicin, Mitoxantrone, Doxorubicin and Cytosine-Arabinoside may induce apoptosis in murine leukemic cells, but whether this cell death pathway is present in fresh human AML cells has not yet been established [5-8]. Vials et al. found that the flow-cytometric method for apoptotic cell detection can be used to evaluate the in vitro effect of drugs on the U937 cell line and suggest that it could be adapted as a predictive assay of the response of blast cells to chemotherapy [9]. The correlation found between in vitro induction of apoptosis by chemotherapeutic drugs and clinical response of patients with leukemia implies that the former factor may be useful to identify tumor cell sensitivity or resistance to particular drugs [10].

The purpose of the present study was to determine whether Cytosine-Arabinoside (ARA-C), Etoposide (VP-16), Idarubicin (IDA), Daunorubicin (DNR), Mitoxantrone (MITOX) and Fludarabine (FLU) can induce apoptosis in human myeloid leukemic cells and to compare the three methods used for the detection of apoptotic cells.

Materials and Methods

Patients and Cell Preparation

Samples of peripheral blood and/or bone marrow were obtained from 23 patients admitted to our institution and undergoing chemotherapy for adult acute myeloid leukemia. Subtypes of AML were defined according to the FAB classification (2 M0, 2 M1, 10 M2, 2 M4, 4 M5, 2 M6, 1 M7).

The mononuclear cells were isolated on a Ficoll density gradient, washed twice and resuspended in Iscove's Modified Dulbecco Medium containing 20% heat inactivated fetal calf serum, 1.250 µl/ml gentamycin and 40.3 µl/ml of a 7.5% sodium bicarbonate solution. Viability was determined by the Trypan-Blue exclusion test and stained smears indicated that there were more than 90% leukemic cells in all cases.

Drug Exposure

The drugs tested were ARA-C, MITOX, DNR, IDA, VP-16. Drug concentrations were 10 mM for DNR, IDA and MITOX, 100 mM for VP-16 and FLU, 1000 mM for ARA-C. Drugs were added into 25 cm^2 culture flasks containing 10 ml of cell suspension (1×10^6 cells/ml) and the cultures were incubated in a humidified atmosphere for 24 h at 37 °C in 5% CO_2.

Light Microscopy

Cytocentrifuge preparations from treated and control suspensions were fixed and stained with May-Grunwald Giemsa. Cells were examined under oil immersion light microscopy and apoptotic cells were defined as cells containing one or more darkly stained pycnotic nuclei.

DNA Gel Electrophoresis

Cells were washed twice in PBS and disrupted by suspension for 20 min at 4 °C in Tris-HCl (5 mM) buffer containing 0.5% Triton X-100 and EDTA (20 mM). The cellular lysates were centrifuged at 40 000 rpm for 20 min to separate low molecular weight DNA. DNA was extracted from the supernatant by treatment with phenol/chloroform/isoamyl alcohol (25:24:1). Sodium acetate and absolute ethanol were added to the upper aqueous layer at 4 °C to precipitate the DNA. The mixture was vortexed and incubated for 60 min at -70 °C. After centrifugation for 30 minutes at 10 000 rpm the pellet was resuspended in Tris EDTA (TE) buffer and incubated for 30 minutes at 37 °C with RNAse A. NaCl was added and the phenol/chloroform/isoamyl alcohol extraction was repeated. DNA was precipitated as above and centrifuged. The pellet, resuspended in TE, was electrophoresed into wells of a 1% agarose gel containing ethidium bromide. DNA was separated over 90 min at 60 V and the bands were visualized by UV illumination.

Flow Cytometric Cell Cycle Analysis

Aliquots of cell suspension were fixed and stained with DNA-Prep reagents (Coulter) containing nonionic detergents RNAse and propidium iodide (50 μg/ml). Analysis was performed on an Epics-XL flow cytometer (Coulter) using Multicycle software (Phoenix Flow Systems). Apoptotic cells were located in the hypodiploid region of the histogram.

Statistics

Chi Square test was used to assess the correlation between the three methods (flow cytometry vs. electrophoresis, flow cytometry vs. light microscopy and electrophoresis vs. light microscopy).

Results

Induction of Apoptosis

Examination of the stained cytospin slides under light microscopy revealed morphological features of apoptosis in the samples treated with FLU, VP-16 and ARA-C including reduction in cell volume, increased cytoplasmic/nuclear ratio, compaction and margination of nuclear chromatin, nuclear fragmentation and apoptotic bodies.

DNA from leukemic cells treated with FLU, VP-16 and ARA-C was subjected to analysis in agarose gel and showed a detectable pattern of DNA fragments at multiples of approximately 200 base pairs. In contrast, there was no evidence of fragmentation in cells treated with MITOX, IDA and DNR (Fig.1).

The cytofluorimetric method demonstrated the presence of a hypodiploid peak when ARA-C, FLU and VP-16 were used. The advantage of this technique is that it provides an easy, rapid and accurate quantitation of apoptosis (Fig. 2). The percentages of samples with apoptotic cells are reported in the Fig. 3.

Correlation Between the Three Methods

Chi Square test was used to analyze the data obtained with the three methods. The correlation between the three methods was demonstrated by $p < 0.00001$ obtained for flow cytometry vs. electrophoresis, flow cytometry vs. light microscopy and electrophoresis vs. light microscopy.

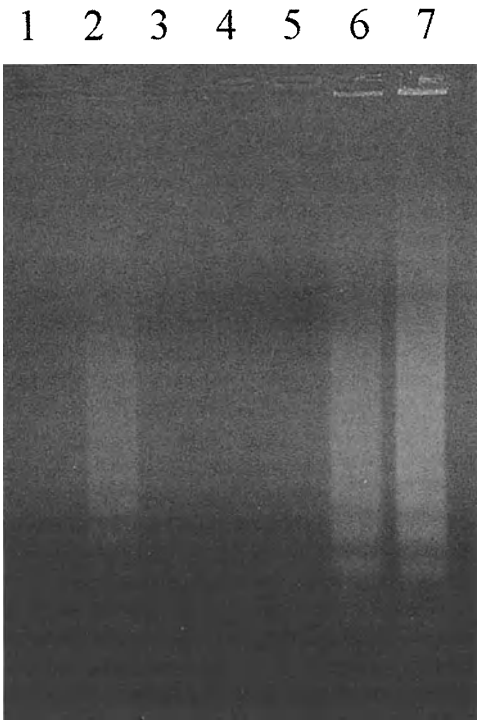

Fig. 1. DNA fragmentation detected by agarose gel electrophoresis (*1* untreated cells; *2, 3, 4, 5, 6, 7* cells treated with ARA-C, DNR, IDA, MITOX, FLU, VP-16)

Discussion

Acute myelogenous leukemia (AML) yields varied morphologic, cytochemical, immunologic and cytogenetic findings and different sensitivity to conventional chemotherapy; 60-70% of patients with de novo AML achieve complete remission but the majority (50-40%) of these patients relapse between 2 years [10].

The biological bases of drug resistance and relapse in AML are not well understood: several studies have shown that autonomous in vitro proliferation of AML cells was associated with poor clinical outcome [11], but few functional analyses of AML samples

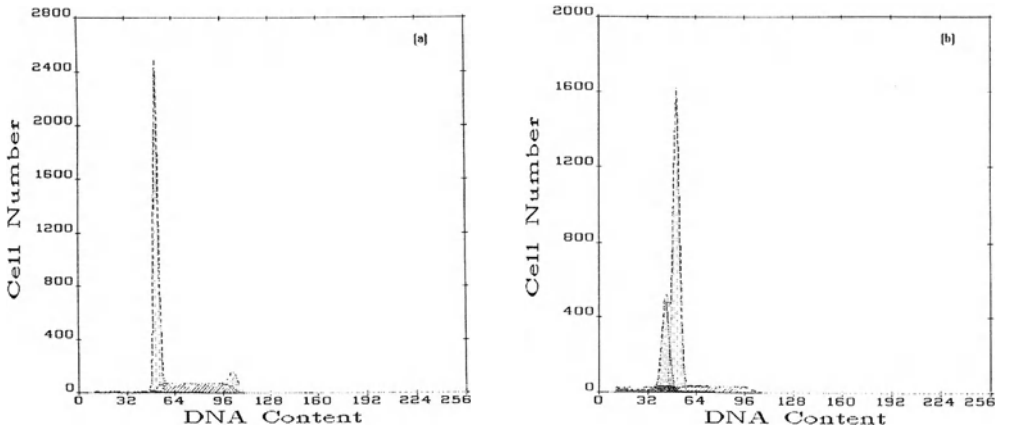

Fig. 2. Epics analysis of DNA content in untreated *(a)* und ARA-C treated cells *(b)*

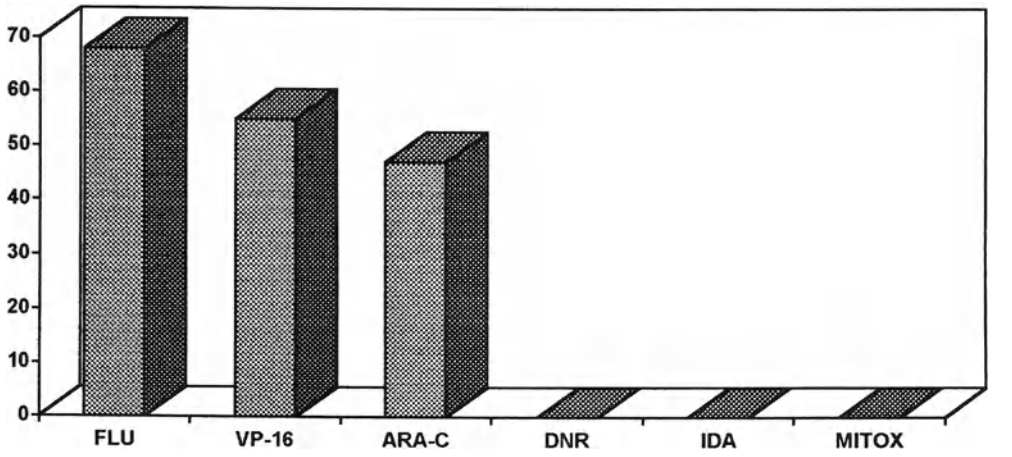

Fig. 3. Percentage of samples with apoptotic cells

have been performed. Some reports indicate that apoptosis plays a role in the response of AML patients to chemotherapy. In particular, a study on a small series of AML samples collected at onset of disease and before treatment suggested that apoptosis constitutes a common but variable response of blast cells to drugs [12].

Other data show that a combination of FLU, ARA-C and GM-CSF can induce apoptosis whereas treatment with any of these drugs alone does not [13].

In our study we investigated the induction of apoptosis by the chemotherapeutic agents used in the treatment of AML patients using three methods which gave comparable results. We found relatively low spontaneous apoptosis in the majority of AML samples analyzed; exposure to drugs (FLU, ARA-C, VP-16, DNR, IDA, MITOX) induced heterogeneous number of apoptotic cells. In particular, in our experiments we found that FLU, VP-16 and ARA-C induce a higher number of apoptotic cells anthracyclines (IDA, DNR) and Mitoxantrone. The heterogeneity of apoptosis induction by each drug could be due to different types of DNA damage and different effects on the cell cycle. In addition, the heterogeneity of the apoptotic phenomenon in AML cells might

depend on other biological characteristics: p53 mutation, BCL-2 expression, cytogenetic abnormalities [t(15;17), t(8;21)] [14,15].

Further studies on a large number of AML patients are needed to determine whether the identification of intrinsic or acquired resistance to apoptosis, evaluated in combination with other biological findings may contribute to therapeutic outcome in AML.

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Comparison of Hydroxyurea, Fludarabine and Cladribine in Modulating Ara-C Pharmacology in the Ara-C sensitive and resistant HL-60 and HL-60/Ara-C Cell Lines

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Abstract

Purpose. To compare the effectiveness of the ara-C modulating agents fludarabine (F-ara), cladribine (2-CdA), and hydroxyurea (HU) in ara-C resistant and sensitive leukemic blast cells in vitro.

Resistance against 1-β-D-arabinofuranosylcytosine (ara-C) is an important cause of treatment failure in pediatric and adult acute leukemias. Development of resistance can involve the multistep process of cellular ara-C-toxification including the rate-limiting first phosphorylation step catalysed by deoxycytidine kinase. The ribonucleotide reductase inhibitors F-ara, 2-CdA and HU are known to potentiate ara-CTP metabolism by an increase of deoxycytidine kinase activity as a consequence of dCTP pool reduction. We compared the efficacy of these drugs in enhancing intracellular ara-CTP accumulation in blast cells of the ara-C sensitive and resistant HL-60 and HL-60/ara-C cell lines.

Results. HL-60 cells preincubated with 5 μM F-ara, 0.1 μM 2-CdA or 0.5 mM HU, respectively, exhibited a significantly enhanced ara-CTP accumulation compared to unpretreated controls. The drugs differed in the preincubation-time dependancy of ara-C modulating efficacy with HU obtaining its maximum effect already after 1 hour of preincubation. A similar 3-4 fold increase of ara-CTP accumulation enhancement was reached after 4 h of preincubation with either of the drugs. In the resistant HL-60/ara-C cell line, however, no ara-C modulatory effect of F-ara and 2-CdA could be observed, while 4 h of preincubation with HU resulted in a 2-fold increase of ara-CTP accumulation compared to control cells. The reason for these striking differences between drugs with a similar presumed way of action is not yet understood. Other factors than dCTP pool reduction are likely to be involved.

Conclusion. These data identifying hydroxyurea as an effective ara-C modulating drug even in ara-C resistant cells provide evidence that this agent should be reincluded into current considerations about modern leukemia treatment schedules to optimize ara-C treatment and to partially overcome ara-C resistance.

Introduction

The pyrimidine nucleoside ara-C plays a major role in the treatment of acute myeloid leukemias (AML) in adults and children. Despite significant advancements in pediatric AML therapy, development of ara-C resistance as a major cause of treatment failure remains a problem. Resistance to ara-C in vitro [2, 3, 4] and in vivo [5-10]

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can involve each of the major metabolic steps in the toxification pathway to ara-CTP [1], the presumably main determinant of ara-C cytotoxicity. Strategies to optimize ara-C treatment and to overcome ara-C resistance have primarily focused on enhancement of cellular levels of ara-CTP. However, ara-CTP and the natural competitor deoxycytidinetriphosphate (dCTP) induce feedback inhibition of the rate-limiting toxifying enzyme, deoxycytidine kinase. This obstacle for further increases of ara-CTP accumulation even under optimized conditions [11] may be overcome by using ara-C-modulating strategies such as inhibition of ribonucleotide reductase which reduces dCTP and thus enhances ara-CTP levels. Combinations of ara-C with the ribonucleotide reductase inhibitors arabinosyl-2-fluoroadenine (F-ara-A) [11-14] and 2-chlorodeoxyadenosine (2-CdA) are already used clinically [15]. Yet, their efficacy in ara-C resistant cells has hardly been tested.

Hydroxyurea (HU), another ribonucleotide reductase inhibitor, has been in clinical use since 1960, primarily in the treatment of chronic myeloid leukemia [16]. In the 1980s, this relatively low-toxic agent attracted attention as an effective ara-C modulator in some solid tumors and in leukemias [17-20]. However, unlike F-ara-A and 2-CdA, HU is hardly used in AML treatment today. Based on these considerations we compared the effectiveness of F-ara-A, 2-CdA and HU in ara-C sensitive and resistant HL-60 cell lines.

Methods

Drugs

F-ara, 2-CdA, HU, natural nucleotides and ara-CTP were obtained from Sigma Chemical Co (St Louis, MO). All chemicals were of the highest purity available.

Ara-C and HU stock solution were prepared in sterile water, F-ara-A was dissolved in ethanol. The final concentration of ethanol in the culture never exceeded 1% and did not affect cell growth. Stock solutions were stored at -20 °C.

Cell Lines

The human acute myeloid leukemic cell line HL-60 was obtained from the European Collection of Cell Cultures, Salisbury, UK. The ara-C resistant HL60/ara-C cell line which was isolated according to a protocol by Bhalla et al. (1984) [25] diplayed 1000-fold ara-C resistance and cross-resistance to F-ara-A and 2-CdA, but not to HU. Both cell lines were tested negative for mycoplasmal infection. Cells were maintained in suspension culture at exponential growth phase in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) under standard conditions with 5% CO_2. Cells were used for 6 weeks. In HL60 cells the 50% inhibitory concentration (IC_{50}) was for ara-C 5-10 μM, for F-ara-A 1-5 μM, for 2-CdA 0.1-0.5 μM and for HU 500-1000 μM, in HL60/ara-C for ara-C 5000-7500 μM, for F-ara-A >100 μM, for 2-CdA >100 μM and for HU 500-1000 μM.

Cellular Pharmacology

Exponentially growing cells (0.5-1 × 10^6/ml) were incubated in HL-60 cells in ara-C 0.5-100 μM and in HL60/ara-C in ara-C 1-10000 μM. Cells were washed twice in ice-cold phosphate-buffered saline (pH 7.2) and counted prior to extraction.

Preincubation experiments were carried out with F-ara-A 0.5 μM, 2-CdA 0.1 μM or HU 500 μM for 1-24 h. Drug concentrations were therapeuticallyrelevant and in accordance with literature [11, 15, 24]. Cells were washed and incubated in ara-C (10 μM) containing RPMI medium in a shaking water bath at 37 °C for 1h. The incubation time (1 h) and the final ara-C concentrations (10 μM for HL-60 and 5000 μM for HL-60/ara-C) were optimal to achieve ara-CTP accumulation below the saturation level.

Nucleotides were extracted using tetrabutyl ammonium hydrogen sulfate. Separation from other nucleotide triphosphates was done by an isocratic ion pair high-performance liquid chromatography (HPLC) method using a reversed phase C18 column (NOVA-PAK, Waters-FRG) and 0.09 M phos-

phate buffer (pH 6, tetrahydrofuran 0.35%, tetrabutyl ammonium hydrogen phosphate 0.01 M) [22]. Quantification was by UV (270 nm); the limit of detection was 25 ng/ml ara-CTP. Anthranilic acid was used as internal standard. Ara-CTP levels were expressed as picogram (pg) ara-CTP per 10^7 cells [22].

Statistical Analysis

Intracellular ara-CTP-concentrations with and without preincubation with F-ara-A, 2-CdA and HU were compared using the Kruskal-Wallis One Way Analysis of Variance on Ranks. To isolate the groups which differed from the others we used the Dunn's All Pairwise Multiple Comparison Procedure.

Results

Accumulation of ara-CTP in ara-C Sensitive and Resistant HL-60 Cells

In HL60 cells 1-h incubation with increasing ara-C concentrations (0.5-100 μM) resulted in highest ara-CTP levels of 387.71 ± 110.99 pM/10^7 at a concentration of ara-C 10 μM (n = 60). In resistant HL-60/ara-C cells, measurable ara-CTP levels were not detected at ara-C concentrations up to 2500 μM. At ara-C 5000 and 10000 μM, mean ara-CTP levels were 44.56 ± 11.09 (n = 3) and 76.11 ± 19.34 (n = 2), respectively (Fig. 1).

Effect of F-ara-A, 2-CdA and HU on Cellular ara-CTP Accumulation in HL-60 Cells

Compared to unpretreated controls preincubation of HL-60 cells with HU 500 μM, F-ara A 5 μM and 2-CdA 0.1 μM for 4 h caused 2.8 ± 0.4 (HU), 2.9 ± 0.3 (F-ara-A) and 3.2 ±

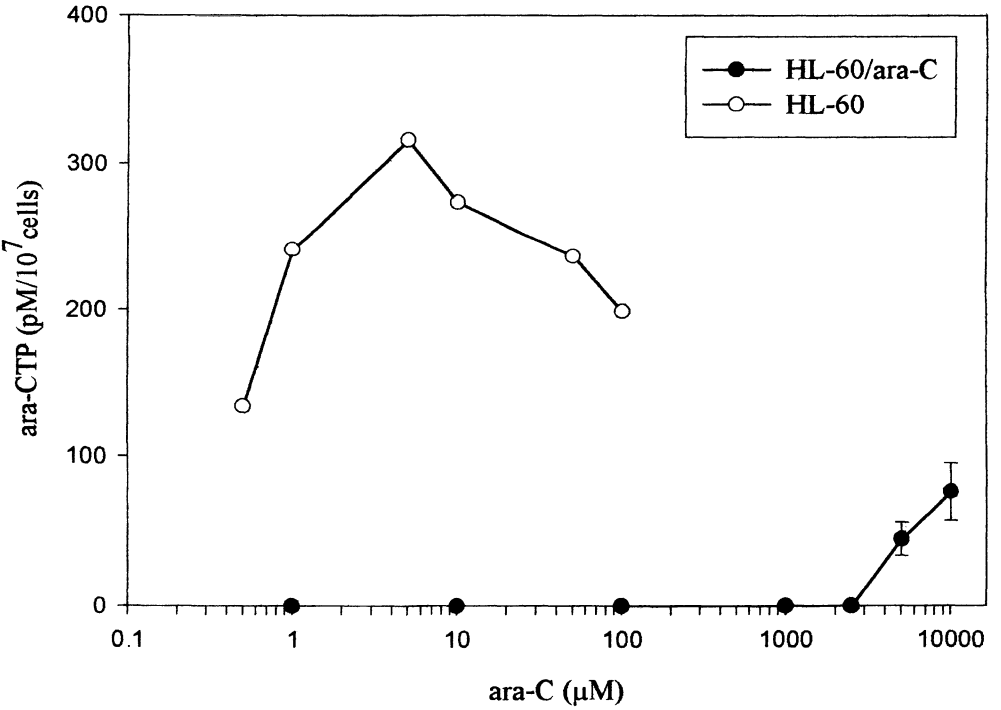

Fig. 1. Ara-CTP accumulation after 1-h incubation with ara-C 0.5-100 μM in HL-60 and ara-C 1-10000 μM in HL-60/ara-C cells. For HL-60 cells, one representative experiment is shown. For HL-60/ara-C cells, *each point* and *error bar* represents the mean and standard deviation of 2-3 experiments

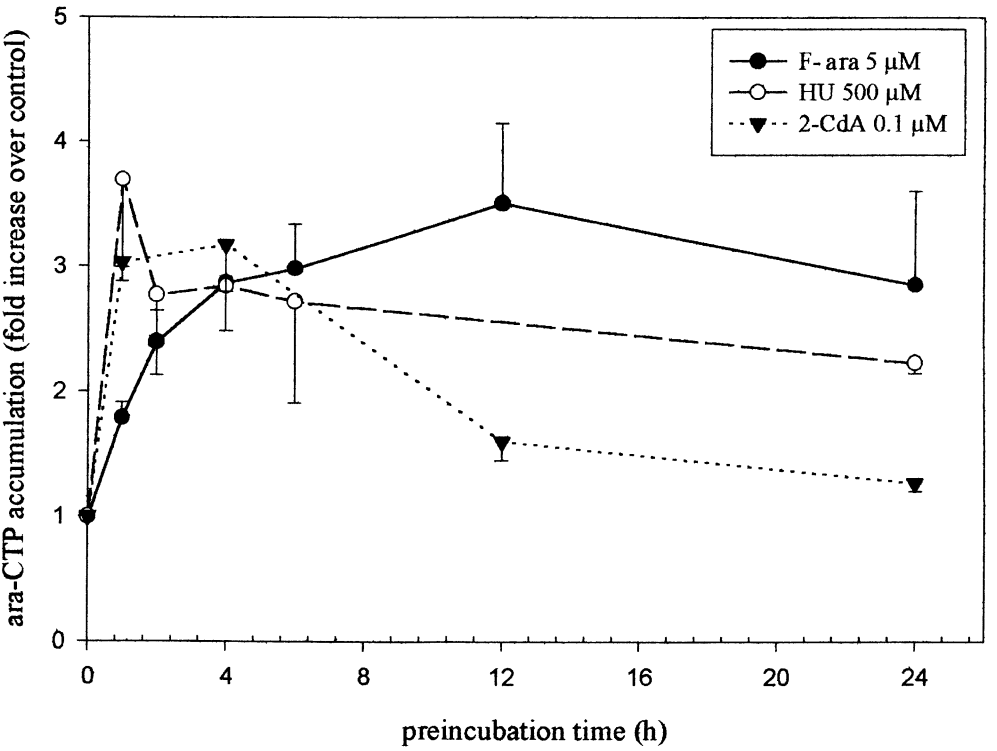

Fig. 2. Effect of preincubation with F-ara-A, HU and 2-CdA on ara-CTP accumulation in HL-60 cells as compared to unpretreated controls. Values are the mean of 3 independent experiments in triplets. Standard deviations never exceeded 20% of mean. Significant ($p < 0.05$) increases of ara-CTP accumulation were observed after preincubation with F-ara-A for 4-24 h, with HU for 1-24 h and with 2-CdA for 1-6 h

0.4 (2-CdA)-fold increases of cellular ara-CTP accumulation (Fig. 2). The three drugs induced highest ara-CTP levels after different preincubation times: HU after 1 h (3.7 ± 0.7-fold), F-ara-A after 6-24 h (2.9-3.5-fold) and 2-CdA after 4 h (3.2 ± 0.4-fold). 2-CdA was shown to be less effective after longer preincubation intervals even at concentrations up to 5 µM (data not shown). Increases of ara-CTP accumulation were statistically significant after preincubation with HU for 1-24 h, F-ara-A for 4-24 h and 2-CdA for 1-6 h.

Effect of F-ara-A, 2-CdA and HU on Cellular ara-CTP Accumulation in Resistant HL-60/ara-C Cells

HL-60/ara-C cells were preincubated for 4 hours with the drug concentration used in the parental HL60 cell line (F-ara-A 5 µM, HU 500 µM, 2-CdA 0.1 µM) and subsequently exposed to ara-C 5000 µM. While preincubation with F-ara-A and 2-CdA did not exert any effect on ara-CTP accumulation, HU was shown to induce 2.0 ± 0.3 fold ara-CTP accumulation compared to cells treated with ara-C alone. This effect was statistically significant ($p < 0.05$).

Discussion

In contrast to the clinically applied ara-C modifiers F-ara-A and 2-CdA, HU at therapeutic concentrations does not only enhance ara-CTP accumulation in ara-C sensitive, but also in ara-C resistant HL-60 leukemia cells.

Ara-C resistance is regarded as an important cause of treatment failure in pediatric and adult AML. Yet, hardly any studies on combinations of ara-C with modulators have been carried out in drug-resistant cell

lines. For isolating our ara-C resistant HL60/ara-C cell line we used the protocol by Bhalla et al. (1984) [25]. Cross-resistance studies let us assume that ara-C resistance is caused by deficiency of deoxycytidine kinase (dCK), the rate limiting enzyme in the generation of ara-CTP. Since dCK-deficiency is also considered to be an important mechanism of ara-C resistance in vivo (1) the HL60/ara-C cell line used by us seems to be a representative model.

F-ara-A, 2-CdA and HU are inhibitors of the ribonucleotide reductase which deplete intracellular dCTP and thus reduce feedback inhibition of dCK. However, to inhibit ribonucleotide reductase 2-CdA and F-ara-A need to be metabolised via dCK to the active triphosphates 2-CdATP and F-ara-ATP [13]. Since our ara-C-resistant HL60/ara-C cell line seems to be dCK-deficient 2-CdATP and F-ara-ATP levels are presumably insufficient for affecting ribonucleotide reductase activity. This might explain that in our study only HU was capable of enhancing ara-CTP levels in HL60/ara-C cells. Bhalla et al. (1991) tested another dCK-deficient, ara-C-resistant HL60/ara-C cell line and found supraadditive growth inhibition by the combination of ara-C and HU, but in contrast to us no significant increment in intracellular ara-CTP accumulation [24, 25].

Our ara-CTP results in HL-60 cells are in accordance with numerous studies which showed ara-CTP enhancing activity of F-ara-A [11-14], 2-CdA [15] and HU [18-20] in ara-C sensitive leukemic cell lines. Combinations of ara-C with F-ara-A and 2-CdA, respectively, have entered AML treatment protocols for refractory disease in adults and children [14, 15]. However, in contrast to the already clinically applied 2-CdA and F-ara-A, only HU was able to increase ara-CTP levels in ara-C resistant HL60/ara-C cells as well. In the light of this HU should be considered as a modifier of ara-C for the treatment of acute myeloid leukemia.

Acknowledgments. This work was supported by the Federal Department of Research and Technology (#1EC9401).

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Primary Resistance to Ara-C in AML Progenitor Cells and its Modulation by Cytokines

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Abstract. An assay for the cytotoxicity of cytosine arabinoside (ara-C) for AML progenitors was validated by correlation of the median cytotoxic dose measured in vitro, D50[ara-C], with the reduction of AML blasts $\leq 5\%$ in the bone marrow after 1 cycle of induction chemotherapy (TAD9).

Bone marrow mononuclear cells from 29 adult patients with de novo AML were isolated on Ficoll-Hypaque prior to therapy, adherence depleted and incubated with 0 to 100 μM ara-C for 12 h at 0.5×10^6 cells/ml. Cells were washed and assayed for AML progenitors in a serum free methylcellulose assay. Dose responses with ara-C were evaluated by logistic regression, yielding a D50 of ara-C to quantify sensitivity or resistance together with an estimate for its standard error. Clinical outcome of each patient was defined as the bone marrow blast content at day 16 following induction therapy with one cycle of TAD9.

Two groups of patients were formed by their in vitro sensitivity to ara-C, separated by the mean D50[ara-C] of 0.507 μM. Of 16 patients with D50 values below this mean, 9 had bone marrow blasts $\leq 5\%$ after one course of TAD as opposed to 2 of 13 patients with D50 above the mean ($p \leq 0.05$, nonparametric test).

The ara-C sensitivity or resistance from this in vitro assay can be validated by the short term clinical results. The assay has been used to screen modulation of resistance in vitro, i.e., with cytokines. The median shifts of D50[ara-C] observed after 48 h modulation with cytokines were ten fold for pIXY321, five fold for G-CSF and two fold for GM-CSF, with G-CSF giving the most consistent results. The resistance assessed and modulated here is of primary type, not secondary as in assays comparing relapsed with de novo AML.

Introduction

The modulation of ara-C sensitivity of leukemic blasts by cytotoxic drugs or by cytokines has been studied in different in vitro assays, showing mostly some positive effect [1-5]. However, in vivo studies on priming with cytokines only gave positive effects for subgroups of patients at best [6-9]. A possible explanation might be an overestimation of possible priming effects by in vitro conditions or a considerable statistical variation of modulatory effects.

Discrepancies between in vitro assays and in vivo studies would not be detectable unless the in vitro assays employed are validated by a correlation to clinical results. This study sought to correlate the ara-C sensitivity for AML progenitor cells as measured in vitro with the qualitative blast reduction in the bone marrow for adult patients with de novo AML after the first course of standard induction chemotherapy.

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Materials and Methods

Bone marrow aspirates were obtained with informed consent from adult patients with de novo AML (bone marrow blasts ≥ 80%). Mononuclear cells were isolated from Ficoll-Hypaque gradients, washed with IMDM, 2% BSA and adherence-depleted overnight in IMDM, 10% FBS (Boehringer). Cells were washed, adjusted to 0.5×10^6/ml and, except for control samples, pretreated with 100 U/ml of either cytokine in IMDM, 10% FBS for 48 h at 37 °C, 5% CO_2 in a fully humidified atmosphere. Cytokines were r-metHuG-CSF (Amgen), or rhGM-CSF (Behring, E. coli-product), GM-CSF plus rhIL-3 (Behring), pIXY321 (Immunex), or rhIL-1β (Boehringer-Ingelheim). Cells were washed three times, counted and seeded at 50 000/ml in a serum-free CFU-L assay based on 1.3% methylcellulose with 20 mg/ml BSA (Sigma), 15 μM water-soluble cholesterol (Sigma, solubilized with β-methyl cyclodextrin), 50 μM water-soluble linoleic acid (Sigma), 2 μg/ml human insulin (Sigma), 50 nM human transferrin, Fe-saturated (Sigma), 100 ng/ml of rhSCF (Amgen), 2 ng/ml each of G-CSF, GM-CSF, rhIL-1β (Boehringer), IL-3, IL-6 (Genzyme, CHO-product) and 2 U/ml of Epo (Cilag) and 50 μM of β-mercaptoethanol (Biorad). After 14 days, colonies > 50 cells were scored on an inverted microscope.

Dose responses yielded D50[ara-C] values using the median effect-principle [10, 11]. The colony number obtained without ara-C is c_0, the number at the given ara-C concentration is c. The formula used for the regression analysis is

$$\log\left(\frac{c_0}{c} - 1\right) = m \log D - m \log D_{50},$$

where m is a scaling factor describing the curvature of the dose response and D is the actual dose of ara-C for each data point. With respect to the colony numbers c this is a logistic regression. Confidence limits for the D50 values were obtained from the regression analysis [12] in MapleV. The D50 values for different pretreatments of individual samples were compared in a t-test adjusted for unequal variances [12]. The D50 values for each factor from the cohort of samples were described and compared by their cumulative frequency distributions.

The induction chemotherapy consisted of ara-C 100 mg/m^2/day continuously i.v., days 1 and 2, and 100 mg/m^2 q 12 h as 30-min infusion days 3 to 8, daunorubicin 60 mg/m^2 as 60-min infusion days 3, 4, and 5 and thioguanin 100 mg/m^2 q 12 h orally days 3 to 9.

Results

The results on 29 samples measured for D50[ara-C] in vitro and the respective therapeutical outcome (marrow blasts reduced to ≤ 5%) are shown in Fig. 1. The two subgroups differ on a significant level of $p < 0.05$ (non parametric tests) and are separated by the mean D50[ara-C] of all samples, 0.507 μM. Quantitative correlations have also been calculated in linear and non linear models, with low correlation coefficients but significance levels of $p < 0.05$ (parametric test).

The modulation by growth factors and its extent in individual samples are shown in Figs. 2 and 3, the statistical results are summarized in Table 1. G-CSF and pIXY321 yielded the largest shifts.

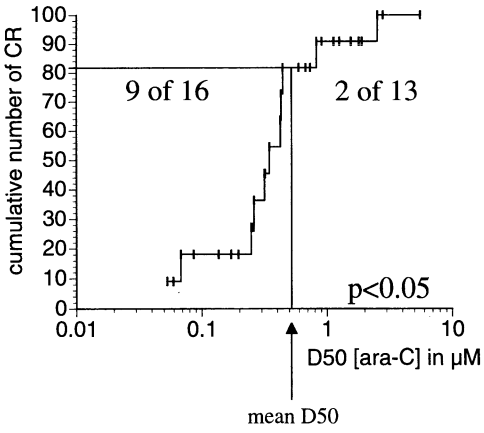


Fig. 1. Validation of assay by short-term clinical outcome. Ara-C sensitivities for AML progenitor cells were assayed in vitro, yielding a D50[ara-C]. These results were grouped by the D50[ara-C] values. Patients with in vitro sensitivities below the mean of the cohort had a higher chance to achieve a blast reduction in the bone marrow to ≤ 5% by one course of induction chemotherapy

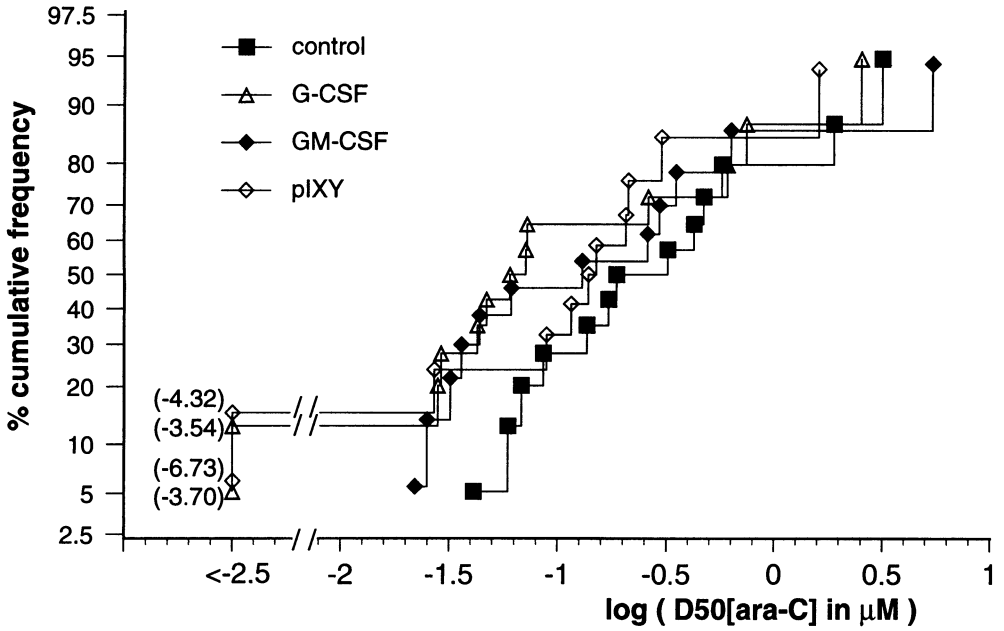
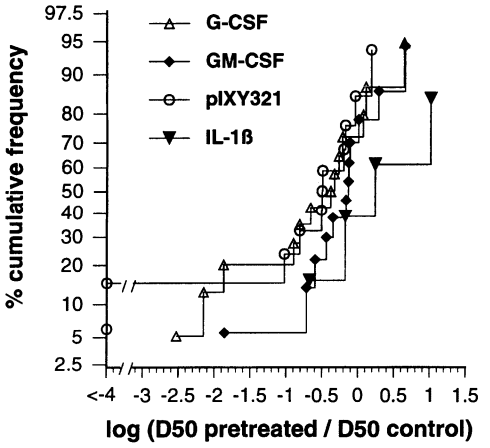


Fig. 2. D50[ara-C] with and without modulation by 48 h pretreatment with cytokines. Ara-C sensitivities were measured in vitro with and without cytokine pretreatment for 48 h. The D50[ara-C] values were stratified by pretreatment and grouped by size. The cumulative frequency distributions show considerable variability of D50[ara-C] values without pretreatment and high interindividual differences by cytokine modulation



Conclusion

The in vitro ara-C sensitivity of AML progenitors correlates qualitatively with a patient's marrow blast reduction ≤ 5% on day 16 after standard dose ara-C induction therapy (TAD9). A quantitative correlation was not to be expected, since the effect of

Fig. 3. Individual shifts of D50[ara-C] by 48 h pretreatment with cytokines. Ara-C sensitivities were measured in vitro with and without cytokine pretreatment for 48 h. The D50[ara-C] values were modulated by cytokine pretreatment with high interindividual variability

Table 1. Descriptive statistics of individual shifts in D50[ara-C]: all shifts compared to control

Growth factor no.	(responsive)	Range (log)	Geometric mean (log) ± SEM	Factor
G-CSF	13 [10]	- 2.531 - 0.649	- 0.711 ± 0.261	5.14[a]
GM-CSF	12 [4]	- 1.858 - 0.665	- 0.268 ± 0.172	1.85
GM-CSF + IL-3	8 [3]	- 3.554 - 0.513	- 0.382 ± 0.373	4.15
QIXY321	11 [7]	- 5.334 - 0.191	- 1.001 ± 0.516	10.02[a]

Shifts, mean, median and SEM were calculated on a log scale.
[a] Shifts with a statistical significance of $p < 0.05$ in non-parametric tests.

daunorubicin in the standard chemotherapy was not addressed in vitro. However, the qualitative result validates the in vitro assay.

The ara-C sensitivity of AML progenitors in vitro shows considerable interindividual variation with a log normal distribution covering 2 logs. A similar range of variation has also been shown in other assays [13-15]. The mean LD50 values reported varied between 0.08 μM and 4.2 μM, depending on the method employed. Here, we report a median D50[ara-C] of 0.5 μM, which has been found in this range for three different data sets with this assay so far [16-18].

Fortyeight hour pretreatment with rhumetG-CSF and pIXY321 increased the ara-C sensitivity by factors of 5 and 10, respectively, while pretreatment with rhGM-CSF was less efficient. These modulatory effects of growth factors are highly variable, explaining some of the contradictions in published in vitro results. Other assays have dealt with ara-C sensitivities measured in vitro for primary versus refractory AML [14, 15]. Thus, the differences in drug sensitivity were of secondary type, i.e., occurring after chemotherapy, not of primary type, as assessed here.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Gemcitabine-Mediated Enhancement of Cellular Cytarabine-5′-Triphosphate (ara-CTP) Accumulation

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Abstract. Gemcitabine (dFdC) is a rather new antimetabolite which potentiates the cellular accumulation of its own 5′-triphosphate (dFdCTP). Self-potentiation of dFdCTP accumulation is specifically induced by an enhanced salvage activity due to inhibition of ribonucleotide reductase, while the cellular retention of dFdCTP is prolonged by inhibition of dCMP deaminase. The present study analyses to which extent the favorable modulatory properties of dFdC can be used to enhance cellular ara-CTP accumulation, an approach which specifically may be applied to overcome cellular ara-C resistance. At the clinically achievable ara-C concentration of 10 μM, HL-60 cells accumulated after 2 h an intracellular ara-CTP concentration of 83 ± 7 μM. Maximal enhancement of ara-CTP accumulation (4.1 ± 0.3-fold) was achieved by preincubation with 0.1 μM dFdC, while greater dFdC concentrations yielded lower enhancement factors. With regard to the duration of preincubation, a 2-3 h intervall appeared to be optimal. At a fixed concentration of dFdC, the enhancement of ara-CTP accumulation increased with ara-C concentration amounting to enhancement factors of 1.8 and 2.6 after exposure to 1.0 μM and 10 μM ara-C, respectively. Cellular elimination of ara-CTP was nearly identical in dFdC-pretreated- and control cells ($T^1/_2$ = 0.8 and 0.7 h respectively). In fact, at a dFdC concentration of 0.1 μM, dCMP deaminase activity was not inhibited. When the incorporation of ara-C into DNA was followed for a 20-h interval after drug washout, [3H]-ara-C_{DNA} was 5-fold greater in dFdC-pretreated cells than in control cells.

Introduction

Gemcitabine is a new antimetabolite characterised by a self-potentiation of cytotoxic activity [1]. Unlike ara-C, gemcitabine inhibits ribonucleotide reductase [2], dCMP deaminase [3,4], and CTP synthetase [5]. This specific interaction with enzymes of the cellular pyrimidine metabolism causes an increased phosphorylation of gemcitabine to the active triphosphate on the one hand and delays its elimination on the other hand [6]. Since gemcitabine induced a self-potentiation of its own activity, it was asked whether the drug could also be used as a modulator of the cellular ara-C metabolism. In fact, Gandhi and Plunkett [7] demonstrated that pretreatment of K562 cells with gemcitabine enhanced the phosphorylation of arabinosyl nucleosides and induced synergistic cytotoxicity. The present study performed in HL60 cells specifically focusses on the effect of gemcitabine-pretreatment on the cellular metabolism of ara-C and its incorporation into DNA. The modulatory effect of gemcitabine is analysed in conjunction with hydroxyurea to differentiate the effect of gem-

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citabine from single inhibition of ribonucleotide reductase.

Materials and Methods

[5-^{3}H] ara-C (specific activity, 29 Ci/mmol) was purchased from Amersham Life Sci. (Little Chalfont, Buckinhamshire, UK). [^{14}C]dCyd (specific activity 48.7 mCi/mmol) was obtained from Sigma Chemicals Co. (St. Louis, MO). Gemcitabine was provided by Eli Lilly Research Laboratories (Indianapolis, IN). Ara-C and all natural nucleosides, nucleoside triphosphates, and [^{3}H]dCyd (specific activity, 20 Ci/mmol) were products of Sigma Chemical Co., Inc. (St. Louis, MO).

Cell Culture

HL-60 cells were maintained in suspension cultures in exponential growth in RPMI 1640 medium (GIBCO Life Technology Ltd., Paisley, UK) supplemented with 10% heat-inactivated fetal calf serum (GIBCO) at 37 °C in a humidified atmosphere containing 5% CO_2. The average cell volume of HL-60 cells was 0.765×10^3 fl.

Nucleotide Extraction and Analysis

Nucleotides were extracted from cells with 0.4 N $HClO_4$ [5], and the nucleoside triphosphates (NTP) in the neutralized acid-soluble extract were analyzed by HPLC as previously described [6]. For determination of deoxynucleotides (dNTP), the NTP in the $HClO_4$-soluble cell extracts were eliminated by periodate oxidation. Subsequently, dNTP were separated and quantitated by the HPLC method described elsewhere [2].

Assay of dCMP Deaminase Activity in Whole Cells

For determination of dCMPD activity HL-60 cells (1 to 3×10^7) were incubated with 0.2 μCi of [^{14}C]dCyd in 5 ml of cell culture medium without fetal calf serum. The incubation was performed for 15 min in the presence of 5 μg of aphidicolin, and the reaction was terminated by washing with ice-cold phosphate-buffered saline and subsequent extraction with 0.4 N $HClO_4$. After neutralization of cell extracts, incorporation of radioactivity into dCTP and dTTP was analysed [3].

Determination of [^{3}H]ara-C Incorporation into DNA

After cell extraction with 0.4 N $HClO_4$, 5 mg calf-thymus DNA was added to the acid-insoluble extract, and the pellet was washed twice with 10 ml of 0.4 N $HClO_4$. DNA was redissolved in 2 ml of 0.3 N KOH, after which 1 ml was transferred for liquid scintillation counting of radioactivity [8] using an LS 1701 Beckmann (Fullerton, CA) liquid scintillation counter and Rotiszint eco plus scintillation fluid (Fa Roth, Karlsruhe, Germany).

Software

For determination of half-lives of cellular drug elimination and for calculation of the area under the concentration × time curve (AUC) the software Topfit was used on an IBM-compatible computer system [9].

Results

Intracellular Accumulation of ara-CTP and Gemcitabine-Triphosphate (dFdCTP) in HL60 Cells

HL60 cells were exposed in separate experiments either to gemcitabine or to ara-C. For drug incubations a time interval of 2 h was generally chosen since longer incubation times resulted in a decrease of intracellular drug-triphosphate concentrations. After 2 h of incubation, cells were extracted and analysed for the respective drug triphosphates dFdCTP and ara-CTP. Within a concentration range of 0.1 to 10 μM, intracellular drug triphosphate concentrations were linearly correlated to the logarithm of dFdC- ($r = 0.979$) or ara-C ($r = 0.962$) concentrations. At drug concentrations of 0.1 and 10 μM, intracellular dFdCTP reached 71 ± 8 μM and 289 ± 3 μM, respectively, while ara-CTP

achieved values of 25 ± 2 μM and 83 ± 7 μM, respectively. At drug concentrations of 0.1 μM and 10 μM, the ratio of dFdCTP/ara-CTP amounted to 2.8 and 3.5, respectively.

Kinetics of dFdCTP Elimination and dFdC-Metabolite Distribution After Exposure to 0.1 μM dFdC

Since the majority of experiments was performed using a dFdC concentration of 0.1 μM, the kinetics of dFdCTP elimination were specifically studied at this drug concentration. After a 2-h incubation with 0.1 μM dFdC, subsequent drug washout, and reincubation in drug-free medium, dFdCTP was eliminated from the cells by log-linear kinetics (r = -0.964) with a half-life of 4.6 h.

Enhancement of ara-CTP Formation as a Function of dFdC Concentration

Cells were incubated for 2 h with dFdC 0.1-10 μM, after which cells were washed out and were reincubated for further 2 h with 10 μM ara-C. Maximal stimulation of intracellular ara-CTP accumulation was observed at a dFdC concentration of 0.1 μM, where an enhancement factor (EF) of 4.1 ± 0.3 was calculated). At greater dFdC concentrations like 1.0 μM or 10 μM, EF-values of 2.9 ± 0.1 or 2.7 ± 0.1 were reached respectively. This observation possibly indicates a feed-back inhibition of dCyd kinase at dFdCTP concentrations greater than 80 μM. By comparison, pretreatment of cells to 5 mM HU caused an EF-value of 2.8.

Importance of the Extracellular ara-C Concentration for dFdC-Mediated Enhancement of ara-CTP Formation

It was asked whether the concentration of ara-C would have an effect on dFdC- or HU-mediated stimulation of ara-CTP synthesis. Using a fixed concentration of dFdC (10 μM) during the preincubation period, ara-C concentrations were varied between 0.1 and 10 μM. At an ara-C concentration of 0.1 μM, ara-CTP formation was inhibited with an EF-value of 0.3. However, at ara-C concentrations of 1.0 and 10 μM, EF-values of 1.8 and 2.6, respectively, were observed. Enhancement of ara-CTP formation is apparently greater when cells are exposed to greater ara-C concentrations.

Effect of the Duration of dFdC Preincubation on Intracellular ara-CTP Accumulation

Cells were incubated with 0.1 μM dFdC for intervals of 0 to 4 h, after which dFdC was washed out, and cells were reincubated with 10 μM ara-C for further 2 h. Enhancement of ara-CTP synthesis reached a plateau after a preincubation period of 2-3 h. All experiments were therefore performed using a dFdC-preincubation period of 2 h.

Effect of dFdC on the Cellular Half-Life and AUC of ara-CTP

When cells were exposed to dFdC 0.1 μM for 2 h, and, after drug washout, were reexposed to ara-C for further 2 h, an ara-CTP concentration of 305 ± 15 μM was reached compared to an ara-C concentration of 83 ± 7 μM in control cells. After a subsequent drug-washout and reincubation in drug-free medium, ara-CTP was eliminated during the first hour at a faster rate which was nearly identical for dFdC-pretreated and control cells ($T^1/_2$ = 0.8 and 0.7 h, respectively). Subsequent elimination was slightly slower in dFdC-pretreated cells ($T^1/_2$ = 3.4 h) than in control cells ($T^1/_2$ = 2.8 h). Pretreatment of HL60 cells with 0.1 μM dFdC produced an $AUC_{ara\text{-}CTP}$ of 841 μmol × h/l which was 4.3-fold greater than in control cells (195 μmol × h/l).

dFdC-Mediated Perturbation of the Intracellular dCTP Pool

In control cells, cellular dCTP amounted to a concentration of 6.44 ± 1.56 μM. After a 2-h incubation with 0.1 μM dFdC, dCTP was decreased to a concentration of 5.26 ± 0.42 μM (82% of control), while 10 μM dFdC depleted the dCTP pool to 0.55 ± 0.57 μM (8.5% of control).

Effect of dFdC on dCMPD Activity

HL-60 cells were exposed to [^{14}C]dCyd for 15 min in the presence of aphidicolin, and incorporation of radioactivity into dCTP and dTTP was assayed. In control cells, the ratio of [^{14}C]dCyd incorporation into dCTP/dTTP was 1:2.8, indicating that in HL-60 cells the majority of dCMP synthesized by dCyd kinase is deaminated by dCMP deaminase and is subsequently channeled into the dTTP pool. Preexposure of cells to 0.1 μM dFdC for 2 h increased the incorporation of radioactivity into dCTP by 4.4-fold, while radioactivity in the dTTP pool only increased by 1.4-fold. The ratio of radioactivity in dCTP/dTTP changed accordingly to 1:1.4. When radioactivity in dCTP and dTTP was taken together as a measure of dCyd kinase activity, 0.1 μM dFdC increased this value by 2.0-fold. At the greater dFdC concentration of 10 μM, incorporation of radioactivity into dCTP was increased by 4.2 ± 0.1-fold, while radioactivity in the dTTP pool was decreased to 29% of control. The sum of radioactivity in dCTP + dTTP was not significantly increased by 10 μM dFdC indicating a presumably unchanged activity of dCyd kinase.

Effect of dFdC on Incorporation of ara-C into DNA

This experiment was performed to evaluate the effect of dFdC or HU on the incorporation of [3H]-ara-C into DNA. HL60 cells were preincubated with 0.1 μM dFdC for 2 h and were subsequently exposed to 10 μM [^{3}H]-ara-C for further 2 h. After cell extraction, the acidinsoluble material was assayed for radioactivity. Preincubation with 0.1 μM dFdC caused a 3.7-fold enhancement of ara-C incorporation into DNA (ara-C_{DNA} = 370 ± 44% of control).

In a subsequent analysis, the kinetics of [^{3}H]-ara-C incorporation into DNA were followed after washout of ara-C and reincubation of cells in drug-free medium. For comparison, ara-C DNA at drug washout was set equal to 1.0 in control cells. In unpretreated cells, the amount of [^{3}H]-ara-C in DNA rose to a value of 3.1 ± 0.15 during 20 h after drug-washout. After pretreatment with 0.1 μM dFdC, [^{3}H]-ara-C DNA achieved a level of 15.6 ± 0.5.

Discussion

This study analyses the effect of gemcitabine as a modulator of cellular ara-C metabolism. The principal goal of modulation consists in the overcoming of cellular resistance to ara-C. The underlying hypothesis of the presented experiments consists in the assumption that gemcitabine modulates the cellular dNTP metabolism to an extent that also the phosphorylation of ara-C may profit from the self-potentiative effect of the drug. In fact, pretreatment of cells with gemcitabine (0.1-10 μM) induced a marked enhancement of intracellular ara-CTP accumulation which was greatest after preexposure to 0.1 μM dFdC [enhancement factor (EF) = 4.1], while greater gemcitabine concentrations resulted in lower EF-values (e.g., EF = 3.3 at 0.3 μM dFdC and EF = 2.9 at 10 μM dFdC). The study demonstrates that dFdCTP concentrations ≥ 145 μM, as achieved after a 2-h exposure to 0.3 μM dFdC, have an inhibitory impact on the enhancement of ara-C phosphorylation which might best be explained by a product inhibition of dFdCTP on dCyd kinase. Competition of gemcitabine and ara-C at the level of dCyd kinase should play a minor role since gemcitabine was washed out prior to ara-C exposure.

When dFdC-mediated modulation of ara-CTP accumulation was analysed as a function of extracellular ara-C concentration, it became apparent that enhancement of ara-CTP accumulation was only observed at ara-C concentrations greater than 1 μM. Similar results were also obtained when HU was used as the modulating agent (data not shown). It may therefore be concluded that enhancement of ara-CTP accumulation as effected by RR-inhibition is greatest at ara-C concentrations clinically achieved by intermediate- or high-dose ara-C treatment [10].

dFdCTP has a marked effect on its own elimination in that the drug-triphosphate inhibits dCMP deaminase, an enzyme playing a major role in dFdCTP catabolism. However, preincubation with gemcitabine had nearly no effect on the cellular elimina-

tion of ara-CTP, an observation also reported by Gandhi et al. [7]. Accordingly, it was concluded that the enhancement of ara-CTP accumulation was essentially due to a stimulation of ara-C phosphorylation rather than to an inhibition of ara-CTP elimination. Relating to the greater peak concentration of ara-CTP after gemcitabine preincubation, the AUC of ara-CTP was increased by a factor of 4.9 compared to control values.

dFdC is a known inhibitor of ribonucleotide reductase. Exposure of cells to 0.1 and 10 μM gemcitabine depleted the dCTP pool to 82 and 8.5% of control, respectively. Interestingly, 0.1 μM gemcitabine caused a greater enhancement of ara-C phosphorylation than 10 μM gemcitabine. Although dCTP supposedly acts as a feed-back inhibitor of dCyd kinase, the rate-limiting enzyme for dCyd analog phosphorylation, the greater dCTP depletion after 10 μM gemcitabine did not result in a correspondingly greater enhancement of ara-C phosphorylation. It cannot be excluded that greater dFdCTP concentrations (289 ± 3 μM) may exert an additional inhibitory effect on dCyd kinase overcoming the activating effect of dCTP depletion.

Since dCMPD takes part in the catabolism of ara-CTP, and since dFdCTP has been demonstrated to inhibit dCMPD in aT-lymphoblastic leukemia cell-line (CCRF-CEM) it was of interest to analyse the effect of dFdC on dCMPD activity in HL-60 cells. The in situ activity of dCMPD activity was analysed by following the incorporation of [^{14}C]dCyd into dCTP and dTTP. It was demonstrated that dFdC 0.1 μM increased the incorporation of [^{14}C]dCyd into dCTP by more than 4-fold, while total phosphorylative activity of dCyd kinase, as determined by the sum of radioactivity in dCTP and dTTP, only doubled. Accordingly, it was concluded that, due to increased dCyd kinase activity, greater amounts of dCMP are available for deamination or phosphorylation. While under control conditions deamination is approximately 3-fold greater than phosphorylation, nearly equal amounts of dCMP were channeled to either pathway in the modulated situation. dFdC 0.1 μM consequently may be regarded as a relative inhibitor of dCMPD, while the overall rate of deamination increases. In conclusion, incorporation of [^{14}C]dCyd into dCTP is enhanced not only because dCyd kinase is activated, but also because less dCMP is deaminated by dCMPD. Since, to a lesser extent, also HU acts as a relative inhibitor of dCMPD, this effect may best be explained by a lack of dCTP, the allosteric activator of the enzyme. When cells were exposed to 10 μM dFdC, incorporation of [^{14}C]dCyd into dCTP showed a 4-fold increase. However, dFdC now caused a major inhibition of dCMP deaminase decreasing the radioactivity in the dTTP pool to 29% of control. dFdC 10 μM did not increase the overall phosphorylation of [^{14}C]dCyd as judged by the sum of radioactivity in dCTP and dTTP. Therefore, the increased channeling of [^{14}C]dCyd into dCTP was due to inhibition of dCMPD rather than to an enhanced activity of dCyd kinase. This observation is in agreement with a previous report stating a rather high IC_{50} (0.46 mM) for dFdCTP-mediated inhibition of dCMP deaminase in cell extracts. The present analysis demonstrates that low concentrations of dFdC (0.1 μM) primarily modulate the cellular metabolism of ara-C by inhibition of ribonucleotide reductase. However, greater concentrations of dFdC (10 μM) may additionally inhibit dCMP deaminase.

At last, the question was asked whether inhibition of DNA synthesis by gemcitabine-pretreatment would affect incorporation of ara-C into DNA. It was demonstrated that preexposure of cells with gemcitabine did not impair the activity of DNA synthesis to an extent which would prevent adequate incorporation of ara-C into DNA. By contrast, gemcitabine enhanced incorporation of ara-C into DNA to an extent correlating with the enhancement of intracellular ara-CTP accumulation.

When the kinetics of ara-C incorporation into DNA were followed during 20 h after washout of ara-C, it became apparent that ara-C_{DNA} increased by 3-fold in controll cells, while in dFdC-pretreated cells ara-C_{DNA} rose by 16-fold. It remains to be clarified if ara-C incorporation into DNA occurs as a result of ongoing DNA replication or if it is due to DNA repair activity. Since the cytotoxic effect of ara-C relates to its incorporation into DNA, a correspondingly greater cy-

totoxic effect may be expected in dFdC-pretreated cells.

In conclusion, gemcitabine enhances intracellular accumulation of ara-CTP and correspondingly increases incorporation of ara-C into DNA. Only low concentrations of gemcitabine (0.1 μM) are needed to achieve maximal enhancement. Gemcitabine concentrations of 0.1 μM are well achieved in patients using a 30-min infusion schedule. Since gemcitabine shows a rather favorable toxicity profile, it appears to be an excellent drug for combination treatment and may serve to overcome ara-C resistance also in vivo.

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Immunotherapy

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Experimental Basis for Immunotherapy of Metastases

V. SCHIRRMACHER

Abstract. Novel biological strategies have emerged in recent years for the treatment of cancer. These include transfer of hematopoietic stem cells for marrow reconstitution (BMT) after high-dose chemotherapy, transfer of activated tumor-reactive lymphocytes for adoptive cellular immunotherapy (ADI) of cancer and metastases, postoperative active specific immunization (ASI) with cancer vaccines, gene therapy, differentiation therapy, antisense therapy, immunotoxins or receptor-based therapies. Some biological treatments have already been established for certain forms of cancer, for instance interferon-α administration for Hairy cell leukemia, BCG (Bacillus Calmette Guérin) for superficial bladder carcinoma and allogeneic or autologous BMT for certain leukemic diseases. ADI was reported to have an overall response rate of about 25% in metastatic melanoma and renal cell carcinoma patients. For the vast majority of human cancer, however, such as carcinomas of lung, gastrointestinal tract, breast or prostate, no effective biological treatments have been found yet.

This report does not attempt to review the whole area of immunotherapy but focuses on progress from the author's laboratory.

Immune T Cells Mediate Effective Immunotherapy of Advanced Established Metastases

Recently, we developed a new cellular cancer therapy with unique efficiency even in late-stage disease [1]. In this model, in situ activated tumor-immune T cells, induced in allogeneic, tumor-resistant, MHC identical but superantigen different donor mice (B10.D2) could transfer strong anti-tumor-reactivity into ESb or ESb-MP lymphoma bearing DBA/2 mice. Systemic immune cell transfer into 5 Gy irradiated DBA/2 mice bearing up to 4 week established syngeneic tumors and macrometastases led to massive infiltration of tumor tissues by CD4 and CD8 donor T lymphocytes [4].

Experiments with magnetic immunobead purified CD4 and CD8 immune T cells which were tested either separately or together revealed that there was a strong synergistic effect in the targeting to liver metastases and their eradication. An approximately 25 000-fold excess of metastatic tumor load could be rejected as revealed quantitatively by FACScan analysis of lac-Z gene transfected tumor cells [1]. In contrast to some previous immunotherapeutic approaches and to clinically applied adoptive cell therapies with in vitro activated LAK or TIL cells, the mechanisms achieved with these in situ activated immune cells were effective without transfected or exogenously added IL-2. When we tested whether additional application of IL-2 could further improve therapeutic effectiveness we found to our surprise a clear-cut negative or antagonistic effect of this cytokine when given systemically in conjunction with already effective in situ activated immune cells.

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ADI therapy of tumor bearing mice was characterized by a dramatic decrease of glycogen and an increase of lipid content in the liver [5]. These biochemical changes had defined kinetics of appearance and disappearance. Serum liver enzyme levels (GOT and GPT), indicating liver damage, changed with similar kinetics.

Future studies are aimed at further analysing the mechanism of this very effective system of antitumor effector cell generation and the mechanism of the function of the cells in the DBA/2 tumor bearing host. To this end we have already established a variety of new techniques including the use of lacZ marked tumor cells for single cell detection by whole organ staining or in frozen tissue sections [1], the ability to distinguish donor from host cells by immunohistology and new non-invasive diagnostic procedures in live intact animals by nuclear magnetic resonance (NMR) spectroscopy for high resolution imaging of metastases and for the analysis of metabolic changes at the primary tumor during ADI. The latter procedure of local 31P-NMR-spectroscopy allows to determine tissue metabolites as well as intra- and extracellular pH values [6].

Viral Superantigens Mediate Augmentation of GvL and Suppression of GvH Reactivity

We made use of new resistant recombinant inbred (RI) strains [7] from a cross between DBA/2 and B10.D2 to generate antitumor immune effector cells and to transfer them back into the tumor bearing susceptible strain. By this adoptive immunotherapy (ADI) we were able to transfer strong graft versus leukemia (GvL) reactivity and only weak graft versus host (GvH) reactivity. While immune cells from B10.D2 were transfering both of these activities, we were recently able to genetically separate in the above described new RI mouse lines D2.D-1/9 and D2.D-1/8 GvL from GvH reactivity. In these RI lines, which lacked Mtv-7 provirus encoding the viral superantigen MIs^a, the anti-tumor response was sufficient to generate effector T cells which were able to transfer GvL reactivity into DBA/2 mice without causing lethal GvH disease. We recently found that Vβ6 T cell receptor-positive T cells from donor mice recognize the Mtv-7-positive tumor cells and contribute to the augmented GvL activity.

Immune T Cells Affect Nitric Oxide (NO) Levels in Liver Endothelial and Kupffer Cells and Induce a Special Host Macrophage Response

We were able to demonstrate in the ESbL-lacZ lymphoma model [8] microenvironmental changes in the liver upon either metastatic progression or immune mediated regression [9, 10]. Periods of tumor growth retardation were associated with the production of the cytotoxic molecule nitric oxide (NO) from arginine with the help of the inducible nitric oxide synthase (iNOS). This was shown for ex vivo isolated liver sinusoidal endothelial cells and for Kupffer cells. A breakdown of this NO synthesis coincided with or preceded the final tumor expansion phase. The induction of NO synthesis by liver sinusoidal cells was dependent on mature T lymphocytes. Transfer of immune T cells before the final tumor expansion phase prevented the breakdown of the NO response and metastatic progression.

A further highlight was the discovery of a strong host response induced by the donor lymphocytes. This remarkable GvL associated phenomenon was made in the liver with regard to a subset of macrophages bearing the adhesion molecule sialoadhesin (SER^+ macrophages): in the livers of tumor bearing but not of normal mice their numbers increased between day 1-12 after ADI by a factor greater 30 [4]. These cells, which were of host origin and interacted in forms of clusters with donor CD4 T lymphocytes, may function as scavengers of the destroyed metastases and as antigen presenting cells in the liver.

Tumor Vaccines Are More Immunogenic After Virus Infection and Attachment of Bispecific Antibodies

Immunogenicity of cancer cells represents, in all likelihood, the total sum of the immu-

nogenicity of a number of immunogenic epitopes. Effective anti-cancer vaccines should therefore include this antigenic multiplicity. Vaccination with multi-component, multivalent vaccines rather than single or oligo-component vaccines appears to be necessary to achieve protection against human cancers with inherent antigenic heterogeneity and potential for immune escape.

The aim for us therefore was to define quality and efficacy criteria for tumor vaccines in metastasizing animal tumors, to develop strategies for increasing tumor vaccine immunogenicity and to translate this knowledge into clinical practice.

Our first efforts were based on tumor cell modifications by virus infection, in particular using Newcastle Disease Virus (NDV) [11]. In the meantime we also performed gene transfection studies using MHC genes ([12], cytokine genes (IL-4 [13], interferon-α), and viral genes (HN of NDV) [14] to augment tumor immunogenicity and to compare the effects of these different genetic manipulations. Furthermore, bispecific antibodies were generated to couple defined costimulatory molecules to the virus-infected vaccines [15].

Important for the clinical application were the findings that the immunogenicity of a tumor vaccine depended strongly on the number of tumor cells, the cell viability [16] and the inactivation method [17] used. While MHC class I expression is important for CD8 cytotoxic T lymphocyte (CTL) stimulation, costimulatory signals are known to be necessary as well as accessory adhesive molecules which will facilitate cell-cell interactions. Transfection of a cDNA for the HN (hemaglutinin-neuraminidase) molecule of NDV into antigen-presenting cells (APC) revealed that HN expression at the cell surface increased APC adhesion with lymphocytes [18] and also increased induction of antigen specific CD8 CTL activity (costimulation) [14]. Further modifications of NDV infection which lead to augmented antitumor function were proven to be the local induction of interferon-β [19] and the increased capacity of such modified vaccines to cause non-specific leukocyte recruitment [20] and to augment delayed type hypersensitivity (DTH) reactions [21].

In an attempt to further improve quality and efficacy of such tumor vaccines, we recently created bispecific hybrid antibodies (HyAbs) which could be attached to NDV infected cells because they anchor to HN. They introduce defined costimulatory molecules such as B7 or anti-CD28. We have successfully created for this purpose respective quadromas as well as single chain scFv-reagents for studies in mouse models and in human.

Recently we obtained also interesting results with vaccines based entirely on either DNA or RNA vectors. We were able to show the induction of antigen specific humoral and cell mediated immune responses as well as of protective antitumor immunity using lacZ as a model tumor associated antigen. Parameters for optimal sites of injection and dosage were established.

Clinical Studies

On the basis of our preclinical findings we started in 1988 to develop a comparable NDV-modified autologous tumor cell vaccine for clinical application in cancer patients. This was done stepwise and systematically. It involved a number of additional steps, such as mechanical dissection and enzymatic digestion of cells from a freshly isolated tumor mass, removal of debris, enrichment of tumor cells by Percoll centrifugation and removal of tumor-infiltrating leukocytes by immunomagnetic beads [16]. This autologous tumor vaccine, designated as ATV-NDV, developed by us and our clinical colleagues, thus represents a live cell vaccine defined by tumor cell number, viability and virus infection, which is inactivated by γ irradiation [21, 23]. It is a multicomponent, multivalent vaccine, which closely matches the individual tumor of a patient.

Immune responses to ATV-NDV were monitored in vivo by the DTH skin response to the vaccine as well as to autologous tumor cells without virus infection (ATV control). Patients whose DTH response to ATV could be significantly increased following vaccination with ATV-NDV were considered immunological responder patients [21-24]. Clinical responses in postoperative, adjuvant, active-specific immunization studies were

based on disease-free and overall survival in comparison to the respective historic controls (phase II studies [16, 22, 24]. In advanced renal cell carcinoma, treated with ATV-NDV plus recombinant interleukin-2 and interferon α2b, we recently reported on clinical tumor response rates and 4-year survival rates [25]. Immunological in vitro studies complemented the clinical studies. In ovarian carcinoma, autologous antitumor CTL responses were found in long-term mixed lymphocyte/tumor cell cultures [26] from ATV-NDV-vaccinated patients [26] while no tumor-specific CTL precursors were detected in peripheral blood lymphocytes from ATV-NDV-vaccinated colorectal carcinoma patients [27]. It was, however, possible to excise vaccination sites from colorectal carcinoma patients, and to expand and analyze T lymphocyte microcultures in vitro for lymphokine production [28].

The majority of past and present clinical studies with tumor vaccines are non-comparative in nature and have not focused on vaccine quality criteria. We recently investigated, in comparative studies, quality and efficacy criteria of the vaccine ATV-NDV by analyzing three independent cohorts of patients vaccinated between 1991 and 1995: primary postoperative breast cancer, metastatic pretreated breast cancer and metastatic pretreated ovarian cancer. The vaccines were standardized well in terms of low debris and leukocyte contaminations but they varied randomly in the numbers of viable cells applied and in dead cell contaminations. We were able to test the hypothesis that (1) the number of viable tumor cells and (2) the median vaccine viability could represent parameters indicating the quality of ATV-NDV. A threshold for these two parameters was defined and if both criteria were above this threshold the vaccine would be classified as type A (high quality) and, if the vaccine was missing one or both of these criteria, it would be defined as type B (low quality). All three patient cohorts could be subdivided into two groups, which were similar in general prognostic parameters but had received vaccine of either type A or type B. In primary postoperative breast cancer, after a median observation time of more than 3 years and with more than 30 patients in each sub-group, the relative risk of dying in group A (high-quality vaccine) as compared to group B (low-quality vaccine) was only 0.2 (univariant Cox model). There was a benefit with regard to overall survival ($p = 0.026$) and with regard to disease-free survival ($p = 0.089$) for group A. In the other two cohorts analyzed there were also trends in favour of group A relative to group B [16].

As to the number of viable cells necessary for efficient induction of antitumor immunity, several investigators suggest an amount of about 10^7 viable cells for optimal efficiency. However, numbers of 3×10^6 and lower also showed an efficiency in certain cases. Our own early data concerning sensitization to non-modified tumor suspensions by tumor cell vaccines indicated that 3×10^6 cells and lower would still have immunological capacities. Taking into account that, in those studies, non-purified vaccines were used and small cell sizes were included, it is conceivable that, in the case of viable tumor cells only, even cell numbers of 1.5×10^6 might have immunogenic activity. High cell viability of a vaccine without sufficient viable tumor cells might not provide enough immunogenic tumor antigen for sensitization, and sufficient numbers of viable tumor cells contaminated with a lot of dead cell material might lead to strong non-specific immune interference. This could explain the relatively poor prognosis of group B in comparison to group A patients and suggests that tumor cell number and vaccine cell viability represent two independent parameters of vaccine quality [16]. Although the data were derived from sophisticated methodological and statistical procedures, they can only indicate trends. They suggest, however, potentially important parameters to be tested and validated in prospective randomized studies. To our knowledge there are at present no randomized or nonrandomized trials that prospectively take into consideration the set of vaccine quality parameters analyzed. Also, except for that of Ockert et al. [23], there are no reports from other groups describing purification procedures for an autologous live tumor cell vaccine. Our data [16] suggest that future clinical vaccine trials should take into account a subset analysis for parame-

ters, such as tumor cell number and vaccine cell viability.

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Acute Leukemias VII
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Gene Marking to Establish the Biology of Minimal Residual Disease and Relapse

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Abstract. Gene marking studies have provided valuable information about stem cell biology, the factors that influence gene transfer efficiency, and the mechanism of relapse in patients receiving stem cell rescue as therapy for malignant disease. Second generation studies are beginning to provide even more information about a wider variety of clinical and biological issues. Although marker studies have been useful, it is becoming apparent that the indicator genes used up to now have a number of undesirable characteristics. Future applications of marking, in the hemopoietic system and elsewhere, will require the use of marker elements that will not produce any modification of the cells' behavior. Finally, marker studies have proved safe so far, but follow up of the treated patients continues.

Introduction

Gene marking may be used to track the in vivo behavior of almost any tissue [1, 2], but for the technique to be successful, the marked cell must be followed not just for its entire lifespan, but also for the lifespan of all its progeny. Hence, vectors must be used that integrate in the host cell DNA or that exist as episomes that stably and efficiently replicate with the cell. In practice, this has meant that all marker studies to date have been performed using retroviral vectors. While these agents stably integrate, they also have many limitations, including a low efficiency of transfer and the ability to integrate only in cells in cycle. Moreover, concerns about their safety still linger. These properties have to some extent dictated the design and limited the interpretation of gene marking studies, but they have not precluded investigations that have provided definitive information about the in vivo behavior of normal and malignant cells.

Material and Methods

We used retroviral marking with two closely related vectors, LNL6 and GINa, kindly provided by Genetic Therapy Inc. Both vectors are based on the Moloney virus and encode the neomycin phosphotransferase gene, neo. In all studies, a 6 hr transduction was used in the absence of growth factors, and the efficiency of gene transfer subsequently determined phenotypically (from G418 resistance) and genotypically (by PCR and RT-PCR. Full details are provided in references [3-7].

Results and Discussion

Gene Marking to Determine the Source of Relapse After Autologous HSC Transplantation

While autologous HSC transplantation appears to result in an improvement in survi-

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val in many malignant diseases, relapse remains the major cause of treatment failure [8-11]. The possibility that residual tumorigenic cells may contaminate reinfused HSC has been debated for many years without any definitive evidence to support this hypothesis. One approach to determining if residual malignant cells do indeed contribute to relapse is to mark the HSC product at the time of harvest and then find out if the marker gene is present in malignant cells at the time of a subsequent relapse. Over the last five years studies using this approach have been initiated in a variety of malignancies treated by autologous HSC transplantation including AML, neuroblastoma, CML, ALL, lymphoma, multiple myeloma and breast cancer [3, 12-21]. In these studies marrow (or peripheral blood) HSC have been marked with either the LNL6 or the closely related G1Na retroviral vector which encode the neomycin resistance gene. This marker gene can subsequently be detected in transduced cells, either phenotypically because it confers resistance to the neomycin analogue G418, or genotypically by PCR.

The first marking studies were undertaken at St. Jude Children's Research Hospital between September 1991, and March 1993, in patients receiving autologous BMT as part of therapy for AML or neuroblastoma [3, 13, 14]. In these studies one-third of the marrow was marked in a simple 6 h transduction protocol in the absence of growth factors. Of 12 patients with AML who received marked marrow, four have relapsed [6, 22]. To definitively prove that the marker gene is in leukemia cells it is necessary to have a collateral leukemia specific marker so it is possible to show both this marker and the marker gene in the same cell. For example in one of the relapsed AML patients, the malignant blasts co-expressed CD34 and CD56, a combination not found on normal hemopoietic cells. The cells also had a complex t(1:8:21) translocation resulting in generation of an AML1/ETO fusion transcript that could be identified by PCR. We were therefore able to sort blasts expressing CD34 and CD56 and show co-expression in a single clonogenic cell of both a leukemia-specific marker (the AML1/ETO fusion protein) and the transferred neomycin gene [6]. In two other patients, the malignant cells have contained the marker gene, while the fourth patient was uninformative. His marrow mononuclear cells had low levels of Neo^R by PCR but no blast colonies grew in G418. Since his blasts did not have a leukemia specific marker, the source of the PCR signal could not be determined.

In the neuroblastoma study, five of nine patients have relapsed, and gene marked neuroblastoma cells were detected in four cases [7, 22]. In these patients, identification of marked neuroblastoma cells was confirmed by detection of co-expression of the neuroblastoma-specific antigen GD2 together with the transferred marker gene. Four of the neuroblastoma patients relapsed in their marrow, but the fifth had disease recurrence in an extramedullary site in his liver. Biopsy of this extra-medullary site showed the presence of gene marked neuroblasts [7]. Similar results have been obtained at MD Anderson Hospital in adult patients receiving autologous BMT for chronic myeloid leukemia using an identical transduction regimen [23]. In this study, marked malignant cells co-expressing the t(9;22) and Neo^R transcripts were found in two patients who relapsed following autologous BMT [23].

Not all efforts to use marker genes in this way have been successful. Studies in adult patients receiving ABMT in second remission of ALL or AML, failed to find marked cells at relapse [24], but for logistic reasons only around 10% of the marrow was exposed to vector, reducing the sensitivity of the study, and the efficiency of marking of normal cells was very low (see below).

The results of marker studies in AML, CML and neuroblastoma show definitively that marrow harvested in these three malignancies may contain residual tumorigenic cells that can contribute to disease recurrence at both medullary and extra-medullary sites. However, it is important to point out that the presence of marked cells does not mean that contamination of HSC is the exclusive reason for disease recurrence; indeed it may well be that it is only those patients with a substantial overall burden of residual disease in whom contamination of HSC occurs. Nonetheless, the implication is

that production of a disease free marrow will be at least one of the steps necessary for improving the outcome of HSC rescue, and that marrow purging will be required to achieve this aim. As will be described, gene marking can also be used to evaluate the purging technologies now available.

Gene Transfer to Normal Cells

These first generation marking studies also afforded an opportunity to evaluate the efficiency of gene transfer to normal progenitors. In the St. Jude study the presence of the gene in hemopoietic progenitor cells in vivo was confirmed by clonogenic assays that showed gene-marked progenitor cells in 15/19 patients at one month after autologous BMT [5]. The marker gene continued to be detected and expressed for up to four years in the mature progeny of marrow precursor cells, including peripheral blood T and B cells and neutrophils. It was also detected in lymphoblastoid cell lines and in cytotoxic T cell lines derived from these patients. The level of transfer varied and was highest in marrow clonogenic hemopoietic progenitors, where an average of 6% of myeloid colonies were G418 resistant. In peripheral blood cells, transfer levels were some five fold lower overall and varied between the lineages, being highest in myeloid cells and lowest in B lymphocytes. The reasons for the discrepancy between blood and marrow is discussed below (What is the Best Marker Gene?). The levels of transfer into marrow progenitors are higher than predicted from animal models and may be attributed to the fact that marrow was harvested during regeneration after intensive chemotherapy, when a higher than normal proportion of stem cells are in cycle. Similar results were seen in the MD Anderson study [23].

Limitations of Early Gene Marking Studies

Although these first marking studies of relapse answered important clinical questions [5, 6], they also confirmed the need to increase the efficiency of gene transfer for future applications. Only modest levels of gene transfer into normal or malignant progenitor cells were obtained, even though these cells were obtained from patients recovering from ablative chemotherapy in whom considerable stem cell division might be anticipated. Because the level of transfer is low, it means that a definitive conclusion can be drawn about the contribution of marrow based disease to recurrence only if a relapse is marked. Unmarked relapses might mean that marrow does not contribute to recurrence, but they may equally well mean that relapse is generated by only a few marrow-derived malignant cells which have escaped being marked because of the inefficiency of the process, or that marrow derived cells make only a small contribution to disease recurrence. These alternative explanations may be particularly relevant when marking is used in patients receiving HSC rescue for solid tumors such as breast cancer [25], or if the marked marrow is also purged. In both cases, the numbers of malignant cells that are marked may drop below the detection threshold of the technique. An increased efficiency of marking is one requirement to overcome this limitation.

Second Generation Marking Studies

The useful information gained from these initial studies has lead to the development of more complex gene marking protocols (Tables 1 and 2). These are intended both to overcome the limitations of the earlier studies, and to extend the range of questions that can be addressed by gene marking. The studies have evolved in three ways. First, it is now feasible to use two distinguishable marker vectors in a single individual, to allow the simultaneous study of two distinctly treated cell populations. Secondly, progressively more purified populations of early progenitor cells are being used, an approach prompted by the desire not only to reduce the consumption of vector, but also to increase the efficiency of gene transfer (by allowing a higher MOI) and to allow the "true" pluripotent stem cell to be phenotypically identified. Finally a variety of growth stimulatory agents have been added into transduction protocols in an attempt to increase the efficiency of gene transfer by inducing stem cells to enter cell-cycle and thereby become more susceptible to retroviral gene transfer. As one might anticipate, the marker

Table 1. Transduction regimens in marker studies in autologous BMT

Institution	Disease	Transduction Regimen
St Jude Children's Research Hospital [3, 12-14, 26, 27]	Acute Myeloid Leukemia Neuroblastoma Pediatric solid tumors	6 h. No growth factors
MD Anderson Cancer Center [12, 16, 17]	Chronic Myeloid Leukemia Chronic Lymphoid Leukemia Lymphoma	6 h. No growth factors or 72 h on stroma
National Institute of Health [18]	Breast cancer Multiple myeloma	72 h in IL3, IL6 and stem cell factor or 6 h. No growth factors or 72 h on stroma
University of Indiana [15]	Acute Lymphoid Leukemia Acute Myeloid Leukemia	4 h. No growth factor
Fred Hutchinson Cancer Institute [19]	Lymphoma Solid tumors	5 days in IL1, IL3, Il6, SCF
University of Southern California [12]	Lymphoma Breast cancer	42-h preincubation in IL3, IL6, or autologous plasma then 6 h in retroviral supernatant with no growth factors
Karolinska Institute [20]	Multiple myeloma	24 h in SCF, Il3, IL6, bFGF
University of Minnesota [12]	Chronic myeloid leukemia	
University of Toronto [21]	Multiple myeloma	3 weeks in long-term bone marrow culture

Table 2. Double marking studies

Comparison	Institution
Blood versus marrow	National Institute of Health [18] MD Anderson Cancer Center [12, 16, 17] University of Southern California [12] St. Jude Children's Research Hospital
Efficacy of purging	St. Jude Children's Research Hospital [26, 27]
Unmanipulated CD34 cells versus ex vivo expanded CD34 cells	St. Jude Children's Research Hospital [12]

studies are at their most powerful when all three approaches are combined.

Multiple Vectors May Increase the Information Gained from Marker Studies

Initial marker protocols used a single vector for each patient so that the safety of each vector could be determined individually. As safety data have accumulated, however, it has become permissible to use two distinguishable vectors in a single individual. This greatly increases the potential of gene marking, since these vectors can be used to compare the effects of particular marrow treatment within, rather than between, individuals. Dual marker studies are in progress to determine the efficacy of purging, the relative contributions of peripheral blood and marrow derived stem cells to repopulation after ABMT and the effects of ex vivo marrow pre-treatment with growth promoting agents on subsequent engraftment. An example of each application follows.

Use of Double Gene Marking to Monitor Purging

We have begun second generation studies of marrow purging using two gene markers to compare either marrow purging versus no purging, or two different purging techniques [26, 27]. We are using two closely related vectors, G1N and LNL6, which can be discriminated by virtue of the differing fragment sizes they produce after PCR amplification. In

the AML study, one-third of the marrow is frozen unpurged as a safety backup. The remaining marrow is split into two aliquots which are marked with G1Na or LNL6 and then randomly assigned to purging with the study drugs. At the time of transplant both aliquots are reinfused. Initially we compared 4HC with IL-2, while current studies use 4HC and CD15 [28]. If the patient should subsequently relapse, detection of either marker will allow us to learn if either of these purging techniques is effective. Eighteen patients have been treated on this protocol as of June 1996. To date only one patient has relapsed and the malignant cells were not marked. Of interest, long term transfer has been seen into normal progenitors, even when 4HC purging had rendered the HSC preparation entirely free of colony forming cells. This result confirms that measurement of gene transfer into committed progenitor cells is an inappropriate surrogate for assessing gene transfer into pluripotent progenitor cells. A similar purging protocol for neuroblastoma has enrolled three patients [26].

Use of Double Gene Marking to Compare Long Term Reconstitution from Different Populations of Hemopoietic Progenitor Cells

Double markers can be used to compare the short and long term repopulating ability of different sources of putative stem cells – such as peripheral blood and marrow, or to determine the function of stem cell subpopulations, for example $CD34^+CD38^+$ versus $CD34^+$ $CD38^-$ progenitor cells.

Source of Stem Cells

Until recently, bone marrow was the sole source of HSC for clinical transplantation. The recent availability of alternate sources such as umbilical cord blood and peripheral blood progenitor cells has provided a need to evaluate their relative content of HSC. Currently available animal and preclinical human models can provide only a surrogate analysis of the behavior of HSC following clinical transplantation. By using two distinguishable retroviral markers it is possible to compare quantitatively in vivo and within each patient the short and long term reconstituting capacity of different populations of HSC. This approach has been adopted in a study at NIH where the reconstitution of blood and marrow has been compared in patients receiving autologous hemopoietic stem cell (HSC) rescue as therapy for myeloma or breast cancer [25]. In this study CD34 selected cells from blood or marrow were randomized to marking with two distinguishable retroviral vectors. Although the levels of transfer detected in vivo were low, the marker gene derived from the blood stem cells has been detected for up to 18 months, demonstrating that peripheral blood and marrow HSC can each contribute to long term recovery [25]. Similar studies are underway in adult patients receiving autologous transplant for lymphoma and CLL [12] and at St. Jude in patients receiving autologous HSC rescue for solid tumors.

CD34+ Cell Subpopulations

There is now good evidence that CD34 selected cells will reconstitute patients after autologous marrow transplantation. The dual justification for $CD34^+$ selection in the context of marking is that it reduces the required volume of vector supernatant and that it may be a component of marrow purging in patients in whom the malignant cells are $CD34^-$ or co-express $CD34^+$ and one or more lineage commitment antigens. The marking approach can be used to discover the relative ability of distinct populations of $CD34^+$ cells to produce long lived, multilineage reconstitution following re-infusion. This strategy is now being used to determine if lineage positive and negative CD34 subsets make a different contribution to short and long term reconstitution. Other possibilities include examination of the activity of $Thy1^+$ and $Thy1^-$ populations and of $CD38^+$ and $CD38^-$ cells in vivo. This information will be useful in targeting gene transfer to the most relevant hemopoietic cell subset.

The potential disadvantages of selecting $CD34^+$ cells or their subsets, is that immune reconstitution may be delayed, since mature lymphocytes are not transferred with the graft. There is a concern that this in turn may increase the risks of subsequent neoplastic change if undetected replication competent retrovirus (RCR) has contaminated the vector. Three of eight primates receiving $CD34^+$ selected autografts transduced with producer lines making RCR – as well as high titer vector – subsequently developed lymphomas containing wild type virus [29]. These animals had evidence of multiple cycles of infection with the replication competent virus. These cycles of infection may have been possible because the $CD34^+$ cell autografted monkeys made a poor immune response to what would normally have been an immunostimulatory inoculum of RCR.

The advantages of CD34 selection may thus be counterbalanced – at least in part – by a decrease in the margin of safety for marker studies. Nonetheless, almost all of the more recently approved marker studies use $CD34^+$ cells as the vector target (e.g., [30] and see Table 1).

Use of Gene Marking to Determine Reconstitution of Ex vivo Manipulated Hemopoietic Stem Cells

One of the aims of clinical stem cell transplantation is to minimize the period of marrow aplasia following the procedure, by increasing the number of progenitor cells infused. This approach will only be of value if progenitor cell manipulation does not induce loss of self renewal capacity when the treated cells are returned to the host. One promising approach is to expand the progenitor cells 50 fold or more ex vivo, using stimulatory cytokines and/or stromal components. For example, patients with solid tumors have received autologous HSC using peripheral blood CD34 cells expanded ex vivo with IL-1β, IL-3, IL-6, SCF and Erythropoietin either alone or in combination with unmanipulated cells [31]. The major concern about this approach is that stimulatory cytokines will not only induce primitive cells to expand but will also cause them to differentiate, and lose their capacity for self renewal. Hence, ex vivo treatment with cytokines might produce faster initial engraftment but at the cost of later graft failure. In the study described above, engraftment was not significantly slower than historical controls. However, these patients were not heavily pretreated and did not receive ablative chemotherapy, so it is uncertain what the relative contribution of infused expanded cells and residual cells were to short and long term recovery.

Gene marking can be used to address these concerns about the effects of growth stimulating agents on pluripotent progenitor cells and hence on long term engraftment. If can also be used to determine whether growth factor treatment also increases the efficiency of gene transfer. Initial studies have suggested that current growth factor regimens may not favor either the expansion of stem cells or their transducibility. Primate and subsequent human studies at NIH, used HSC from marrow or peripheral blood and transduced the cells with retroviral vectors after 72 h culture in the presence of IL-3, IL-6 and stem cell factor. Although the level of transfer into committed progenitor cells post transduction was as high as 50%, the levels of the marker gene present in hemopoietic cells post transplant was lower than in the earlier St Jude studies in which growth factors were not used [32, 33], and engraftment with marked cells was not accelerated. This may reflect the different patient population (adults versus children and breast cancer/myeloma versus leukemia/neuroblastoma), but alternatively it may indicate that culture with growth factors commits transduced cells to differentiation, so that high level, long term engraftment with transduced cells is not obtained. To try to resolve this issue, we are selecting $CD34^+$ cells from harvested marrow and splitting them into two aliquots which are marked with LNL6 or G1Na [12]. One aliquot is frozen without manipulation while the other aliquot is expanded ex vivo for 7 days in IL-3, IL-6 and SCF. Both aliquots are infused at the time of transplant. By tracking the two marker genes following transplant we will be able to determine whether ex vivo expansion results in faster initial engraftment and

whether this aliquot contributes to long term reconstitution. Because each patient acts as their own control, it should be possible to discern the effects of any given growth factor regimen on stem cells, even in a small cohort of patients.

The use of growth factors is only one means by which progenitor cells may be expanded and their transducibility increased. Another promising method includes the use of long term cultures, since studies in a canine model have shown high levels of long term gene transfer if transduction is carried out in long term bone marrow culture [34]. This strategy is currently being evaluated in a marking study in myeloma patients receiving autologous BMT [21]. Other groups are studying the effects of adding stromal support components to culture. For example, addition of a recombinant fragment of fibronectin serves to bring retroviral vector and target cell into close apposition and increases the efficiency of progenitor cell marking [35]. The effects of this fragment on marrow repopulating cells and on the efficiency of gene transfer will be tested in at least two human HSC transplant settings, using either neo or the MDR1 gene to ascertain the fate of the treated cells. Finally, recent data suggest that treatment of animals with a combination of G-CSF and stem cell factor, mobilizes marrow progenitor cells that are more primitive and more readily transduced than after treatment with G-CSF alone [36]. Again, marking studies will rapidly allow investigators to determine the true effect of such treatment on the survival and transducibility of the repopulating stem cell.

Gene Marking Studies after Allogeneic BMT

As described earlier, the very first gene marker studies made use of reinfused tumor infiltrating lymphocytes [37]. More recent studies have shown the feasibility and apparent clinical efficacy of adoptive transfer of CTL directed at viral or tumor antigens [38-42]. Gene marking provides a means to evaluate the biological efficacy of such an adoptive transfer approach, particularly in the recipients of allogeneic bone marrow.

What is the Best Marker Gene?

All of the clinical studies reported to date have used *neo* as the reporter gene. *Neo* has the advantage that its safety has been widely studied in many different animal models, and that it can be detected both phenotypically and genotypically. It is unlikely, however, that *neo* is the best marker to use. Integrating and expressing any new gene can modify the behavior of the cell being studied. The activity of neomycin phosphotransferase, the product of the *neo* gene, is not entirely restricted to its named substrate. The enzyme is also likely to phosphorylate cellular proteins, and may thereby modify the growth or differentiation of cells expressing the gene. Such an effect has certainly been observed in the HL60 hemopoietic cell line in vitro [43]. Evidence that a similar phenomenon occurs in vivo comes from the almost uniform observation that the proportion of cells positive for the *neo* marker gene in the circulation is 0.5 to 1 log below the number of cells that are positive as determined by in vitro colony assays in the presence of the neomycin analogue G418 [22]. It may well be that *neo* expression retards progenitor cell growth and differentiation in vivo. Even a modest retardation of progenitor cell development would rapidly diminish the numbers of *neo* progeny detectable. Only under the ex vivo selective pressure delivered by agents such as G418 may it be possible to obtain a true estimate of the frequency of transduced progenitor cells.

There is an alternative explanation for the discrepancy observed between the proportion of cells that are *neo*-positive in vivo and ex vivo. It is now evident that when a new molecule is expressed by a transduced cell, an immune response may result that eliminates the transduced population. For some gene products, such as the hygromycin-thymidine kinase fusion protein, the potency of this response may be striking, eliminating large numbers of transduced cells in less than 48 h [41]. It is uncertain whether an immune response occurs after *neo* transduction. If it does, it is less effective than the response to the Hy-Tk product, since *neo* positive cells can be detected for at least 4 years after infusion, and *neo* marked T cells can

readily be expanded in vivo by appropriate antigenic stimulation [39]. Nonetheless, the possibility of a weak immune response that produces a steady state destruction of mature neo positive cells in vivo cannot yet be excluded.

Because of the limitations of neo as a marker, several alternatives have been proposed. Amongst the most widely studied of these are green fluorescence protein and cell surface markers such as truncated nerve growth factor [44] and CD24 [45]. Unfortunately, many of the same limitations described above will likely apply to these other options. Moreover, aberrant expression of cell surface molecules may lead to unwanted cell trafficking or harmful inter-cellular contacts, even if the cell surface molecule itself has been modified to preclude intracellular signalling. For the moment therefore, neo seems the most appropriate marker gene. Notwithstanding its limitations, the gene has proved its ability to act as a marker and it will continue to be used to address many issues concerning the biology of hemopoietic cells and their progeny.

Safety

A remaining concern about retrovirus-mediated gene transfer into long-lived cells is the possibility of insertional mutagenesis. This mutagenesis could occur at the time of initial exposure to the vector or thereafter, if wild-type retrovirus contaminates the vector or is formed by recombination. This concern has increased following the development of thymomas in monkeys injected with a vector contaminated with wild type virus [29]. So far, no events attributable to mutagenesis have occurred in more than 200 patient years of study, with a maximum follow up of 5 years, but all patients will have prolonged follow-up, including genetic analysis of any tumors that may appear within the next 15 years.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Adoptive Immunotherapy in the Treatment of Post-Transplant Relapse and Epstein-Barr Virus Lymphoproliferative Disorders

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Abstract. Adoptive immunotherapy has recently been recognized as an effective treatment for both post-transplant relapse of chronic myelogenous leukemia (CML) and the treatment of post-transplant Epstein-Barr Virus lymphoproliferative disorders (EBV-LPD). Sixteen patients have received donor-derived leukocytes containing a dose of $CD3^+$ cells ranging between 0.2-1.0 $\times 10^6$ $CD3^+$ cells/kg for treatment of EBV-LPD. Fifteen of the sixteen patients responded with complete eradication of lymphoma. Eight of thirteen evaluable patients developed either acute and/or chronic graft-vs.-host disease (GvHD). Of these eight, six exhibited mild GvHD and two patients developed extensive chronic GvHD. Nine of the sixteen patients are alive and well approximately 6-50 months post-treatment. No patient has had recurrence of lymphoma.

Donor leukocyte infusions have also been effective in the treatment of post-transplant relapse of chronic myelogenous leukemia. We have utilized a dose escalation approach attempting to achieve a graft-vs.-leukemia effect without a graft-vs.-host reaction in patients with hematologic, cytogenetic, or molecular relapse of CML following T cell depleted transplants. Thirty-six patients have been treated with escalating doses of donor leukocytes ranging from 1×10^5-5×10^8 $CD3^+$ cells/kg. Of these patients with hematologic (chronic phase or accelerated phase), cytogenetic, and/or molecular disease, thirty-two have responded. GvHD was seen in eleven of the responding patients and none of the four nonresponders. Of the eleven patients developing GvHD, five developed limited chronic GvHD and six developed extensive GvHD. The dose of T cells required to achieve a response correlated with the status of disease at the time of the donor leukocyte infusion. In addition, the development of GvHD correlated with the cumulative dose of T cells administered. Dose escalations of donor derived lymphocytes result in durable remissions following post-transplant relapse of CML. Patients requiring a smaller dose of donor leukocytes to achieve remissions had a lower incidence of GvHD complicating this therapy.

Introduction

Recently, donor leukocyte infusions have been utilized for the treatment of numerous post-transplant complications, in particular, Epstein-Barr virus lymphoproliferative disorders (EBV-LPD) [1] as well as other post-transplant viral complications [2], and for the treatment of relapsed disease [3, 4, 5]. Although the exact effector cell responsible for these effects is not known, T cells or certain subsets of T cells are thought to be among the primary mediators of these responses.

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Epstein-Barr Virus Lymphoproliferative Disorders

Post-transplantation lymphoproliferative disorders are an uncommon but often lethal complication of both allogeneic bone marrow and solid organ transplantation [6,7]. In the majority of cases, expression of Epstein-Barr virus proteins such as EBNA-1, EBNA-2, EBNA-3, and LMP-1 has linked this virus to the development of these post-transplant lymphoproliferations [8]. Epstein-Barr virus associated lymphoproliferative disorders (EBV-LPD) occur in approximately 1-3% of bone marrow, kidney, or liver transplant recipients, and in 5-13% of heart or heart-lung transplant recipients. In all cases, they arise as a result of profound cell-mediated immunodeficiency. Although "benign" proliferations have been documented in both solid organ and bone marrow recipients, EBV-LPD arising after allogeneic marrow transplantation is most often a monoclonal, malignant proliferation which is refractory to standard therapies. Spontaneous regressions have been noted in some cases but are more commonly seen in the setting of polyclonal or oligoclonal proliferations in solid organ recipients. In these patients, cessation of immunosuppressive therapy can result in resolution of the lymphoproliferations [9].

EBV-LPD following BMT usually presents as a malignant lymphomas with high grade histology, i.e., diffuse large cell or immunoblastic types. These are most often of B-cell immunophenotype and donor cell origin. The onset is generally within the first 4-6 months post-BMT, a period during which EBV-specific cytotoxic responses remain low or undetectable [15]. In addition, they tend to occur in the setting of increased immunosuppression, i.e., in recipients of mismatched or unrelated grafts, recipients of T-cell depleted transplants, or patients receiving immunosuppressive therapy for the prevention or treatment of graft rejection or graft-vs.-host disease (e.g., cyclosporine, steroids, anti-thymocyte globulin or monoclonal antibodies) [10,11].

Until recently, the development of EBV-LPD was an often fatal complication of BMT. Treatment with chemotherapy or radiotherapy has been unsuccessful in the treatment of EBV-LPD. The combination of IFN-α and intravenous gammaglobulin has been reported to cause regressions of these lymphoproliferative disorders but has not been consistently reproducible [12]. Treatment with anti-B-cell monoclonal antibodies has shown some efficacy, but this has been most promising in BMT patients with polyclonal or oligoclonal proliferations [13].

The period during which these patients are at greatest risk for developing EBV-LPD (i.e., the first 4-6 mos. post-BMT) is a time of profound deficiency of T-cell function induced by immunoablative cytoreduction or concomitant immunosuppressive therapy. Therefore, major-histocompatibility-complex-restricted cytotoxic T cell responses to viruses are delayed in these patients. Since the frequency of EBV-specific cytotoxic T cells in normal, seropositive donor is approximately 1/400-1/25 000, and since these lymphoproliferations are EBV-transformed donor cells growing in the host, we hypothesized that infusions of donor-derived leukocytes containing cytotoxic T cell precursors that have been presensitized in the donor to EBV might effectively control or eradicate the EBV-transformed donor type cells.

We previously reported on the efficacy of donor leukocytes in the treatment of 5 patients with post-transplant EBV-LPD who achieved complete and durable remissions of disease following infusions of small doses of peripheral blood mononuclear cells derived from their EBV seropositive marrow donors [1]. We have since treated an additional 11 patients with donor leukocytes. Results of treatment and patient outcomes will be presented.

Chronic Myelogenous Leukemia

Since the original reports of Kolb et al. [14] demonstrating the efficacy of large numbers of donor leukocytes in inducing remissions in CML patients relapsing post-transplant, numerous investigators have reported similar findings, thus confirming the efficacy of DLI in treating post-transplant relapse of CML [3, 4, 5]. However, although highly effective, with

nearly 80% of patients treated in a chronic phase or cytogenetic relapse responding to therapy, most series have reported a high incidence of GvHD in patients achieving remission.

In an attempt to limit GvHD following these infusions, we investigated whether substantially lower numbers of donor leukocytes could induce remissions, and if so, whether this would result in a lower incidence of GvHD than had been reported following DLI therapy. We therefore designed a dose escalation study to assess whether the effect of donor leukocyte infusions could be achieved at T cell doses lower than that which would induce clinical GvHD. Forty-one patients have been treated on such a dose escalation trial. The results will be discussed.

Patients – Methods – Results

EBV-LPD

Sixteen consecutive patients developed EBV-LPD occurring a median of 113 days post-transplant. All patients were recipients of T cell depleted transplants by either the soybean lectin agglutination and sheep red blood cell rosette method (SBA^-E^-) (n = 15) or by a monoclonal antibody (T10B9) depletion method (n = 1). The source of marrow in these patients was as follows: 8-HLA identical siblings, 1-mismatched sibling, 7-unrelated donors. Fourteen of the 16 patients were on no immunsuppressive therapy when they developed the EBV-LPD. Two patients were receiving cyclosporine and/or steroids. Clinically these patients presented with fever, localized or diffuse adenopathy, tonsillar enlargement, extranodal tumors involving the CNS, nasopharynx, lungs, gut, and liver.

All 16 patients presented with diffuse, large cell lymphomas of B-cell phenotype. Eight of 10 evaluable specimens were of donor cell origin, and two were mixed donor/host but primarily donor. Clonality of the specimens was documented by either clonal rearrangement of the IgH chain gene or by size homogeneity of genomic termini of EBV episomal DNA. By one or both of these methods, 9 of 11 evaluable samples were monoclonal.

Patients were treated with single infusions of peripheral blood mononuclear cells from their normal seropositive donors providing doses of $0.2\text{-}1.0 \times 10^6$ $CD3^+$ T-cells/kg to recipients of unrelated grafts and $0.5\text{-}1.0 \times 10^6$ $CD3^+$ T-cells/kg to recipients of HLA-matched sibling grafts. The doses administered were calculated to provide a dose of T-cells 10-fold higher than the threshold dose for acute GvHD in HLA-matched sibling recipients given at the time of the primary BMT, but still 10-fold lower than that provided by an unmodified graft. The infusions were well tolerated. Complete pathological and/or clinical resolution of the EBV lymphomas were observed in 15 of the 16 patients. These responses were documented pathologically as early as 8-16 days post-infusion. Clinical remissions were achieved within 14-30 days. Nine of 16 patients have survived in sustained remission without further treatment for approximately 6 to 50 months since leukocyte infusion. Two patients died 8 and 16 days post-infusion from sepsis and interstitial pneumonia, respectively. At autopsy, there was no microscopic evidence of residual lymphoma in either patient. Five other patients have died from leukemic relapse (n = 3), late sepsis with chronic GvHD (n = 1), or persistent EBV-LPD (n = 1). Eight of thirteen evaluable patients developed GvHD following DLI: 2 pts with acute GvHD (Gr I, II); 5 pts with limited chronic GvHD; and 2 pts with extensive chronic GvHD.

Patients were also studied both pre and post DLI infusions for cytotoxic T-lymphocyte responses to autologous EBV transformed B-lymphoblastoid cell lines (BLCL) derived from the normal marrow donor and the transplanted host using

1. a bulk culture assay to measure cell mediated cytolysis against ^{51}Cr labelled EBV-transformed lines and
2. a limiting dilution assay to measure the frequencies of EBV-reactive CTL precursors.

Our results suggest that in some recipients of both unmodified and T-cell depleted marrow grafts, re-emergence of EBV-BLCL

reactive T-cell populations may be observed as early as three months post-BMT, while in others, such CTLp frequencies may not develop until late after transplant [15]. In addition, these studies have demonstrated that infusions of donor leukocytes can not only induce a marked increase in the overall number of T-cells in the transplant patient, but can also induce a rapid expansion of EBV-specific cytolytic T-cells increasing their frequency to levels equal to or in excess of those detected in immunologically normal seropositive individuals. Patients studied at the time of EBV-LPD demonstrated no ability to lyse EBV-transformed targets derived from the donor. However, within two weeks of receiving a dose of donor leukocytes, these patients exhibited EBV specific cytotoxicity and demonstrated EBV-specific CTLp frequencies in the range of normal seropositive donors.

Although this approach has proven efficacious in the treatment of post-transplant EBV-LPD, the usefulness of this approach in both recipients of unrelated and mismatched related transplants where one would anticipate greater allodisparity has not been proven. Therefore, future strategies must focus on isolation of HLA-restricted, virus-specific T-cells selected on the basis of their inability to react presence of alloreactive T cells in the infusion, with their potential for inducing GvHD, limit the against uninfected target cells of host or donor origin [16]. Alternatively, genetically modified, drug susceptibility gene positive, alloreactive T-cells or virus-specific T-cells could circumvent these problems by providing a mechanism for in vivo destruction due to drug sensitivity [17].

CML

Thirty-six patients with either molecular, cytogenetic, or hematologic relapse of CML following a T cell depleted allogeneic transplant were enrolled in a dose escalation trial of donor derived leukocytes containing targeted doses of $CD3^+$ cells/kg of recipient weight. All patients were recipients of SBA^-E^- grafts derived from either related (n = 33) or unrelated (n = 3) donors. The first 10 patients enrolled on study with either cytogenetic or hematologic disease began treatment at a T cell dose of 1×10^5/kg. There were 8 planned dose levels ($CD3^+$ cells/kg of recipient weight): 1×10^5, 5×10^5, 1×10^6, 5×10^6, 1×10^7, 5×10^7, 1×10^8, 5×10^8. The starting dose of T cells was chosen because it represented the number of clonable T cells that, if administered on the day of transplant, is capable of causing GvHD. Dose escalations during the earlier part of this trial were performed at a median of 6 weeks (range, 4-33 weeks).

No responses in these patients with cytogenetic or hematologic disease were documented at doses lower than 1×10^7/kg. However, 12 of the first 30 patients with cytogenetic or hematologic relapse responded to doses of 1×10^7/kg, with only 2 of the responders developing GvHD.

Earlier studies by Mackinnon et al. [18] had demonstrated that the presence of minimal residual disease, detected using reverse transcriptase-polymerase chain reaction (PCR) able to identify *bcr-abl* transcripts, had a high predictive value for subsequent relapse after T cell depleted transplants. Given this information, and noting that GvHD could be separated from graft-vs.-leukemia activity by using small, incremental doses of T cells, modifications were made to the original study design such that patients with evidence of molecular relapse (defined as a positive RT-PCR for *bcr-abl* on both blood and bone marrow samples on two occasions, three months apart) were eligible for entry on this trial. Therefore, although patients with evidence of hematologic disease were enrolled at a starting dose of 1×10^7/kg, patients with evidence of molecular or cytogenetic relapse only were entered at a lower starting dose of 3×10^6/kg.

Ten patients with either molecular (n = 4) or cytogenetic (n = 6) relapse of CML received DLI containing T cells at 3×10^6/kg. Of these, 6 have responded with complete cytogenetic and molecular remission. The 4 patients failing this dose have been escalated to the next dose level of 1×10^7/kg.

Overall, 32 of the 36 patients have responded to DLI. The cumulative dose required to achieve a response correlated with disease status at the time of the DLI. For ex-

ample, patients with either chronic or accelerated phase required higher cumulative doses of T cells to achieve response than did patients with either molecular or cytogenetic relapse only. Of the 32 responders, 11 patients have developed GvHD. Of these 11, 5 patients developed limited chronic GvHD and 6 extensive chronic GvHD. All patients developing extensive cGvHD received cumulative doses $\geq 5 \times 10^7$/kg. Seven patients have died following DLI: one of progressive leukemia, one of idiopathic interstitial pneumonitis occurring at the time of cytogenetic remission, two of infection in the setting of bone marrow aplasia following DLI, one of fungal infection in the setting of cGvHD, one of gastrointestinal hemorrhage while on steroids for treatment of GvHD, and one splenectomized non-responder died of pneumococcal sepsis after becoming noncompliant with penicillin prophylaxis. Bone marrow aplasia was seen in six patients. In four, all recipients of sibling donor grafts, the aplasia was transient and did not require a bone marrow boost. In two, both recipients of unrelated donor grafts, aplasia resulted in death before donor marrow could be obtained.

In conclusion, our data indicate that patients treated at a time of minimal residual disease, or with evidence of cytogenetic relapse only, may achieve remission with little or no toxicity when treated with DLI containing $\leq 1 \times 10^7$/kg. Therefore, our current strategy in the treatment of patients with CML is to perform T cell depleted transplants using the SBA^-E^- method, where the incidence and severity of acute GvHD is significantly reduced and where there is virtually no chronic GvHD. Patients are then followed for evidence of molecular relapse or persistence of disease at 3-month intervals post-transplant. If molecular disease is identified on two consecutive samples of both blood and bone marrow, patients receive a single dose of 3×10^6/kg. If after a 4-month observation period, they remain persistently PCR positive or have developed evidence of cytogenetic relapse, they proceed with further DLI escalation. Further study of this approach is needed to determine if this strategy will yield improved long term disease free survival in patients undergoing allogeneic transplants for the treatment of CML.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Induction of Graft-versus-Leukemia-Activity after Peripheral Blood Progenitor Cell Transplantation (PBPCT)

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Abstract. Using a murine transplantation model we have investigated the induction of GvL activity with allogeneic peripheral blood progenitor cells (PBPCs). We compared the influence of allogeneic PBPC and BM grafts on GvHR and leukemic relapse in mice bearing a B-lymphoblastic leukemia (A20). Furthermore, we evaluated the impact of T cell depletion on the risk of relapse and determined the effectiveness of ex vivo treatment with NK-cell activating cytokines as a compensation for the loss of T-cell derived factors stimulating natural cytotoxicity. Methods: After pretreatment of Balb/c (H-2^d) recipients with 7.5 Gy of total body irradiation, 2×10^7 rhG-CSF-mobilized PBPCs of syngeneic or MHC-identical DBA (H-2^d) mice were transfered. Selective T-cell depletion (TCD) was performed by immunomagnetic purging with a monoclonal antibody directed against CD3. In some experimental groups, T-cell-depleted PBPCs were incubated with 200 U/ml IL-2 and 100 U/ml IL-12 for 24 hrs. To investigate antileukemic activity in vivo, recipient mice were inoculated with 1×10^5 A-20 cells (a B-lymphoblastic leukemia of Balb/c origin) 2 days prior to PBPCT. Results: The mortality rate due to GVHD was identical after allogeneic BMT and allogeneic PBPCT, although PBPC grafts contained the fourfold amount of CD3+ T cells than BMC grafts (61% vs. 15%). The relapse rates were 80% after syngeneic PBPCT and 60% after allogeneic BMT. After allogeneic PBPCT, a relapse rate of 34 % was observed, indicating significantly ($p < 0.05$) superior GVL activity of PBPCT. After TCD of allogeneic grafts with anti-CD3, the incidence of GvH-related mortality was below 5% but leukemia free survival was decreased to 25% and thus was similar to syngeneic PBPCT (17%, $p < 0.05$). When CD3-depleted grafts were incubated with IL-2 and IL-12, 45% of the animals remained free from leukemia. However, the difference was statistcally not significant. Our results suggest that stronger GVL effects can be induced by transplantation of PBPC as compared to BM grafts and that the ex-vivo activation of residual MHC-matched NK-cells with IL-2 and IL-12 does not fully compensate for the abrogation of GvL-activity after depletion of CD3-positive T-cells.

Introduction

Clinical and experimental studies indicate that the transfer of allogeneic marrow cells can exert a beneficial antileukemic effect [1, 2], a phenomenon called graft-versus-leukemia (GvL) effect. Unfortunately, the antileukemic effect is often associated with clinically overt graft-versus-host disease (GvHD) [3]. During the last years, several attempts have been made to retain or augment

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the antileukemic potential of cells in the graft while avoiding GvHD. We have previously described GvL effects without graft-versus-host reaction in a murine transplantation model [4]. In this model, antileukemic efffects were correlated with NK-cell-mediated lysis determined in vitro.

Recently, first successful allogeneic peripheral blood progenitor cell transplantation (PBPCT) studies have been reported [5, 6] and it is possible that the transplantation of PBPCs will replace BMT after myeloablative chemotherapy for treatment of hematologic malignancies. The major concern with allogeneic PBPCT was related to the assumption that the large numbers of T-cells contained in PBPC grafts [7] might give rise to an increased incidence or severity of GVH reactions. However, the experience with larger cohorts of patients receiving allogeneic progenitor cell transplants [8, 9] suggests that the risk of developing acute GVHD may not be substantially higher after transplantation of PBPCs as compared to BMT.

Following transplantation of T-cell-depleted bone marrow grafts, increased frequencies of graft failure and leukemia relapse were observed [10]. We have demonstrated that the increased risk of engraftment failures is a consequence of the loss of progenitor cells after the technical maneuvers necessary to deplete the T-cells [11, 12]. Consequently, this problem should be avoidable with higher numbers of progenitor cells as contained in PBPC harvests. The increased risk of leukemia relapse [13] was assumed to result from the loss of T-cells. Recent data, however, suggest that NK cells can also exert high cytotoxic activity against leukemia targets [14, 15]. Due to the high number of NK cells contained in leukapheresis products (up to 20 times more than in a bone marrow harvest) [7], the number of antileukemic effector cells could be significantly higher in T-cell depleted PBPC harvests than in T-cell depleted BM grafts resulting in the conservation of GvL activity following transplantation of T-cell-depleted allogeneic PBPC grafts.

The present preclinical studies were performed to compare GvL and GvH reactivity after PBPCT and BMT and to investigate the influence of T cell depletion on GvL activity after PBPCT. Finally, ex vivo treatment of the PBPC grafts with NK-cell-activating cytokines was tested for its effectiveness to compensate for the putative loss of T-cell-derived stimulatory factors.

Materials and Methods

Animals. Balb/c (H-2^d) and DBA (H-2^d) mice were bred and kept at the animal facilities of the University of Kiel. All animals were housed in conventional cages, 7 to 10 animals to a cage, and were given non-sterilized food and water ad libitum. Cotrimoxazole was given for 40 days after PBPC transplantation.

Peripheral Blood Progenitor Cell Transplantation. All recipients received a lethal dose of 7.5 Gy total body irradiation (TBI) delivered by two opposing Cs^{137} sources at a dose rate of approximately 1.5 Gy/min. Mice intended to serve as donors of PBPCs had their spleens removed at 8-10 weeks of age. Splenectomy was performed under general anaesthesia at least 14 days prior to transplantation. Starting 5 days prior to collection of PBPCs, 5 μg of rhu-met-G-CSF (Filgastrim, AMGEN, Thousand Oaks, CA) were injected subcutaneously twice daily. The last injection of Filgastrim was given 2 h before harvesting the PBPCs. The mice were then anticoagulated with heparine, anesthezied, and killed by cervical dislocation. The peripheral blood was collected under sterile conditions by dissection of both carotid arteries. For each single experiment, the peripheral blood from 4 to 5 donors was pooled. Erythrocytes were lysed by incubation of PB in 0.15 M ammoniumchloride buffer at 20 °C for 5 min. The cell number was adjusted to 6×10^7 nuclear cells (NC)/ml. The cells were injected intravenously 2 h after irradiation.

T-Cell Depletion. Donor T-cells were removed from the PBPC graft by immunomagnetic separation. Magnetic beads (Dynal, Oslo), coated with goat-anti-mouse immunoglobulin were coupled with anti-murine CD3 MoAb (KT-3, Serotec, Germany). The CD 3 MoAb was used at a concentration of

11 µg/4 × 10^8 beads. Coupling of MoAb and magnetic beads was performed at 4 °C for 30 min. The ratio of beads / target cell was 6:1. After coincubation of the magnetic beads with the PBPC graft at 21 °C for 1h, CD3-positive cells were removed with a permanent-magnetic device (Dynal, Oslo). Untreated and T-cell-depleted grafts were examined by FACS analysis for their content of residual CD3-, CD4-, CD8-positive T-cells using directly conjugated antibodies against these antigens (145-2C11, L3T4, Ly-2, Pharmingen, Germany). In addition, the number of myelocytic cells (Gr-1/11-26c.2a, Pharmingen, Germany), and of Ly49c-positive NK-cells (5E6, Pharmingen, Germany) was determined. In general, vitality (determined by propidiumiodid) was > 98%.

Cytokine Treatment. T-cell depleted PBPCs were incubated in 25-cm^2 tissue culture flasks (Greiner, Frickhausen, Germany) at a concentation of 1-2 × 10^6 / ml with 200 U/ml IL-2 (Genzyme, Munich, Germany) and 100 U/ml IL-12 (Genetics Institute, Massachusets) in RPMI 1640 + 5% FCS for 24 h at 37 °C with 5% CO_2. Previous experiments have indicated that these concentrations induce nearly maximum effects with regard to cytotoxic activity of purified allogeneic NK-cells in vitro. Effector cells were washed two times with RPMI before injection.

Progenitor Cell Colony Assay. The progenitor cell content of the grafts was determined with the Methocult GF M3434 kit (Stemcell Technologies, Vancouver, Canada). The assays were performed according to the protocol of the manufacturer. CFU-GM were counted 12 days after plating of 1.5 × 10^4 nucleated cells per dish. The cell concentration was 1.5 × 10^5 NC / ml.

Leukemia Cells. A20 is a B-cell leukemia/lymphoma of Balb/c origin that occured spontaneously in a 15 month old mouse [16]. It is nonimmunogeneic in syngeneic hosts. A20 cells were continuously maintained in culture in RPMI 1640 + 5% FCS at 37 °C and 5% CO_2. For in vitro cytotoxicity tests, fresh cultured cells were used. For in vivo experiments, spleen cell suspensions were obtained from tumor-bearing balb/c mice. After inocculation with A20 cells and development of hepato-splenomegaly, donor mice were killed and their spleens were removed. Spleen cell suspensions containing close to 100% of these in vivo passaged leukemia/lymphoma cells were stored in liquid nitrogen and used for further experiments.

Assessment of Leukemia Relapse, GvHD and Graft Rejection. All animals were examined daily and necropsied after death. Signs of GVHD (weight loss, rough fur and gibbus) were documented. Periperal blood counts were performed every 3 days from day 7 to full recovery of hematopoiesis or death.

Death due to leukemic relapse was defined as death with macroscopic evidence of tumor and liver weight > 1.5 g and spleen weight > 0.15 g. For some animals in each group histologic examination of liver and spleen was performed. Animals with hepato-splenomegaly were without exception found to harbour leukemic cells. Healthy mice of the same age were found to have a liver weight of 1.3 g ± 0,2 g and a spleen weight of 0.1 g ± 0.02 g.

Death due to GVHD was defined as death with recovery of hematopoiesis (leukocytes > 3/nl and thrombocytes > 50/nl) and clinical signs of GVHD as weight loss, rough fur, hair loss, and gibbus. In some animals with clinically overt GVHD histologic examination of skin and liver was performed which revealed changes compatible with acute GVHD.

Death due to graft failure/graft rejection was defined as death between day 6 and 30 after transplantation with leukocytes < 1/nl and granulocytes < 0.5 / nl.

Assessment of NK-Cell Activity. Effector cells were prepared by density gradient centrifugation of peripheral blood cell suspensions of syngeneic Balb/c and allogeneic DBA mice. Target cells were obtained from cell culture and were labeled with 7.4 MBq $Na^{51}CrO_2$ (Amersham-Buchler, Braunschweig, Germany) in 0.5 ml complete medium for 1 h. They were washed 3 times with complete medium and added at a concentration of 1 × 10^4 cells/well in round bottomed microtiter plates (Nunc, Denmark). Effector cells were added at various effector:target

ratios in a final volume of 200 µl/well. The plates were incubated for 4 h at 37 °C in a humidified atmosphere with 5% CO_2. Maximum chromium release was ensured by addition of 10% Triton. The culture supernatant was harvested with a Scatron Titertek System (Scatron, Suffolk, GB) and counted in a gamma counter (Beckmann, Heidelberg, Germany). The percentage of specific lysis was calculated as (Experimental cpm - spontaneous cpm) / (Maximum cpm - spontaneous cpm) × 100. All determinations were made in hexaplicate and data were calculated as mean ± SE. Each experiment was done 7 times.

Assessment of Long-Term Chimerism. In a number of surviving animals, long-term chimerism was investigated by FACS analysis determining the presence of the Lyt 1.1 (CD5, clone H11 86.1, Pharmingen, USA) antigen on spleen cells. This antigen exists in two different forms: Balb/c mice express the isoform Lyt 1.2, whereas DBA/2 mice are known to express the Lyt 1.1 isoform of this antigen. Animals were killed on day 100 post transplant, the spleens were removed and a single cell suspension was produced. Untreated animals of Balb/ c and DBA/2 origin were used as negative and positive controls, respectively. If the recipient mouse showed the same fraction of splenocytes (± 6%) positive for Lyt 1.1 as the DBA control animal, it was assumed to be a long-term chimera.

Statistical Analysis. Survival and freedom from leukemia were calculated according to the method of Kaplan and Meier. The experimental groups were compared using the Wilcoxon test. The calculations were done on a PC with Statistica statistical software.

Results

Cellular Composition of the Graft. Following treatment of DBA (H-2^d) mice with subcutaneous injections of 125 µg/kg rhu-G-CSF twice daily for 5 days, the number of white blood cells increased from 14.900/µl to 51.200/µl. In parallel, the number of CFU-GM rose from 2.3/µl to 25.0/µl. We could demonstrate that 2×10^7 G-CSF-mobilized PBPCs are sufficient to ensure engraftment of allogeneic MHC-identical grafts (data not shown). and this number of cells was used in all subsequent experiments. Following immunomagnetic purging, the amount of CD3-positive T-cells was reduced from 21.6 to 0.3% (Table 1). In particular, the number of CD3/CD4-positive cells decreased from 1.4×10^8/kg to 3×10^6/kg and the number of CD3/CD8-positive cells fell from 8.4×10^7/kg to $< 1 \times 10^6$/kg. Conversely, the percentage of transplanted NK-cells was slightly increased.

GvL and GvHD After BMT and PBPCT. To investigate GVL effects, Balb/c mice bearing the lymphoid leukemia A20 were tranplanted. After i.v. injection of 1×10^5 A20 cells, all untreated animals died after two months (median 28 days). TBI with a dose of 7.5 Gy and subsequent syngeneic PBPCT resulted in a prolongation of the time to relapse up to 43 days and 20% of the animals remained free from leukemia. Transfer of allogeneic MHC-matched PBPC after the same pretreatment

Table 1. Cellular composition of untreated and T-cell depleted PBPC grafts mobilized by subcutaneous injection of 2 × 125 µg G-CSF given for 5 days

Donor	T-cell depletion	Cytokine incubation	Percentage of nucleated cells positive for					CFU-GM/µl[b]
			CD3/CD4	CD3/CD8	CD19	5E6[a]	Gr-1	
Balb/c	None	None	19.5 (4.1)	9.6 (2.8)	12.4 (3.0)	3.9 (0.8)	55.0 (6.4)	23.6 (6.7)
DBA	None	None	14.5 (2.8)	8.4 (2.1)	14.3 (2.2)	4.6 (2.1)	57.4 (5.6)	25.0 (4.5)
DBA	Anti CD3	None	0.3 (0.2)	0.1 (0.1)	18.0 (1.9)	7.4 (2.4)	63.8 (6.6)	n.d.
DBA	Anti CD3	IL-2/IL-12[c]	0.3 (0.3)	0.1 (0.1)	19.6 (3.4)	8.6 (3.0)	66.9 (7.4)	n.d.

[a] The monoclonal antibody 5E6 marks a subpopulation of NK cells.
[b] CFU-GM were counted on day +12 post seeding.
[c] T-cell depleted PBPCs were incubated with 200 U/ml IL-2 and 100 U/ml IL-12 for 24 h.

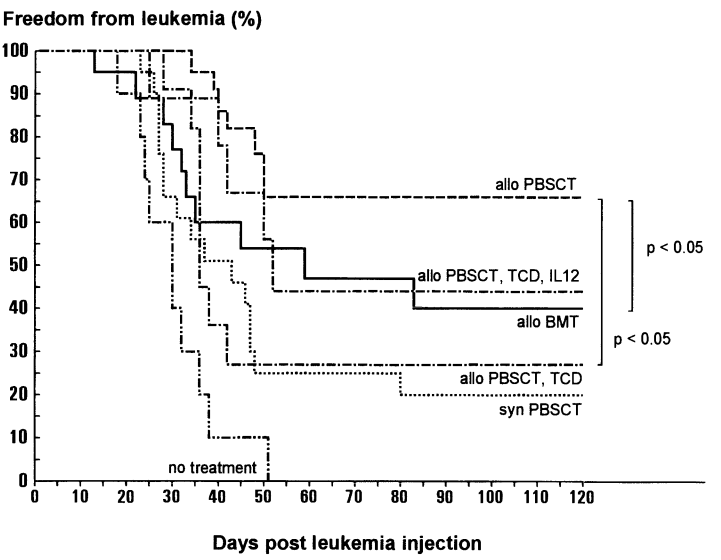

Fig. 1. Freedom from leukemia of Balb/c mice injected with 1 x 10^5 cells of the B-lymphocytic leukemia cell line A20. Two days after leukemia cell injection, recipients were treated with 7.5 Gy of TBI followed by transplantation of either syngeneic or allogeneic (DBA) BM cells or PBPCs. In some experimental groups, allogeneic grafts were T-cell-depleted and activated with IL-2 (500 U/ml) and IL-12 (100 U/ml). Transplantation of untreated allogeneic PBPCs resulted in a significantly lower relapse rate as compared to syngeneic PBPCs, allogeneic BM cells or allogeneic T-cell-depleted PBPCs ($p < 0.05$)

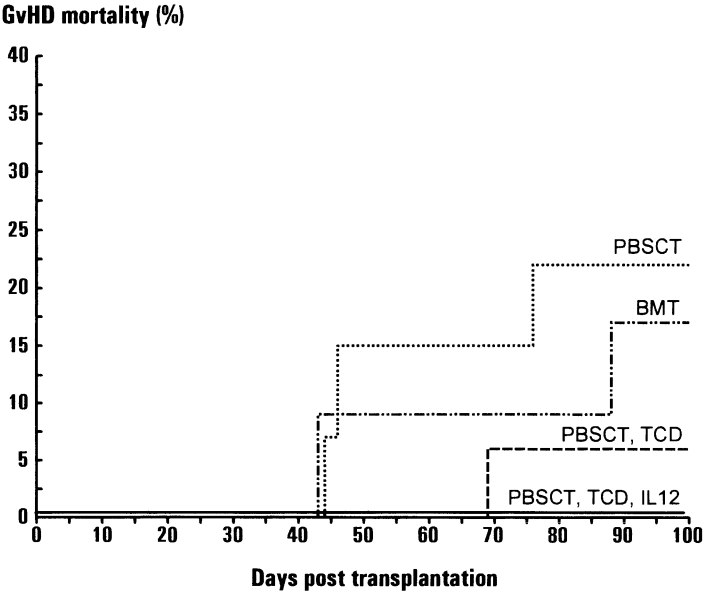

Fig. 2. Mortality after transplantation of allogeneic (DBA) grafts. Balb/c mice were irradiated with 7.5 Gy and received 2 x 10^7 G-CSF-mobilized BM cells or PBPCs which were either untreated, T-cell depleted only, or T-cell-depleted and activated with IL-2 (500 U/ml) and IL-12 (100 U/ml). T cell depletion was performed by immunomagnetic removal of CD3-positive cells by anti-CD3 coated magnetic beads, resulting in less than 1% CD3+ cells as shown by flow cytometry. All animals died with signs of GvHD (weight loss, rough fur and gibbus)

of the recipient caused a significantly ($p < 0.05$) lower relapse rate with freedom from leukemia of 66% demonstrating a strong GVL activity. Freedom from leukemia is 40% with a median time to relapse of 58 days after allogeneic BMT and thus significantly lower than after allogeneic PBPCT ($p < 0.05$) (Fig. 1).

However, the antileukemic activity of allogeneic grafts was associated with the occurence of GvHD (weight loss, rough fur, hair loss and gibbus). The cumulative incidence of mortality due to GvHD was comparable in recipients of allogeneic PBPCs and BMT (Fig. 2). All surviving animals showed donor-type hematopoiesis.

T-Cell Depletion and Antileukemic Efficacy of Allogeneic PBPC Grafts. Immunomagnetic purging of allogeneic PBPC grafts reduced the proportion of CD3-positive cells to 0.4% (Table 1). However, the number and function of graft-derived NK-cells was not negatively affected by this procedure. As indicated in Fig. 2, the incidence of GvH-related mortality decreased to 5%. All of the animals engrafted and showed donor-type hematopoiesis.

The GvL effect was significantly ($p < 0.05$) reduced after depletion of CD3+ cells from the PBPC harvest (Fig. 1). Whereas 71% of the animals remained leukemia free after unmanipulated PBPCT, freedom from leukemia was only 25% after T-cell-depleted allogeneic transplants and thus similar to unmanipulated syngeneic grafts.

Ex-Vivo Activation of T-Cell Depleted PBPC Grafts with IL-12 and IL-2. In order to restore the graft-versus-leukemia activity lost after T-cell depletion, we have used IL-2 and IL-12 to activate NK-cell-mediated antileukemic cytotoxicity (Fig. 3). After removal of CD3-positive cells, the remaining PBPCs were incubated with 500 U/ml IL-2 and 100 U/ml IL-12 for 24 h. None of the animals died due to GvHD (Fig. 2) or graft rejection and all surviving animals proved to be chimeras. The GvL activity of unmanipulated transplants, however, was not reached by transplantation of cytokine-incubated CD3-negative PBPC grafts (Fig. 1). Only 45% of the animals survived free from leukemia. The median survival time was slightly prolonged from 35 to 50 days after pretreatment of the grafts with IL-12 and IL-2.

Discussion

We did not find substantial differences between BM or PBPC grafts with respect to their GVHD-inducing potential. Roughly the same rates of mortality due to GVHD (25%) were observed after allogeneic BMT and PBPCT. With approximately threefold more T-cells in a PBPC graft as compared to marrow this is a surprising yet unexplained finding which is in line, however, with clinical observations reported so far [8].

Our data suggest an advantage of PBPC grafts over BM grafts with regard to antileukemic activity which could be caused by higher number numbers of T cells and NK cells in the PBPC harvests. Both cell types have been shown to be involved in GVL activity [14].

GvHD is caused by alloreactive T-cells and removal of CD3-positive cells can prevent GvHD-related mortality in animal models [12, 16]. Clinical trials, however, have

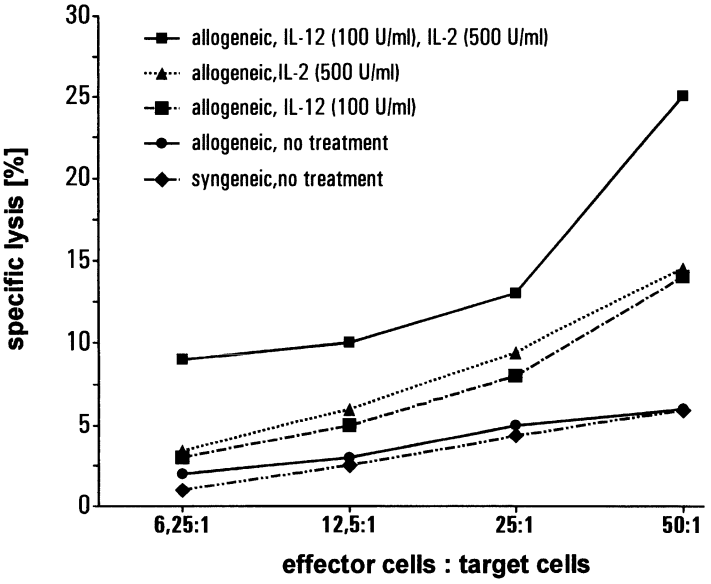

Fig. 3. Cytolytic activity of allogeneic (DBA) and syngeneic (Balb/c) effector cells against cultured A20 leukemia cells. Effector cells were splenocytes which were either untreated or activated with IL-2 (500 U/ml) and/or IL-12 (100 U/ml) for 24 h. The cytolytic activity was determined in a conventional 4 h Cr-release assay. Data were pooled from 6 separate experiments

revealed that transplantation of T-cell depleted bone marrow was followed by increased graft failure and relapse rates leading to inferior overall survival [10].

The use of PBPCs instead of BM cells may help to overcome the two major problems of purged bone marrow transplants. In contrast to BM cells, PBPCs can be harvested in virtually unlimited numbers to ensure engraftment. Recent clinical data show that the transfer of high numbers of G-CSF mobilized CD34+cells allows successful engraftment even in the haplo-identical setting [17]. The increased risk of leukemia relapse [10] after T cell depletion could also be positively affected by the use of PBPCs instead of BM cells. Due to the number of NK-cells in PBPC harvests, GvL activity may be partly conserved if peripheral progenitor cells are selectively depleted from CD3-positive T-cells.

Our experimental data clearly indicate that the depletion of CD3-positive T-cells can result in complete loss of antileukemic activity exerted by allogeneic G-CSF-mobilized PBPCs (Figure 1). NK cells remaining in the graft were not able to exert significant antileukemic activity in vivo. Since this may be due to the lack of T-cell derived stimulatory signals, PBPCs were incubated with NK-stimulatory cytokines prior to grafting. Although treatment with IL-2 and IL-12 resulted in significanty enhanced natural cytotoxicity in vitro, it was not able to restore the GVL effect of unmanipulated grafts in vivo. No increase in the incidence of GvHD was observed in animals treated with cytokine-activated CD3-negative transplants which confirms previous data suggesting that allogeneic NK-cells do not exert biologically significant reactivity against other than hematopoietic tissues of the host [18, 19].

There are some limitations with regard to the conclusions that can be drawn from preclinical animal models. Although our model resembles a common clinical setting (MHC-identity, T-cell sensitivity and NK-cell resistance of the malignancy), the results are limited to this specific situation. There is increasing evidence that NK-cells derived from MHC-mismatched individuals exhibit higher lytic activity because not all of their inhibitory receptors will bind to self-type HLA-molecules [20]. In such a situation, activated NK-cells might be able to compensate for the loss of GvL activity after T-cell depletion [21].

Taken together, our findings suggest that increased graft-vs-leukemia (GVL) effects following transplantation of allogeneic MHC-matched PBPCs as compared to BM grafts will be completely abrogated even after selective (specific) T-cell depletion. Activation of MHC-matched NK-cells with IL-2 and IL-12 in vitro does not fully compensate for the loss of GvL activity after depletion of CD3-positive T-cells.

Acknowledgment. This work was supported by a grant from Deutsche Krebshilfe (Uh-0958-Uh I).

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Induction and Post-Remission Therapy in Acute Myeloid Leukemia: Experience of the Eastern Cooperative Oncology Group (ECOG)

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Introduction

Over the past few decades 2222 patients have entered seven consecutive ECOG studies for acute myeloid leukemia (AML) for an overall complete remission rate of 64% (Table 1). Accurate follow-up data are available on almost all patients and indicate a steady improvement in long-term survival, generally paralleling increasing the intensity of post remission therapy [1]. However, this difference is generally not observed among older adults over age of 55. The complete remission rate has not changed over much of this period using a fairly uniform induction regimen for most of the studies. While most adults with AML can achieve complete remission the challenge has been to maintain the duration of the disease-free survival especially in older adults. In this latter group, little progress appears to have been made.

Materials and Methods

The following data have been collected from seven consecutive ECOG studies of patients with AML since 1976: E2476 (n = 293); E1479 (n = 310); E3483 (n = 483); PC486 (n = 115); E3489 (n = 808); E1490 (n = 119); E2491 (n = 94) for a total 2222 patients, and is a follow-up to previously published data [2, 3]. Data are also described separately for patients above and below age 55 (Tables 2 and 3) since 1979 the induction regimens have been almost identical, allowing for a reliable assessment of any effect that may be due to changes in supportive care and in the various modalities used as post remission therapy.

Table 1. AML studies, 1976-1991 newly diagnosed patients (n)

E2476	293
E1479	310
E3483	483
PC486	115
E3489	808
E1490	119
E2491	94
Total	2222
CR rate	64%

Table 2. CR by protocol: patients < 55 years

Study	Patients (n)	CR rate (%)
E2476	187	56
E1479	181	67
E3483	353	68
PC486	115	73
E3489	808	68
E2491	70	79

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Table 3. CR by protocol patients > 55 years

Study	Patients (n)	CR rate (%)
E2476	106	40
E1479	129	53
E3483	130	55
E1490	119	54
E2491	24	71

Table 4. Induction regimens

Study	Induction regimen		(mg/m^2)
E1479, E3483, PC486	DAT	Daunorubicin	60 × 3 days
		Ara-C	200 × 5 days
		6-thioguanine	100 × 5 days
E1490	DA	Daunorubicin	60 × 3 days
		Ara-C	100 × 7 days
E3489	IA	Idarubicin	12 × 3 days
		Ara-C	100 × 7 days

One to two courses to CR (day 10-14 marrow)

Table 4 summarizes the induction regimens used since 1979 for each of the protocols. Fundamentally, these are very similar consisting of three days of an anthracycline together with cytarabine. The APL ATRA protocol (E2491) is not included.

E1479

This was a study evaluating the role of moderate consolidation therapy prior to maintenance therapy (Fig. 1). In this study all patients had de novo AML, ages 15-70. Induction therapy consisted of DAT (Table 4) and all patients who entered complete remission were randomized to either go directly to maintenance therapy or to receive two courses of moderate-dose consolidation therapy consisting of: daunorubicin 45/mg/m^2 IV/day × 2 days; cytarabine 100/mg/m^2 IV/q12h × 5 days; 6-thioguanine 100/mg/m^2 po q12h × 5 days. Maintenance therapy was given for two years employing the following regimen; 6-thioguanine 40/mg/m^2 po b.i.d. 4 days per week and cytarabine 60/mg/m^2 sc one day per week.

E3483

This was a randomized study of more intensive post remission therapy and was the first ECOG study to attempt to evaluate the role of bone marrow transplantation (Fig. 2).

Importantly, an observation arm was also included and the overall age of patients in this study (except allo BMT) was 15-65. Induction therapy consisted of DAT as in the previous study (E1479) and consolidation therapy consisted of cytarabine 3/mg/m^2 IV q12h over 1 h on days 1-6 and amsacrine 100/mg/m^2 IV days 7-9.

PC486

This was a pilot study designed to test the feasibility of using purged autologous transplants (Fig. 3).

In this pilot, patients received induction therapy with DAT and those in complete remission (CR) were directly assigned to allogeneic transplantation if they had a histocompatable sibling and were less than 41 years of age. If no HLA sibling was available,

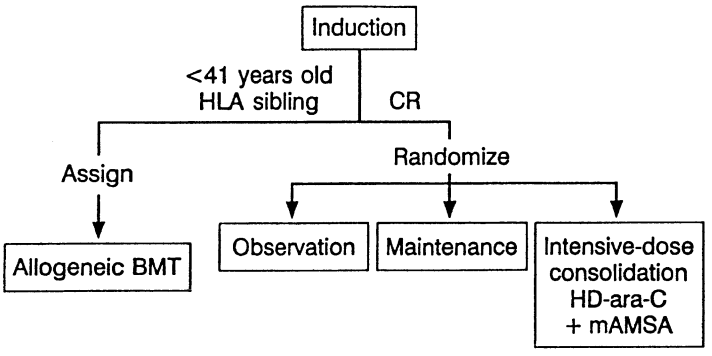

Fig. 1. E3483: randomized study of postremission therapy

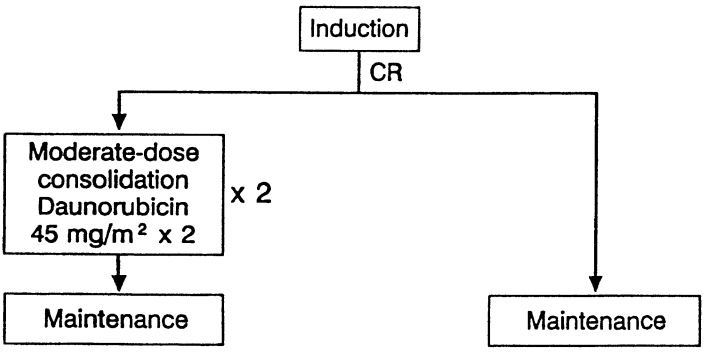

Fig. 2. E1479: randomized study of maintenance ± moderate consolidation

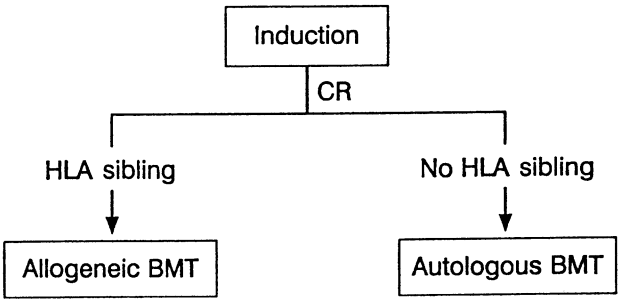

Fig. 3. PC486: Study of postremission BMT (age < 55 years)

the patients went on to receive autologous transplantation after conditioning with busulphan (16 mg/kg) and cyclophosphamide (200 mg/kg). The harvested autologous marrow was purged with 4-hydroxyperoxy cyclophosphamide. It is important to note that no consolidation was offered these patients prior to bone marrow transplantation.

E3489

This is a major recently completed intergroup study (Fig. 4) led by ECOG, and included a post remission randomization to consolidation using high dose cystosine arabinoside alone at a dose of 3 g/m^2 q12 h over 1 h × 6 days. The autologous transplants were performed as in the previous study (PC486).

E1490

This was a study designed primarily to evaluate the role of hematopoietic growth factors in older adults (ages > 55-70) with AML (Fig. 5). The induction regimen, even in this age group, used daunorubicin 60/mg/m^2 for 3 days and cytosine arabinoside 100/mg/m^2 × 7 days. Consolidation therapy was offered with cytosine arabinoside 1.5 g/m^2 q12h over 1 h for 6 days.

E2491

This was a recently completed intergroup study of all-trans-retinoic acid (ATRA) in newly diagnosed patients with acute promyelocytic leukemia. While this study included approximately 400 patients, only ECOG patients are used in this analysis.

Results and Discussion

The overall complete remission rate for all 2222 patients is 64% (Table 1). However, when this is broken down by age (Tables 2 and 3) it is clear that the response is significantly higher among patients less than 55 years old. It is interesting to note that the complete response rate has not significantly

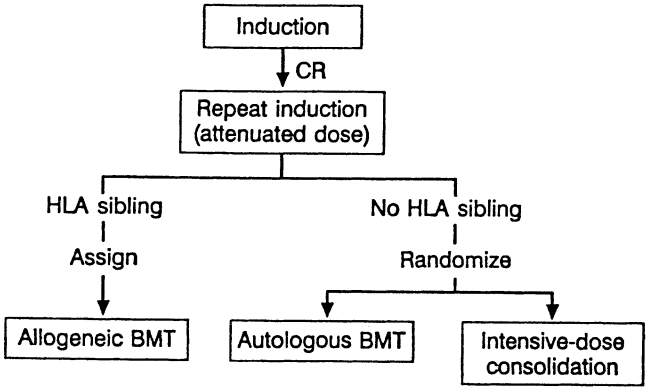

Fig. 4. E3489: randomized study of postremission therapy (age < 55 years)

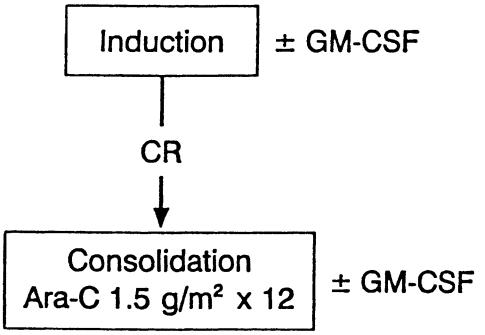

Fig. 5. E1490: randomized study of GM-CSF in induction (age > 55 years)

changed – at any age group – between 1979 and the present day when similar induction regimens are used (Table 4). This is clearly shown in Tables 2 and 3 if one leaves out the results for E2476 – a study that used a significantly less intensive induction regimen – and also excludes the APL study (E2491) which used ATRA for induction. In fact the response rate is very consistent over this period of time, suggesting that any improvement in the long-term survival is not likely to be due to major changes in supportive care but rather to differing post remission therapies. In this context, it is also important to note that the overall complete remission rate was not altered when idarubicin was introduced in place of daunorubicin in E3489 [4].

The overall survival of all patients entered on ECOG studies for AML since 1976 – excluding patients from the latest study (E3489), is illustrated in Fig. 6. This figure include all patients entered on these studies whether or not they achieved complete remission. It is perhaps important to note that even in the pre-ATRA era patients with APL were singled out as the only morphologic subtype with a clearly improved overall survival [5] (Fig. 7).

Looking at the overall survival since 1976 it is clear that there has been a slow but steady improvement in the overall survival with sequential studies (Fig. 8).

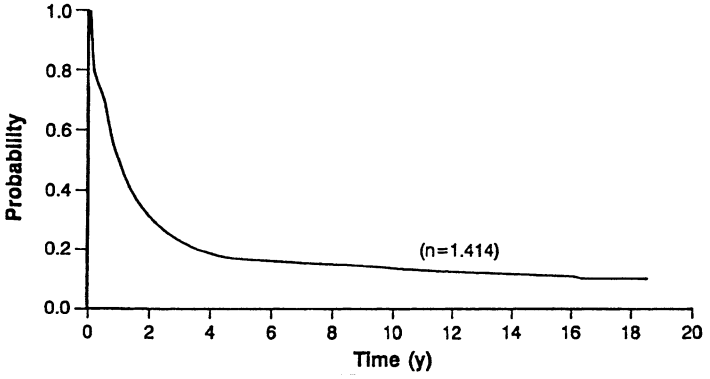

Fig. 6. Overall survival from time of diagnosis: all protocols

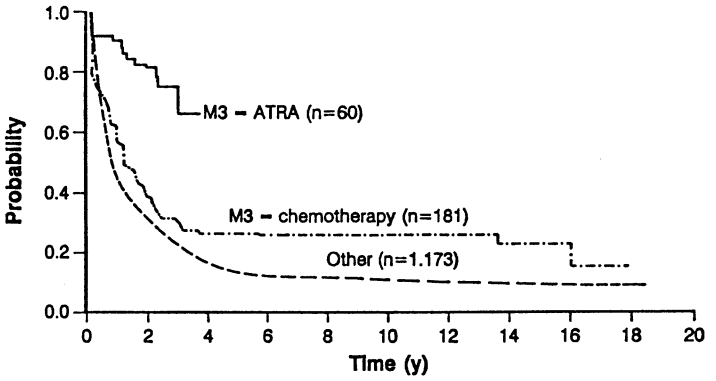

Fig. 7. Overall survival from time of diagnosis: all protocols

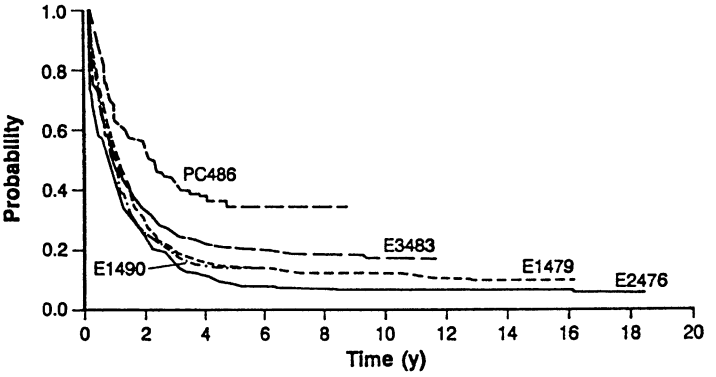

Fig. 8. Overall survival from time of diagnosis, by protocol

However, this may be meaningless without subdividing this by age and Table 5 illustrates what appears to be a continuous improvement in the 2-year and 5-year overall survival with sequential studies for patients less than 55 years. On the other hand, it is not clear that there has been a significant improvement in the overall survival for patients greater than 55 years (Table 6). If one considers the different treatment modalities offered in these sequential studies it clear that intensifying the post remission therapy markedly improves the few-year disease-free survival and overall survival in patients < 55 (Table 7).

It can be noted, as has previously been described [1] that at least some form of post remission therapy is required for there to be any significant disease-free survival. The observation arm on E3483 was closed early due to a high relapse rate.

The disease-free survival is clearly related to the intensity of post remission therapy in patients less than 55 years (Fig. 9), though there is no convincing evidence from these historical data that intensifying the post remission therapy for patients older than 55 years is significantly more effective than maintenance therapy alone as given in the above studies (Fig. 10).

Table 5. Survival by protocol patients < 55 years

Survival from time of diagnosis			
Study	Median (y)	2-Year (%)	5-Year (%)
E2476	0.89	25	9
E1479	1.09	27	17
E3483	1.03	33	22
PC486	1.88	49	33

While some of these data are based on sequential studies and not on prospective comparisons much can be learnt from the overall pattern of response in the varying age groups and the differing protocols. Ana-

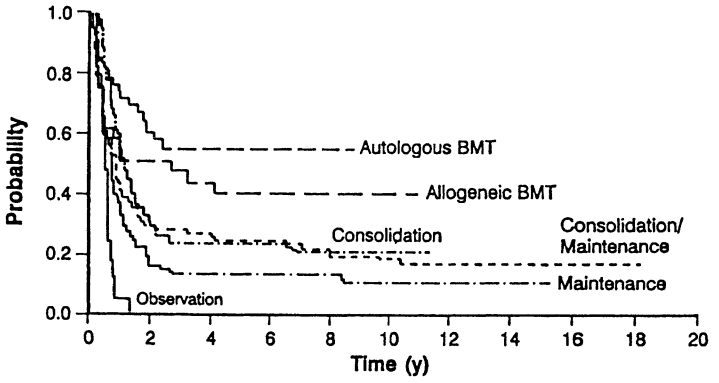

Fig. 9. Disease-free survival: patients < 55 years old

Table 6. Survival by protocol: patients > 55 yrs

Survival from time of diagnosis			
Study	Median (y)	2-Year (%)	5-Year (%)
E2476	0.20	13	6
E1479	0.63	23	9
E3483	0.53	22	13
PC486	0.72	23	13

Table 7. Escalating intensity of postremission therapy: patients 55 < years

Postremission therapy	5-Year DFS (%)	5-Year Survival (%)
Observation	0	10
Maintenance	13	19
Maintenance + intermediate-dose consolidation × 2	24	33
Intensive-dose consolidation × 1	24	29
Autologous BMT	55	53
Allogeneic BMT	40	45

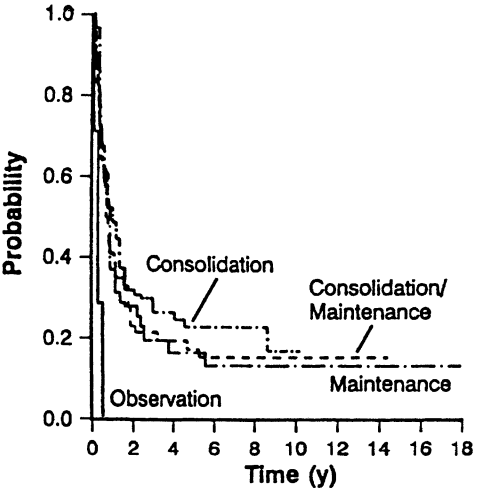

Fig. 10. Disease-free survival: patients > 55 years old

lysis of E3489, as well as other similar large studies, should provide important prospective data regarding the role of bone marrow transplantation in patients with de novo AML. It is likely, however, that ultimately therapeutic decisions will need to be made based on prognostic factors such as cytogenic analysis, immunophenotyping, molecular genetic analysis, as well as assessment of multidrug resistance. Clearly, these mostly historical data provide valuable information, but much remains to be learnt.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Cellular Immunotherapy of Cancer: Adoptive Transfer of Monocyte-Derived Tumorcytotoxic Macrophages

R. ANDREESEN, B. HENNEMANN, and S.W. KRAUSE

Abstract. Basic studies into the role of immune cells to combat malignant disease and the recent reports on complete remissions upon the application of allogeneic cells have created renewed enthusiasm in the potential of cellular immunotherapy. Lymphocytic effector cells (donor-derived buffy coat cells or specific T-cells) have been successfully used in the treatment of patients with relapsed CML and acute leukemia, with EBV-associated lymphoma and also to provide anti-CMV immunity. Ex vivo grown cytotoxic macrophages (MAC) able to recognize and destroy tumor cells but not normal cells are effective in murine models of metastasizing tumors. Upon the development of large scale technology to generate MAC in vitro from circulating blood monocytes (MO) clinical trials in cancer patients had proved the feasibility and safety of infusing $> 2 \times 10^9$ MO-derived MAC activated by IFN-g or LPS. Various modalities of adoptive therapy with human MAC have been realized: routes of application used were IV, IP, intrapleural and through selective hepatic artery perfusion. In addition, MAC had been generated from MO collected after GM-CSF treatment in vivo. Biodistribution studies using ^{111}Indium labelled cells revealed localization of MAC to sites of bulk tumor growth upon regional infusion as well as to liver metastases upon systemic application. Malignant ascites disappeared in about 50% of patients after ip treatment, yet no other evidence of therapeutic efficacy of MAC could be demonstrated. Further developments of adoptive transfer of MO-derived cells are developed focusing on the generation of antigen-presenting cells primed in vitro with tumor cells or specific peptides. Clinical trials with tumorantigen-pulsed dendritic cells either generated from blood MO or from CD34+ progenitor cells are in progress.

Introduction

There is substantial evidence to prove that host defense mechanisms are operating to control the development of malignant tumors. Among the various attempts to restore or augment an ineffective anti-tumoral response of the host the use of immune effector cells has long been investigated [1-46]. This approach, i.e., transferring in vitro expanded and activated immune cells to patients, is termed adoptive immunotherapy and has been realized both with effector cells of the innate and the specific immune system, respectively, as well as with cells being either directly cytotoxic or acting as inducers of specific immunity by presenting tumor antigens to T-cells (Table 1).

Furthermore, the clinical use of such cellular products has recently been extended to confer antiviral immunity [7, 8], e.g., to treat CMV infection [8, 9, 10] as well as EBV-asso-

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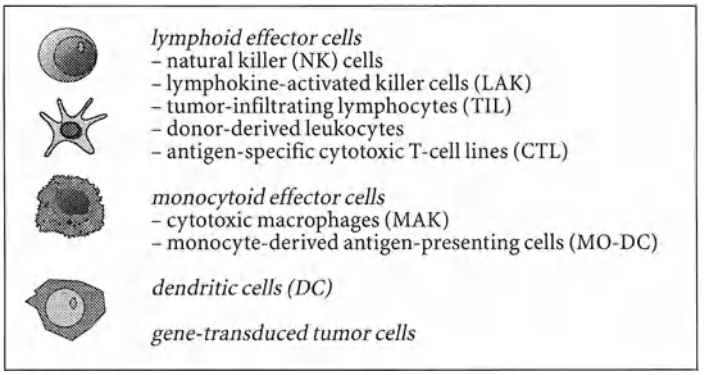

Table 1. Clinical use of cellular products in the immunotherapy of cancer

ciated lymphomas arising in recipients of allogeneic T-cell depleted bone marrow transplantation [11].

A landmark study in the field of adoptive immunotherapy was published by Kolb et al. in 1990 [5] who for the first time proved that not only patients with minimal residual disease but also with extensive tumor loads could respond with complete resolution of their malignancy. They reported the induction of complete hematological remissions by infusion of donor-derived buffy coat cells in CML patients relapsed after allogeneic BMT. In this study whose observation was subsequently confirmed in large cohorts of patients with CML and acute leukemias [6, 12-16] alloreactive T-cells of donor origin are the putative effectors, yet also autologous T-cells are highly effective in overt disease: in bone-marrow transplant patients with EBV-associated lymphomas infusion of ex vivo expanded specific T-cell clones resulted in a dramatic response and complete pathological remission in all of 5 patients [11, 17].

Macrophages as Effector Cells in Cellular Immunotherapy

Cell Sources

Macrophages as part of the non-specific immune system against microbial pathogens and tumors are able to recognize and destroy malignant cells through several mechanisms including direct cell-contact mediated killing, antibody-dependent cellular cytotoxicity (ADCC) and through the release of soluble cytotoxic mediators like oxygen radicals and tumorcytotoxic proteins (TNF-alpha, MTC170 [18]). In murine models, repeated infusions of activated MAC elicited from the peritoneal cavity or grown ex vivo from bone marrow precursor cells can induce regression of pulmonary metastases [19]. In humans, blood MO were first introduced to adoptive immunotherapy by Stevenson et al. [20, 21]. However, blood MO are rather immature precursor cells, a fact that thus may seriously impair their efficacy as is also evident from their weak anti-tumor effects on human xenografted tumors in nude mice [22]. On the other side, competent cytotoxic effector cells can be generated in vitro from blood MO by incubation under appropriate conditions [23, 24]. When cultured in the presence of autologous serum blood MO mature to MAC, e.g. they acquire a maturation-associated phenotype in terms of morphology and antigen expression [25], become more responsive to IFN-g [26], increase their cytotoxic potential of both cell-mediated and antibody-dependent cytotoxicity and secrete considerable amounts of supportive colony-stimulating factors (M-CSF, GM-CSF) as well as tumortoxic proteins (TNF-alpha and MTC170) when stimulated with LPS (Fig. 1A to B [27, 28, 29]).

Based on the technique established to generate mature MAC by suspension culture on hydrophobic teflon foils [23] large-scale technology was developed to allow the production of MAC sufficient for first clinical trials [30]. Mononuclear cells (MNC) purified from apheresis products by ficoll gradi-

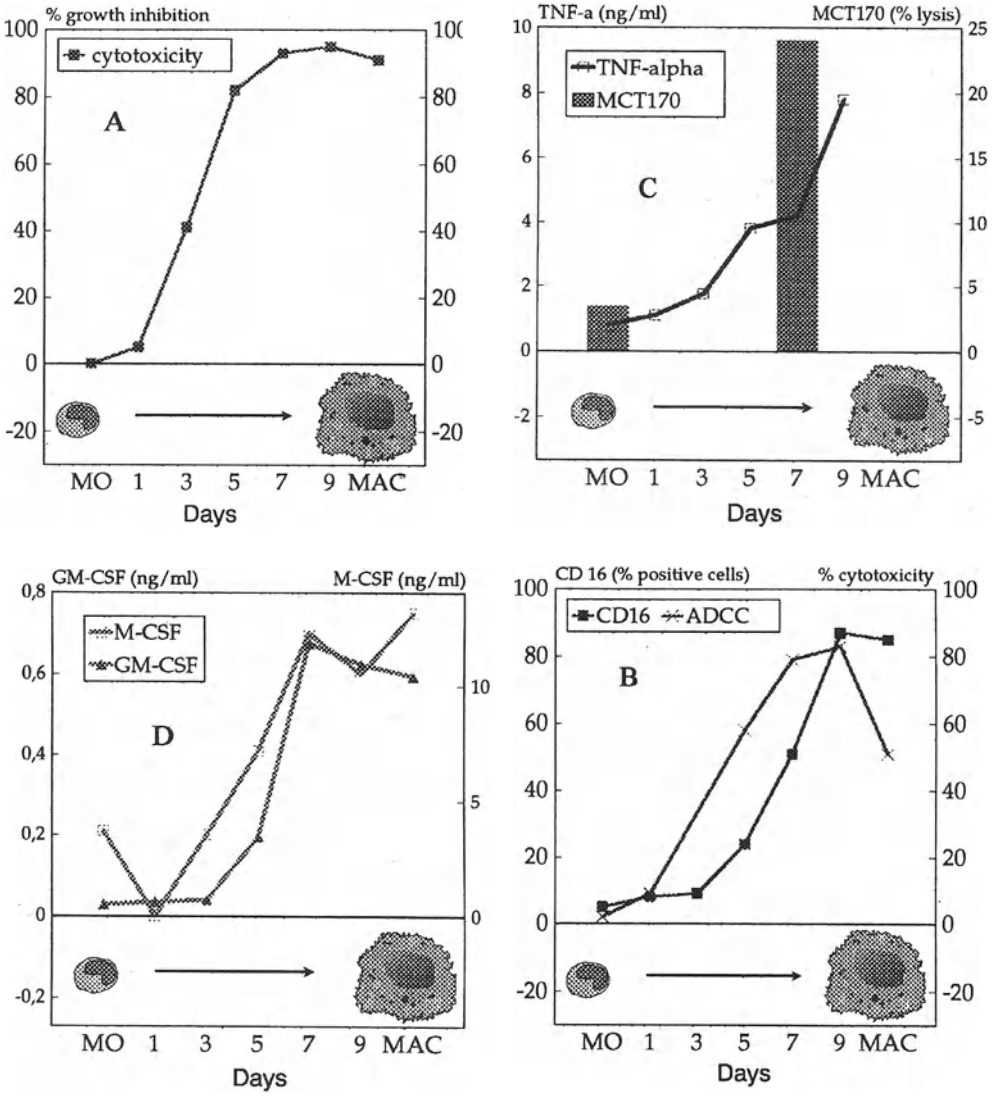

Fig. 1 A–D. Functional competence of MAC derived in vitro from blood MO. Different functions are depicted during differentiation of blood MO into MAC *A* Cellular toxicity against a tumor cell line (U937) after stimulation of MO/MAC with IFNγ. *B* Parallel increase of low affinity IgG receptor CD16 and activity of MO/MAC in an assay for ADCC. *C* TNFa production and of secretion of a novel MAC cytotoxic factor (MCT170). *D* Production of hematopoietic growth factors GM-CSF and M-CSF

ent centrifugation are cultured for 7 days, activated either overnight with IFN-g or 2 h with LPS *(S. abortus equi)*, harvested and either subjected to counter-current centrifugal elutriation to separate mature MAC from remaining lymphocytes or used as an unseparated MNC population (see Fig. 2). On average about 50% of the initially seeded MO can be recovered from the Teflon bags as activated MAC which are > 90% pure after elutriation.

Clinical Trials

Autologous MAC generated in the way described above have been retransfused into patients without major side effects, the ma-

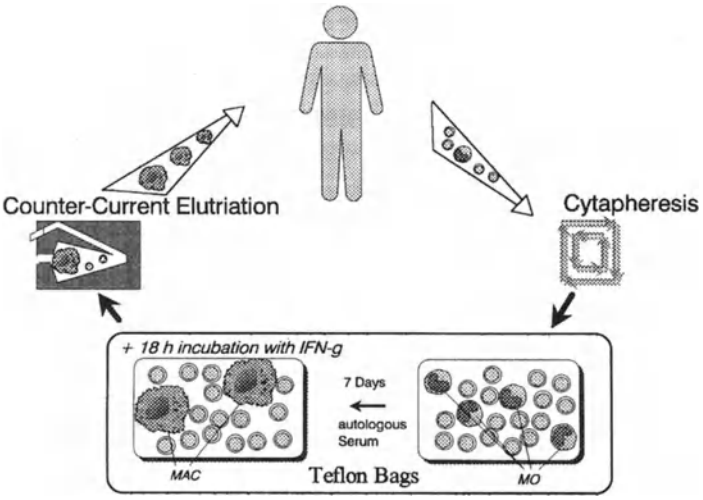

Fig. 2. Schematic treatment schedule of macrophage adoptive immunotherapy

ximal number per therapy being 1.9×10^9 MAC without patients' pretreatment and 2.7×10^9 MAC when the MO were derived from patients pretreated with GM-CSF [32]. Low-grade fever (Fig. 3), a dose-dependent increase of circulating thrombin-anti-thrombin complexes (TAT), a rise in serum neopterin and a short-lived induction of the acute-phase C-reactive protein (CRP) were seen upon IV, IP and intrahepatic infusion.

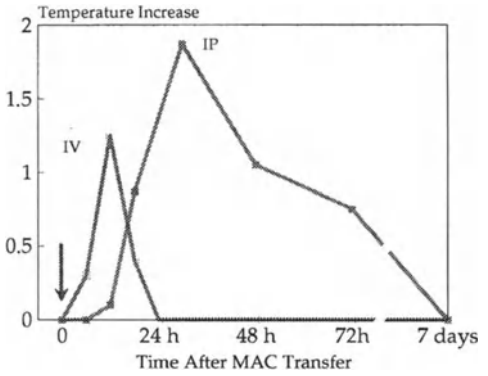

Fig. 3. Low-grade fever induction by infusion of MO-derived MAC

None of these side effects was dose-limiting, with regard to the procoagulant activity no clinically evdient thromboembolic events occurred. Trafficking of transfused MAC were followed with the use of ^{111}Indium-labelled cells and revealed a remarkable long transit time through the lungs (about 2 h) and pooling of activity in spleen and liver after 18 h with no preferential labelling of lung metastases. Regional application, however, could clearly demonstrate an accumulation of cells at sites of tumor bulk in the peritoneal cavity and around metastatic lesions in the liver upon infusion via the hepatic artery [33]. Even with IV administration MAC pooling into the liver after 4 days is more intensive at metastases than in normal liver.

When LPS-stimulated cytokine activated MAC were transferred, both induction of

Table 2. Phase I studies of adoptive immunotherapy with tumorcytotoxic macrophages: the Regensburg experience

	IV	IV, LPS activated MAC	IV, grown from GM-CSF mobilized MO	IP	a. hepatica infusion
Patients (n)	12	9	10	14	7
Therapy cycles (n)	52	61	20	68	35
Max. cell count/infusion (10^8)	17	14.9	27	9.9	13.4
Max. cell count/patient (10^8)	64	53	36	50	57

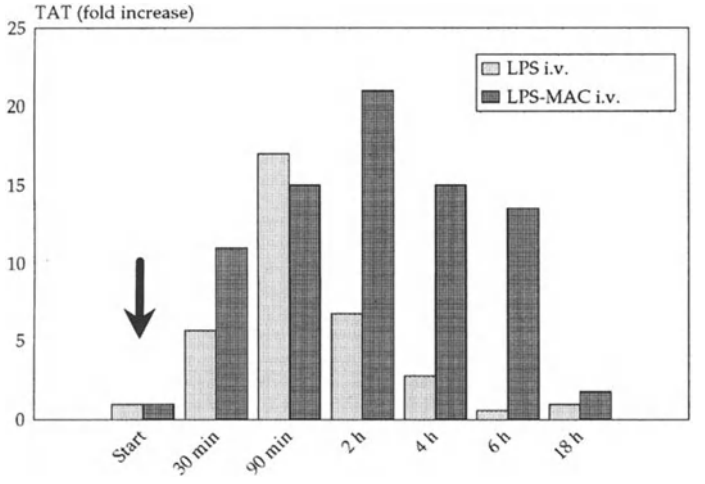

Fig. 4. Induction of TAT complexes by injection of LPS as compared to the infusion of LPS-stimulated MO-derived MAC

TAT complexes as well as pro- and anti-inflammatory cytokines (TNF-alpha, IL-1RA) were observed, yet with a different time course when compared to the injection of LPS alone (Fig. 4 [34]).

Table 2 summarizes our own experience with MAC adoptive immunotherapy (first published in 1990 [31]). No objective tumor response has been documented uptodate, the reduction of malignant ascites in about half of the patients treated IP being interpreted to be a non-specific inflammatory reaction rather than a true anti-tumoral effect. All of the available data on MAC cellular therapy reported from the groups in Strasbourg, Paris and Reims are depicted in Table 3 [35-39].

Future Perspectives on the Use of MO-Derived Cells in Cellular Immunotherapy

Reviewing the available data on the use of MAC in adoptive immunotherapy, it is evident that on the basis of generating a cellular product being directly tumorcytotoxic a clinical benefit has not been proven and continuation along this line of development is debatable. However, much enthusiasm has been created by the possibility to derive highly potent antigen-presenting cells from blood MO which exhibit most if not all the features of dendritic cells. Dendritic cells and the related cell types of Langerhans cells and interdigitating reticulum cells are rare and difficult to obtain but can be grown in vitro at large quantities either from blood

Table 3. Macrophage adoptive immunotherapy: Current clinical experience

Center	Pat. (N)	Stimulus	Route	Clinical response	Reference
Freiburg	8	IFN-g	IV	NO	Andreesen et al. (1990)
	7	IFN-g	IP	Ascites[a]	Andreesen et al. (1990)
	10	IFN-g	a.hepatica	NO	Hennemann et al. (1996)
Strasbourg	11	IFN-g	IV	NO	Faradji et al. (1991)
Paris	12	IFN-g	IV	1 PR	Lopez et al. (1993)
	8	IFN-g	intrapleural	NO	to be published (1997)
Reims	15	IFN-g	IV	NO	Eymard et al. (1996)
Regensburg	9	LPS	IV	NO	Hennemann et al. (1995)
	10[b]	IFN-g	IV	NO	to be published (1997)

[a] Reduction of malignant ascites in 3/7 patients.
[b] Pretreatment with 10 μg/kgGM-CSF for 7 days s.c.

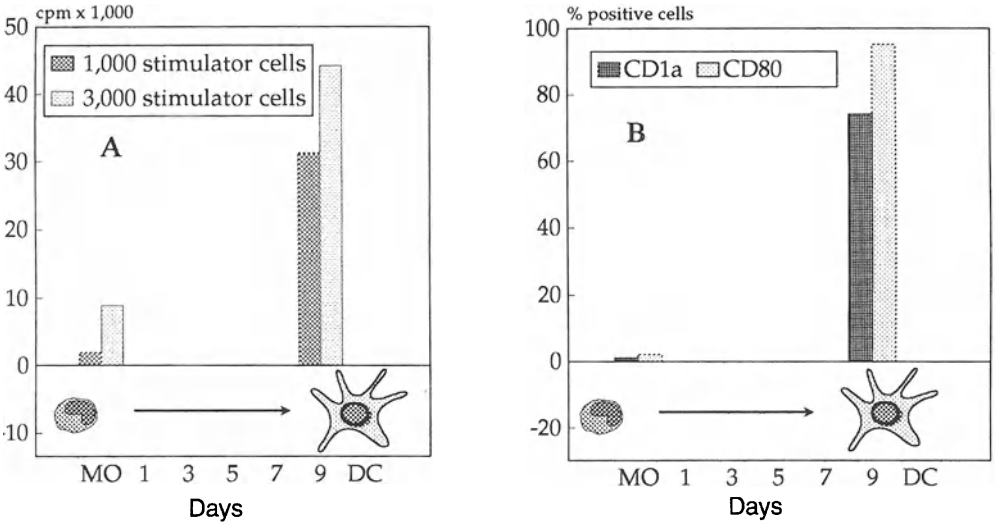

Fig. 5 A, B. Differentiation of MO into DC. *A* Stimulation of allogeneic lymphocytes by MO or MO-derived DC. *B* Detection of typical surface antigens on MO-derived DC versus blood MO by flow cytometry

MO or hematopoietic CD34+ precursor cells [40] under the influence of GM-CSF, IL-4 and TNF-alpha. Indeed, when IL-4, GM-CSF and IFN-g are added to our culture system and human serum is replaced by small amounts of FCS an DC-typical antigenic phenotype develops and a potent allostimulatory activity is acquired (Fig. 5 [41]).

First clinical trials are in progress using these DC pulsed either with tumorlysates or natural and synthetic specific peptides of tumor-associated antigens. These primed DC should induce a cytotoxic T-cell response when retransfused to the patient based on animal studies [42, 43]. Initial reports on DC therapy are promising [44] but await further confirmation. In addition, several questions remain to be answered: first of all, antigen-presentation can induce both T-cell activation leading to induction of an immune response and T-cell anergy and tolerance induction. Thus, it may be of crucial importance which type of DC are being used for cellular therapy, especially as recent publications suggest an essential role of DC which apparently decide on an immunogenic or a tolerogenic response of the recognizing T-cell [44, 45]. By means of function and phenotype DC cultured from CD34+ hematopoietic precursors or MO, respectively, are quite similar [41], but little is known so far which cytokines can modulate DC biology in a way to optimize the induction of anti-tumor immunity in vivo. A similar concern is expressed with respect to cell dose, optimal treatment schedule as well as the route of application.

To take the concept of antigen-presenting cells a step further, it is tempting to also explore them for their ability to induce cytotoxic T-cells specific for tumor-associated antigens in vitro which subsequently upon expansion and activation can be used as the therapeutic cellular product (Fig. 6).

Conclusion

Large quantities of highly competent tumor-cytotoxic MAC can be generated in vitro from circulating blood MO and are well tolerated when reinfused into the autologous host. Although active in limiting the metastatic growth in murine tumor models no objective clinical response has yet been documented in more than 90 patients treated at 5 different European centers. It is suggested that future perspectives in the use of cells of the mononuclear phagocyte system in adoptive immunotherapy clearly has to be developed towards generating antigen-presenting DC derived from blood MO. These DC

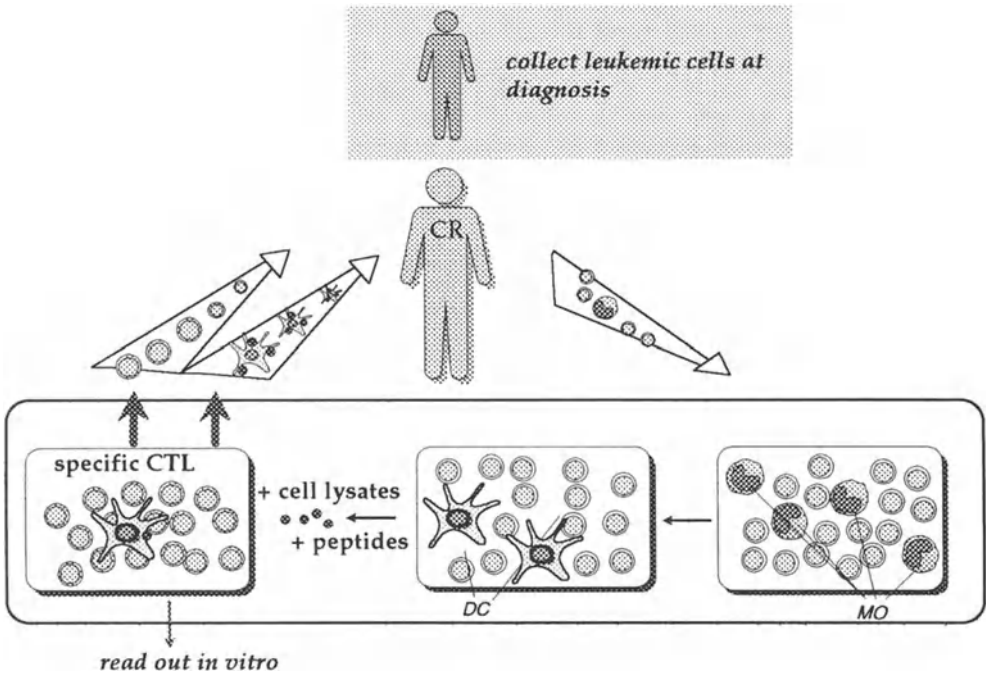

Fig. 6. Use of MO-derived dendritic cells in cellular immunotherapy: Schematic description (further details see text)

could be primed with tumor-relevant antigens, either by incubation with tumor cell fragments, native or synthetic peptides or by transduction with genes encoding for tumor antigens. Primed DC can be directly infused or used to induce and expand cytotoxic T-cells which ultimately are used for cellular immunotherapy.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

GM-CSF Stimulation of Immune Response in Minimal Residual Cancer

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Abstract. Minimal residual disease in cancer can be detected by identification of epithelial tumor cells in bone marrow using monoclonal antibodies directed against cytokeratin. The presence of cytokeratin positive (CK+) tumor cells in bone marrow of gastric cancer patients is associated with a significantly higher relapse rate than patients without CK+ cells. Monocytes and macrophages can recognize and destroy tumor cells. The granulocyte-macrophage colony-stimulating factor (GM-CSF) was found to induce and enhance the tumoricidal activity of peripheral blood monocytes. The aim of our study was to evaluate whether GM-CSF is able to reduce the number of CK+ cells in bone marrow. 23 patients with resectable gastric cancer and presence of CK+ cells in their bone marrow were randomized to receive either GM-CSF (2 μg/kg subcutaneously, n = 16) or placebo (n = 7) on days 1-14 and 29-42. The leucocytes, C-reactive protein, neopterin (signs of an acute phase reaction), complement receptor CR3 expression and HLA-DR-expression on monocytes (characteristics of monocyte activation) increased during GM-CSF application. The values remained constant in the placebo group. The CK+ cells in bone marrow were analyzed prior to treatment and 8 weeks after commencement of therapy. There was a decrease of CK+ cells in bone marrow in 14/16 patients treated with GM-CSF and 4/7 patients in the placebo group. GM-CSF treated patients showed a significant reduction of the CK+ cell number in bone marrow ($p = 0.01$). 21 patients were evaluable for follow-up analysis (mean follow-up 35 months, 11-54). To date 7 relapses have occured in 14 GM-CSF treated patients whereas 5 relapses were observed in the 7 placebo patients. The Kaplan-Meyer analysis yields a slightly better relapse free survival rate in the GM-CSF treated patients. Due to the low number of patients there is no significant difference between the two groups ($p = 0.08$). The conclusions of our trial are: (1) GM-CSF at a dose of 2 μg/kg daily is well tolerated. (2) GM-CSF activates monocytes and macrophages despite the low dose administered. (3) GM-CSF can reduce the number of CK+ cells in bone marrow. (4) GM-CSF has a positive influence on disease free survival, however this difference is not significant due to the low patient number. Further prospective randomized clinical studies are warranted.

Introduction

The treatment of most malignant tumors has hardly improved during the last decade. This overall negative balance is mainly due to the early systemic dissemination of tumor cells occuring prior to the diagnosis or

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resection of the primary tumor. Thus, the detection and elimination of micrometastases in patients with small resectable tumors poses a major challenge to cancer research.

Monoclonal antibodies (Mab), directed against epithelial antigens are able to identify epithelial tumor cells or small carcinoma cell clusters in mesenchymal organs, which are undetectable by conventional diagnostic methods. The bone marrow is an easily accessible mesenchymal organ from which epithelial cells appear to be excluded in non-malignant conditions. The antibody used should be directed against an antigen preferentially expressed on all tumor cells and should not react with haematopoetic or other autochthonous cells of the bone marrow. These conditions are fulfilled by monoclonal antibodies directed against cytokeratin epitopes (CK), which as an abundant intracellular protein antigen appears to be a target for cell identification superior to cellular membrane antigens [1].

Gastric cancer accounts for approximately 8% of all malignant tumors. In the adjuvant setting there is no standard therapy proven to improve survival rates for patients with resectable tumor. The presence of cytokeratin positive (CK+) tumor cells in the bone marrow of gastric cancer patients is associated with a significantly higher relapse rate compared to patients without CK+ cells [2, 3, 4].

Experimental data support the possibility of using GM-CSF in an immunotherapeutic approach for cancer patients [5-10]. Monocytes and macrophages can recognize and destroy tumour cells in vitro and in vivo [5, 11, 12, 13]. Human granulocyte-macrophage colony-stimulating factor (GM-CSF) was found to induce and enhance the tumoricidal activity of peripheral blood monocytes [5-8]. Several clinical trials studied the anticancer effects of GM-CSF, mainly in patients with advanced metastatic malignancy [5, 14, 15]. There is evidence that GM-CSF treatment initiated in an earlier stage of cancer disease is more successful. The question arose whether tumoricidal activity of GM-CSF activated monocytes/macrophages can be directly demonstrated by monitoring CK-positive cell numbers.

Methods

The design of a randomized, placebo-controlled and double-blinded trial has been chosen. Included were 23 patients with resectable gastric cancer with or without nodal involvement and proven presence of cytokeratin positive cells in bone marrow, aged between 18 and 75 years and given written informed consent according with the ethical standards of the responsible committee. Admission to the study and patient monitoring included physical examination, monitoring of vital signs, determination of performance status, ECG, chest x-ray, laboratory evaluations. All patients had histologically proven resectable (R0) stomach carcinoma. No patient has received previous chemotherapy, radiotherapy or immunotherapy. Excluded were patients with significant intercurrent medication, infections or complicating factors for the drug trial and patients with Karnofsky performance status < 40%. Bone marrow samples, aspirated from the iliac crest at the time of primary surgery and 8 weeks after commencement of therapy with GM-CSF, were examined with the monoclonal antibody Mab CK2 directed against the cytokeratin-component 18 [1].

The proportion of patients receiving active drug to placebo was 2:1. Recombinant human (rh)GM-CSF (Leucomax-Sandoz Pharma AG Basel Switzerland and Schering-Plough International Kenilworth NJ USA) 2 μg/kg (16 patients) or placebo (7 patients) was administered once a day by subcutaneous injection in two 14-day cycles (day 1-14 and 29-42). For achieving a better tolerability and compliance there was a treatment stop for two weeks between the two cycles (day 15-28).

There were selected blood and urine sampling at baseline and throughout the study as part of the safety and efficacy evaluations (haematology, chemistry, C-reactive protein, neopterin, CR3-expression, HLA-DR-expression).

Serum levels of C-reactive protein were measured in 11 patients by an immunologic turbidity test (Boehringer Mannheim). Neopterin in serum was analyzed by ELISA (Merck), CR3-expression and HLA-DR-ex-

Table 1. Patients included

Pat. No.	Age (yrs)	Sex (m/f)	Tumor stage	Therapy (verum/placebo)
1	64	m	T3N2M0G3	Placebo
2	59	m	T1N0M0G3	Verum
3	55	m	T3N0M0G3	Verum
4	58	m	T3N0M0G2	Verum
5	50	m	T2N2M0G3	Verum
6	50	m	T2N1M0G3	Placebo
7	43	m	T3N1M0G3	Verum
8	62	m	T2N0M0G3	Placebo
9	66	m	T2N1M0G2-3	Verum
10	65	m	T2N0M0G3	Verum
11	52	m	T3N1G2M0	Verum
12	65	m	T2N1M0G3	Placebo
13	54	m	T3N0M0G2	Placebo
14	63	m	T3N1M0G2	Verum
15	52	m	T2N1M0G3	Verum
16	60	f	T2N1M0G3	Placebo
17	60	m	T4N2M1G3	Verum
18	60	m	T2N0M0G2	Verum
19	49	m	T2N0M0G3	Placebo
20	49	m	T2N0M0G3	Verum
21	70	f	T4N2M0G4	Verum
110	52	m	T2N2M0G3	Verum
111	48	f	T2N1M0G3	Verum

pression on monocytes were determined by FACS-analysis.

The data were analyzed with a non-parametric test (Wilcoxon matched-pair rank test). We estimated relapse free survival by the Kaplan-Meier method, differences were tested by the log-rank test.

Results

The characteristics of the patients enrolled are shown in Table 1. Two patients were added instead of two drop-outs.

In 23 patients evaluation of drug safety and efficacy on CK+ cells in bone marrow was performed. Therapy with GM-CSF was well tolerated without major side effects. During the treatment phase we observed cutaneous allergies like rash and hives WHO grade 1 and 2, fever and myalgia alleviated by paracetamol. No capillary-leakage-syndrome was found.

As expected, we observed an increase of leucocytes under treatment with GM-CSF despite the low dose administered. In five patients GM-CSF was temporarily discontinued because the leucocyte count was > 25000 WBC/mm^3 (maximal value observed 33.4). In contrast, the placebo group achieved no increase. Figure 1 shows the mean values of each group.

C-reactive protein levels increased during GM-CSF administration and decreased after cessation of GM-CSF. In the placebo group serum-levels remained constant (Fig. 1).

The mean neopterin level was 6.64 nmol/l before GM-CSF therapy and went up to 13.24 nmol/l after the first GM-CSF cycle respectively 8.76 nmol/l after the second cycle. No increase was demonstrable in the placebo group.

Similar results revealed the determination of HLA-DR-expression and complement receptor CR3-expression on the surface of monocytes. The HLA-DR expression remained constant in the placebo group, under therapy with GM-CSF a rise of 31% was detected. The values of CR3 expression on monocytes increased about 45% after therapy with GM-CSF, no increase could be demonstrated in the placebo group.

CK-positive cells were found mostly as individual cells at a frequency of one cell per 10^4 to 10^5 nucleated bone marrow cells. The number of cytokeratin positive cells in bone

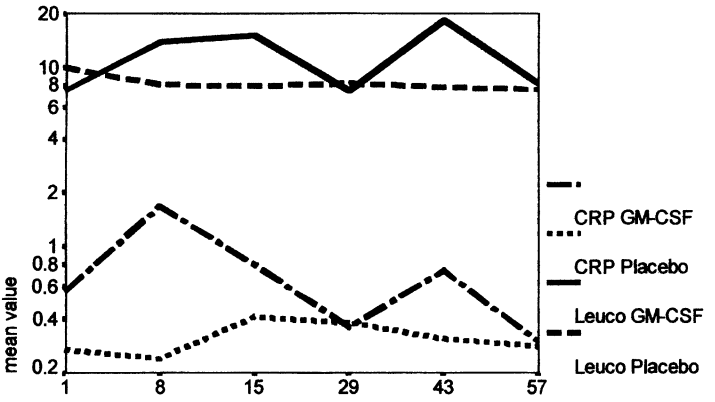

Fig. 1. Leucocytes (/nl, n = 23) and C-reactive protein (mg/dl, n = 7) in GM-CSF-treated and placebo patients. The graphic shows the mean values of each group

marrow decreased in 14 of 16 GM-CSF treated patients and in 4 of 7 placebo patients. The Wilcoxon test revealed a significant reduction of CK+ cells in the GM-CSF treated patients ($p = 0.01$), whereas there was no significant difference in the placebo group ($p = 0.23$). The course of CK+ cells in both groups is presented in Fig. 2.

Twenty one patients were evaluable for follow-up analysis. Two patients could not be considered (drop-outs): One patient was found to have liver metastasis (No. 17) and one patient died after therapy with GM-CSF due to surgical complication (No. 20). The mean observation time was 17.33 months (1-53). To date 7 relapses have occurred in the 14 evaluable patients treated with GM-CSF (50%), one patient died due to a tumor-independent cause, 5 relapses were observed in the 7 placebo patients (71%).

The Kaplan-Meier analysis yields a slightly better relapse free survival rate for the GM-CSF treated patients ($p = 0.08$). However, due to the low number of patients there is no significant difference between the two groups (Fig. 3).

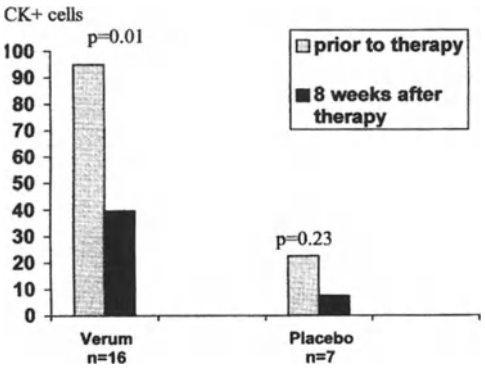

Fig. 2. Course of CK+ cells in bone marrow. Presented are the mean values of each group. (Data analyzed with the Wilcoxon test)

Discussion

GM-CSF is a growth factor with a wide range of activities. GM-CSF acts on myeloid progenitor cells that appear early in the haematopoetic hierarchy. GM-CSF also has the capacity to stimulate the production of neutrophils, patients with neutropenia after chemotherapy treated with GM-CSF show a distinct increase in leucocyte count.

In addition to stimulating myelopoesis, GM-CSF has an effect on the immune response by a wide range of mechanisms. GM-CSF stimulates the proliferation, mobilization and differentiation of dendritic cells, the expression of B7 and major histocompatibility (MHC) class II antigen in these antigen-presenting cells, endocytosis and antigen presentation by monocytes and macrophages. GM-CSF can activate important effector functions of granulocytes and monocytes / macrophages. Monocytes stimulated with GM-CSF demonstrate an increased number of surface membrane receptors [16], enhanced oxidative metabolism (respiratory burst) [17], increased cytotoxic activity [11], enhanced antimicrobial activity [18] and enhanced cytotoxicity against tumors [19]. The major mechanism of indirect antitumor cytotoxicity is an increase of ADCC activity of monocytes /

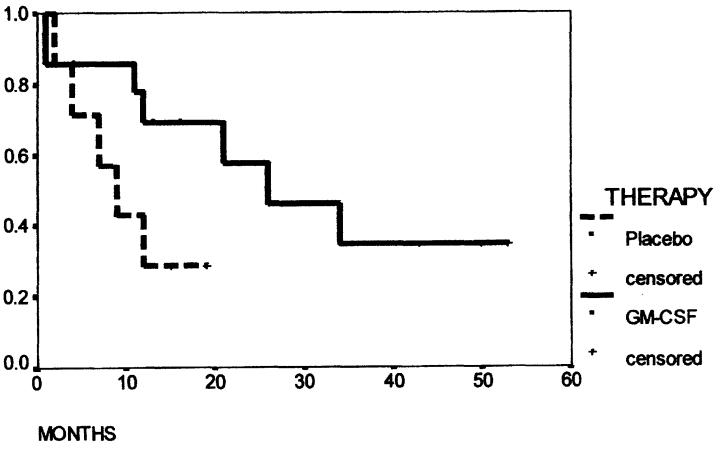

Fig. 3. Relapse free survival. Mean follow-up: 17.33 months (1-53) $p = 0.08$

macrophages, T-lymphocytes and neutrophils [6, 7]. Cannistra et al. [8] and Wing et al. [5] showed, that GM-CSF may stimulate monocyte cytotoxicity by enhancing expression of tumor necrosis factor (TNF). GM-CSF administered together with tumor antigens is able to induce an antitumor immunity. Experiments with GM-CSF transfected melanoma cells in mice conferred 100% protective immunity against wild-type melanoma cells [20]. Therefore GM-CSF does not only promote cell proliferation and differentiation but also alters the immunoregulation.

Tumoricidal monocytes and macrophages can recognize and destroy neoplastic cells in vitro and in vivo, leaving non-neoplastic cells uninjured [5, 8, 11, 14, 21, 22, 23, 24, 25]. In contrast, tumour growth stimulation by GM-CSF was not shown in in vivo experiments [26, 27, 28] and may therefore not be clinically relevant.

Therapy with GM-CSF has been studied in patients with advanced malignancy. Steward et al. [14] could demonstrate a stabilisation of metastatic disease, one patient underwent a tumor reduction of a soft tissue sarcoma after receiving GM-CSF. The results of other trials were disappointing [5, 15]. The reason for this negative outcome might be the advanced stage of disease in the involved patients. However these trials have shown that therapy with GM-CSF can be administered without serious side effects.

The most effective role of GM-CSF in the treatment of cancer can theoretically be expected in patients with minimal residual disease, a stage where the disseminated tumor cells are rare. The outcome of the interaction between macrophages and tumor cells is mainly determined by the ratio of effector cells to target cells. Treatment with GM-CSF should be most successful when metastases are small and infiltrated by activated tumoricidal monocytes.

Tumor cells or small cell clusters can now be visualized immunocytochemically in bone marrow by cytokeratin antibodies. Bone marrow contains a large number of monocytes/ macrophages, so we chose this compartment as a model for demonstrating the tumoricidal activity of GM-CSF activated monocytes/macrophages in vivo.

The presence of cytokeratin positive tumor cells in bone marrow is associated with a significantly higher relapse rate compared to patients without CK+ cells [2, 3, 4].

Gastric cancer seems to be favourable, because there does not exist an accepted standard therapeutic regimen in the adjuvant setting. A well tolerated therapy is required in the adjuvant situation, a condition which is fulfilled by low dose GM-CSF therapy. The aim of our study was to evaluate the tolerability of GM-CSF, to demonstrate the activation of monocytes and to document the efficacy of GM-CSF in reducing the amount of disseminated tumor cells in bone marrow of patients with resectable gastric carcinoma.

The dose of 2 μg/kg GM-CSF day 1-14 and 29-42 was well tolerated. Despite the low

dose administered we could show a distinct activation of monocytes. The activation of monocytes/macrophages induces the release of monokines, which is accompanied by an acute phase response. As signs of monocyte activation we measured the levels of C-reactive protein and neopterin in serum and could demonstrate an increase of these parameters during treatment with GM-CSF. Similarly, we found an elevation of complement receptor CR3 expression and HLA-DR-expression on the surface of monocytes during GM-CSF application, both also characteristics of monocyte activation.

We were able to show a significant reduction in the number of CK+ cells in the patients treated with GM-CSF. GM-CSF treatment was associated with a trend to a prolonged disease free survival in spite of the higher number of CK-positive cells in the treated subgroup (Fig. 3). As Jauch et al. [4] could show, the extent of tumor-cell contamination in bone marrow correlates with prognosis in curatively resected patients, one would expect a worse prognosis in our GM-CSF treated group. The difference in disease-free survival cannot be addressed to cancer stage since staging of the patients revealed no significant differences between the two groups concerning age, lymph node involvement and tumor grading.

Further adjuvant clinical trials with GM-CSF in cancer patients should provide more data concerning the effect of GM-CSF on micrometastasis and reduction of mortality.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

T-Cell-Mediated Immunity against B-Cell Malignancies: Preclinical Results and Translation into a Novel Immunotherapeutic Approach for B-Cell Malignancies

J. L. SCHULTZE

Abstract. The paucity of a clinically significant immune response to B cell malignancies is striking. Although expressing MHC class I and II molecules, these cells seem to lack the capability to serve as efficient tumor-antigen presenting cells to induce a tumor-specific T cell mediated immune response. Evidence from in vitro studies suggest that the paucity of a clinically relevant T cell mediated immune response is due to numerous defects in antigen presentation by the malignant B cells, expression of immunosuppressive factors in the tumor microenvironment and defects in T cell activation and effector function. A novel approach to overcome these defects is to modify the malignant B cells in vitro by CD40 activation in the presence of IL-4. These CD40-activated B cells are now very potent APCs and can be used to prime autologous T cells. A clinical application of this approach, vaccination with CD40-activated lymphoma cells, will be discussed.

Introduction

Most B cell malignancies including low grade and high grade non-Hodgkin's Lymphoma (NHL), chronic lymphocytic leukemia (CLL), plasmocytoma as well as adult pre-B acute lymphocytic leukemia (ALL) are not curable in most patients using standard therapy, including high dose chemotherapy with stem cell support. Therefore novel therapeutic approaches have to be developed as alternative or additional therapy modalities to improve the outcome of these diseases. Recent advances in basic immunology and molecular biology have revived tumor immunology. With the identification of tumor antigens and their specific recognition in the context of MHC class I and II molecules by T lymphocytes, immunotherapy has become once more a very attractive treatment modality. Although most work in the past few years has been focused on solid tumors and melanoma there is evidence that B cell malignancies might be a suitable target for immunological approaches [1]. At least for the more differentiated B cell malignancies the tumor-specific idiotype has been proven to be recognized by the hosts immune system as a tumor-specific antigen and could be targeted by immunotherapy [2, 3]. Indeed, vaccination with this antigen has already been clinically tested and there are several clinical trials currently ongoing [4]. However, there are some very important disadvantages to this approach. Since the antigen is patient-specific one has to produce a patient specific vaccine for each patient. Although novel molecular approaches have simplified the methodology to produce such a vaccine, it is still time consuming and cost intensive. Moreover it is not clear if other tumor antigens might exist in B cell malignancies which might be better targets than the

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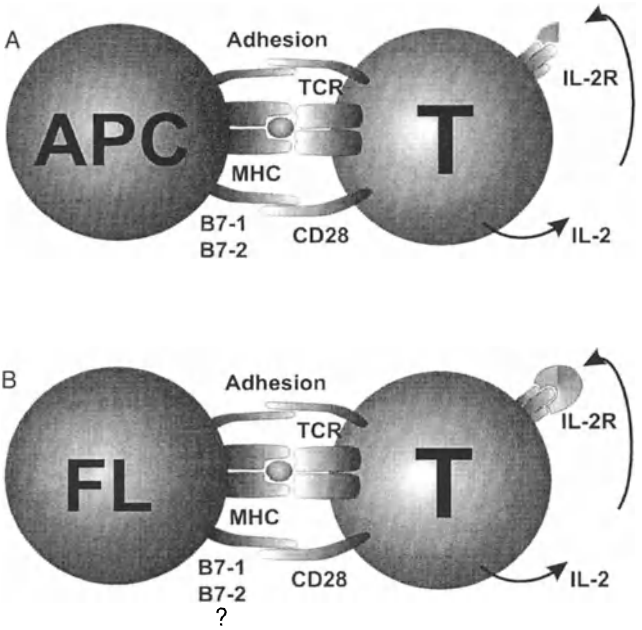


Fig. 1 A, B. Two-signal model of T-cell activation. *A* Professional antigen presenting cells (APC) provide the necessary signals to efficiently activate T cells to proliferate and produce cytokines. APC provide signal 1 via MHC/peptide complexes to the T cell receptor on the T cell in context of adhesion. The second necessary signal (Signal 2) is called costimulatory signal and one of the most important costimulatory signals is provided by members of the B7 family (B7-1 and B7-2) to the CD28 molecule on the T cell. *B* Since most malignant B cells express MHC molecules the expression of adhesion and costimulatory molecules is a decisive factor for their capacity to present antigen

tumor-specific idiotype. We, therefore, were interested whether a more broadly applicable approach could be identified for the B cell malignancies which circumvents these disadvantages.

Antigen-specific T cell proliferation and expansion is only induced if the T cells receive an antigen specific signal through their T cell receptor in the presence of a costimulatory signal via the CD28 molecule on the T cell surface (Fig. 1A; [5-7]). Antigen presenting cells (APC) including dendritic cells (DC), activated B cells and macrophages are capable to deliver both signals, an antigen-specific signal by complexes of major histocompatibility complexes (MHC) and antigen-derived peptides in the groove of the MHC molecules (signal 1), and an antigen-independent costimulatory signal by members of the B7 family (B7-1 - CD80, B7-2 - CD86) (signal 2) (Fig. 1A; [8-10]). Since normal B cells have been shown to function as professional antigen presenting cells (APC) once they are activated appropriately, we sought to determine whether malignant B cells function as APCs in vivo and if not whether we could modulate them ex vivo to become efficient antigen presenting cells so that they could present their own antigens to T cells. Clearly, for such an approach to function, we also have to study if the T cells in the tumor bearing host can be stimulated by these ex vivo modulated B cells to recognize the primary tumor cells. To answer these important questions we have first studied Follicular Lymphoma as a model (Fig. 1B). It has been demonstrated in several studies that malignant B cells express high levels of MHC class I and II molecules [11-15] indicating that these cells should be readily able to express their own tumor antigens. In addition, we [16, 17] and others have been able to demonstrate by immunohistochemistry that some Follicular Lymphoma (FL) also express low levels of B7 molecules on their surface. Moreover, since FL cells are the malignant counterpart of activated B cells these tumor cells seemed to us

the prime candidate to study whether malignant cells could function as fully competent APC. Therefore, we initiated our studies about T cell mediated immunity against B cell malignancies with this subset of B cell tumors.

Once we had established a system to study the interactions of T cells and these malignant B cells we have repeated the same experiment for other B cell malignancies including CLL, mantle cell lymphoma (MCL), plasmocytoma and pre-B ALL with very similar results. We will focus in this report on the results for Follicular Lymphoma, however we want to stress that most of the results can readily be broadened to other B cell malignancies. Here, we demonstrate that malignant B cells are poor APC for autologous and allogeneic cells but can be modified in vitro by stimulation with CD40 ligand and IL-4 to become highly efficient APCs. These CD40 activated B cells (CD40-B) can be used for the amplification and the generation of tumor-directed cytotoxic autologous T cells. Moreover malignant CD40-B cells can overcome the inhibitory effect of IL-10 [18-20] and TGFβ [21-28] shown to be produced in many B cell malignancies. Based on these preclinical results a clinical trial has been initiated using ex vivo CD40 activated FL (CD40-FL) cells as a vaccine. The approach is based on the hypothesis that these ex vivo modified tumor cells can function as professional APC to present efficiently their own tumor antigen(s). Therefore this approach is independent of the individual tumor antigens expressed by the tumor cells.

Material and Methods

Normal and Neoplastic Tissues

Lymph node specimens were obtained under sterile conditions from patients with follicular lymphoma (FL) undergoing diagnostic lymph node biopsies. By the Working Formulation all samples were of follicular, predominantly small-cleaved cell type (type B). Ten patients were selected with tumor cells that expressed a t(14:18) translocation detectable by polymerase chain reaction (PCR). This provided a marker that could be used to demonstrate that cultured FL cells belonged to the malignant clone. For control studies peripheral blood was obtained from all patients to obtain normal T and B cells. These cells were only used as controls if found to be negative for the t(14;18) translocation. Normal autologous B cells from peripheral blood could only be obtained in sufficient quantities from two patients (FL4 and FL6). Splenic B cells were purified from normal human spleen obtained from organ donors. Allogeneic T cells from healthy donors were purified from peripheral blood. All discarded specimens and blood samples were obtained after approval by Institution Review Committees.

Purification of B and T Cells

Lymph node or splenic samples were mechanically homogenized and mononuclear cells isolated by Ficoll-Isopaque density centrifugation. Purification of B cell and T cell populations was performed as previously described [29,30], and purity assessed by immunophenotyping. Isolated B cells were uniformly > 90% $CD20^+$ $CD40^+$ and < 5% CD3, CD14 or CD56. $CD20^+$ cells were > 97% lambda or kappa light chain monoclonal. $CD3^+$ T cells or CD3+CD4+ T cells were obtained from peripheral blood mononuclear cells (PBMC) of healthy donors as previously described (Gribben, PNAS, 1995). $CD3^+$ T cells were > 98% $CD3^+$ and $CD4^+$ T cells were > 98% $CD4^+$. The total number of purified $CD3^+$ tumor infiltrating T cells (T-TIL) after isolation and purification ranged between 1.9×10^7 (FLIO) and 2.5×10^8 cells (FL4) depending on the size of biopsy material available.

Immunofluorescence Studies

Surface expression of molecules was detected using the following mAbs conjugated with FITC or PE; CD3-FITC, CD4-PE, CD8-PE, CD19-PE, CD20-PE, CD56-PE, anti-MHC class II-PE (Coulter, Miami, FL), CD54-PE (Becton Dickinson, Mountain View, CA), CD58 FITC (Southern Biotechnology, Bir-

mingham, AL) CD14-FITC, anti-kappa and anti-lambda FITC (Dako, Carpinteria, CA). For detection of B7-1 mAbs 133, GB10.F3 and YB2.C4 (Repligen Corporation, Cambridge MA) were used and for B7-2 (B70-PE, clone IT2.2) (Pharmingen, CA). CTLA4-Ig FITC and control-Ig fusion proteins (FP-Ig) were provided by Repligen. Hybridomas for anti-MHC class I and CD40 were obtained from ATCC. MAbs for the alpha, beta and gamma chain of the IL-2 receptor were a kind gift from Dr. J. Ritz (Dana-Farber Cancer Institute, Boston, MA).

Cytokines

Optimal concentrations of cytokines were established in proliferation assays using allogeneic T cells (10^5/well) as effector cells and CD40-activated follicular lymphoma cells (2×10^4/well) as stimulator cells in the presence of increasing concentrations of the cytokines. Interleukin 2 (1L-2) was a generous gift of Dr. Ritz (Dana-Farber Cancer Institute, Boston, MA) and was used at 100 IU/ml. Human recombinant human Interleukin 4 (IL-4) was a generous gift of Dr. Widmer (Immunex, Seattle, WA) and used at 5 ng/ml. Human Interleukin 12 (IL-12, 1 ng/ml) and Interferon gamma (IFN-g, 10 ng/ml) were purchased from Genzyme (Cambridge, MA). IL-7 was purchased from Sigma (St. Louis, LA) and used at 5 ng/ml.

CD40 Ligand Transfected NIH3T3 Cells

The CD40L coding region was amplified from human activated T cell cDNA by PCR using sense primer, GTAGGAATTCGTCGACGCCGCCACCATGATCGAAACATACAACC containing EcoR I and Sal I sites, a strong translational start site, and the first 20 nucleotides of the CD40L coding sequence and antisense primer, GACTAGTGTCGACGAATTCAGAGTTTGAGTAAGCCAAAG containing the last 23 nucleotides of the CD40L coding sequence including the stop codon and EcoR I, Sal I, and Spe I sites. PCR conditions were 94 °C, 1 min, 50 °C, 1 min, 72 °C, 1 min for 40 cycles followed by a final extension period at 72 °C for 10 min. The 0.8-kb PCR product was digested with EcoR I, gel purified, and ligated into EcoR I digested pSP65_SRa2 expression vector. NIH3T3 cells were stably transfected via electroporation with Fsp I linearized CD40L plasmid and Pvu I linearized pSP65-Neo plasmid. Transfectants were selected by growth in 200 μg/ml G418 and subcloned. Subclones were selected on the basis of their capacity to stimulate B cell proliferation in the presence of IL-4. CD40 ligand transfectants (tCD40L) were cultured in 45% DMEM (Gibco BRL), 45% F12 (Gibco BRL) supplemented with 10% FCS, 2mM Glutamine, 15 μg/ml Gentamicin and 200 mg/ml of G418. For B cell cultures, 10^5 irradiated (96 Gy) t-CD40L were plated as feeder layer cells onto 24-well tissue culture plates (Falcon). Cells were incubated overnight at 37 °C in 5% CO_2. The plates were then washed twice with PBS before B cells were plated on these feeder layers.

Activation and Culture of B-Cells onto t-CD40L

Purified normal or malignant B cells were stimulated by culture on murine NIH3T3 fibroblasts transfected with the human CD40 ligand (t-CD40L) at a concentration of 5×10^5 cells/ml in IMDM (Gibco BRL) supplemented with 2% FCS, 0.5% BSA (Sigma), 50 μg/ml human transferrin (Boehringer Mannheim), 5 μg/ml bovine insulin (Sigma) and 15 μg/ml Gentamicin (Gibco BRL) at 37 °C in 5% CO_2. B cells were transferred at day 3 to plates with fresh irradiated t-CD40L cells and harvested on day 5 for functional studies. After harvesting, B cells were washed 2× in IMDM, kept on ice for 1 h in IMDM and then finally washed and resuspended in RPMI supplemented with 5% human serum, 2 mM Glutamine, 15 μg/ml Gentamicin (RPMI-5). To ensure that cytotoxicity is not directed against serum components used in the culture conditions, HLA-$A2^+$ CD40-activated B cells were pulsed with a known immunogenic peptide binding to HLA-A2. After repetitive stimulation of autologous T cells with these peptide pulsed CD40-B cells cytotoxicity was induced only directed against peptide pulsed target cells but not the CD40-B cells themselves. This

demonstrates that serum components are not presented sufficiently to autologous T cells to induce cytotoxicity in this system (unpublished results).

Allogeneic Mixed Lymphocyte Reaction (Allo-MLR)

$CD3^+$ T cells or $CD4^+$ T cells from healthy individuals were plated at 1×10^5 T cells/well with 10^4 to 10^5 irradiated (25 Gy) FL or B cells/well, or irradiated (96 Gy) Epstein Barr Virus transformed lymphoblastoid cell lines (EBV-LBL) as stimulators in 96-well round-bottom plates (Nunc, Roskilde, Denmark) in a final volume of 200 ml. Cultured B and FL cells were washed twice at 4 °C in PBS before use. Cultures in triplicate were incubated for 5 days in RPMI-5 at 37 °C in 5% CO_2. Cells were pulsed with [^{3}H] Thymidine (1 mcCi, Du Pont, Boston, MA) for the last 16 h of the 5-day culture period. Cells were then harvested onto filters and the radioactivity measured in a beta plate liquid scintillation counter (Pharmacia, Piscataway, NJ). IL-2 concentrations in culture supernatants collected at day 5 of the culture were assayed by ELISA (Endogen, Cambridge) or CTLL-20 bioassay.

Polymerase Chain Reaction (PCR) of t(14:18) Translocation

Malignant B cells carrying the t(14;18) translocation in fresh lymph node samples and cultured cells in the tCD40L system were detected by PCR. To estimate the amount of malignant cells in each sample under study cells were serial diluted from 10^6 cells to single cell level. From these samples DNA was extracted by standard techniques and nested PCR amplification performed and visualized on 2.5% agarose gel containing ethidium bromide as previously described.

In Vitro Cytotoxic T Lymphocyte Response Induction

Purified T cells from lymph nodes with Follicular Lymphoma (T-TILs) were stimulated with irradiated (64 Gy) CD40-FL cells or with FL cells at different Effector:Target (E:T) ratios ranging from 4:1 to 1:2. For CD40-FL cells an optimal ratio for T cell expansion at 4:1 was established. T cells were stimulated with irradiated stimulator cells on day 0, 7 and 14. Cytokines were either added at initiation of culture or first added at day 3 and every third day thereafter until cytotoxicity was assessed. Were indicated, CD28 mAb (10 μg/ml), irradiated (64 Gy) B7-1 or B7-2 transfectants (t-B7-1, t-B7-2) were added on day 0, 7 and 14 together with FL cells. Co-cultures were cultured in RPMI supplemented with 5% human AB serum, 2 mM Glutamine, 50 μg/ml Penicillin-Streptomycin (Gibco BRL), 15 μg/ml Gentamicin (Gibco BRL) at 37 °C in 5% CO_2 at a concentration of 1 to 3×10^6 cells/ml. Before each restimulation and cytotoxicity test T cells were Ficoll-density centrifuged to increase cell viability.

Cytotoxicity Assay

A previously published method to assess T cell mediated cytotoxicity as adapted to measure T cell cytotoxicity against human FL. As target cells CD40-FL cells, FL cells autologous normal CD40-B cells or allogeneic CD40-B cells were used. Autologous PHA blasts or autologous T cells were used as control targets only when autologous B cells were not obtained in sufficient numbers. CD40-B cells were harvested from culture, washed twice by centrifugation in PBS and resuspended in RPMI-5. The target cells were incubated with [^{3}H] thymidine overnight at 37 °C. Target cells (10^4 cells/well) were again washed by centrifugation and mixed with various numbers of effectors in a final volume of 0.2 ml of RPMI-5 in round-bottom microtiter plates. At a concentration of 10^4 cells/well thymidine incorporation ranged between 800 and 2300 cpm for FL cells, 2000-15 000 cpm for CD40 activated FL and normal B cells, 1500 and 13 000 cpm for PHA blasts. Where indicated, T cell mediated lysis was measured in the presence of antibodies to MHC class I (W6/32, 10 mg/ml) and/or MHC class II (949, 10 mg/ml) to block the interaction of T cells and target cells. After 6-8 hr of incubation, the plates were harvested and the radioactivity was determined in a

beta counter. Spontaneous lysis was determined by incubating the targets alone, in the absence of effector T cells. Maximum lysis was determined by incubating the target cells with 0.2% Triton X-100 (Sigma). All determinations were done at least in triplicates and the standard errors of the means were always < 10% of the value of mean. Percent specific cytotoxicity was determined using the following equation. % specific DNA loss = (S-E)/S × 100 where E = experimentally retained DNA in the presence of T cells (in cpm), S = retained DNA in the absence of T cells (spontaneous). The JAM test was validated with allogeneic T cell lines specifically killing FL cells as well as with an autologous T cell line established from patient FL4 by performing standard chromium release assays. The results from these chromium release assays were almost identical to the results obtained in JAM tests performed at the same time (data not shown). To further confirm that the target cells lysed were indeed FL cells we have analyzed by flow cytometry using directly conjugated mAbs and HOECHST 33342 staining which cell population was killed after 4 h of co-culture with the autologous T cell line. This analysis revealed that 50% of $CD19^+$ $CD38^+$ $CD10^+$ cells had undergone apoptosis or were already dead (data not shown) whereas FL cells co-cultured with unstimulated T cells showed no cell death during this time period.

Statistical Analysis

Differences between experimental groups were analyzed by the X^2 test and the Student's *t* test.

Results

Defects in Recognition of FL Cells by Autologous T Cells

Lack of Sufficient Functional Adhesion and Costimulatory Molecules on FL Cells Is Responsible for Their Poor APC Function

Our principle concept is to use FL cells as APC presenting their own tumor antigens. Therefore, we first sought to determine whether these FL cells could indeed present antigen efficiently to T cells. To answer this question we used an allogeneic system were highly purified allogeneic T cells were assessed for their ability to proliferate and secrete cytokines due to stimulation with FL cells. Allogeneic T cells from healthy donors were used mainly because they should be fully functional and because of their high frequency.

The results of these earlier experiments described here serve as background information for experiments in the autologous system described later (see below). Surprisingly, when allogeneic T cells were stimulated with highly purified FL cells no significant T cell proliferation could be detected (Fig. 2A), although these FL cells expressed very high levels of MHC class I and II molecules (Fig. 2B; [29]). Although we could detect adhesion molecules and costimulatory molecules on the surface of FL cells by immunohistochemistry using a technique with high signal amplification [17], the level of expression as determined by FACS analysis was either very low or undetectable (Fig. 2B), indicating FL cells did not express sufficient amounts adhesion and costimulatory molecules to stimulate allogeneic T cells to proliferate. Some FL cells expressed sufficient amounts of B7-2 molecules, still did not induce allogeneic T cell proliferation. In context with recent data in a murine tumor model, there is strong evidence that the lack of function of B7 molecules might be due to conformational changes as demonstrated by the lack of binding to some mAbs of the B7 panel on murine tumor cells [17].

T Cells from FL Lymph Nodes Show a General Unresponsiveness to T Cell Mediated Signals Probably Due to Lack of TCR ζ

Before studying a potentially specific T cell response to the autologous FL cells we first assessed whether T cells from the tumor environment could be activated via their T cell receptor.

Tumor infiltrating T cells (T-TIL) were isolated and assessed for their ability to proliferate to stimulation with CD3 monoclonal antibodies (mAbs) (light gray bars) in the presence of costimulatory CD28 mAbs (dark gray bars). At the same time T cells from the

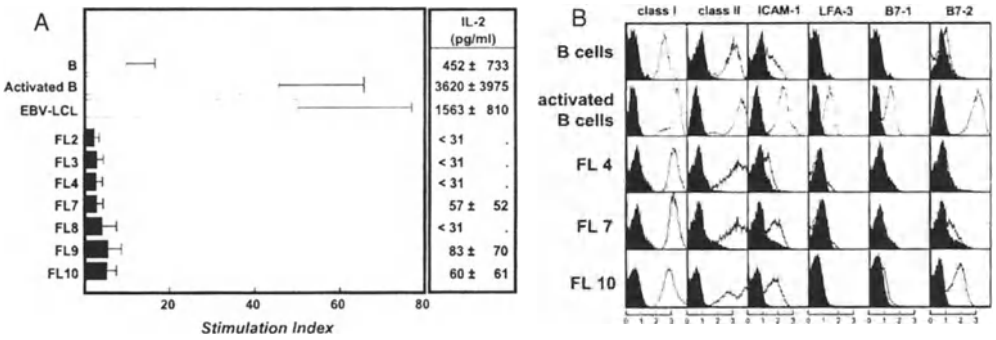


Fig. 2 A, B. FL cells are poor APC although they express high levels of MHC molecules. *A* Response of allogeneic $CD3^+$ T cells to FL cells. $CD3^+$ T cells from seven normal donors were co-cultured with unstimulated FL cells from seven patients. As controls, T cells were stimulated with freshly isolated resting B or CD40-activated (CD40-B) splenic B cells or with an EBV-LBL. [^{3}H] Thymidine incorporation was assessed for the last 16 h of a 5-day culture. The stimulation index for every donor was calculated as $cpm_{T\ cells\ +\ FL\ cells}/cpmT_{cells}$. Results are expressed as means ± SDs of the stimulation index of the seven normal donors. IL-2 was assessed by ELISA. *B* Cell-surface expression of MHC class I and II molecules ICAM-1 and LFA-3 and costimulatory molecules B7-1 and B7-2 on unstimulated FL cells. Phenotype of three representative FL cells is compared with normal resting B cells and CD40-B cells. Shaded area indicates fluorescence of isotype-matched antibody

peripheral blood of the same patient as well as T cells from healthy donors were analyzed. Whereas the normal control T cells and the peripheral blood T cells of the patient showed minor proliferation to CD3 stimulation which was significantly increased by the addition of CD28 mAbs, T-TIL showed no proliferation to both stimuli (Fig. 3A). T cell proliferation induced by phorbol myristate acetate (PMA) in the presence of CD28 mAbs (dark gray bars) demonstrated that T-TIL were able to proliferate (Fig. 3B). Since PMA activates protein kinase C (PKC) directly these data indicated that T-TIL were defective in proximal T cell signaling.

To further study this possibility, Western blot analysis of components of the proximal T cell receptor signaling cascade in T-TIL were performed. As shown in Fig. 4, although T-TIL showed reduced levels of TCR ζ, they expressed normal amounts of the CD3 molecule on the cell surface. These findings confirm results in other human tumors [31-34] and in several murine tumor model systems [35, 36] suggesting that T cells are altered in the tumor microenvironment. The lack of TCR ζ might partially explain the lack of T-TIL to proliferate to T cell mediated signals. To test this hypothesis we first sought to determine signals that might be capable of overcoming this defect. Incubation of T-TIL, with exogenous IL-2 for 24 h restored the expression of TCR ζ already and further incubation did not induce significantly higher expression. These IL-2 preincubated T-TIL could proliferate to CD3 mAbs in the presence of CD28 mAbs, indicating that proximal T cell receptor signaling defects were reversed by exogenous IL-2 (data not shown). Whether other cytokines are capable of reversing this defect is currently under investigation.

T-TIL in FL Show no Spontaneous Cytotoxicity and Show Either Very Low or Undetectable Expression of Granzyme B and Perforin

Since T-TIL could not even be stimulated by an antigen-independent TCR signal, we were not surprised that these cells did not show any FL-directed cytotoxicity (data not shown) as has been shown for freshly isolated T-TIL in some other human tumors. Preincubation with IL-2 for up to 7 days did not induce any FL-directed cytotoxicity (data not shown), indicating that either other defects are still present in these cells or alternatively the frequency of FL-specific cells is rather low. The lack of cytotoxicity by autologous T cells can not be explained by the inability to induce T cell mediated apoptosis in FL cells since allogeneic T cell lines (TCL) in-

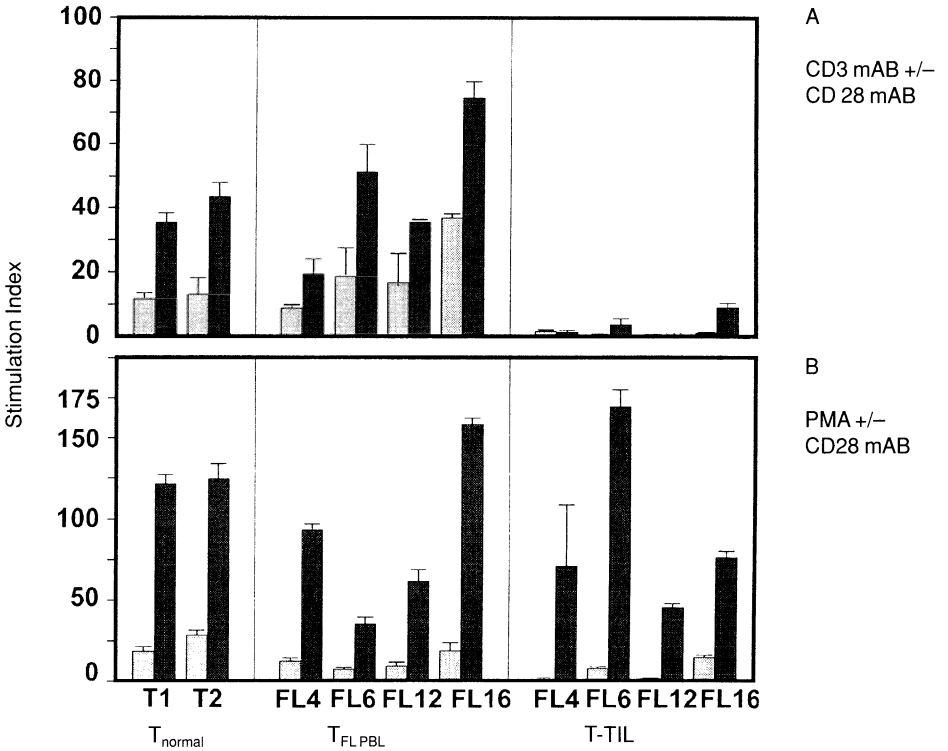


Fig. 3 A, B. Proliferation of T-TIL to TCR mediated and mitogen mediated stimulation. *A* Stimulation with CD3 mAbs ± costimulatory CD28 mAbs. Highly purified peripheral blood T cells from two healthy donors (T1, T2), peripheral blood T cells from 4 patients with FL (FL4, FL6, FL12, FL16) and highly purified T-TIL from the same four patients were stimulated with submitogenic CD3 mAbs with (dark gray bars) or without (light gray bars) costimulatory CD28 mAbs. [^{3}H] Thymidine incorporation was assessed for the last 16 hrs of a 3-day culture. *B* The same T cells were stimulated at the same time with PMA with (dark gray bars) or without (light gray bars) costimulatory CD28 mAbs

duced by multiple stimulations with CD40-FL cells could kill both CD40-FL cells and FL cells, suggesting that FL cells are susceptible to T cell mediated killing (data not shown).

Therefore, we next sought to determine if T-TIL express sufficient amounts of the molecules necessary for cytotoxic effector function. The major cytotoxic pathway in T cells has been identified [37, 38] and perforin [39] and granzyme B [40] have been proven in knockout experiments [41] to be essential for this pathway. The lack of one of both molecules is sufficient to completely block the cytotoxic function of T cells to targets that cannot be killed by alternative pathways [42-44] including interaction between Fas ligand [45] and Fas [46] and TNF-mediated cytotoxicity [47].

Perforin and granzyme B were analyzed by immunohistochemistry in FL samples. Whereas there was virtually no expression of perforin detectable in any of the FL cells tested, we could see staining for granzyme B in T-TIL in some but not all FL tested (data not shown). To further quantitate the production of granzyme B in these samples quantitative RT-PCR for granzyme B was performed and the amount in T-TIL compared with peripheral blood T cells from the same patient and from healthy donors. In T-TIL granzyme B was either not detectable or significantly lower than in T cells from peripheral blood (data not shown). Although these results strongly suggest that the expression of these important molecules is not sufficient in T-TIL in FL they do not rule out that very low amounts of these molecules might be already sufficient for T cells to be highly cytotoxic. However, when cardiac [48-50], liver [51] or renal [52, 53] allografts in murine model

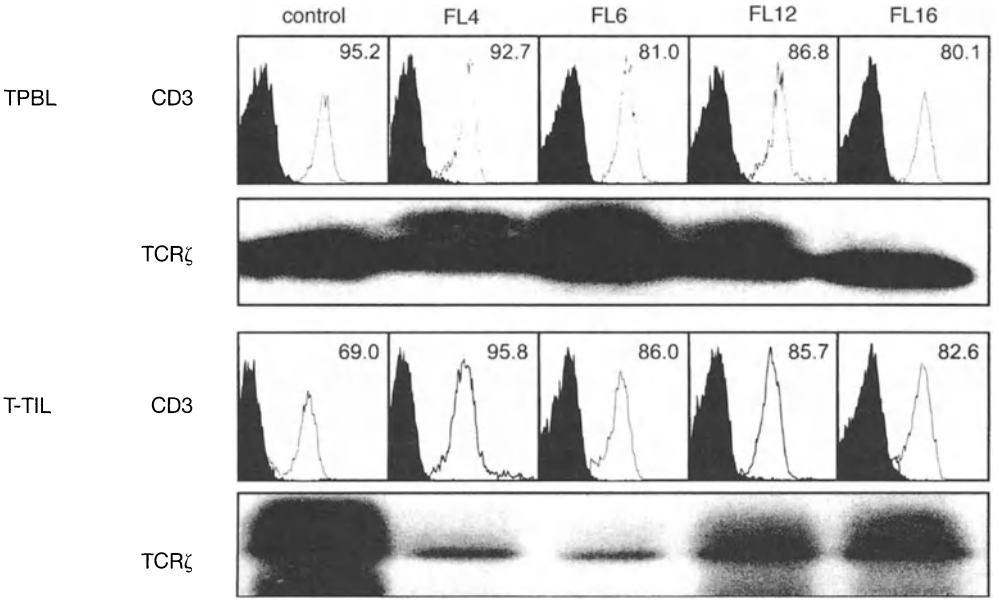


Fig. 4. T-TIL in FL express decreased levels of TCR ζ. Peripheral blood T cells from one healthy donor and 4 patients (FL4, FL6, FL12, FL16) were compared with T cells from a hyperplastic LN and T-TIL from the same patients for their expression of surface CD3 and total TCR ζ. Surface expression of CD3 was measured by FACS analysis. Shaded area indicates fluorescence of isotype-matched antibody. For analysis of TCR ζ T cells were lysed and Western blot analysis performed using equal amounts of whole lysates

systems are analyzed for granzyme B expression at time of allograft rejection, these tissues are infiltrated by huge numbers of T cells exceptionally expressing high levels of granzyme B suggesting that the expression of this molecule in T-TIL is insufficient [54].

Expression of IL-10 and TGFβ in Non-Hodgkin Lymphoma

Several studies have demonstrated that IL-10 and TGFβ are produced by a variety of B cell tumors including pre-B ALL, CLL, plasmocytoma and a variety of NHL. We have analyzed FL cells and T-TIL for the expression of TGFβ by quantitative RT-PCR and could measure mRNA for this cytokine in FL cells as well as in the T-TIL (Fig. 5A). The amount was higher than in normal T cell control samples suggesting that the TGFβ could be expressed in higher quantities in the tumor microenvironment. In addition IL-10 was expressed by all FL samples tested to date and could be shown not only by PCR analysis, but also by intracytoplasmic staining on FACS analysis (Fig. 5B) indicating that both inhibitory cytokines are indeed present at the tumor site.

Taken together, several defects in the interaction of tumor-infiltrating T cells and the tumor cells are demonstrated, which might, at least in part, explain the lack of a clinically significant T cell mediated immune response against this tumor. We strongly believe that the knowledge about these defects is extremely important to identify new successful strategies for immunotherapy in this disease and other B cell malignancies.

Repair of the Defects in T-TIL

Since we could demonstrate that FL cells can be modified in vitro via CD40 activation to become effective allo-APC able to prime allogeneic T cells against the primary FL cells [29], we next sought to determine if these cells could overcome the defects in T-TIL cells and could stimulate autologous T-TIL cells to recognize the autologous FL cells and subsequently kill the tumor cells.

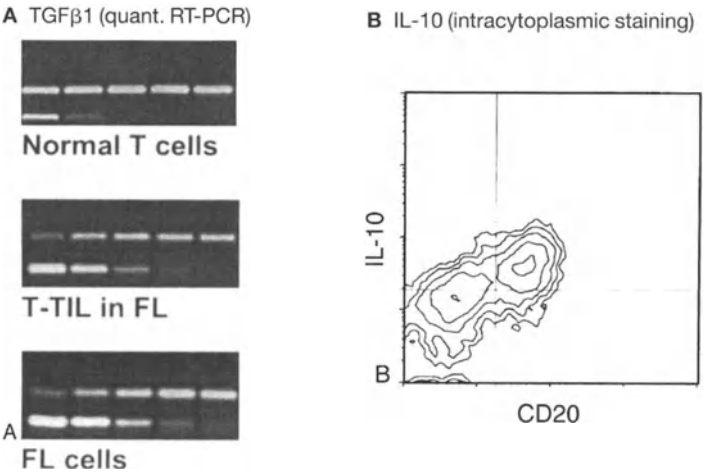


Fig. 5 A, B. IL-10 and TGFβ are expressed in FL. **A** TGFβ was measured by quantitative RT-P in normal control samples (PBL derived T cells), in T-TIL and in the tumor cells themselves. A representative experiment is shown here. 6/6 T-TIL and 10/10 FL cells were highly positive for TGFβ1. **B** Intracellular staining for FL cells. Although all FL expressed mRNA for IL-10 (data not shown), protein could only be measured in some of the samples analyzed so far. One representative sample of IL-10$^+$ FL cells is shown here

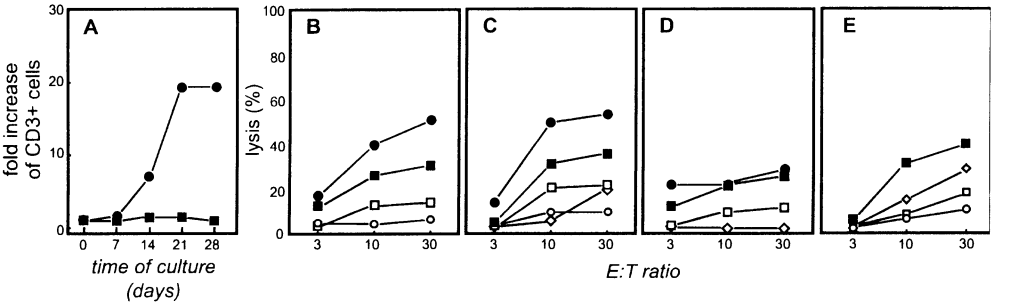


Fig. 6 A-E. Highly enriched T-TILs expanded in the presence of CD40-FL and exogenous IL-2 exhibit cytotoxicity against autologous lymphoma cells. **A** T-TILs from patient FL4 were stimulated with CD40-FL cells *(solid squares)* or CD40-FL cells in the presence of 100 IU/ml IL-2 *(solid circles)*. Total number of viable cells was assessed by Trypan blue exclusion test and number of CD3$^+$ T cells by immunophenotyping on day 0, 7, 14, 21 and 28 of culture. Viable cells were > 97% CD3$^+$ T cells as assessed by phenotypic analysis. **B** Cytotoxicity of TCL cells after 3 stimulations with CD40-FL with addition of IL-2 at day 3 of co-culture. Cytotoxicity was assessed on day 28 of culture. As targets autologous CD40-FL cells *(solid circles)*, FL cells *(solid squares)*, autologous CD40-B cells *(open squares)*, allogeneic CD40-B cells *(open diamonds)* and allogeneic CD40-FL cells *(open circles)* were used in **B** chromium release assay and **C** JAM test. Similar results were obtained using cells from patient FL6. **D** TCL lines derived from T-TILs depleted of CD8$^+$ T cells exhibit reduced cytotoxicity against FL and CD40-FL cells. **E** Blockade of MHC class I (open diamond), MHC class II *(open square)* by mAbs decreases anti-FL directed T cell cytotoxicity and the combination of both *(open circle)* almost abrogates cytotoxicity of autologous TCL to FL4

Identification and Optimization of Culture Conditions for T-TIL to Overcome the General Unresponsiveness and to Induce FL-Directed Cytotoxicity

To determine whether CD40 activated FL (CD40-FL) cells could be used to expand T-TILs they were purified and stimulated with CD40-FL cells weekly. Viable CD3$^+$ cells isolated throughout these cultures are shown in Fig. 6A.

No expansion occurred when the T-TILs were cultured without the addition of cytokines. However, under these culture conditions, the addition of exogenous IL-2 from day three of culture induced a more than 20-fold increase of CD3$^+$ T cells after 28 days. Cytotoxicity of T cells isolated from day 28 of culture with CD40-FL and exogenous IL-2 are shown in Fig. 6B and C. At this time point T cells lysed autologous CD40-FL cells (solid circles) as well as unstimulated FL cells (solid squares). There was only minor cytotoxicity against allogeneic CD40 acti-

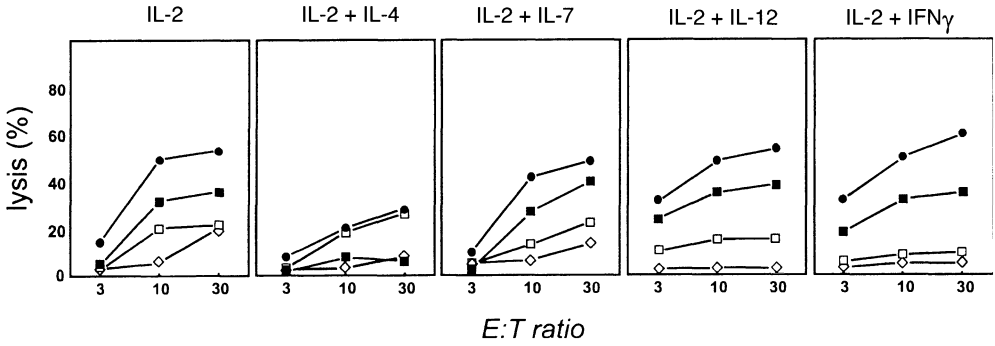


Fig. 7. Cytotoxicity of TCL cells under different culture conditions. T-TILs (5×10^6 cells) from FL4 were isolated, purified and stimulated with CD40-FL cells in the presence of IL-2 (100 IU/ml) either alone or in combination with IL-4 (2 ng/ml), IL-7 (5 ng/ml), IL-12 (1 ng/ml) or IFNγ (10 ng/ml). Cytotoxicity was measured by JAM-test. As targets autologous CD40-FL cells *(solid circles)*, FL cells *(solid squares)*, autologous CD40-B cells *(open squares)* and allogeneic CD40-FL cells *(open diamonds)* were used. Cytotoxicity was analyzed after three stimulations with CD40-FL and exogenous cytokines added at day three of culture

vated B cells (open diamonds) at high E:T ratios and no cytotoxicity against allogeneic CD40 activated FL cells (open circles) cultured under the same culture conditions. More important autologous CD40 activated normal B cells (open squares) cultured under the exact same culture conditions as the CD40-FL cells were not lysed at low E:T ratios and significantly less than the CD40-FL cells at the highest E:T ratio. This indicates that the cytotoxicity is directed against tumor-associated antigens and rules out that CD40-FL cells presented antigens captured during culture. Moreover these results indicate that common B cell antigens do not seem to be a major target for these T cells. When T cell lines were established which were depleted of $CD8^+$ T cells prior stimulation and expansion, cytotoxicity against both FL cells (24% at E:T = 30:1) as well as CD40-FL cells (24% at E:T = 30:1) was diminished but not abrogated indicating that both $CD4^+$ and $CD8^+$ T cells were responsible for cell death of the FL cells (Fig. 6D). Blockade by MHC class I or II mAbs decreased FL-specific cytotoxicity and the combination of both almost completely abrogated cytotoxicity (Fig. 6E) indicating that both $CD4^+$ and $CD8^+$ T cells are exhibiting FL-directed cytotoxicity.

T cell expansion induced by CD40-FL cells in the presence of IL-2 (Fig. 6A) would likely be insufficient for adoptive transfer strategies. Therefore, we examined whether the addition of other exogenous cytokines could enhance T cell proliferation and/or cytotoxicity. When autologous T cells were stimulated with CD40-FL cells in the presence of either IL-4, IL-7, IL-12 or IFNγ, no expansion could be detected (data not shown) suggesting that IL-2 was a prerequisite for expansion of these T cells. However, as seen in Fig. 5, when T-TILs were cultured with CD40-FL and exogenous IL-2 alone their expansion was increased by further addition of IL-4 (70 fold increase), IFNγ (52 fold increase) or IL-7 (28-fold increase). In contrast, the further addition of IL-12 led to a five fold decreased expansion compared to IL-2 alone (data not shown).

We next assessed cytotoxicity of the T cells expanded under the various culture conditions described above. After 28 days of culture and three consecutive stimulations with CD40-FL cells (Fig. 7), TCL expanded with IL-2 resulted in killing of CD40-FL cells at 52% (solid circle) and FL cells at 31% (solid square) at an effector to stimulator ratio of 30:1 whereas there was significantly less killing of autologous (open square) and allogeneic activated B cells (open diamond). Although the combination of IL-4 and IL-2 was the most efficient combination to expand T cells stimulated with CD40-FL cells, somewhat surprisingly this led to greatly diminished cytotoxicity against CD40-FL cells (28% at a E:T = 30:1) and loss of cytotoxic activity against unstimulated FL cells (Fig.

7). Moreover, there was loss of specificity since they now killed autologous activated B cells (27% at an E:T = 30:1) but not allogeneic activated B cells. T cell lines expanded in the presence of IL-2 and either IL-7 or IL-12 killed CD40-FL, unstimulated FL cells and normal B cells comparably to T cells expanded with IL-2 alone. The most specific cytotoxicity was induced by TCL expanded with the combination of IL-2 and IFNγ. Although these TCL did not kill more efficiently than TCL expanded in IL-2 alone, there was now negligible non-specific killing of normal B cells (Fig. 7). Taken together, these results suggest that culture conditions for efficient expansion of lymphoma specific cytotoxic T cells includes culture of the T-TILs with CD40-FL followed by addition of exogenous IL-2 and IFNγ.

Stimulation with CD40 Activated FL Cells in the Presence of IFNγ and IL-2 Induces FL-Specific T Cells in 4 of 5 Patients

Using these optimized culture conditions we next assessed whether T-TILs could be isolated and expanded from four additional patients with FL. As can be seen in Fig. 8, T cells could be expanded from T-TILs in each patient after 28 days of culture from 21.8 to 250.2 fold (calculated values).

Since we could purify T cells from these patients ranging from 1.9×10^7 cells up to 2.5×10^8 cells this would translate into a potential number of expanded T cells ranging from 4.14×10^8 to 1.23×10^{10} cells, if all cells isolated would be used entirely for expansion (Fig. 8). Since these tumor samples were not obtained for therapeutic use, these numbers might be even higher in a therapeutic setting. Cytotoxicity of these cells after 28 days in culture was assessed (Fig. 9). At an E:T ratio of 30:1, specific cytotoxicity from four patients ranged from 38 to 82% against autologous CD40-FL (closed circles) and from 25.6-39.1% against the autologous FL cells (closed squares). No detectable cytotoxicity against the unstimulated autologous FL cells was generated from patient FL15. T-TILs expanded from FL12 had slightly elevated cytotoxicity against allogeneic CD40 activated B cells (open diamond) at a E:T ratio of 30:1. Non-specific killing against autologous B cells (open square) could not be tested from FL10, FL12 and FL15. However, autologous PHA blasts (open circles) or autologous T cells (data not shown) were not lysed by the expanded T cell lines from these patients.

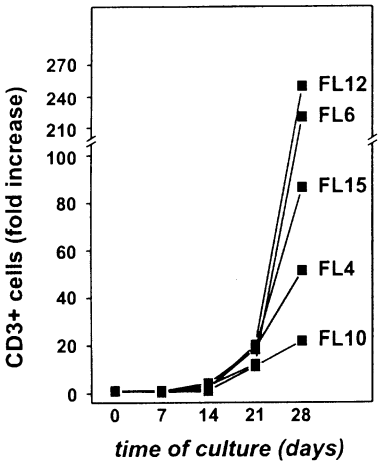
Taken together, these data indicate that FL cells can function as APC in all autologous in vitro system once they are pre-activated via the CD40 pathway. Moreover T-TIL can be primed to recognize and kill the primary FL cells in vitro. The expression of granzyme B by T-TIL or TCL generated from T-TIL correlates with their cytotoxic effector function in vitro (data not shown).

Overcoming Immunosuppression by TGFβ and IL-10

For immunotherapy using CD40-FL cells as a tumor vaccine it is important to know if these cells could also stimulate T cells in the presence of inhibitory cytokines which are produced in the tumor bearing host. To answer this question, experiments were performed using CD40-FL cells as allo-APC in the presence of inhibitory cytokines. As controls we used the unstimulated FL cells in the presence if costimulatory CD28 mAbs (data not shown) or dendritic cells (data not shown). Only CD40-FL cells were fully competent APC in the presence of IL-10 and TGFβ or their combinations, neither FL cells with exogenous costimulation nor dendritic cells could overcome the effect of IL10 and TGFβ on the T cells.

Even if vaccination would be performed after cytoreduction therapy, it has to be established if autologous CD40-FL cells could stimulate T cells which are unresponsive to T cell receptor signals. To answer this issue allogeneic T cells were preincubated with several cytokines and subsequently stimulated with FL cells, FL cells plus CD28 mAbs or CD40-FL cells (data not shown).

Similar results as described above were obtained. Preincubation with IL-2, IL-4 or IFNγ compared to medium alone did not decrease T cell proliferation induced by allogeneic FL cells (data not shown). As expected, proliferation induced by FL cells was very low, addition of CD28 mAbs increased proliferation and CD40-FL cells were the most potent stimulators. Preincubation with IL-10 and/or TGFβ did not decrease T cell proliferation induced by CD40-FL cells but



T cells isolated	Maximal number of T cells after 28 days
$1.2x10^7$	$4.24x10^9$
$1.5x10^7$	$3.23x10^9$
$5.0x10^7$	$4.24x10^9$
$2.5x10^8$	$12.25x10^9$
$1.9x10^7$	$0.41x10^9$

Fig. 8. Calculated maximal expansion of T-TILs from FL4, FL6, FL10, FL12 and FL15 stimulated with autologous CD40-FL cells with addition of IL-2 and IFNγ from day 3 of co-culture if all T cells isolated from the tumor specimen would have been cultured only for expansion and usage in a therapeutic setting. Total number of viable cells was assessed by Trypan blue exclusion test and number of $CD3^+$ T cells by immunophenotyping on day 0, 7, 14, 21 and 28 of culture. Expanded cells were always > 97% CD3 T cells and therefore expansion of total cells equals the expansion of T cells

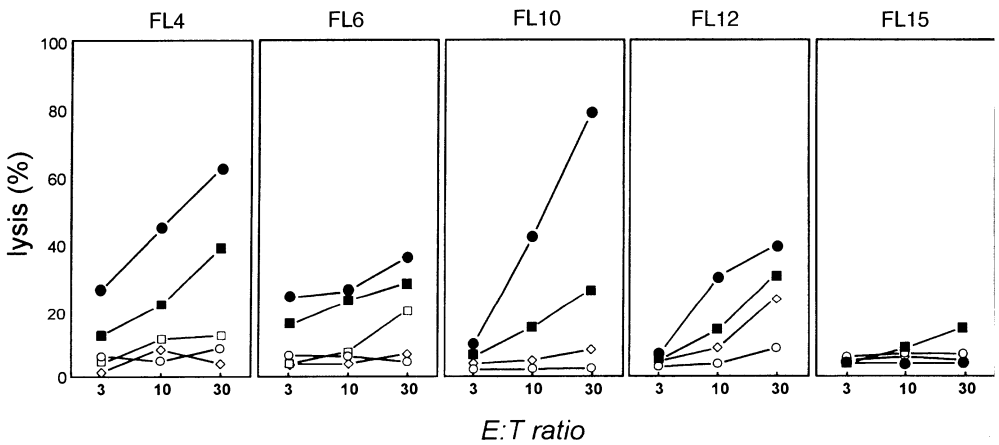


Fig. 9. Cytotoxicity of TCL from FL4, FL6, FL10, FL12 and FL15 expanded by stimulation with autologous CD40-FL in the presence of IL-2 and IFNγ. TCL were stimulated three times with autologous CD40-FL and cytotoxicity was measured by JAM-test at day 28. Cytokines were added after 3 days of initiation of co-culture of T cells and CD40-FL cells. As targets autologous CD40-FL cells *(solid circles)*, FL cells *(solid squares)*, autologous CD40-B cells *(open squares)*, autologous PHA blasts *(open circles)* and allogeneic CD40-FL, cells *(open diamonds)* were used

reduced proliferation induced by FL cells and FL cells plus CD28 mAbs significantly (data not shown), suggesting that only CD40-FL cells were capable to overcome T cell unresponsiveness induced by these cytokines. Interestingly the addition of IL-2 during preincubation rendered the T cells responsive (data not shown) indicating that the effect of IL-2 on the T cells is dominant over the effect of IL-10 and TGFβ.

Translation of the Results into the Clinic: Vaccination with CD40-Activated Follicular Lymphoma Cells

Based on the above described data we have initiated a phase I trial using CD40 activated FL cells as a tumor vaccine. This trial is a phase I trial to determine the feasibility and maximal tolerated dose of CD40 ligand activated follicular lymphoma cells to patients with relapsed Follicular Lymphoma. Pa-

tients eligible are less then 70 years of age with NHL, Working Formulation B-C, who failed at least one chemotherapy regimen. They must be ineligible for, or refused entry to, available institutional bone marrow transplantation protocols. Patients must have adequate tumor tissue available for harvesting. They must have evaluable disease by physical exam or radiographic studies after harvesting tissue samples necessary for vaccine preparation. Life expectancy has to be greater than 6 months and ECOG performance status of 0-1. Eligibility includes also no evidence of pulmonary or cardiac compromise. Patients with evidence of active infection requiring therapy, infection with HIV, pregnancy or nursing, as well as chemotherapy, immunotherapy or radiation therapy within 4 weeks of protocol entry, ongoing immunosuppressive therapy or prior history of autoimmune disease are not eligible.

Patients will be treated with 2-6 vaccinations of each 10^8 live CD40 activated autologous FL cells intravenously. This trial includes a dose escalation schema. Three patients will be treated with 2 vaccination 2 weeks apart, 3 further patients with 4 vaccinations and another 3 patients with 6 vaccinations, each 2 weeks apart. Dose limiting toxicity (DLT) will be defined as grade 3 or 4 non-hematologic toxicity as defined by the Common Toxicity Criteria. Up to 3 patients will be initially enrolled at each dose level; additional patients can be added at that dose level if any of the 3 are invaluable. If no DLT is seen in the first 3 evaluable patients, the next patients will be registered at the next dose level. If one of 3 patients experiences DLT, then additional patients must be added to the current dose level. The decision concerning escalation will be delayed until the appropriate number of individuals is evaluable. If two or more patients of one dose level experience DLT, no more patients will be entered at the current dose level and the lower dose will be declared the MTD. With this design, the probability of escalating for specified values of the true but unknown probability of experiencing DLT is given in the table below.

To date 5 patients have been enrolled in the study, vaccines have been prepared for 4, and 3 patients have been treated. Therapy has shown no side effects. All three patients have been enrolled with progressive disease and huge tumor load. Clearly, it is very unlikely that this approach will result in any clinical response. At the moment it is to early to anticipate, whether these 3 patients have benefited from the therapy.

Discussion

As exemplified for the B cell malignancies by Follicular Lymphoma, we have started to uncover some of numerous defects in the interaction of the hosts immune response and B cell malignancies. Both the tumor cells and the T cells in the tumor microenvironment exhibit defects responsible for the lack of recognition by the hosts' T cells. Poor APC capacity by the tumor cells, lack of important signaling molecules in the T cells TCR as well as insufficient expression of important effector molecules indicate that a dysregulation of control mechanisms in the interaction of T and B cells in these B cell malignancies might be involved in the pathogenesis of the disease. Increased expression of immune inhibitory cytokines is another indication for this dysregulation. Novel immunotherapy has to be based on the understanding of the pathophysiology of the disease. It can not be our goal only to induce an immune response against the tumor if we are not successful in overcoming the defects induced by the tumor or its microenvironment. Therefore we have to include into the establishment of novel approaches their efficacy under such circumstances. Here we demonstrate that the use of B cell tumor cells as antigen presenting cells to present their own tumor antigens is successful in vitro even if the effector T cells are inhibited by downregulatory cytokines. We further demonstrate that the technology is easy and feasible for a clinical setting. Based on the in vitro findings and after optimization for a clinical protocol we have begun to translate our findings into the clinical arena. Here we shall test our principle concept of whether malignant B cells can function as APC in vivo. These ongoing and future trials will answer the questions if this strategy is

safe and successful in vivo in the human. Based upon our results in follicular lymphoma, we aim to broaden these studies into all B cell malignancies.

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Treatment of Childhood ALL

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Treatment of Children with Relapsed Acute Lymphoblastic Leukemia Studies of the BFM Study Group

H.-P. Adams and G. Henze for the ALL-REZ BFM Study Group

Introduction

Whereas the probability of event-free survival at 5 years of children with initial ALL approaches 75% the outcome of children with relapsed ALL is still poor [1-4]. The probability of event free survival at 5 years is about 30%. Therefore, the Berlin-Frankfurt-Münster (BFM) study group designs and performs clinical multi-center trials to optimize therapy for children with relapsed (REZ) acute lymphoblastic leukemia (ALL).

In all, four main studies have been carried out, namely ALL-REZ BFM 83, 85, 87, and 90. Additionally, seven pilot studies have been executed to check for feasibility of the main studies as well as to test new approaches. These were the studies ALL-REZ BFM P87, P88, P89, P91, P92, P94, and P95. The current main study is named ALL-REZ BFM 96 and started January 1st 1997. Each successive study included the knowledge gained during the previous trials as well as the experiences of the studies with children presenting with the initial disease [5, 6].

Patients and Methods

This report presents the results of patients who have been treated according to ALL-REZ BFM protocols. Patients enrolled into study ALL-REZ BFM 96 are not included. The inclusion criteria were: patients presenting with the first relapse of a non B acute lymphoblastic leukemia; patients had to be younger than 18 years at relapse diagnosis; informed written consent had to be obtained from guardians (and patients if appropriate) prior to treatment. During the study ALL-REZ BFM 90 children with a very poor prognosis were excluded and treated according to pilot protocols.

Three independent risk factors have been identified that predict the outcome of children with relapsed ALL, namely timepoint, immunophenotype, and site [6]. The duration of first remission is categorized into three classes. Timepoint "late" is defined as relapse free-survival longer than 6 months after cessation of front-line therapy, timepoint "early" denotes relapses occurring earlier then 6 months after cessation of front-line therapy but later than 18 months after initial diagnosis. All other relapses are defined as "very early" relapses. The current ALL-REZ BFM stratification scheme entails these three risk factors, see Table 1.

Survival time was computed from the date of relapse diagnosis to the last follow-up. Event-free survival (EFS) was calculated from date of second remission to the last date the patients were reported to be in second continuous remission or the date of an adverse event. In case of nonresponse to therapy, EFS was set to 0. In patients undergoing bone marrow transplantation (BMT), survival time and EFS were censored with

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Table 1. Therapy groups defined in ALL-REZ BFM 96

Site	Immunophenotype: non-T			Immunophenotype: (pre-) T		
Time point	Extra-medulary	BM combined	BM isolated	extra-medulary	BM combined	BM isolated
Very early	S2	S4	S4	S2	S4	S4
Early	S2	S2	S3	S2	S4	S4
Late	S1	S2	S2	S1	S4	S4

BM = bone marrow.

the date of the transplantation for survival analyses showing results of ALL-REZ BFM chemotherapy. For survival analyses including chemotherapy and BMT, patients undergoing autologous BMT were censored with the date of BMT [7].

Statistical analysis entailed univariate statistics [8] and survival analysis [9]. The level of significance was set to $\alpha = 0.05$ for all tests. All computations and statistics were performed on a PC using SAS, version 6.11 (SAS Institute, Cary, NC, USA).

Results

Altogether 1175 children, 770 boys and 405 girls, have been treated according to the aforementioned protocols; 777 patients were enrolled into the main studies and 398 into the pilot studies. Their median age at initial ALL diagnosis was 63 months (5^{th} percentile 16, 95^{th} percentile 169 months). The median age at relapse diagnosis equaled 99 months (5^{th} percentile 35, 95^{th} percentile 198 months). The median duration of first remission lasted 27 months (5^{th} percentile 8, 95^{th} percentile 72 months). In terms of timepoints, 513 children suffered a late relapse, 344 an early, and 318 a very early relapse. In 1097 instances immunophenotyping of lymphoblasts was performed, 161 samples were of (pre-) T and 936 of non-T lineage. EFS by the three major risk factors are shown in Figs. 1-3. Event-free survival by therapy group as defined in Table 1 is displayed in Fig. 4.

A second remission could be achieved in 991 children (84.3%), 124 (10.6%) did not respond to therapy, 60 children (5.1%) died during induction therapy. As can be noted in Fig. 4 most induction failures occur in the patient groups with poorest prognosis, namely S3 (41/174) and S4 (105/298). Additionally, after a second remission is achieved their relapse-free survival can be expected to be very short.

Allogeneic bone marrow transplantation was performed in 178 instances. The benefi-

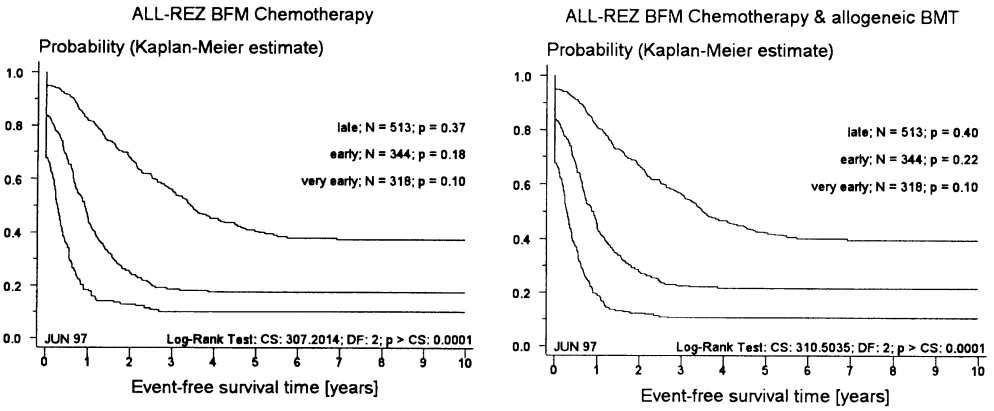

Fig. 1. Event-free survival by time point of relapse

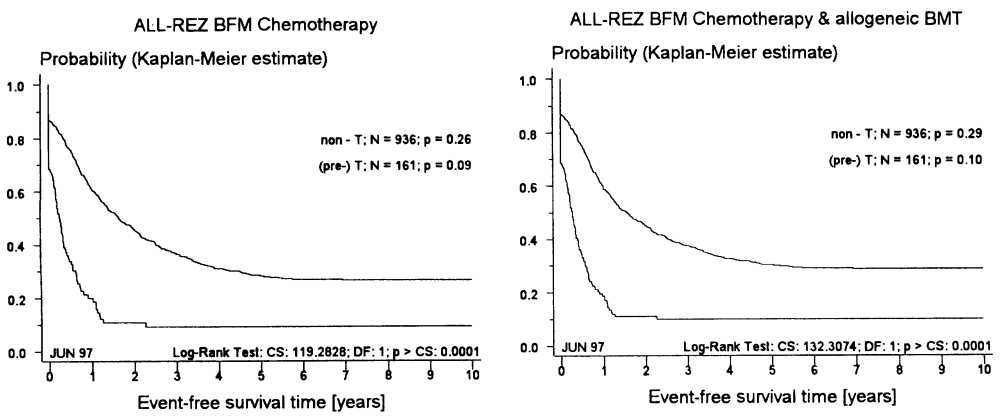

Fig. 2. Event-free survival by immunophenotype of relapse

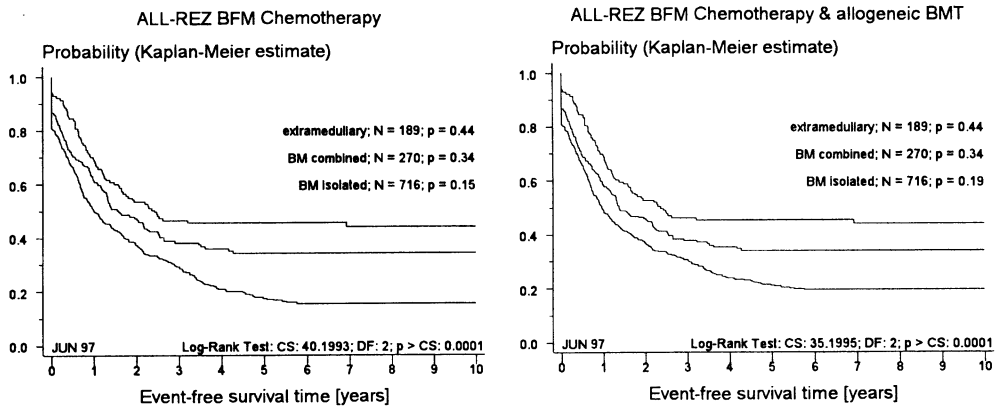

Fig. 3. Event-free survival by site of relapse

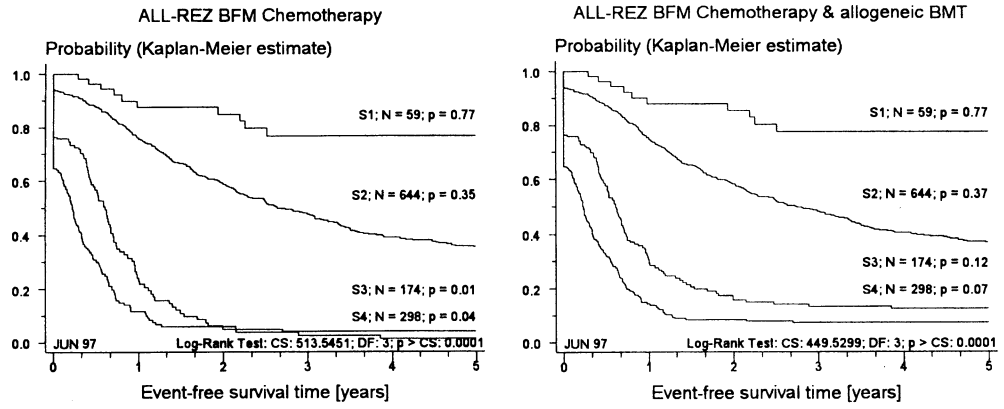

Fig. 4. Event-free survival by therapy group

Table 2. Events in second remission

Event	Chemotherapy	Allogeneic BMT	Autologous BMT	Total
No event	307	92	11	410
Relapse	427	55	38	520
Second malignancy	2	1	1	4
Death in remission	27	30	0	57
Total	763	178	50	991

BMT = bone marrow transplantation.

cial effect can be seen clearly in Fig. 4, therapy group S3. In fact, children with early, non-T, isolated bone marrow relapses seem to benefit most from allogeneic bone marrow transplantation. It has been shown already that the current procedure of autologous bone marrow transplant is not effective [7].

Death in second remission is a rather rare event (27/763 = 3.54%) in patients receiving chemotherapy only. The risk to die after allogeneic bone marrow transplantation is considerably higher (30/178 = 16.85%). But the frequency of relapse after allogeneic bone marrow transplantation is definitely lower. The events in second remission are compiled in Table 2.

Discussion

Despite many efforts the outcome of children with relapsed ALL is poor. The overall probability of 5-year event-free survival is as low as 30%. Still, a small group can be found with an excellent outlook, these are children with late extramedullary relapses. Another group with similar good outcome are TEL-AML1 positive ALL (data submitted for publication). Some progress has been made by adding preventive CNS radiation to the therapy plan of children with late isolated bone marrow relapses [10].

The induction of a second remission is the first hurdle that has to be conquered. From trial ALL-REZ BFM 90 there is evidence that the time intervals between the first courses of induction therapy might influence both remission rate and long term outcome [11]. Hence the current trial ALL-REZ BFM 96 addresses the question whether the intervals between therapy blocks can be shortened using defined rules for dose reductions and/or the randomized administration of G-CSF. For therapy groups S2-S4 the maintenance of a remission remains a problem. A recently published meta-analysis demonstrated the benefit of reinduction pulses during maintenance therapy [12]. Therefore, in the current trial ALL-REZ BFM 96 reinduction pulses have been added to the maintenance therapy for therapy groups S2, and for therapy groups S3 and S4 bone marrow transplantation has been made obligatory as soon as a second remission is achieved.

Even though the tailored-therapy approach (ALL-REZ BFM P92) was not as successful as expected [13] assessment of in vitro resistance to cytostatics may be of help to predict toxicity of the therapy individually [14]. Finally, the monitoring of minimal residual disease might help to identify patients that need a more intensive treatment, and those who need less.

A comparison between the presented results and reports from other group are always difficult. The big advantage of the ALL-REZ BFM studies is the high compliance of all participating centers guaranteeing unbiased results.

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Experience with BFM 1990 Protocol Treatment of Recurrent Childhood Lymphoblastic Leukemia. Report of the Polish Children's Leukemia/ Lymphoma Study Group

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Abstract. Between 1993 and 1996, 54 children aged from 6 mths-18 years (16 girls and 38 boys) with first relapse of acute lymphoblastic leukemia were included to the study. The children were treated according to the BFM 90 relapse protocol. There were 40 cases with early (including 14 children with very early) and 14 cases with late relapse (BM-30, local-12, combined 12). The probability of EFS were calculated according to the Kaplan-Meier method.

The overall second complete remission (CR) rate in very early was 71.42%, in early relapse was 73.07% and in late relapse 78.57%. The probability of overall event-free survival (EFS) after 2 years was 39.9%. The EFS achieved in children with late relapses was four times higher when compared with early and very early relapses 73.2% vs. 59% vs. 29.2% ($p = 0.05$).

The results obtained with BFM 90 chemotherapy in children with first late relapse are acceptable. One must conclude that for children with early relapses, other chemotherapy methods together with BMT in second remission should be applied.

Introduction

Many factors within the past 10 years have contributed to a marked improvement in the prognosis of childhood acute lymphoblastic leukemia (ALL): an increasing number of effective chemotherapeutic agents, combination chemotherapy, modified dose schedules, intensive treatment during remission, CNS prophylaxis and vigorous supportive care. Over 75% children now become long-term survivors [1, 2]. Relapse has to be considered as the most important obstacle in the way of curing all children with ALL. It is still unclear how best treatment for children with ALL, who are in a second remission [3, 4, 5].

Fifty-four of 600 children treated for ALL in seven centers of The Polish Children's Leukemia Lymphoma Study Group between 1993-1996 according to the BFM protocols, relapsed during therapy or after its discontinuation.

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Table 1. Initial characteristics of children with first ALL relapse treated according to BFM-90 protocol

Sex	Boys	38 (68.03%)	
	girls	16 (31.97%)	
		First diagnosis	Relapse
Age	< 2 years	3 (5.56%)	–
	2-10 years	41 (75.92%)	34 (69.39%)
	> 10 years	10 (18.52%)	20 (29.25%)
Clinical classification	LRG	14 (25.92%)	
	MRG	26 (48.16%)	
	HRG	14 (25.92%)	
FAB classification	L1	26/42 (61.90%)	10/28 (35.71%)
	L2	15/42 (36.10%)	17/28 (60.14%)
	L3	1/42 (2.00%)	1/28 (4.15%)
Immunological classification	B	1/31 (3.22%)	1/32 (3.12%)
	Pre B	15/31 (48.39%)	17/32 (53.63%)
	T	2/31 (6.45%)	1/32 (3.12%)
	Common	11/31 (35.49%)	11/32 (34.08%)
	NonT nonB	2/31 (6.45%)	2/32(6.15%)

Material and Methods

Patients and Definition

Between June 1993 and April 1996, 54 children aged from 2-18 years (16 girls and 38 boys) with first relapse of acute lymphoblastic leukemia were included to the study. Initial characteristics of the children at first presentation of the disease and at relapse are presented in Table 1.

T cell ALL was recognized in 6.45% for children with first diagnosis and 3.12% at relapse. B cell ALL was diagnosed in 3.22% both at first diagnosis and at relapse.

At their first presentation children were treated according to the BFM 86 protocols for LRG and MRG groups and New York protocol for HRG group [6, 7]. At relapse the BFM 90 relapse protocol was used. In ten children megachemotherapy with BMT was applied (in 8 allo BMT in 1 auto BMT in 1 PBSCT).

Very early relapses were those, which occurred at the patients at first 18 mths of first line therapy.

The early relapses were diagnosed when occurred still on therapy or up to 6 months after stopping of front line treatment. The remeinder were estimated as late relapses.

Isolated bone marrow relapse was diagnosed in the presence of 25% bone marrow blasts with the absence of any other clinically proven leukemic extramedullary infiltrations.

Isolated extramedullary marrow relapses were diagnosed in children with clinically overt manifestations of leukemia or histologically/cytologically proven leukemic infiltrations at any site except bone marrow. Marrow involvement was diagnosed in children with proven leukemia at extramedullary sites and at least 5% blasts in bone marrow.

Types and time of first ALL relapse in childhood are presented in Table 2. The predominance of early and very early relapses was observed in our material (73.77%). There were 26 cases with early, 14 with very early relapse and 14 cases with late relapse. Most of early relapses were isolated BM (57.41%), followed by isolated CNS (12.96%) and testes (7.43%). The proportion of early mixed relapses was 22.22%. In the group of late relapses predominated also isolated BM (57.14%) involvement followed by isolated CNS (21.43%) relapse, combined BM+tests (14.28%).

In boys with unilateral testicular relapse the involved testis was surgically removed and biopsy was taken from clinically non involved testis. If the biopsied testis proved histologically to be free of leukemia, radiotherapy was administered to the remaining testis at dose of 18 Gy. In patients with bilateral testicular relapse either both testicles were to be removed or irradiated at a dose of 24 Gy.

Table 2. Type and time of first ALL relapse treated according to BFM-90 protocol

Type of relapse	Very early relapse n = 14 (25.63%)		Early relapse n = 26 (48.14%)		Late relapse n = 14 (25.63%)		Total n = 54 (100%)	
	n	[%]	n	[%]	n	[%]	n	[%]
Isolated								
BM	10	71.43	13	50	8	57.14	31	57.41
CNS	2	14.28	3	11.54	2	21.43	7	12.96
testes	1	7.14	3	11.54	–	–	4	7.43
Razem	13	91,67	19	73.08	10	71.43	42	77.78
Mixed								
BM+CNS	–	–	4	14.29	1	7,14	5	9.25
BM+testes	1	7.14	2	7.14	2	14.28	5	9.25
	–	–	–	–	1	7.14	1	1.85
BM+mediastinum	–	–	1	3.57	–	–	1	1.85
BM+CNS+bones								
Total	1	7.14	7	26.92	4	28.57	12	22.22

Statistical analysis.
The probability of EFS was calculated according to the Kaplan-Meier method [8].

After the end of the intensive phase of treatment the children received maintenance therapy with daily 6-tioguanine (50 mg/m^2) and biweekly i.v. Mtx (50 mg/m^2).

The duration of maintenance therapy was 2 years for children with BM relapse and 1 year for patients with isolated extramedullary relapse. Triple intrathecal therapy in 6-week intervals up to the end of the first year of treatment were administered to children with CNS relapse.

Response Criteria

In children with BM relapse remission was diagnosed, if there were less than 5% blast cells in an otherwise normocellular marrow. CNS remission was defined by the absence of leukemic cells in CSF.

Treatment

Patients were stratified into two treatment groups AB and C (Fig. 1).

Regimen AB was given to children with early and late isolated and combined BM relapse, and regimen C to children with isolated extramedullary relapses, irrespective of the time of occurrence. In all patients treatment was started with a 5-day phase of prednisone (2 mg/kg/d). This was done to reduce the initial mass of leukemic cells and to stabilize the patients general condition.

Table 3. Treatment courses

	Drug	Dosis	Administered on day
Block R1	Prednisone	100 mg/m^2	1-7, 15-21
	Mercaptopurine	100 mg/m^2	1, 8, 15, 22
	Vincristine	1.5 mg/m^2	1
	Metotrexate i.v.	1 g/m^2	15, 16
	Metotrexate i.t.	12 mg*	2, 3, 17, 18
	Cytarabine	300 mg/m^2	
	Teniposide	165 mg/m^2	
	L-asparginase	10000 U/m^2	
Block R2	Dexamethasone	20 mg/m^2	1-5
	Thioguanine	100 mg/m^2	1-5
	Vindesine	3,0 mg/m^2	1
	Metotrexate i.v.	1 g/m^2	1
	Metotrexate i.t.	12 mg*	1
	Ifosfamide	400 mg/m^2	5
	Daunorubicin	50 mg/m^2	5
	Lasparginase	25000/m^2	6-8
Block R3	Dexamethasone	20 mg/m^2	1-5
	Thioguanine	100 mg/m^2	1-3
	Vindesine	3.0 mg/m^2	1
	Metotrexate i.v.	1 g/m^2	1
	Metotrexate i.t.	12 mg*	1
	Ifosfamide	400 mg/m^2	1-5
	Daunorubicin	50 mg/m^2	5

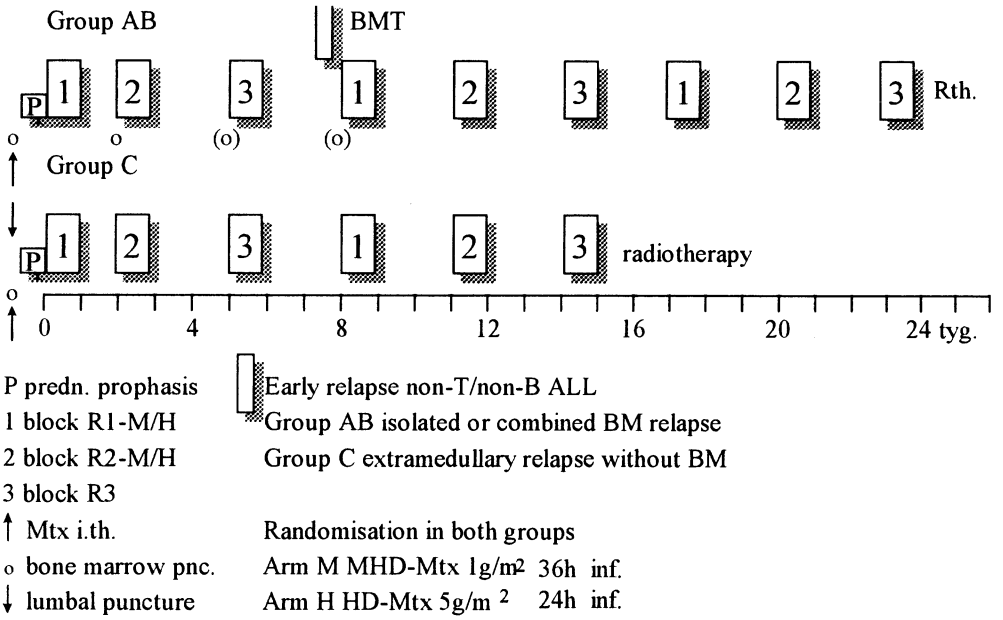

Fig. 1. Relapse protocol ALL BFM-90

Main elements of treatment for induction and consolidation were alternating courses of intensive polychemotherapy (R1, R2 and R3) (Table 3). All children with BM relapse were treated with nine R-blocks, whereas only six such courses were administered to children with isolated extramedullary relapse. After third block (R3) of chemotherapy allo BMT is recommended. If patients has no BMT donor, other 6 blocks of chemotherapy should be applied.

At the end of the intensive phase the systemic chemotherapy was supplemented by cranial irradiation in children with CNS

Table 4. Summary of results to treatment of first ALL relapse treated BFM-90 protocol

Type of relapse	N 54	CR 39 (75.59%)		No. CR 15 (26.41%)		Second II relapse		Death		Still in CR	
		n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Very early:	14	10	71.42	4	28.58	3	21.42	8	70.33	5	35.71
BM	10	7	70.00	3	30.0	2	27.27	7	57.14	3	27.27
Extramedulary	3	3	100	–	–	–	–	–	–	2	100
CNS	1	1	100	–	–	–	–	–	–	1	100
Testes	1	1	100	–	–	–	–	–	–	1	100
Mixed	1	–	–	1	100	1	–	1	100	–	–
Early:	26	19	73.07	72	26.3	5	19.23	11	42.30	15	57.67
BM	13	8	61.54	5	28.46	3	25.00	5	41.67	8	66.67
Extramedulary	6	6	100	–	–	2	26.67	4	80.00	3	42.86
CNS	3	3	100	–	–	1	20.00	2	40.00	3	60.00
Testes	3	3	100	–	–	1	20.00	2	40.00	–	–
Mixed	7	5	71.42	2	28.58	–	–	2	28.57	4	57.14
Late:	14	11	78.57	3	21.42	1	7.14	3	21.42	9	64.29
BM	8	6	75.00	2	25.00	1	14.28	2	28.57	4	57.14
Extramedullary	2	2	100	–	–	–	–	–	–	3	100
CNS	2	2	100	–	–	–	–	–	–	3	100
Mixed	4	3	75.00	1	25.00	–	–	1	25.00	2	50.00

Table 5. Early death (first 4 weeks) analysis in children with first ALL relapse treated acc. to BFM-90 protocol (n = 7/54)

	Very early relapse		Early relapse		Late relapse	
	n	(%)	n	(%)	n	(%)
Progression of the disease	1	13.3	1	1.85	1	1.85
Aplasia + infection	0	0	0	0	0	0
Therapy toxicity diathesis haemorrhagica	1	1.85	0	0	0	0
Infection	1 CNS + *Str. pyog. E. coli*	1.85	1 Sepsis *Pseudom. aerug.*	1.85	0	0
Haemorrhagia ad CNS	0	0	0	0	1	1.85
Invaginatio ilei	0	0	1	1.85	0	0
Total	2	3.70	3	5.55	2	3.70

relapse, as well as by testicular irradiation in boys with testicular relapse. Cranial irradiation was administered at dose 24 Gy to non-pre-irradiated patients. Standardized dose reductions were made according to age of patients and doses previously applied.

In boys with isolated testicular relapses remission was achieved by orchiectomy, or in non-orchiectomised patients if the size of the involved testis(es) had returned to normal. Complete remission (CR) had to be achieved after application of two treatment Block. Patients in non-remission at that time were termed non-responders, even if CR was achieved later.

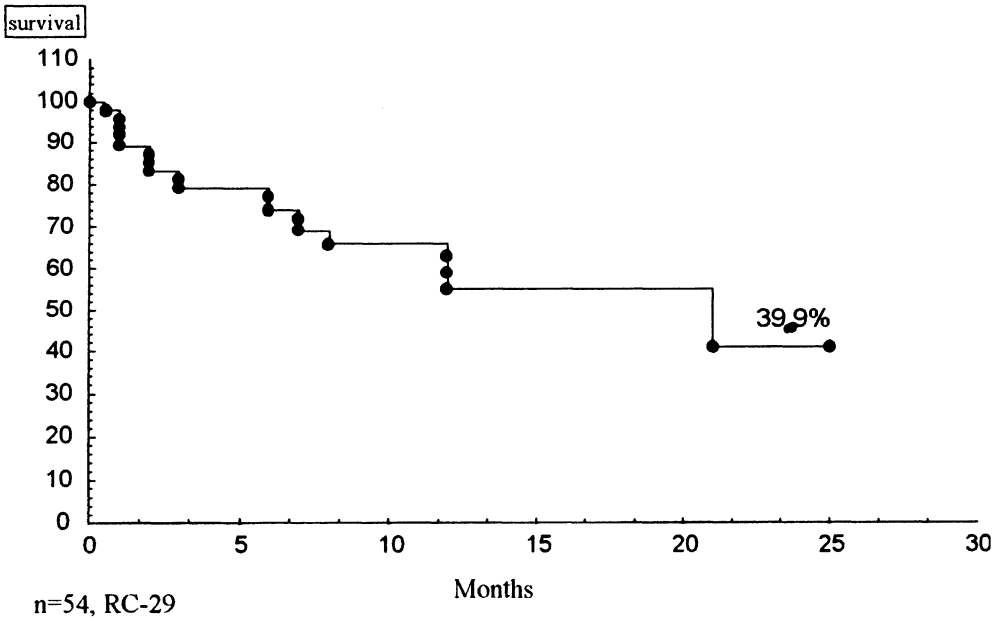

Fig. 2. Probability of event-free survival of children with first relapse of ALL treated according to BFM-90 protocol (n = 54)

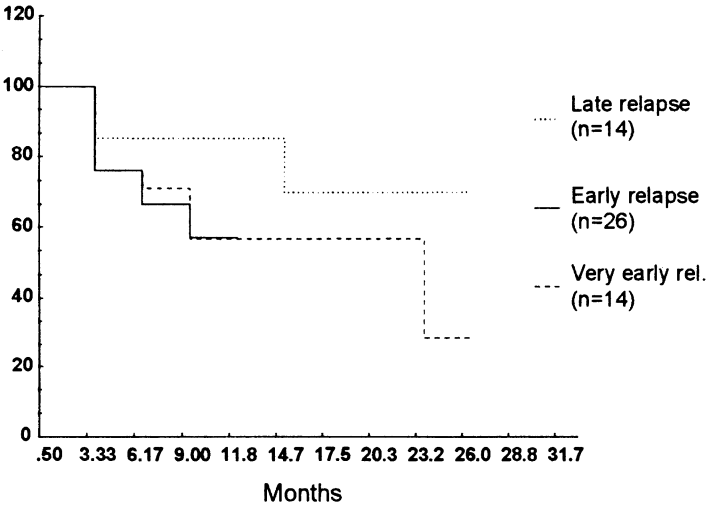

Fig. 3. Probability of event-free survival of children with very early, early and late first relapse of ALL treated according to BFM-90

Results

A summary of the treatment response is shown in Table 4.

Overall Results

The second complete remission (CR) was achieved in 39 of 54 patients (75.59%)

The second complete remission was observed in 71.42% of children with very early relapse, in 73.07% with early and in 78.77% with late relapse. Five children with very early relapse, 15 children with early and 9 with late are still in II CCR.

Seven from 54 children died during the first 4 weeks of therapy (Table 5).

Late death analysis of 15/54 children with first ALL relapse is presented in Table 6.

Clinical evolution of ALL relapse in children with I relapse of ALL treated according to BFM 90 protocol including bone marrow transplantation is presented in Table 7.

The probability of overall event-free survival (EFS) of relapsed children after 2 years was 39.9% (Fig. 2).

The EFS achieved in children with late relapses was higher when compared with early and very early relapses 70.55% vs. 57.47% vs. 25.33% ($p = 0.05$) (Fig. 3).

The EFS achieved in children with isolated late BM relapses was higher when compared with isolated early and very early BM 51.20% vs. 42.55% vs. 19.47% ($p = 0.05$) (Fig. 4).

Table 6. Late death analysis in children with first ALL relapse (n = 15/54)

	Very early relapse		Early relapse		Late relapse	
	n	(%)	n	(%)	n	(%)
Disease progression	6	11.1	8	13.98	0	0
I relapse						
II relapse	2		5	9.2	0	0
	4	3	5.55	0	0	
Death in CR	0	0	1	1.85	0	0
	0	0	1	1.85	0	0
Infection						
Total	6	11.1	9	16.67	0	0

Table 7. Clinical characteristics of children with I relapse of ALL treated according to BFM 90 protocol including bone marrow transplantation (n = 10)

lp	NN	Age	Sex	Type of relapse	Type of BMT	Time from beg. of rel. to BMT	II relapse	Alive days after BMT
1.	MM	12	m	Very early BM	Allo BMT	8 mths	No	235
2.	DS	14	m	Very early BM	Auto PBSCT	12 mths	No	338
3.	AS	10	f	Early BM	Allo BMT	6 mths	No	448
4.	AK	3	f	Early BM	Allo BMT	10 mths	No	147
5.	HSz	6	f	Early BM + CNS	Allo BMT	3 mths	No	102
6.	LH	2	m	Very early BM	Allo BMT	3 mths	114 d. after BMT	Died
7.	PA	6	m	Late BM + testes	Allo BMT	6 mths	160 d. after BMT	Died
8.	EY	5	m	Late BM + testes	Allo BMT	5 mths	No	240
9.	RM	3	m	Late BM + testes	Allo BMT	3 mths	No	360
10.	DM	8	m	Very early BM	Allo BMT	4 mths	180 d. BM	Died

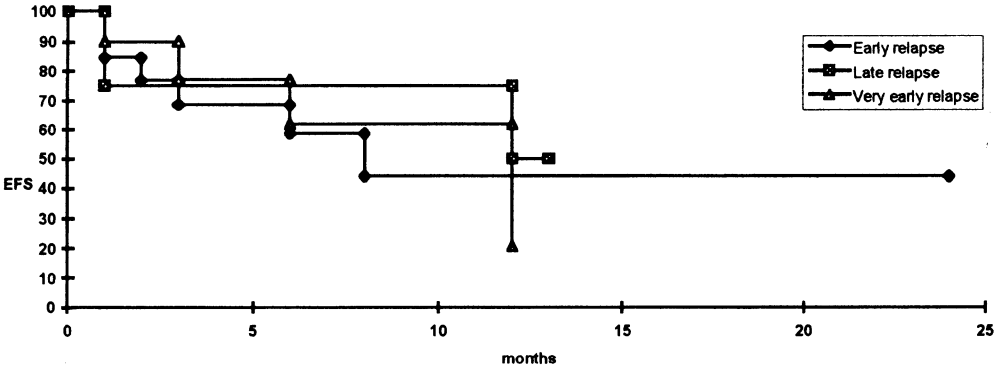

Fig. 4. Event-free survival of children with first BM relapse of ALL treated according to BFM-90 protocol

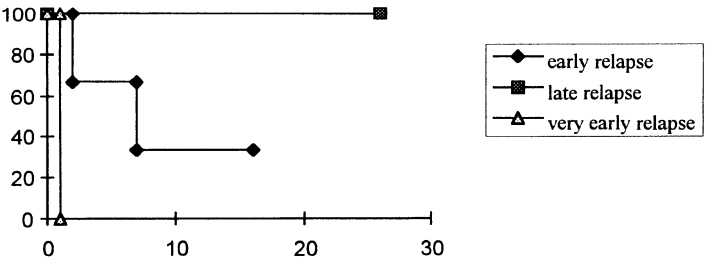

Fig. 5. Event-free survival of children with mixed first relapse of ALL treated according to BFM 90 protocol

The EFS achieved in children with mixed late relapses was one and half times higher when compared with mixed early relapses 40.00% vs. 25.00% ($p = 0.1$) (Fig. 5).

The best EFS (78%) was achieved in children with first relapse of ALL treated according to BFM-90 protocol including bone marrow transplantation (Fig. 6).

Discussion

This report presents the results of the multicenter study and the efficacy of the BFM relapse protocol 1990. The trial was performed in years 1993-1996 in children with first recurrence of ALL. This study confirm the results of previous studies on

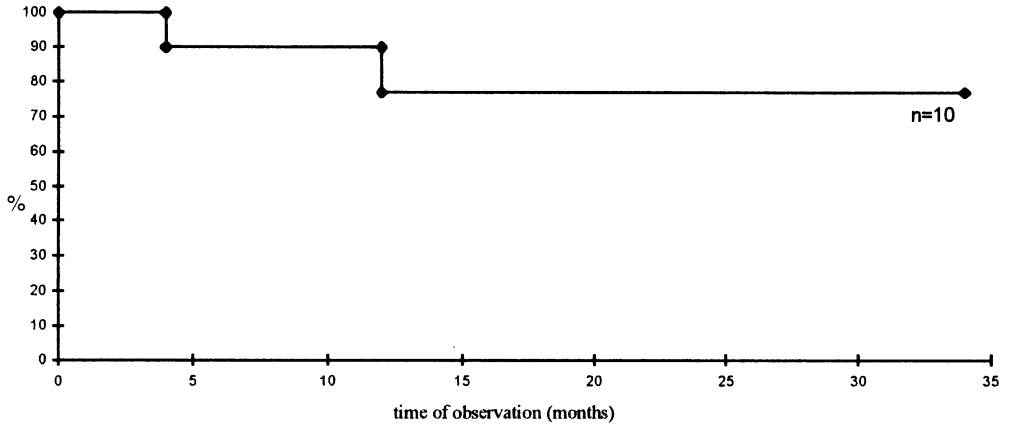

Fig. 6. Event-free survival of children with first relapse of ALL treated according to BFM-90 protocol including bone marrow transplantat

children with first ALL relapses, which showed that these patients do not make up a homogenous group [9–11]. It became clear that children, who developed their relapse during initial maintenance treatment and up to 6 months after the end of therapy (early relapse) have a worse prognosis compared with children, who relapse more than 6 months after cessation of therapy (late relapse). EFS achieved in children with late relapses was higher when compared with early and very early relapses (70.55% vs. 57.47% vs. 25.33%) ($p = 0.05$).

EFS in children with late isolated BM relapses was 51.20%. Others have reported equally bad results in similar subgroup [12–14]. Until recently, the outcome in these children was uniformly dismal, with most patients succumbing to progressive and refractory disease within 12 months from the time of relapse [15–18].

It was shown in our previous study that the BFM protocol produced improvement of EFS in children with first relapse in comparison with chemotherapy previously used by the Polish Children's Leukemia Lymphoma Study Group [5].

The best therapy for children with late relapses, who achieved second remission is still controversial [19, 21–23]. Our results suggest that the therapy including bone marrow transplantation (BMT) is better than standard chemotherapy for children with late and early relapses.

Conclusions

The results obtained with BFM 90 chemotherapy in children with first late relapse are acceptable. One must conclude that for children with very early and early relapses megachemotherapy together with BMT in second remission should be applied. The treatment results obtained in children with early relapse, although gradually improving are far from satisfying. New treatment strategies together with BMT in second remission must be designed for this group of children.

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Acute Leukemias VII
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Modified New York Regimen for Children with High Risk Acute Lymphoblastic Leucemia (HR-ALL). Preliminary Results

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Introduction

Therapy of acute lymphoblastic leukemia (ALL) is based on risk assessment at diagnosis, so currently only those patients at high risk (HR) of relapse are treated aggressively [1,2].

The improved projected event-free survival rates observed in this group of patients justify the risk of this aggressive approach [3,4].

Since 1987 in several pediatric hematologic centres in Poland we have used a modified New York protocol for treatment of high-risk ALL children [5].

In this study we report our preliminary results of such a treatment which was held on 24 HR-ALL patients in the Department of Pediatric Haematology and Oncology in Dzialdowska Street in Warsaw.

Materials and Methods

From May 1987 to November 1996 24 patients with newly diagnosed HR-ALL were studied on the modified New York protocol. There were 16 female patient and 9 male patients with median age of 7.2 years .

The diagnosis was obtained by bone marrow aspiration biopsy. WBC ranged from 50 to 549 × 10 9/l.

Surface marker analysis of bone marrow blasts revealed calla (+) antigen in twelve patients. Two children had lymphoma syndrome, 9 patients had L 1, 10 had L 2, 5 had L 1/L2 FAB morphology. CNS involvement was seen in two patients and one had testicular involvement .

The method of Kaplan and Meier was used to construct the life time projection of event-free survival on data available November 1996.

Results are shown in Table 1.

Twenty one patients achieved a bone marrow remission. All of our patients survived induction but four died during consolida-

Table 1. Results of the New York protocol

Patients entered	24
Evaluable for induction	24
I CR	21
PR	3
Died during induction	0
Evaluable for consolidation	24
Died during consolidation	4
sepsis	2
cerebral haemorrhage	2
Lost to observation in consolidation	1
Evaluble for maintenace therapy	19
Relapse during maintence therapy:	3
Bone marrow relapse	2
CNS relapse	1
II CR	2
Death in relapse	1
Completed maintence	9
During maintence therapy	7
Alive	18
in I CR	16
in II CR	2

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tion because of the toxic effects of treatment.Two of them had cerebral haemorrhage due to thrombocytopenia and two children died because of sepsis during consolidation caused by *Staphylococcus epidermidis.*

One girl with Down's syndrome emigrated from Poland and was lost to observation.

Remission duration was, therefore evaluated in nineteen patients who entered maintenance therapy.

16 (66%) are in CCR (8 to 87 + months). Nine patients that completed their maintenance New York therapy are still in first CCR between 43 to 87 months. Eight of our patients are still on maintenance therapy.

In two patients remission was terminated by a marrow relapse 12 to 14 months from diagnosis.

One patient had an isolated CNS relapse after 19 months on study.

Two children who had bone marrow relapse achieved II CCR and one who had CNS relapse died; 18 patients of our group 24 HR ALL children are still alive.

The event-free survival of this group is 76%.

Toxicity During Therapy

Induction

No death occurred during remission induction among the 100 patients. All patients completed induction in a median of 34 range (28 to 45 days). Four patients needed more than a week longer than intended to complete induction (between 8 to 16 days).

Six patients had a positive blood culture *(Pseudomonas aeruginosa, Streptoccosus viridans, Staphylococcus epidermidis).* Two patients had purulent skin infections due to *Staphylococcus aureus.* Two had pneumonia, three FUO, five had significant mucositis and oesophagitis. One had ilu es paralyticus, one had urinary tract infection.

Granulocyte recovery to more than 1000/ml was seen on day 17 (median) and platelet recovery to more than 100 000 on day 19 (median).

Consolidation

Four of our 24 children died during consolidation (3 of them without first CR). Two of them had cerebral haemorrhage due to thrombocytopenia and two children died because of sepsis caused by *Staphylococcus epidermidis.*

Five patients had mucositis, three had FUO, one had meningitis due to Candida, one had mycoplasmal pneumonia, and one had urinary tract infection.

The other patients completed consolidation in a median of 42 days (range 30 to 66 days).

The lowest granulocyte number occurred on day 17 (median).The lowest platelet count was observed on day 16 (median).

Most of the complication occurred between the 10th and 20th day of consolidation.

Anemia, leukopenia, thrombocytopenia and stomatitis were observed during consolidation therapy in most of our 24 patients.

Other consolidation toxicities included transient liver enzyme or bilirubin elevation in 2 patients. Toxic heart injury was observed in one child. Cushing syndrome was seen in 2 patients.

Maintenance Therapy

The maintenance therapy was generally well tolerated.

Complications during maintenance in our 19 patients included : 2 episodes of bacteriemia (*Klebsiella* and *Proteus*), 4 episodes of fever while neutropenic, 3 pneumonia, 1 viral meningitis, 2 otitis, 2 urinary tract infections, 1 infection of central venous catheter (*Staphylococcus aureus*), 3 stomatitis (*Candida alb.*), 1 anemia; 3 of our patients had recurrent neutropenia after oral tioguanine.

All recovered without sequelae.

Discussion

Intensive chemotherapy combinations have improved the outlook for children with

acute lymphoblastic leukemia who would have been at high risk of early relapse on former therapies [3, 8]. The past therapies predicted EFS of less than 50% for such a group of children. At the same time, protocols have become more complex. There are more short-term toxicities and increased potential for delayed side-effects. The improved projected event-free survival rates observed in patients with HR ALL justify the risk of this aggressive approach.

The New York I and BFM protocols yield EFS rates of 77 and 79% at 3 years, respectively, in high-risk patients [7].

Polish Pediatric Leukemia/Lymphoma Study Group (PPLLSG) have made several changes in the original New York 1 protocol [6].

No radiation to bulk disease in induction was administered. The cranial irradiation was delayed to consolidation. We gave L-Asparaginase $3 \times 25\,000$ j/m during induction, $3 \times 25\,000$/m during consolidation and 12 times during maintenance therapy. Instead of intrathecal Mtx during induction, consolidation and maintenance therapy we administered TIT (Mtx + Ara-C +Prednison). After the limiting dose of anthracycline has been reached, we gave VM-26 during maintenance.

Our preliminary results based on a small 24 patients group, are promising with an 76% 6-year EFS. The modified New York protocol with its intensive induction-consolidation and cross-resistant drugs in maintenance, where doses are pushed to the patients individual tolerance, was in general well tolerated. It is difficult to assess hematologic toxicity in our group of patients during induction because most of the patients began with neutropenia, anemia and thrombocytopenia.

Very dangerous for our patients was the first phase of consolidation, when severe complications were seen. The toxicity was due to prolonged periods of myelosupression and pushes the limit of tolerance. The incidence of bacterial and fungal infections was troublesome. We think that the use of hematopoietic growth factors may prove beneficial not only in reducing periods of neutropenia and infections complications but also may reduce the number of delays between therapy phases.

Despite of all complications and toxicities we think the modified New York protocol is a promising regimen for the treatment of high-risk ALL patients. Our preliminary results will be compared with that achieved by the PPLLSG.

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Treatment of Adult ALL

Acute Leukemias VII
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Treatment of High-Risk Acute Lymphoblastic Leukemia. Preliminary Results of the Protocol PETHEMA ALL-93

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Abstract

Objective. To analyze the results of a multicenter prospective randomized protocol, PETHEMA ALL-93, for high-risk acute lymphoblastic leukemia (HRALL).

Design. Induction (5-wk): vincristine (VCR), prednisone, daunorubicin, asparaginase (ASP) and cyclophosphamide (CFM). Early intensification: 3 cycles of intensive chemotherapy including VCR, dexamethasone, mitoxantrone, high-dose ASP, CFM, high-dose methotrexate (MTX), high-dose cytosine arabinoside, teniposide and mercaptopurine (MP). CNS prophylaxis: intrathecal MTX, cytarabine and DXM. Patients with a histocompatible sibling received allogeneic bone marrow transplantation (BMT) and the remaining were randomized to receive autologous BMT or the same chemotherapy used in the early intensification phase followed by maintenance treatment (MP, MTX) up to 2 yrs.

Parameters Analyzed. (June 1993-January 1997). Early response to treatment, complete remission (CR), leukemia-free survival (LFS) and overall survival (OS) by intention-to-treat.

Characterisitics of the Series. Eighty three patients included, 72 evaluable patients diagnosed with ALL (ALL L3 excluded) in 20 Spanish hospitals. The median (range) age of the series was 24 (1-50) years and leukocyte count 30 (2-822) $\times 10^9$/L. ALL L1/L2 was present in 23/49 patients, early pre-B in 22, common+pre-B in 28 and T in 22 cases. Myeloid markers were present in 36 cases. Cytogenetics (44 patients): normal 19, hypodiploidy 3, pseudodiploidy 22 (Ph ALL 10).

Response to Therapy. Early death 4 patients, no response 8 (11%), CR 60 (83%). Median LFS was 23 months, with a 3-yr probability of 46% (95%CI 28-74), whereas median OS was 24 months and 3-yr probability 47% (31-63%). LFS and OS for children (n = 15) were not significantly different than for adults. There were no differences in LFS for any postintensification type of treatment. Slow response to therapy (BM14 > 10%) was the only feature with negative influence on CR (OR 30.2, 95% CI 3.6-248.8), LFS (3.3, 1.4-8.0) and OS (2.2, 1.1-4.7).

Conclusion. The preliminary results of PETHEMA ALL-93 are encouraging. Up to now there are no differences in LFS for HRALL patients treated with allogeneic BMT, autologous BMT and intensive chemotherapy. Slow response to induction therapy is the main prognostic factor.

Indroduction

Recent clinical trials have shown that 65-85% of adults with acute lymphoblastic leukemia (ALL) may achieve complete remission (CR) using four or five cytotoxic drugs in the remission induction phase [1-10]. However, these remissions have been disappointingly short, and there is agreement that postremission is a critical and still open issue in the therapy of adult ALL [11-13]. The

Table 1. PETHAEMA ALL-93. Inclusion criteria

Children (age< 15 yr): One or more of the following:
Age< 1yr
WBC count >100 × 10^9/l and T-cell phenotype
WBC Count >300 × 10^9/l
t(9;22) or bcr-abl rearrangement
t(4;11)
t(1;19)
Adults (age ≥ 15 yr). One or more of the following:
Age 30-50 yr
WBC count >25 × 10^9/l
t(9;22) or bcr-abl rearrangement
t(4;11)
t(11;19)

attempts to eliminate residual disease in CR patients include allogeneic or autologous transplantation of hematopoietic progenitors (THP) and intensive postremission therapy. Ongoing multicenter trials are currently testing and comparing these three approaches, especially in adult ALL patients with adverse prognostic factors.

We report the preliminary results of the ongoing prospective multicenter randomized trial, PETHEMA ALL-93, designed to evaluate and compare the results of intensification chemotherapy, allogeneic and autologous BMT in high-risk ALL patients.

Patients and Methods

Patients and Diagnostic Criteria

From June 1993 to January 1997, 81 previously untreated ALL patients from 20 Spanish centers were prospectively included in the PETHEMA (Program for the Study and Treatment of Malignant Hemopathies, Spanish Society of Hematology) ALL-93 protocol. Diagnosis of ALL was made according to morphologic (FAB classification) [14, 15], immunologic and cytogenetic criteria. Four immunologic subtypes of ALL were considered. For the B lineage: early pre-B (CD19+, CD10-, intracytoplasmatic μ chain [μIC]-), common (CD19+, CD20+/-, CD10+, μIC-) and pre-B (CD19+, CD20+/-, CD10+/-, μIC+), and for the T lineage (CD7+, cCD3+, CD5+/-, CD2+/-, CD1+/-). The presence of myeloid antigens was also evaluated [16-19]. Cytogenetic studies were routinely performed (short-term culture and G-bands) [20]. Inclusion criteria for children and adults (age 15yrs) are referred in Table 1. Patients with prior malignancy, previous treatment for ALL, ALL-L3 morphology or with surface membrane immunoglobulin expression, cardiac, renal or liver failure not due to ALL or psychiatric disease or those who did not fulfill the inclusion criteria were excluded from the protocol. Patients provided informed consent before entering the study.

Treatment and Criteria for Response

Treatment of ALL is referred in Table 2. Briefly, induction treatment included a 5-week conventional therapy with vincristine, prednisone, L-asparaginase, daunorubicin and cyclophosphamide. Patients not acheiving CR received the first intensification cycle

Table 2. PETHEMA ALL 93. Chemotherapy schedule

Phase	Week no.	Route	Dose	Days
Induction				
Vincristine	1-4	IV	2 mg	1, 8, 15, 22
Daunorubicin	1-4	IV	30 mg/m^2	1, 8, 15, 22
Prednisone	1-4	IV/PO	60 mg/m^2	1-28
	5	IV/PO	30 mg/m^2	29-33
	5-6	IV/PO	15 mg/m^2	34-38
L-asparaginase	3,4	IV/SC	10 000 IU/m^2	16-20, 23-27
Cyclophosphamide	5	IV	1 000 mg/m^2	36
CNS propylaxis				
Methotrexate	1, 4, 7, 11, 15 21, 25, 29, 33 37, 41, 45	IT	15 mg	1, 28, 49, 77, 105, 175, 203, 231, 259, 287, 315

Table 2. PETHEMA ALL 93. Chemotherapy schedule (continued)

Phase	Week no.	Route	Dose	Days
Cytosine arabinoside	idem	IT	30 mg	idem
Hydrocortisone	idem	IT	20 mg	idem
Early intensification-1				
Vincristine	7-8	IV	2 mg	1, 8
Dexamethasone	7-8	IV/PO	20 mg/m^2	1-5
		IV/PO	10 mg/m^2	6
		IV/PO	5 mg/m^2	7
		IV/PO	2.5 mg/m^2	8
Methotrexate	7	IV	3 g/m^2	1
Cytosine arabinoside	7	IV	2 g/m^2/12 h	5
L-asparaginase	7	IV/IM	25 000 IU/m^2	5
Mercaptopurine	7	PO	100 mg/m^2	1-5
Early intensification-2				
Vincristine	11-12	IV	2 mg	1, 8
Dexamethasone	11-12	IV/PO	20 mg/m^2	1-5
		IV/PO	10 mg/m^2	6
		IV/PO	5 mg/m^2	7
		IV/PO	2.5 mg/m^2	8
Methotrexate	11	IV	3 g/m^2	1
Cyclophosphamide	11	IV	150 mg/m^2	1-5
L-asparaginase	11	IV/IM	25 000 IU/m^2	5
Mitoxantrone	11	IV	12 mg/m^2	5
Early intensification-3				
Dexamethasone	15-16	IV/PO	20 mg/m^2	1-5
		IV/PO	10 mg/m^2	6
		IV/PO	5 mg/m^2	7
		IV/PO	2.5 mg/m^2	8
Cytosine arabinoside	15	IV	2 g/m^2/12 h	1-2
Teniposide	15	IV	150 mg/m^2	3-4
L-asparaginase	15	IV/IM	25 000 IU/m^2	5
Delayed intensificaction-1[a]				
Vincristine	19-20	IV	2 mg	1, 8
Dexamethasone	19-20	IV/PO	20 mg/m^2	1-5
		IV/PO	10 mg/m^2	6
		IV/PO	5 mg/m^2	7
		IV/PO	2.5 mg/m^2	8
Methotrexate	19	IV	3 g/m^2	1
Cytosine arabinoside	19	IV	2 g/m^2/12 h	5
L-asparaginase	19	IV/IM	25 000 IU/m^2	5
Mercaptopurine	19	PO	100 mg/m^2	1-5
Delayed intensification-2[a]				
Vincristine	23-24	IV	2 mg	1, 8
Dexamethasone	23-24	IV/PO	20 mg/m^2	1-5
		IV/PO	10 mg/m^2	6
		IV/PO	5 mg/m^2	7
		IV/PO	2.5 mg/m^2	8
Methotrexate	23	IV	3 g/m^2	1
Cyclophosphamide	23	IV	150 mg/m^2	1-5
L-asparaginase	23	IV/IM	25 000 IU/m^2	5
Mitoxantrone	23	IV	12 mg/m^2	5
Delayed intensification-3[a]				
Dexamethasone	27-28	IV/PO	20 mg/m^2	1-5
		IV/PO	10 mg/m^2	6
		IV/PO	5 mg/m^2	7
		IV/PO	2.5 mg/m^2	8
Cytosine arabinoside	27	IV	2 g/m^2/12 h	1-2
Teniposide	27	IV	150 mg/m^2	3-4
L-asparaginase	27	IV/IM	25 000 IU/m^2	5
Maintenance (until 24 mo from diagnosis)[a]				
Mercaptopurine	31-104	PO	60 mg/m^2	Daily
Methotrexate	31-104	IM	15 mg/m^2	Weekly

[a] Only for patients randomized to receive chemotherapy.

(see below) and if no CR was then achieved, they were excluded from the protocol.

HLA study was performed to patients and their siblings once CR was achieved. These patients received three monthly cycles of early intensification chemotherapy including cytotoxic drugs active against ALL at intermediate or high-doses (Table 2). Central nervous system (CNS) prophylaxis consisted in intrathecal chemotherapy with methotrexate, cytosine arabinoside and hydrocortisone beginning during the induction phase and given throughout the first year of treatment in addition to high-dose intravenous methotrexate given in the first two cycles of early intensification therapy. Patients with a histocompatible sibling received allogeneic bone marrow transplantation (BMT) and the remaining were randomized to receive autologous BMT or the same three cycles of chemotherapy used in the early intensification phase followed by conventional maintenance treatment (daily mercaptopurine and weekly methotrexate) until two years after CR achievement (Table 2). Randomization was performed after CR, when the results of the HLA study were known. Hematopoietic growth factors (G-CSF) were used after each intensification cycle [21]. Conditioning regimens for BMT consisted in cyclophosphamide and fractioned total body irradiation (TBI) [22], busulfan and cyclophosphamide [23], or high-dose etoposide and TBI [24]. Methotrexate and cyclosporine were used for prophylaxis of GVHD in allogeneic BMT [25]. No additional cytotoxic or immunomodulatory treatment was given to patients submitted to allogeneic or autologous BMT. The use of hospitalization, the prophylaxis and management of infections and the transfusion policy were not prescribed by the protocol and were performed according to the specific protocols of each participating hospital.

Patients were considered to be in CR when all the extramedullar disease had resolved, the neutrophil count was higher than 1.5×10^9/l, the platelet count was greater than 100×10^9/l, and there were fewer than 5% blast cells in bone marrow examination. Two patterns of response were considered: slow, defined as the presence of peripheral blood blast cells (PBBC) on the 8th day of therapy or >10% blast cells in a bone marrow aspirate performed at day 14 of treatment, and fast, defined as the absence of PBBC on the 8th day and ≤10% BM blast cells (BMBC) at day 14. Leukemia-free survival (LFS) was defined to be the time from achieving CR to relapse, death or date of last follow-up. Overall survival (OS) was defined as the time from study entry to death or date of last follow-up.

Parameters Evaluated

In each patient the following initial parameters were recorded: age, gender, lymphadenopathy, organomegaly and mediastinal mass, CNS or testicular involvement at diagnosis, Hb, WBC and platelet counts, biochemical parameters including liver function tests (AST, ALT, alkaline phosphatase and gammaglutamyltranspeptidase), serum albumin and serum lactodehydrogenase (LDH) levels, as well as morphologic (ALL L1, ALL L2) and immunologic (early pre-B, common, pre-B, T and ALL with myeloid antigens) subtypes of ALL and results of cytogenetic study. In addition, the pattern of response (slow or fast), CR attainment, LFS and OS were also evaluated. Analysis was based on all the data evaluable as of January 31, 1997 and was performed on the basis of intention-to-treat.

Statistical Methods

Descriptive statistical study (mean, standard deviation, median, range) was first performed. Bivariant tests (Student t-test, Mann Whitney U-test, when appropriate) were used to compare quantitative variables and χ^2 or Fisher's exact test and variance analysis were employed to assess differences in proportions. Actuarial curves for LFS and OS were plotted according to the Kaplan-Meier method [26] and were compared by the log-rank test [27]. The statistically significant ($p < 0.05$) variable or those with borderline significance ($0.05 < p < 0.1$) identified in univariate studies were included in multivariate analyses. A logistic regression model was used to identify predictive factors for CR attainment, whereas mutivariate analyses for LFS and OS were per-

formed using the Cox proportional hazards regression model [28]. In multivariate analyses a logarithmic transformation of WBC count was performed to reduce the influence of extreme values. Ninety-five percent confidence intervals for probabilities and median survival times were calculated [29]. The significance level was fixed at $p = 0.05$ and all p values were two sided unless otherwise stated. Statistical analyses were carried out using the SPSS (Statistical Package for Social Sciences) package, version 6.0 for Windows.

Results

Patient Accrual

From June 1993 to January 1997 83 patients from 20 Spanish hospitals were entered in the PETHEMA LAL-93 protocol. Eleven patients were excluded from the study. Causes of exclusion were age > 50 yrs in one case and no eligibility for the study (after review) in the remaining 10. Thus, up to now 72 patients have been found eligible and evaluable for this report.

Patient Characteristics

The 72 patients had a mean (SD) age of 25 [13] years, with a median age of 24 (range 0.1-50) years. Fifty-seven patients (79%) were adults and 15 children. There were 43 men (60%) and 29 women (40%). Thirty-three patients (45%) had palpable lymphadenopathies, 33 (46%) hepatomegaly and 38 (54%) splenomegaly. Mediastinal mass was present in 10 (14%) cases. CNS disease was present at diagnosis in 6 cases (8%) and testicular infiltration in 2 (4%). The main hematological and biochemical characteristics as well as the distribution of morphologic, immunologic and cytogenetic subtypes of ALL are referred in Table 3. There was a predominant L2 morphology (49 cases, 68%), and common+pre-B phenotype (28 cases, 39%). Myeloid markers were present in 36 cases. Cytogenetic study was performed in 44 cases. Pseudodiploidy was present in 22 cases, being Ph ALL in 10.

Results of Therapy

Table 4 summarizes the main results of the PETHEMA ALL-93 protocol. Four patients died before CR could be ascertained, 8 (11%) were resistant and the remaining 60 (83%) attained CR (55 with conventional induction therapy and 5 with the addition of the first intensification cycle). PBBC on the 8th day of treatment were observed in 15 out of 57 cases (26%) and BMBC > 10% at day 14 were seen in 27 out of 70 (38%). By January 31, 1997 19 patients (32%) had relapsed (17 in

Table 3. Main hematologic, biochemical, immunophenotypic and cytogenetic characteristics of ALL patients

Parameter	n (%)	Mean (SD)	Median	Range
Hemoglobin (g/l)	72 (100)	93 (26)	88	35-152
WBC count ($\times 10^9$/l)	72 (100)	20 (167)	31	1-822
Platelets ($\times 10^9$/l)	72 (100)	67 (62)	39	4-279
LDH (U/l)	69 (90)	1957 (2177)	1145	119-10037
ALL L1	23 (32)			
ALL L2	49 (68)			
Early pre-B	22 (31)			
Common+pre-B	28 (39)			
T	22 (30)			
Myeloid markers	36 (50)			
Cytogenetic study				
Not performed/not evaluable	28 (38)			
Normal	19 (26)			
Hypodiploidy	3 (5)			
Pseudodiploidy[a]	22 (31)			

[a] t(9;22) in 10 patients.

Table 4. Main results of therapy

Patients entered	83
Patients eligible	72
Induction deaths	4 (6%)
Refractory disease	8 (11%)
Complete remission (CR)	60 (83%)
Patients with a HLA-identical sibling	22
Pts randomized to intensification chemotherapy	15
Pts randomized to autologous BMT	15
Total number of relapsed patients	19 (32%)
Dead patients	28 (39%)
Alive in first continuous CR	7 (51%)

the bone marrow, one in the CNS and one in the mediastinum) and 28 (39%) had died. Median LFS for the whole series was 23 months, with a projected LFS of 46% at 3 yrs (95% CI 28-64%) (Fig. 1). The median follow-up of alive patients in first CR was 16 months. In turn, 44 out of 72 patients are alive (37 in first CR). Median OS was 24 months, with a 3-yr projected OS of 47% (95% CI 31-63%) (Fig. 1), being the median follow-up of alive patients 17 months. LFS and OS for children were not significantly different than those for adults. By analysis based on intention-to-treat, 22 patients were assigned to allogeneic BMT, 15 were randomized to autologous BMT and 15 to intensification and maintenance chemotherapy. Up to now there are no differences in LFS between patients treated with intensification therapy, autologous or allogeneic BMT (Fig. 2). The 3-yr projected LFS were 54% (95%CI 24-84), 31% (95%CI 0-77%) and 48% (95%CI 22-74%), respectively.

Prognostic Factors

Univariate and multivariate analyses of prognostic factors for CR attainment showed that slow response to the treatment (presence of >10% BMBC on the 14th day) was the only parameter associated with a lower probability of CR (Table 5).

The results of univariate analysis of predictive factors for LFS showed that the only variables with an unfavorable influence on LFS were the presence of PBBC on the 8th day, BMBC >10% at day 14 of treatment and high WBC count. The Cox proportional hazard regression model isolated one variable

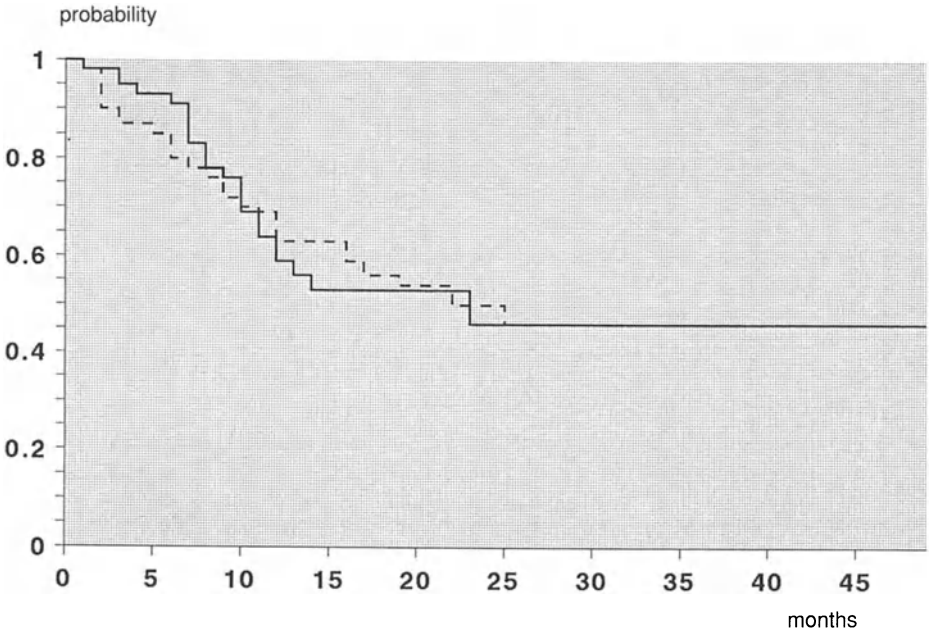

Fig. 1. Actuarial curves of leukemia-free survival *(continuous line)* and overall survival *(dotted line)* for the whole series

Table 5. Multivariate analyses for complete remission attainment, leukemia-free survival and overall survival

Variable and order of entrance in the model	Beta coefficient	Odds ratio	95% CI	*p*
CR attainment				
BMBC>10% 14th day	3.41	30.23	3.6-248.8	0.002
Leukemia-free survival				
BMBC >10% 14th day	1.19	3.31	1.37-8.03	0.008
Overall survival				
BMBC>10% 14th day	0.81	2.24	1.07-4.73	0.03

BMBC: Bone marrow blast cells.

associated with a shorter LFS: BMBC >10% at day 14. In turn, the only variable negatively influencing the probability of OS in both uni and multivariate analyses was BMBC >10% at day 14 (Table 5).

Discussion

The results of the PETHEMA ALL-93 protocol confirm those of the most recent studies which have reported high CR rates after intensive induction chemotherapy in HRALL, with a low rate of refractory disease [1-10, 30-37]. The high CR rate (83%) in this study was mainly due to the combination of vincristine, prednisone, daunorubicin and asparaginase, whereas the influence of cyclophosphamide was minimal due to the fact that it was administered in the fifth week of induction therapy.

There is little doubt about the benefit of postremission therapy in ALL. However, the optimal postremission regimen remains uncertain [11-13]. The most widely used for HRALL patients is multiagent chemotherapy with cytotoxic drugs active against ALL at intermediate or high doses given in con-

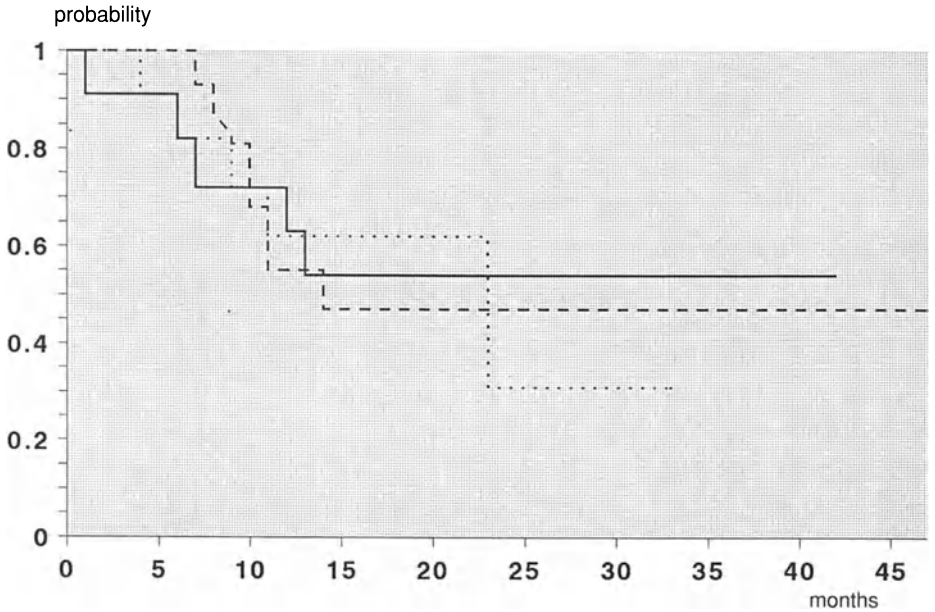

Fig. 2. Leukemia-free survival actuarial curves for CR patients randomized to receive delayed intensification therapy *(continuous line)*, autologous BMT *(dotted line)* and allogeneic BMT *(thick line)*

junction with the drugs used in the remission induction period. Similar to other reports [4, 9], high-dose methotrexate, cytosine arabinoside and L-asparaginase, combined with mercaptopurine, dexamethasone, vincristine and daunorubicin or mitoxantrone have been employed in our study.

The main goal of this ongoing prospective and randomized study was to compare the usefulness of delayed intensification therapy, allogeneic and autologous BMT in HRALL patients. Patients included in this trial presented the most common features defining HRALL. For children, age < 1 yr, hyperleukocytosis and cytogenetic abnormalities associated to bad prognosis; for adults, advanced age, high WBC counts, chromosomic translocations carrying unfavorable prognosis and the presence of myeloid markers. The inclusion of the latter feature was based on our previous experience [16], but it may be questionnable since in recent large studies the presence of myeloid markers has not been associated to bad prognosis [17-19]. Patients with ALL-L3 were excluded from this protocol because of the improvement observed in these patients with the use of specific therapeutic trials [38, 39].

To answer the question regarding the best postremission treatment several options shoud be compared in a prospective, randomized study. There are three possible types of early postremission therapy. First, the administration of improved multiagent chemotherapy; second, allogeneic THP either from related or unrelated donors [40-42]; and third, autologous THP [43]. The prospective randomized study of the French BGMT group compared allogeneic and autologous BMT performed early after an induction chemotherapy based on Berlin-Frankfurt-Münster (BFM) protocols [44]. They found allogeneic BMT significantly more effective than autologous BMT. In addition, the use of interleukin-2 after autologous BMT did not decrease the relapse rate. However, another multicenter prospective French study (LALA87) did not find differences in disease-free survival between allogeneic, autologous and chemotherapy arms [5]. Several retrospective studies have shown better results with autologous BMT than those reported by the French BGMT group [45] and, in fact, the overall results of allogeneic and autologous BMT have improved over time [46]. Furthermore, the similar results of autologous BMT with respect to those of allogeneic BMT recently reported by the GMALL group [47] are encouraging and, consequently, this therapy deserves further evaluation. Recently, several multicenter trials testing the aforementioned three approaches of postremission therapy have been initiated, but the results are still pending, due to the large number of patients required in each arm. Interpretation of the results of such trials could be difficult due to the fact that there is a tendency to treat the different clinicobiologic subtypes of ALL differently. For example, ALL L3 is currently treated with specific protocols [38, 39] and the results for patients transplanted in first CR require comparison with modern dose-intensive regimens [48]. T-ALL is the subtype with the best prognosis in patients treated with chemotherapy and the inclusion of these patients (at least those of late stage T-ALL) in these types of trials could be questionnable. On the contrary, the results of chemotherapy in Philadelphia-positive ALL (Ph ALL) are disappointingly bad and there is little doubt about the better value of allogeneic TPH over intensification chemotherapy in this ALL subtype [49-52]. It has been recently suggested that the presence of additional chromosome abnormalities in adult patients with Ph ALL may have unfavorable prognostic significance [53].

The results of our study are preliminary, due to the low number of evaluable patients and their short follow-up. Based on intention-to-treat analysis, no postremission arm has proved to be better than the other. The increase in the number of patients and their longer follow up will allow the results of patients treated with each specific arm to be analyzed as well as subgroups of HRALL such as Ph ALL to be studied.

The only prognostic factor isolated in this trial was the slow reponse to treatment, defined as the presence of > 10% BMBC at day 14 of treatment. The effect of high WBC counts on LFS has only been evident in univariate analysis. On the other hand, no differences in prognosis were found between

children and adults. This fact can be explained because only children with very high-risk ALL have been included, and, on the other hand, adults over 50 yrs (the subgroup with the worst prognosis, with < 10% of long-term survivors [54]) have not been entered in this protocol. The speed of response to therapy influenced CR attainment, LFS and OS [55,56]. BMBC on the 14th day of induction treatment was superior to the presence of PBBC at day 8 to predict treatment failures as can be inferred from the results of multivariate analyses.

In conclusion, the preliminary results of the PETHEMA ALL-93 trial for HRALL patients are encouraging. Up to now, there are no differences in LFS for patients treated with intensive chemotherapy, allogeneic or autologous BMT, but a higher number of patients and a longer follow-up are needed to confirm these results. Slow response to induction treatment is the only adverse prognostic factor.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Idarubicin and Ifosfamide in the Treatment of Refractory and Relapsed Acute Lymphoblastic Leukemia

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Abstract. 22 adult patients with relapsed ALL and 2 with refractory ALL were treated in a cooperative trial with an association of idarubicin, ifosfamide, and dexamethasone. Complete remission was achieved in 7 relapsed patients (29%, 95% confidence interval, [CI] 13% - 51%). Twelve patients (50%) did not respond to treatment and 5 (21%) died during induction. All of the patients experienced profound myelosuppression. Median time to recovery of neutrophils $> 0.5 \times 10^9$/l was 28 days (range 17-52), and of platelets $> 50 \times 10^9$/l 21 days (range 13-58). Although gastrointestinal toxicity was common (12%), no patient developed severe cardiac, hepatic, or renal complications. Sepsis was documented in 19 cases, of which 4 developed fatal infectious disease. One patient died from complications of central nervous system involvement. Median time to CR was 38 days. We conclude that our schedule combining idarubicin, ifosfamide and dexamethasone has an acceptable toxicity in adult ALL. However, a more intensive form of therapy appears warranted for pts with poor prognosis ALL.

Introduction

Despite substantial progress in the treatment of acute lymphoblastic leukemia (ALL), only 20% to 35% of adults treated with chemotherapy alone are long-terms leukemia-free survivors [1, 2, 3]. Patients who relapse or are resistant to remission induction generally have a poor prognosis [4]. New drugs or alternative schedules including conventional agents given alone or in combination, and at different dosages, have been employed as induction therapy to increase complete remission (CR) and disease-free survival (DFS) rates in these patients. Of the newer anthracyclines, idarubicin has shown substantial antileukemic effect, and less cardiotoxicity than conventional anthracyclines [5]. Furthermore, a potential advantage for its effectiveness is that its breakdown product idarubicinol reaches measurable levels in the central nervous system (CNS). Ifosfamide, an alkylating agent, has exhibited remission induction potential at least comparable to that of cyclophosphamide [6], and is now included in highly effective B-ALL regimens. Dexamethasone is used in VAD chemotherapy that has allowed complete responses in lymphoid blast crisis of chronic myeloid leukemia [7] and in refractory ALL [8].

We summarize here our experience with idarubicin, ifosphamide and dexamethasone combination chemotherapy in relapsed and refractory ALL.

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Material and Methods

Patient Selection

Twenty-four adult patients aged more than 15 years with relapsed (16 first relapses and 6 second relapses) or refractory (2 patients) ALL were treated in this cooperative trial. The median of first CR was 6.5 months (-range, 0 to 36 months). Only patients with a performance status of 2 or less and no grade > 2 organ failure according to the World Health Organization (WHO) grading system could enter the study. Diagnosis of ALL was established on the basis of morphologic, cytochemical, and immunologic studies. ALL was classified morphologically into 3 groups according to the French-American-British (FAB) criteria [9]. Studies of cell-surface markers were available for all patients. Cytogenetic study was available in 22 patients.

Treatment Regimen

The induction phase included idarubicin, 10 mg/m^2/day on day 1, day 3 and day 5, ifosfamide, 2 g/m^2/day from day 1 to day 5, and dexamethasone, 40 mg/day from day 1 to day 5. In case of persistance of blast cells on day 14 bone marrow (BM) aspirate, patients who did not experience major complications during aplasia received a fourth infusion of idarubicin (10 mg/m^2) at day 14. All patients received gastrointestinal decontamination and prophylactic red blood cell and platelet transfusions. Broad spectrum empirical antibiotherapy was initiated as soon as the patient became febrile. CNS prophylaxis was left to center decision. Consolidation therapy consisted in one or two courses of chemotherapy combining idarubicin 10 mg/m^2 on day 1, ifosfamide 2 g/m^2 on day 1, and dexamethasone 40 mg/day from day 1 to day 3. Two patients who had a suitable familial donor received allogeneic bone marrow transplantation (BMT) after induction therapy. One patient received autologous BMT.

Response Criteria

To assess response to therapy, bone marrow aspirations were made on day 14 and around day 28 of induction therapy. Patients were considered to be in CR if they had less than 5% leukemic blast cells in BM aspirate at time of evaluation, no symptoms or physical findings suggestive of leukemia, and normal blood cell counts [10].

Statistical Methods

95% confidence intervals on proportions of CR patients and toxic deaths were calculated using the exact binomial formula. Overall survival and DFS were estimated by the Kaplan-Meier method. Patients undergoing BMT while in CR were conventionally censored at the time of grafting.

Results

Patient Population

Main clinical and laboratory characteristics of patients at diagnosis are shown in Table 1. The 24 patients ranged in age from 17 to 66 years (median age, 48 years). Fourteen patients (58%) were of male and 10 (42%) were of female sex. Initial median WBC count was 3.4×10^9/l (range, 0.4 to 78.4×10^9/l), median granulocyte percentage 32% (range, 0 to 74%), and median peripheral blood (PB) blast percentage 15% (range, 0 to 97%). Twenty-two patients could be classified according to FAB criteria. Fifteen of these patients were L1, and 8 were L2. Immunophenotypic analysis was available in all cases. Twenty patients were diagnosed as B-cell lineage ALL, and 4 as T-cell lineage ALL. Karyotypes were analyzable in 22 cases. Among them, no abnormality was seen in 5 cases, while 6 patients presented with Ph+ chromosome.

Treatment Effectively Received and Efficacy of Therapy

All patients received the full dose of cytotoxic agents involved in the induction course.

Table 1. Clinical and laboratory characteristics of the 24 patients

Characteristics		
Age (years)		48 (17-66)[a]
Sex (male/female)		14/10
Hepatomegaly (yes/no)		1/23
Splenomegaly (yes/no)		2/22
Superficial adenopathy (yes/no)		4/20
Mediastinal adenopathy (yes/no)		1/23
CNS involvement (yes/no)		3/21
White blood cell count (x 109/l)		3.4 (0.4-78.4)
Peripheral blood granulocytes (%)		32 (0-74)
Peripheral blood blast cells (%)		15 (0-97)
Bone marrow blast cells (%)		91 (22-100)
FAB classification	L1	15/24
	L2	8/24
	unclassified	1/24
Immunophenotype	B-cell lineage	20
	T-cell lineage	4
Karyotype	normal	5
(in 22 cases)	monosomy 5 or 7	2
	t(4; 11)	1
	Ph chromosome	6
	other translocation	8
LDH (μ/l)		1023 (229-5870)

[a] Values are median (range)

Seven patients (29%, 95% CI 13-51%) achieved CR: 7 of the 22 relapsed patients and none of the 2 refractory patients. Twelve patients (50%) did not respond to treatment and 5 (21%) died during induction, of whom one died from complications of CNS involvement. Six of the 20 pts with B-cell lineage ALL and 1 of the 4 with T-cell lineage ALL responded. Only 1 of the 6 patients with Ph+ chromosome responded. Fifteen patients (62%) presented blast cells on day 14 BM aspirate. Among them, only 8 patients received an extra dose of idarubicine on day 14. The reasons for non receiving idarubicin in 7 cases were a WHO grade 3 performance status or toxicity. The median time to CR was 38 days (range, 27 to 58 days). Five of the 7 patients who achieved CR received consolidation therapy: 2 underwent allogeneic bone marrow transplant (BMT), 1 autologous BMT, and 2 consolidation chemotherapy. Two patients did not receive any consolidation therapy because of a WHO grade 3 performance status after induction. Median DFS was 12 months. Four patients relapsed at a median time of 5 months (range 1 to 13 months), and 1 patient died while in CR. Median overall survival for the entire cohort was 4 months.

Toxicity

Chemotherapy-related toxicity was mainly hematologic. All of the patients experienced profound myelosuppression. Median time to granulocyte recovery to $> 0.5 \times 10^9$/l was 28 days (range 17-52 days). Median time to thrombocyte recovery to 50×10^9/l was 21 days (range 13-58 days). Sepsis was documented in 19 cases, of which 4 developed fatal infectious disease (toxic deaths during induction therapy, 16%, 95% CI 5-37%). Only 5 patients (21%) completed induction without becoming febrile. Severe gastrointestinal toxicity (grade 3 or more) occurred in 3 patients (12%). No patient developed severe cardiac, hepatic, or renal complications. Four patients (16%) developed neurotoxicity (grade 3 or more): 2 developed fungal CNS infections, 1 had optic neuropathy related to therapy and 1 had subdural hemorrhage.

Discussion

The prognosis of refractory and relapsing adult ALL is generally considered to be poor. There are few therapeutic experiences based on small and heterogeneous patient popula-

tions (reviewed by Welborn [4]). A second complete response occurred generally in 25 to 50% of patients with a median DFS of less than 6 months. In that field, new therapeutic agents and new drug combinations must be employed with the aim of overcoming drug resistance. In the present study, the idarubicin, ifosfamide, and dexamethasone combination showed antileukemic efficacy with a CR rate of 29%. The predominent side effect was profound myelosuppression, which caused 4 fatal infections. However, hematologic toxicity remained easily manageable, and extra-hematologic toxicity was acceptable.

In refractory or relapsed ALL, the most encouraging CR rates were achieved with high-dose cytarabine [4], and second line treatments are often compared to these regimens. Regarding CR rate, our results are lower than most earlier reports, but all series included only a small number of patients and most of them have excluded patients with Ph-positive ALL. However, our trial compared more favorably regarding treatment toxicity and DFS since Welborn [4] reported an average treatment related mortality of 15%, and an average median DFS of 4.2 months.

There were also differences when comparing our results to those of trials using similar drugs. We did not confirm the previous very encouraging study combining ifosfamide and an anthracycline. Indeed, the combination of ifosfamide and doxorubicin yielded a CR rate of 89% in 9 patients with minimal toxicity [11]. Our results were closer to those from a study using ifosfamide alone at 1200 mg/m^2/day for 5 days that reported 33% of CR [6]. More recently, a phase II study combining ifosfamide with etoposide and mitoxantrone has reported 73% of complete responses [12], but only 11 patients were included in the study of whom only 1 was Ph-positive. Idarubicin has been used mainly in relapsed ALL in 2 studies combining it with high-doses cytarabine [13, 14]. They reported respectively 54 and 64% of CR with 15 and 8% of toxic deaths during induction.

Despite heterogeneous results regarding CR rates probably related to the small number of patients in published series and to the degre of selection of treated patients, the major problem in relapsing ALL remains the limited duration of DFS. There is no study with markedly different results in this respect. We conclude that our schedule combining idarubicin, ifosfamide and dexamethasone has a certain effect on relapsed adult ALL and an acceptable toxicity. However, a more intensive form of therapy is warranted for patients with poor prognosis ALL.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Treatment of Ph+ and t(4;11)+ Acute Lymphoblastic Leukemia in Adults

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Philadelphia Positive Adult ALL

Introduction

About 20-25% of adult patients with acute lymphoblastic leukemia (ALL) present with a Philadelphia chromosome t(9;22) as cytogenetic marker and/or on a molecular level with the corresponding BCR-ABL-rearrangement [1-4]. The proportion of ALL-patients with Ph+/BCR-ABL+ ALL increases with age. The median age of patients with BCR-ABL+ ALL in the German multicenter adult ALL (GMALL) trials is 45 years [4].

Results of Chemotherapy in BCR-ABL-Positive ALL

With recent intensive induction regimens relative high complete remission rates of approximately 70% as defined with conventional morphological criteria can be achieved. The prognosis of these patients after intensive conventional chemotherapy remains extremely poor. Long-term survival ranges between 0-10% [4]. Attempts to improve the results, such as intensification of chemotherapy by high-dose treatment with high-dose AraC (HdAraC), high-dose methotrexate (HdMTX) resulted in only minor improvements.

Minimal Residual Disease After Induction/ Consolidation Chemotherapy

Residual BCR-ABL+ cells can be detected in bone marrow and in peripheral blood samples by RT-PCR with a high sensitivity of approximately $1:10^6$ [5-6]. In our experience residual BCR-ABL+ cells were detectable by RT-PCR in remission marrow samples from all 21 patients tested so far. By semiquantitative RT-PCR we found a median titer of 4 log above limit of detection after induction and/or consolidation therapy [6].

Allogeneic BMT in BCR-ABL+ ALL

The best outcome is achieved in patients receiving an allogeneic bone marrow transplant [7-12]. The group from Stanford achieved a 46% event-free survival after allogeneic BMT in first complete remission and 28% for BMT in CR 2 [12]. The International Bone Marrow Transplant Registry (IBMTR) reported a retrospective analysis with an event-free survival of > 38% after 2 years [10]. Thus, at present, allogeneic BMT is accepted as the best first line therapy in patients with an HLA-identical family donor.

Unrelated Donor Transplants

Patients without a matched family donor

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may receive an autologous marrow or stem cell graft, or they are candidates for a matched unrelated donor (MUD) transplant. The priorities of these options have not yet been established. Data on long term results after autologous or MUD transplants in BCR-ABL-positive ALL are limited. One series including 12 children transplanted from unrelated donors has been presented [13] with a DFS of 68%.

These encouraging results, however, will certainly not translate to patients with a median age of 45 years. In addition, since relapse occur early in BCR-ABL-positive ALL-patients this might reflect a patient selection. In contrary the positive results could lead to the hypothesis that in unrelated BMT in BCR-ABL positive ALL patients a graft versus leukemia effect - otherwise not or only marginally seen in other ALL subtypes - might play a decisive role.

Autologous Transplantation in BCR-ABL+ ALL

Due to the higher age of patients with BCR-ABL-positive ALL, the still limited availability of a matched unrelated donor and the time required to complete a search, a majority of BCR-ABL-positive ALL patients remain candidates for autologous transplants. Some groups reported small autotransplant series including 1 to 11 patients [8, 9, 14, 15]. The event-free survival (weighted mean) of all 36 patients reported by these authors was 31%. A series of 25 patients diagnosed and initially treated according to GMALL protocols have been autografted in Frankfurt or cooperating BMT centers [16]. More detailed results from the GMALL series are as follows:

Purging of Bone Marrow Autografts. The rationale for in vitro purging is based on the relatively high titer of residual BCR-ABL-positive cells as monitored by semiquantitative RT-PCR. The median titer in autologous bone marrow grafts was 4 log above the limit of detection. Purging using a cocktail of CD10, CD19 and AB4 monoclonal antibodies and two cycles of immunomagnetic beads depleted median 3 log of BCR-ABL+ cells from autologous bone marrow grafts. None of the purged BM-grafts, however, was completely PCR-negative [6].

Peripheral Blood Stem Cells (PBSC) for Transplantation. After high dose-AraC/mitoxantrone (part 2 of induction therapy in of the GMALL 05/93 high-risk protocol) median 9 (range 0.8-23) x 106/kg CD34+ cells can be collected by one leukaphereses [17]. In non-mobilized peripheral blood samples 1-2 log (median 1.7) less residual BCR-ABL+ cells were found compared to control bone marrow samples (n = 6) [18]. The median RT-PCR titer in leukapheresis-derived autografts (n = 8) was median 2 log below the median titer of to BM-autografts (n = 14). In most patients it is not feasible to further reduce residual BCR-ABL-positive cells in PBSC-autografts by "in vivo purging" using additional chemotherapy since the yield of CD34+ cells declines rapidly after subsequent cycles [17]. However, immunomagnetic bead purging yielded completely BCR-ABL-negative PBSC-grafts in 4 of 8 patients [17].

Outcome After ABMT in BCR-ABL+ ALL. The event-free survival after ABMT in first CR was about 30% after > 2 years, whereas all patients autografted beyond CR1 relapsed within 6 months [16]. Thus relapse remains the most frequent adverse event.

Future Aspects

At least in a proportion of patients it is possible to achieve apparently BCR-ABL-negative PBSC autografts by in vitro purging. However, relapse after transplantation remains the major cause of treatment failure. It is likely that relapses originate from leukemic cells resisting the conditioning regimen in vivo. After collection and purging of autografts these cells might be eliminated by further escalating the cumulative dose of in vivo therapy. One option of further intensification is to sequentially apply cycles of myeloablative high-dose therapy with subsequent stem cell transplantation. Sequential autotransplant protocols have already been pioneered in patients with multiple myeloma, metastatic breast cancer and

small cell lung cancer. In patients with multiple myeloma "tandem"-autografts with conditioning protocols using high-dose melphalan followed by TBI + melphalan are feasible with acceptable toxicity.

A double autograft protocol is now proposed for patients with BCR-ABL-positive ALL. This intensified autograft protocol is illustrated in Fig. 1. Patients with age < 60 years, WHO-performance status ≤ 2 and complete or partial morphological remission after the first cycle of induction therapy (vincristine, prednisone, daunorubicin, asparaginase) arc eligible.

Transplantation is scheduled in first complete remission. Within 3 months patients with age < 45 years may opt for a MUD transplant. Thus HLA-typing of the patient, his siblings and eventually the search for an unrelated donor are initiated as soon as possible. Unless an allogeneic/MUD transplant is definitively scheduled, patients are treated according to the double autograft protocol. Peripheral stem cells are collected after the second cycle of induction therapy (HdAraC/Mitox.), since mobilization is likely to be impaired after subsequent chemotherapy. At least 4×10^6 CD34+ cells/kg should be collected to enable 2 successive transplants. The autografts are purged in vitro using immunomagnetic beads. Subsequently the patients receive consolidation chemotherapy with HD-MTX according to the GMALL protocol while PBSCT is scheduled. The first conditioning regimen includes thiotepa and melphalan. This combination is expected to provide antileukemic efficiency with low pulmonary and hepatic toxicity. The second conditioning regimen consists of hyperfractionated total body irradiation (TBI) and etoposide (VP-16), as used previously in single transplant patients. Eight weeks after the second transplant patients receive maintenance therapy with 6-MP/MTX based on the beneficial experience by the Royal Marsden Group [15].

Preliminary Results After Double Autografting. Four patients have completed this double autograft protocol. Hematopoietic recovery was rapid in all cases. The most prominent toxicity was prolonged mucositis. No further unexpected toxicity and no mortality was seen so far. Longer follow-up

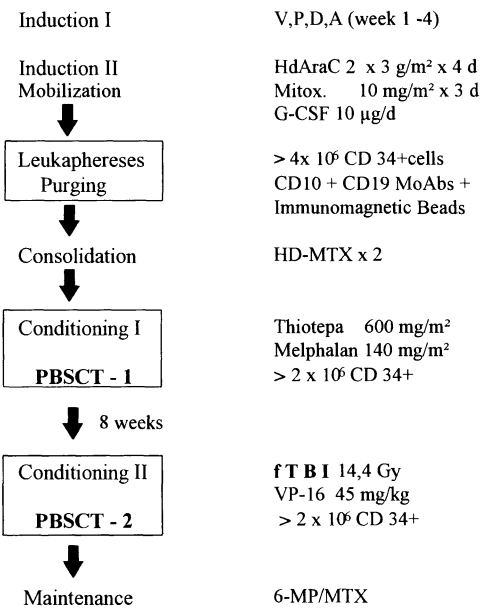

Fig. 1. Double autografting protocol for patients with BCR-ABL-positive ALL

and more patients are needed to conclude on event-free survival.

Ph+/BCR-ABL+ ALL is a subtype in which unfortunately so far – at least by chemotherapy – no progress in outcome could be achieved. In contrary in adult ALL patients with translocation t(4;11) results apparently seem to be improvable by inclusion of high dose chemotherapy.

Translocation t(4;11) in Adult ALL

Introduction

The translocation t(4;11) (q21;q23) is beside t(9;22) the most frequent specific translocation in ALL and occurs in approximately 2-5% of all childhood ALL with a peak (42-66%) in infant ALL [19]. In adult ALL the incidence of t(4;11) is slightly higher compared to childhood ALL and ranges between 3-6%.

t(4;11) identifies a subset of ALL with characteristic features such as hyperleukocytosis, young age, female predominance, high incidence of initial CNS involvement, organ involvement, early pre-B phenotype and lineage heterogeneity [19-22].

Molecular Genetics

t(4;11) (q21;q23) is the most frequent form of 11q23 abnormalities in ALL. The involved gene on chromosome 11 was cloned and named MLL gene for mixed lineage leukemia [23]. Synonyms are ALL-1, HRX and HTRX1. Various breakpoints occur and result in the generation of a series of chimeric proteins that are thought to play a crucial role in leukemogenesis. In t(4;11) translocations the MLL gene is fused to a gene located on chromosome 4 which is named AF-4 (also referred to as FEL). So far little is known about the role of the MLL gene in normal hematopoiesis and the role of MLL fusions genes in leukemic transformation. Interestingly, the molecular breakpoints of 11q23 are similar in ALL and AML. This finding suggests that MLL gene plays a regulatory role in both lineages [19, 20].

A great variety of heterogeneous fusion transcripts results from alternative splicing events and differential exon usage from the MLL and AF4 gene. It has been speculated that differential fusions genes might be correlated with prognosis. Seven of 13 adult patients with HRX (MLL) rearrangements showed HRX exon 6 fusions compared to 1 of 4 infants. HRX exon 7 fusions were more common in infant ALL (3 of 4) compared to adults (6 of 13). In a literature review these results appeared to be confirmed since in a total of 30 infants exon 6 was involved in 23% compared to 52% in adults [24]. The question whether those differences can be confirmed in larger patient numbers and may be associated with different prognosis in infant and adult ALL requires further investigation.

Immunophenotype

Although some reported cases of t(4;11) ALL expressed myelomonocytic characteristics or mixed-lineage markers most patients 71% (50-94%) show early B-precursor phenotype (Table 1).

In earlier studies the term Null-ALL referred to a heterogenous group of CD10 negative B-lineage leukemias without expression of further differentiation markers. In more recent trials a relatively homogenous

Table 1. Incidence of Pre-pre-B phenotype in ALL with t(4;11)/HRX-rearrangement

	HRX rearrangement or t(4;11)	Pre-pre B or Null ALL
Pui 1991 [25]	33	26 (79%)
Cimino 1995 [26]	25	14 (56%)
Charrin 1996 [22]	16	15 (94%)
Secker-Walker 1997 [21]	10	5 (50%)
Weighted mean	84	71%

definition had been applied to identify early B-precursor ALL (also referred to as pre-pre-B-ALL, B-I, pro-B-ALL, early B-ALL). It is generally characterised by the expression for HLA-DR and CD19 and the lack of CD10. The EGIL group has recently proposed an uniform definition for immunologic subtypes of ALL. Pre-pre-B-ALL was named B-I (pro-B) ALL and defined by the expression of at least two of early B-Cell markers (CD19, CD79a or CD22) without expression of further differentiation antigens [27].

It became evident that pre-pre B-ALL occurs more frequently in adult ALL (11-12%) compared to childhood ALL (5-6%) [28-30] and the incidence is highest in infant ALL (43%) [28].

Pre-pre B-ALL is associated with a high incidence of HRX rearrangements and of myeloid coexpression (50%) compared other immunophenotypic subgroups in children (10%) and adults (20%) [31]. The most commonly expressed myeloid antigens are CD15 and CDw65 whereas the latter may be a more discriminative marker. Thus a combination of pre-pre-B-ALL with coexpression of CDw65 is highly predictive for t(4;11) or an HRX rearrangement [24].

Molecular Markers for Detection of MRD in t(4;11) Positive ALL

Recently, reverse transcriptase chain reaction (RT-PCR) assays have been developed for the comfortable identification of chimeric m-RNA (HRX-FEL or ALL-1/AF4) resulting from t(4;11) with a sensitivity of 1 in 105 cells. The RT-PCR assay is successful in detection approximately 90% of MLL rearrangements.

Althogether there is a good correlation between t(4;11) detected by conventional cytogenetics and MLL rearrangement. In the great majority of patients with t(4;11) an MLL rearrangement can be detected by PCR (Table 2). It fails however to detect fusion genes in unusual translocations t(4;11) [32]. These cases can only be identified by Southern blotting with a far lower sensitivity of about 1 in 100 cell arrangements (reviewed in [20]).

Table 2. Correlation of t(4;11) and MLL rearrangements

Author	t(4;11)	MLL rearrangement
Hilden 1995 [32]	8	6[2] (75%)
Behm 1996 [33]	18[1]	18 (100%)
Griesinger 1996 [24]	13	13 (100%)

[1] Including t(4;11, t(9;11), t(11;19).
[2] MLL not detected in 2 patients with unusual t(4;11).

Screening for 11q23 abnormalities with PCR leads to the identification of a considerable number of additional positive cases. Thus in infant ALL PCR screening boosts the frequency of 11q23 aberrations to 70-80% [19-20] including many cases that have not been detected cytogenetically.

Only preliminary results are available for monitoring of HRX rearrangement for detection of minimal disease (MRD). So for there is a good correlation between the lack of MRD and continuous complete remission. All investigated patients in CCR were MRD negative whereas in relapsed or resistant cases the rearrangement was consistently present (Table 3). This is in contrast to MRD monitoring in other subtypes where in a considerable number of patients MRD was present despite continuous CR or in few patients relapses occurred in MRD negative patients. The findings in t(4;11) positive ALL have, however, to be confirmed in prospective studies of greater patient numbers.

Outcome in Patients with t(4;11)/MLL Rearrangements

The close relationship between t(4;11), MLL-rearrangements and the immature pre-pre-B ALL subtype correlates with similarly poor results for both entities. The first reports on outcome in t(4;11) positive ALL revealed extremely poor results [1] (Table 4). t(4;11) is the major factor accounting for the poor outcome in infant ALL [19].

MLL rearrangements as well proved to exert a strong negative prognostic impact. In 5 studies reviewed by Rubnitz the EFS was 9-19% in children with MLL rearrangement

Table 3. Detection of MRD in t(4;11) positive ALL

		CCR	MRD+	Rel./Res.	MRD+
Janssen 1994 [34]	18	7	0	n.r.	n.r.
Cimino 1996 [35]	12	5	0	7	7
Ida 1997 [36]	3	2	0	1	1

Table 4. Outcome in ALL with t(4;11)/MLL rearrangement

Author	Patients	N	Aberration	Survival/CCR
Bloomfield 1986 [1]	Children	9	t(4;11)	0
Pui 1991 [25]	Children	40	t(4;11)	16 (40%)
Janssen 1994 [34]	Children	4	ALL-1/AF4	2 (50%)
Cimino l996 [35]	Infants	7	ALL-1/AF4	3 (43%)
Bloomfield 1986 [1]	Adults	9	t(4;11)	0
Schardt 1992 [37]	Adults	5	t(4;11)	0
Janssen 1994 [34]	Adults	16	ALL-1/AF4	7 (44%)
Schoch 1995 [38]	Adults	23	t(4;11)	9 (39%)
Cimino 1996 [35]	Adults	5	ALL-1/AF4	2 (40%)
Charrin 1996 [22]	Adults	16	t(4;11)	0
Secker-Walker 1997 [21]	Adults	10	t(4;11)	1

compared to 46-100% in those with germline configurationt [20].

There is some evidence that outcome of t(4;11) ALL may be age related. In a literature review by Pui in 100 patients the outcome in infants and adolescents (> 10 years) was inferior compared to children at intermediate age (1-9 years). Twenty adult patients with t(4;11) had the worst outcome and all failed to achieve remission or relapsed within the first year [25, 39]. Two recently published trials in adult t(4;11) ALL also showed poor results [21, 22]. There is evidence for an inferior outcome in Null/pre-pre-B-ALL compared to common and T-ALL as well [40] although no difference was found by others [28, 41]. The majority of the reported patients with poor outcome has, however, been treated before 1990.

In t(4;11) as well as in pre-pre-B-ALL evidence increases that treatment results are improving by the introduction of treatment intensification. Thus the majority of CCR patients (15 of 16) reported by Pui had been treated to more contemporary protocols initiated after 1987 [42]. In the series reported by Schoch 9 of 23 patients with adult de novo ALL remained in first CR (10-83 mo), including eight patients with initial WBC > 100 000 [38]. Further, 7 of 16 adult patients with MLL rearrangements remained in long-term CCR [34]. In a preliminary report on 67 adult patients with pre-pre-B ALL with a high incidence of t(4;11) (48%) outcome was improved compared to previously published Null-ALL. The median remission duration and survival were 35 months and 23 months respectively compared to 15 months of median remission duration in the earlier trial [31, 40]. The majority of these patients have been treated according to the high risk arm of the intensive risk-adapted GMALL protocol comprising intensified consolidation with high dose cytosine-arabinoside/mitoxantrone or high dose methotrexate/asparaginase. Furthermore, allogeneic BMT in first complete remission was scheduled for all t(4;11) positive patients with sibling donor. The improved outcome of t(4;11) ALL is underlined by the above-mentioned findings of MRD analyses since in contrast to Ph/bcr-abl positive ALL in all CCR patients MRD was not detectable [34].

Conclusions and Future Perspectives for t(4;11) Positive ALL

- t(4;11) identifies a subgroup of ALL with distinct clinical and biologic features.
- Pre-pre-B-ALL phenotype with myeloid coexpression (CD15, CDw65) is highly predictive for t(4;11).
- PCR screening for MLL rearrangements identifies a considerable number of additional t(4;11) positive cases compared to conventional cytogenetics alone. Prospective studies are ongoing therefore in the GMALL study group.
- In a subgroup with formerly fatal prognosis now long-term remissions can be achieved in a considerable proportion of patients.
- MRD evaluation is important in t(4;11) ALL since negative MRD status is apparently highly predictive for low probability of relapse in contrast to other ALL subtypes
- Treatment with high dose cytosine-arabinoside appears to be effective in t(4;11) positive ALL. The application of a second cycle of high dose cytosine-arabinoside/mitoxantrone in order to further improve results is therefore investigated in the ongoing GMALL trial 05/93.
- Allogeneic bone marrow transplantation in first complete remission, although not reviewed here appears to be profitable.
- The identification of additional risk factors within t(4;11) positive ALL e.g. additional aberrations, differential splice variants may lead to the definition of unfavourable subgroups for selection of matched unrelated BMT.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Philadelphia (Ph)-Chromosome-Negative Peripheral Blood Stem Cells can be Mobilized in the Early Phase of Recovery after a Myelosuppressive Chemotherapy in Ph-Chromosome-Positive Acute Lymphoblastic Leukemia

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Introduction

Allografting is the only recognized therapy able to cure adult acute lymphoblastic leukemia patients with Ph-chromosome. Unfortunately, suitable HLA-matched related or unrelated donors are not available for the majority of leukemic patients. Transplantation of stem cells derived from the patient's own marrow (autografting) avoids the need of an HLA-matched donor, but relapses are extremely frequent [1-5]. In our department, none of the Ph-positive adult patients with early pre B or pre-B cell treated with intensive chemotherapy were alive after 2 years. In analogy to the encouraging results achieved by our team with mobilization of Ph1-negative PBPC and autografting in patients with Ph-positive chronic myelogenous leukaemia [6-8], we have used the same approach on patients with Ph-positive acute lymphoblastic leukemia.

Methods

Twelve patients were enrolled in this pilot study. Eight patients were male whose ages ranged from 17 to 59 years (median 38 years). Six patients were in first relapse, four in second relapse and two patients in first hematologic remission but not in cytogenetic remission. The median time from diagnosis to mobilizing therapy was 10 months (range 4-16 months). All patients had previously received conventional chemotherapy according to the German protocol [4] or to the Memorial Hospital protocol [9]. Patients relapsed at a median time of 5 months (range 4-11 months) from remission achievement (Table 1).

Mobilizing Therapy. All patients received a regimen of idarubicin 8 mg/m^2/d on days 1-5, arabinosylcytosine at 800 mg/m^2 2 h infusion daily on days 1-5, etoposide at 150 mg/m^2 2 h infusion daily on days 1-3 [6-8]. Recombinant G-CSF was given daily at 5 μg/kg until the total neutrophil count was over 1 x 10^9/l for three consecutive days. Peripheral blood counts were monitored daily and leukapheresis were begun as soon as WBC exceeded > 0.8 × 10^9/l. The next leukapheresis was done when the WBC reached > 1 × 10^9/l and leukapheresis were subsequently performed daily until WBC exceeded 3.0 × 10^9/l. The collected cells were counted and studied for chromosome analysis, bcr-abl m-RNA transcripts, in vitro culture and phenotypic characteristics. The total product was frozen using Planer R-203. When peripheral blood counts had fully recovered, a sample of bone marrow was taken for cytogenetic analysis.

Cytogenetic Analysis. Cytogenetic studies were carried out on blood cells and on bone marrow (BM) using short-term (24-48 h) cultures without the addition of mitogens. Chromosomes were analysed using G and Q

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Table 1. Clinical characteristics and outcome

Patient	Age/ Sex	WBC × 10^9/l at diagnosis	Length of first CR (months)	Pre-chemo BM Ph+ metas (%)	PBSC collection Ph+/ metas	BM after mobilization Ph+/ metas	Clinical response after mobilization	Outcome
1 (RLP-1)	20/M	410	5	100	0/48	20/50	CR	PBSCT D/L
2 (RLP-2)	17/M	9	4	100	50/50	50/50	NR	D/L
3 (RLP-2)	37/F	380	10	100	36/36	42/42	NR	D/L
4 (RLP-1)	59/M	390	4	100	38/38	ND	NR	D/L
5 (RLP-1)	47/M	180	5	100	42/42	48/48	NR	D/L
6 (RLP-1)	38/M	280	5	100	0/38	10/48	CR	PBSCT A/R (CCR +18)
7 (RLP-2)	36/M	435	11	100	30/30	36/36	CR	D/L
8 (RLP-1)	39/M	65	5	100	0/28	0/40	CR	PBSCT D/L
9 (RLP-2)	40/F	220	6	100	0/36	10/26	CR	PBSCT D/L
10 (RLP-2)	44/M	540	4	100	0/40	20/54	CR	PBSCT D/L
11 (CHR)	23/F	10	30	100	0/70	ND	CR	PBSCT A/W (+9)
12 (CHR)	58/F	16	1	100	0/67	0/80	CR	A/W*

RLP1: first relapse; RLP2: second relapse; CHR: complete hematologic remission; CR: complete remission; BM: bone marrow; PBSC: peripheral blood stem cell; Ph+: Philadelphia chromosome positive; PBSCT: autografting with peripheral blood stem cell; D/L: died of leukemia; A/R: alive in relapse; A/W: alive and well; ND: not done; * autografting ongoing.

banding techniques and classified according to ISCN [10]. Karyotype studies were done on bone marrow before therapy, on blood stem cells collected by leukapheresis and on bone marrow after mobilizing therapy. At least 20 metaphases were analysed when available. When a lower number of metaphases was found in a particular analysis, a second analysis was done in 2-4 weeks, and the results of the two specimens were pooled. All Ph-negative patients were evaluated by PCR amplification for bcr-abl transcripts. The method used consisted of two round "nested" PCR [11].

Results

In 7/12 (58%) patients cytogenetic analysis failed to reveal Ph-positive translocation in peripheral blood stem cells and one of them (patient 6) also became bcr/abl negative (Table 1). Only one patient had complete Ph1-negative hematopoiesis in the bone marrow. Complete hematological remission was achieved in 8/12 patients. One remitting patient relapsed and died of leukemia before transplantation (patient 7, Table 1). The median numbers of mononuclear cells, CFU-GM, CD34+ and CD34+/DR-cells collected were 3×10^8/kg (range, 0.8-7.6), 2.1×10^4/kg (range, 0-21), 3.4×10^4/kg (range, 1.4-28.1) and 0.8×10^6/kg (range, 0.1-21), respectively (Table 2).

In six patients, so far, the cryopreserved Ph-negative peripheral blood stem cells have been used as autotransplants. The conditioning regimen consisted of etoposide 800 mg/m^2/d for 2 days, cyclophosphamide 60 mg/kg/d for 2 days or idarubicin (50 mg in single dose in 3 h infusion) and total body irradiation (8.5 Gy) at a single dose rate of 16-18 cGy/min. Within 48 h of radiation, the

Table 2. Details of biological results on peripheral blood stem cells collected

	MNC $\times 10^8$/kg	CD34+ $\times 10^6$/kg	CD34+/DR- $\times 10^6$/kg	GM-CFU $\times 10^4$/kg
	1.8	3.6	0.5	2.8
	2.1	4.4	0.1	0
	2.9	3.2	0.8	2
	3.8	2.8	0.8	2.2
	7.6	6.4	2	21
	4.1	3.1	0.9	3.8
	3.8	3.8	1.1	2
	1.8	2.6	1	0.9
	2.8	1.4	0.3	0.6
	2.9	1.9	0.4	1.3
	5.9	28.1	4.8	9.6
	3.1	5	0.3	9.3
Median	3	3.4	0.8	2.1
range	(1.8-7.6)	(1.4-28.1)	(0.1-4.8)	(0-21)

cryopreserved Ph-negative peripheral blood stem cells were thawed and reinfused. Recombinant G-CSF was given after cell infusion at 5 μg/kg/d until neutrophil counts reached > 1 x 10^9/l for 3 consecutive days. The median time to ANC count recovery > 0.5 × 10^9/l and to platelet recover of > 20 × 10^9/l after autografting was 24 days (range, 18-36 days) and 45 days (range, 29-65 days), respectively. All patients showed substained engraftment. Two patients are alive; one in relapse after 18 months of complete cytogenetic remission and another patient is in complete cytogenetic remission 9 months after autografting (Table 1). Both these patients were maintained after transplantation with IFN-AAA/low doses IL-2. Molecular studies to detect bcr-abl transcripts were performed on one Ph-negative patient and were negative for the first 3 months but, subsequently, became positive.

Discussion

Philadelphia-chromosome-positive ALL is a subgroup of ALL having a dire prognosis in children and adults which almost invariably leads to death. On more than 200 ALL patients recently analysed, survival was worse in patients with bcr-abl transcripts compared to the brc-abl negative group [12, 13]. A possible cure has been recently reported in four patients (three adolescents and one child) treated with extensively reinforced, early chemotherapy followed by rotational treatment with pairs of non-cross-resistant drugs. The median duration of leukaemia-free-survival in this subgroup was 6 years (range, 6-8 years) [14]. The same results have been recently confirmed in Italy in a small number of patients [15]. A part from these results, there is no doubt that conventional chemotherapy is inadequate to maintain a durable remission. Allogeneic bone marrow transplantation remains the only procedure capable of curing this group of patients and therefore is mandatory for patients with compatible donors. A review of BMT results revealed a 2 years leukemia-free-survival probability of 38, 41 and 25% for patients transplanted in first remission, after relapse and after failure to achieve initial remission, respectively [16]. Similar results were achieved in Stanford [17]. Patients without compatible donors have been offered autografts, but this procedure does not seem very promising [9, 18-20]. The main problem is to be able to purge marrow of Ph-positive leukemic cells. Concerning the first point, one possible solution might come from the development of techniques able to induce an overshoot of diploid hemopoietic progenitor cells which can be used as "rescue" after intensive high-dose therapy. Using this approach, we were able to confirm the presence of diploid cells during early recovery of hemopoietic function after intensive conventional chemotherapy in chronic myelogenous leukaemia [6-8]. Likewise, we have employed the same technique in 12 patients with Ph-positive ALL, mainly during their first or second relapse. The results were very interesting in terms of kariotypical conversion especially considering that this group of patients was at high risk. In seven out of 12 patients the cytogenetic analysis on peripheral blood cells demonstrated the absence of Ph-chromosome. The bone marrow evaluation analyzed in the same days revealed Philadelphia-chromosome translocation in variable amount and only in two cases the complete absence of it.

The proposed mechanism of the "normal" haemopoietic rebound during early recovery phase following intensive conventional chemotherapy is uncertain. The basic mechanism underlying the achievement of remission probably lies in the fact that normal cells regrow faster than the leukemic ones [21]; therefore, the overshoot of "normal" haemopoietic progenitor cells in the blood during early recovery represents a process that is occurring in the bone marrow, and this might be amplified by the use of growth factors. The reason why very primitive hemopoietic progenitors cells enter the blood, reflects a homeostatic mechanism aimed to re-establish the hemopoietic progenitors and to repopulate homogenously all hemopoietic sites.

Finally, the quality and quantity of stem cells detected in each patient and in each apheresis might depend upon the combination of different features, such as the degree of residual normal bone marrow, the de-

bulking of Ph-positive cells, and - last but not least - whether such a debulking reaches the same degree in each haemopoietic site.

In conclusion, further work is needed to confirm these preliminary results but it is possible that if we could mobilize these patients in earlier phases of their disease we could satisfy two different aims:

1. the achievement of more diploid hemopoietic progenitor cells, as we are now observing in chronic myelogenous leukaemia mobilized at diagnosis and
2. to achieve more cures after high-dose therapy followed by autografting with these normal cells.

More patients and more time, can confirm the place of this procedure in the treatment of high-risk acute leukemias.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Prolonged Remission in Adult B-ALL (L3) and Advanced Burkitt's Lymphoma Using Acute Leukaemia Regimens: Study of 34 Patients

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Abstract. Thirty-four adults with B-ALL (n = 23) and advanced-stage Burkitt's lymphoma (n = 11) received their primary treatment with one of five consecutive regimens developed for adult ALL over a 15-year period. The complete remission rate was 62%, with median duration 1.6 years and 2-13 years probability 0.46. Factors associated with higher probability of response were a diagnosis of B-ALL, normal serum creatinine, uninvolved central nervous system, and use of idarubicin-containing regimens. Remission duration was favourably affected by blast cell count $< 1 \times 10^9$/l and/or age < 50 years (59% after 5 years, $p < 0.025$ by the log-rank test) and the most recent consolidation programmes employing very high-dose chemotherapy supported by autotransplants. Further improvement in poor-risk subgroups is expected by more aggressive and timely prevention of neuromeningeal spread and increased-dose consolidation with blood cell autograft.

Introduction

B-ALL (acute lymphoblastic leukaemia) accounts for less than 5% of adult ALL. This discrete ALL syndrome exhibits a so-called L_3 or Burkitt-like blast cell morphology, nonrandom translocations of chromosomes 14, 18, 2, and 8, and a mature B-cell phenotype with monoclonal surface immunoglobulin. Until very recently, B-ALL was considered a very aggressive disease associated with an extremely bad prognosis. In a recent German Multicenter Acute Lymphoblastic leukemia (GMALL) study [1], the remission rate and leukaemia-free survival increased from 44 and 0%, obtained with ALL-type treatment, to 74 and 71% using an intensive regimen for childhood B-ALL and the strictly related entity Burkitt's lymphoma (B-NHL). Prior studies with ALL-directed chemotherapy, reviewed by Hoelzer [1], involved less than 10 total patients and reported remission rates between 0-67% (median 35%) and leukaemia-free survival between 0-33%. From 1979-1995 we conducted five subsequent chemotherapy trials for adult ALL, enrolling 346 total patients aged 15-78 years and including B/L3-ALL as well as B-NHL. We review retrospectively initial response rate and long-term outcome of 34 adults with B/L_3-ALL and advanced B-NHL, homogenously treated with these adult ALL-type protocols. To the best of our knowledge this is the largest series of its kind to be compared with smaller ones reviewed by Hoelzer and the GMALL study results. The present report is the 1997 update of a previously published study [2].

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Materials and Methods

Diagnosis

B-ALL was diagnosed when > 30% bone marrow cells were lymphoid blasts expressing mature B-lineage differentiation markers with surface light chain immunoglobulin (SIg+) of either the kappa or lambda type. An L_3 blast cell morphology according to the French-American-British subclassification was not mandatory in cases with SIg+ phenotype, but it was necessary in those without the immunophenotype study. Cases with L_3 morphology and non-SIg+ phenotype were excluded. Advanced-stage B-NHL was defined as clinical stage III/IV by the Ann Arbor and St. Jude's staging systems or C/D by the National Cancer Institute staging classification for B-NHL, with bone marrow involvement < 30% to rule out a diagnosis of B-ALL [2].

Treatment

Details on adult ALL protocols between 1979-1995 were published [2]. A schematic overview of induction and consolidation phases is reported in Table 1. Note that prophylactic cranial irradiation was given at end of early consolidation in 4 programmes, after a median of 3 months from remission achieved, or was not included in the protocol containing high-dose cytosine arabinoside (ara-C).

At presentation all cases underwent a diagnostic lumbar puncture an were put on oral allopurinol 300-600 mg/die and hyperydration/urine alkalinization to correct or prevent uric acid nephropathy and kidney failure. If serum creatinine or uric acid were > 1.6 g/dl or > 8 mg/dl, respectively, chemotherapy was deferred for 24-72 hours with the exception of corticosteroids 0.5-1 mg/kg/die. Management of anaemia, neutropenia, thrombocytopenia and related complications was as for ALL patients at time of study. Prevention of neutropenic infectious complications was with oral paromomycin-nystatin or ciprofloxacin-nystatin or ciprofloxacin-fluconazole depending on study period.

Definitions and Statistics

A complete remission (CR) was defined as the disappearance from the bone marrow and other involved tissues and the cerebrospinal fluid of lymphoblastic/L_3 cells with a normocellular or moderately hypocellular

Table 1. Treatment regimens

Program	Induction CNS phase	Consolidation	Autograft	Maintenance
HEAV'D	A,V,As,P	A,V,C/it MTX,RT	-	MP,MTX,C
OPAL-HDara-C	A,V,As,P	A,V,HDaraC/it MTX +araC	-	MP,MTX
HEAV'D-2	A,V,P,C	A,V,C,araC,T/ it MTX,RT	-	MP,MTX[a]
IVAP	I,V,As,P	I,V,As,C,araC,T/ it MTX+araC,RT	B,E,M[b]	MP,MTX[c]
07/93	I,V,As,P	I,V,E,araC,C,Dx/it araC+MTX,RT	C,E,M[d]	

Definitions: A, adriamycin; V, vincristine; As, L-asparaginase; P, prednisone; C, cyclophosphamide; RT, cranial radioprophylaxis; MP, 6-mercaptopurine; MTX, methotrexate; it, intrathecal; HD, high-dose; araC, cytosine arabinoside; T, teniposide; I, idarubicin; B, BCNU; E, etoposide; M, melphalan.
CNS: central nervous system.
Maintenance: 3 years unless otherwise indicated.
[a] 2 years.
[b] High-dose therapy with bone marrow autograft (marrow harvest at end of early consolidation, unpurged).
[c] 6 months (post-autograft) or 12 months (no autograft).
[d] High-dose therapy with blood cell autograft (leucapheresis after first consolidation +G-CSF, unmanipulated)

bone marrow showing normal trilineage haematopoiesis. In B-NHL patients, CR was confirmed by ultrasound scan, computed tomography, or nuclear magnetic resonance as indicated. A recurrence was defined as the detection of > 5% SIg+ L_3/blast cells in the bone marrow, L_3/SIg+ blasts in the spinal fluid, or biopsy-proven B-NHL in swollen lymphodes or other clinically suspect tissues. The comparison of treatment outcome among different patient groups was by means of the Fisher exact text and the log-rank test. Multivariate analysis was by means of logistic regression and the Cox proportional hazard model. Survival was taken from date of diagnosis to death or last follow-up. Disease-free survival (DFS) was calculated from date of CR to recurrence, death in CR, or time of last follow-up in CR. Survival and CR estimates were calculated and plotted by the method of Kaplan-Meier.

Results

Patients

As of December, 1995, 23 out of our 346 adult ALL patients had B/L3-ALL (7%). Immunophenotype was diagnostic in 18 out of 19 cases studied (one technical failure). Four SIg+ B-ALL were not L3. Five cases not immunophenotyped were included on the basis of L3 morphology. Altogether, 18 out of 23 patients (78%) were immunophenotypically SIg+ B-ALL. Median age of BALL patients was 43 years (range 15-65) and median blast count was 0.9 x 109/l (range 0-31). Two had overt CNS disease. Stated inclusion criteria were met by 11 out of 18 patients with histologically proven B-NHL. Median B-NHL patient age was 51 years (range 17-70). Six patients (54%) had bone marrow involved with < 30% blast cells, 7 had extensive gastrointestinal disease (64%) and 8 had involvement of other extranodal sites including CNS (73%). Median follow-up from diagnosis to longest follow-up was 5 years (range 14 months-13.1 years). No patient had HIV-related disease.

Treatment Outcome

The number of patients entered into each of 5 subsequent treatment regimens and their respective outcomes are detailed in Table 2.

A CR was achieved in 21 patients overall (62%), 13 patients died early of refractory disease (n = 6, 18%), pancytopenic complications (n = 5, 15%), and kidney failure (n =

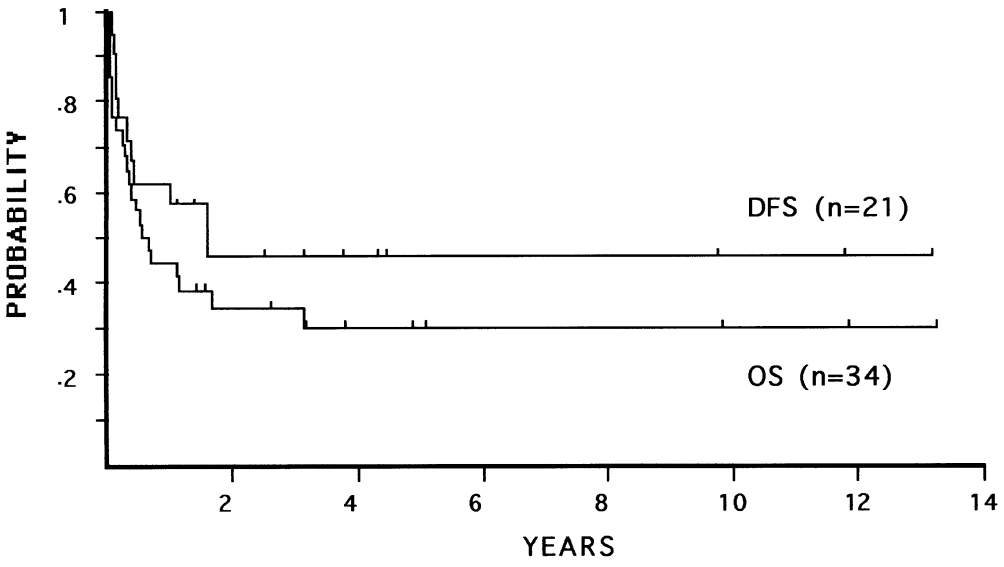

Fig. 1. Overall survival (OS) and DFS

Table 2. Response to treatment by protocol

Treatment	No. of patients	CR (%)	Recurrence (%)
HEAV'D	7 ALL, 1 NHL	6 (75)	3 (50)
OPAL-HDara-C	2 ALL	1 (50)	–
HEAV'D-2	1 ALL, 9 NHL	2 (20)	–
IVAP	6 ALL	5 (83)	4 (80)
07/93	7 ALL, 1 NHL	6 (87)	2 (33)

2, 6%). Median survival from diagnosis was 6.7 months, and projected overall survival between 4-13 years was 30%. Eleven patients are alive, 10 of whom in first CR (32%). For CR patients, median DFS was 1.6 years and projected DFS between 2-13 years was 49%. Overall survival and DFS curves are shown in Fig. 1.

Two patients, one of whom in program 07/93, died in CR of invasive fungal infection and pulmonary thromboembolism associated with septic complications and acute renal failure, respectively. Nine CR patients had a recurrence in the bone marrow (n = 5), bone marrow plus central nervous system (CNS, n = 1), and CNS (n = 3) after a median of 4 months (range 1.2-19 months), and within 6 months in 7 out of 9 cases. Median survival from relapse was only 22 days (range 4 days-1.4 years). One patient who underwent an allogeneic bone marrow transplant at relapse remains well after 12 months. All eligible patients in protocols IVAP (n = 1) and 07/93 (n = 5) underwent an autograft. All engrafted promptly and none relapsed.

Role of Prognostic Variables

Early response rates and long-term DFS and survival rates by prognostic factors and se-

Table 3. Presentation features and outcome (CR, complete remission; DFS, disease-free survival of patients; OS, overall survival)

		CR	DFS		OS	
Variable	No. of cases	no. (%)	Median (yr)	5-yr %	Median (yr)	5-yr %
Age (yr),						
< 50	20	12 (60)	NR	56	1.6	36
> 50	14	9 (64)	0.3	33 (4-yr)	0.3	21
L3 cells (x 10^9/l),						
< 1	23	13 (56)	NR	60[a]	1	37
> 1	11	8 (73)	0.2	25 (2-yr)	0.4	18 (2-yr)
LDH (U/l)						
< 500	6	3 (50)	1	33	1.5	25
> 500	20	13 (65)	NR	51	0.4	33
Creatinine (mg/dl),						
< 1.6	18	12 (66)	NR	56	1.5	36[b]
> 1.6	4	1 (25)	1.1	100 (1-yr)	0.1	25 (1-yr)
CNS (no.),						
Negative	30	20 (67)	1.6	43	0.6	30
Positive	4	1 (25)	3.7	100 (3-yr)	0.1	25 (3-yr)
Diagnosis (no.),						
B/L_3-ALL	23	18 (78)[c]	1.5	43	0.7	37
B-NHL	11	3 (27)	NR	67	0.5	18
Anthracycline (no.),						
Adriamycin	20	9 (45)	NR	55	0.6	25
Idarubicin	14	12 (86)[d]	1.5	37 (3-yr)	1.5	37 (3-yr)
Programme (no.),						
07/93	8	7 (87)	1.6	47 (3-yr)	1.5	42 (3-yr)
Others	26	14 (54)	1	43	0.5	26

NR, not reached.
[a] $p < 0.05$ by univariate and multivariate.
[b] $p < 0.025$ by univariate.
[c] $p = 0.005$ by univariate and 0.007 by multivariate.
[d] $p = 0.0018$ by univariate.

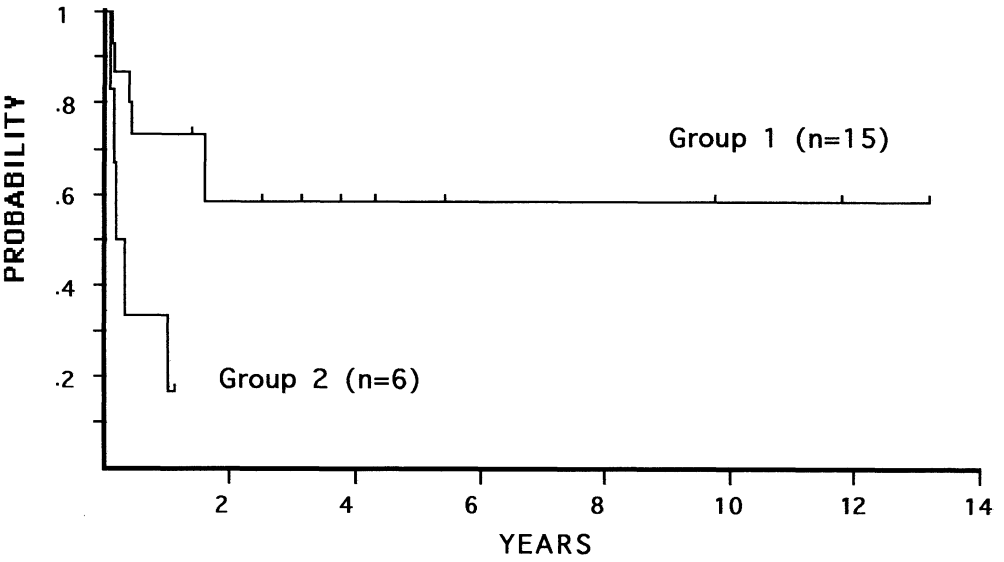

Fig. 2. DFS by age > 50 years and blast count > 1×10^9/l. Group 1: no or single feature; Group 2: both features. $P < 0.025$ by the log-rank test

lected treatment variables are presented in Table 3.

CR rates were lower in patients with elevated serum creatinine, CNS disease, and B-NHL. Three BNHL patients were refractory (27%). The incidence of refractory disease in B/L_3-ALL was 13%. All refractory patients received adriamycin-based regimens, while use of idarubicin was apparently correlated with lack of induction resistance. DFS was positively affected by age < 50 years, a low tumoral burden as expressed by a peripheral blast cell count < 1×10^9/l and, in view of reduced relapse rates (Table 2), by programme 07/93 where only 2 patients aged > 60 years developed a recurrence. By combining age and blast count (Fig. 2), long-term DFS probability of patients aged < 50 years and/or with < 1×10^9/l blasts was 0.59 (median not reached), but only 0.17 at 1 year (median 1.8 months) in those aged > 50 years and with higher blast count ($p <$ 0.025).

Discussion

What we obtained in B/L_3-ALL and advanced-stage B-NHL of adults using ALL-directed regimens was better than previously reported by others and must be discussed in the light of state-of-the-art results from GMALL trials [1]. Median patient age was higher in our series than in GMALL studies (44 vs. 34 years), as was the incidence of documented CNS involvement (17 vs. 12%). The number of cases with L_3 morphology without confirmatory immunophenotype was quite similar (22 vs. 19%). On the contrary the peripheral blood cell count was lower in our cases, but while we referred to absolute blast cell count and included B-NHL patients who necessarily did not have a peripheral blood contamination by tumoural cells, GMALL studies considered total white cell counts. Sometimes both mature and immature circulating myeloid cells are increased in B-ALL patients, so that evaluation of true blast cells instead of total white cells seems advisable for adequate inter-series comparison. Altogether, we had more patients with renal failure and an overall 7% incidence of B-ALL/L_3 among adult ALL. In GMALL study 01/81 the incidence of B-ALL was only 2% (3) and is not reported for the most recent trials [1]. We suspect either an augmented incidence of B/L3-ALL in our region or, rather, a very low exclusion rate at diag-

nosis, considered our patient age range and incidence of renal insufficiency.

Our results support separate conclusions for the remission induction and postremission consolidation phases. During induction, we found that refractory disease was associated with the adriamycin regimens (9/20 cases or 30%) but not with idarubicin regimens (none of 14 patients), an intriguing observation since other drugs and drug scheduling were basically the same. Theoretically, a greater therapeutic index of idarubicin over adriamycin in B-ALL could be explained by two synergistic pharmacologic effects. First, the idarubicin metabolite idarubicinol, that is cytotoxic even at low concentrations and exhibits a long plasmatic half-life in excess of 50 h [4], might inhibit, by virtue of this peculiarity, B-ALL cell proliferation during the 48-72 hours estimated for every B-ALL cell to transverse the cell cycle [5]. Second, idarubicin appears from in vitro studies to be less vulnerable to cellular drug efflux mediated by P170 membrane-associated glycoprotein [6], a multidrug resistance mechanism frequently expressed by B-ALL [7]. GMALL induction trials with daunorubicin confirmed a relatively high refractoriness rate (44% in study ALL 01/81 to 17% in study B-NHL 86) [1, 3]. The exact place of idarubicin in B-ALL requires further clinical and experimental in vitro evaluation.

In selected circumstances, represented by patient age < 50 years and/or nearly absent leukaemic blood contamination, our ALL-type protocols induced DFS rates close to GMALL B-NHL 83/86 results [1]. Our favourable group, with long-term DFS 59%, included 71% of all CR patients. In the GMALL experience, all patients with $< 50 \times 10^9$/l white blood cells (83% of evaluable cases) fared very well (DFS 71%). This probably means that outcome of several high-risk patients, as those with higher B-ALL cell count and older age, can be significantly improved by GMALL B-NHL regimens, whilst the advantage would probably not be as clear in low-risk patients.

The analysis of postremission chemotherapy indicated a possible benefit by protocol 07/93. This consisted of six short pulses alternating idarubicin-vincristine-cyclophosphamide with idarubicin-vincristine-etoposide-ara-C, along which cranial irradiation, medicated lumbar punctures, and an autologous unmanipulated peripheral blood cell graft. The efficacy of this programme and the safety of the peripheral blood autograft, in terms of blood cell recovery and disease contamination, would be preliminarily confirmed since none of 5 autografted patients relapsed after a minimum follow-up of 18 months. It was therefore regrettable that one of these few patients died in CR causing a drop of actuarial DFS estimate. Additionally, the fact that 07/93 was not as promising for other ALL subtypes (unpublished data) is another argument, in line with GMALL indications [1], for the adoption of specific treatment schedules in adult B-ALL.

Analysis of recurrence indicated a very short time to relapse with high incidence of neuromeningeal progression, greater than that observed in GMALL studies (19 vs. 13%). This difference can be partially explained by a delayed cranial radio-prophylaxis, starting 8-12 weeks from remission achieved. Retrospectively, this was not unexpected since it is now well recognized that spread to CNS can occur very early in B-ALL, contrary to other ALL types. In GMALL trials skull irradiation was delivered after the first two chemotherapy courses, that is a minimum of one month earlier than in our study [1]. Nevertheless, CNS progression rates remain the highest among ALL subsets, to underline the difficulty of obtaining an adequate CNS sterilization in patients whose bone marrow can be rendered disease-free. Our current programme is derived from 07/93 and considers, immediately after the induction course, early brain irradiation plus increased-dose intrathecal ara-C (75 mg) and methotrexate (15 mg). Systemic high-dose treatment including cyclophosphamide, ara-C and methotrexate in a tight sequence is given thereafter.

In view of recent GMALL data the meaning of our report is mainly historical but, at variance with prior studies, it contributes to define the prognostic heterogeneity of B-ALL and generates some speculations of potential interest. Such are the use of idarubicin during induction, the enforcement of

early chemo-radioprevention of CNS disease, and the administration of high-dose chemotherapy with autologous blood cell rescue.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Autologous Bone Marrow Transplantation in Adult Lymphoblastic Leukaemia – Single Centre Experience

J. Holowiecki, J. Wojnar, M. Krawczyk, P. Latacz and T. Kruzel

Abstract. Among 200 BMT procedures performed since 1991, ABMT was carried out in 34 high-risk ALL patients aged 15-50 y, median 27 y; 22 pts in CR1 and 12 pts in CR2 or > 2. The mean number of 1.74×10^8 nucleated cells/kg (1.61×10^6 $CD34^+$ cells/kg) was transplanted after myeloablative regimen consisting of Cyclophospamide, Ara-C and Etoposide (CAV). The mean regeneration time of granulocytes up to > 0.5 G/L and G > 1.0 G/L equaled 15 and 19 days, respectively. The recovery of platelets > 50 G/L occurred on day 14, and > 100G/L on day 17. Two pts died (5.8%) in the early post-transplant period. 6/22 pts in CR1 and 9/12 pts in CR ≥ 2 relapsed 2-26 months after ABMT. DFS survival was significantly longer in patients who had obtained a full dose remission induction treatment and high dose consolidation compared to those who received a time/dose reduced therapy ($p < 0.01$). DFS was also significantly longer in CR1 patients than in patients transplanted in CR2 or beyond ($p < 0.01$).

Introduction

A remarkable improvement in the treatment results of acute lymphoblastic leukaemia (ALL) has been observed during the last two decades with complete remission rate about 75% (64 to 85%) in adult patients. However, despite of intensive remission induction and consolidation therapy, most patients relapse and long-term remission are achieved only in approximately 33% (18 to 40%) of adult patients [1]. The further progress has been achieved by the use of bone marrow transplantation (BMT). The allogeneic bone marrow transplantation (alloBMT) may result in 40%-60% disease free survival (DFS) with an actuarial relapse rate of 10%-40% and 90% of the recurrences occurring within the first 2 years [2]. There is no doubt that allogeneic BMT is an effective antileukemic treatment, specially in acute and chronic myelogenous leukaemia. However, the risk of complications related to transplantation raises the question whether it should be performed in ALL patients in first or in subsequent remission [3, 4, 5, 6]

The lack of an optimal sibling donor in 80% of patients in need as well as the uncertainty concerning the indications for allogeneic transplantation in ALL patients in first remission, cause a growing interest in various forms of autologous transplantation. Their value in the treatment of ALL is still not finally established, but there are several proofs indicating that this procedure is effective.

The aim of the study was to determine direct and long-term results of autologous bone marrow transplantation (ABMT) in the ALL patients.

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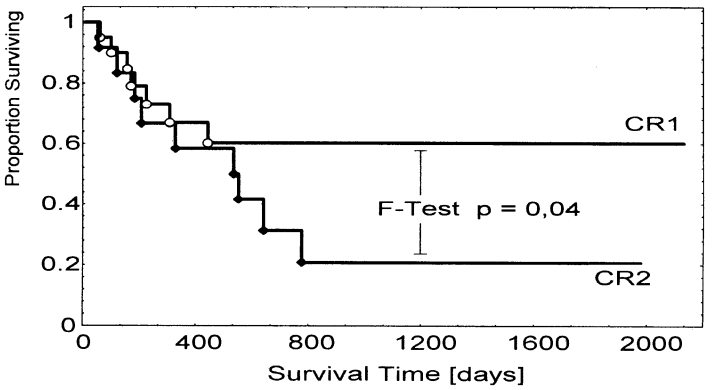

Fig. 1. Survival in CR1 (22 patients, median observation time 550 days) versus CR ≥ 2 (12 pts, med. observ. time 898 days)

Materials and Methods

Among 200 BMT procedures performed in our department since April 1991, ABMT was done in 34 ALL patients (24 males and 10 females aged 15-50 years, median 26 years); 22 high risk patients in CR1 and 12 patients in CR2 or > 2.

There was no significant difference in the age between CR1 and CR ≥ 2 group (28.6 + 18 versus 24.1 + 8.6 years, $p = 0.18$). The distribution of particular ALL subtypes in the CR1 and CR2 groups was following: pre-pre B; 7/22 and 4/12, "common"; 3/22 and 5/12, T-type; 7/22 and 2/12, undetermined; 5/22 and 1/12 respectively. Two patients in the CR1 group expressed bcr/abl rearrangement. The mean observation times after ABMT equaled 789 ± 644 days in the CR1 group and 896 ± 586 days in the CR ≥ 2 group.

The conditioning regimen consisted of Cyclophosphamide 60 mg per kg body weight on day -3 and -2, Ara-C 1000 mg/m^2 every 12 h on days -3, -2, -1 and Etoposide 700 mg/m^2 on day -3, -2, -1. Bone marrow was transplanted on day 0 through a central vein catether. In 4 patients growth factors were administered after ABMT: GM-CSF (Leucomax-Sandoz) in 3 patients and G-CSF (Neupogen-Roche) in 1 patient.

The number of CD34$^+$ cells and the minimal residual disease were determined using flow fluorometry (FACS-STAR plus, Becton-Dickinson). The method was controlled within the international standardisation system [8].

The primary end points were survival and disease free survival (DFS), calculated with Kaplan-Meier product limit estimate and compared by paired log-rank test and Cox's test. Multiple regression analysis was used to test the correlations between both the transplanted cell numbers and the duration of last complete remission and the DFS.

Results

Table 1 presents data concerning the transplanted cell numbers and the regeneration times after ABMT.

Two of 34 patients (5.9%) died in the early post-transplant period < 100 days because of sepsis. Both the survival (Fig. 1) and the DFS (Fig. 2) were significantly longer in patients autografted in CR1 than in CR ≥ 2. The 3-year probabilities of survival equaled 70% for autotransplantations done in CR1 and

Table 1. Transplanted cell numbers and the recovery time after ABMT in ALL patients.

		Mean	Range
	Transplanted cell number		
Nucleated cells	(× 10^8/kg)	1.74 + 1.12	0.48-4.7
CD34$^+$ cells	(× 10^6/kg)	1.61 + 1.23	0.44-3.9
	Regeneration		
Neutrophils > 0.5 g/l	(day)	15	12-26
Neutrophils > 1.0 g/l	(day)	19	13 - 29
Platelets > 50 g/l	(day)	14	9 - 22
Platelets > 100 g/l	(day)	17	12 - 27

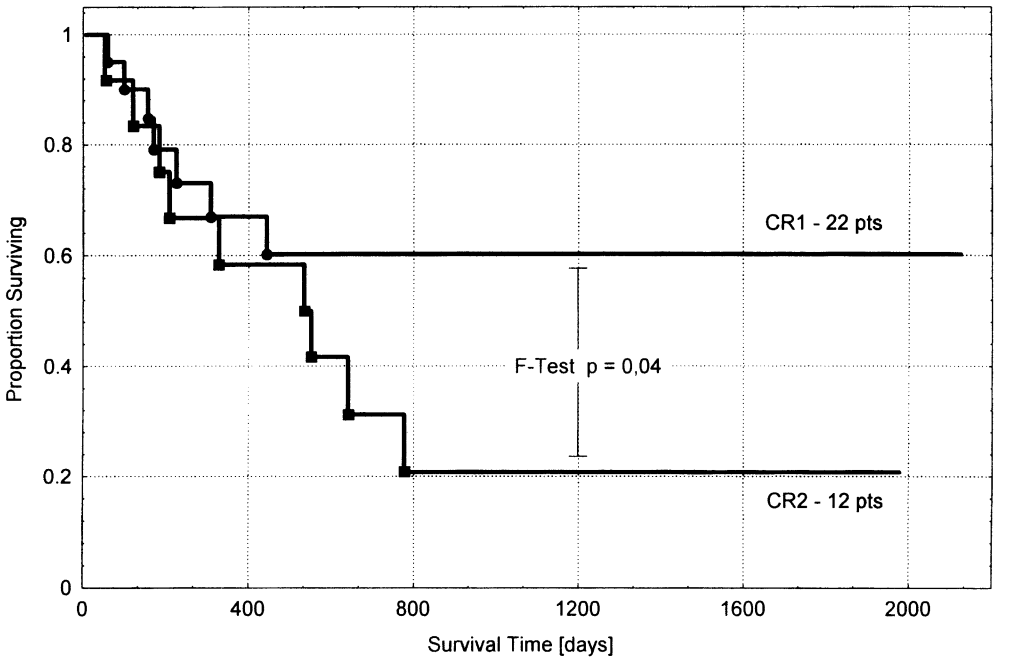

Fig. 2. DFS in CR1 (22 patients, median observation time 341 days) versus CR ≥ 2 (12 pts, med.observ. time 545)

23% for patients autografted in CR ≥ 2 ($p < 0.05$), while the coresponding DFS probabilities accounted for 60% and 21%, respectively ($p = 0.04$). In CR1 patients (Fig. 3) the DFS was longer (78%) in 14 patients who obtained a full dose induction and a high dose

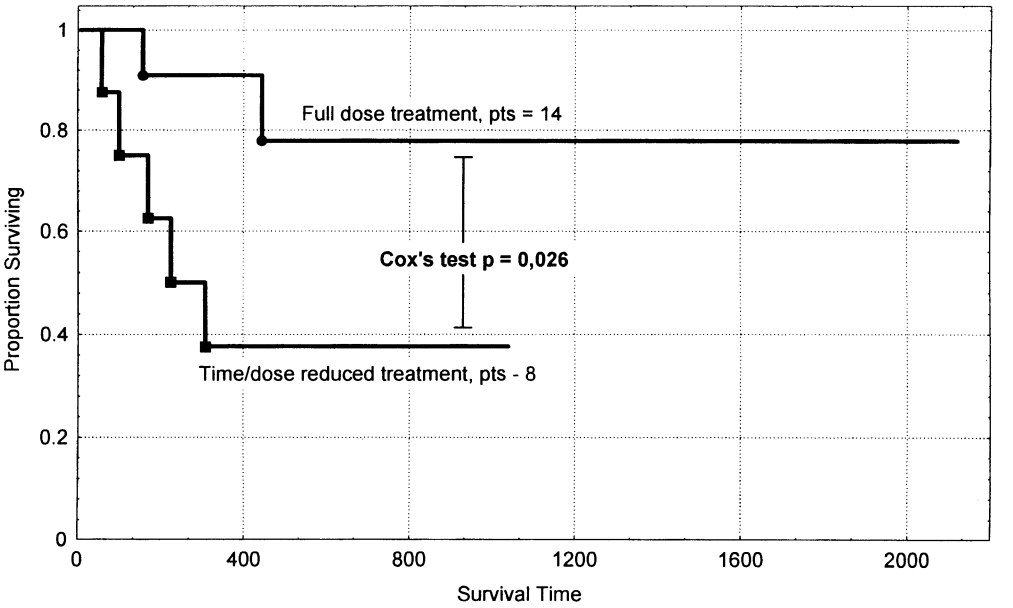

Fig. 3. DFS in CR1 patients who obtained before ABMT a full dose induction and consolidation (14 patients, med. observ. time 750 days) versus treated with postponed or time/dose reduced cycles (8 pts, med. observ. time 404 days)

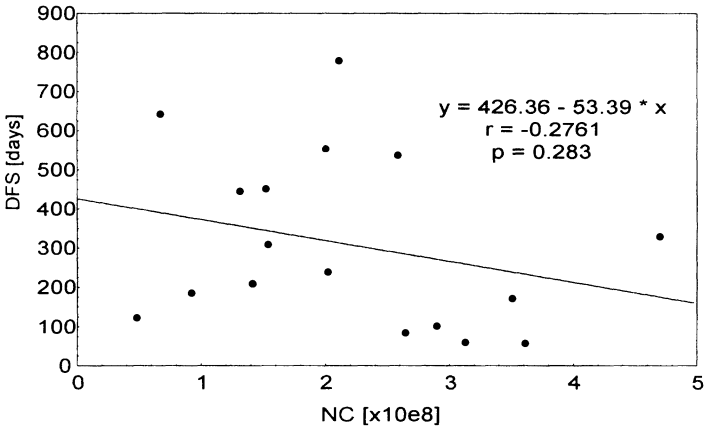

Fig. 4. Correlation between transplanted nucleated cell (NC) number and DFS

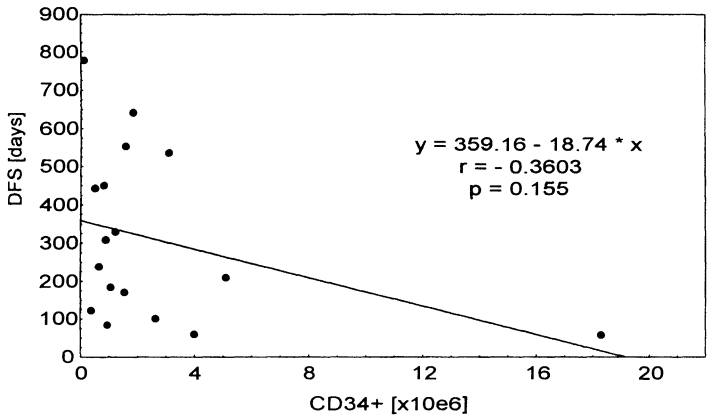

Fig. 5. Correlation between transplanted $CD34^+$ cell number and DFS

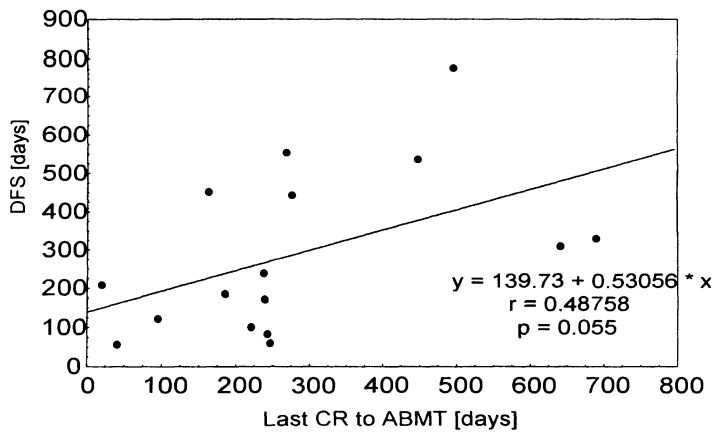

Fig. 6. Correlation between the duration of the last CR before ABMT and DFS

consolidation before ABMT if compared to 8 patients treated with delayed or dose/time reduced cycles (38%) $p = 0.026$.

A tendency toward a negative correlation was observed between the number of transplanted nucleated cells (Fig 4) as well as the number of $CD34^+$ cells (Fig 5), and the duration of DFS, but the results are not significant.

A positive correlation was found between the duration of the last complete remission

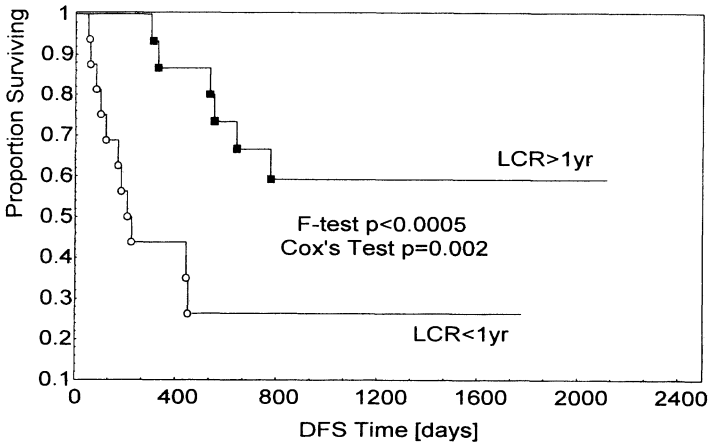

Fig.7. DFS time in patients transplanted in remission lasting shorter than one year versus patients autografted after one year of complete remission (LCR>1 yr)

before ABMT and the time to relapse after ABMT, $p < 0.05$ (Fig. 6). In the whole group of patients (CR1 + CR $\geq$ 2) a better outcome in terms of DFS was found in those autografted after a complete remission lasting one year or longer if compared to patients autografted earlier in complete remission, $p < 0.002$ (Fig. 7).

Discussion

Combination chemotherapy of adult ALL including vincristine, prednisone, anthracyclines, L-asparaginase, cytosine arabinoside and cyclophosphamide results in a relatively high complete remission rate ranging 60-80% but the leukaemia free survival remains low at 13-44% with majority of patients dying from relapsed disease [9, 10].

Intensive consolidation treatment appears to play an essential part in prolonged leukaemia free survival, but the optimal strategy is as yet not established. The role of BMT in first remission, either for all patients or for defined high-risk groups is still belong investigeted. Some particular subgroups of adult ALL such as those with the t(9:22) or a t(4:11) are virtually incurable by chemotherapy alone, and there are reports demonstrating the potential of allogeneic BMT in producing a cure in these subtypes.

Allogeneic BMT from HLA identical donor allows to achieve long-term DFS in 45-60% of adult patients with ALL in CR1 [9] and 22-36% in CR $\geq$ 2 [11]. This is confirmed by IBMTR data from 1996; 3 year probabilities of LFS: 54 ± 4% for CR1 patients, 40 ± 13% for CR $\geq$ 2, and 20 ± 5% for patients in relapse. The results obtained in first CR by ABMT are surprisingly similar, whereas those in CR $\geq$ 2 are inferior. According to the ABMTR data (1996) the 3 year LFS after ABMT in CR1, CR > 2 and in relapse equalled 43 ± 12%, 25 ± 6% and 17 ± 7%, respectively. The wider application of allogeneic BMT is restricted by the biologically low probability of possessing a compatible donors. The results observed after unrelated donor BMT are promissing in comparison to ABMT only for patients in CR2 or > 2; LFS in CR1 - 37 ± 14% in CR $\geq$ 2 - 36 + 6% (IBMTR 1996). There are some reports aiming to compare the results of allogeneic BMT to those of chemotherapy given alone, as well as to compare the results of allogeneic to autologous BMT [6, 9]. No significant superiority of allogeneic over the autologous BMT has been shown in adult patients with high risk ALL [12].

The success of ABMT appears to depend on the following factors: (1) a good quality of remission at the time of harvesting cells for transplantation, confirmed by the absence of minimal residual disease [13]; (2) an effective conditioning therapy, potent to eliminate cloister sites of neoplasmatic growth; (3) a proper function of the bone marrow stroma; and (4) a satisfactory general condition of the patient.

Failures following the ABMT are caused mainly by the leukaemia relapse. In our ma-

terial 2 patients from the CR1 group died during the posttransplantation period and only one patient died due to the relapse after ABMT. In CR $\geq$ 2 group 9 patients relapsed after ABMT and 8 of them died.

Our results indicate that a better outcome was achieved if ABMT was performed in first remission, confirming the data reported by other authors [13]. The results of ABMT in ALL patients in CR $\geq$ 2 are better when compared to chemotherapy given alone [3]. According to Herve at al. [10] the 3 year LFS in ALL patients in CR2 or beyond treated with chemotherapy, ABMT and allo-BMT equaled; < 5, 20 and 25-30%, respectively.

Our single institution results confirm the utility of ABMT in high risk ALL in CR1 [15]. The results in CR2 and beyond were significantly inferior and correspond to the values reported by ABMTR and some other authors [14]. It appears however that further improvements of pretransplant, transplant and posttransplant procedures are necessary. Our observations indicate that a consequent and radical pretransplant chemotherapy and a rather longer duration of CR before ABMT correspond with better results after ABMT. This is probably due to a more effective elimination of the residual leukaemia as a result of the so called biological purging. In our experience also a lower number of transplanted cells seems to correlate with a longer DFS, suggesting the potential negative role of proportionally higher number of residual leukaemic cells in cell rich transplant. It has to be determined whether ex vivo purging would be effective in such cases.

ALL consists of few distinct subgroups and a properly adjusted regimens should be evaluated in prospective trials. Our observations suggest that ABMT is an effective post remission treatment in high risk adult ALL patients in first CR. A full dose induction and high dose consolidation appears to be important for achieving a high DFS rate. For CR $\geq$ 2 patients lacking a matched donor, ABMT offers a salvage therapy that needs further improvement.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Cranial Computed Tomographic Scans at Diagnosis in Children with Leukemia

T. JACKOWSKA, E. WAGIEL, and R. ROKICKA-MILEWSKA

Abstract. The cranial computed tomographic scan (CT) was performed in 68 leukemia children aged from 3 month to 15.6 years. The CT scan had been taken before chemotherapy started. Out of 21 (31%) patients with changes on the CT scan: 16 had a cerebral atrophy, 6 ventricular dilatation, 2 hypodense areas of white matter and septum pellucidum cysts, in one case asymmetry of ventricular width and quadrilocular hydrocephalus was found. In some of the children more than one abnormality was noted. We did not observe any abnormalities on the examination of cerebrospinal fluid in the children with abnormal CT scan. It is impossible to determine if the abnormal CT scan was related to the basic disease. Even normal CT taken in the early phase of disease did not exclude brain involvement. Routine CT scan in early state of leukemia can be useful for comparison with CT scan following therapy in order to assess the late side effects of therapy or possible central nervous system (CNS) involvement.

Introduction

The significant improvement in the treatment results of children with leukemia has been observed in the last few years due to intensive therapy in some cases, combined with cranial irradiation [1-3].

The central nervous system (CNS) prophylaxis, performed to eliminate the residual disease in this area, constitutes one of the treatment methods of childhood leukemia. However, the long-term results of new methods of effective CNS prophylaxis have to be taken into consideration. The majority of retrospective studies evaluates computed tomography (CT) findings in CNS after CNS prophylaxis [4-9]. The relationships between the results of intensive chemotherapy, CNS irradiation, CNS metastases and the CT findings on brain images are not exactly defined [7, 8]. The aim of this study was to evaluate the CNS in children with leukemia using CT scan before chemotherapy and correlate these findings with CNS disease.

Materials and Methods

The study included 68 patients (37 boys and 31 girls, mean age 6.2 years, range from 3 months to 15 years and 6 months). All children were treated between 1989 and 1996 in the Department of Pediatrics, Hematology and Oncology in Warsaw. Fifty-five of them were diagnosed with ALL, 12 and 1 with AML and CML respectively (Table 1).

During the first few days of chemotherapy with steroids, diagnostic lumbar puncture and CT of the cranium were performed in all children to define CNS disease. Spinal fluid

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Table 1. Characteristics of patients

No. of patients	68
Boys	37 (54%)
Girls	31 (46%)
Age at diagnosis	3 months-15.6 years
Mean	6.2
Diagnosis:	
ALL	55 (81%)
AML	12 (17.6%)
CML	1 (1.4%)

was analyzed for glucose, protein content and WBC count. The CNS disease was diagnosed on the basis of the WBC count above 5/mm^3 and presence of leukemic cells in the spinal fluid. CT scan was performed using Picker 1200 Expert or Somaton apparatus.

The section thickness was from 5 to 10 mm. In the majority of patients CT scan was performed with and without contrast enhancement. Ventricular dilatation, cerebral atrophy, thickening of menninges and hypo- or hyperdensity areas were assessed on the CT scan.

Results

Forty-seven of 68 children had the normal CT scan. In the 21 remaining children a variety of abnormalities was detected (Table 2).

Cerebral atrophy (Fig. 1) and ventricular dilatation were observed in 16 and 6 children, respectively. In 2 children with septum pellucidum cyst and hypodensity areas in the white matter (Fig. 2) contrast injection did not cause disappearance of these findings. Hydrocephalus and asymmetry of ventricles (Fig. 3) were detected in one child with cerebral palsy. In 6 children more than one CT scan abnormality was detected.

None of children with CT findings had spinal fluid abnormalities. WBC count from 11 to 643 cells/mm^3 was found in children with CNS disease. The CT scan of the brain of these children was normal (Table 3).

Fifty-three of total 68 children are still alive. Treatment was completed in 24 of them. Twenty of 21 children with CT abnormalities are alive and in none of them CNS

Table 2. Changes in cranial CT scan findings

CT changes in 21 children	No. of patients[a]	%[a]
Cerebral atrophy	16	76.2
Ventricular dilatation	6	28.6
Septum pellucidum cyst	2	9.5
Hypodense area of white matter	2	9.5
Asymmetry of ventriculi width	4	7
Quadrilocular hydrocephalus	1	4.7

[a] In 6 children more than one changed was observed.

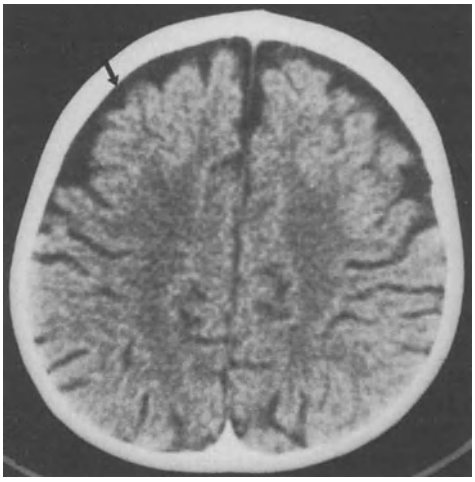

Fig. 1. Cerebral atrophy

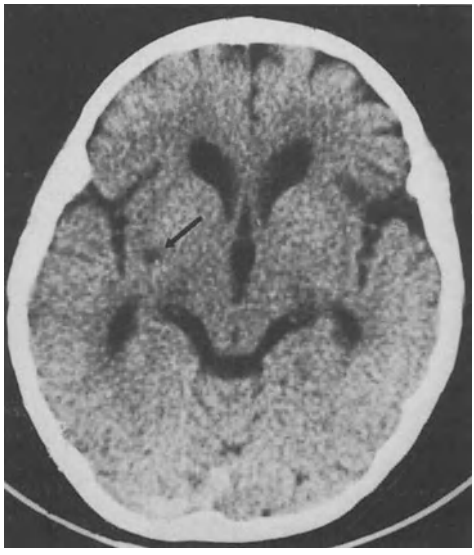

Fig. 2. Hypodense area of white matter

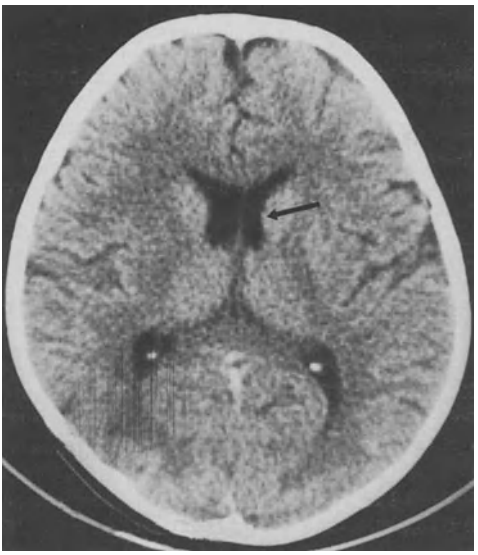

Fig. 3. Asymmetry of ventriculi width

Table 3. Changes in cranial CT scan findings and cerebrospinal fluid

No. of patients	Normal CT	Changes in cranial CT	Total
Normal cerebrospinal fluid	43	21	64
Abnormal cerebrospinal fluid	4	0	4
Total	47	21	68

Table 4. Clinical follow-up

	No. of patients	%
Total group	68	100
Remain alive in remission	53	78
After treatment cessation	24	45.3
Death	15	22
Abnormal CT brain scan	21	31
Remain alive in remission	20	95
After treatment cessation	13	65
Death	1	4.8
Abnormal cerebrospinal fluid	4	6
Remain alive in remission	2	50
After treatment cessation	0	0
Death	2	50

disease was diagnosed. Treatment was completed in 13 of these patients. Two of 4 children with spinal fluid abnormalities are alive and are on a maintenance therapy. Two remaining children died in the course of the disease (Table 4).

Discussion

CNS leukemia produces minimal signs and symptoms in the majority of cases. Neurologic examination, spinal puncture and CT scan or magnetic resonance imaging (MRI) are done routinely at the moment of diagnosis of leukemia [10].

CNS abnormalities on CT scan are detected in 18% to 62% of children with leukemia at this time [5, 11-15]. In our studies CT abnormalities of the brain were detected in 31% of patients and most of them presented as cerebral atrophy. Numerous studies have been published on the relationship between CT abnormalities and CNS disease and poor outcome [11]. Not all the studies confirm these findings [13, 17]. In our patients a correlation between CT and spinal fluid abnormalities was not found. During the course of the disease none of children with initial CT abnormalities developed CNS relapse. In the course of the disease CNS relapse occurred in two children with normal CT scan at the time of diagnosis.

CT is not a reliable method for CNS disease diagnosis in leukemia. Performing CT before starting chemotherapy may be useful for late event evaluation in the course of leukemia treatment (CNS irradiation, chemotherapy). It allows avoiding diagnosis of poststeroidal cerebral atrophy in children with primary ventricular dilatation [16, 17].

Irradiation and chemotherapy affect mainly the white matter of the brain. This is why MRI would be a more accurate and sensitive modality for CNS disease detection compared to CT imaging [18-20].

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Treatment of Childhood AML

Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Autologous Bone Marrow Transplantation in First Complete Remission as Intensification Therapy in Children with High Risk AML – Results of the Pediatric Cooperative AML Trial 1987-1992 in East Germany

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Abstract

Background. The reported results of autologous bone marrow transplantation (ABMT) in childhood AML differ widely. The event free survival (EFS) in different pediatric studies ranges from 21 to 87%. In two CCG trials (USA) the EFS worsened from 51% in 1993 to 36% in 1996. In a short series of Bonetti (1995), all 10 patients are alive relapse-free after ABMT. We here report on the results of the East German cooperative AML trial 1987-1992.

Patients and Methods. In a nonrandomized fashion 41 patients with high risk AML in first CR were treated with maintenance chemotherapy (12 pts.), alloBMT (8 pts.) or ABMT (21 pts.) respectively, after BFM based induction/consolidation therapy, an 8-week course of IDMTX (1 g MTX/ 36 h × 4) and a 5-day intensification. Bone marrow for ABMT was harvested at various times of remission (3 to 62 wks.). The conditioning regimen consisted of busulfan (16 mg/kg) and cyclophosphamide (50 mg/kg × 4). After hematologic recovery autografted patients received a maintenance therapy (thioguanine, MTX) for 1 year.

Results. The pEFS after 5 years is 45% in the chemotherapy group, 63% in the alloBMT groups and 71% in the ABMT group, respectively. There was no transplantation related mortality in the ABMT group. In 11 patients bone marrow was harvested after 3 to 15 wks. of remission. In this group 5 pts. relapsed. In 10 pts. bone marrow was harvested after 30 wks. of remission with only one relapse.

Conclusions

1. The results of ABMT in patients with high risk AML in first CR in our group are encouraging.
2. Bone marrow should not be harvested in early remission (in vivo purging effect).
3. Because of the different pretreatment the results of ABMT in first CR of various investigators are not comparable.
4. If the event free interval in the high risk group of the current BFM AML 93 protocol does not exceed 50%, our BFM AML 83 based results should be confirmed in a prospective randomized manner in the next BFM AML protocol.

Introduction

Acute myeloid leukemia (AML) in children requires intensive, almost myeloablative induction therapy for achieving a remission. Improvement in both remission rate and long term survival has been slow and not as impressive as in acute lymphoblastic leukemia. More aggressive induction chemotherapy has led to remission rates of 70 to 90% of

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patients (1-5). However, many children in complete remission (CR) experience relapse of disease so that the survival rate is approximately 40 to 50% at 5 years.

Several strategies have been explored to avoid the leukemic relapse, including intensive postremission therapy/maintenance therapy and allogeneic or autologous bone marrow transplantation. Among these strategies, allogeneic bone marrow transplantation (alloBMT) is associated with a considerable procedure related morbidity and mortality, but has a maximal antileukemic effect. Therefore alloBMT in first CR is regarded as the treatment of choice for young patients with AML who have an HLA-compatible sibling donor. However, only one third of patients with AML who achieve CR will have a suitable matched donor. So autologous BMT (ABMT) is frequently proposed as "megadose" therapy with stem cell rescue for children who lack a histocompatible donor.

The reported results of autologous bone marrow transplantation in childhood AML are extremely different. The event free survival (EFS) in different pediatric studies ranges from 21% [6] to 61% [7], 63% [8], 75% [9] and 87% [10]. In two CCG trials (USA) the EFS worsened from 51% in 1993 to 36% in 1996 [11, 3]. In a shot series of Bonetti [12] all 10 patients are alive and free from relapse after ABMT.

In 1987 the East German Pediatric Cooperative AML group started the second AML protocol (AML II/87). In this protocol patients with high risk for relapse (see Table 1) with a suitable donor received an alloBMT in first CR. Patients without donor were offered autologous BMT or further chemotherapy in a nonrandomized fashion.

We here briefly report the results of the chemotherapy and BMT groups with special regard for the results of autologous BMT.

Patients and Methods

Diagnosis

The diagnosis of de novo AML was based on the criteria of the French-American-British (FAB) cooperative group [13-15]. According to slightly modified BFM 83 criteria [16] patients were assigned to standard or high risk group for relapse (see Table 1).

Table 1. Acute myeloid leukemia. Risk factors: AML II/87

FAB	
M1	Auer neg. + leucocytes $> 20 \times 10^9$/l
M2	Auer neg. + leucocytes $> 20 \times 10^9$/l
M3	?
M4	Eo < 3%. + leucocytes $> 20 \times 10^9$/l
M5	All patients
M6	All patients
M7	All patients
M1, 2 + 4	> 80 days till first remission

Chemotherapy

All patients received an intensive BFM 83 based induction and consolidation therapy as previously described [1]. Briefly, patients with more than 20×10^9/l leucocytes received a pretreatment with low doses of thioguanine and ara-C for up to 7 days.

Induction therapy consisted of a seven day course of prednisolone, thioguanine, ara-C, adriamycin and VP16. The 6-week consolidation protocol started between day 21 and 28 and consisted of prednisolone, vincristin, adriamycine, thioguanine and ara-C. After 2 weeks rest the patients received thioguanine and four times intermediate dose MTX (1 g MTX/m^2 over 36 h with leucovorin rescue) over 8 weeks. After another 2 weeks rest the patients received a 5-day reinduction with adriamycin, ara-C and thioguanine. Patients in the chemotherapy group received a maintenance therapy with thioguanine daily and 4 days ara-C monthly and CNS irradiation (18 Gy). Patients with alloBMT received no further chemotherapy. After ABMT a 12-months maintenance therapy with thioguanine and weekly MTX for 1 year was started after complete hematological recovery.

Response Criteria

CR was defined as disappearance of clinical signs of leukemia, marrow showing maturation of all cell lines and fewer than 5% blast cells. Relapse was defined as more than 5%

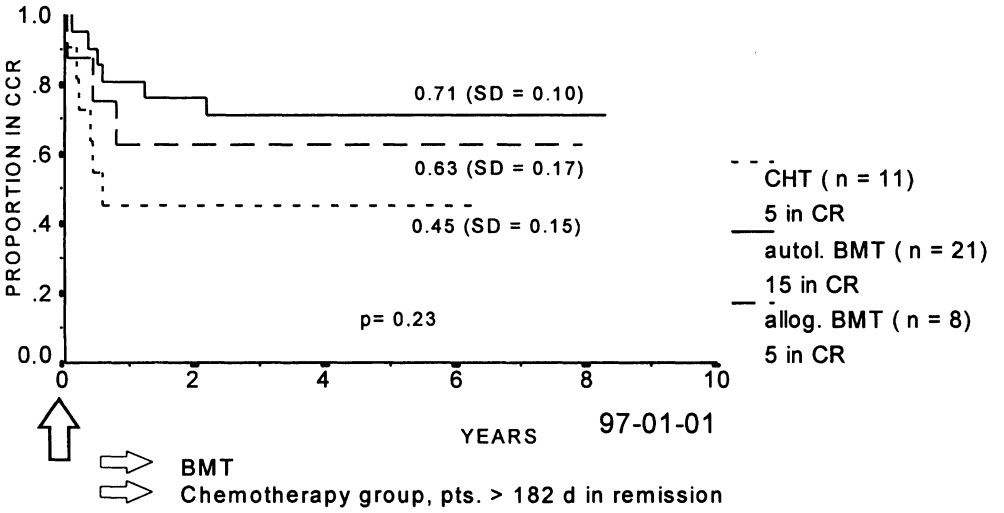

Fig. 1. Pediatric AML-Study II/87 – East Germany. EFI. BMT vs. Chemotherapy (HR; 182 d in Remission)

blasts in the bone marrow or the presence of extramedullary disease.

Patients

From January 1987 to December 1992 out of 41 patients with high risk criteria 12 patients were treated with chemotherapy and CNS irradiation (18 Gy), 8 patients with HLA matched siblings received an alloBMT. In the group of 21 ABMT patients were included 2 patients with AML as a second malignancy, 1 patient with incomplete AML therapy and 2 patients with a longer or insufficient pretreatment.

Bone Marrow Harvest

Bone marrow was collected at various times in remission. The earliest time was two weeks after the end of the first consolidation therapy.

Bone marrow was obtained by aspiration from the dorsal iliac crests under general anesthesia. For both allogeneic and autologous BMT we collected 3 to 5 x 10^8 mononuclear cells / kg body weight of the recipient. For allogeneic BMT the marrow was infused immediatly after harvesting. Bone marrow processing and cryopreservation for autologous BMT was described elsewhere [17,18]. All patients were autografted with unpurged marrow.

Supportive Care

All transplanted patients were nursed in laminar air flow rooms and received total decontamination. Trimethoprim/ sulfameracin therapy to prevent Pneumocystis carinii pneumonitis was given from day - 14 to day + 180.

Conditioning Regimen and GVHD Prophylaxis

Cytoreduction for BMT in first CR was similar for patients transplanted allogeneic or autologous and consisted of busulfan 4 mg / kg × 4 days and cyclophosphamide 50 mg / kg × 4 days. Prophylaxis for graft versus host disease in patients with allogeneic BMT consisted of MTX and prednisolone, since 1989 of cyclosporine A, MTX and prednisolone.

Statistical Analysis

Event free interval (EFI) in the chemotherapy group and event-free survival (EFS) after BMT were calculated by the product limit estimates of Kaplan and Meier [19].

Table 2. Results of allogeneic and autologous BMT

		Allogeneic BMT	Autologous BMT
No. of patients		8	21
BMT related deaths		2	0
Relapses after BMT		1	6
Time to relapse (months)	Range	10	2-26
Time to relapse	Median	10	7
In CR after BMT		5 (= 63%)	13 (= 62%)
In CR after BMT (months)	Range	42-95	45-101
In CR after BMT	Median	83	73
pCCR		0.63	0.71

Results

The overall results of chemotherapy (high risk group), allogeneic and autologous BMT are illustrated in Fig. 1. Table 2 summarizes the results of allogeneic and autologous BMT.

Chemotherapy group

In the chemotherapy group, 6 of 11 high risk patients relapsed and died. 5 patients are in CCR 33 to 81 months after remission (median: 59 months). The event free interval (EFI) in this group is 0.45 (SD = 0.15).

Table 3. Characteristics of transplanted bone marrow

		MNC $\times 10^8$/kg body weight	CFU-GM $\times 10^4$/kg body weight
Allogeneic BMT	Median	4.47	57.50
	Range	0.90-7.62	1.50-186.0
Autologous BMT	Median	2.08	1.46
	Range	0.18-4.86	0.10-18.38

Allogeneic BMT

Eight patients received an allogeneic BMT in first CR. The numbers of transplanted mononuclear cells and CFU-GM, respectively, are shown in Table 3. Of these 8 patients 2 died of transplantation related complications. One patient relapsed 10 months after transplantation, 5 patients are living in complete remission 42 to 95 months after BMT (median: 83 months). The EFS in this group is 0.63 (SD = 0.17). The Kaplan-Meier plot of this group is illustrated in Fig. 1.

Autologous BMT

Twenty one children received an autologous BMT in first CR. The numbers of reinfused mononuclear cell and CFU-GM, respectively, are shown in Table 3.

Time of Bone Marrow Collection and Relapse Rate. In 11 children bone marrow was harvested between 3 and 15 weeks in remis-

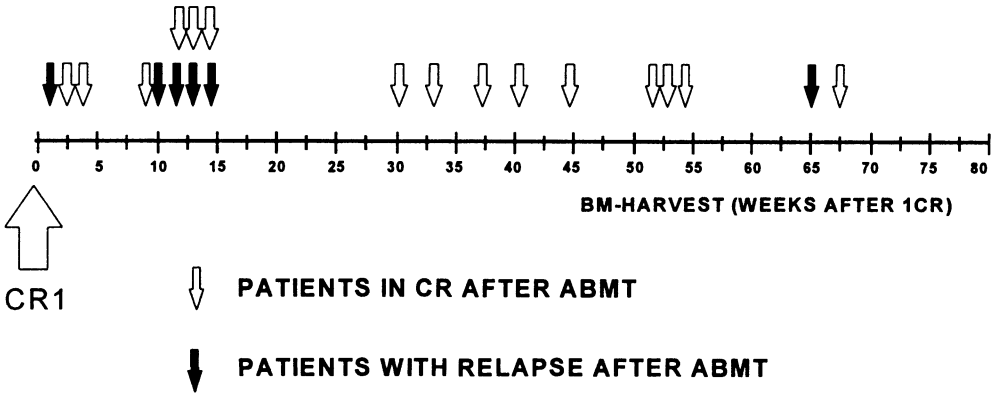

Fig. 2. AML-Study II/87: Correlation of duration of CCR; bone marrow harvesting and probability of relapse after ABMT

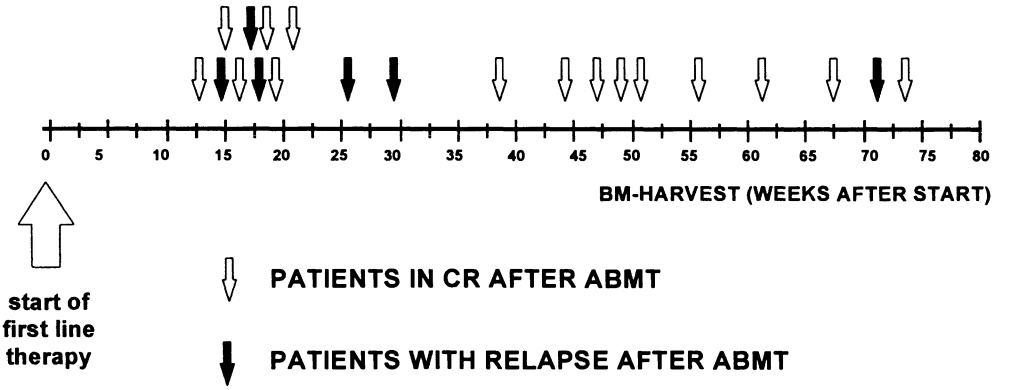

Fig. 3. AML-Study II/87: Correlation of duration of first line therapy, bone marrow harvesting and probability of relapse after ABMT

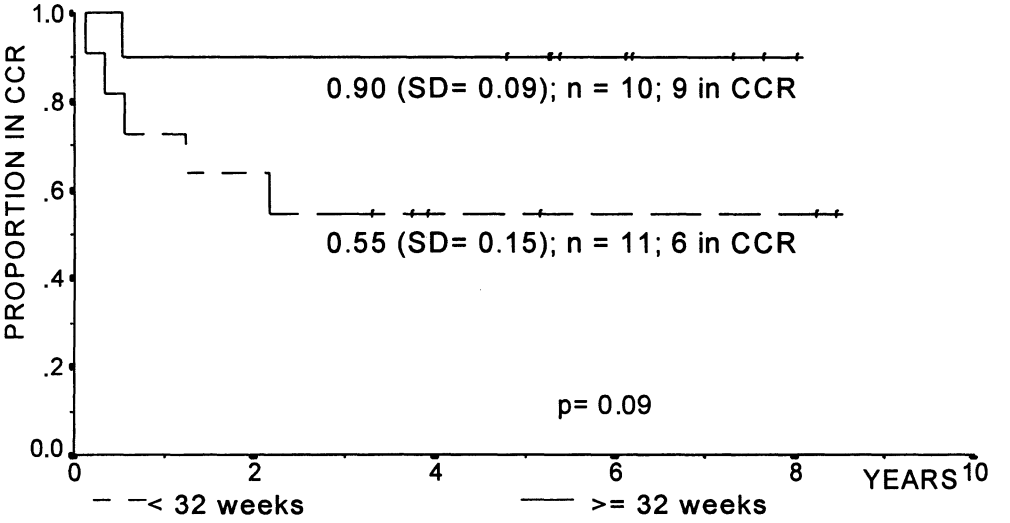

Fig. 4. AML II/87: ABMT in 1st CR. EFS; BM-Harvest < 32 weeks vs. ≥ 32 weeks after beginning of therapy

sion (early harvest). In this group 5 patients relapsed and 6 are in CCR. In 10 patients bone marrow was harvested between 30 and 70 weeks in remission (late harvest). Only one patient in this group relapsed (Fig. 2).

Because the date of remission is often not exactly to define, we analysed in a second step the correlation between the time from diagnosis to bone marrow collection and relapse rate (Figs. 3 and 4). In 11 children the bone marrow was harvested between 12 and 30 weeks from diagnosis (early harvest). Five of these patients relapsed. In the group of 10 patients in whom bone marrow was harvested more than 38 weeks after diagnosis there was only one relapse.

Late Effects of Autologous BMT. None of the autografted patients experienced late cardiac or pulmonary complications. Two patients suffer from diffuse alopecia as a consequence of busulfan therapy. Late endocrinological sequelae are under investigation.

Discussion

In contrast to childhood ALL the results of chemotherapy in children with AML only

slightly improved in the past 20 years. It is generally agreed that early intensive chemotherapy is necessary to achieve high complete remission rates in AML patients. The relative roles of intensive continuation therapy, allogeneic and autologous BMT in children with AML in first CR remain controversial.

In this report we give a short update of our results with chemotherapy, allogeneic and autologous unpurged bone marrow transplantation in children with high risk AML in first CR and focus on the results of autologous BMT.

According to the results of the AML-BFM 83 protocol Creutzig et al. [16] could identify two risk groups. The risk group I had an EFS of more than 80% and the risk group II an EFS of less than 45%. The East German AML protocol II/87 is based on this protocol. Therefore we adopted this risk definition with only slight modifications (Table 1). BMT was only offered to patients with high risk for relapse. In other series [8, 10, 11] all patients with AML were included in the respective transplantation programs.

The EFS from the time of remission in the chemotherapy group is 0.42 (SD = 0.14). To make this group comparable to the transplantation groups we only evaluated patients who were 6 months in CR. The EFS of these patients is 0.45 (SD = 0.15).

Of the 8 patients with allogeneic BMT 5 are in CCR with an EFS of 0.63 (SD = 0.17). Current results of other groups show that 50-70% of young patients with AML who undergo allogeneic BMT in first CR experience prolonged EFS and may be cured [21-23].

In our study, ABMT results were remarkably good. The EFS for 21 children is 0.71 (SD = 0.10). It is noteworthy that none of our autografted patients died of ABMT complications. In contrast to most other groups our autologous transplanted children received maintenance therapy with thioguanine and MTX for 1 year as an equivalent for a theoretical graft versus leukemia effect in allografted patients.

Six patients relapsed after ABMT, at a median time of 7 months (range 2 to 26). We analyzed the correlation between the time from diagnosis to bone marrow harvest and the relapse rate. In five of 6 children who relapsed the bone marrow was collected in the first 6 months of therapy. After this time only one out of 10 children relapsed. This observation was previously made by other investigators too [8, 24] and has been defined as in vivo marrow purging. It is uncertain as to whether any ex vivo bone marrow purging may be avoided with this approach. In most series ex vivo purging was associated with impaired hematopoietic reconstitution [8, 10]. It also is unknown if the good results with ABMT could be reproduced with autologous peripheral blood stem cells.

Conclusions

The reasons for the extremely different results of ABMT are different pretreatment characteristics. Therefore the results are not comparable. In contrast to other investigators our encouraging results of ABMT are based on a patients' population with a bad prognosis.

Our results are based on a AML-BFM 83 derived protocol in a nonrandomized study. They should be confirmed in a prospective randomized manner in the next BFM AML protocol.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Risk Factors for Survival in Children with Refractory AML Treated According to AML Relapse Strategies

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Abstract. About one third of the children treated within the national studies for AML in Germany (BFM studies) experience recurrence of their disease. In order to estimate the value of intensive salvage therapy for children with AML, we analysed the relapse regimens of the BFM study group for outcome and risk factors.

Patients and Treatment. 102 patients (pts.) with first relapse of AML were treated according to BFM relapse strategies: study REZ91 with double induction (mitoxantrone [MITOX]/VP-16 twice, 15 pts.), study REZ93 with induction MITOX/VP-16 plus HD-Ara-C/MITOX (HAM, 28 pts.) followed by a 6-week consolidation and either allogeneic or autologous bone marrow transplantation (BMT). Another 59 patients received either elements of these protocols or other intensive salvage therapy.

Results. Time to relapse was in median 1.1 years, range 0-15 years. Fifty-two of 102 patients (51%) achieved 2nd remission (CR), 10 (10%) partial remission, 37 (36%) were nonresponders, and 3 (3%) died early during salvage therapy. Twenty-seven were alive, in median 2.5 years, range 0.4-7.0 years after relapse, overall survival was 21%, SE 5% after 5 years. The response and survival rate was similar in all treatment groups. MITOX/VP-16 induction was well tolerated: 6 of 26 patients in study REZ93 suffered severe infections after 1st induction, compared to 9 of 12 patients after HAM. Fifty patients were transplanted, 43 in 2nd CR, and 7 with residual blasts; 27 patients received an allograft: Twenty-one from a matched sibling (MSD), 1 from a haploid and 5 from a matched unrelated donor (MUD); 23 received an autograft. Results were poor for patients in partial remission, however, promising for autologous BMT in 2nd CR with 10 of 22 patients surviving. Seven of 16 were alive after MSD in 2nd CR and 1 after haploid BMT, 4 of 5 patients died after MUD BMT. Multivariate risk factor analysis for survival after relapse revealed duration until relapse to be the most important factor. The maximum risk-ratio was obtained with a threshhold value of 1.5 years after diagnosis resulting in a 5-year survival of 10%, SE 4% for early relapse, and 40%, SE 10% for late relapse, p logrank 0.0001.

Conclusion. Second CR can be achieved in half of the patients, however, only children with late relapse (>1.5 years after diagnosis) have a realistic chance to be cured.

Introduction

About 20% of children with de novo AML do not achieve remission and nearly 30% relapse. Relapse in AML is in itself the most

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important factor indicating an unfavorable outcome. However, with intensive salvage therapy these patients can achieve remission again, and at least some of them have a second chance of cure [1].

The aims of our study were to analyse prognosis after intensive salvage therapy and the influence of the factors leukocyte- and thrombocyte count, hemoglobin, peripheral blasts, FAB type (all factors initially and during relapse) and duration until relapse on outcome of children with first relapse of AML.

Patients and Methods

From February 1988 until July 1996, 134 children suffering from first relapse of AML were reported to the study center of the AML-BFM group in Münster. Twenty-eight of 134 patients relapsing were treated palliatively and 4 transplanted without attempting to induce a 2nd remission prior to bone marrow transplantation (BMT). These children were excluded from the analysis.

Treatment During Relapse (Fig.1)

Treatment in relapse study 91(REZ-91) consisted of double induction with mitoxantrone (MITOX), VP-16 (MITOX 10 mg/m ^{2}/d day 1 and 2 + VP-16 100 mg/m ^{2}/12h days 1-6). In relapse study 93 (REZ-93) first induction with MITOX/VP-16 was followed by high dose Ara-C/MITOX (HAM) with Ara-C 3 g/m^2 /12h, days 1-3 + MITOX 10 mg/m ^{2}/d, day 3 and 4. In both studies a subsequent 6-week consolidation and either allogeneic or autologous BMT or maintenance therapy was added. The conditioning regimen for autologous BMT consisted of busulfan (16 mg/kg) together with VP-16 (50 mg/kg).

Fifty-five of the patients evaluated received elements of these protocols, e.g. HAM twice or high dose Ara-C/VP-16 or similar intensive induction regimens, like IDA-FLAG [2].

Statistics

Survival was calculated from date of diagnosis to death of any cause or last follow-up. The Kaplan-Meier method [3] was used to estimate survival rates with comparisons based on the two-sided log-rank test. Time to relapse was measured from diagnosis and not from the date of 1st remission (CR). The day of CR was often difficult to specify since chemotherapy was continued even if the neutrophils criterion of peripheral remission (>1500/µl neutrophils) was not ful-

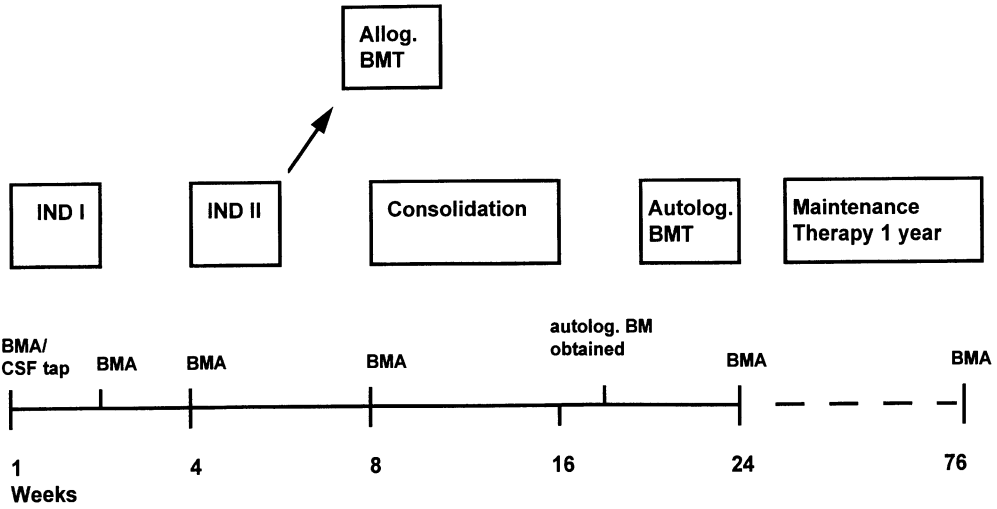

Fig. 1. Treatment plan of relapse study AML BFM REZ-93; *autolog.* autologous; *allog.* allogeneic; *BMA* bone marrow aspiration; *BMT* bone marrow transplantation; *CSF* tap cerebral spinal fluid tap; *IND* induction

filled. For analysis of risk factors a CART-(Classification And Regression Trees) analysis was performed [4,5]. All quantitative variables suspected to have an influence on survival where scanned for a threshhold value which divided the 102 intensively treated patients into two subgroups with the highest risk-ratio, a minimum p-value below 0.01 and a minimum group size of 20 patients.

Results

Patient Data

The FAB types of the 102 children treated intensively according to our relapse strategies are shown in Table 1. Their distribution in patients with relapse was different compared to de novo AML, mainly due to the higher percentage of M5 types (31% compared to 23% in de novo AML), reflecting the relatively high risk of relapse in the FAB types of the high risk group [6,7].

The median age was 8.1 years, range 1 to 19 years. Initially most of the children had been treated according to study BFM-87 [8] (71 patients) or study BFM-93[9] (26 patients), and 5 patients with other intensive induction regimens.

Table 1. Patients treated after relapse

Patients		n	%
Total		102	
Age (years) median	8.1		
range	1–19		
FAB types	M0	3	3
	M1	11	11
	M2	28	28
	M3	3	3
	M4	19	19
	M5	31	30
	M6	2	2
	M7	5	5
First-line treatment	BFM 87	71	70
	BFM 93	26	25
	other	5	5
Time to relapse since diagnosis (years)	<0.5	9	9
	0.5–1.0	36	35
	1.0–1.5	18	18
	1.5–2.0	16	16
	≥2.0	23	23

Table 2. Results after relapse treatment

Therapy	n	ED	NR	PR	CR	Alive
Intensive	59	1	21	5	32 (54%)	13 (22%)
REZ1	15	1	6	2	6 (40%)	5 (33%)
REZ93	28	1	10	3	14 (50%)	9 (32%)
Total	102	3	37	10	52 (51%)	27 (26%)

Abbreviations: ED = early death, NR = nonresponse, PR = partial remission, CR = complete remission.

Treatment Results (Table 2)

Fifty-two of 102 (51%) patients achieved remission (CR) after one or two induction courses. Three (3%) patients died early during induction. Thirty-seven were nonresponders (36%) and 10 (10%) achieved partial remission. Overall 27 of 102 patients (26%) were alive in median 2.5 years (range 0.4 -7.0 years) after relapse. Overall survival was 21%, SE 5% after 5 years (Fig. 2).

Efficacy of Various Therapeutic Regimens

All of the different treatment modalities induced similar response and survival rates (estimated probability of 5-year survival: REZ-91 0.24, SE 13%; REZ-93 0.28, SE 0.9%, other intensive regimens 0.20, SE 0.6%).

A total of 50 patients had been transplanted, mainly in 2nd CR (Table 3). All of the 7 patients transplanted in partial remission died from BMT-related complications or relapse. Seven of 16 patients transplanted from a matched sibling donor in 2nd CR were still alive, with a longer duration of 2nd remission than initially. In contrast only 1 of 5 patients survived after unrelated BMT. Ten of 22 patients receiving an autologous transplantation in 2nd CR stayed alive, 4 of them with a longer 2nd remission. Another 7 patients remained in CCR without BMT after intensive chemotherapy, with 4 of them experiencing a longer duration of 2nd remission than initially.

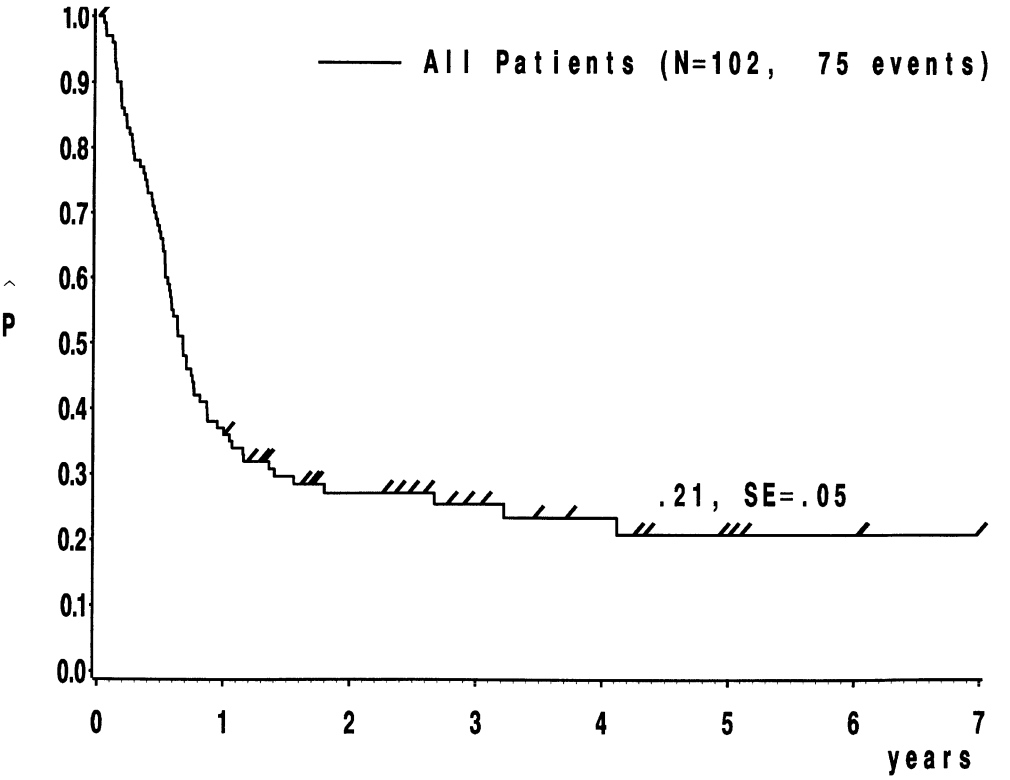

Fig. 2. Probability of survival after first relapse of 102 patients with AML receiving intensive treatment. *Slash* indicates patients alive

Table 3. Results after BMT and chemotherapy only

BMT		n	TRD	2. Relapse	Alive
MSD	2.CR	16	3	6	7 (7)[a]
	PR	5	1	4	
MUD	2.CR	1		1	
Haploid. BMT	2. CR	1			1 (1)[a]
Autolog. BMT	2. CR	22	2	10	10 (4)[a]
	PR	1		1	
Total BMT		50	8	23	19 (12)[a]
Chemotherapy only		2. CR 10		3	7 (4)[a]

[a] Indicates the number of patients with a longer duration of second remission than initially.
Abbreviations: BMT = bone marrow transplantation, CR = complete remission, PR = partial remission, MUD = matched unrelated donor, MSD = matched sibling donor, haploid. = haploidentical, TRD = treatment related death.

Risk Factors

Risk factor analysis for survival after relapse revealed the duration until relapse to be the most important factor. The best threshhold value was 1.5 years after diagnosis, resulting in a 5-year survival of 10%, SE 4% for early relapses and 40%, SE 10% for late relapses (*p*-logrank test 0.0001, Fig. 3). Most of the relapses occurred during the first 18 months (N = 63, 62%). Time to relapse was in median 1.1 years, range 0-15 years.

The influence of time to relapse on the CR rate is shown in Fig. 4. A significant rise in CR rate can be noted with increasing duration of first remission. Overall, the CR rate was 50%. In children relapsing after more than one year the CR rate exceeded 70%. Figure 5 demonstrates the increase in the survival rate depending on the duration until relapse.

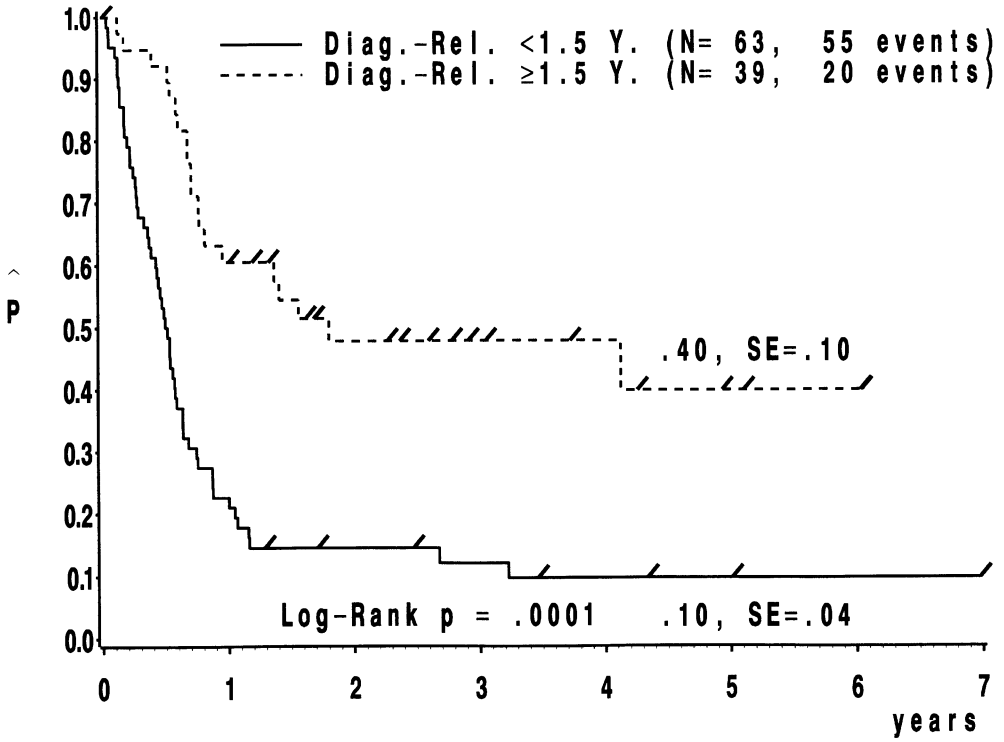

Fig. 3. Probability of survival of 63 patients with early relapse (< 1.5 years after diagnosis) compared to 39 patients with late relapse (≥ 1.5 years after diagnosis). *Slash* indicates patients alive

Toxicity

Three patients died during induction from measles related pneumonia (1 pt.) or sepsis in aplasia.

Severe infections occurred in study REZ-93 in 6 of 22 patients during first induction with MITOX/VP-16, and more often (9 of 12 patients) after the 2nd induction with HAM therapy. The incidence and severity of toxicity during first induction was comparable to that ob-served during induction treatment of study AML BFM-93 for de novo AML.

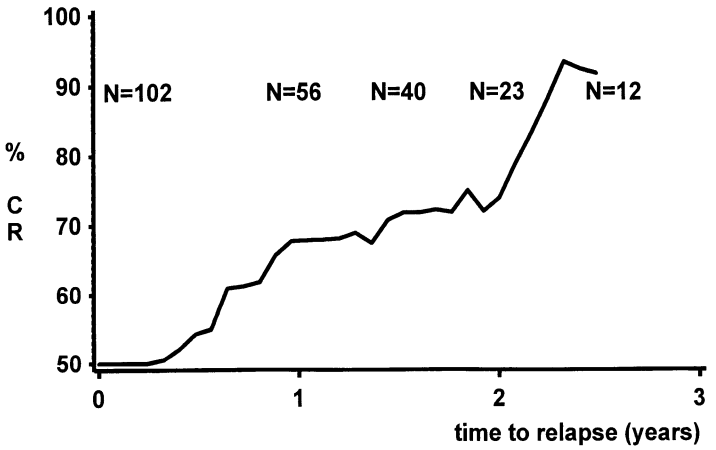

Fig. 4. Influence of time to relapse on the probability of achieving remission

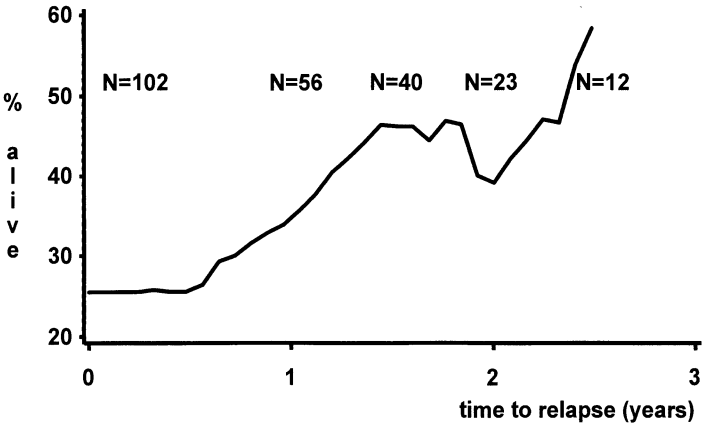

Fig. 5. Influence of time to relapse on the probability of survival

Discussion

Although results of first-line treatment in children with AML have substantially improved during the last 15 years, nearly half of the patients achieving CR still relapse [10]. Prognosis after relapse is generally poor due to the development of drug resistance [11]. Therefore, mainly different and more toxic therapy elements including new agents or higher dosages of drugs known to be effective in relapsed AML were given. Further intensive therapy necessary to maintain remission was often administered as allogeneic or autologous BMT.

The aim of our analysis was to evaluate not only the outcome after relapse according to treatment modalities, but also factors influencing prognosis after relapse.

These data show:

1. An overall CR rate of 50% achieved with different intensive chemotherapy courses, comparing favorably with other studies of children with relapse of AML [12, 13]. These results may be attributed to the efficacy of our two-drug regimen like MITOX/VP-16 and HAM. The majority of patients had not been treated with the combination of high dose Ara-C/MITOX (HAM) during first-line treatment (study AML BFM 87). Furthermore, the good response may be dose-related to the 1000 mg/m ^{2} VP-16 dosage over 5 days, considering the lower VP-16 dosage applied during first-line treatment in these patients.
2. A 5-year survival was seen in 21% of all patients. For those achieving CR, survival increased to 42% by further treatment with allogeneic or autologous BMT or continuous chemotherapy. A better outcome compared to the 3- to 5-year survival of 10% reported in children [13] and adults [14].
3. Results after BMT were favorable in patients achieving a confirmed second remission, in contrast to the poor results of patients with partial response. The 5-year survival was similar for allogeneic or autologous BMT, which may be explained by the higher transplant-related mortality in allogeneic BMT (19%) compared to 9% after autologous BMT, thus neutralizing the better antileukemic effect of allogeneic BMT. In addition patient numbers were small in both groups and so far, only in patients allografted, duration of 2nd remission exceeded that of 1st remission (Table 3).
4. The most important factor influencing survival was the duration until relapse. Clearly demonstrating the dependency of outcome on the duration of first remission. This finding corresponds with previous reports by Kantarjian et al., Hiddemann et al., and Estey et al. [14, 15] in adults with AML. Recently, Tomas et al. [16] could demonstrate in young adults achieving a 2nd remission after relapse, that duration of first remission was the main factor influencing final outcome after BMT.

Our results in more than 100 children with first relapse of AML after similar first-line

treatment regimens may influence the clinical management of children with AML. Children with late relapse of AML have a 2nd chance of cure with intensive chemotherapy either followed by allogeneic or autologous BMT or by further intensive chemotherapy.

Experimental drugs should be used mainly in the treatment of early relapses since 70% of patients with late relapses achieve remission with most of the intensive induction regimens applied by therapy trials. However, more intensive induction courses including non-cross-resistant drugs may have an impact on the quality of remission (eradication of residual blasts), and hence influence the final outcome after BMT regimens.

Another interesting finding, which needs to be investigated further, is the remarkable biology of late relapsed AML, sustaining it's disease sensitivity to chemotherapy [15] thus contradicting the general hypothesis of multidrug resistance of refractory leukemia [17].

More studies are also necessary to identify patients at high risk for late relapse already during first remission. And, by improving first-line chemotherapy eradicating the residual leukemic clone during intensification or maintenance therapy, late relapses may be avoided.

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Treatment of Adult AML

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Intensified Therapy for AML: The Role of the HAM Combination

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Abstract. HAM as a combination of high-dose AraC and mitoxantrone produced 53% complete remissions in patients with refractory AML according to rigid criteria. Its sequential version S-HAM even proved successful in salvage treatment for patients pretreated with HAM. In first line treatment TAD-HAM double induction improved the long term remission rate by more than 10% over that following conventional induction. As part of postremission therapy one single course of S-HAM given after TAD consolidation exhibited an antileukemic long term effect equivalent to that from prolonged maintenance treatment. With 38% long-term disease-free survival on the basis of intent to treat in patients < 60 years S-HAM ranges among the most effective intensification regimens.

The principle of intensified therapy of AML has recently found support by demonstrating dose effects of induction [1, 2] and postremission [3, 4] chemotherapy on patients outcome.

Escalation to high-dose AraC is an approach to overcome cellular resistance against this substance and proved active as a single drug treatment in refractory AML [5]. A similar single drug salvage effect was shown for mitoxantrone [6]. The HAM combination was designed in order to combine both single drug effects. In addition, by its special timing the regimen utilizes an accumulation of leukemic cells in S-phase [7] as a conditioning effect to subsequently given anthracyclines. Actually, HAM (AraC 3 g/m^2 q 12 h × 8 + Mitox starting day 2 or 3, 12 mg/m^2/d × 3 escalating to 10 mg/m^2/d × 4 and to x 5) produced complete remissions in 53% of patients who were refractory to two courses of TAD conventional dose induction therapy or relapsed within 6 months or relapsed later and were resistant to again receiving TAD [8].

After proving successful in salvage treatment HAM (AraC 3 g/m^2 q 12 h x 6 with Mitox 10 mg/m^2 d 3-5) was introduced into first line treatment and incorporated into the double induction strategy. Preceding the AMLCG specific TAD consolidation and prolonged maintenance the sequence of TAD-HAM double induction in patients aged 16-60 years resulted in a 71% CR and 35% 5 year disease-free survival. When ran-

Table 1. Response to double induction TAD-TAD versus TAD-HAM

	TAD-TAD	TAD-HAM	*p*
All patients	360	365	
CR	65%	71%	n.s.
Patients with > 40 % b. m. blasts after 1st course	33	30	
CR	25%	45%	.03
Patients with unfavorable karyotype	22	20	
CR	36%	70%	.029
CCR 5 Y	0	21%	n.s.

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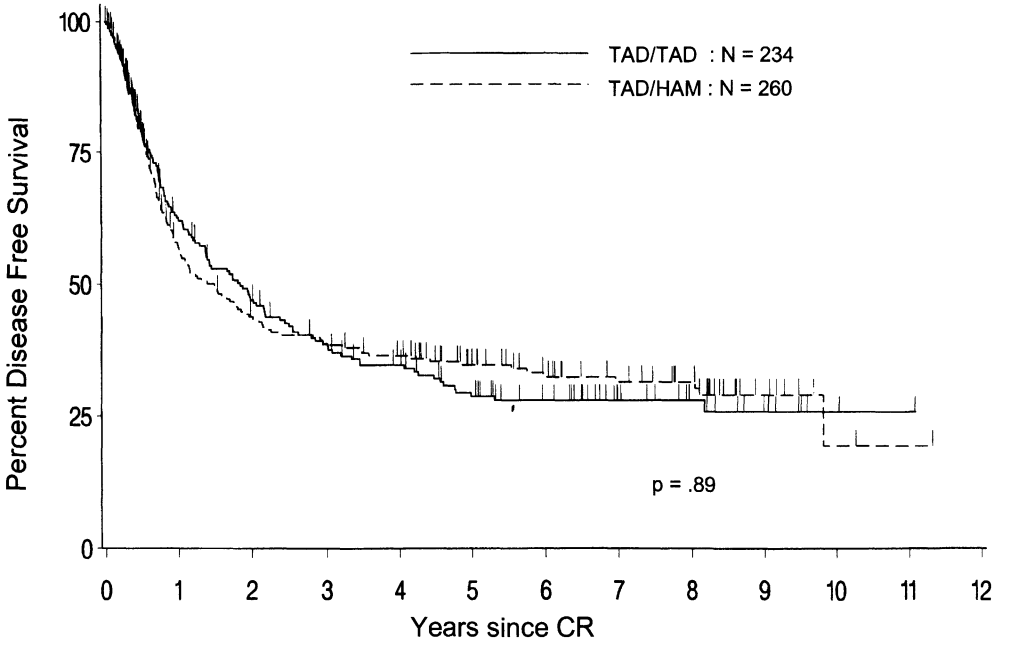

Fig. 1. AMLCG study on double induction in patients aged 16-60 years randomly comparing the two sequences TAD-TAD with TAD-HAM. Disease-free survival with *tick marks* indicating patients alive and in remission

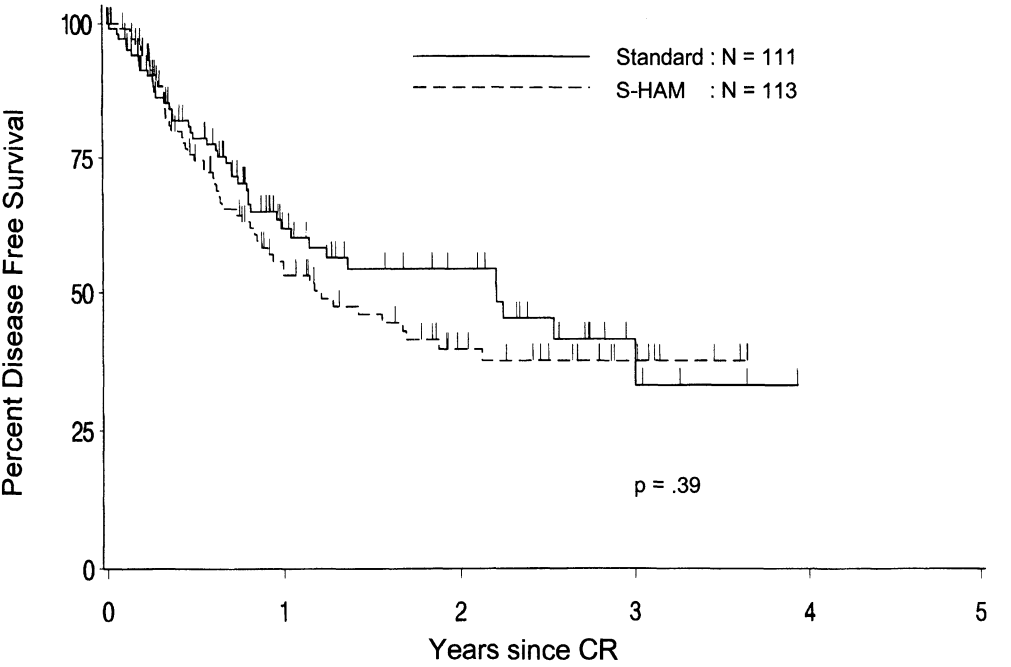

Fig. 2. AMLCG study randomly comparing postremission treatment by TAD consolidation and prolonged maintenance (standard) with TAD consolidation followed by one course of S-HAM instead of maintenance. Disease-free survival in patients aged 16-60 years with *tick marks* indicating patients alive and in remission

domly compared with the TAD-TAD sequence TAD-HAM was superior in patients with > 40% residual bone marrow blasts after the first induction course [1] and in patients with unfavorable karyotype [9] (Table 1). While more patients – especially in high risk groups – went into remission after TAD-HAM the overall disease-free survival was not clearly different between TAD-HAM and TAD-TAD with 32% patients alive and in remission at five years (Fig. 1).

Based upon AraC selfpotentiating effects [10, 11] HAM was further modified in a time sequential fashion (S-HAM, AraC day 1-2 and 8-9 + Mitox day 3-4 and 10-11) where it produced 64% CR in patients with relapsed and refractory AML half of them pretreated by non-sequential HAM [12].

Incorporated into first line treatment S-HAM has been administered as second consolidation course instead of prolonged maintenance. In patients aged < 60 years the three year disease-free survival is similarly 35% in the S-HAM and in the maintenance arm suggesting equivalent long-term effects [13] (Fig. 2). On the other hand, S-HAM in this position was followed by a long lasting myelosuppression with a median recovery time of neutrophils and platelets as long as six weeks from the end of S-HAM. Thus, S-HAM intensified consolidation after TAD-HAM induction and TAD consolidation requires support by autologous stem cell reinfusion in order to improving its practicability.

In summary, HAM and his modification S-HAM have been increasingly established as regimens with high antileukemic activity non-cross resistant with standard dose combinations. A new trial by AMLCG will investigate S-HAM as part of an escalating consolidation strategy with autologous stem cell transplantation.

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Recent Studies in AML, RAEB-t, and RAEB at M. D. Anderson Hospital

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Introduction

This chapter will describe M. D. Anderson trials, conducted since 1995, in newly diagnosed AML or MDS. The background for these trials were studies conducted between 1991 and 1995 using (1) idarubicin (12 mg/m^2 daily days 1-3) + continuous infusion high-dose ara-C (1.5 g/m^2 daily days 1-4), called IA, (2) IA + G-CSF given 1 day before, during, and for 3 days after completion of IA (IA + G), (3) Fludarabine (30 mg/m^2 daily days 1-5) + ara-C (2 g/m^2 daily days 1-5), called FA, (4) FA + G-CSF given 1 day before FA and continuing until recovery of the neutrophil count to > 1000/μL [FLAG], and (5) FLAG + idarubicin (FLAG + ida) in which fludarabine and ara-C were each administered for 4 days, idarubicin was given at 12 mg/m^2 daily in days 2-4, and G-CSF was given as in FLAG but continued only until 8 days after the start of chemotherapy. Patients were assigned to these regimens according to their leukemia cell cytogenetics. Patients with an inv(16), t(8;21), or normal karyotype were considered as being in a "better" prognostic group, and those with abnormalities of chromosomes 5 and/or 7 or + 8 in a „worse" group. Other patients were classified as better it they had no documented abnormality of blood count for ≥ 1 month before M. D. Anderson presentation (AHD) and worse if they had an AHD. Patients with APL were treated separately. Regimens given the better group were, successively, IA, FA, FLAG, and IA + G while the worse group successively received FA, FLAG, and FLAG + ida. We analyzed outcome in 520 patients. Cox analyses indicated that, as previously, karyotype, AHD status, performance status, age etc were determinants of event-free survival, and survival. After accounting for these covariates IA, IA + G, and FLAG gave equivalent results while FA and FLAG + ida were worse than IA, IA + G, or FLAG. Of note, diagnosis, i.e., AML, RAEB-t, or RAEB, had no effect on outcome with the possible exception that patients with RAEB-t did better [1]. At any rate, it appears that if patients with RAEB or RAEB-t have a poor prognosis if untreated, as those we treated did, there is no more reason not to give them AML-type chemotherapy than there would if they had AML, rather than RAEB or RAEB-t.

Patients and Methods

The 1995 studies are exploring 2 principal modalities, lisofylline (LSF) and all-trans retinoic acid (ATRA). Since IA and IA + G were identical, LSF is being combined with IA. Patients are eligible for this study if they have AML, RAEB-t, or RAEB, are under age 71, have no AHD, have not received chemotherapy, e.g., for another malignancy, and have normal kidney and liver function. This definition of a "better" prognosis group is thus at variance with our prior definition

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based on cytogenetics. One advantage of the new definition is that patients in the lisofylline study are largely identical and thus easily comparable, to those treated on most cooperative groups protocols. In reality given the close association between age and AHD status on the one hand, and karyotype on the other, the two systems identify similar patients as "better" (and therefore "worse") prognosis. For example, 86% of inv16 patients fall into the better group with the current system and 75% of the -5, -7 patients into the worse group. Patients eligible for the LSF study are randomized to receive IA + LSF or IA + placebo. In CR they are separately randomized to continue ara-C alternating, with IA either ± ATRA. The worse group of patients (age > 70, with an AHD or secondary AML or MDS, or abnormal organ function) are randomized to receive either

a) chemotherapy alone (fludarabine + ara-C + idarubicin, as previously describe, = FAI),
b) FAI + G-CSF starting 1 day before chemotherapy and continuing until neutrophil recovery,
c) FAI + ATRA 45 g/m^2 daily beginning, 2 days before, and continuing 3 days after completion of, FAI or
d) FAI + G-CSF + ATRA.

Once in CR patients continue to receive, for 6 months, the arm assigned initially. Patients with APL are ineligible for the LSF or FAI ± G-CSF and ATRA studies.

The rationale for use of LSF are data obtained in recipients of allogeneic transplants that, compared to placebo, LSF decreased major infection rate and improved 100-day survival rate [2]. ATRA is being used because it can decrease concentrations of BCL-2 and BCL-XL in cultured AML cells, thereby increasing their sensitivity to ara-C-induced apoptosis and perhaps apoptosis induced by other drugs [3-5]. Despite its failure to equal the results of FLAG in the 1991-1995 experience, we are using FAI + G-CSF again, because we are now giving the G-CSF until neutrophil recovery (as with FLAG but unlike the original FLAG + ida regimen). Although we have conducted relatively few randomized studies before, at least one observation suggests there may be some reason for doing both the lisofylline, and ATRA trials in such fashion. While the proportion of patients with PS > 2 ranged from 13-18 between 1991-1994, it fell to 8% in 1995 and 6% in 1996 ($p < 0.01$). While it may be that the proportion of patients with worse PS has decreased, it is also possible that patients with PS > 2 as assessed in 1991-1994 are being assessed as PS < 3 currently. Since PS > 2 is a powerful predictor of outcome, the current patients could be concluded to be doing worse than previous patients if in fact the change in PS is artifactual and if trials were not being done in randomized fashion.

Results

The IA ± LSF and IA ± ATRA trials are currently double-blinded (the former trial) or too early (the latter) so the focus will be on the "4-arm" study in worse prognosis patients (FAI, FAI + G-CSF, FAI + ATRA, or FAI + G-CSF + ATRA). Table 1 illustrates pretreatment characteristics of the patients; 20% have complex abnormalities involving chromosomes 5 and/or 7, and 52% have other presumably unfavorable abnormalities including simple abnormalities of chromosomes 5 and/or 7 (-5, -7, 7q-, 5q- as a single clone); 69% have an AHD and 31% have secondary AML. The 4 groups appear balanced

Table 1

	FAI	FAI + G	FAI + ATRA	FAI + G + ATRA
PTS	27	26	24	27
Median age	66	65	62	66
PS > 2	2[a]	3	0	3
PE[b]	17	17	16	17
AHD	20	18	19	15
Prior chemo[c]	8	11	4	9
AML	18	16	16	16
RAEB-t	4	9	5	11
RAEB	5	1	3	0
1 Outside cours[d]	5	3	3	4
Complex -5, -7	6	5	5	5
INV16 or t(8;21)	0	2	0	1
Other abnormal	15	12	14	13
Normal	6	7	5	8

[a] Numbers of patients with given characteristic.
[b] PE = treated in laminar air flow room.
[c] For another malignancy.
[d] For AML/MDS.

Table 2

	FAI	FAI + G	FAI + ATRA	FAI + G + ATRA
PTS	27	26	24	27
CR (%)	10 (37)	11 (42)	13 (54)	16 (59)

with respect to these and other prognostic variables. Table 2 indicates the current (as of 1/29/97) CR rates. The CR rates for FAI + G-CSF (arms 2 and 4) vs. FAI without G-CSF (arms 1 and 3) are 51 vs. 45% (p = ns) while those for FAI + ATRA (arms 3 and 4) vs. FAI without ATRA (arms 1 and 2) are 40% vs. 57% (p = 0.08, 95% CI for the difference [-0.02, 0.36]).

Why the seemingly higher success rate in the patients given FAI + ATRA? Table 3 indicates the causes of failure. Resistant patients are those in whom treatment was changed after 1 or 2 courses, who died on courses 2, or who died on course 1 with either (a) 20% blasts (10% MDS) in a marrow that was > 10% cellular on day ≥ 28, (b) > 50% blasts (> 20% MDS) in a > 10% cellular marrow on day ≥ 21, (c) a WBC count ≥ 1500 with > 30% blasts on day > 14 or (d) persistent cytogenetic abnormalities on day 21 of course 1 since this predicts for failure to respond. A similar number of patients eligible for course 2 (neither dead nor in CR after course 1) received course 2 in the arms with and without ATRA, with second course CR rates being 3/8 in arms with, and 1/11 in arms without, ATRA. When recurrences are taken into account and resistant patients are considered as those who were resistant during induction or whose disease recurred the proportion of resistant patients is as illustrated in Table 4. The suggestion is that the FAI + G + ATRA is associated with less resistance. Current EFS data indicate superiority for the arms with ATRA, with $p = 0.22$.

Table 3

	FAI	FAI + G	FAI + ATRA	FAI + G + ATRA
Failures	17	15	11	11
Died days	1	1	1	4
Died hypoplastic	1	4	1	3
Equivocal	4	3	1	2
„Resistant“	11 ($6^a, 5^b$)	7 ($5^a, 2^b$)	8 ($7^a, 1^b$)	2 ($2^a, 0^b$)

[a] Resis. after 1 co.
[b] Resis. after 2 co.

Table 4

	FAI	FAI + G	FAI + ATRA	FAI + G + ATRA
PTS	27	26	24	27
Eval. for resis.	21	17	21	18
Resis.	11	7	8	2
	67%	71%	62%	39%
Recurred	3	5	5	5

Eval for resis. = PTS - ED - hypo - equivocal - death in CR.

Discussion

We are planning to formally analyze the study after 116 induction responses have been evaluated. The study design called for randomization of 232 patients so as to detect with global p = 0.05 and power 0.80 a 20% absolute difference in response rate, after covariate adjusting between the arms with and without G-CSF, with and without ATRA, and also to test for an interaction effect between G-CSF and ATRA. Covariates to be adjusted for include those illustrated in Table 1 as well as diagnosis, and various CBC and SMA values.

Although conclusions about the 4-arm study are premature, the study raises a question we are discussing among ourselves: should patients not in CR after one course get a second course of the same therapy or should treatment be changed? As noted above, 4 of 19 patients given a second course on the 4-arm study entered CR. These 4 differed from the 15 who did not get a CR on course 2 in having had a better response to the first course (e.g., all 4 had decreases in marrow blasts vs. 7 of the 15 second course failure), rather than starting the second course earlier or later (median start day = day 39 of course 1 for the 4, day 43 of course 1 for the 15).

Furthermore, we have observed that the CR rate, at least on course 1, does not appear to decrease until day 35 or later. Although these facts might argue for a delay until at

least day 35 to starting course 2, we have also observed that residual survival after achievement of CR declines dramatically before then. This puts a premium on quick achievement of CR, or, identically, using treatment to which the AML cells are very sensitive. Under these circumstances we plan to give patients not in CR by day 28-35 of course 1 alternative therapy and suggest this as a matter for future discussion. It should be noted that although double induction strategies have seemed useful in children and adults, those strategies may be of value only in patients with sensitive disease, e.g. no AHD, de novo AML, given 3 + 7 type regimens and not in patients given high-dose ara-C initially or who because they are older or have an AHD or secondary AML might be less tolerant of, or less responsive to, a second identical course beginning on day 21.

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FLAG-Ida, an Effective Regimen for High Risk AML

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Abstract. A number of cytogenetic patterns have been identified as being disadvantageous for response to treatment in AML. In addition, the presence of an antecedent hematologic disorder (AHD) also consistently has a negative impact on prognosis being closely related with the incidence of myelodysplasia. Since 1991, the MDACC has elected to treat patients (pts) with adverse cytogenetics and/or an AHD (AML, poor risk) with a combination of regimens based on the synergism between the fludarabine (Fludara) and cytosine arabinoside (ara-C). Fludara enhances the ara-CTP formation in leukemic blast cells. Thus, a combination regimen of Fludara and ara-C (FA) was developed in 1991. Subsequently, GCSF was added being given before, during, and after the FA to form the FLAG regimen. In more recent times, idarubicin has been added. Thus, the FLAG-Ida regimen is Fludara 30 mg/m^2/day for 5 days followed each day 4 hours later by ara-C 2 g/m^2 over 4 h. GCSF at a dose of 200 μg/m^2 is given 1 day before and during the therapy. Use following completion of chemotherapy is according to need of the patient. Idarubicin is given at a dose of 12 mg/m^2/day for 3 days. Pts treated with prior regimens in 1980-1990 had a complete remission rate of 38%. The combined results of the FA, FLAG, and FLAG-Ida regimens is 66 CRs of 131 pts (50%). The CR rate to FA was 12/30 (40%), FLAG 27/49 (55%), and FLAG-Ida 27/52 (52%). There was no increase in the mortality during remission induction caused by the addition of idarubicin. Comparison to the historical past of patients treated between 1986-1990 demonstrates an advantage in CR rate which is not statistically significant for the FA, FLAG, and FLAG-Ida regimens. The CR duration at 2 and 3 years, however, is substantially higher with these regimens than previous experience. A multivariate analysis for response and survival will be carried out on this patient population to identify risk factors. Age, poor performance status, hypoalbuminemia, and treatment outside a protected environment all appear to be adverse at this time.

Introduction

A large number of prognostic factors have been identified in acute myelogenous leukemia (AML) [1, 2]. Consistently, the age of the patient, history of a myelodysplastic onset or antecedent hematologic disorder (AHD), organ dysfunction, and white blood cell count have been identified as prognostically significant for complete remission (CR). However, at our institution, the most important prognostic factor has been the cytogenetic pattern of patients [3]. Patients can be classifed as having relatively favorable (low risk) karyotype which includes patients with inv16, t8;21, t15;17, and a diploid karyotype (Table 1). Adverse cytogenetic groups include those with abnormalities in chromosomes #5 and #7 and trisomy chromo-

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some #8. Patients with insufficient metaphases and other miscellaneous groups have relatively favorable or unfavorable prognosis depending on whether they have an antecedent hematologic disorder (AHD) or not. This classification assigns patients with AML into low risk and high risk. In our historical control group from 1980-1990, patients in the high risk group had a probability of response of 38 vs. 67% for those in the low risk group. Thus patients with high risk disease are in urgent need of new approaches to treatment [4].

Previous laboratory and in vivo studies have demonstrated that pretreatment with fludarabine enhances the formation of the triphosphate form of cytosine arabinoside (ara-CTP) in patients with AML [5]. This observation gave rise to the concept of fludarabine/ara-C regimens. Our previous experience with Fludara and ara-C (FA) or Fludara + ara-C combined with GCSF (FLAG) has been published [4]. The response rate with FA was similar to that seen with previous combinations of anthracyclines and ara-C and the addition of GCSF was associated with a favorable but not statistically significant improvement in response rate [6]. Thus the decision was made to build on the FLAG experience by the addition of Idarubicin. This formed the regimen FLAG-Ida. The results of therapy with FLAG-Ida in frontline therapy of high risk AML patients is reported in this manuscript.

Patients and Methods

Fifty-two previously untreated patients with AML were treated with FLAG-Ida between July 1993 and May 1995. All patients were classified as high risk according to Table 1.

Our past experience with 224 high risk patients treated between 1980 and 1990 had a CR rate of 38% with 38% patients dying during remission induction and 24% of patients being declared resistant. The percentage of patients who were five year survivors was 4% [4]. Immediately preceding the FLAG-Ida study, 30 patients were treated with FA and 49 patients with FLAG. The FA regimen was administered from January 1991 to March 1992 and FLAG from March 1992 until July 1993 prior to the initiation of FLAG-Ida. In FA the dose of fludarabine was 30 mg/m^2 i.v. and ara-C 2 g/m^2 i.v. on each of days 1-5. FLAG added GCSF 400^2 µg/m i.v. subcutaneously daily from day -1 until CR. FLAG-Ida added Idarubicin at a dose of 12 mg/m^2 i.v. on days 2, 3, and 4 but Fludara and ara-C were only given on days 1-4 and GCSF was given only on days -1 through +8. Patients in whom the white blood cell count was > 50 000/µl who were assigned to the GCSF containing regimens FLAG or FLAG-Ida began the GCSF on the same day as chemotherapy rather than on the day store.

Table 1. Classification of cytogenetically defined risk groups in AML

Karyotype	Good risk	Poor risk
t(8;21)	All	-
t(15;17)	All	-
Inv16	All	-
Diploid	All	-
Miscellaneous	No AHD	AHD
Insuff. metas.	No AHD	AHD
+8	-	All
-5, -7	-	All

Patients were preferentially treated in the Protected Environment if they agreed to this approach. Second courses of FLAG-Ida were administered if patients had more than 20% blasts in a bone marrow sample that was more than 20% cellular on day 21 after the first course of chemotherapy. Complete remission (CR) was diagnosed when the bone marrow had less than 5% blasts and peripheral blood showed more than 1000/µl of granulocytes in more than 100 000/µl of platelets at the same time [4]. Patients were removed from study if they were not in CR after two courses. In the FLAG-Ida group, after achieving CR, patients received one course of ara-C at a dose of 100 mg/m^2 per day by continuous infusion on days 1-5. This was followed by a course of fludarabine 30 mg/m^2 i.v. and ara-C 1 g/m^2 i.v. on days 1 and 2 and Idarubicin 8 mg/m^2 i.v. on day 2. GCSF was given during and for the 3 days after completion of each course of chemotherapy. These conventional ara-C dose courses and FLAG-Ida courses were alternated until the

Table 2. FLAG-Ida in poor risk AML - characteristics

Patients	52
Age - med. (range)	63 (26-82)
Male	29 (56%)
Prior AHD	30 (52%)
Perf. status 2-3	23 (44%)
-5/-7	28 (54%)

Table 3. FLAG-Ida response in poor risk AML by prognostic group

Characteristic	Value	Patients	%CR	%Died
Age (yrs)	< 60	21	62	19
	60-69	21	48	33
	≥ 70	10	40	50
Performance status	0-1	29	72	17
	2-3	23	26	48
Albumin	< 3.5	31	36	42
	≥ 3.5	21	76	14
Protected environment	Yes	36	56	28
	No	16	44	38
LDH	Normal	13	85	–
	Elevated	39	41	41
WBC	< 5000	25	72	16
	≥ 5000	27	33	44
Cytogenetics	-5/-7	28	43	39
	+8	9	44	22
	11q	2	100	–
	Misc.	5	40	40
	Insuff.	8	88	13

patients relapsed or until they had been in remission for 12 months.

The patient characteristics are shown in Table 2. The median age was 63 years with 10 patients being over 70 years of age. Forty-four percent of the patients were performance status 2 or 3. Thirty-six patients (69%) were treated in protected environments. The overall CR rate was 27/52 (52%) with 16 (31%) dying during remission induction, 11 in the first course. Twenty-five patients achieved CR after a single course. Only 11 patients received a second course of treatment and only two of these obtained a CR with 5/11 (46%) of patients dying during the second course. The CR rate was not significantly associated with age of the patient but strongly correlated with performance status, albumin level, serum LDH, and white blood cell count levels (Table 3). Analysis by cytogenetic subgroup showed favorable response rates in patients with 11q23 abnormalities and insufficient metaphases, whereas patients with trisomy of chromosome #8 or abnormalities in chromosomes #5 and #7 and other miscellaneous abnormalities had response rates between 40 and 45% (Table 3). The major causes of failure to respond were deaths due to infections mortality. Three patients died with documented bacterial infections, four with documented fungal infections, six with pneumonias of unknown pathogen and 11 patients with multi-organ dysfunction. Three patients had hemorrhage as a significant contributing factor. Many patients had multiple contributing causes.

The remission duration of the patients was disappointing. The median CR duration was 39 weeks with only 25% of patients projected to still be in remission at one year (Fig. 1). The overall survival of the patients was a median of 28 weeks with one-third of patients living for one year and approximately one in six patients living at two years (Fig. 2). No cardiac toxicity was noted. Hy-

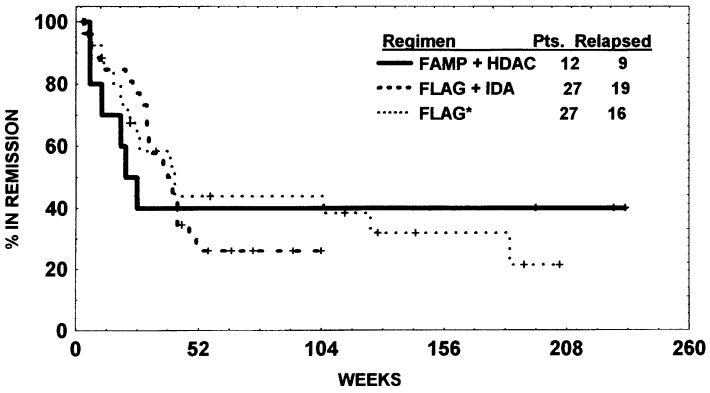

Fig. 1. CR duration poor risk FA by regimen

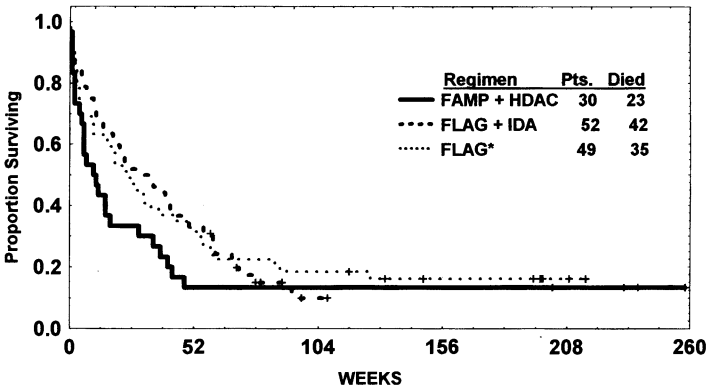

Fig. 2. Survival poor risk FA by regimen

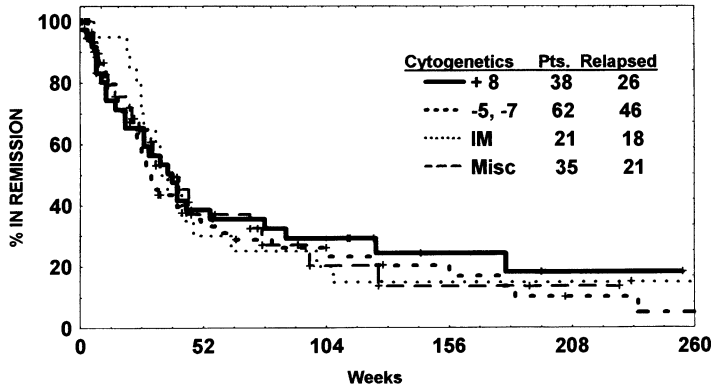

Fig. 3. CR duration poor risk by cytogenetics

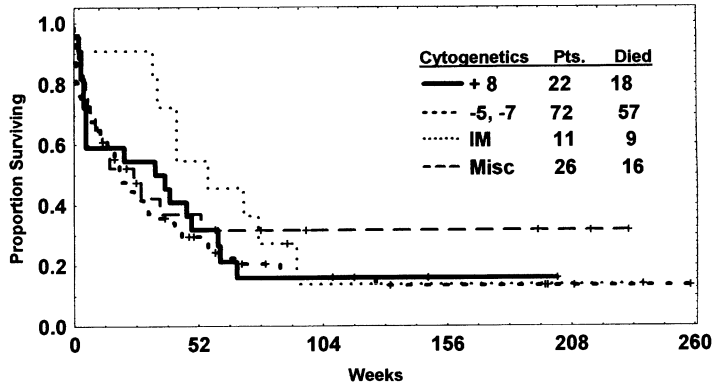

Fig. 4. Survival poor risk by cytogenetics

perbilirubinemia (> 2.5 mg%) was noted in 15 patients and a significant elevation of creatinine (> 2.5 mg%) in nine patients. Comparison of the results with FLAG-Ida with FA and FLAG in comparable patients showed that FLAG and FLAG-Ida had similar response rates and similar mortality (Table 4). Thus no difference in response rate, remission duration or survival was noted between FLAG and FLAG-Ida. Somewhat lower response rates were noted for FA. The remission duration and survival by cytogenetics demonstrated that there was no significant difference between the cytogenetic subcategories (Figs. 3, 4). As demonstrated in Figs. 5 and 6, the CR duration and survival

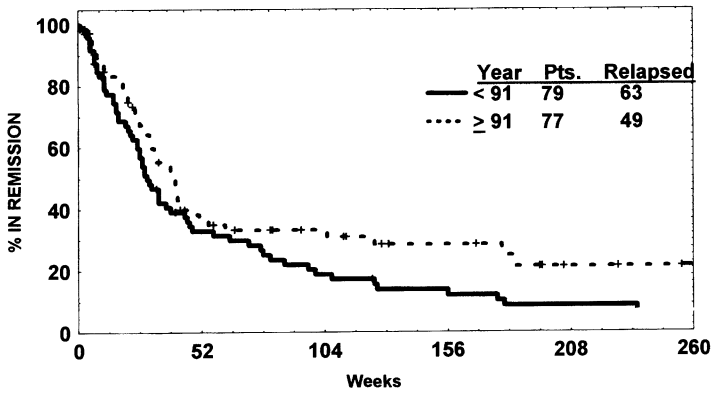

Fig. 5. CR duration poor risk by year

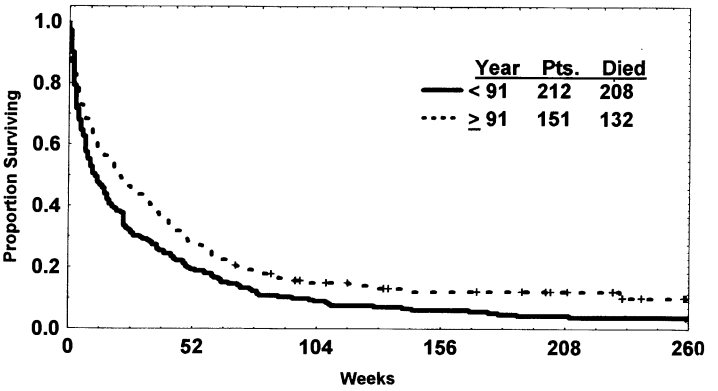

Fig. 6. Survival poor risk by year

of patients during the period 1991-1995 is somewhat superior to that noted in the immediately proceeding decade 1980-1990 (Figs. 5, 6).

Discussion

The fludarabine/ara-C combinations have demonstrated that they are able to obtain CR rates comparable to anthracycline ara-C combinations in high risk patients with AML compared to our historical experience. The addition of idarubicin to the regimens called FLAG-Ida has not been associated with an increase in CR rate and CR duration or survival. The reasons for this are uncertain. FLAG-Ida differs from FLAG in that the GCSF is given only until day 8. In addition there is one less couplet of fludarabine/ara-C as this treatment is only given for four days rather than five days in the FLAG. Thus the beneficial contribution of idarubicin to the protocol may have been lost by these changes in the delivery of the FLAG component. The major risks to patients with adverse cytogenetic subgroups continue to be twofold. The mortality during remission induction associated with this group is very significant usually due to fungal infection. In addition, the risk of relapse in patients who achieved complete remission remains very high. New attempts to improve supportive care and development of new techniques for post-remission therapy are desperately needed in this adverse subset of patients. It is of interest that while the overall remission duration and survival remains disappointing, in the FA, FLAG, FLAG-Ida, comparison with our historical past with treatments prior to FA, the long term CR fraction appears to be higher. Therefore we continue to build on this approach by the addition of ALL-trans-retinoic acid as reported by Dr. Estey elsewhere in this publication [6]. Similar approaches of the treatment of patients with de

novo myelodysplastic syndrome with adverse cytogenetic features has been associated with a similar response rate to that reported in AML [4]. The difference in response rate, CR duration, and survival between AML and MDS in our experience is minimal so that patients with both diagnosis are being registered on the frontline protocols.

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Chemotherapy with Idarubicin, Ara-C, VP-16, Amsacrine, Followed by G-CSF and Maintenance Immunotherapy with Interleukin-2 for Patients with High-Risk Acute Myeloid Leukemia: a 3-Years Follow-Up

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Abstract. To improve the complete remission (CR) rate and to prolong CR duration in patients with advanced MDS, AML evolving from MDS, and secondary AML, a phase-III trial of aggressive chemotherapy followed by G-CSF was initiated in January 1992. Pts. achieving a CR were randomized to receive either high-dose or low-dose IL-2 to evaluate the potential of this cytokine to eliminate residual leukemic cells and to prolong the CR duration.

A total of 110 pts aged 18 to 76 yrs (median: 58 yrs) were treated with idarubicin (Ida, 10 mg/m^2 IV, 3 days), Ara-C (100 mg/m^2 CIVI, 7 days), and VP-16 (100 mg/m IV, 5 days) as induction therapy. Patients in CR after two induction courses (induction 2: Ida 10 mg/m^2 IV, 2 days, Ara-C 100 mg/m^2 CIVI, 5 days, and VP-16 100 mg/m^2 IV, 5 days) received 2 consolidation courses, the first being identical to induction 2, and the second consisting of Ara-C 600 mg/m^2 twice a day, 5 days, and amsacrine 60 mg/m^2, 5 days. G-CSF (5 µg/kg SC) was given from the day after the end of chemotherapy until neutrophil recovery. 20 CR patients have been randomized to start with IL-2 therapy within 8 weeks after the end of consolidation (9 pts: 9 × 10^6 U/m IV; 11 pts. 0.9 × 10^6 U/m IV; days 1-5, days 8-12; 4 courses).

Forty nine pts (45%) achieved CR (<60 yrs: 52%; > 60 yrs: 35%; p = 0.06), and 10 pts (9%) obtained PR. 39 pts (35%) were treatment failures. Early death occured in 12 pts (11%); 9 pts unterwent allo-BMT. The median overall survival was 8 mths, 21 mths for the CR pts (95%-CI: 17-25 mths) after a median observation period of 36 mths (range: 14-56). The median RFS for CR pts including those not receiving all consolidation courses and IL-2 maintenance therapy was 12.5 mths (<60 yrs: 19 mths; >60 yrs: 7 mths). Respectively median RFS is 19 mths in the high-dose and 13 mths in the low-dose IL-2 arm (p = 0.8).

We conclude that chemotherapy with Ida,

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Ara-C, and VP-16 followed by G-CSF is effective and well tolerated with a low early death rate. IL-2 therapy seems to be of benefit with no apparent difference between the two dose levels.

Introduction

High-risk acute myelogenous leukemia (AML, such as AML evolving from myelodysplastic syndromes (MDS) or secondary AML) and de novo AML are considered to differ with regard to response rate and remission duration after chemotherapy. Patients suffering from de novo AML can be cured by conventional chemotherapy whereas those with advanced MDS and secondary AML are considered incurable.

Treatment of MDS with AML-type chemotherapeutic regimens is capable of inducing complete remission. Because of the age of the patients and the individual duration of MDS a high rate of early complications was reported during the use of standard AML cytotoxic regimens [1, 2]. Trials in elderly AML-patients have demonstrated that the rate of infection-related deaths during induction therapy can be reduced by using hematopoietic growth factors to accelerate hematopoietic recovery [3]. Treatment of CR-patients after induction chemotherapy with interleukin-2 (IL-2) has resulted in the induction of an antileukemic immune response by lymphokine activated killer cells (LAK) and elimination of residual leukemic cells [4–6].

A phase III trial of intensive chemotherapy followed by G-CSF was initiated in patients with high-risk AML. Patients achieving a CR were randomized to receive either high-dose or low-dose IL-2 to evaluate the potential of this cytokine to eliminate residual leukemic cells and thereby to prolong the CR duration. The present report showes the feasibility of this therapeutic approach and the first results of a 3-year follow up analysis.

Material and Methods

A total of 110 patients (Table 1) with refractory anemia with blast excess in transformation to acute leukemia (RAEB-T), AML evolving from MDS or AML secondary after previous cytotoxic chemotherapy or radiotherapy were entered in this study from January 1992 until May 1995. The diagnosis was established by examination of peripheral blood and bone marrow aspirate and trephine biopsie.

Table 1. Patients' characteristics

No. of patients	110
Male/female	49/61
Age (years)	
Median	58
Range	18-76
Diagnoses	
RAEB-T	18
AML after MDS	86
Secondary AML	6

The chemotherapy regimen for induction consisted of cytosine-arabinoside (ara-c) as a continous IV infusion at a daily dose of 100 mg/m ^{2}/day for 7 days, idarubicin administered as IV bolus injection at 10 mg/m ^{2}/day on days 1, 2, and 3, and VP-16 as 1-h infusion at 100 mg/m ^{2}/day on days 3-7. Two early consolidation courses included ara-c, 100 mg/m ^{2}/day IV, days 1-5, idarubicin, 10 mg/m ^{2}/day IV, days 1 and 2, and VP-16 100 mg/m ^{2}/day IV, days 1-5. A late consolidation course within 6 weeks after this therapy contained amsacrine, 60 mg/m^2/day, administered by IV short infusion on days 1-5, and ara-c at a dose of 600 mg/m^2 as 2-h infusion every 12 h on days 1-5. After each course of chemotherapy, patients received granulocyte colony-stimulating factor (G-CSF) 5 µg/kg SC until recovery of granulocyte counts (Fig. 1). Eligible patients (below 55 years of age, HLA-identical donor) received an allogeneic bone marrow transplant after achieving complete (CR) or partial (PR) remission.

Starting 4-6 weeks after the late consolidation course, patients were randomized to four cycles of either high-dose (9×10^6 IU/m^2 /day) or low-dose (0.9×10^6 IU/m ^{2}/day) recombinant human interleukin-2 (IL-2, provided by Hoffmann-La-Roche, Grenzach-Wyhlen, Germany) as 1-hour infusion on days 1-5 and 8-12. Cycles were repeated at 6-week intervals.

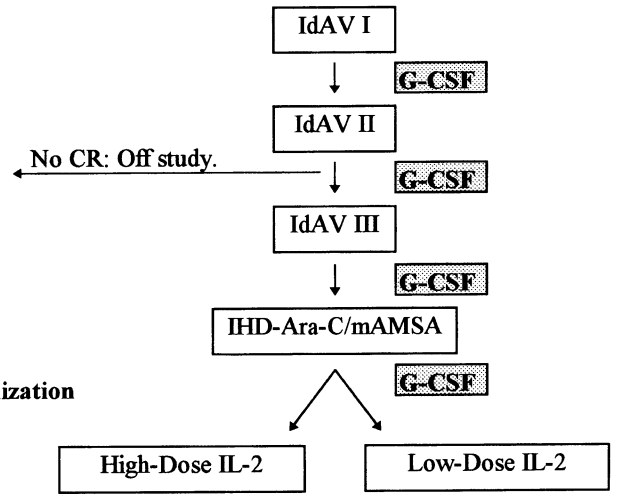

Fig. 1. Schedule of treatment for high-risk AML. *IdAV* idarubicin, ara-c, VP-16; *IHD-Ara-C/mAMSA* intermediate high-dose ara-c, amsacrine.

According to FAB-criteria CR was defined as less than 5% blasts in normocellulare bone marrow and normal peripheral differential counts (absolute neutrophil count > 1000/µl, platelets > 100 000/µl). Patients who did not achieve CR were considered as non-responder. Patients who died during the induction phase within four weeks of starting therapy and who were not evaluable for response were considered as early death. Remission duration was calculated from the time of achieving CR to the date of the date of the most recent follow-up or documented relapse or death in remission.

Results

All of the patients entered into the study were assessable for response to the treatment. A total of 49 patients (45%) achieved a CR, 35 patients after IdAV I, 14 others required treatment with the second induction cycle. 13 patients (12%) obtained a partial remission after two induction courses. 41 patients (38%) were treatment failures. Early death occured in six patients (5%) during induction therapy. Six patients died in CR, five of these patients were older than 60 years. The overall toxic death rate was 10%. Nine patients, six in CR and three in PR, underwent allogeneic bone marrow transplantation (Table 2).

20 CR patients were randomized to receive either high-dose or low-dose IL-2 as maintenance therapy to induce an antileukemic immune response and to reduce minimal residual disease.

Table 2. Treatment results

CR	49 (45%)
PR	13 (12%)
Failure	41 (38%)
Toxic death	11 (10%)
Early (day 28)	5 (5%)
Treatment related	6 (5%)
Overall survival (months)	8.3
Relapse-free survival (months)	12.5
High-dose IL-2	19
Low-dose IL-2	13

The median overall survival (OS) was 8.3 months after a median observation period of 36 months (range: 14-56), OS for CR patients was 21 months. The median relapse free survival (RFS) for CR patients including those not receiving all consolidation courses and IL-2 maintenance therapy was 12.5 months. Respectively median RFS is 19 months in the high-dose and 13 months in the low-dose IL-2 arm (Table 2), the difference is nonsignificant ($p = 0.80$).

Discussion

It is known that a long-term benefit in advanced MDS and high-risk AML, can only be achieved by eradication of the abnormal

clone and restoration of normal hematopoiesis. The present study was prompted by reports that idarubicin is effective in patients with high-risk AML and that the use of hematopoietic growth factors, e.g., G-CSF, will not only accelerate neutrophil recovery but will also reduce the rate of severe infections and early death. Our results in 110 patients illustrate that the combination of idarubicin (at a total dose level of 50 mg/m ^{2} in two induction courses), ara-c, and VP-16 is effective for remissions induction and well tolerated [7, 8, 9]. Compared with previous studies evaluating intensive therapy (reviewed in[1, 2] the high median age of 58 years and the low rate of toxic death in the present study deserves notice. The low rate of early death in our patients, which was even lower than in many trials with younger patients treated for de novo AML, could be due to the consequent use of G-CSF after each chemotherapy cycle [10].

With a total of 20 patients treated with either high-dose or low-dose IL-2, no significant differences have become apparent between both treatment arms with regard to relapse free survival. Because of the lower rate of side effects the described lower dosage of 0.9×10^6 IU/m ^{2} will be evaluated in future studies. Whether IL-2 is beneficial as a maintenance in CR patients and prolongs remission duration by the elimination of residual leukemic cells, cannot be decided due to the design of our trial, and no randomized trial has been finished yet to settle this question.

In conclusion the effectiveness with a CR rate of 45% and the low early death rate makes our protocol a suitable treatment regimen for the mainly elderly patients with advanced MDS and high-risk AML.

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Time Sequential Therapy (TAD-HAM) for Secondary AML with and without G-CSF Priming and Support – a Randomized Study

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Abstract. Secondary acute myeloid leukemias are generally treated like primary AML, yet with inferior remission rates and shorter remission duration. Unfavorable cytogenetics and expression of MDR have been identified as adverse factors, so far. We have tried to extend the concept of double induction chemotherapy from the AMLCG study on primary AML towards secondary AML. This preliminary report deals with 22 patients, age 23 to 73, treated since 1994 in two centers. One patient was excluded after randomization for comorbidity (G-CSF arm). The short term results show 6 out of 9 patients in CR in the G-CSF group, 2 death as opposed to the group without G-CSF, containing 6 responders, and 4 early deaths

Table 1. Patient data

No.	Sex	Age	G-CSF 1:yes	FAB	Result	CR	OS	Description
1	f	53	1	M4	CR	5	5	AlloBMT, †
2	m	56	0	M2	CR	4	9	Relapse, †
3	f	65	0	M5	CR	3	3	Death in CR, †
4	f	23	1	M2	CR	4	13	AlloBMT, relapse, †
5	m	66	0	M4	ED	0	2	With evidence of leukemia, †
6	f	48	0	M5a	ED	0	2	Sepsis, †
7	m	56	1	(excluded)				
8	f	68	1	M2	CR	14	16	
9	m	41	1	(RAEB-T)		CR	4	AlloBMT, †
10	m	72	0	M2	ED	0	1	Sepsis, †
11	m	70	0	M5a	CR	2	5	Relapse, †
12	m	57	1	M2	CR	9	12	Relapsed MDS
13	f	47	0	M2	CR	7	9	
14	m	71	0	M0	CR	5	8	Relapsed MDS
15	f	46	1	M2	NR	0	6	AlloBMT
16	m	71	1	M2	ED	0	2	†
17	m	68	1	M2	CR	14	17	
18	f	52	0	M2	NR	0	1	Sepsis, †
19	m	69	0	M4	CR	1	3	Relapse, †
20	f	68	1	M1	CR	3	5	
21	f	69	0	M1	NR	0	1	†
22	f	59	0	M0	ED	0	2	i.c. hemorrage, †

Patient number 7 was excluded, due to contraindications against aggressive chemotherapy occurring after randomization.

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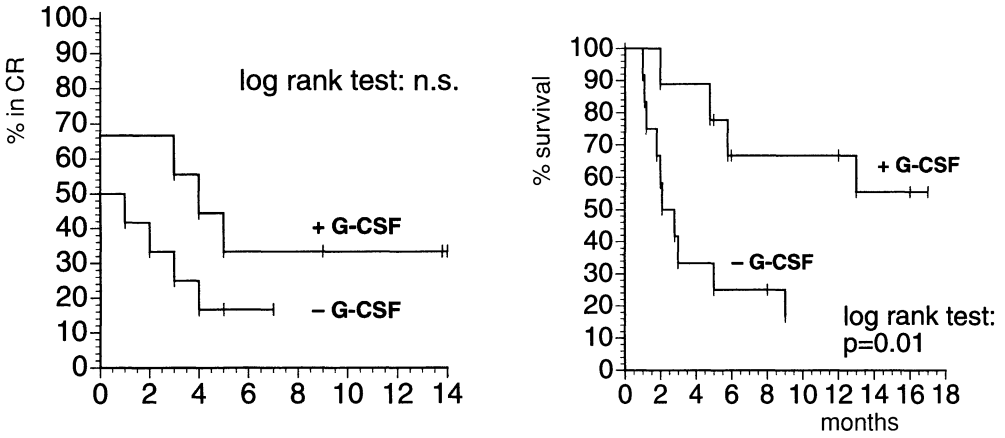

Fig. 1A, B. *A* Remission duration. *B* Survival

Table 2. Statistical summary

	G-CSF	no G-CSF
Patients/average age	9	12
Median age	56.9 y	62.2 y
Complete remission	6	6
Non-responder	2	2
Early death	1	4

Twelve out of 21 patients reached a complete remission. The differences between both arms are not significant, although the early death rate is higher and the CR rate lower without G-CSF treatment. No major toxicity one to G-CSF was detected. Pretreatment with G-CSF seems to have no adverse effects. More significant numbers are expected with continuation of the study.

Introduction

Secondary acute myeloid leukemias are generally treated like primary AML, yet with inferior remission rates and shorter remission duration [1]. Unfavorable cytogenetics and expression of MDR have been identified as adverse factors, so far [2, 3]. We have tried to extend the concept of double induction chemotherapy from the AMLCG study on primary AML towards secondary AML. Pretreatment with r-metHu G-CSF was randomized in this study. G-CSF has been employed as supportive measure or concomitant to chemotherapy in previous trials, without negative effect [4, 5].

Materials and Methods

G-CSF pretreatment for 48 h and continuous G-CSF support were added in a randomized fashion for half of the patients. Induction chemotherapy consisted of ara-C 100 mg/m^2 continuously i.v. for 48 h, 100 mg/m^2 q12h day 3 to 8, daunorubicin 60 mg/m^2/day day 3 to 5, 6-TG p.o. 100 mg/m^2 day 3 to 9. For patients under 60 y, this was followed by HAM, ara-C 3g/m^2 q12h day 1 to 3, mitoxantrone 10 mg/m^2 day 3 to 5 at day 21 of treatment. For older patients, HAM was only given when bone blasts on day 16 exceeded 5%, and ara-C was reduced to 1 g/m^2 q12h. Consolidation was TAD as above. Maintenance therapy consisted of ara-C 100 mg/m^2 q12h day 1 to 5, combined with either daunorubicin 45 mg/m^2 day 3 and 4 (first cycle) or 6-TG 100 mg/m^2 day 1 to 5 (second cycle). TAD, HAM and maintenance therapy were preceeded by 48 h r-metHu G-CSF, 5 μg/kg qd, with further continuation during therapy and thereafter, until recovery from aplasia, only for patients randomized into the G CSF arm.

Results

Patients' characteristics were comparable in both groups. The median age was 56.9 y in the group with G-CSF versus 62.2 y in the

standard group. This preliminary report shows a tendency for longer remission duration for the group with G-CSF pretreatment, which currently is not significant. The log rank-test shows a significance level of $p < 0.02$ for a survival difference. However, this result is not adjusted for serial testing and might still be subject to a sampling error.

Conclusion

Due to the small sample sizes, no definitive conclusions can be drawn from these data yet. However, the current data suggest that G-CSF pretreatment and support might improve survival, and also the CR rate and the remission duration. Further corroboration by higher patient numbers is needed for definitive results. Positive effects of G-CSF have been described in higher patient numbers for chemotherapy of MDS and AML, albeit without randomized control group [6].

A sequential test is instituted for the earliest possible detection of statistically significant criteria for discontinuation of this trial.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Oral Chemotherapy for Acute Myeloid Leukaemia

T. RUUTU on behalf of the Finnish Leukaemia Group

Abstract. Two studies have been carried out to evaluate the role of a totally oral combination of etoposide, 6-thioguanine, and idarubicin (ETI) in the treatment of acute myeloid leukaemia (AML) in patients with advanced disease or in elderly patients. The drug dosages in ETI were: etoposide 80 mg/m^2 and thioguanine 100 mg/m^2 twice daily on days 1-5, and idarubicin 15 mg/m^2 on days 1-3, all given orally. Sixteen patients with advanced AML were given one to six ETI cycles. Four patients (25 per cent) achieved remission, and in addition clearing of the blood of leukaemic cells was seen in six other patients. In another study ETI was compared with 5-day TAD (thioguanine, cytarabine, and daunorubicin) in the treatment of patients over 65-years of age with newly diagnosed AML. According to randomization the patients were given two cycles of ETI or TAD, followed by maintenance with mercaptopurine and methotrexate. Fifteen of 25 patients randomized to ETI (60%) achieved remission with two ETI cycles, compared with 6 of 26 patients (23%) randomized to receive TAD ($p = 0.007$). The survival was significantly longer in the ETI arm. There were no significant differences in side effects. In conclusion, the totally oral ETI regimen has proven to be an effective and useful combination in the treatment of AML in patients with advanced disease and in elderly patients.

Introduction

Chemotherapy for acute myeloid leukemia (AML) is usually given intravenously. An alternative is oral administration of the drugs. This approach has been limited by the fact that most of the drugs used for the treatment of AML cannot be administered orally. The situation changed when idarubicin, a relatively new anthracycline which can be given by mouth, became available. The main disadvantage of oral treatment is variable absorption from the gut and therefore a potentially unpredictable effect. When the treatment is intended to be fully intensive with curative aim, the oral route is not the preferred way of administration. However, there are situations where oral induction and consolidation chemotherapy may have an important role. The toxicity of fully intensive treatment is considerable, and in some groups of patients this has to be avoided. For example, many elderly patients do not tolerate fully intensive treatment, and as cure is in those cases not a realistic goal, the quality of life becomes even more important than among younger patients who may be cured with intensive toxic treatments. The quality of life may be improved by avoiding infusions and by giving the treatment orally, and by treating the patient as far as possible on an ambulatory basis. A moderately intensive oral treatment might also be useful in the management of patients with advanced acute leu-

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Table 1. ETI treatment

Etoposide	80 mg/m^2	Twice daily	Days 1-5
Thioguanine	100 mg/m^2	Twice daily	Days 1-5
Idarubicin	15 mg/m^2	Once daily	Days 1-3

All drugs given p.o.

kaemia when the possibility of cure is remote. With these considerations in mind we designed a totally oral combination regimen consisting of etoposide, 6-thioguanine and idarubicin (ETI) (Table 1). We have studied the effects of ETI in the treatment of patients with advanced leukaemia [1] and elderly patients with newly diagnosed AML [2].

ETI for the Treatment of Advanced AML [1]

Patients

Sixteen patients with AML were treated. The age range was from 20 to 69 years, median 50 years. Two patients were in first relapse, eight in second relapse, two had primarily refractory disease and three refractory relapse, and in one case the disease was preceded by myelodysplastic syndrome. The FAB subtypes were: M1 2, M2 8, M3 1, M4 3, and M5 1. At diagnosis, 10 patients had a normal karyotype, four an abnormal karyotype, and in two cases the analysis was unsuccesful. The patients described as refractory did not respond to standard combinations of cytarabine and daunorubicin (7 + 3 or 9 + 3, daunorubicin in the dose of 50 or 60 mg/m^2).

Chemotherapy

ETI treatment was given at 3-week intervals. The aim was to give as many ETI cycles as needed to achieve remission up to a maximum of six cycles.

Results

A total of 51 ETI courses were given. In several cases the treatment had to be discontinued earlier than planned because of progressive disease or poor clinical condition. In most cases the dosages of the drugs had to be reduced in the later courses, usually due to cytopenias.

Four of the 16 patients achieved complete remission. Three patients with second relapse entered remission after 1-2 ETI cycles. One patient judged to have primarily refractory AML achieved remission after six ETI cycles. The patients who entered remission represented FAB types M1, M2, M3, and M5, one each. The relapse-free survival of the patients who entered remission was 1, 3, 4, and 7 months. Three patients relapsed and one died of intracerebral haemorrhage in remission. In addition to the remissions, marked antileukaemic effect, clearing of the blood of leukaemic cells, was seen in six other patients. The median survival in the whole patient group was four months, range 1-11 months.

The side effects, graded according to the WHO scale, are presented in Table 2.

Table 2. Side effects of ETI treatment

		Percentage of ETI cycles
Infection	grade I-II	19
	grade III-IV	36
Vomiting	grade I-II	37
	grade III-IV	9
Diarrhoea	grade I-II	32
	grade III-IV	7
Positive fecal blood test		25

Infection was clearly the most serious side effect. An infection was encountered after 55 % of the ETI cycles. Severe grade III-IV infection was seen after slightly more than a third of the cycles. Marked vomiting or diarrhoea, grade III-IV, was seen after less than 10% of the courses. Fecal blood was demonstrated after 25% of the cycles, but macroscopic melena was not observed in any case. Congestive heart failure was seen in one patient. It responded well to standard treatment. The patient had a major general infection which was probably the main contributing factor. The patient had previously received daunorubicin 450 mg/m^2. No heart problems were seen following further ETI courses in this patient. No clear liver toxicity was observed in any patient.

The main problem during ETI treatments was cytopenias which were largely caused by the advanced disease. In cases where granulocytes and platelets recovered to levels over 0.5×10^9/l and 50×10^9/l, respectively, this occurred after a mean of 17 days from the beginning of the treatment for both cell lines.

ETI for the Treatment of Newly Diagnosed AML in Elderly Patients: a Randomized Comparison with 5-Day TAD [2]

Based on the promising results with ETI in the treatment of advanced AML, the Finnish Leukaemia Group launched a randomized trial comparing ETI with a conventional treatment of moderate intensity, TAD with 5 days of intravenous cytarabine in standard doses and 1 day of daunorubicin, in the induction and consolidation treatment of newly diagnosed AML in elderly patients who were judged not to tolerate full-scale intensive treatment.

Patients

Patients over the age of 65 years with newly diagnosed AML were considered to enter the study. If the physician in charge judged the patient fit enough to receive moderately intensive treatment aiming at remission but not able to receive fully intensive standard induction and consolidation, the patient was eligible for the study. Patients with one or more of the following disorders were excluded from this study:

- Another disease which would essentially impair the prognosis
- Congestive hear failure which would prevent the administration of anthracyclines
- Severe liver or kidney disorder
- Another serious disease which would prevent the administration of the planned treatment
- Disorders which would prevent oral treatment or the absorption of drugs from the intestine
- Gastric or duodenal ulcer

The randomization was done centrally at the coordinating center, separately for each participating hospital and separately for those who had de novo AML and those who had preceding chemo-therapy or another haematological disorder.

Table 3. Patient characteristics

	ETI (n=25)	TAD (n=26)
Age		
Range	65-85	67-87
Median	72	74
Sex		
Male	10	14
Female	15	12
Performance status		
0	6	5
1	12	14
2	2	5
3	4	2
4	1	0
Disease		
New AML	19	19
Treatment-related AML	1	1
AML after myelodysplasia	5	6
FAB		
M1	7	4
M2	10	7
M3	0	1
M4	5	8
M5	3	4
M6	0	1
Unknown	0	1
White blood cell count (x10^9/l)		
Median	5.8	5.3
Range	0.8-305	1.4-206
Blast cell count (x10^9/l)		
Median	2.2	1.4
Range	0.0-295	0.0-206

Fifty-one patients were included in the study (Table 3). Their median age was 73 years, range 65-87 years. There were no significant differences between the treatment arms. Among patients with cytogenetic information, 11 of 21 patients in the ETI group and 10 of 23 in the TAD group had a normal karyotype. There were no clear differences in the presence of poor prognostic karyotype findings.

Chemotherapy

The patients were randomized to receive two cycles of either ETI or TAD (thioguanine 100 mg/m^2 p.o. and cytarabine 100 mg/m^2 i.v. twice daily on days 1-5, daunorubicin 60 mg/m^2 i.v. on day 5). If the number of leu-

kaemic cells had increased after the first cycle of treatment or if there was no reduction of leukaemic cells after two cycles as compared with the original findings, the patient was crossed over to receive one cycle of the induction treatment of the other arm. If the number of leukaemic cells had decreased or remained stable after the first cycle of the other randomization arm, another cycle of the same treatment was given. Thus, the patients could receive a total of two to four induction and consolidation treatments. Treatments were initiated at three weeks after the beginning of the previous cycle, or, if there was no recovery of the marrow at that time, immediately after the recovery of the marrow had clearly begun.

After the induction and consolidation treatments the patients received maintenance with mercaptopurine, 70 mg/m^2 orally once daily, and methotrexate, 12 mg/m^2 orally once weekly. The doses were modified in case of marked cytopenia. In patients with remission in the bone marrow the maintenance was initiated when peripheral blood cell counts fulfilled the criteria for remission. In patients not in remission, maintenance was started as soon as the peripheral blood cell counts permitted. Maintenance was continued until relapse, progression of the disease, or for three years.

Results

Twenty-five patients were randomized to receive ETI and 26 to receive TAD. In the ETI arm six patients out of the 25 entered remission after the first cycle and nine more patients after the second cycle. A total of 15 patients out of 25 (60%) had achieved remission after two cycles. In the TAD arm four patients entered remission after the first cycle and two more after the second cycle. A total of six patients out of 26 (23%) achieved remission with one or two cycles of TAD. One patient randomized to ETI and six randomized to TAD died during the first induction cycle. The difference between the two arms in the remission rate was significant (p=0.007). Three patients originally randomized to receive ETI were later crossed over to the TAD arm and one of these patients entered remission with one cycle of TAD. Eight patients were crossed over from the TAD arm to the ETI arm, and two of these patients entered remission with ETI, one with one cycle and the other with two cycles. Two patients randomized to the ETI arm and one randomized to TAD entered remission during the maintenance treatment, and one patient originally randomized to TAD achieved remission later with another treatment. Thus, three patients randomized to ETI and four randomized to TAD achieved a remission with a treatment other than the originally randomized induction-consolidation treatment.

Figure 1 shows the survival in the two arms. The median survival in the ETI arm was 9.9 months and in the TAD group 3.7 months. The survival in the ETI arm was significantly better (p=0.042). Two patients in the ETI arm died in remission of non-leukaemic causes, one by suicide and the other of another cancer.

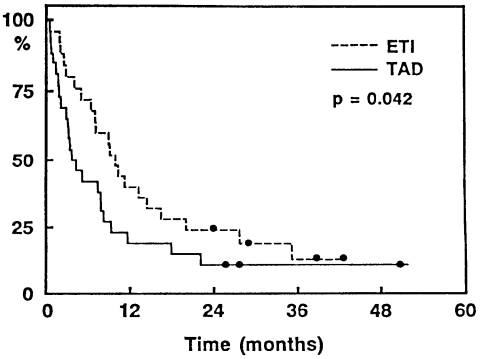

Fig. 1. Survival in the two treatment arms. The *black circles* indicate surviving patients [2]

Figure 2 shows the relapse-free survival in the two arms. The median relapse-free survival was 7.2 months in the ETI arm and 2.7 months in the TAD arm. The trend towards better relapse-free survival among the ETI patients was not statistically significant.

There were no significant differences in the toxicity of the two randomized treatments. No differences were observed in infections, gastrointestinal toxicity, duration of neutropenia or thrombocytopenia, days with infusions, days in hospital, or numbers

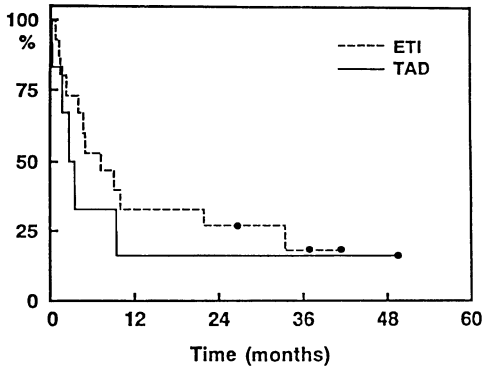

Fig. 2. Relapse-free survival in the two treatment arms. The *black circles* indicate patients alive and in first remission [2]

of red cell or platelet units transfused (Table 4). Neither were there any differences in performance status, bleeding, nausea or vomiting, blood cell nadirs, days with fever, presence of fecal blood, or cardiotoxicity.

Discussion

These studies have shown that totally oral induction and consolidation treatment of AML is a feasible approach. Twenty-five % of patients with advanced leukaemia achieved a complete remission, and a 60% remission rate was observed among patients over 65 years of age with newly diagnosed AML. The latter result compared favourably in a randomized study with the results obtained with a conventional mainly intravenous treatment of moderate intensity.

The high remission rate in the ETI group of the randomized trial was somewhat unexpected. The patients in the study were selected in that patients in poor general condition were excluded. On the other hand, patients in best condition, between 65 and 70 years of age and considered capable of tolerating full scale intensive treatment, were also excluded, and therefore the patient population represents elderly patients in relatively good condition excluding the most fit ones.

If the unexpectedly good antileukaemic effect of ETI, originally planned to be an only moderately intensive treatment for patients who cannot be treated fully intensively, is confirmed in later studies, what might

Table 4. Toxicity of the first cycle

	ETI	TAD	
Infection[a]			
Grade 0	1	2	
Grade 1	4	3	
Grade 2	9	9	NS
Grade 3	10	9	
Grade 4	1	3	
Gastrointestinal toxicity[a]			
Grade 0	7	9	
Grade 1	5	6	
Grade 2	11	8	NS
Grade 3	2	2	
Grade 4	0	1	
Days with neutrophil count <0.5 x 109/l			
Median	22	21	NS
Range	13-64	0-52	
Days with platelet count <50 x 10^9/l			
Median	18	15	NS
Range	0-50	6-47	
Days with infusions			
Median	19	18	NS
Range	0-52	0-78	
Days in hospital			
Median	26	23	NS
Range	20-64	15-82	
Red cell transfusions (units)			
Median	7	6	NS
Range	3-11	2-19	
Platelet transfusions (units)			
Median	23	24	NS
Range	0-216	6-68	

[a]WHO scale

the good efficacy be due to? Thioguanine has been widely used in the induction and consolidation of AML. Etoposide has also been used extensively for this disease, but mainly administered intravenously [3]. Idarubicin is a relatively new anthracycline that has been increasingly used in the treatment of AML. Most studies comparing intravenous idarubicin with daunorubicin have indicated that idarubicin is more effective in the doses used [4-8]. Idarubicin differs in several respects from daunorubicin. Idarubicin is more lipophilic and its uptake into cells is higher [9]. It seems to pass the blood-brain barrier more effectively, although the clinical relevance of this is unknown [10]. Its main metabolite idarubicinol is present at a high concentration, and the cytotoxicity of

this metabolite is greater than that of daunorubicinol [10]. Idarubicinol may have an important role in the effect of idarubicin treatment [9]. Idarubicin may be less affected by P-glycoprotein mediated multidrug resistance than daunorubicin [11]. Mainly preclinical evidence indicates that idarubicin may be less cardiotoxic than daunorubicin in relation to haematopoietic toxicity [12].

Idarubicin is the first anthracycline that can be given orally. Its absorption from the gut is variable, the bioavailability being about 30% in the average [9]. The pharmacokinetics of oral idarubicin differs somewhat from that after intravenous administration [9]. Idarubicinol is formed to a considerably greater degree after oral than intravenous administration [9]. It has been suggested that gastrointestinal toxicity might be more common after oral administration [9], but we did not see any increased toxicity as compared with a conventional intravenous treatment. There is both preclinical and clinical evidence that cardiotoxicity might be less after oral than intravenous administration of idarubicin [9]. It is not clear why this is the case, but the reason might be lower plasma peak concentrations [13]. The production of high amounts of a idarubicinol with its half-life longer than that of the parent compound might be one explanation for the good efficacy of oral idarubicin [9], and therefore also of ETI.

The toxicity of ETI did not differ from that of 5-day TAD. The length of cytopenias was similar, and there was no difference in non-haematological toxicity. Therefore the higher remission rate and better survival were not achieved at the cost of increased toxicity.

Oral idarubicin has been used either alone or in combination with other usually parenteral drugs, often cytarabine, to treat newly diagnosed or relapsed AML [14-21]. Oral idarubicin has also been used in the treatment of myelodysplastic syndromes [17, 22-25]. The results have varied according to the stage of the disease and the combination used (reviewed in [9]. To our knowledge there are no reports in addition to our studies of the use of oral etoposide and oral idarubicin in combination.

The choice of treatment for AML in elderly patients is often problematic. AML in this age group is characterized by unfavourable prognostic features which are related both to the patient and the disease [26, 27]. The patients often tolerate less well intensive chemotherapy due to underlying diseases, organ dysfunction or age-related modification of drug pharmacokinetics. AML at advanced age is often preceded by a myelodysplastic syndrome, and the proportion of patients with unfavourable cytogenetic findings is higher than among younger patients [27]. For these reasons the results of chemotherapy in this age group are relatively poor. Achieving complete remission is prognostically favourable and should be the aim of the treatment [26]. However, the intensity that can be applied has to be considered individually. Some elderly patients are in good general condition and can be given full intensive treatment, but in many cases this is not possible, and then a moderately intensive treatment protocol is preferable. As cure is in these cases not a realistic goal, quality of life aspects become especially important, and oral treatment becomes attractive.

The original idea in designing the ETI regimen was to have a treatment that could be given on an outpatient basis. We wanted to compare resource utilization as well as quality of life aspects in our randomized study. However, this was not successful. The first chemotherapy cycle is not optimal for the demonstration of potential benefits of oral treatment in these respects as the hospitalization of a patient with fresh acute leukaemia is usually necessary regardless of the mode of treatment, and there are initially often other causes for intravenous infusions in addition to the administration of cytotoxic drugs, such as the treatment of infection. Comparisons between subsequent cycles in the two arms were invalidated by the difference in remission rate after the first cycle. In general, an advantage of oral over intravenous treatment in the length of the stay at hospital and the need for infusions would be more likely to appear in the consolidation phase than in the induction.

To study further the possible advantages of oral consolidation treatment, the Finnish Leukaemia Group has initiated a new study of the treatment of AML in patients over 65

years of age with de novo AML. The inclusion criteria are as in the previous randomized study. To guarantee a uniform first treatment and to avoid any variability in the absorption of oral drugs, all patients first get the same intravenous treatment, cytarabine 100 mg/m^2 twice daily with 12 h intervals on days 1-6, and idarubicin 12 mg/m^2 on days 4 and 6. Thereafter the patients are randomized to receive either two cycles of ETI or two cycles of TAI (thioguanine and cytarabine as in 5-day TAD, and in addition idarubicin 12 mg/m^2 on day 5). The randomization is stratified according to whether the patient is in remission after the first cycle or not. Maintenance treatment is given with mercaptopurine and methotrexate. In addition to parametres reflecting antileukaemic effect, days spent at hospital, days with infusions, and toxicity are evaluated.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Oral Idarubicin in the Treatment of AML

J.-L. Harousseau

Abstract. The current treatment of Acute Myeloid Leukemia (AML) is based on intensive chemotherapy at different steps (induction treatment, consolidation, treatment of relapse). Since these strategies induce prolonged and severe myelosuppression, all patients are nursed in specialized units and receive a number of intravenous supportive treatments (antibiotics, antifungal treatments, red blood cell and platelet transfusions, parenteral nutrition, etc.) which are administered through a central line. Therefore the place of oral chemotherapy appears very limited in the overall management of AML.

However, in some cases, giving an orally effective antileukemic drug partly on an outpatient basis could be an attractive alternative. These conditions include palliative therapy of relapsed or refractory AML, consolidation (or maintenance) therapy and induction therapy in poor-risk patients. Elderly patients represent the most important cohort in the latter category.

Treatment Strategies in Elderly Patients with AML

The treatment of AML in patients over the age of 60 remains very disappointing [1]. While remission rates of 80% are commonly achieved in younger patients, complete remission (CR) rates decline with increasing age. This is partly due to a lower tolerance of infectious and hemorrhagic complications and to extrahematologic side-effects of chemotherapy [2, 3]. However, the poorer prognosis seen in elderly patients is also explained by intrinsic differences in the biology of leukemia. Unfavorable FAB subtypes, unfavorable cytogenetic abnormalities, cytological evidence of trilineage abnormalities and preexisting myelodysplasia are more frequent in the elderly [2, 3].

Early studies suggested that age had no effect on the duration of remission. However, with the increasing use of intensive post-remission therapy, increasing age adversely affects the duration of remission because elderly patients cannot tolerate the repeated courses of intensive consolidation therapy used in younger patients [3].

Thus, while most cases of AML occur in patients over the age of 60 [4], the prognosis for these patients remains dismal. This explains why many older patients are not included into clinical trials and why, in a substantial proportion of cases, no treatment is offered beyond supportive care [3]. This purely palliative approach is justified by an unacceptable toxic death rate and by the fact that even if a remission is obtained after prolonged hospitalization and life-threatening complications, it is often very short-lived and spent mostly in hospital. Economical

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considerations as well as ethical issues must be raised when deciding the type of treatment in the elderly.

A careful analysis of prognostic factors should help to make a decision. Beside age, these factors include performance status, white blood cell count, preexisting myelodysplasia, adverse prognosis cytogenetic abnormalities.

Three strategies are currently debated.

Intensive Chemotherapy. The aim of the treatment is to achieve and maintain CR whatever the risks and the costs. This would be the first choice for elderly patients with a good performance status and with an acceptable expected cure rate.

Attenuated Dose Chemotherapy. The aim of the treatment is to prolong survival by achieving complete or partial remission, but with reduced risks and at lower costs. This would the first choice for patients in relatively good condition but with a poor prognosis.

Palliative Approach. The aim of the treatment is not to induce remissions but to reduce the time spent in hospital and to favour quality of life. This approach would be preferable in patients with a poor performance status and unable to tolerate any chemotherapy.

Intensive Induction Chemotherapy in Elderly Patients with AML

The high toxic rate during aplasia following intensive chemotherapy partly explains the lower remission rates seen in elderly patients [5]. Hematopoietic growth factors (G-CSF or GM-CSF) have been introduced in the treatment of elderly patients with AML with the objectives of shortening the period of chemoinduced neutropenia, reducing the incidence and the severity of infections and increasing the CR rate.

At least six placebo-controlled trials [6-11] have recently addressed the issue of the impact of G-CSF or GM-CSF in the induction treatment of AML in the elderly (Table 1). In all these studies a significant reduction of time to neutrophil recovery has been observed in the groups of patients receiving the growth factor. However, in only 2 of them was this pharmacodynamic effect associated with a higher CR rate [6, 8].

The Role of Attenuated-Dose Regimen in the Treatment of Elderly Patients with AML

In an attempt to reduce the early death rate observed with intensive induction treatments, dosage reduction has been investigated in several trials. Fifteen years ago, Yates et al. reported a higher remission rate and a lower toxic death rate in patients treated with reduced doses of daunorubicin (47% and 41% respectively with 30 mg/m^2/day versus 31 and 54%, respectively, with 45 mg/m^2/day) [12]. Similarly Kahn observed a lower incidence of early death with an attenuated dose DAT regimen as compared to the full dose DAT regimen (15 versus 55%) [13].

Low-dose cytarabin (LD-Ara-C) has been considered as an attractive approach in the treatment of myelodysplastic syndromes and of AML in the elderly. In vitro studies had shown that LD-Ara-C could induce differentiation of leukemic cells. Thus, it was hoped that CR could be achieved using LD-ARA-C without the life-threatening complications of chemoinduced bone marrow aplasia, with less transfusions and shorter hospital stays. However, in a meta-analysis of 53 publications on the role of LD-Ara-C in AML, Cheson et al. found an overall CR rate of only 32% [14]. When focusing on the compiled results achieved in elderly patients, Powell et al. found a CR rate of 35% [15]. Moreover, in a majority of cases, LD-Ara-C did not induce differentiation of leukemic cells but rather had a cytotoxic effect.

Tilly et al. conducted a randomized multicenter trial comparing LD-Ara-C with an intensive conventional chemotherapy [16]. Forty one patients over the age of 65 received LD-Ara-C (20 mg/m^2/day for 21 days) and 46 received a combination of rubidazone and conventional dose Ara-C (200 mg/m^2/day for 7 days). The CR rate was higher after intensive chemotherapy (52 versus 32%). However, the toxic death rate

Table 1. G or GM-CSF in the therapy of AML in elderly patients. Result of placebo-controlled studies

Reference	No. of patients	Type of chemotherapy	Growth factor	Start of study	Results
Rowe [6]	117 elderly patients (55-70)	DNR + ARA-C (3 + 7)	GM-CSF 250 Êg/m^2	D11 (bone marrow aplastics without blasts)	- Shorter time to neutrophil recovery (13 vs. 17D $p = 0.001$) - Higher CR rate (60 vs. 44% $p = 0.08$) - Lower therapy-related morbidity and mortality - Longer median survival (10.6 vs. 4.8 m, $p = 0.048$)
Stone [7]	388 elderly patients (> 60)	DNR + ARA-C (3 + 7)	GM-CSF 5 µg/K	D8	- Shorter time to neutrophil recovery (15 vs. 17D $p = 0.02$) - No difference in CR rate or in treatment-related mortality rate
Dombret [8]	173 elderly patients (> 65)	DNR + ARA-C	G-CSF 5 µg/K	D8	- Shorter time to neutrophil recovery (21 vs. 27D $< p$ 0.001)- Higher CR rate (70 vs. 47% $p = 0.002$)
Godwin [9]	193 elderly patients (> 55)	DNR + ARA-C (3 + 7)	G-CSF	D11 (< 5% blasts in bone marrow)	- Shorter time to neutrophil recovery - No difference in morbidity and mortality - No difference in CR rate
Witz [10]	244 elderly (55-75)	IDR + ARA-C	GM-CSF 5 µg/K	D + 1 → end neutropenia	- Reduced time to neutrophil recovery ($p = 0.0001$) - Longer DFS ($p = 0.011$) - Longer survival in patients < 65 y
Lowenberg [11]	326 elderly (> 60)	DNR + ARA-C	GM-CSF 5 µg/K	DO → end neutropenia or D28	- Reduced time to neutrophil recovery ($p = 0.001$) - No increase of CR rate, DFS or overall survival

was also higher (31 versus 10%) while partial remissions were more frequent with LD-Ara-C (22 versus 2%). As a consequence, there was no difference in overall survival between the two groups. Patients treated with LD-Ara-C required fewer transfusions and had a shorter hospital stay.

Rationale for Using Oral Idarubicin in Elderly Patients with AML

Anthracyclins are the cornerstone of induction therapy in AML. Intravenous Idarubicin (IDR) has proven effective and in three randomized studies was superior to daunorubicin administered at conventional doses [17-19].

Idarubicin is the only anthracyclin in current use that can be orally administered. Idarubicin is rapidly absorbed following oral administration and peak plasma concentrations are achieved between 2 and 4 h after ingestion [20-22].

Although interpatient variability for bioavailability of oral IDR covers a wide range, most studies have reported an average bioavailability of around 30% [20-22]. However, the actual bioavailability should take into consideration both IDR and its active metabolite idarubicinol. The active metabolite may compensate for reduced bioavailability, yielding to an overall cytotoxic activity not inferior to that obtained by the i.v. route [22]. In a randomized trial on 100 patients with de novo AML (median age 41) Bezwoda et al. compared oral IDR (20 mg/m^2/day for 3 days) with intravenous doxorubicin (30 mg/m^2/day for 3 days) with identical dosages of Ara-C (100 mg/m^2/day for 7 days). The CR rate was 67% with IDR and 58% with doxorubicin. Although the difference was not significant, the results were in favour of the oral anthracyclin.

Phase II Studies of Oral Idarubicin in Elderly Patients with AML

In a pilot study in poor-risk patients, Lowenthal treated 9 patients aged 69-86 with oral IDR at a dose of 20-25 mg/m^2/day on 3 consecutive days and obtained only 6% CR [24]. Keldsen treated 18 patients aged 63-86 with lower doses (15-20 mg/m^2/day for 3 consecutive days) and obtained 22% CR [25]. Pagano used a combination of oral IDR (25 mg/m^2/day for 3 consecutive days) plus Ara-C (100 mg/m^2/day given subcutaneously for 7 consecutive days) [26]. Out of 17 patients aged 62-86, including 6 patients with relapsed AML, 47% achieved CR.

Finally, we conducted two consecutive studies of oral IDR in patients over the age of 65 [27, 28].

In the first study, IDR was administered at a daily dosage of 30 mg/m^2 for 3 consecutive days [27]. Twenty patients aged 65 to 79 (median age 72) with previously untreated de novo AML were included in this study. Five patients whose bone marrow was still blastic on day 14 received a second course. Eight patients (40%) achieved CR (6 after a single course). There were 1 early death, 4 deaths in aplasia and 7 failures.

Extrahematologic toxicity was mild, with 6 patients experiencing grade 3-4 WHO nausea and vomiting, and only 2 patients suffering from grade 3 mucositis.

For patients who attained CR the median duration of neutropenia was 19 days (range 12 to 34) and the median duration of thrombocytopenia was 18 days (4 to 40 days). All but one patient had to stay in hospital.

We concluded that oral IDR was an effective therapy for AML in elderly patients but that the total dose of 90 mg/m^2 was too toxic to be administered safely outside the hospital.

In the second study, we used a combination of oral IDR at lower doses (20 mg/m^2/day for 3 consecutive days) and standard dose Ara-C (100 mg/m^2/q 12 h given subcutaneously for 10 consecutive days) [28]. Thirty-two patients aged 65 to 82 years (median 76) with de novo AML were included in this study. Eight patients whose bone marrow was still blastic on day 20 received a second course with 3 days of IDR and 5 days of Ara-C. Complete remission was achieved in 13 cases (40.5%) (12 patients after a single course). There were 1 early death, 3 deaths in aplasia, 2 partial remissions and 13 failures. Again, the extrahematologic toxicity was mild with only 1 patient experiencing grade 3 nausea and vomiting. All but 5 patients

were entirely managed in hospital. The median duration of neutropenia was 18 days (0-36) and the median duration of thrombocytopenia was 7 days (2-40). We concluded that these results were comparable to those obtained with conventional dose chemotherapy and that this combination could be considered as an effective attenuated dose regimen.

Randomized Comparison of a Totally Oral Induction Treatment with an Attenuated Dose TAD

Ruutu et al. addressed the issue of the efficacy of an oral induction and consolidation regimen in patients fit enough to receive an attenuated dose regimen but not able to receive fully intensive standard induction and consolidation [29].

Fifty-one patients with a median age of 73 years (range 65-87) were included in the study. They were randomized to receive either a totally oral ETI regimen (25 patients) or conventional 5-day TAD regimen (26 patients). Thirty-eight patients had de novo AML, 11 patients had AML subsequent to myelodysplastic syndrome and 2 patients had therapy-related AML. ETI consisted of VP16 (80 mg/m^2/day) and thioguanine (100 mg/m^2/day twice dally) on days 1 through 5, and IDR 15 mg/m^2/day on days 1 through 3. TAD consisted of oral thioguanine and IV Ara-C, both in the dose of 100 mg/m^2 twice daily on days 1 through 5, and daunorubicin 60 mg/m^2 on day 5. The two groups were well balanced regarding the initial clinical characteristics. In the ETI group, CR was achieved in 15 patients (60%) (6 after the first cycle, 9 after a second identical cycle). In the TAD group, only 6 patients (23%) achieved CR (4 after the first cycle, 2 after the second cycle). The difference between the two induction treatments was significant (p = 0.007). There were no difference in gastrointestinal toxicity, duration of neutropenia or thrombocytopenia, days in hospital, days with IV infusions, number of red cell or platelet transfusions. Patients in CR received a consolidation with the same treatment. The median survival was 9.9 months in the ETI arm and 3.7 months in the TAD arm. The survival was significantly longer in the ETI arm (p = 0.042).

The authors concluded that the totally oral ETI regimen resulted in a significantly higher CR and longer survival than the 5-day TAD regimen with no more toxicity.

Conclusion

Some patients with AML are suitable only for attenuated chemotherapy regimen. Within this group, elderly patients probably represent the largest cohort.

Before deciding the type of treatment to use in patients over the age of 60-65, a thorough analysis of prognostic factors is necessary. These factors include performance status, FAB classification, preexisting myelodysplasia and cytogenetics. They help to define which patients are candidate to an intensive treatment given with a curative intent, which are candidate to an attenuated dose chemotherapy, and which are suitable only for a palliative approach. If an attenuated regimen is chosen, oral IDR offers the opportunity to propose an effective and well-tolerated induction therapy and could contribuate to a reduction of the time spent in hospital.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Acute Myeloid Leukemia in Patients Aged Over 75 Years: Clinico-Hematological Characteristics and Treatment Results

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Abstract. We retrospectively analyzed a cohort of 62 consecutive patients with AML aged more than 75, diagnosed between January 1986 and December 1995. The median age was of 79 (range 76-86). Thirty-nine patients were male and 23 female. In 9 patients a myelodysplastic syndrome preceded the onset of AML. Trilinear myelodysplasia was present in 26 patients (42%). In eleven cases (18%) a diagnosis of hypocellular leukemia was made. An active infection was found in 10 patients (16%). Three patients were in PS 1, 24 in PS 2, 27 in PS 3, and 8 in PS 4. An aggressive chemotherapy regimen, i.e.an anthracyclin plus cytarabine (ARA-C), was given to 22 patients (35%); 7 patients (11%) received low-dose ARA-C (LD-ARA-C) and, finally, 33 (53%) were managed with supportive care only and/or hydroxyurea (HU) in case of leukocytosis higher than 30×10^9/l and/or severe bone pain. Therapeutic choice was significantly influenced by PS ($p = 0.03$), absence of infection ($p = <0.0001$), severe co-morbid disease ($p = <0.0001$), and diagnosis of hypocellular AML ($p < 0.001$). Complete remission (CR) was obtained in 7/22 patients aggressively treated (32%), 0/7 in the group receiving LD-ARA-C, and in one patient treated with HU. The median survival for the whole patient population was of 14 weeks. There was no significant difference among the three treatment groups (12, 12 and 15 weeks, respectively, $p = 0.23$). However, the outcome was significantly better for patients achieving CR (median survival 32 weeks). All patients managed with aggressive induction experienced profound cytopenia needing intensive support and prolonged hospitalization (median 41 days) that is significantly longer as compared to the remaining two groups (12 and 7 days, respectively, $p = 0.0002$). We conclude that intensive chemotherapy aiming at CR achievement is not appropriate for most of very elderly patients with AML being costly, unpleasant and requiring prolonged hospitalization. However, a minority of patients seems to take advantage from an aggressive approach, therefore any effort should be made to preliminary identify this subset at diagnosis.

Introduction

Acute myeloid leukemia (AML) occurs predominantly in the elderly, more than half of all cases being diagnosed in patients aged over 60 years and more than one third over 70 [1-5]. The chronological age limits for identifying an AML patient as "elderly" are still controversial since thresholds of 55 to 70 years have been adopted in different series [6]. However, even in the absence of a general agreement, most investigators would agree that is reasonable to consider as "elderly" those AML patients aged 60 years or more. As matter of fact, most reports deal-

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ing with elderly AML patients adopt a cut-off point of more than 60 years [6, 7]. Given the progressive aging of the general population, the frequency of the disease in the elderly is expected to further rise in the future, namely in individuals with very advanced age, i.e. over 75 years old. In this latter subset, although clinical experience suggests that hematologists tend to be rather reluctant to submit patients to intensive treatment programs, quantitative information concerning the rate of inclusion into chemotherapy trials as well as the clinical outcome is substantially lacking [8]. Because the proper management of these patients is likely to gain increasing clinical relevance in the future, it is important to provide data on the clinical characteristics and therapeutic results currently achieved in these extremely aged AML patients. Here we report our experience on a series of 62 consecutive AML patients older than 75 years observed at our institutions during the last decade.

Patients and Methods

Sixty-two patients aged > 75 were diagnosed as having AML between January 1986 and December 1995 at our institutions. This figure represents 10% of the whole AML patient population observed during the same period. Diagnosis was based on conventional morphologic and cytochemical FAB criteria [9]. In 38 cases (61%), immunophenotypic studies, performed as previously described [10], were also made. There were 39 males and 23 females. The median age was 79.3 years (range 76-86). Thirty-eight patients (61%) were aged between 76 and 80, while 24 (39%) over 80. Main clinical and hematological characteristics at diagnosis are summarized in Table 1. Three patients were in performance status (PS) 1, 24 in PS 2, 27 in PS 3 and 8 in PS 4, as assessed according to WHO criteria. In 9 patients (14%) a previously diagnosed myelodysplastic syndrome (MDS) preceded the onset of AML (3 AREB, 5 AREB-t and 1 CMML, respectively). However, in 26 patients (42%) morphological trilineage myelodysplasia (TrMDS), as defined according to the criteria of Brito-Bapapulle et al [11], was found. Eleven patients (18%) had a diagnosis of acute hypocellular leukemia defined by markedly reduced bone marrow cellularity, pancytopenia and low count or absence of blast cells in the peripheral blood [12, 13]. A decrease in serum albumin concentration (< 3 g/dl) was observed in 17 patients (27%), while a serum creatinine value > 2.5 mg/dl was found in 4 patients (6%). Seventeen patients (27%) presented with antecedent chronic disease requiring specific therapeutic intervention, while 10 (16%) had a documented active infection.

Table 1. Clinical and hematological characteristics at diagnosis

No.	62
Sex (M/F)	39/23
Age (years)	79 (76-86)
MDS	9 (14.5 %)
TrMDS	26 (42 %)
Hypocellular AML	11 (17.7.8 %)
PS 1 (WHO)	3 (4.8 %)
PS 2	24 (38.7 %)
PS 3	27 (43.5 %)
PS 4	8 (14.8 %)
Active documented infection	10 (16.1 %)
FAB M1-M2	26 (41.9 %)
FAB M4-M5	36 (58.1 %)
Blasts in PB ($\times 10^9$/l)	27 (0.04-240)
Hemoglobin (g/dl)	8.5 (5.5-12.2)
Platelets ($\times 10^9$/l)	55 (9-580)
Serum albumin < 3 g/dl	17 (27.4 %)
Serum creatinine > 2.5 g/dl	4 (6.4 %)
Serum LDH (IU/l)	792 (178-3581)

Twenty-two out 62 patients (35%) were selected for intensive induction therapy. Depending on the period of observation, i.e., before or after June 1991, induction treatment consisted of a combination of either daunorubicin (DNR) at the dose of 40 mg/m^2 intravenously (i.v.) on day 1 plus cytosine-arabinoside (ARA-C) at the dose of 100 mg/m^2 every 12 h from day 1 to 5 in 12 cases, or of idarubicin (IDA) (10 mg/m^2 i.v.) days 1 to 3 plus ARA-C in 10 patients, respectively. Post-remission treatment was given to all patients achieving CR. In patients treated with DNR, a second identical induction course was administered, while in the group treated with IDA consolidation consisted of one course of IDA (10 mg/sqm on day 1), ARA-C (100 mg/m^2 every 12 h from day 1 to 5) and etoposide (100 mg/m^2 from day 1 to 5). Seven patients (11%) received

Table 2. Clinical and hematological characteristics at diagnosis by different therapeutic approach

	intensive induction	LDARA-C	Supportive treatment	*p* value
No.	22	7	33	
Age	78.8	80.1	79.6	0.74
PS 1-2	12	2	11	
PS 3	10	4	15	0.03
PS 4	0	1	7	
Severe co-morbid disease	0	0	15	<0.0001
Active infection	0	0	10	<0.0001
Hypocellular AML	0	0	11	<0.0001
Blasts in PB ($\times 10^9$/l)	21	34	26	0.15
Serum LDH (IU/l)	753	721	811	0.24
Days of hospitalization	40	12	7	<0.0001

low-dose of ARA-C (LDARA-C) that is 10 mg/m^2 every 12 h for 15-21 days subcutaneously, and finally 33 patients (53%) were treated by supportive care only and/or hydroxyurea in the case of leukocytosis higher than 30 $\times$ 10^9/l. The reasons for exclusion from any treatment including LDARA-C were: (a) diagnosis of hypocellular leukemia in 11 patients (18%); (b) severe renal failure in 4 (6%); (c) preexisting co-morbid disease in 15 (24%); (d) active infection in 10 (16%); (e) PS 4 in 8 (13%). More than one of these features were simultaneously present in some patients. No well established criteria were adopted to assign patients to LDARA-C; possibility to administer the treatment in a outpatient setting, geographical reasons (distance from our institution) and relatives attitude were mostly discriminant in selecting between LDARA-C and supportive care. Age did not differ among the three groups (78.8 for intensive chemotherapy subset, 80.1 for LDARA-C patients, and 79.6 for patients receiving supportive treatment only, $p = 0.84$). On the contrary, PS was significantly different when the three groups were compared ($p = 0.03$). Table 2 summarizes main patient characteristics at diagnosis by different therapeutic approach. Survival curves were calculated according to the method of Kaplan and Meier [14], differences between survival curves were evaluated by the log-rank test.

Results

The median overall survival (OS) of the whole patient population was of 14 weeks and is depicted in fig 1. Complete remission (CR) was obtained in 7 out of 22 patients receiving intensive induction (32%). All patients achieving CR experienced profound pancytopenia requiring intensive support in terms of both blood and platelet transfusions. Among the 15 patients who failed to achieve a CR, hypoplastic death (death occurring during marrow hypoplasia) occurred in 5 cases (33%). Infection was the cause of death in 3 patients, while two patients died of cerebral hemorrhage. Primary drug resistance (defined either as remission induction therapy failing to produce significant bone marrow hypoplasia in patients surviving 15 or more days after therapy, or leukemic regrowth following aplasia) oc-

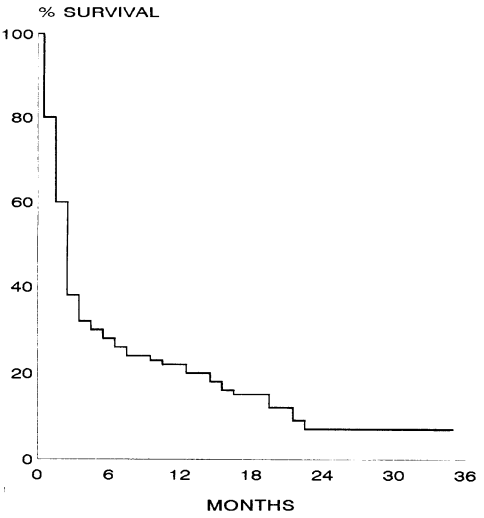

Fig. 1. Overall survival of the whole patient population (median 14 weeks)

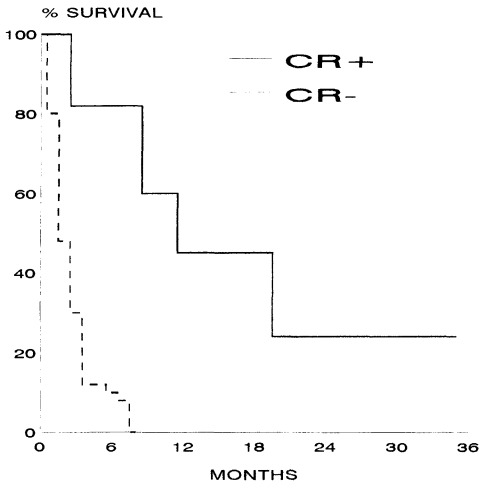

Fig. 2. Survival according to CR achievement (p = 0.009)

curred in 10 patients (45%). Extrahematological toxicity of grade > 2 occurred in 18 out of 22 patients and consisted of 15 episodes of mucositis, 13 of infections, 2 of neurologic toxicity, 1 of acute fatal renal failure and 1 of severe cardiac arrhythmia. The median OS and disease free survival (DFS) for this group of patients were of 12 and 28 weeks, respectively. One patient is still alive and in first CR at 36 months from diagnosis. The median survival for patients achieving CR was 32 weeks; CR achievement had a significant impact on OS, as showed in Fig. 2.

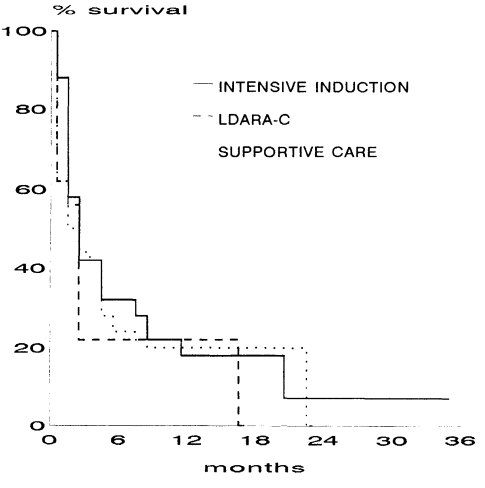

Fig. 3. Survival according to treatment (p = 0.43)

No patient treated with LDARA-C achieved CR. One died early in induction, while 6 of them were refractory. Five out of 7 patients were managed as outpatients. Median OS for LD-ARA-C patients was 12 weeks. Among patients receiving only support one patients achieved CR while in treatment with hydroxyurea; the median survival for this group was of 15 weeks. No patient with hypocellular variety received any kind of chemotherapy and median survival was of 8 months, that is significantly better as compared to the remaining patient population (p = 0.01). In Fig. 3 survival for different therapeutic approach is shown; the difference is not statistically significant (p = 0.43, according to the log-rank test). Patients receiving aggressive induction had a median hospitalization of 40 days (range 3-83), while those treated with LDARA-C or supportive therapy were hospitalized for a median of 12 and 7 days, respectively (range 2-28). The difference is highly statistically significant (p = 0.0001).

Discussion

A number of studies and reviews dealing with the management of elderly patients with AML have been published in the recent years. In these reports elderly is identified as age > 55, > 60 or, more rarely, > 70 years and no specific information is usually given as specifically concerns very elderly patients, i.e. those aged over 75 years [8, 15]. The incidence of AML is increasing in this latter age category due to either the progressive aging of the general population or to an improved referral of elderly patients to hematological departments for diagnostic work-up. Nonetheless, one should also consider that an undetermined number of these patients may not be referred to hematological institutions because of decisions stemming from either the general practitioner, or the family members (extremely advanced age, critical comorbid disease, very low-life expectancy).

In this study, we performed a critical analysis on a series of 62 consecutive AML patients aged more than 75 years observed at two different institutions in the south of Italy during the last decade. As concerns the

clinico-hematological characteristics at diagnosis, typical features of elderly AML patients were found. As matter of fact, high incidence of preceding MDS was present (14%) as well as a high frequency of trilinear dysplastic abnormalities also in apparently de novo cases, suggesting that in some patients a subclinical undiagnosed MDS could have preceded the onset of AML. In addition, 11 patients (14%) presented with hypocellular AML, an AML variety almost exclusively seen in individuals older than 50 years [12-13]. All these patients showed markedly hypocellular bone marrow and profound peripheral pancytopenia with low blast cell count. Although some authors suggested that aggressive treatment may be of benefit in selected patients with hypocellular AML [12], in the elderly the clinical behavior strictly resembles that of MDS (smoldering acute leukemia) rather than of typical AML. In our series, no patient with hypocellular variety received any kind of chemotherapy and median survival was of 8 months, that is significantly better as compared to the remaining patient population. Hence, we confirm that a watch and wait approach is probably the treatment of choice for elderly patients with hypocellular AML. Finally, a considerable number of patients presented with various degrees of hypoalbuminemia and renal failure. The latter in 4 cases (6%) was a main reason of exclusion from any aggressive treatment.

Twenty-two of 62 patients (35%) were selected for aggressive induction treatment. There was no difference as concerns the median age of patients who received intensive chemotherapy as compared to those managed with LDARA-C or supportive management. This is not surprising if we consider the restricted age range of our patient population (76-86 years). On the contrary, PS did significantly differ between the two groups, more than 50% of patients aggressively treated being in stage < 3, as compared to 72% of the remaining patients. The presence of co-morbid disease requiring specific treatment, namely cardiac or respiratory failure, was the additional main criteria addressing the therapeutic choice. Finally, possibility to administer the treatment in an outpatient setting, geographical reasons (distance from our institution) and relatives' attitude were usually discriminant for selecting between LDARA-C and supportive care. Thus, as usually happens in the management of elderly leukemic patients [16, 17], during our process of clinical decision making, we operated a relevant selection as concerns inclusion into chemotherapy trials, although these had been specifically developed for elderly AML patients. This may account for the relatively low rate of induction death (22%) observed in this study as compared to other series dealing with very elderly patients [15]. Notwithstanding, the CR rate was low (32%), mainly due to a relevant percentage of refractory cases (45%), confirming that the unsatisfactory therapeutical results usually obtained in AML of the elderly, apart from the impaired patient capacity to withstand the side effects of chemotherapy, are also due to peculiar biologic characteristics of leukemic cells [5, 18-20]. The median survival for the whole patient population was of 14 weeks. Overall, patients aggressively treated did not fare better than those receiving a less intensive approach. However, patients achieving CR had a significantly longer survival and one patient is still alive and in first CR at the time of writing at 36 months from diagnosis. In conclusion, while these results seem to confirm the general feeling that aggressive chemotherapy leading to bone marrow aplasia is not an appropriate therapeutic option for the majority of very elderly AML patients even though a relevant selection is operated at diagnosis, on the other hand they suggest that selected patients can take substantial advantage from intensive chemotherapy. We feel that the adoption of PS and absence of co-morbid disease as unique criteria to assign patients to intensive regimens is misleading. Therefore, any effort should be made to precisely identify additional prognostic tools useful to select at diagnosis the elderly patients in whom chemotherapy may result in a true improvement of CR achievement and survival. The low number of patients treated with intensive induction regimens in this series do not allow to draw any conclusion as concerns association between pretreatment characteristics and response. However, in a cohort of patients

aged more than 60, we have recently demonstrated that unfavorable karyotype and high serum LDH level are significantly related to CR achievement and duration in AML [21]. Other authors have investigated additional parameters resulted as having high predictive prognostic value [22-24]. Thus, it seems now possible on the basis of pre-treatment characteristics at diagnosis to identify a specific subset of elderly AML patients with substantially absent chance of achieving CR of prolonged duration. Alternative approaches including differentiating agents, new drugs able to circumvent the multiple drug resistance, growth factors in combination with old drugs [25] should be offered to the above patients, because conventional chemotherapy is in most cases ineffective as well as costly, unpleasant and associated with high mortality and morbidity rate.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Improved Results of AML Treatment in Adults during a 12-Year Period. A Population-Based Study

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Abstract. In the period 01. 01. 1984-31. 12. 1995 a total of 317 adult AML patients were diagnosed in the counties of Funen and Ribe, corresponding to an annual incidence rate of $4.8/10^5$. The median age of these patients was 68 years. Curative chemotherapy was administered to 203 patients (64%) with a median age of 62 years. The three consecutive 4-year periods were characterized by somewhat different principles of AML treatment. In the periods 1984-1987 and 1988-1991 most patients received daunorubicin-AraC, and aclarubicin-AraC, respectively, as induction therapy. In both periods three fourths of CR patients received high-dose AraC based post-remission therapy. In the period 1991-1995 almost all patients were treated with repeated courses of mitoxantrone and high-dose AraC. The CR rate increased from 43% to 77% in the study period. The duration of CR did not change over time, whereas a prolongation of survival was seen, indicating that around 20% of all patients with AML can now be cured.

Introduction

It has been estimated that, using standard induction therapy, comprising an anthracycline and cytosine arabinoside (AraC), and a post-remission regimen including high-dose AraC (HD-AraC), 25% of all adults with de novo AML will be cured [1]. This estimate presumably applies to younger patients in particular. Population-based data obtained before the introduction of HD-AraC as post-remission treatment point to much lower cure rates [2-4]. Notwithstanding the importance of randomized studies, population-based data from uniformly treated patients may contribute valuable background information regarding distribution of prognostic subgroups and outcome of therapy.

The present study describes the overall results of treatment in three differently treated complete cohorts of AML patients diagnosed in two Danish counties in three consecutive 4-year periods.

Material and Methods

Patients

We reviewed all cases of AML (de novo and non-de novo) diagnosed in the counties of Funen and Ribe (~550 000 inhabitants ≥ 15 years) from 01.01.1984 to 31.12.1995. The patient records were retrieved via the files of the hospitals and departments of pathology in the two counties.

Diagnosis

In both counties, AML was diagnosed by haemopathologists, often in collaboration

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with consultant haematologists. A morphological diagnosis of all patients treated with curative intent was made by or in collaboration with haemopathologists at Odense University Hospital (OUH).

Referral Pattern and Treatment

The Department of Haematology, OUH, is a tertiary referral center in a teaching hospital, situated in the county of Funen. As applies to the diagnostic evaluation, AML treatment of patients from the county of Ribe took place in collaboration with the Department of Haematology, OUH, which took care of all patients undergoing curative chemotherapy.

In the three consecutive 4-year periods from 1984 to 1996, we employed three different principles of chemotherapy: In period I (1984-1987) most patients received daunorubicin and AraC as induction therapy. In period II (1988-1991) most patients received aclarubicin and AraC. In both periods the majority of patients received HD-AraC based post-remission therapy. Almost all patients in period III (1992-1995) were treated with repeated courses of mitoxantrone and HD-AraC.

Statistical Evaluation

Survival curves were drawn according to the method of Kaplan-Meier. The logrank test and the logrank test for trend were used for comparison of survival and continuing complete remission times. Comparisons of proportions were performed using the Chi-square test.

Results

Patient Characteristies

A total of 317 adult AML patients were diagnosed in the 12-year period (1984-1996). This corresponds to an annual incidence rate of $4.8/10^5$.

De novo AML was seen in 299 cases, non-de novo AML in the remaining 18 patients (periods I, II, and III: 5, 3, and 10 patients, respectively). The patients are further characterised in Table 1.

Table 1. Patient's characteristics

	Period I	Period II	Period III
Diagnosed patients			
Number	106	106	105
Median age	67 years	67 years	67 years
Age range	19-92	15-89	19-90
Sex (M/F)	1.12	1.26	1.19
Treated patients			
Number (%)	67 (63%)	66[a] (62%)	70 (67%)
Median age	61 years	62 years	62 years
Age range	19-78	15-77	19-82
Sex (M/F)	1.03	1.87	1.12
Median follow-up time (months)	127	81	30

[a] Excluding two patients treated elsewhere.
Period I: 01.01.1984-31.12.1987.
Period II: 01.01.1988-31.12.1991.
Period III: 01.01.1992-31.12.1995.

Treatment

Overall, 203 patients received chemotherapy with curative intent. Except for two patients in period II who were treated elsewhere (and who died without attaining a CR) these patients make up all intensively treated AML patients from the two counties, corresponding to 64% of all patients with an AML diagnosis. The remaining patients received palliative treatment with hydroxyurea or supportive care only.

In period I (1984-1987) two-thirds of the treated patients received daunorubicin-AraC as induction therapy, one fourth received aclarubicin-AraC, and the remaining patients received HD-AraC/amsacrine/AraC/VP16 in various combinations. HD-AraC based post-remission therapy was administered to three fourths of CR patients.

In period II (1988-1991) two-thirds of the treated patients received aclarubicin-AraC as induction therapy, one fifth received mitoxantrone-AraC; the remaining patients received HD-AraC ± mitoxantrone/amsacrine. As in period I, three-fourths of the CR patients received HD-AraC based post-remission therapy.

In period III all patients except three received repeated courses of mitoxantrone and HD-AraC (20 mg mitoxantrone/d for 3 days and AraC 2 g every 12 h for 5 days). The remaining three patients received acla-AraC, dauno-AraC, and HD-AraC, respectively, as induction therapy. The CR patients in period III received on average 3 courses (range: 1-5) of mitoxantrone-HD-AraC.

Complete Remission Rates

Period I: 43%; period II: 50%; period III: 77%. In patients less than 60 years: period I: 56%; period II: 61%; period III: 88%. In patients over 60 years: period I: 31%; period II: 42%; period III: 68%. The differences between the CR rates in period III and those of periods I and II are highly significant (p= <0.001).

Toxic Deaths

Toxic deaths (including early death, death in hypoplasia, and therapy-related death in CR) were seen in 21, 17, and 9% in periods I, II, and III, respectively, the difference between period I and period III being statistically significant ($0.025 < p < 0.05$). No toxic deaths were seen in patients under 60 years of age in periods II and III.

Duration of Complete Remissions

The CR duration in the three study periods are shown in Fig. 1. It can be seen that the curves stabilize, corresponding to 30-40% of the CR patients being in continuing complete remission, with no difference between the periods ($p = 0.93$; logrank test).

Survival times

Figure 2 shows the survival curves of the patients receiving curative chemotherapy. Applying the logrank test for trend, a prolongation of survival in the course of the study period was seen ($p = 0.021$). The curves of periods II and III did not differ significantly.

The survival curves corresponding to all diagnosed patients are shown in Fig. 3. Again, application of the logrank test for trend showed prolongation of the survival over time. The curves of periods II and III did not differ significantly.

Discussion

The very few population-based data available suggest that 5-10% of all adult AML patients are cured [2-4]. Treating two-thirds of all patients in a well-defined AML population during three 4-year periods character-

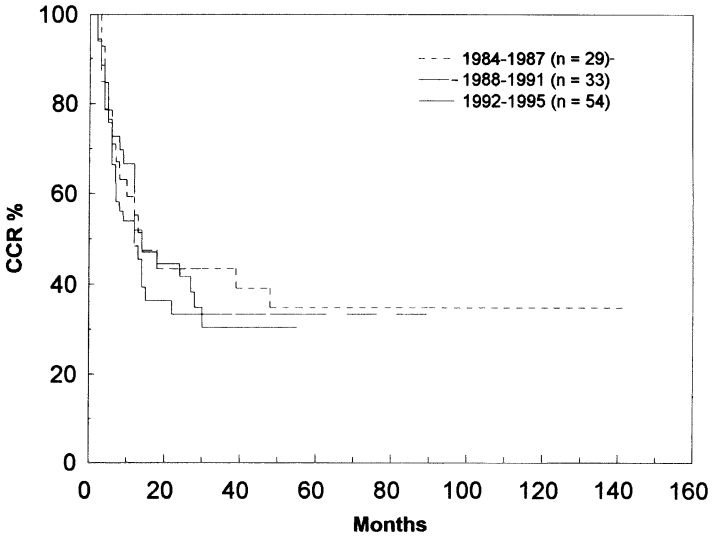

Fig. 1. Duration of complete remissions. *CCR* continuing complete remission

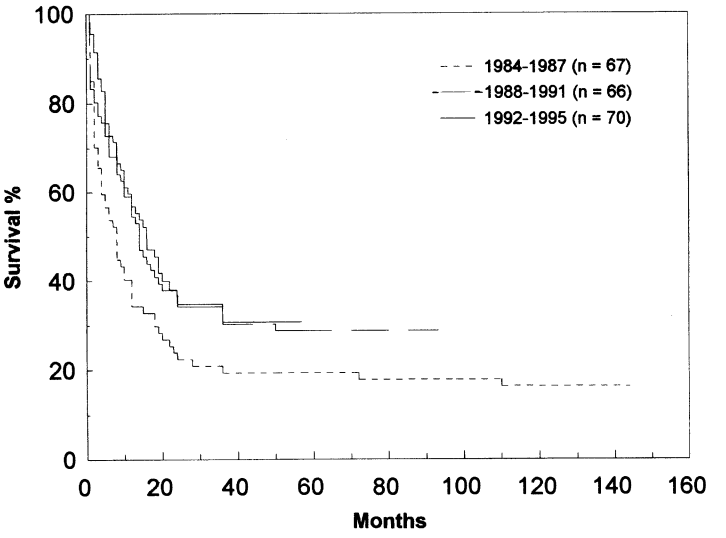

Fig. 2. Survival curves of all AML patients receiving curative chemotherapy

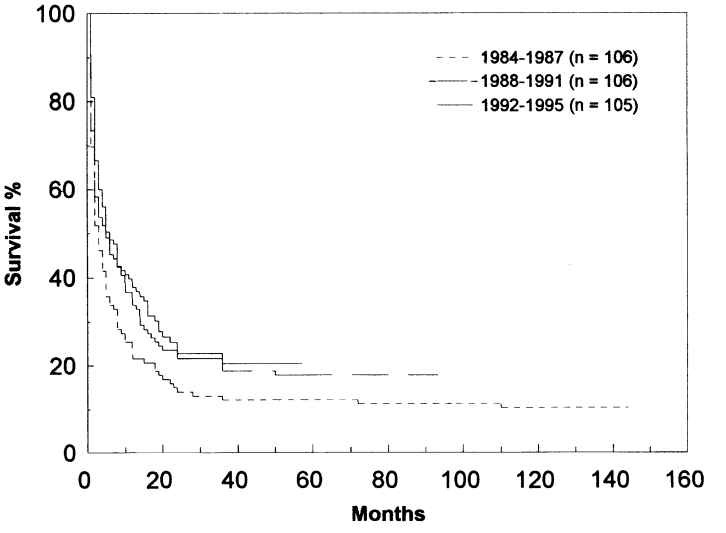

Fig. 3. Survival curves of all patients with a diagnosis of AML, whether treated or untreated.

ized by somewhat different principles of AML treatment, we obtained encouraging results in the last two periods, indicating that around 20% of all patients with AML can now be cured.

In the period 1984-1996, the CR rate increased from 43 to 77%. This remission rate is higher than expected in an AML population with a median age of 62 years [3-7]. The improved CR rate in period III was due to fewer patients with resistant disease and fewer toxic deaths. Uncontrolled observations suggesting a higher CR rate in patients treated up-front with HD-AraC [8] have not been substantiated by the results of randomized studies [9,10], and there are no data from randomized studies indicating that mitoxantrone is associated with a higher CR rate than achieved with generally used anthracyclines. However, a study comparing daunorubicin with mitoxantrone showed that patients in the mitoxantrone group were more likely to achieve CR with one induction course [5]. The declining toxic death rate in our patients may be a consequence of unappreciated changes in supportive care. It

is likely, however, that the favourable extramedullary toxicity profile of mitoxantrone [11] is of greater importance.

Our fixed AraC dose, roughly corresponding to 10 doses of 1.2 g/m^2, combines favourable cellular pharmacology [12] with acceptable toxicity.

There is reason to suggest that intensified induction therapy prolongs remission duration [9]. The unchanged CR duration over time in the present study may be a consequence of more high risk patients achieving a temporary, maybe clonal, remission in period III.

The promising survival rate in period II is to some extent a result of successful salvage therapy. Thus 45% of patients from period II being in CR at the end of the follow-up period had been salvaged following induction failure or relapse. One third of these salvaged patients had received an allogeneic transplant. Corresponding salvage figures in periods I and III were 30 and 12%, respectively.

The effect of patient selection and exclusions on remission and survival rates has been demonstrated previously [13]. The pattern of referral and registration of Danish AML patients makes us confident that the AML population of the present study is truly representative of this disease. This suggestion is further supported by the high median age of our patients, by the uniformity of patient characteristics over time, and by the high annual incidence rate.

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Experimental Approaches and Novel Therapies
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Stratification of Postremission Therapy in Adult Acute Myeloid Leukemia According to the Karyotype – Preliminary Results of the Multicenter Treatment Trial AML HD93

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Abstract. Specific chromosome aberrations are one of the most important predictive factors for response rate and remission duration in acute myeloid leukemia (AML). In June 1993, we initiated a multicenter treatment trial for adult AML (AML HD93) in which the karyotype is used for stratifying postremission therapy. The patients, except those with acute promyelocytic leukemia, receive an intensive double induction chemotherapy consisting of 2 cycles of idarubicin, standard-dose cytarabine and etoposide (ICE), followed by an early consolidation therapy with high-dose cytarabine and mitoxantrone (HAM). For second consolidation, patients $\leq$ 55 years are stratified into three treatment arms:

1. low-risk, defined by t(8;21), inv(16), t(15;17): HAM;
2. intermediate-risk, normal karyotype: allogeneic bone marrow transplantation (BMT) for patients with an HLA-identical sibling, or S-HAM; and
3. high-risk, all other chromosome aberrations: allogeneic or autologous BMT.

Patients older than 55 years are treated according the low-risk arm. At present, 172 patients (median age: 44 years; range: 16-60 years) are eligible. Central cytogenetic diagnosis which included chromosome banding analysis and molecular screening by fluorescence in situ hybridization was successful in 167 of the 172 (97%) leukemias. Complete remission (CR) rates were 98, 81 and 56% for the low-, intermediate- and high-risk group, respectively. The estimated disease-free survival (DFS) at 1.5 years is 67, 58 and 45% for the respective groups. In conclusion, the results confirm the high predictive value of the karyotype for achieving a CR. Although the median follow-up time is short, our preliminary data suggest that intensive double induction chemotherapy with ICE and postremission therapy that is stratified according to the karyotype may improve DFS for the intermediate- and high-risk leukemias.

Introduction

In acute myeloid leukemia (AML), numerous chromosome aberrations have been identified that, in part, define distinct bio-

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logical subgroups of leukemias [1,2]. In retrospective and prospective treatment trials, it has been shown that these chromosome aberrations are one of the most important predictive factors for response rates and remission durations [2]. Although such prognostic factors may highly depend on the therapy used, some consistent features have emerged over time. Leukemias which carry the translocation t(8;21) or the inversion inv(16) almost invariably have been shown to be associated with high complete remission (CR) rates and long survival times, whereas leukemias e.g. with monosomies or deletions of chromosomes 5 and 7 have a very poor outcome. Furthermore, subgroups of leukemias defined by specific chromosome aberrations may benefit from certain chemotherapeutic agents in postremission therapy [3]. It therefore seems attractive to use these prognostic factors to individualize the treatment of patients with AML. This could avoid excessive toxicity related with allogeneic or autologous stem cell transplantation in a group of patients which has a high probability of being cured by conventional chemotherapy. On the other hand, patients with leukemias which have poor prognosis karyotypes may be assigned to more intensified treatment regimens, i.e. high-dose chemotherapy with hematopoietic stem cell transplantation.

In June 1993 we initiated a multicenter treatment trial for patients with AML aged 16 to 60 years, in which postremission therapy is stratified according the karyotype. The objective of this ongoing treatment trial is to evaluate whether this strategy will result in an improvement of disease-free (DFS) and overall survival in genetically defined subgroups of AML.

Materials and Methods

Patients

Between June 1993 and February 1997, 194 patients from 11 institutions, 16 to 60 years old, with newly diagnosed AML were entered. Patients with secondary AML following a primary malignancy were included, while patients with preexisting myelodysplastic or myeloproliferative syndrome were excluded from the protocol. Twenty-two patients were ineligible: 7 patients were diagnosed as having acute lymphoblastic leukemia, 8 had a preexisting myelodysplastic or myeloproliferative disorder; and there was protocol violation in 7 cases. Leukemias were diagnosed according to the criteria of the French-American-British (FAB) classification [4]. Of the 172 eligible patients, 158 had de novo AML and 14 had secondary AML. The study was approved by the local Ethical Review Committee, and written informed consent was obtained from all patients.

Central Cytogenetic Analysis

Cytogenetic analysis, including conventional chromosome banding analysis and interphase cytogenetics using fluorescence in situ hybridization (FISH), were done centrally for all patients as previously described [5,6].

Treatment Plan

Double Induction Therapy. The treatment plan is shown in Fig. 1. Patients received a double induction therapy consisting of 2 cycles of ICE (idarubicin 12 mg/m ^{2}, day 1,3,5 i.v.; cytarabine 100 mg/m ^{2} continuously i.v., day 1-7; etoposide 100 mg/m^2, day 1-3 i.v.). The second cycle ICE which included the administration of R-metHuG-CSF (filgrastim) (300 µg s.c. daily 24 h after completion of chemotherapy) was started on day 28 provided that at least a partial remission was achieved. Patients with leukemias which were refractory to the first cycle of ICE (i.e., more than 30% blasts in the BM) received the second induction cycle according to the HAM protocol (cytarabine 3 g/m^2/12 h, day 1-3 i.v.; mitoxantrone 12 mg/m^2, day 2-3 i.v.). Patients with acute promyelocytic leukemia (APL) received induction therapy according to the AIDA protocol (all-trans retinoic acid 45 mg/m p.o., day 1-28; idarubicin 12 mg/m, day 2, 4, 6, 8 continuously i.v.) [7] followed by the second induction cycle with ICE.

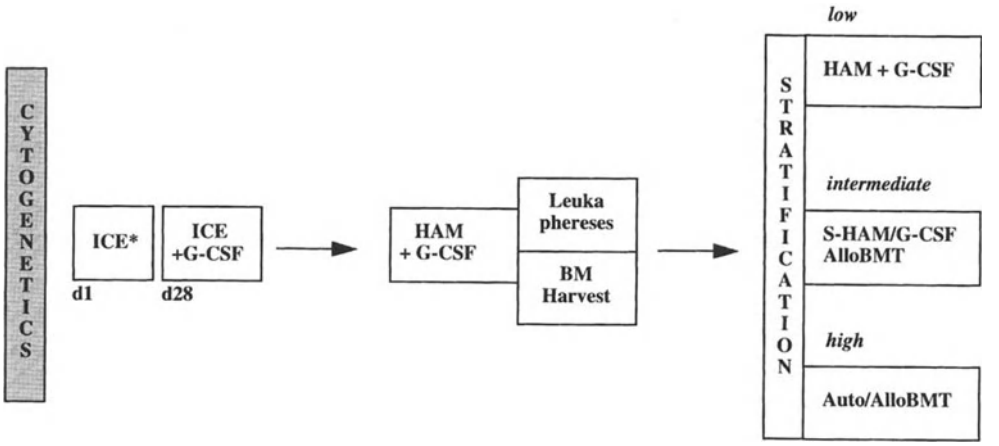

Fig. 1. Design of AML HD93 treatment trial. Low-risk: t(8;21), inv (16), t(15;17); intermediate risk: normal karyotype; high risk: all other abnormalities. * Patients refractory to ICE receeiive HAM as second induction cycle. * Patients with AML FAB M3 receive AIDA as first induction cycle

Early Consolidation Therapy and Autologous Stem Cell Harvest. All patients in CR or PR after double induction therapy were given a first consolidation therapy with HAM supported by G-CSF 24 h after completion of chemotherapy. Optional peripheral blood progenitor cell aphereses were performed during the G-CSF supported hematological recovery [8]. Two to 3 weeks after the hematological recovery a bone marrow harvest from the posterior iliac crest was performed in all patients in CR below the age of 56 years lacking an HLA-identical sibling.

Stratification of Late Consolidation Therapy according to the Karyotype. Late consolidation chemotherapy in patients younger than 56 years was stratified according to the karyotype. The patients aged 56 to 60 years were treated in analogy to the low-risk arm of the study. The cytogenetically risk groups were defined as follows:

1. Low-risk: t(8;21), inv(16), t(15;17). Patients within this group received a second consolidation therapy with HAM supported by G-CSF.
2. Intermediate-risk: normal karyotype. Patients within this group received either S-HAM (cytarabine 3 g/m^2/12 h, day 1+2 and day 8+9 i.v.; mitoxantrone 10 mg/m^2, day 3+4 and day 10+11 i.v.) [9], or allogeneic bone marrow transplantation (BMT) if an HLA-identical sibling was available.
3. High-risk: all other cytogenetic abnormalities. These patients were assigned to either allogeneic or autologous BMT.

Evaluation of Response

CR was defined as <5% blasts in a normocellular BM, and PR was defined as >5% and <30% blasts with >0.5 $\times$ 10^9/l neutrophils and >100 $\times$ 10^9/l platelets in the blood and disappearance of all other signs of leukemia. Relapse was defined as >5% leukemic blasts in the BM.

Statistical Methods

The data were analyzed with a close-out date of March 6, 1997. The remission status of all but 3 patients was known at this date. The DFS was measured from the date of achieving CR to the date of relapse or the date of the last contact. Patients who died in CR were included as failures. Overall survival was measured from the date of diagnosis to the date of death for all patients who died.

The data were analyzed on an intention-to-treat basis. Leukemias with t(8;21), inv(16) or t(15;17) and additional chromosome aberrations initially were classified as high-risk leukemias and therefore, between June 1993 and April 1995, 5 of these patients

had allogeneic or autologous BMT. After the meeting of the protocol committee in May 1995, all leukemias with t(8;21), inv(16) or t(15;17) with or without additional chromosome aberrations were classified as low-risk leukemias and treated with HAM for late consolidation. Statistical computations were performed using the statistical software packages SAS, Version 6.11 (SAS Institute Inc., 1995) and S-Plus, Version 3.3 (MathSoft Inc., 1995). Survival curves were estimated using the Kaplan-Meier product-limit method.

Results

Central Cytogenetic Analysis

The cytogenetic analysis was succesful in 167 of the 172 (97%) leukemias. In five cases, no evaluable metaphase spreads were obtained on chromosome banding analysis and no clonal chromosome aberrations were detected by interphase cytogenetics. Thus, these five cases could not be assigned to one of the cytogenetically defined risk groups and they were excluded from further considerations.

Remission Rates

Complete remission (CR) was achieved in 126 of the 164 (77%) patients who are evaluable for response after double induction chemotherapy. 102 (81%) patients achieved the CR after one induction cycle, the remaining 24 patients after the second cycle. The median time interval from day 0 of the first induction cycle to the start of the second induction cycle was 32 days (range: 19 to 152 days).

The response rates for the specific cytogenetic subgroups are shown in Table 1. The CR rates for the low-, intermediate- and high-risk leukemias were 98% (39 of 40), 81% (56 of 69), and 56% (31 of 55), respectively. In the low-risk group there were no leukemias refractory to induction chemotherapy, whereas the rate of induction failures was 6% (4 leukemias) and 35% (19 leukemias) in the intermediate- and high-risk group, respectively. Twenty-five of the 31 patients who did not achieve remission upon the first cycle received the second induction chemotherapy with HAM and 7 (28%) achieved a CR. Three patients with leukemias refractory to double induction achieved the CR after allogeneic BMT.

Table 1. Patient characteristics at diagnosis

No. of patients			167
Age (years)	Median		44
	Range		16-60
Sex	Male		80
	Female		87
Morphology (FAB)			
	M0		4%
	M1		13%
	M2		26%
	M3		5%
	M4		22%
	M4Eo		7%
	M5		19%
	M6		3%
	M7		1%
Hematological parameters	Median	Min	Max
Hemoglobin (g/dl)	8.5	4.3	15.7
White cell count (x109/l)	13.3	0.6	345.0
Platelet count (x109/l)	57	7	488
Lactate dehydrogenase (IU/l)	387	89	2992
% blasts in blood	33	0	100

Survival Analysis

Figure 2 shows the overall survival times of the patients according to the cytogenetic risk groups. The median follow-up time is 18 months ranging from 0.5 to 45 months.

Low-Risk. Thirty-one of the 39 patients achieving CR received HAM as late consolidation. Prior to the protocol revision in May 1995, 5 patients were treated according to the high-risk arm, i.e., 3 patients had allogeneic and 2 patients had autologous BMT as late consolidation. The leukemic cells of these patients exhibited secondary chromosome aberrations in addition to the specific translocation or inversion. One other patient had allogeneic BMT because the leukemic cells of this patient had a normal karyotype on

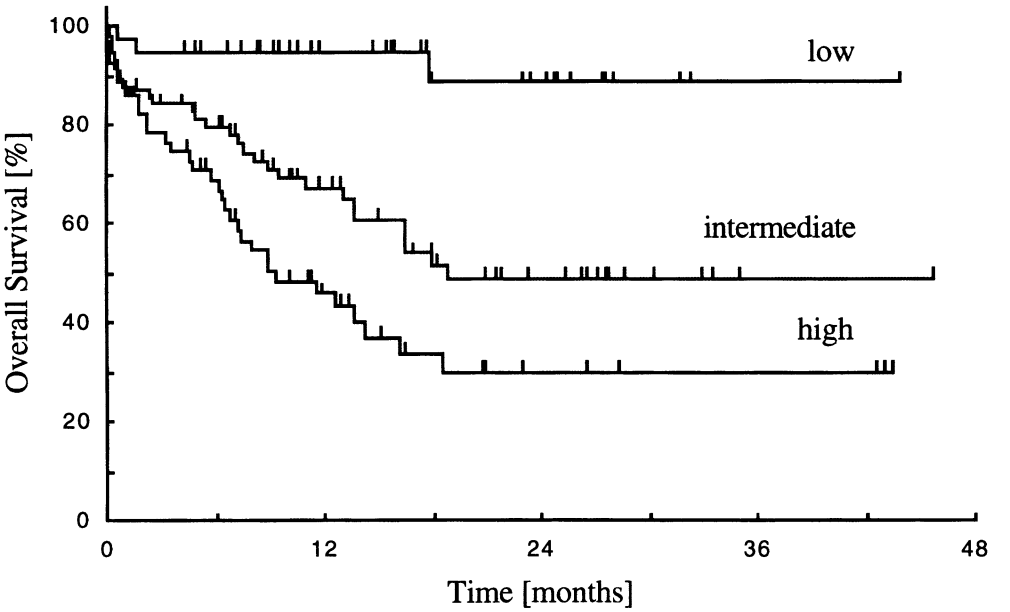

Fig. 2. Kaplan-Meier plots of overall survival of all patients according to the three cytogenetic risk groups

chromosome banding analysis, but were later shown by molecular screening to carry an inv(16). One patient died during hypoplasia following the second cycle ICE.

Six of 18 (33%) patients with inv(16), 1 of 12 (8%) patients with t(8;21) and 1 of 8 (13%) patients with t(15;17) relapsed between 6 to 13 months after achieving CR. All 6 patients with inv(16) achieved a second CR following reinduction therapy containing high-dose cytarabine. One of these patients died from complications of allogeneic

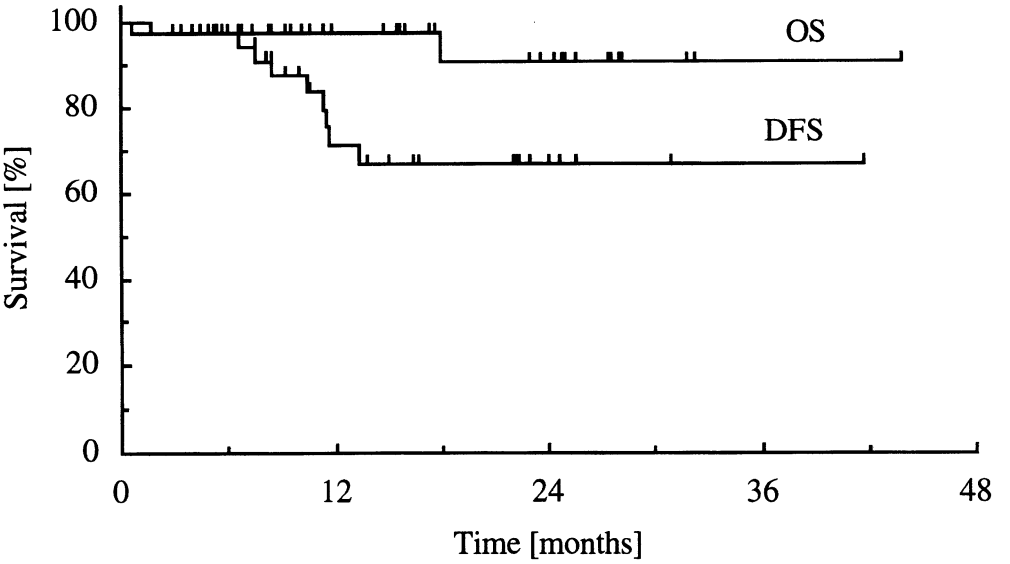

Fig. 3. Disease-free (DFS) and overall survival (OS) of the 39 complete responders in the low-risk group

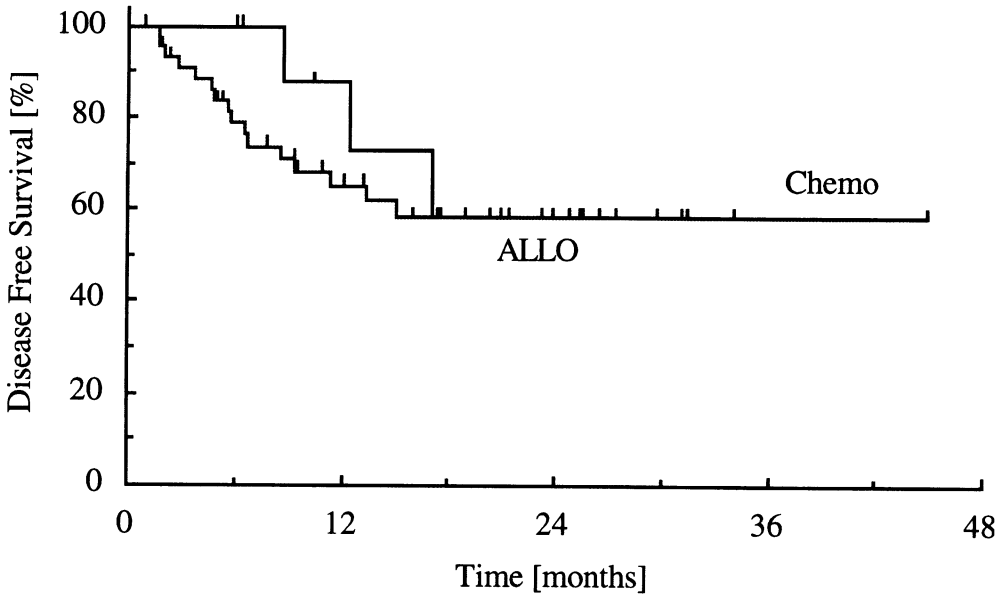

Fig. 4. Disease-free survival of the 53 complete responders in the intermediate-risk group, according to whether they were assigned to allogeneic BMT or intensive consolidation with S-HAM or HAM.

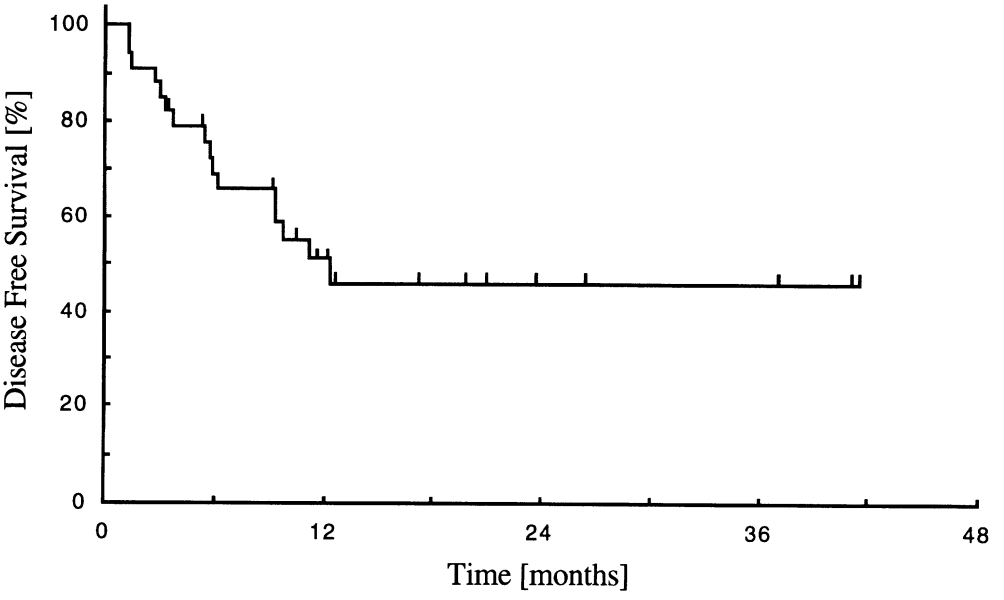

Fig. 5. Disease-free survival of the 31 complete responders in the high-risk group

BMT. The remaining 5 patients are in continuous second CR: 3 after autologous blood stem cell transplantion (ABSCT), and two after repetitive cycles of high-dose cytarabine. One patient with t(8;21) relapsed 9 months after autologous BMT and did not achieve a second CR. One patient with t(15;17) had reinduction therapy with AIDA and is in continuous second CR after ABSCT. The DFS and the overall survival at 1.5 years of the complete responders in the low-risk group is 67 and 92%, respectively (Fig. 3).

Intermediate-Risk. Of the 56 patients achieving CR, 16 were 56 to 60 years old and received HAM as late consolidation. Of the remaining 40 patients younger than 56 years, so far 10 received allogeneic BMT, 16 S-HAM, and 5 HAM as late consolidation. There were seven deaths in CR. Twelve of the 56 (21%) patients within the intermediate-risk group relapsed, one after allogeneic BMT, 3 after S-HAM, 6 after HAM and 2 before late consolidation. All 12 patients who relapsed died without achieving a second CR. The DFS at 1.5 years is 58% for both the patients receiving allogeneic BMT and intensive chemotherapy (Fig. 4).

High-Risk. Of the 31 patients achieving CR following induction therapy, 8 were 56 to 60 years old and received HAM as late consolidation. Of the 24 patients younger than 56 years, so far 13 received autologous BMT, 5 allogeneic BMT and 3 either S-HAM or HAM. The three patients who were refractory to induction chemotherapy but achieved CR after allogeneic BMT were included in the survival analysis. Four patients died in CR, 2 of them during aplasia after early consolidation and 2 after autologous BMT. Twelve patients relapsed, 11 of them did not achieved a second CR, and one patient is during reinduction. The DFS at 1.5 years of the 34 patients is 46% (Fig. 5).

Discussion

The objective of this ongoing multicenter treatment trial in AML is to evaluate whether stratification of postremission therapy according to cytogenetic risk groups will result in an improvement of disease-free and overall survival.

All patients received an intensive double induction chemotherapy consisting of two cycles of idarubicin, cytarabine and etoposide (ICE). The rationale for the use of this drug combination was twofold. First, the initial randomized trials comparing standard-dose cytarabine plus either daunorubicin or idarubicin have uniformly demonstrated a higher efficacy of the idarubicin containing regimen [11-13]. A recent meta-analysis of randomized trials of idarubicin in AML suggests that this anthracyclin not only improves CR rate but also survival compared with daunorubicin at least in younger patients with de novo AML [14]. Secondly, the Australian Leukaemia Study Group (ALSG) has shown that etoposide when given in addition to cytarabine and an anthracyclin in induction therapy significantly improves remission duration in patients younger than 55 years [15].

The overall remission rate in our study was 77%. This rate is similar to the remission rates reported by others for idarubicin containing induction regimens [11-13] and compares favorably to the CR rates reported by large leukemia study groups in which daunorubicin was used for induction therapy [16-19]. In the latter studies, the CR rates for similar age groups ranged between 58 and 71%. Our study confirms the high predictive value of the karyotype for achieving a CR. In the low-risk group, there were no leukemias refractory to induction treatment. The rate of induction failures was also low (6%) in the intermediate-risk group but increased to 35% in the high-risk group. Within the latter group, the highest CR rate was seen in leukemias with translocations involving chromosome band 11q23 (80%), whereas the CR rates remained low for leukemias exhibiting -5/5q- (38%), -7/7q- (31%), and -17/17p- (13%) (Table 2).

For postremission therapy, all patients in our study first received early consolidation with high-dose cytarabine in combination with mitoxantrone (HAM), followed by a second intensive consolidation therapy which was stratified according to the karyotype. The design of the stratification was primarily based on the results of the study of the Cancer and Leukemia Study Group B (CALGB) reported by Mayer et al. [16] and Bloomfield et al. [3]. In this study the CALGB could show that the outcome of patients with the most favorable chromosome aberrations, t(8;21) and inv(16), as well as of patients with a normal karyotype, but not with most other aberrations, can be significantly improved by the use of high-dose cytarabine consolidation therapy. Accordingly, in our study patients within the low- and intermediate-risk groups received a second cycle containing high-dose cytarabine.

Table 2. Distribution of patients with complete remission (CR), early death (ED), and refractory disease (RD) after two induction cycles according to specific chromosome abnormalities

	No. of Pts.	CR	ED	RD
Low-risk	40	39 (98%)	1 (2%)	—
inv(16)	19	18 (95%)	1 (5%)	—
t(8;21)	12	12 (100%)	—	—
t(15;17)	9	9 (100%)	—	—
Intermediate-risk	69	56 (81%)	9 (13%)	4 (6%)
High-risk	55	31 (56%)	5 (9%)	19 (35%)
t(11q23)	15	12 (80%)	2 (13%)	1 (7%)
-5/5q-	13*	5 (38%)	1 (8%)	7 (54%)
-7/7q-	16*	5 (31%)	3 (19%)	8 (50%)
+8/8q+	13*	7 (54%)	2 (15%)	4 (31%)
-17/17p-	8*	1 (13%)	1 (13%)	6 (75%)
Others	22	15 (65%)	1 (4%)	7 (31%)
	164	126 (77%)	15 (9%)	23 (14%)

[a] Because of complex karyotypes some leukemias are listed in more than one of these categories.

The sensitivity of the low-risk leukemias to high-dose cytarabine is reflected in a DFS of 67% at 1.5 years in our study (Fig. 3). This is somewhat lower than the 84% DFS at 5 years reported by the CALGB in a group of 18 patients who had received four cycles of cytarabine (3 gm/m^2, q12 h, d1-3) compared to only 2 cycles in our study. Interestingly, in our study 7 of the 8 patients in the low-risk group who relapsed achieved a second CR using reinduction therapy with cytarabine. One of the complete responders died from infectious complications after allogeneic BMT, however, the remaining 6 patients are in continuous second CR following transplantation with autologous blood progenitor cells collected in first CR (4 patients) or following repetitive cycles of high-dose cytarabine (2 patients). The overall survival of the low-risk patients is excellent with an estimated probability of 90% at 1.5 years (Fig. 3). This is higher than the 71% survival at 5 years that was reported for the AML-10 trial of the Medical Research Council (MRC) in which allogeneic and autologous BMT was part of the postremission therapy in first CR [20]. From these preliminary data of our study and those of other multicenter trial we conclude that consolidation therapy with high-dose cytarabine will likely cure the majority of patients with low-risk leukemias. High-dose chemotherapy with allogeneic or autologous progenitor cell transplantation should be reserved for relapsed patients.

The 58% DFS of the intermediate-risk group in our study compares favorably to all published series. Our patients were assigned to either allogeneic stem cell transplantation or to the S-HAM protocol which had previously been shown to have considerable antileukemic activity in refractory leukemias [9]. Interestingly, so far we did not find a difference of DFS between the patients who received allogeneic BMT and those who received S-HAM (Fig. 4). Our results provide further evidence that these leukemias, in analogy to the leukemias in the low-risk group, may benefit from high-dose cytarabine consolidation. The reported probabilities of DFS for this risk group vary between 17% and 46% at 3 years [21-27]. In these studies, by far not all patients were treated with dose intensified cytarabine in postremission therapy. In the CALGB study, the patients in the intermediate-risk group who received high-dose cytarabine for consolidation therapy had a 37% DFS at 5 years [3]. This lower DFS may in part be explained by the fact that this analysis also included the elderly patients (>60 years). In the MRC AML-10 trial [27], 672 of 1613 (42%) children and adults up to 55 years of age had a normal karyotype. The DFS at 5 years in this study was 41%.

In the CALGB study the outcome of the high-risk leukemias remained poor with a DFS at 5 years of only 15%, regardless of whether they had received cytarabine in a

dosage of 100 mg, 400 mg or 3 g for consolidation [3]. In our group of high-risk patients younger than 56 years, 21 of 26 (80%) patients received the intensified postremission therapy with either autologous or allogeneic BMT. The estimated DFS at 1.5 years of approximately 45% is promising. However, it seems that only specific subgroups within the high-risk group most likely benefit from this intensified consolidation therapy, whereas the outcome of the leukemias associated with, e.g., -5/5q- or -7/7q- remains very poor.

Although the median follow-up period is still short, the treatment strategy of our study will possibly result in an improvement of DFS for the high- and intermediate-risk leukemias. A subanalysis of the different genetic entities within the high-risk group may identify patients who benefit from our treatment approach and those who do not. New treatment strategies are required for leukemias, e.g., associated with abnormalities of chromosomes 5 and 7.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Continuous Infusion of Mitoxantrone Combined with High Dose Cytarabine (c-HAM) in de Novo AML

S. Sormann, P. Neumeister, M. Konrad, and W. Linkesch

Introduction

The treatment of acute myelogenous leukemia (AML) has improved over the past two decades with the introduction of postremission bone marrow transplantation and better induction chemotherapy regimens [1,2]. As the majority of patients for different reasons are not eligible for bone marrow transplantation many efforts have been taken to improve induction and postremission chemotherapy resulting in a higher response rate as well as a longer remission duration [2,3]. Cytarabine (Ara-c) has been an essential component of combination induction therapy as it first produced long term survivors in AML. Recent data suggest that high dose cytarabine regimen (HD-Ara-c) as initial treatment prolongs remission duration and disease-free survival in patients with de novo AML [2]. The high antileukemic potential of HD-Ara-c in combination with Mitoxantrone (HAM) could be demonstrated in several studies [4,5]. In an attempt to reduce early death rates and nonhematological toxicity a modified HAM protocoll (c-HAM) was developed for AML patients. In contrast to bolus administration the continuous infusion of mitoxantrone leads to a constant high long lasting plasma concentration of mitoxantrone up to 15 days after initiating therapy, is above a level necessary to kill human cells in vitro[6]. Furthermore cell kinetic studies revealed a conditioning effect of cytosine arabinosid for the subsequent administration of daunorubicin or mitoxantrone [7,8].

In a pilot study comprising 23 patients promising results concerning response rate and early deaths resulting in a high therapeutic index prompted us to evaluate the c-HAM protocol as induction as well as postremission chemotherapy regimen in a larger cohort of patients with de novo AML [9].

Patients and Methods

The prospective phase II study comprised 66 consecutive patients (median age 55; range 18-83 years). Patients who met the following criteria were included: (1) patients age > 18 yrs.; (2) de novo AML; (3) induction treatment with one of the following regimen: Cytarabin/Idarubicin (n = 43) or Cytarabin/Daunorubicin/Thioguanine (n = 23). c-HAM was either used as second induction cycle(n = 19), consolidation therapy (n = 43) or both (n = 4). The age and sex was well balanced with 31 < 60 and 35 > 60 years of age, 34 women and 32 men. FAB-subtypes: M1 n = 9; M2 n = 14; M3 n = 2; M4 n = 32; M5 n = 8; M6 n = 1

Treatment Protocols

Induction therapy consisted of: cytarabine 200 mg/m^2 as continuous infusion on days 1-5 and idarubicin 12 mg/m^2 as bolus infusion

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on day 1-3 or DAT-regimen: daunorubicin 50 mg/m^2 as bolus infusion on days 1-3, cytarabine 200 mg/m^2 as continuous infusion on days 1-5 and thioguanine 100 mg/m^2 every 12 h per os on days 1-5. cHAM consisted of HD-AraC 2 g/m^2 for pts. ≤ 60 yrs and 1 g/m^2 for pts. > 60 yrs. given every 12 h by a 3 h infusion on days 1-3 and mitoxantrone 10 mg/m^2 given by an 18 h infusion on day 1-3. CR was defined according to the cooperative acute leukemia group B (CALGB) criteria.

Results

Complete remission (CR) was achieved in 92% of pts. ≤ 60 yrs. and in 56% of pts. > 60 years. After a median observation period of 10 (3-66) months, seventeen out of 31 pts. ≤ 60 yrs. are alive, 13 pts. in continuous complete remission (CCR), 12 out of 35 pts. >60 yrs. are alive, 4 of them in CCR. The disease-free-survival rate is shown in Fig. 1 and 2 for the different age groups. The overall-survival is shown in Fig. 3.

The main nonhematological side effects included diarrhea, vomiting, nausea and mucositis. We observed no significant toxicity (WHO grade III-IV) concerning kidney, liver, CNS or heart, as previously described [6]. During the treatment of cHAM we did not observe any death. Early deaths were observed in 3% of the younger pts. and in 20% of the older patients group. There was no objective relation between observed early death and application of cHAM regimen.

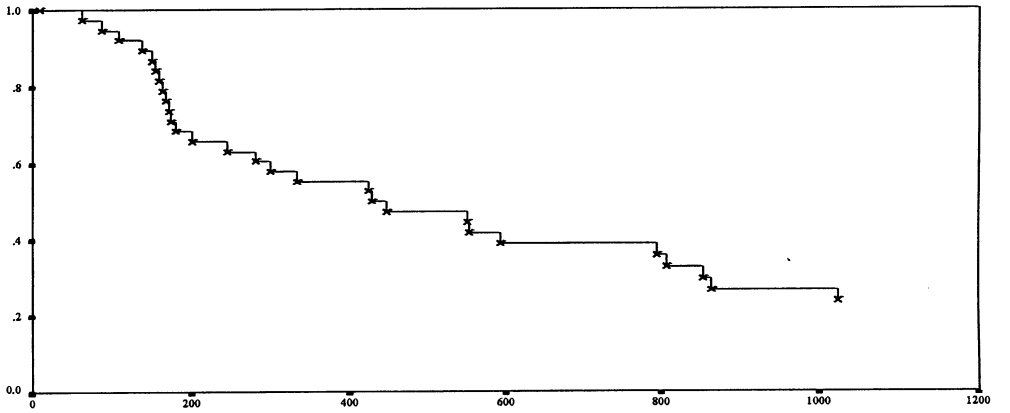

Fig. 1. Disease-free-survival pts. < 60 years censored for BMT days

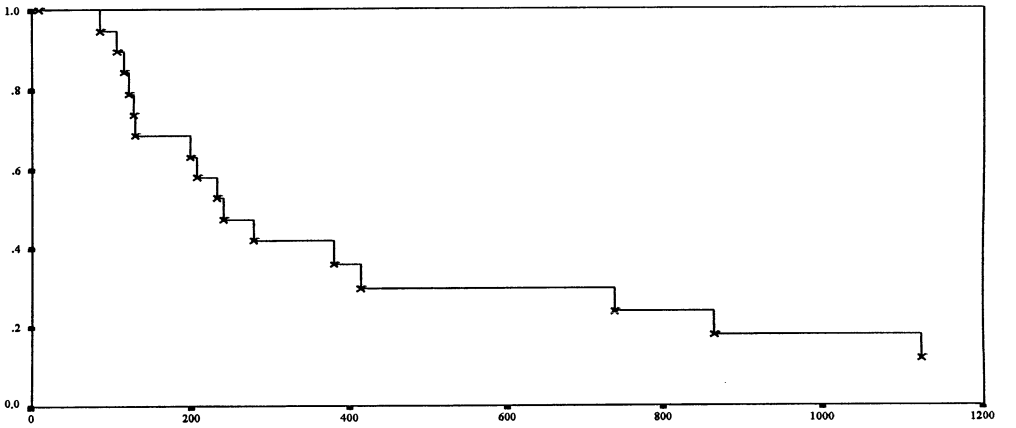

Fig. 2. Disease-free-survival for pts > 60 years censored for BMT days

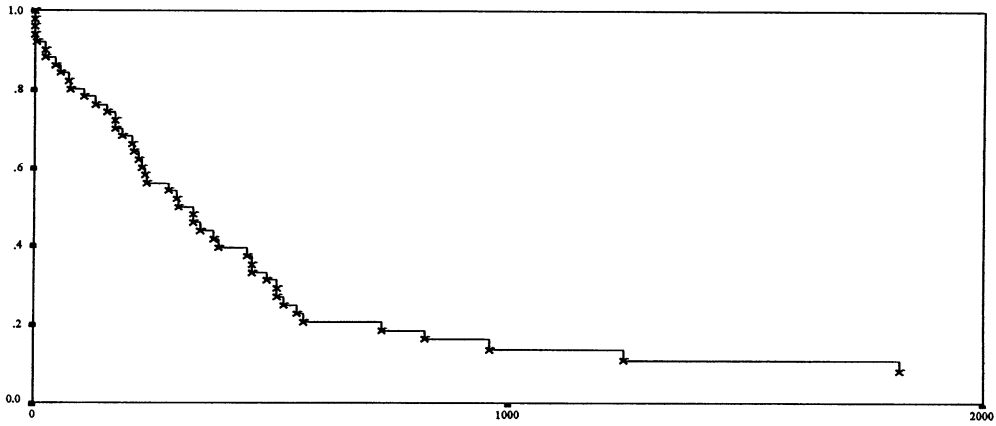

Fig. 3. Overall-survival censored for BMT days

Relapse was the main cause of death in this study population(26) followed by infection 7; bleeding 3; cardiac arrest 1. As previously described the cHAM regimen shows a myeloablative effect comparable to other regimens [6].

Discussion

As suggested in our previous study the high antileukemic potential of the c-HAM regimen could again be demonstrated in a larger cohort of patients resulting in a total CR rate of 74%. Especially for elderly AML patients a complete response rate of 56% combined with a remarkable low toxicity seems to offer some advantages compared to the original HAM protocol. Similar, in patients $\leq$ 60 years a CR rate of 92% compares favorably to other conventional treatment regimens. However, due to the short mean observation period it is at present not possible to asses whether or not the excellent response rates will result in a improved DFS or increased overall survival.

In contrast to continuous infusion, in vitro pharmacokinetic studies reveal evidence that bolus administration of mitoxantrone leads to plasma concentration below mean lethal concentration for human cells 1 h after infusion [10]. Furthermore recent data suggest that patients achieving CR show higher plasma levels of mitoxantrone compared to patients with resistant disease [11]. For our study we could demonstrate a constant high level of mitoxantrone ranging about 200 ng/ml two weeks after initiating therapy [12, 13]. In conclusion we consider the c-HAM regimen to be at least equieffective to other standard therapy or the original HAM protocol with respect to CR-rates and the low toxicity resulting in a high therapeutic index especially in elderly patients with de novo AML.

Therapy: c-HAM

HD-Ara-C 2 g/m^2 (for pts. $\leq$ 60 yrs.) 1 g/m^2 (for pts. $\leq$ 60 yrs.) every 12 h as 3 h infusion on day 1–3 combined with Mitoxantrone 10 mg/m^2 as 18 h infusion on day 1–3.

Properties: constant mitoxantron plasma level over a long time, low non hematological tocixity, obvious myelosuppression

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

All-Trans Retinoic Acid (ATRA) Combined with Double Induction Strategy in Acute Promyelocytic Leukemia (APL). Preliminary Results

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Abstract. In November 1994, the AML Cooperative Group (AMLCG) started a still ongoing trial for treatment of patients with acute promyelocytic leukemia (APL). The therapy consists of TAD/HAM double induction chemotherapy in combination with all-trans retinoic acid (ATRA) until complete remission, followed by TAD consolidation and monthly maintenance. After starting with ATRA alone, the beginning of chemotherapy is stratified according to the improvement of coagulopathy and to the level of the white blood cell counts. By now, 35 patients are included. Twenty one of 23 (91%) of the presently evaluable cases responded to induction therapy. Nineteen (83%) patients reached complete remission (CR), one (4%) partial remission, one (4%) hypoplasia without persistence of blast cells. Two (9%) early deaths occurred during induction therapy due to bleeding complications. Grade $\geq$ 3 bleeding complications and infections were 15% and 36%, respectively. ATRA syndrome occurred in 3 (13%) patients. In no patient were blast cells resistant to therapy. The high antileukemic activity of this treatment strategy was further demonstrated by the disappearance of RT-PCR positivity for PML/RAR alpha after induction and by the persistence of negativity (4 to 24 months) in all 14 patients monitored during follow-up.

Introduction

Prognosis of acute promyelocytic leukemia has impressively improved due to the introduction of the differentiating agent all-*trans* retinoic acid (ATRA) [1]. With the combination of ATRA and chemotherapy, a reduction of the relapse rate, an improvement of overall survival and a reduction of the early mortality rate was observed compared to chemotherapy alone [2-6]. Response to ATRA is also characterized by an improvement of coagulopathy within the first days of treatment [7]. The probability of cure, however, seems to depend on the antileukemic effectivity of the chemotherapy [8]. Despite the advances in treatment of APL, the optimal combination of chemotherapy and the best timing of ATRA and chemotherapy are still unknown.

From 1985 to 1992 the AML Cooperative Group (AMLCG) included patients with newly diagnosed APL in their protocol for treatment of acute myeloid leukemia with

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TAD/HAM (TAD) double induction, TAD consolidation and 3 years monthly maintenance (AMLCG 85 study) [9, 10]; 39 (74%) of 50 adult APL patients of all age groups responded to therapy (68% complete (CR), and 6% partial remissions). Only 2 patients (4%) were nonresponders. Early death rate was 22%. Relapse free survival was 55% after 9 years.

On the basis of these results suggesting a high antileukemic effectivity of the AMLCG chemotherapy strategy in newly diagnosed APL, the AMLCG decided in November of 1994 to combine their protocol with ATRA. Preliminary results of the still ongoing study are presented in this chapter.

Patients and Methods

Included are adult patients with newly diagnosed APL of all age groups. Diagnostic criteria are AML M3 morphology, t(15;17) and/or positive RT-PCR of PML/RAR alpha. Induction therapy consists of ATRA (45 mg/m^2/day) until CR in combination with TAD/HAM, followed by consolidation and maintenance as in the previous study.

Timing of ATRA and Chemotherapy

After an initial phase of ATRA alone for maximal 14 days to use the positive effect of ATRA on coagulopathy, the start of chemotherapy is stratified according to the normalization of significant coagulopathy and to the level of the white blood cell counts (WBC). In patients with initial WBC counts over 5000/µl, rapidly increasing WBC or M3 variant, TAD is added immediately to prevent ATRA syndrome.

Chemotherapeutic Regimens

Drug dosages and combinations are given as follows:

TAD. 6-thioguanine (6-TG) 100 mg/m^2 q12 h day 3 to 9, daunorubicin (DNR) 60 mg/m^2 day 3 to 5, cytosine arabinoside (ara-C) 100 mg/m^2 by continuous i.v. infusion day 1 and 2, and 100 mg/m^2 q12 h by 30 min i.v. infusion day 3 to 8.

HAM. Ara-C 3 g/m^2 q12 h by 3 h i.v. infusion day 1 to 3, mitoxantron 10 mg/m^2 by 30 min i.v. infusion day 3 to 5.

Monthly Maintenance Courses. Ara-C 100 mg/m^2 s.c. q12 h day 1 to 5, alternatingly combined with either DNR 45 mg/m^2 day 4 and 5, or 6-TG 100 mg/m^2 q 12 h day 1 to 5, or cyclophosphamide 1 g/m^2 day 3.

If there are no contraindications, the HAM cycle is started on day 21 after the beginning of TAD, independently of neutropenia. Patients over 60 years receive a dose reduced HAM with 1 g/m^2 Ara-C instead of 3 g/m^2. In these patients HAM is only given if CR was not achieved after TAD. Consolidation with TAD is started 4 weeks after CR.

Salvage Strategies for Patients with High Risk of Relapse

Monitoring of minimal residual disease by RT-PCR of PML/RAR alpha is performed after the induction and consolidation therapies and during the maintenance phase. Patients with persistence or reappearance of positive PCR for PML/RAR alpha receive an allogeneic transplant if a family donor is available. Otherwise, ATRA 45 mg/m^2 day 1 to 7 is added to the maintenance courses.

Results

Until February of 1997, 35 patients were recruted for the study. One patient died before the start of treatment. One patient had contraindications against the study medication. 10 patients were not yet evaluable. In the 23 evaluable patients (median age 43 years, range 16 to 46) M3 morphology was seen in 19 and M3 variant in 4 patients, respectively. Translocation t(15;17) and/or PML/RAR alpha was found in all but one patient. Mean duration of ATRA treatment before the start of chemotherapy was 7 days (range 0 to 19). At diagnosis 17 of 21 (76%) had significant coagulopathy (reduced fibrinogen or prothrombine time).

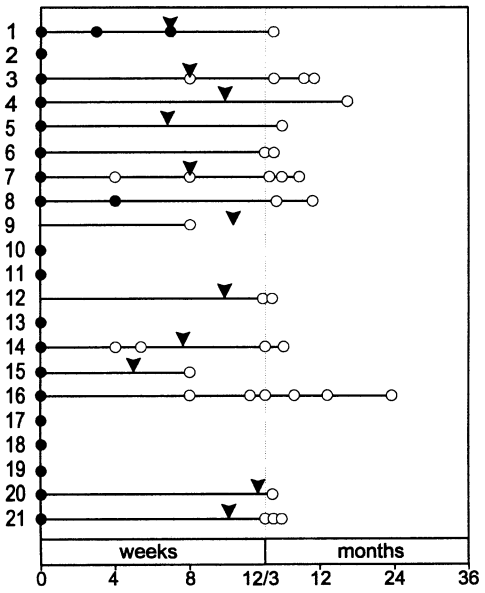

Fig. 1. Monitoring of minimal residual disease by RT-PCR of PML/RAR alpha. ○ PCR negative, ● PCR positive, ▼ date of hematologic complete remission after TAD/HAM

ing a disappearance of the initially detectable PMURAR alpha transcript after TAD/HAM (Fig. 1).

Causes of the two (9%) early deaths were bleeding complications during induction therapy. ATRA syndrome occurred in 3 patients (13%). No increased toxicities, but a reduction of WHO grade ≥ 3 bleeding complications (15%) by ATRA plus chemotherapy compared to the historical control group with chemotherapy alone (28%) were seen. The rate of WHO grade ≥ 3 infections was reduced from 55 to 36%.

The preliminary results of overall survival and of relapse free survival are shown in Figs. 2 and 3. At the present time, the median overall survival is 388 days (range 3 to 746) and the median relapse free survival 385 days (range 25 to 707).

During follow up, in 14 patients the minimal residual disease was monitored by RT-PCR (Fig. 1). By now, no patient had a reappearance of positive PCR after 4 to 24 months.

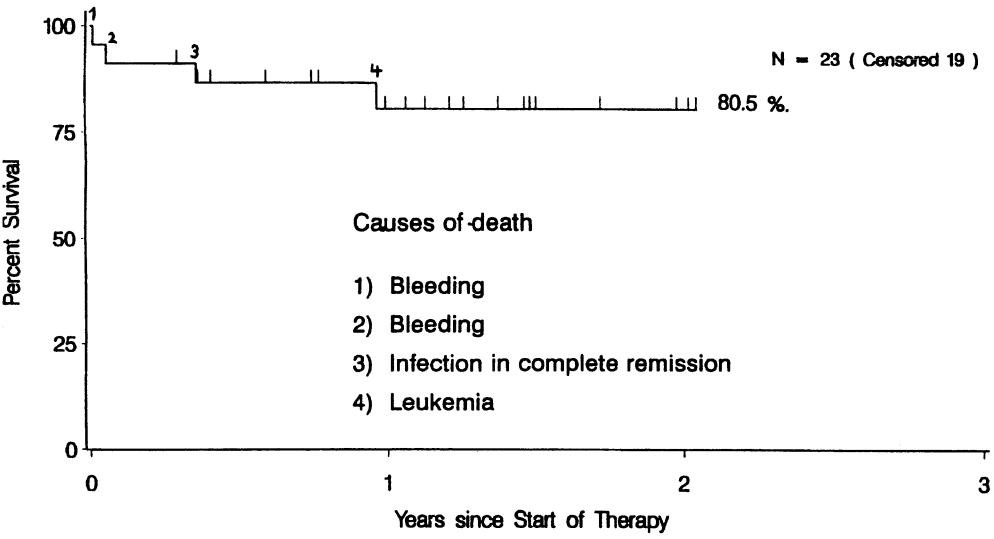

Fig. 2. Overall survival after ATRA and chemotherapy

Twenty one patients (91%) responded to induction therapy. CR was achieved in 19 (83%) patients. Partial remission (4%) and hypoplasia without persistence of blast cells (4%) were seen in one patient each. In 11 patients CR was confirmed by RT-PCR show-

Discussion

The main objective of our study is to improve the prognosis of patients with newly diagnosed APL by combination of ATRA with a chemotherapy strategy that proved

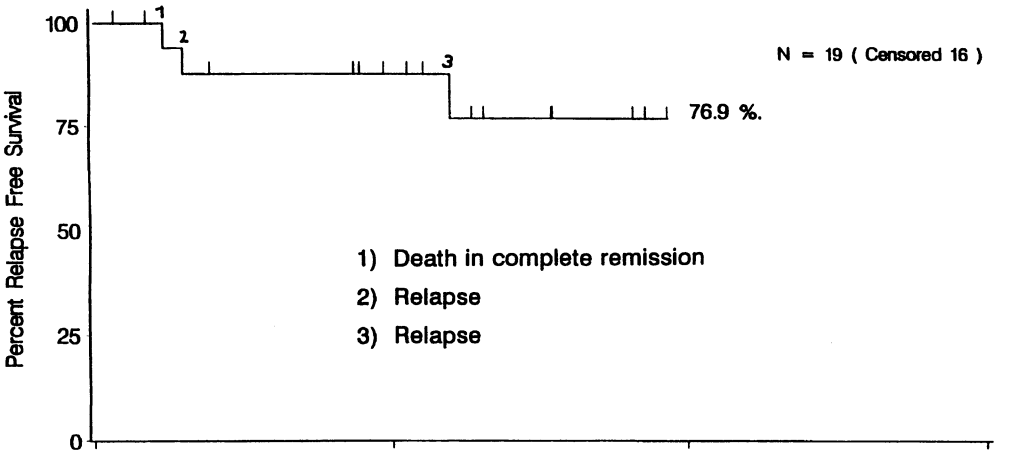

Fig. 3. Relapse free survival after ATRA and chemotherapy

highly effective when given alone before the widespread use of ATRA [9, 10]. A second goal is to identify patients with a high risk of relapse despite the combination with ATRA and to cure them by the early application of salvage strategies.

The high antileukemic activity of the AMLCG chemotherapy concept alone (AMLCG 85 study) has been demonstrated by the low rate of nonresponders of 4% and by the high rate of patients with relapse free survival of 55%. These results are superior to the results of most APL studies with chemotherapy alone achieving median CR durations between 10 and 25 months and relapse free survival rates of about 25% after 5 years [7, 11].

Preliminary results of the present study show a good practicablity and high antileukemic effectivity of ATRA combined with double induction therapy. Compared to chemotherapy alone, the CR rate was improved and the early death rate was impressively reduced from 22 to 9% by the combination with ATRA. No increased toxicities but a reduction of grade ≥ 3 bleeding complications and infections were seen. The rate of ATRA syndrome of 13% is comparable to the rate observed in the presently ongoing APL 93 trial, that also combines ATRA and chemotherapy for remission induction [12].

The high sensibility of APL blast cells to double induction plus ATRA is also supported by the results of RT-PCR. Persistence or reappearance of a positive PCR for PML/RAR alpha after a negative period is correlated with a subsequent relapse [13]. By now, all patients tested before and after induction therapy converted to negativity of PML/RAR alpha and remained negative during follow up. It should also be emphasized that in no patient were the blast cells resistant to induction therapy. Preliminary results of overall and relapse free survival predict an improvement by ATRA.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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The Effect of ATRA (Roche) on Clinical and Laboratory Parameters in 36 APL Patients – Polish Multicenter Retrospective Analysis

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Introduction

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML). It is characterized by an arrest of differentiation at the promyelocytic stage. APL is consistently associated with translocation between the long arms of chromosomes 15 and 17 - t(15;17). The translocation leads to fusion of the genes for PML (promyelocytic leukemia) on chromosome 15 with the gene encoding the retinoic acid receptor a (RARa). The fusion protein PML/RARa is believed to cause APL. At initial presentation the coagulopathy associated with APL is often present. Standard chemotherapy (Daunorubicine + ARA-C) increases the risk of early fatal hemorrhage by exacerbating coagulopathy and due to decreases of the platelet count.

All-*trans* retinoic acid (ATRA, Vesanoid) is a differentiating agent of APL blasts and has been shown to an effective drug, which could give complete remission (CR) in 69-94% of patients without worsening of coagulopathy and symptomatic pancytopenia. However, ATRA is associated with some adverse events, the signs and symptoms of the so-called hypervitaminosis A syndrome and the retinoic acid syndrome.

The aim of the study was a retrospective evaluation of the influence of ATRA on clinical course, hematological and biochemical parameters, and side effects in the group of newly diagnosed and resistant APL patients in Poland.

Material and Methods

Forty patients were included to the study, twenty male and twenty female in age from 7 to 72 with the mean of 43.3. The diagnosis of APL was established based on widely accepted hematological, immunophenotypic and cytogenetic criteria. Eight pts were previously treated by standard induction therapy comprizing daunorubicin and ARA-C without obtaining a complete hematological remission, and 32 pts were newly diagnosed. At the presentation patients showed weakness (77%), hemorrhage diathesis (75%), including DIC (17%), fever (32%), infections of upper respiratory tract (17%), diaphoresis (15%), weight loss (7%), headache (7%), ostealgia (7%). The FBC showed anemia with a mean of 5.30 mmol/l (8.22 g/dl), leukopenia – median 2.15 $\times 10^9$/l and thrombocytopenia with a median 29.5$\times 10^9$/l. Seven pts (17%) revealed DIC confirmed by laboratory data. ATRA was given in doses 10-60 mg/m^2 per os for a median 44 days (range 16-360 days). The first group of patients (9 pts.) received ATRA alone as induction ther-

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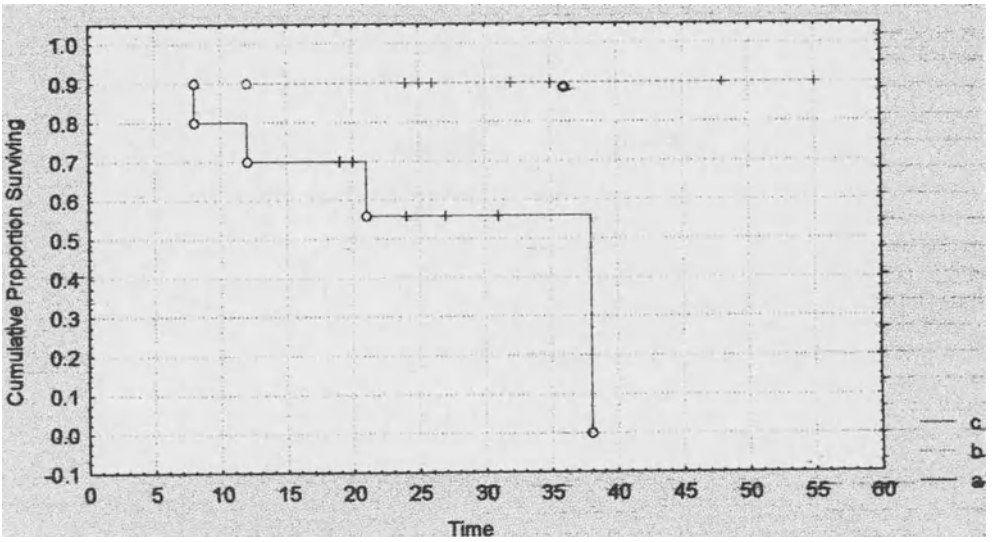

Fig. 1. Cumulative Proportion Surviving (Kaplan-Meier). ○ Complete + Censored

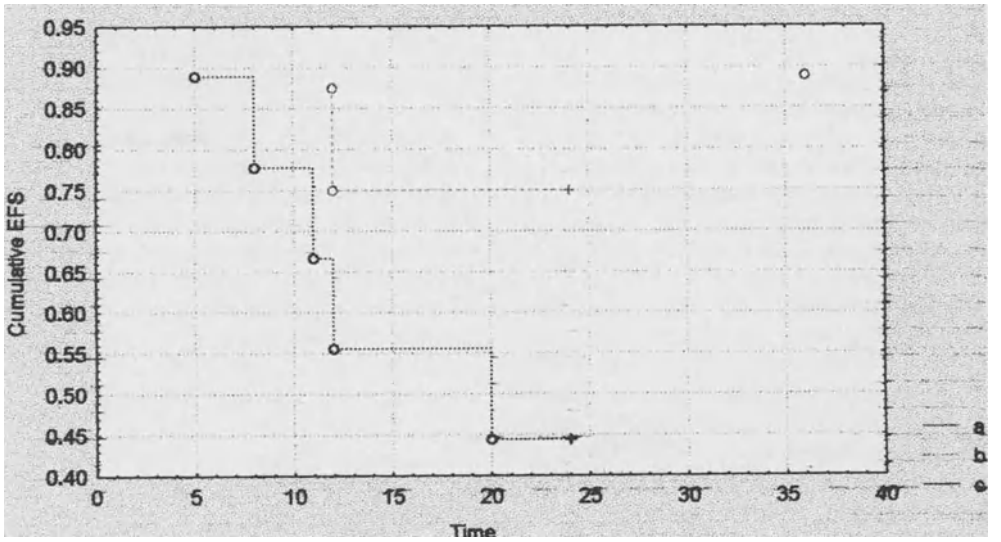

Fig. 2. Cumulative Event Free Surviving (Kaplan-Meier). ○ Complete + Censored

apy and after obtaining complete remission received induction chemotherapy (daunorubicine and ARA-C) with subsequent consolidation and maintenance. The second group (13 pts) following induction with ATRA alone and obtaining a CR received only standard maintenance therapy. In the third group of patients chemotherapy was introduced in 1 patient on day 1 concomitantly with ATRA, 4 patients received chemotherapy because of increased white blood count above 5000/μl by day 5 and 10 000/μl by day 10, in 13 pts chemotherapy was started between day 9 and 11. We evaluated complete remission rate in three groups of patients, event-free survival (EFS) including as events: resistance, relapse and death in CR, and long-free survival (LFS) with a mini-

mum follow up of 24 months after CR achievement.

Results

Complete remission was obtained in 30 (75%) out of 40 patients. In the first and second group of patients (ATRA alone followed by 3+7 induction schedule and ATRA alone followed by maintenance therapy as in AML) the 18 (86%) out of 22 pts, achieved CR in the third group (ATRA and chemotherapy concomitantly achieved CR 12 (66%) out of 18 patients. The analysis showed an EFS in group I, II and III 100%, 77 and 44% respectively ($p > 0.05$, $p = 0.019$) at 2 years of and LFS of 100, 88 and 66%, respectively at 2 years, with no statistical significance (due to the short observation period).

Discussion

Two main classes of adverse reactions are distinguished after administration of ATRA in APL patients: the so-called hypervitaminosis A syndrome and the retinoic acid syndrome (RAS). Hypervitamonosis A syndrome is usually mild and reversible and includes dryness of skin and mucosae of the mouth, nose and eyes, erythema, pruritis, dermatitis, flaking and peeling, cheilitis, rhagades. Moreover, headache, bone pain, hypertriglyceridemia, hypercholesterolemia, transient hepatic dysfunction with elevated serum transaminases, alkaline phosphatase and bilirubin, increased serum creatinine are observed. RAS is a more dangerous event than hypervitaminosis A syndrome and can lead to death, if left untreated. The incidence of RAS varies from country to country and occurs in an average of 25% of patients. It is characterized by fever, hyperleukocytosis, hypotension, dyspnoea, pulmonary infiltrates, acute respiratory distress syndrome (ARDS), multiorgan failure especially hepatic and renal.

In our group of 40 patients we observed the following side effects related to ATRA including: hyperleukocytosis (17.5%), fever (12.5%), erythema (10%), dermatitis (10%), bone pain (10%), headache (7.5%), dryness of skin and mucosae (5%), flaking (5%), elevated serum transaminases (5%), hypertriglyceridemia and hypercholesterolemia (2.5%), hairy loss (2.5%), ulceration of the hand's skin (2.5%), ARDS (2.5%), Multiorgan failure in 20 patients (50%) grade I to III according to WHO scale were observed, making necessary 5-50% reduction of doses in 6 pts (15%) or withdraw the drug in 5 pts (12.5%).

Conclusion

Our clinical retrospective results shows that the combination of ATRA and subsequent standard induction therapy followed by consolidation and maintenance can increase the EFS (100%) and the LFS (100%) compared to the group treated with ATRA alone followed by maintenance – 77% and 88% respectively, and companed to the group treated with ATRA with chemotherapy concomitantly – 44 and 66% respectively. This should be confirmed by randomized, double blind, clinical trial.

The most important advance of ATRA, as a differentiating agent, is its safety and high response rate,which is achieved with a relatively low morbidity. APL patients usually present with coagulopathy at diagnosis, often worsening with standard chemotherapy (high mortality rate), which is improved by therapy with ATRA.

ATRA (Vesanoid) is nowadays the drug of choice in the treatment of APL patients.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Abundance of Auer Bodies and Bilobed Nuclei of Leukemic Cells may be Related to Longer Disease Free Survival of Patients with Acute Promyelocytic Leukemia (M3)

D. IMANISHI, K. KURIYAMA, S. YOSHIDA, and M. TOMONAGA

Abstract. Acute promyelocytic leukaemia (AML-M3) has a unique morphology as defined by FAB classification. Although M3 belongs to good risk AML in general, early deaths and relapses are not infrequent in patients treated with chemotherapy alone or in combination with all trans retinoic acid (ATRA). We evaluated morphology of bone marrow leukemic cells at diagnosis and carried out statistical analysis to make clear the relationship between morphology and prognosis. We investigated 47 adult patients with diagnosis of AML-M3. Twenty two patients were treated with chemotherapy plus ATRA and 25 with chemotherapy alone. Median follow up time was 2.9 years. Thirty one patients (66%) showed Auer body at least in one cell among two hundred leukemic cells (ranging: 0.5~18%). All patients showed unique bilobed nuclei but its positive rate varied widely (ranging: 1~45%). Nineteen (40%) patients showed abundance of the cells with bilobed nuclei defined as more than 15% of leukemic cells. Disease free survival (DFS) was significantly better in the Auer body-positive group and in group with > 15% bilobed nuclei ($p = 0.0498$, $p = 0.0062$, respectively) than in the Auer body-negative group and group with less than 15% bilobed nuclei, respectively. Overall survival was not statistically significant between these comparable groups. These results may suggest biological differences of leukemic cells in M3 which otherwise seems to be a rather homogeneous leukemia entity. However, further confirmation is needed in a much larger-scale M3 clinical trial.

Introduction

Acute promyelocytic leukemia (M3) of the French-American-British (FAB) classification system [1, 2] exhibits some characteristic features: cytoplasmic hypergranularity, Auer body, bilobed nuclei, the specific cytogenetic abnormality t(15;17) [3], and the complication of disseminated intravascular coagulation. Although M3 belongs to good risk AML in general [4, 5], early death and relapse are not infrequent in patients treated with chemotherapy alone or in combination with all trans retinoic acid (ATRA). We investigated 47 adult patients with newly diagnosed M3 and evaluated morphology of bone marrow leukemic cells at diagnosis and carried out statistical analyses to make clear the relationship between morphology and prognosis.

Material and Methods

Patients and Diagnosis

A total of 49 adult patients with newly diagnosed M3 were treated with chemotherapy

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plus ATRA or chemotherapy alone at our institute and eleven cooperative hospitals between September 1987 and May 1996. Diagnosis of M3 was made according to the FAB classification and confirmed in part by the presence of t(15;17) or PML/RARA fusion transcript. Informed consent was obtained from the patients or their families before the treatment.

Morphology

May-Grunwald-Giemsa (MG) stained bone marrow smears were used for morphological analysis. Percentages of cells with Auer body were counted among 200 leukemic cells. Percentages of cells with bilobed nuclei and percentages of hypergranular cells were counted among 100 leukemic cells, respectively.

Response Criteria

Response was evaluated by standard criteria generally used for chemotherapy. CR was defined as less than 5% of blasts and promyelocytes with normal hematopoiesis in the bone marrow, and more than 1.5×10^9/l neutrophil counts and more than 100×10^9/l platelet counts in the peripheral blood.

Statistical Analysis

To test factors for predicting CR, the chi-square test was used for univariate analysis, and the logistic regression model for multivariate analysis. Overall survival was measured from the first day of therapy to death. Disease free survival (DFS) for patients who had achieved CR was measured from the date of CR to relapse or death. Overall survival and DFS were determined according to Kaplan-Meier's method and compared by the log-rank test for univariate analysis, and the Cox regression model for multivariate analysis. SAS programs were used for analysis.

Table 1. Clinical hematological and cytogenetic findings

	No. of patients
Age (years, n = 47)	48[a] (19~78[b])
Sex (male/female)	22/25
the use of ATRA	22
WBC (x 10^3 μl, n = 46)	3.0[a] (0.6~129.3[b])
Plt (x 10^4 μl, n = 46)	4.05[a] (0.5~18.6[b])
t(15;17)	28/31
CR rate (%, n = 47)	85.1

[a] Median [b] Range

Results

Patients' Characteristics and Treatment Outcome

Forty seven patients could be evaluated for bone marrow smears and treatment outcome. Two patients were excluded because of poor bone marrow smears. Cytogenetic analysis were available in 31 patients; 28 patients of them had t(15;17) and 1 of the remaining 3 patients had PML/RARA fusion transcripts. ATRA was given in 22 patients. Forty patients (85.1%) achieved CR. Median follow-up time was 2.9 years. Clinical, hematological and cytogenetic data on patients are summarized in Table 1.

Morphological Analysis

Fifteen (32%) and five (11%) patients showed abundance of blast forms and mature neutrophils defined as more than 10% of 100 white blood cells (WBC), respectively. Thirty six (77%) patients showed abundance of promyelocytes defined as more than 70% of 100 WBC. Increase of basophils defined as more than 3% was observed in six patients (13%). Increase of monocytes was not observed in all patients. Thirty one patients (66%) showed Auer bodies at least in one cell among two hundred leukemic cells. The maximum positive rate was 18%. All patients showed bilobed nuclei among one hundred leukemic cells but its positive rate varied widely (ranging: 1-45%). Nineteen (40%) patients showed abundance of the cells with bilobed nuclei

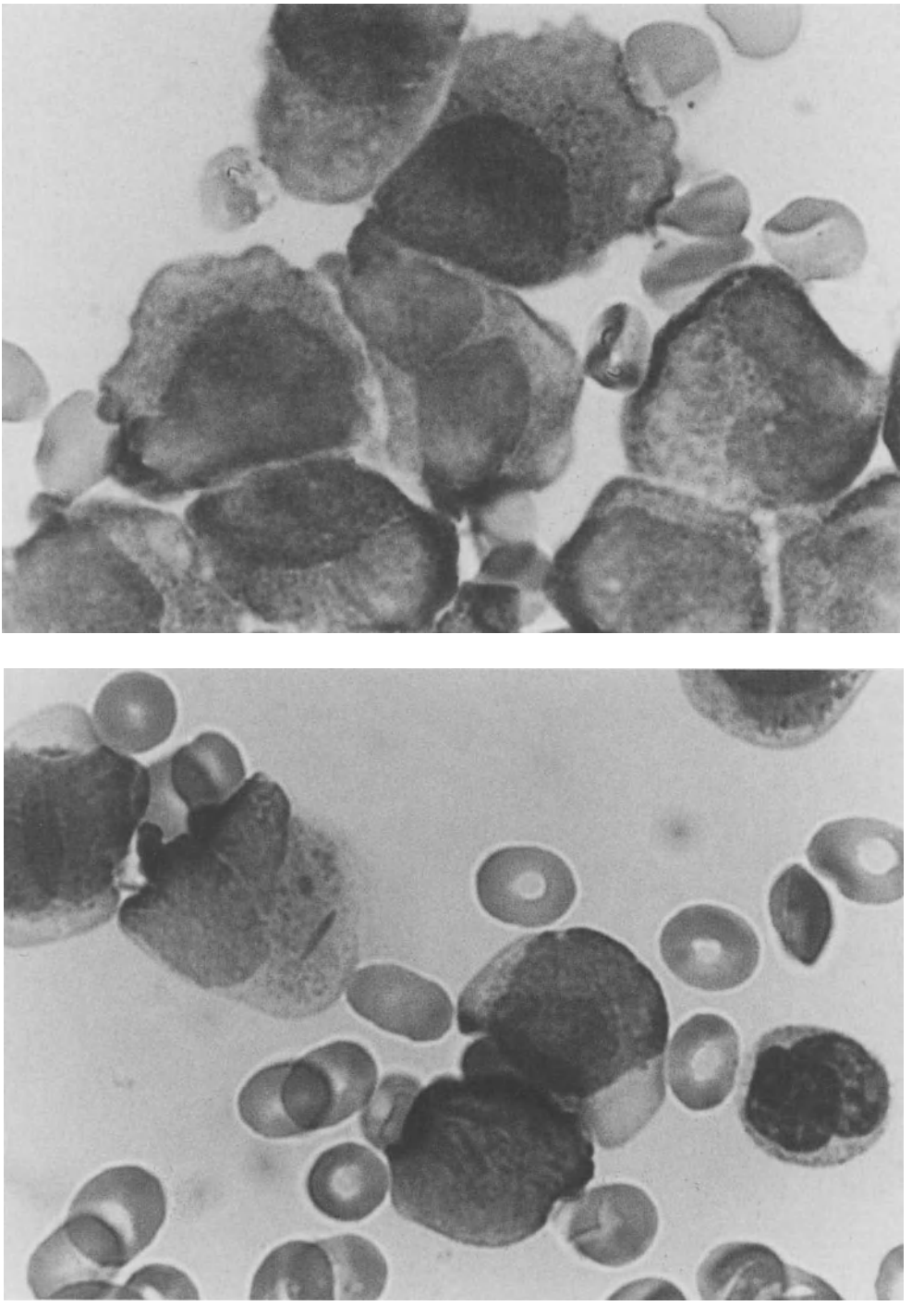

Fig. 1. Auer body and/or bilobed nuclei

Table 2. Results of morphological analysis

		No. of patients	(%)
Analysis			
Blast		10% <	15 (32)
Promyelocyte		70% <	36 (77)
Mature neutrophils		10% <	5 (11)
Basophil		3% <	6 (13)
Monocyte		1% <	2 (4)
Auer body		1% <	31 (66)
Bilobed nuclei	{	1% <	47 (100)
	{	15% <	19 (40)
Hypergranular cell		70% <	28 (60)

defined as more than 15% of leukemic cells. Twenty eight (60%) patients showed abundance of hypergranular cells defined as more than 70% of leukemic cells. These data are summarized in Table 2 and cells with Auer body and/or bilobed nuclei are shown in Fig. 1.

Statistical Analysis

Univariate analysis showed that CR rate and overall survival rate were not significantly different between all investigated factors (age, WBC and platelet count, percentages of blast, promyelocyte, mature neutrophil, basophil, Auer body, bilobed nuclei and hypergranular cell). DFS was significantly better in the Auer body positive group and in the group with 15% < unique nuclei ($p = 0.0498$, $p = 0.0062$, respectively) than in the Auer body negative group and the group with less than 15% unique nuclei, respectively (Fig. 2). Multivariate analysis could not be done due to the small numbers of patients in each category.

Discussion

Acute promyelocytic leukemia (M3) is considered to be a rather homogeneous leuke-

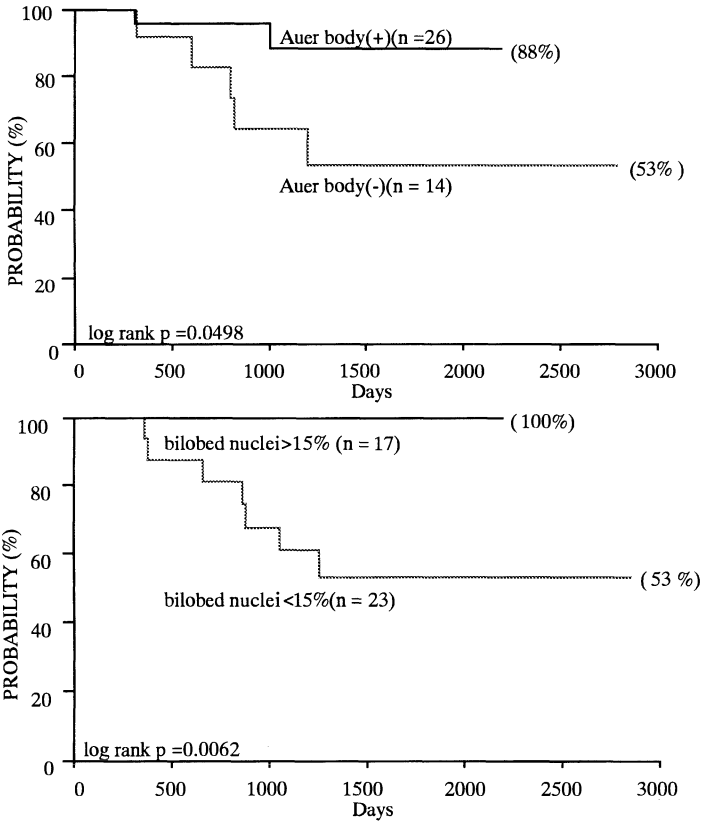

Fig. 2. Kaplan-Meier curves for DFS. Disease-free survival (DFS) was significantly better in Auer-body-positive group (top) and in group with 15% < bilobed nuclei (bottom) ($p = 0.0498$, $p = 0.0062$, respectively) than Auer-body-negative group and group with less than 15% < bilobed nuclei, respectively

mia entity with some characteristic features [1, 2] and believed to have good prognosis [4, 5]. However, unusual morphology are occasionally observed in some patients [6, 7, 8] and early death and relapse are not infrequent. For this reason, we carried out this study to elucidate the relationship between morphology and prognosis. In this study, unusual morphology such as a relatively high percentages of blast forms or mature neutrophils or basophils and relatively low percentages of promyelocytes or hypergranular cells were found in some patients. Only 31 patients (66%) showed Auer bodies in at least one cell among 200 leukemic cells and the maximum positive rate was 18%. These percentages were lower than expected. In statistical analysis, disease free survival (DFS) was significantly better in the Auer-body positive group and in the group with 15% < unique nuclei ($p = 0.0498$, $p = 0.0062$, respectively) than in the Auer body negative group and the group with less than 15% unique nuclei, respectively. These results may suggest biological differences of leukemic cells in M3. However, further confirmation is needed in a much larger M3 clinical trial.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

The Influence of Dysplastic Hematopoiesis on the Outcome in Patients with de Novo AML

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Abstract. The detection of dysplastic features of hematopoiesis in de novo acute myeloid leukemia (AML) by light microscopy is defined as AML with trilineage myelodysplasia (AML/TLMD). The prognostic relevance of these dysplastic features for patients with de novo AML still remains unclear. In order to evaluate the role of dysplasia in de novo AML, bone marrow aspirates from 95 patients were analyzed prospectively and investigated separately for erythropoiesis, granulopoiesis and megakaryopoiesis by three independent investigators.

The overall complete remission (CR) rate was 48.8% and partial remission (PR) or non responder were 52.2% of the patients investigated. The median overall survival time was 5 month with a disease free interval of 3.5 months for all patients. Dysgranulopoiesis (DysG) was observed in 28.4%, dysmegakariopoiesis in 40.0%, dyserythropoiesis was observed in 37.5% respectively. Out of all patients 20.0% of the patients showed trilineage dysplastic features and, thus, were classified as AML/TLMD.

A significant worse prognosis was calculated for those patients with detection of only dysgranulopoiesis ($p = 0.002$), dysmegakariopoiesis ($p = 0.02$) or dyserythropoiesis ($p = 0.04$) as compared to patients without any dysplastic signs. An unfavourable karyotype was correlated with patients showing dysgranulopoiesis ($p = 0.02$) and dysmegakariopoiesis ($p = 0{,}04$). For the patients with an unfavourable karyotype, the occurrence of any dysplastic features had no additional prognostic impact.

Dysplastic features (DysG, DysM, DysE) seem to be an important prognostic factor in de novo AML correlating with short overall survival time. Dysgranulopoiesis and dysmegakariopoiesis correlated well with the appearance of unfavourable chromosomal abnormalities. It may be reasonable to assume that patients with dysplastic features should be considered for more aggressive treatment schedules at the time of diagnosis.

Introduction

Aggressive chemotherapeutic schedules in patients with de novo acute myeloid leukemia (AML) have increased the rate of patients achieving complete remission (60-80% for de novo AML) [4]. Despite several post-remission strategies, only 20-30% of patients with CR achieved have an overall survival rate of more than 5 years [13]. Although different risk factors predicting stable disease have been discussed (e.g., age, leukemic cell mass and abnormal cytogenetics) [9, 12], these factors are not established and still under debate.

Recently, in a large study it was shown that dysplastic features likewise observed in

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myelodysplastic syndromes are also observed in de novo AML. According to these findings, clinical outcome of this particular group of de novo AML seems to be more closely related to the prognosis of advanced myelodysplastic syndromes [8, 10]. These cases of AML defined as AML with trilineage myelodysplasia (AML/TLMD) have been reported to account for 10-15% of de novo AML [3, 6, 8]. Furthermore, in patients with AML/TLMD who achieved CR relapse occurred much earlier than patients without dysplastic features [11]. The lack of prospective studies is one of the important reasons that the prognostic relevance of TLMD detection remains unclear. Whether AML/TLMD is an acute transformation of MDS and whether AML/TMD in fact has to be defined as poor prognostic subtype of AML is under debate.

Thus, it was the aim of this study to investigate by prospective analysis patients with de novo AML with regard to dysplastic features. For this purpose from 1990 to 1997 all patients with de novo AML were enrolled and investigated for trilineage myelodysplasia.

Materials and Methods

Patients. Ninety five patients. (median: 60.5 years ranged from 17 to 85 years, female: n = 52 and male: n = 43) with de novo AML were classified according to the FAB classification [2] [M0 (1,9%), M1 (22.8%), M2 (22.8%), M3 (26.6%), M4 (4.9%), M5 (4.7%), M6 (4.7%), M7 (2.8%), for details see Table 1]. The patients, were treated with the following induction therapies: TAD-VP (thioguanine, cytarabine, etoposide) 19.9% (n = 21), DAE (daunoblastine, cytarabine, etoposide) 22.8% (n = 24), DA (daunoblastine, cytarabine) 29.3% (n = 31), the remaining 18% (n = 19) of patients. achieved no induction chemotherapy protocol.

Morphological Evaluation. Bone marrow aspirates were investigated by conventional cytochemical methods (May-Giemsa, myeloperoxidase, alpha-naphtyl butyrate esterase, and chloroacetate esterase), multiparametric flow cytometry (CD33/ CDw65/ CD13, CD34/CD117/HLA-DR, CD45/ CD14/CD20, CD19/CD14/CD64, CD2/ CD56/CD7 and myeloperoxydase/lactoferrin) and cytogenetic analysis (chromosomal banding analysis). The bone marrow smears were examined independently by three experienced morphologists. If morphologically differing assessment was achieved by two investigators a panel discussion was started and only those cases with confirmation by all investigators were included in this study.

AML/TLMD (for details see Table 1) was diagnosed if dysplastic features were detected in the erythroid, granulocytic and megakaryocytic lineages, essentially according to Brito-Babapulles's criteria [3]. Dyserythropoetic features were defined as more than 50% dysplastic features in at least 25 erythroblasts, i.e., megaloblastoid changes, multinuclearity, nuclear fragments, or karyorrhexis. Dysgranulopoetic features were defined as three or more hyposegmented nuclei (pseudo-Pelger-Huet'sche anomaly) or as more neutrophils being agranular or hypogranular. Dysmegakaryocytic features

Table 1. Distribution of dysgranulopoiesis, dyserythropoiesis and dysmegakariopoiesis in patient suffering from acute myelogenous leukaemia (AML, n = 95) according the FAB classification [2].

FAB-subtype	Number	Dysgranulopoiesis	Dysmegakariopoiesis	Dyserythropoiesis	Trilineage dysplasia
M0	2	2	2	2	2
M1	24	8	12	11	6
M2	24	4	10	7	4
M3	26	1	2	1	0
M4	6	3	6	6	2
M5	5	0	1	0	0
M6	5	4	4	4	4
M7	3	0	0	0	0

were defined as three or more megakaryocytes being micro-nuclear (mono- or bi-nuclear), multi-separated nuclear, or large mononuclear forms.

Cytogenetic Analysis. The cytogenetic analyses were performed on bone marrow cells; karyotypes were analysed using Q- and G-banding method [5].

Statistical Analysis. Clinical and haematological data as well as remission rates were analyzed using the chi-square test (SPSS software). The Kaplan procedure was performed to determine survival and disease free survival (DFS). Overall survival was calculated from the first day of therapy until the day of death. DFS was calculated from day achieving CR until relapse or death. To test single factors that predict CR and overall survival, the logistic progression model and the Cox regression model were use for multivariate analysis (SPSS software).

Results

After induction chemotherapy of all patients the overall complete remission rate was 48.8% and partial remission as well as non-responder rate was 52.2%, respectively. The median overall survival was 5 months with a disease free interval of 3.5 months (Fig. 1).

Dyserythropoiesis (DysE) was found in 31 (29.3%) cases, dysgranulopoiesis (DysG) was found in 22 (20.9%) cases and dysmegakariopoiesis was found in 38(36.1%) cases. Among the 95 patients enrolled a total of 38 cases had no dysplastic features. Trilineage Myelodysplasia (AML/TLMD) was not observed in AML FAB M3 and M7 subtype and had a numeric increased frequency in AML FAB M6 subtype.

Disease-free survival was analyzed for all patients according to the presence or absence of dysplastic features. The estimated median (50% alive) overall survival for patients with dysgranulopoiesis (DysG) was 2 month as compared to 4 months for patients without dysgranulopoiesis (DysG). If dysmegakariopoiesis (DysM) was observed the overall survival for those patients was 1 months versus 5 months for patients without dysmegakariopoiesis (DysM). Patients with dyserythropoiesis (DysE) have had also a disease-free survival of 1 month as compared to 5 month for those without dyserythropoiesis (DysE). These results were significant for all patients with dysplastic features (DysG $p = 0.002$; DysM $p = 0.02$ and DysE $p = 0.04$; for details see Figs. 2-4).

The disease-free survival for patients with AML/TLMD was not significant different from patients without any dysplastic features. It should be mentioned that despite the lack of significance a (numeric) worse disease free survival was observed for patients with trilineage dysplasia (AML/TLMD, Fig. 5).

Cytogenetic findings were available in 78 cases (71.4%) and among the 35 karyotypes

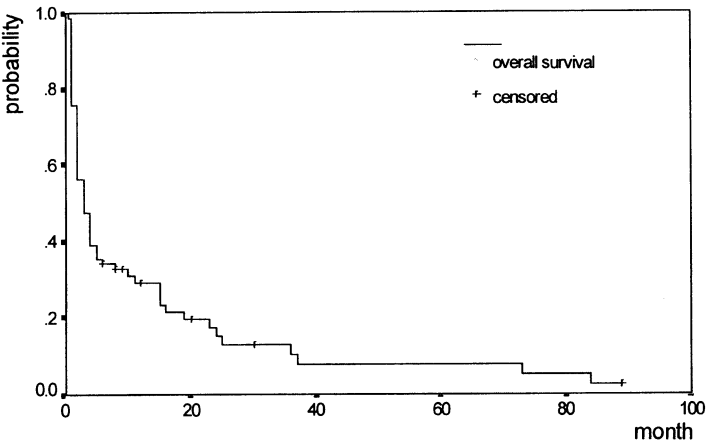

Figure 1. Overall survival in patients with de novo AML and dysplastic features

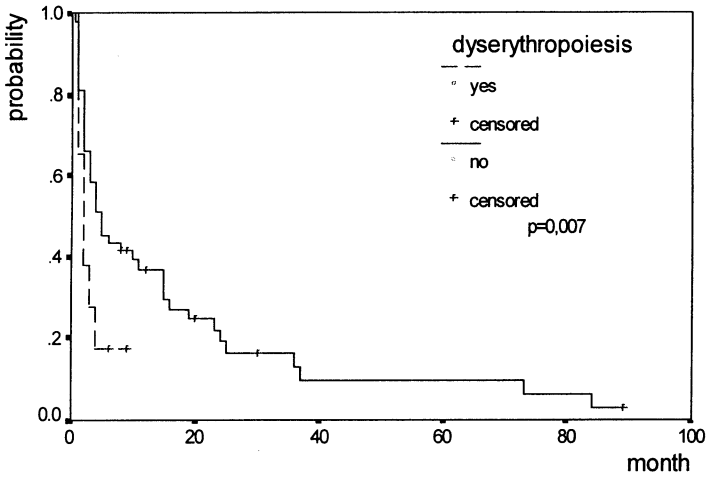

Fig. 2. Disease free survival in patients with de novo AML and dyserythropoiesis and patients without dysplastic features

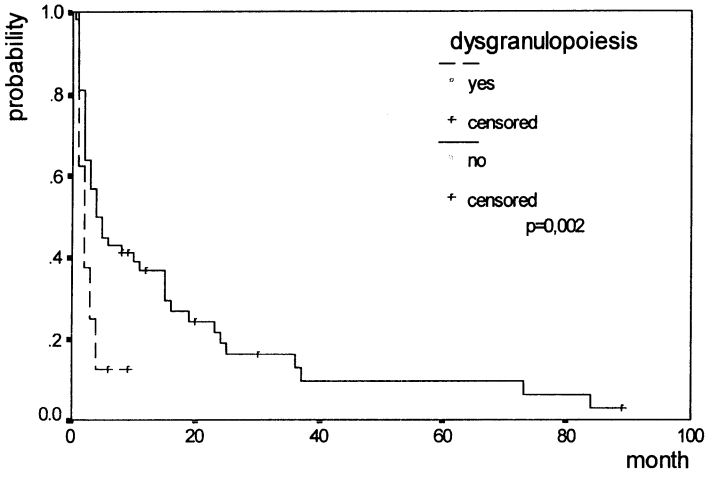

Fig. 3. Disease-free survival in patients with de novo AML and dysgranulopoiesis and patients without dysgranulopoietic features

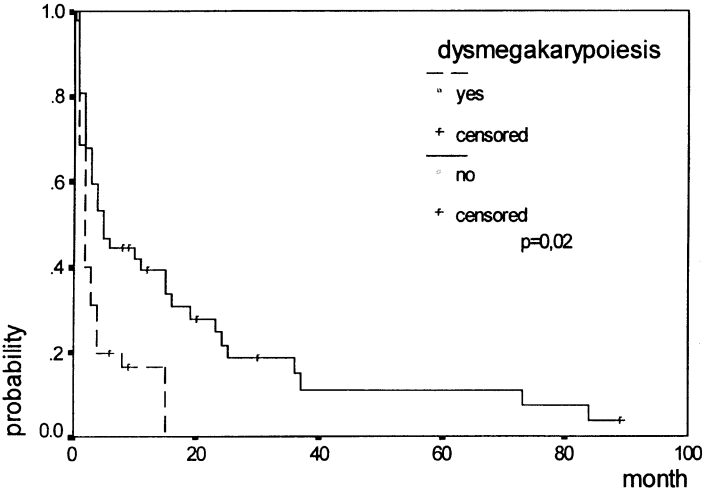

Fig. 4. Disease free survival in patients with de novo AML and dysmegakaryopoiesis and patients without dysmegakariopieticc features

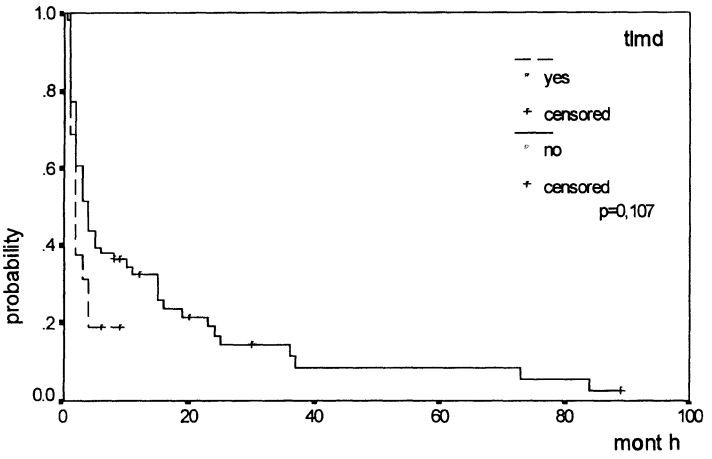

Fig. 5. Disease-free survival in patients with de novo AML and trilineage myelodysplasia (AML/TLMD) and patients without TLMD

of patients with DysM 15 had an unfavourable karyotype (i.e., -7q, -5q, t(1;7), inv.3, t(3;3), complex chromosome aberrations). Out of the 31 patients with DysG 13 patients. had unfavourable chromosome abnormalities. Unfavourable karyotype correlated significantly with patients showing DysG ($p = 0.02$) and DysM ($p = 0.04$). For those patients with an unfavourable karyotype, the occurrence of any dysplastic features had no additional prognostic impact for the overall survival.

Discussion

In a large study Brito-Babapulle and coworkers [3] showed that patients with AML and trilineage dysplasia should be defined as a separate group of patients with de novo AML and do not reflect secondary AML. For these patients a close relationship could be demonstrated between blast cell counts and occurrence of dysplastic features (TLD) (14). Thus, it was of special interest to investigate prospectively the occurrence of dysplastic features in de novo AML and to correlate overall survival of this distinct AML population. With regard to the definitions of dysgranulopoiesis (DysG), dysmegakariopoiesis (DysM) and dyserythropoiesis (DysE) 74 patients out of 95 patients with de novo AML could be analyzed to clarify the clinical relevance of dysplastic features [3].

The short median overall survival time of the patients is related to an unselected patient group and is in accordance to recent findings by others [1]. A significant < 0.05, using Student's t-test) worse prognosis could be demonstrated in those patients with dysgranulopoiesis ($p = 0.002$), dysmegakaryopoiesis ($p = 0.02$) or dyserythropoiesis ($p = 0.04$) as compared to those patients without any dysplastic signs.

Furthermore, this study confirms previous data that AML/TLMD was observed in an increased frequency of FAB-M4, M2 and M6 subtype. Trilineage dysplasia was not observed in AML FAB M3 subtype [3, 6, 8]. Although in this study no patient was observed with FAB M5 and TLMD, the occurrence for this subtype has been reported by others [3, 6].

However, some important conclusions could be drawn from the results obtained: first, dysplastic features (DysG, DysM, DysE) could be indeed demonstrated in patients with proven de novo AML. Second, dysplasia is an important prognostic factor for de novo AML correlating with an decreased overall survival time as well as disease free survival as compared to patients without dysplastic features. Dysplasia of granulocytes and megakaryocytes correlated well with the appearance of unfavourable chromosomal karyotypes.

The results strongly suggest it may be reasonable to assume that patients with dysplastic features should be considered as prognostic risk group. To identify these patients at diagnosis, detection of dysplastic features by light microscopy is an easy and

simple tool. In this way these patients could be stratified early in the course of risk adapted treatment strategies.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Trial to Extract Prognostic Factors Prior to the Start of Induction Chemotherapy for Adult AML

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Abstract. To predict prognoses of AML patients prior to the start of chemotherapy and to classify them according to their predicted prognoses, we analyzed the data on 545 (90%) patients out of 606 patients who were enrolled in the JALSG AML-87 or -89 protocol. Median age was 48 years. AML subtypes were as follow; AML-M1 was 96 (17.6%), M2 196 (36.0%), M3 107 (19.6%), M4 95 (17.4%), M5 31 (5.7%), M6 17 (3.1%) and M7 3 (0.6%). Eighty-nine patients(16%) were diagnosed as having trilineage dysplasia (TLD). We could elucidate seven good prognostic factors using multivariate analysis and gave them the weighted scores such as +2; ≤50 years of age, ≤ 20 000/mm^3 of WBC, ≥ 50% of myeloperoxidase (MPO) positivity of blasts and presence of Auer bodies and +1; absence of TLD, FAB subtypes of AML-M2 or -M3 or -M4 with eosinophilia and ≤30 IU/l of serum glutamic oxaloacetic transaminase (GOT). A scoring system could classify the patients into three groups such as good (total scores; +9~+11) (149 cases, 53% at 5 years-survival), intermediate (+5~+8) (175 cases, 29%) and poor risk (0~+4) (95 cases, 10%). This system will be tested for feasibility in the next AML-92 protocol (about 700 cases) prospectively. If this procedure proves feasible, clinical trials could be designed based on such a risk group-stratification.

Introduction

Over half of AML patients who have undergone allogeneic BMT during the first remission can survive for a long time. On the other hand, the long-term survivors who received chemotherapy alone have not reached 50%, though prognosis has gradually improval. The long survivors include many patients having karyotypes such as t(15;17), t(8;21) or inv(16), or having lower WBC counts or being younger, and they are regarded as good prognostic factors [1]. If we can predict the prognostic factors, including the karyotypes, in AML patients before chemotherapy, we could treat them according to a treatment strategy for AML based on these factors. However, it is difficult to obtain the karyotypes prior to the start of induction chemotherapy. Therefore we analyzed the data excluding the karyotypes on the patients enrolled in the JALSG-AML protocols before chemotherapy with respect to prognosis; we could classify AML into good, intermediate or poor risk groups according to the prognostic factors.

Materials and Methods

Five hundred forty-five (90%) of 606 patients enrolled in the AML-87 or -89 protocol

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[2, 3] were reviewed morphologically by the central committee and were analyzed in this study. The diagnosis and classification for AML were done according to the FAB criteria [4]. Clinical data were obtained from the data sheets sent by 23 institutions. Survival and disease free survival (DFS) were calculated according to the Kaplan-Meier method and compared by the two sided log rank test, with the use of Phreg procedure in the SAS program. Prognostic factors for the achievement of complete remission (CR) and for longer survival and DFS were determined by multivariate analysis. The AML-87 or-89 protocol is described in detail elsewhere [2, 3]. It is summarized as follows, the induction therapy of AML-87 consisted of Behenoyl-ara-C (BHAC), Daunorubicin, 6-mercaputopurine and prednisolone and was randomized with regard to the addition or not of vincristine. After three courses of consolidation therapy, maintenance therapy was also randomized to either 4 or 12 courses. BHAC in AML-89 protocol was compared with ara-C in the induction and consolidation therapies. After six courses of maintenance therapy, the randomization of immunotherapy was done.

Table 1. Comparison of clinical findings between AML-87 group and AML-89 group

	AML-87 (n = 234)	AML-89 (n = 311)
Male/female	128/106	165/146
Age (yrs, median)	48 (15-79)	48 (14-82)
RBC (x 10^4/mm^3, median)	255 (29-535)	251 (2-480)
WBC (x 10^3/mm^3, median)	12.5 (0.4-360)	16.7 (0.3-657)
Plt. (x 10^4/mm^3, median)	5 (0-54)	4 (0-45)
Blasts in BM (%, median)	65 (5-99)	66 (8-99)
PS (0-2) (cases %)	196 (90.7)	276 (90.5)

Table 2. Frequency of AML subtypes according to the FAB classification

FAB	AML-87	AML-89	Total
M1	43 (18.4%)	53 (17.0%)	96 (17.6%)
2	83 (35.5)	113 (36.3)	196 (36.0)
3	42 (17.9)	65 (20.9)	107 (19.6)
4	46 (19.7)	49 (15.8)	95 (17.4)
5	13 (5.6)	18 (5.8)	31 (5.7)
6	6 (2.6)	11 (3.5)	17 (3.1)
7	1 (0.4)	2 (0.6)	3 (0.6)
Total	234	311	545

Results

Table 1 shows the clinical findings of the patients. The median age was equal and the other findings were not different in both groups.

AML subtypes according to the FAB classification [3] were as follow; AML-M1 was 96 (17.6%), M2 196 (36.0%), M3 107 (29.6%), M4 95 (17.4%), M5 31 (5.7%), M6 17 (3.1%) and M7 3 (0.6%) (Table 2). Eighty-nine patients (16%) were diagnosed as having trilineage dysplasia (TLD) (Table 3) [5, 6]. They were compared with the patients without TLD. Both groups were different statistically in platelet counts, blast percentage in bone marrow (BM), myeloperoxidase (MPO) positive blasts in BM and the cases having Auer bodies in blasts.

We analyzed 25 factors in regard to CR, survival and longer DFS by multivariate

Table 3. Comparison of clinical findings between AML with trilineage dysplasia (TLD) and AML without TLD

	AML with TLD (n = 89)	AML without TLD (n = 452)	*p* value
Male/female	46/43	244/208	$p = 0.69$
Age (yrs, median)	52 (16-79)	48 (14-82)	$p = 0.26$
RBC (x 10^4/mm^3, median)	257 (107-532)	252 (2-480)	$p = 0.54$
WBC (x 10^3/mm^3, median)	18.2 (0.7-235)	13.9 (0.3-657)	$p = 0.17$
Plt. (x 10^4/mm^3, median)	8 (1-54)	4 (0-39)	$p < 0.001$
Blasts in BM (%, median)	47 (8-91)	69 (5-99)	$p < 0.001$
MPO + blasts in BM (%, mean)	40 + 30	78 + 30	$p < 0.001$
Auer + blasts (cases) (%)	24 (27)	260 (58)	$p < 0.001$
PS (No. of 0~2/No. of cases) (%)	80/87 (92)	390/431 (90)	$p = 0.40$

Table 4. Prognostic factors for the achievement of CR and for longer survival and DFS, determined by multivariate analysis

	Favorable variable	*p* value
Achievement of CR		Logistic procedure
GOT	≤ 30 IU/l	0.0113
PO positivity of blasts	> 50%	0.0541
Total protein	> 7 g/dl	0.0722
TLD	Without	0.1899
Survival		Phreg procedure
Age	≤ 50 yrs	0.0001
PO positivity of blasts	> 50%	0.0120
Auer bodies	Present	0.0189
TLD	Without	0.0703
FAB classification	M2, M3, M4e	0.0723
DFS		Phreg procedure
Age	≤ 50 yrs	0.0002
WBC	≤ 20 000/mm^3	0.0035
PO positivity of blasts	> 50%	0.0079
Auer bodies	Present	0.0233

Table 5. The scoring system for predicting longer survival and DFS of the patients with AML

	Favorable variable	Score
Patient characters		
Age	≤ 50 yrs	+2
Laboratory findings		
WBC	≤ 20 000/mm^3	+2
GOT	≤ 30 IU/l	+1
Morphological findings		
MPO positivity of blasts	> 50%	+2
Auer bodies	Present	+2
TLD	Without	+1
FAB classification	M3 or M2 or M4e	+1

Total score is +9~+11; Good risk group
+5~+8; Intermediate risk group
0~+4; Poor risk group

analysis. The strongest for achieving CR impact factor was a single course of induction. However, this factor was excluded because it cannot be adapted to the patients before induction therapy. Prognostic factors for the achievement of CR and for longer survival and DFS were determined by multivariate analysis (Table 4). We could elucidate seven good prognostic factors and gave them the weighted scores such as +2; ≤ 50 years of age, ≤ 20000/mm^3 l of WBC, ≥ 50% of MPO positivity of blasts and presence of Auer bodies and +1; absence of TLD, FAB subtypes of AML-M2 or -M3 or -M4 with eosinophilia (M4e) and ≤ 30 IU/l of serum glutamic oxaloacetictransaminase (GOT) (Table 5). We classified the patients into three groups such as good, intermediate and poor risk according to total scores of +9~+11, +5~+ 8 and 0~+4, respectively (Table 5). Both survival and DFS of the three groups which were classified according scoring to this system differed significantly (Fig. 1). Five year-survival and -DFS were 53 and 54% in the good risk group, 25 and 29% in the intermediate group and 13 and 10% in the poor risk group, respectively. Frequency of the FAB subtypes in each risk group is shown in Table 6. In gene-

Table 6. Relationship between AML subtypes and risk groups according to the scoring system

FAB classification	Risk group			Total
	Poor	Intermediate	Good	
AML-M1	25 (32%)	49 (63%)	4 (1%)	78
AML-M2	19 (13)	50 (33)	82 (54)	151
AML-M3	1 (1)	26 (30)	61 (69)	88
AML-M4	35 (44)	43 (54)	1 (1)	79
AML-M5	11 (92)	1 (8)	0	12
AML-M6	3 (30)	6 (60)	1 (10)	10
AML-M7	1 (100)	0	0	1

Table 7. Relationship between each factor and risk groups according to the scoring system

Factor	Risk group			Total
	Poor	Intermediate	Good	
Age				
≤50 yrs	15 (6%)	102 (44%)	115 (50%)	232
> 50 yrs	80 (43)	73 (39)	34 (19)	187
WBC				
≤20 000/mm^3	27 12)	73 (32)	126 (56)	226
>20 000/mm^3	68 (35)	102 (53)	23 (12)	193
GOT				
≤30 IU/l	51 (17)	113 (38)	137 (46)	301
> 30 IU/l	44 (37)	62 (53)	12 (10)	118
Auer bodies				
present	4 (2)	85 (37)	138 (61)	227
absent	91 (47)	90 (47)	11 (6)	192
MPO positivity of blasts				
≤50%	69 (65)	36 (34)	1 (1)	106
> 50%	26 (8)	139 (44)	148 (47)	313
TLD				
without	59 (17)	144 (41)	147 (42)	350
with	36 (52)	31 (45)	2 (3)	69

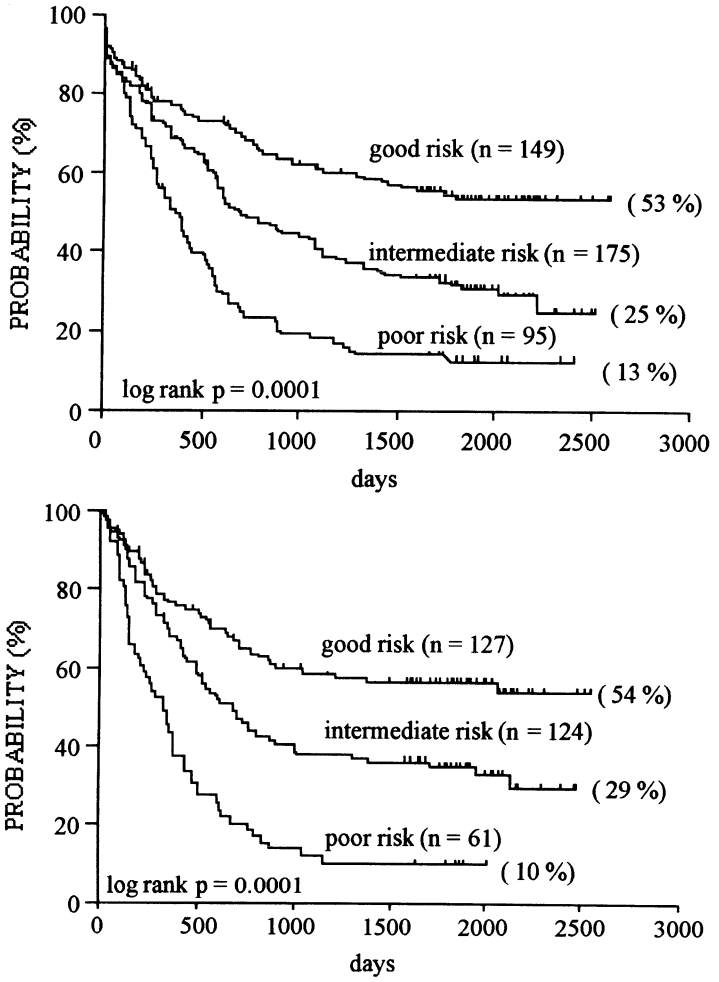

Fig. 1. Kaplan-Meier curves for survival *(top)* and DFS *(bottom)*

ral over 50% of AML-M2 and -M3 were included in the good risk group, on the other hand most patients of the other subtypes belonged to the inter mediate or the poor risk group. The risk factors excluding the FAB subtypes are as shown in Table 7. The good risk factors were $\leq$ 50 years of age, 20000/mm^3 of WBC, $\leq$ 30 IU/l of GOT, presence of Auer bodies, $\geq$ 50% of MPO positivity of blasts and absence of TLD.

Discussion

Our scoring system based on the factors of the clinical and morphological data could classify AML patients into three risk categories. The karyotypes, which are regarded as one of the most important risk factors are excluded in this study because cytogenetic results can usually not be obtained before the start of chemotherapy. Over 90% of the good prognostic karyotypes such as t(8;21), t(15;17) or inv(16) were included in AML-M2, -M3 or -M4, respectively, so that the karyotypes are well reflected in the scoring system. It is controversial whether the patients achieving CR should continue chemotherapy or undergo allogeneic BMT. But if we can predict prognoses of AML patients before chemotherapy, the good risk group may continue chemotherapy whereas the intermediate and the poor risk groups may be chosen for allogeneic BMT. The induction

therapy for the good risk group except AML-M3 should be done by the conventional chemotherapy, because the CR rate was 90% (data not shown). AML-M3 is now treated with all-*trans* retinoic acid with or without chemotherapy and over 90% of the patients can achieve CR [7]. The post-induction therapy for this group should be more intensive, incorporating high dose ara-C, in order to further improve the long-survival rates. In the other two groups, the patients who are younger than 50 years old should be given a more intensive induction and consolidation chemotherapy with or without various cytokines, in order to improve the CR rates and the long-survival rates. We cannot find any reports about successful chemotherapy convincing in the elderly so that the strategy for this age group remains most difficult one. This fact means that palliative therapy is important a until useful chemotherapy is developed. We propose that allogeneic BMT is indicated for the younger patients in 2nd CR or more in the good risk group and those of 1st CR in the intermediate and poor risk groups.

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Stem Cell Transplants in Acute Myeloid Leukaemia (AML)

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Abstract. The 10th United Kingdom Medical Research Council (MRC) AML trial showed a reduced overall relapse rate compared to previous trials, attributed largely to increased treatment intensity. Autologous bone marrow transplantation (ABMT) was also associated with a reduced rate of relapse. These benefits, however, were partly offset by slow neutrophil and platelet recovery after later courses of chemotherapy, and in the context of ABMT, the resulting high procedural mortality (12%) from infection and/or haemorrhage led to no overall survival benefit for ABMT. The 12th MRC AML trial aims to assess the benefit of further treatment intensification through randomisation to 4 or 5 chemotherapy courses for standard risk patients, and to examine further the role of ABMT. In an attempt to overcome some of the previous problems associated with delayed haemopoietic recovery, the trial incorporates the option to collect peripheral blood stem cells (PBSCs) and administer them as additional support after course 4, 5, or in association with ABMT, with the aim of facilitating more rapid haemopoietic reconstitution. Factors associated with optimal PBSC collections will be analysed as the trial proceeds. The theoretical risk of tumour contamination of PBSC samples will be assessed, where possible, by molecular techniques, and at close of trial in terms of relative relapse rate.

Introduction

In the management of acute myeloid leukaemia (AML), the availability of peripheral blood stem cell transplantation (PBSCT) has made this an attractive alternative to autologous bone marrow transplantation (ABMT) as a means of stem cell rescue following high-dose therapy. It seems unlikely that a prospective study of PBSCT versus ABMT in AML will be performed; indeed there is generally an increasing switch towards total or subtotal use of peripheral blood stem cells, rather than bone marrow, for autologous stem cell rescue. The potential benefits of PBSC following high-dose therapy in AML are twofold. Firstly, there is the possibility that the addition of PBSC in place of marrow alone will reduce the morbidity and mortality seen in prospective randomised studies of high-dose therapy in AML. This may allow the improvement in relapse rate after ABMT, compared to conventional chemotherapy, to be translated into improved overall survival. Secondly, there is a theoretical possibility, for which no data is currently available, that PBSCs may be less contaminated with clonogenic leukaemic cells than autologous bone marrow.

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Materials and Methods

Previous Data and Rationale

The rationale for the use of peripheral blood stem cells in the new UK adult leukaemia trial, MRC AML-12 [1], is derived from the structure and findings of the previous MRC AML trial, now closed (AML-10). The AML-10 trial [2,3] ran between 1987 and 1994 and accrued almost 2000 patients including both adults and children. The trial involved an initial chemotherapy randomisation between DAT and ADE, each of which was given as two courses. Following this, two further courses of conventional chemotherapy were administered, MACE and MidAC. After the fourth course of chemotherapy, those patients having a matched sibling were allocated to allogeneic bone marrow transplantation in first remission, while those patients for whom a matched sibling was not available were randomised, either to stop chemotherapy, or to have a fifth course of treatment in the form of cyclophosphamide and total body irradiation (TBI), rescued by autologous bone marrow.

In this study, the relapse rate of ABMT versus Stop is currently 37% vs 58%, with a highly significant p value (0.0007). However, because of problems of a death rate in remission of approximately 12% in the ABMT arm, i.e, 15 patients out of 126 randomised patients, the overall survival for ABMT vs Stop is currently 57% vs. 46% at 7 years, which is not significant ($p = 0.2$). The increased death rate in CR in the ABMT patients has been mainly attributed to slow recovery of neutrophil and platelet counts. This is demonstrated in Table 1, where median days to recovery following allogeneic transplantation are compared with those following autologous transplantation.

Out of a total of 226 patients who had ABMT for AML, some of whom were not randomised, 27 patients died in CR. Analysis of the data available on these 27 patients shows that: (1) 21/21 patients (100%) never achieved platelet recovery to $>50 \times 10^9$/l; (2) 10/23 (44%) did not achieve neutrophil recovery to $> 0.5 \times 10^9$/l, while a further 10/23 (44%) took longer than 30 days to recover their neutrophils; (3) the causes of death in 21 of the 27 patients were infection (8/26 = 31%); bleeding (6/26 = 23%); graft failure (3/26 = 11.5%); cardiac failure (2/26 = 7%), and multiple causes (2/26 = 7%).

Table 1. Comparison of neutrophil and platelet recovery times for allogeneic and autologous BMT in AML-10. All differences were highly significant (AML-10 data)

	Allogeneic BMT	Autologous BMT
Median days to neutrophils $>0.5 \times 10^9$/l	19	31
Median days to neutrophils $>1.0 \times 10^9$/l	23	48
Median days to platelets $>50 \times 10^9$/l	25	71
Median days to platelets $>100 \times 10^9$/l	42	146

Given the suggestion that slow haemopoietic recovery after ABMT in AML accounted for the considerable death rate in remission in these patients, the basis for the new MRC AML-12 protocol was derived.

Design of MRC AML-12 Trial

In the new trial, patients are divided into three main risk groups. Those patients assigned to the good risk group have the cytogenetic abnormalities t(8;21), inv 16 or t(15;17). Patients remain in this group irrespective of their marrow status after the first course of chemotherapy. Patients in the standard risk group are those who are neither in the good risk nor the poor risk group, i.e. who have no favourable karyotypic abnormalities, but not more than 20% blasts in the bone marrow after course 1. Patients in the poor risk group are those with more than 20% blasts in the bone marrow after course 1 and without favourable karyotypic abnormalities, or patients who are not in remission after course 2. Failure to achieve less than 20% blasts after course 1 has previously been shown to be important in terms of prognosis [3].

In AML-12, new randomisations:

1. compare ADE vs. MAE chemotherapy as courses 1 and 2;
2. compare the effect of four versus five courses of chemotherapy, and
3. continue to explore the role of ABMT.

The structure of the trial is as follows. With the first randomisation, to ADE vs. MAE, patients are also randomised to G-CSF or not. Following the first course of chemotherapy the patients are then divided into one of the above three risk groups – good, standard and poor. The good risk patients are not subjected to transplantation in first remission, but are randomised instead to four courses of chemotherapy (where course 3 is MACE and course 4 is MidAC) vs. five courses of chemotherapy (where MACE is the 3rd, ICE the 4th, and MidAC the 5th course). The standard risk patients are randomised four ways, either to four courses, in which the fourth course is BMT or MidAC, or five courses, in which the fourth course is ICE, and the fifth, BMT or MidAC. For those in the BMT arms, an allograft is carried out if a matched sibling is available, and an autograft if not. The poor risk patients are treated on a relapse protocol (MRC AML-R [4]). This compares standard versus sequential ADE (three courses), with a further randomisation to cyclosporin versus no cyclosporin as potential resistance modification. Allogeneic transplant with sibling or unrelated donor is then carried out as soon as possible.

Peripheral blood stem cells are collected and used in AML-12 for a variety of reasons:

1. to establish the adequacy of stem cell mobilisation after AML chemotherapy;
2. to analyse factors influencing stem cell yields;
3. in an attempt to shorten the time to haemopoietic recovery after courses 4 or 5, and/or after high-dose therapy;
4. to assess the effects of PBSC use on morbidity and mortality overall.

In the trial, peripheral blood stem cells are used in addition, rather than as an alternative, to autologous marrow as stem cell rescue. This is because when the study was being conceived it was not certain whether stem cells alone would sustain the grafts.

Options for PBSC Collection and Use

PBSCs may be collected either after course 2 or course 3 of chemotherapy. PBSCs may be returned after conventional chemotherapy to shorten recovery after course 4, course 5 and/or after ABMT. There is no specification in this study as to the number of leucaphereses, the number of cells to be collected, or the number of cells to be returned at each stage.

Results

From December 1994 to December 1996, 561 patients were enrolled into MRC AML 12, from 132 UK centres. Of these patients, 205 have had PBSCs collected. The preliminary collection data from 99 of these 205 patients shows that 57 patients were collected after course 2, 38 patients after course 3 and for the four other patients the data is not fully gathered. The average number of PBSCs collected per apheresis is shown in table 2, using an arbitrary threshold of 2×10^8 /kg mononuclear cells. Patients in whom the data collection is not complete are excluded. These results show that, using this threshold, the majority of patients achieve adequate total yields after course 2 or 3, although the yields appear to be greater after course 2 (Table 2). Similarly, there may be a tendency to require more collections after course 3 (Table 3).

Table 2. Number of patients obtaining adequate and inadequate PBSC yields, using an arbitrary threshold of 2×10^8 /kg mononuclear cells, when harvested after course 2 and 3 (AML-12 preliminary data)

Average PBSC yield	$<2 \times 10^8$ /kg	= or $> 2 \times 10^8$ /kg
After course 2 (n = 50)	11 (22%)	39 (78%)
After course 3 (n = 32)	10 (31%)	22 (69%)
Total (n = 82)	21 (26%)	61 (74%)

Table 3. Number of patients who underwent 1, 2 or 3+ aphereses after courses 2 and 3 (AML-12 preliminary data)

No. of aphereses	1	2	3+
After course 2 (n = 52)	37 (71%)	14 (27%)	1 (2%)
After course 3 (n = 36)	16 (44%)	13 (36%)	7 (19%)
Total (n = 88)	53 (60%)	27 (31%)	8 (9%)

Multivariate analysis of factors influencing the average PBSC yield suggests a possible influence of:

1. the course after which PBSCs are collected ($p = 0.0479$);
2. the patient risk group, standard vs good vs poor ($p = 0.0219$);
3. randomisation to either ADE or MAE for courses 1 and 2, ($p = 0.0014$). The direction of influence is not revealed in this preliminary data, because the trial is still open.

The PBSC yield was not affected by the age of the patient. In the 34 out of 99 patients who had bone marrow harvested in addition to PBSCs, there was no correlation between the PBSC yield and the number of cells collected at bone marrow harvest. Nor was the bone marrow harvest yield affected by the factors that apparently influence stem cell yield.

At the present time, only 19 out of 99 patients have received PBSCs back, in four patients after course 4, in five patients after course 5, and in ten patients following ABMT.

Discussion

The UK MRC AML-12 trial represents an attempt to analyse the utility of PBSCs in AML. Data from the UK MRC AML-10 trial, and others including the EORTC AML-8 trial [5], show that high-dose therapy with autologous rescue, following conventional chemotherapy, significantly reduces relapse in patients with AML. The problem has been the morbidity and mortality of ABMT in this situation, i. e. a loss of about 12% of patients in complete remission. Although the initial single-centre pilot studies of ABMT in AML showed a transplant-related mortality of only 5-6%, it has been of considerable concern that the multi-centre trials looking prospectively at the utility of ABMT in AML have shown a transplant mortality twice as great as in initial pilot studies. This could have happened for a number of reasons. Firstly, there is the difficulty of translating the efficacy and safety of single-centre studies to a multi-centre prospective trial. Secondly, the level of intensity of the four courses of chemotherapy which precede ABMT is far greater than in the pilot studies. Thirdly, some of the pilot studies had been performed using high-dose chemotherapy, without TBI, as the ablative regimen, and it may be that recovery following ABMT is greater after a TBI-based ablative regimen than after high-dose chemotherapy alone.

In any case, it appears from AML-10 that the significant reduction in relapse achieved by high-dose therapy has been almost totally mitigated by the loss of patients through delayed recovery from the transplant. Thus was the rationale for the development of AML-12 derived. This trial aims to achieve the benefit of reduction in relapse of additional therapy, whilst making that additional therapy safer. The use of PBSCs seemed an obvious method of shortening the recovery from high dose therapy; however, at the time of initiation of the new study AML-12, it was not clear that peripheral blood stem cells alone would be sufficient to sustain and maintain the graft for an indefinite period. For this reason it was decided to use PBSCs to supplement autologous marrow, thus bringing about the possibility of shortening the recovery time, whilst reducing the possibility of failure to sustain the graft by adding autologous marrow as well. The structure of the study in this fashion, however, means that the issue of potential contamination of PBSCs, compared to autologous BM, will not be fully assessable. However, where a suitable marker is present, the quality of PBSCs in terms of tumour contamination will be evaluated by laboratory studies.

Another explanation for the improved relapse rates seen in AML-10 after ABMT, is that it is not the high-dose therapy per se that reduces relapse, but the administration of an additional course of therapy. The AML-12 trial examines this question by looking at a fifth course of chemotherapy in some patients who do not receive high-dose therapy. Again, there is concern about haemopoietic recovery after later courses of chemotherapy - significant delays in platelet recovery, in particular, were seen in AML-10 after course 4. As an additional option of the AML-12 trial, therefore, some or all available PBSCs may be added back in the recovery phase of courses 4 and/or 5 of conventional

chemotherapy, to improve recovery times, and reduce morbidity and mortality.

In summary, PBSCs represent a potential means of reducing morbidity and mortality in the treatment of AML, while allowing the benefits of longer and more intensive therapy to be further explored. The MRC AML-12 trial is designed, in part, to exploit these advantages of PBSCs. Whether there are any disadvantages – for example, through tumour contamination of stem cells – may also be revealed by laboratory studies, and by analysis of relative relapse rates.

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Experimental Approaches and Novel Therapies
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Blood Stem Cell Transplantation in Acute Myeloid Leukemia

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Abstract. Autologous blood cell transplantation (BCT) is an alternative to autologous bone marrow transplantation (BMT) for patients with acute myeloid leukemia (AML) in first remission. Its main advantage is that it is followed by a quicker engraftment than that observed after BMT. From the retrospective analysis of the European Blood and Marrow Transplantation (EBMT) registry, the 2-year disease-free survival (DFS) (44 ± 6% versus 51 ± 2%), relapse incidence (RI) (50 ± 6% and 43 ± 1%) and survival (53 ± 6% and 58 ± 1%) are similar after either BCT or BMT. In the BGMT 87 and BGMT 91 prospective studies, no statistically significant differences were seen between BCT and BMT for DFS, RI and survival. Thus, we conclude that the use of blood cells for autologous transplantation has more advantages than disadvantages.

Introduction

Autologous stem cell transplantation (ASCT) is increasingly used for the treatment of Acute Myeloid Leukemia (AML) in first remission (CR1), although its superiority over intensive chemotherapy has been shown in only one study [1]. The main cause of failure following ASCT is leukemia recurrence which could be due, at least in some cases, to the reinfusion of malignant cells present in the graft [2]. When blood stem cells were initially proposed to be used instead of bone marrow to perform ASCT, the hypothesis was that their use could lead to a decrease in the relapse incidence, as they could be less contaminated by residual leukemia cells than bone marrow. Up to now, this hypothesis has neither been confirmed nor refuted but the advantage of blood cell transplantation (BCT) over bone marrow transplantation (BMT) is that hematopoietic recovery is significantly quicker after BCT than after BMT [3]. To analyse possible advantages and disadvantages of BCT over BMT, we have reviewed the data of the European Blood and Marrow Transplantation (EBMT) registry as well as those of our own BGMT studies in which autologous BCT and BMT were prospectively compared.

Patients and Methods

EBMT Registry

Among the patients who were transplanted for AML in CR1 and reported to the EBMT Registry during the period 1985-1995, we retrospectively reviewed the data of those for whom main characteristics were available. These characteristics included age and sex of patients, FAB morphology, initial white blood cell (WBC) count, the intervals between diagnosis and CR1 or CR1 and ASCT, the dates of CR1, transplant and, fol-

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lowing ASCT, the dates of relapse or death or last follow-up. Thus we were able to compare 100 patients who underwent BCT with 1293 patients who were treated with BMT.

BGMT Studies

In the BGMT study including 204 AML patients under the age of 55 years, 33 patients were allocated to ASCT after induction chemotherapy and intensification (with high dose Aracytine: 3 g/m^2 x 8 and Daunorubicin: 45 mg/m^2 x 3) which was followed by stem cell collection (blood cells: 17; bone marrow: 16); the 33 patients subsequently underwent ASCT after a preparatory regimen including Busulfan (4 mg/kg/day x 4) and Melphalan (140 mg/m^2) [4]. No growth factors were used after either intensification or ASCT. In the BGMT 91 study, 26 patients allocated to BCT were given the same treatment as in the BGMT 87 study but G-CSF (Lenograstim: 263 μg/day) was given from the first day after the end of intensification to the completion of leukaphereses. No G-CSF was used after BCT.

Statistical Analysis

In the EBMT registry and BGMT studies, we compared the disease-free survival (DSF), relapse incidence (RI) and survival (OS) of patients undergoing either BCT or BMT. The Kaplan Meier method was used to calculate DFS, RI and OS and the log rank test to compare these parameters for BCT and BMT patients. We also compared the median number of days to reach absolute numbers of 500 polymorphonuclear cells (ANC 500) or 50 000 platelets (P 50 000) per mm^3.

Results

EBMT Retrospective Study

Some characteristics of BCT and BMT differed significantly as the BCT patients were older ($p < 0.0003$), had less incidence of promyelocytic leukemia ($p < 0.02$), a shorter interval between CR1 and ASCT ($p < 0.04$) and to a lesser extent a longer interval between diagnosis and CR1 ($p > 0.08$). The 2-year DFS, RI and OS were 44 ± 6 and 51 ± 2%, 50 ± 6 and 43 ± 1%, 53 ± 6 and 58 ± 1% for BCT and BMT respectively (Table 1). These differences were not significantly different. Using multivariate analysis performed on the 1393 ASCT patients (BCT = 100; BMT = 1293), the factors which significantly influenced DFS were the intervals between CRI and ASCT or diagnosis and CR1 ($p < 0.005$ and $p < 0.0003$ respectively), promyelocytic leukemia ($p < 0.005$) and age ($p < 0.05$).

Table 1. Results of the EBMT registry BCT versus BMT in patients with AML in first CR1

	BCT	BMT	*p* value
Two-year DFS	44 ± 6%	51 ± 2%	0.12
Two-year RI	50 ± 6%	43 ± 1%	0.045
Two-year S	53 ± 6%	58 ± 1%	0.59

The median time to ANC 500 was significantly shorter after BCT (13 days; 1-60) than after BMT (24 days; 2-389) ($p < 0.0001$); similar differences were seen for platelet reconstitution as P 50 000 was obtained after 42 days (9-713) versus 60 days (2-1332) following BCT and BMT, respectively ($p < 0.007$).

BGMT Studies

In the BGMT study, the 3-year DFS and RI were similar following either BMT (52.3 ± 12.1% and 43.7 ± 12.4% respectively) or BMT (56.3 ± 12.4% and 39.4 ± 12.6% respectively) (p = NS). In the BGMT 91 study, the three-year DFS and RI of the 26 BCT patients were 53.6 ± 9.8% and 46 ± 9.8% respectively and were similar to those found in the BGMT 87 study for BCT patients. These results did not differ significantly from those found for the BMT patients of the BGMT 91 study. As reported elsewhere [5], survival DFS or RI were not influenced by the number of cells infused for autologous transplantation.

Hematopoietic recovery was analysed in the patients transplanted in our institution (CHU Bordeaux). After BCT, the median

times to ANC were 13 (11-51) and 14 days (11-55) in the BGMT 87 and BGMT 91 studies, respectively (p = NS); however, platelet reconstitution was quicker in the BGMT 91 study (57 days; 10-635) than in the BGMT 87 study (92 days; 12-293), but the difference was not statistically different.

Discussion

It is now very clear that hematopoietic recovery is significantly quicker after BCT than after BMT. The data from the EBMT registry confirm this point. However, it has not been demonstrated that this could lead to a decrease in transplant-related mortality. In many reports, the time to ANC 500 is very close to 13-15 days and seems to be similar after chemotherapy-mobilized or chemotherapy + G-CSF mobilized BCT (Table 2); however, platelet reconstitution seems to be quicker when G-CSF is combined with chemotherapy for blood cell mobilization as compared to the use of chemotherapy alone (Table 2). However, this has not been demonstrated prospectively. Hematopoietic reconstitution following transplantation of blood cells mobilized with G-CSF alone (16-32 μg/kg) during steady state is as quick as that observed after chemotherapy (± G-CSF) mobilized BCT, suggesting that chemotherapy is not needed for collecting blood cells in AML.

Some preliminary reports involving a low number of patients have suggested that the RI could be higher after BCT than BMT. Laporte et al. reported four patients transplanted with blood cells, of whom three had relapsed within four months from transplant [12]. Mehta et al. also reported four patients who presented with early relapse after BCT, and suggested that the higher cell dose used for BCT (with more residual leukemic cells) could explain an increase in the RI [13]. Körbling et al. reported 20 patients transplanted with unpurged blood cells for whom the 2-year DFS was 35% (14-56%, 95% CI) which was lower (although not significantly) than that observed in a group of 23 patients with similar characteristics transplanted during the same period with mafosfamide-purged marrow [7].

Two larger series of patients have more recently been published. Sanz et al. transplanted 24 AML patients with blood cells reinfused after a preparatory regimen of Busulfan and Cyclophosphamide. Actuarial DFS and RI at 30 months were 35 (25-45%; 95% CI) and 60% (50-72%; 95% CI) respectively [14]. In the study by Schiller et al., 48 patients were transplanted with blood cells after TBT and Cyclophosphamide; after a median follow-up of 24 months, 29 of them were still in continuous complete remission [9]. Interestingly in this latter study, although blood cells were collected after mobilization with chemotherapy and G-CSF (whereas G-CSF was not used in other studies), the RI was not higher than that observed in the studies where G-CSF was not used for mobilization [7, 12, 13, 14].

Table 2. Hematopoietic recovery in patients with AML undergoing autologous transplantation (according to the source of stem cells)

	ANC 500[a]	P1 20000[a]	Reference
Purged BMT	42 (not given)	46 (not given)	7
Unpurged BMT	27 (9-389)	50 (10-700)	6
CT-Mobilized BCT	15.5 (9-60)	58.5 (11-713)	6
	14 (not given)	30 (not given)	7
	13 (11-51)	92 (12-293)[b]	8
HGF + CT-Mobilized BCT	15 (11-35)	16 (8-153)	9
	14 (10-27)	11 (5-210)	10
	14 (11-55)	57 (10-635)[b]	Reiffers et al.; unpublished
HGF-Mobilized BCT	12 (8-27)	15 (8-103)	11

[a] ANC 500 and P1 20000 signify the median number of days to achieve an absolute neutrophil count and platelet count of 500 and 20000 per mm^3 respectively.
[b] Time to recover to 50000 platelets per mm^3.

From these studies, there are no apparent differences in the long-term results of BCT versus BMT for AML patients in first CR. To address this point in a larger number of patients, we retrospectively reviewed the data of 1393 patients reported in the European Blood and Marrow Transplantation (EBMT) registry and found that there was no statistical difference in the clinical results of BCT versus BMT. In the prospective BGMT studies, we also found that DFS, RT and survival of patients undergoing BCT or BMT were similar. Thus, despite disappointing results observed in a small series of patients undergoing BCT for AML in CR1, no clinical results to date indicate that the results of BCT and BMT are very different.

Conclusion

In AML patients in first remission, the use of blood cells has the advantage of a quicker neutrophil and platelet engraftment which could lead to a decrease in morbidity but not mortality. The long-term survival of patients after either BMT or BCT is similar.

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Peripheral Blood Stem Cell Transplantation During Postremission Treatment of de Novo Acute Myelogenous Leukemia

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Abstract. Intensification of induction and consolidation treatment in de novo AML lead to a continuous improvement of disease-free survival in the last 20 years. To increase dose-intensity during consolidation, patients in continued CR (n = 16) which did not qualify for allogeneic BMT, received busulfan/cyclophosphamide conditioning and peripheral blood stem cell transplantation (PBSCT) after double induction treatment with TAD (thioguanine, daunorubicin and AraC) and HAM (high-dose AraC and mitoxantrone) and an early consolidation with a second course of TAD. G-CSF treatment was started on day two after each chemotherapy as well as on the day of PBSCT. PBSC were collected after mobilization with HAM for back-up and after the second course of TAD for transplantation. PBSCT could be performed in all patients being in continued CR (1.33×10^6 CD34+ cells per kg (range 1.0 to 2.9×10^6); 1.38×10^5 CFU-GM/kg (range 0.14 to 2.8×10^5)) except one patient with persistent pancytopenia after the second course of TAD. A further mobilization with cyclophosphamide was necessary in three patients after the second course of TAD. Leukocyte counts of 10^9/l were achieved within 10.4 days (range 8-12). In five cases the increase in thrombocyte counts was biphasic but remained stable > 100×10^9/l after 59 days (range 16-92) in all patients. Severe toxicities (grade IV) were observed in 4 patients (peumonia 2 ×, pericarditis 1 ×, VOD 1 ×). One patient died of pneumonia. Relapse-free survival was 66% (95% confidence interval 58 to 74%) at a mean observation time of 27 months. Cytogenetically patients were found to be at good (n = 1) intermediate (n = 14) and high risk (n = 1). Of the four patients relapsing three only achieved CR after a second induction cycle, one was at high risk cytogenetically and two had an excessively high initial LDH level (> 1000 U/l). One patient developed a myelodysplastic syndrome. Thus, myeloablative chemotherapy with PBSCT has tolerable toxicity and may be performed routinely as consolidation treatment following TAD-HAM-TAD. The risk of relapse seems to be increased if achievement of CR is delayed. The observed relapse-free survival justifies a randomized trial with standard versus high-dose chemotherapy combined with the transplantation of in vivo purged PBSCs.

Introduction

Dose intensity in the treatment of acute myeloid leukemia has been continuously escalated during the last 20 years and has improved the outcome steadily [1]. Dose escalation during induction and consolidation treatment as well as during postremission treatment resulted in improved disease-free survival [2-5]. However, high-dose cytosine arabinoside (AraC) combined with anthracyclines or an anthrachinone in the postremission treatment is associated with an in-

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creased risk of long-lasting myelotoxicity, with a significant morbidity and mortality as well as with prolonged therapy-free intervals which may increase the risk of an early relapse [4-6]. Therefore, tolerance of this kind of postremission-therapy is significantly reduced and further decreases with age implicating an undertreatment of elderly patients.

Aim of our pilot study was to increase both tolerance and dose-intensity of postremission chemotherapy by the use of autologous PBSC following myeloablative chemotherapy.

Patients and Methods

Patients

Between November 1993 and December 1995, all patients aged 15 to 60 years with de novo AML entering our institution were enrolled onto the study protocol on the basis of intent to treat. Diagnosis of AML was made according to French-American-British (FAB) group revised criteria. Patients without an HLA-identical sibling who achieved CR after double induction chemotherapy were considered eligible to receive consolidation chemotherapy followed by an intensive maintenance chemotherapy with a myeloablative conditioning regimen and PBSCT. All patients included had a forced expiratory volume $\geq$ 70% predicted, a diffusion capacity of carbonmonoxide of $\geq$ 60%, an adequate renal and liver function as determined by a serum creatinine $\leq$ 2 times normal, and a bilirubin, serum glutamine oxaloacetic transaminase and alkaline phosphatase $\leq$ 2 times normal, and no heart insufficiency > II° (NYHA), respectively. Promyelocytic leukemias were excluded and treated according to a specially adapted protocol.

A total of 23 consecutive patients were entered onto the protocol. 16 of 23 eligible patients (Table 1) were subsequently treated with high-dose chemotherapy and aPBSCT (69%). One patient was excluded due to an early relapse, one patient died of heart failure in complete remission after induction chemotherapy, one patient died of cerebral bleeding during induction chemotherapy due to a leukostasis syndrome and four patients with HLA identical siblings received allogeneic bone marrow transplantation.

Table 1. PBSCT in de novo AML: patients' characteristics

Total no. of patients	16
Sex (no. of patients)	
Male	7
Female	9
Age (years)	
Median	47
Range	34-59
FAB subtype (no. of patients)	
M1	4
M2	6
M4	5
M5	1
WBC count ($\times 10^9$/l)	
Median	48
Range	1-139
Hemoglobin (g/dl)	
Median	9.2
Range	6.1-12.6
Platelet count ($\times 10^9$/l)	
Median	32
Range	11-196
Lactate dehydrogenase (U/l)	
Median	735
Range	254-1884
Cytogenetic aberrations [a]	
normal karyotype	11
aberrations	4
metaphases not available	1
Interval diagnosis to PBSCT (months)	5
Median	4.5
Range	4-5
Interval CR to PBSCT (months)	
Median	3
Range	2-4

Abbreviations:
CR: complete remission; PBSCT: peripheral blood stem cell transplantation
[a] t(1;7), inv 16, t(8;16), complex aberrations

Treatment Schedule

Double-induction chemotherapy [7] consisted of one cycle TAD, daunorubicin (60 mg/m^2 day 1 to 3), Ara C (100 mg/m^2 day 1 and 2, twice daily 100 mg/m^2 day 3 to 8) and thioguanine (twice daily 100 mg/m^2 day 1 to 3) combined with mitoxantrone (20 mg/m^2

day 3 to 5). When complete remission was achieved patients received a second TAD cycle as consolidation treatment. A further mobilization course with 2 g/m^2 cyclophosphamide (CTX) day 1 was applied when not enough CD34 positive cells could be harvested during HAM and TAD II mobilization (n = 3). As intensive maintenance therapy high-dose busulfan (4 mg/kg day -9 to -6) combined with CTX (60 mg/kg day -5 to -2) was applicated followed by PBSCT on day +1 [8].

Stem Cell Mobilization

G-CSF (Neupogen) was given after each cycle of chemotherapy. Daily G-CSF application was started on day 2 after chemotherapy (5 µg/kg s.c.).

Harvesting of PBSC

Stem cells were collected with a continuous flow blood cell separator, either a Cobe Spectra Apheresis System or a Fenwal CS 3000. During leukapheresis a total volume of 8-12 l at a flow rate of 50-70 ml/min was processed. Leukaphereses were performed during recovery following HAM and TAD II when CD34 counts were > 4 x 10^3/ml. Additional leukapheresis cycles were performed in 3 patients following cyclophosphamide mobilization. Daily leukaphereses were performed during 2 to 4 subsequent days. A median of 2.25 aphereses per cycle and a median of 1.7 cycles have been performed per patient following mobilization with HAM and TAD II.

Cryopreservation and Retransfusion of PBSC

The apheresis product was mixed with an equal volume of minimal essential medium containing 20% dimethylsulfoxide (DMSO, Merck, Darmstadt, Germany) and immediately transfered into freezing bags (Cryocyte freezing container, Fenwal) and frozen to -100 °C with a computer-controlled cryopreservation device (Cryoson BV-6, Cryoson Deutschland GmbH, Schöllkrippen, Germany). The frozen bags were stored in liquid nitrogen at -196 °C until use.

Thawing was performed in a 40 °C waterbath. For retransfusion the cell suspension was aspirated into 50 ml syringes and immediately injected into a central line at high flow rate. To avoid anaphylactic complications patients received premedication consisting of 100 mg prednisolone and H1-blocker 1 h prior to transplantation. A mean of 1.33×10^6 CD34 positive cells per kg (range 0.1 to 2.9×10^6) and 1.38×10^5 CFU-GM/kg (range 0.14 to 2.8×10^5) were retransfused. Only leukapheresis products collected following TAD II or CTX were used for transplantation.

In Vitro Colony Assay

Granulocyte-macrophage progenitor cells (colony-forming unit granulocyte-macrophage, CFU-GM) and erythroid progenitor cells (burst forming units BFU-E) were analyzed in each apheresis product. Mononuclear cells were plated in 35-mm Petri dishes in triplicate. Human placenta-conditioned medium was used as source for colony-stimulating factors. Cultures were incubated in a closed humidified atmosphere at 37 °C with 5% $C0_2$.

Quantification of PBSC

Stem cell counts were performed using Li-Heparin anticoagulated peripheral blood samples or leukapheresis products. In a first step whole blood (100 µl) or the concentrate diluted with PBS (10^6 cells in 100 µl) were incubated for 15 min on ice with saturating concentrations of a R-phycoerythrin-conjugated CD34 antibody (HPCA-2, Becton Dickinson, San José, CA) and a PerCP conjugate of CD45 (Hle-1, Becton Dickinson). FITC-conjugated reagents with specificity for previously identified abnormal blast populations were added at the same time were necessary. Following incubation the cell samples were incubated for 10 min at room temperature with a lysing reagent (FASClyse, Becton Dickinson) followed by washing twice (1500 g, 4 °C) with PBS and re-

suspending in 300 µl PBS. The samples were then analyzed using forward scatter as the trigger parameter on a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Peripheral blood stem cells were indentified using LYSYS-II software based on cellular forward and light scatter characteristics corresponding to CD45++ lymphocytes simultaneously with a low expression of CD45 similar to neutrophils and an only bright expression of CD34. The absolute concentration of stem cells was calculated from the relative percentage of stem cells in the flow cytrometric analysis of nucleated cells and absolute leukocyte counts determined from an EDTA-anticoagulated blood sample in a H*3 hematology analyzer (Bayer Diagnostics, Miles Division, Tarrytown, NY).

Supportive Care, Defenitions and Statistical Analysis

Patients were nursed in reverse-barrier isolation when leucocytes were < 1000/µl and received decontamination with ciprofloxacin p.o. and oral amphotericin suspension. Chemotherapy and all infusions were applicated on a central line. All cellular products were irradiated to 1.5 Gy and filtered before transfusion. To prevent busulfan-induced side effects oral diphenylhydantoin or oral diazepam was given.

Complete remission (CR) was defined according to the Cancer- and Leukemia Group B [9]. Complications related to the conditioning treatment were graded corresponding the WHO criteria. Hepatic venoocclusive disease (VOD) was suggested when two of the following symptoms were observed in addition to hyperbilirubinemia symptoms (bilirubin > 34 mmol/l): hepatomegaly, ascites, weight gain [10].

Event-free survival was defined as the length of time until the first event – that is, treatment failure (relapse or failure to enter remission) or death. Calculation of event-free survival started from consolidation treatment. Actuarial curves showing event-free survival were constructed according to the Kaplan-Meier method. Time to relapse was calculated from the time of first CR to relapse.

Results

Mobilization of CD34-Positive Cells

CD34 positive cells were sequentially measured by flow cytometry during mobilization to determine the maximal increase. The median number of CD34 positive cells/kg collected per leukapheresis cycle (n = 15 following HAM, n = 15 following TAD II) was 1.61×10^6 (range 0.4 to 25×10^6), the median number of CFU-GM/kg was 4.9×10^4/kg. The increase of CD34 positive cells in peripheral blood was paralleled by increasing leukocyte counts. However, no correlation could be found between the number of circulating CD34-positive cells and the corresponding mononuclear cells, lymphocyte or polymorphonuclear cells count both during TAD and HAM mobilization (data not shown).

The time intervals to maximal CD34 release into the peripheral blood were highly variable and independent of the mobilization schedule (TAD or HAM). Maximal CD34 counts were measured after 11 to 24 days (mean 14 days). In most cases CD34 cell counts were maximally increased on day 11 to 15 (n = 20, 66%), irrespective of the mobilization schedule used. However, a delay was observed in 10 mobilization cycles (33%). A delayed mobilization was not associated with a decreased yield in CD34 positive cells.

Hematopoietic Reconstitution

Complete hematopoietic bone marrow recovery was observed in all 16 patients following myeloablative chemotherapy and PBSCT (Table 2). No back-up leukapheresis

Table 2. Hematopoietic reconstitution following myeloablative chemotherapy and PBSCT (n = 16)

	Days to achieve			
	PMN/µl		Platelets/µl	
	>500	$>20 \times 10^3$	$>50 \times 10^3$	$>100 \times 10^3$
Mean	10.4	22.7	36.3	59
Range	8-12	10-35	13-64	16-92

product had to be retransfused. Median duration of severe neutropenia was 14.7 days (6 to 22 days) after TAD I, 12.5 days (7 to 22 days) after HAM, 8 days after TAD II and 9.8 days (6 to 14 days) after Bu/Cy. Duration of neutropenia following TAD I or HAM differed significantly from TAD II or Bu/Cy ($p < 0.01$). In 5 patients reconstitution of thrombopoiesis was biphasic.

Toxicity of Myeloablative Chemotherapy and aPBSCT

Transplantation was well tolerated without severe side effects. No BU-associated seizures were observed. The most frequent toxicity was mucositis, however no grade III or IV mucositis was seen. 13 patients developed at least one fever episode with > 38.5 °C. In one patient mild clinical VOD was observed 12 days after aPBSCT.

The etiology of a pericarditis observed in one patient could not be clarified. Two patients suffered from pneumonia. One of these patients developed severe respiratory insufficiency, was ventilated and died of progressive pneumonia despite hematopoietic reconstitution.

Clinical Status After PBSCT

EFS and the probability of relapse at a mean observation time of 27 months are shown in Fig. 1 and in Fig. 2. Five patients relapsed within 4 to 14 months, one developed a myelodysplastic syndrome. Important clinical characteristics of these patients are shown in Table 3. Only one relapsing patient (No. 5) has shown no risk factors. In three patients a second CR could be achieved. Meanwhile all relapsed patients have died.

Late Complications

One patient developed a pneumonia within the first year after PBSCT. The pneumonia resolved with antimicrobial therapy. In one patient multiple granulomas of unknown etiology were found in the bone marrow. The granulomas disappeared within 13 months after PBSCT.

Discussion and Conclusion

In AML sequential application of submyeloablative chemotherapy schedules is a necessary prerequisite to induce and maintain complete remission. Despite the high dose-intensity during AML treatment it was possible to mobilize in all patients sufficient CD34 positive cells during consolidation treatment to rescue hematopoiesis after myeloablative chemotherapy in postremission. Myeloablative chemotherapy followed by retransfusion of PBSC was associated with a significantly shorter duration of severe days with PMN count (0.5×10^9/l) aplasia than following TAD I and HAM. The median duration of severe neutropenia of 9.8 days compares with the results of other studies [11-14]. Thus, dose-intensive postremission chemotherapy may become more tolerable also for elderly patients who are frequently undertreated because of the high risk of morbidity and mortality due to long-lasting pancytopenia associated with dose-intensive postremission treatment schedules.

Table 3. Relapses following PBSCT in AML (n = 16)

Patient	CR	Initial LDH (U/l)	Cytogenetic aberrations	Duration of CR
1	Following TAD I	1860	No	7
2	Following HAM	436	No	14
3	Following HAM	293	Complex aberrations	9
4	Following HAM	1621	n.a.	4
5	Following TAD I	324	No	8
6[a]	Following HAM	671	t(8;16)	10

[a] Myelodysplastic syndrome at relapse

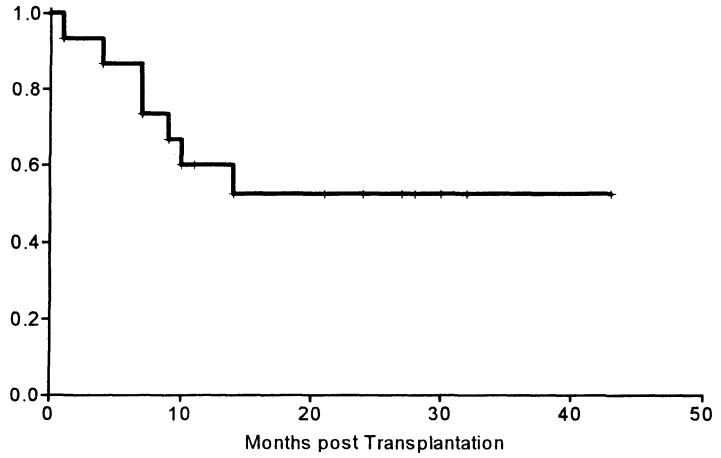

Fig. 1. Event-free survival of patients with de novo AML after autologous stem cell transplantation

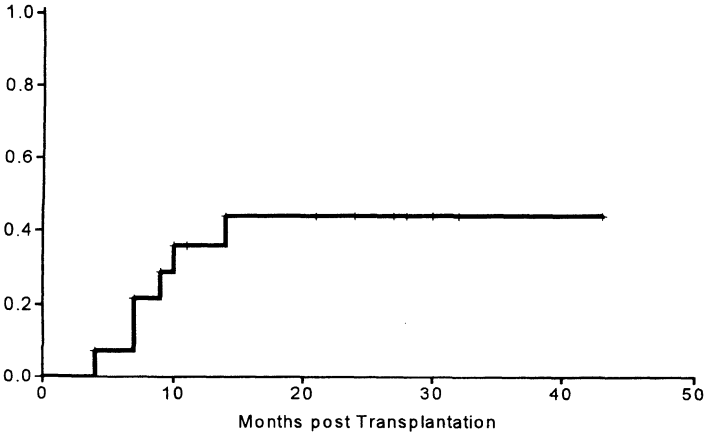

Fig. 2. Probability of relapse after autologous stem cell transplantation for de novo AML

The question whether myeloablative post-remission chemotherapy supported with autologous stem cells results in superior survival remains to be answered by randomised trials, comparing dose-intensive induction and consolidation treatment with PBSCT [1]. Studies are in progress to prove feasibility of further dose-intensification by sequential stem cell-supported chemotherapy [1].

Characteristics of relapsing patients in our study indicate that risk factors for standard chemotherapy as well as for myeloablative chemotherapy supported with PBSC seem to be similar. Therefore, further randomised studies should not be exclusively restricted to high risk AML patients.

EFS in our study compares to results of other phase II trials. The promising results justify to do PBSCT without ex vivo purging. Larger studies may clarify which patients are at a high risk of relapse. In those subgroups with high risk of relapse purging procedures may be evaluated for their effectiveness.

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Acute Leukemias VII
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Myeloablative Chemotherapy and Peripheral Blood Stem Cell Transplantation vs. Consolidation Therapy of Acute Myelogenous Leukemia in First Remission

A. Reichle and R. Andreesen

Abstract. Historical data about dose intensity during induction and/or consolidation treatment as well as the evaluation of randomized clinical trials comparing different dose-levels of anthracyclines or Ara C demonstrate a close dose-response relationship in terms of CR rate and/or survival. However, repetitive application of submyeloblative regimens results in prolonged and severe therapy-associated pancytopenia. On the other hand, only few patients (17 to 37%) with HLA-identical siblings may be treated with allogeneic bone marrow transplantation (BMT). New dose-intensification concepts during consolidation for patients up to 60 years are now under evaluation using myeloablative or sequentially applied submyeloablative consolidation cycles supported with autologous peripheral blood stem cells (PBSC), which may be routinely collected in 1st CR during subsequent consolidation cycles. These PBSC give rise to leucocyte reconstitution about 10 days post transplantation (> 1.0/nl) as well as to long-term hematopoietic recovery. Stem cell collection and transplantation offers new insights in leukemic disease biology: stem cell kinetics in the peripheral blood significantly differ from that of lymphomas and solid tumors. Additionally, chemotherapy may produce both irreversible as well as reversible stem cell damage. Further, studies to determine the clinical significance of minimal residual disease are of major importance.The risk of reinfusion of residual leukemic cells seems to be of minor relevance to autologous transplantation than control of the disease in the host:

1. Myeloablative chemotherapy (CT) in 2nd CR supported with autologous BM collected in 1st CR shows significantly superior long-term results than conventional CT.
2. Results of allogeneic BMT in syngeneic twins in 1st CR compare with autologous BMT or PBSCT.
3. Superior survival data of autologous transplantation concepts are observed when standard CT with relatively low dose-intensity has been used and outcome seems to be at least equivalent when compared to highly dose-intensive standard treatment schedules.
4. Risk factors for standard CT seem to be the same for autologous and allogeneic transplantation.

Nevertheless, gene marking experiments have shown that transplantation of clonogenic leukemia cells remains a possible risk. When myeloablative CT supported with autologous PBSC is used as late consolidation more than 80% of the relapses are observed during the first year post transplantation. Thus, myeloablative chemotherapy results in another relapse behaviour compared with standard CT probably indicating better disease control in standard risk groups. This

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observation is supported by the analysis of risk factors in relapsing patients after myeloablative CT supported with PBSC. In conclusion, PBSC supported dose-intensification during consolidation seems to be a feasible treatment concept which may result in less severe therapy-associated side effects due to shortened pancytopenia and an improved leukemia control.

Dose-Intensive Treatment of AML

Dose intensity in the treatment of acute myelogenous leukemia (AML) has been continuously escalated during the last 20 years to improve steadily outcome (Table 1) [1-14]. At the same time, many study groups focused on supportive care to reduce the increasing risk of therapy-associated morbidity and mortality due to dose-intensive treatment schedules applicated during remission, induction, consolidation and postremission treatment [15].

Despite these efforts tolerance of dose-intensive regimens remained low in large multicenter studies and toxicity increased especially with age [13]. The reasons are prolonged therapy-related pancytopenia, prolonged therapy-free intervals with the risk of relapse because time schedules can not be kept. Additionally in up to 30% of the AML patients HLA-identical allogeneic transplantation is chosen as the treatment of choice for most AML, [16]. When colony stimulating factors (CSF), such as G-CSF, were available in the late eighties they were initially estimated to be clinically not suitable for shortening the duration of leukopenia during AML treatment: G-CSF may stimulate simultanously regrowth of leukemic blast cells as shown in vitro and in vivo [17]. Ohno and Estey first compared in a randomised and in a retrospective study, respectively, leucocyte reconstitution and outcome of AML patients receiving G-CSF following dose-intensive chemotherapy [18,19]. Both demonstrated no negative impact on CR rates and disease-free survival but granulocyte recovery was significantly faster in the groups receiving G-CSF. A multicenter study with 521 AML patients confirmed these early results [20].

The most frequently used drugs during induction, consolidation and postremission treatment are cytarabine (Ara-C) and anthracyclines. Dose-escalation of these two drugs with the combined application of G-CSF post chemotherapy seems to be limited due to their dose-limiting non-hematologic toxicity. Many dose-escalation studies using drugs with dose-limiting hematologic toxicity followed by the application of G-CSF have shown a very limited range for dose-intensification, frequently not large enough to significantly improve outcome [21].

Nevertheless, in recent years several randomised multicenter studies impressively demonstrated the importance of Ara-C and anthracylines for dose-escalation within standard chemotherapy during induction, consolidation and postremission chemotherapy, respectively, with respect to disease-free survival (Table 2) [4, 13, 14, 22].

Table 1. Dose intensity and outcome in multicenter randomised AML studies

Study group	Induction	Consolidation	Maintenance	Complete remission	5 years-DFS
CALGB 81, 87, 91 [1–3] ECOG 92 [4]	++	–	+++	60% (53–68%)	15% (12–22%)
SECSG 84 [5] EORTC 86 [6]	++	++	+	65% (64–66%)	14% (10–17%)
AMLGC 85 [7], GIMEMA 92 [8] MRC 86 [9], SECSG 92 [10]	++	++	++	66% (59–71%)	22% (18–25%)
AMLGC 92 [11]	+++	++	++	62%	30%
ALSGM4 96 [12]	+++	++	++	71%	41%
CALGB 94 [13]	++	+++	++	64%	37%
SWOG 96 [14]	+++	++	+++	55%	34%

+ reduced intensity
++ intensity as in standard treatment

Table 2. Dose intensity during postremission treamtnet in AML (CR1): randomised studies

Disease-free survival at 4 years						
Dose intensity		ECOG 92 [4] n = 143 < 60 y	SWOG 96 [14] n = 242 < 50 y	50–64 y	CALGB 94 [13] n = 467 < 60 y	GIMEMA 92 [22] n = 422 10–45 y
Low dose maintenance		14%	–	–	–	–
Ara C	100 mg/m^2	–	–	–	24%	–
	200 mg/m^2	–	24%	4%	–	30%*
	400 mg/m^2	–	–	–	29%	–
	2 g/m^2	–	14%	17%	–	–
	2 g/m^2	–	34% °	25% °	–	–
	3 g/m^2	28%	–	–	44%	–
Autologous BMT		–	–	–	–	48%*
Allogeneic BMT		–	–	–	–	55%
P – values		0.047	0.13		0.02	0.05*

° induction HD-AraC

Table 3. Hematopoietic reconstitution following postremission chemotherapy in AML

	n	Purging	ANC $> 0.5 \times 10^9$/l (range)	Platelets $> 50 \times 10^9$/l (range)
Intensive chemotherapy				
Büchner et al. (AMLCG) [52]	65	–	42	56
Autologous BMT				
McMillan et al. [24]	76	–	BMT 1:22 (11–73)	34 (13–300)
			BMT 2:27 (12–48)	38 (21–98)
Stein et al. [25]	44	–	28 (11–68	87 (19–322)
Linker et al. [26]	32	–	32	79
Cassileth et al. [27]	35	+	31 (17–78)	47 (22–638+)*
Löwenberg et al. [28]	32	–	39	63*
Autologous PBSCT				
Sanz et al. [29]	24	–	11 (8–817)	19 (12–213
Lévy et al. [30]	15	–	14 (10–27)	11 (5–210)
Reichle et al. [31]	16	–	10 (8–12)	36 (13–64)
Schiller et al. [34]	44	–	14 (11–36)	15 (8–72)
Linker et al. [35]	43	–	9	10 platelet independ.

* $> 20 \times 10^9$/l

Patients <60 years with de novo AML attaining complete remission (CR) have now a disease-free survival (DSF) at 5 years of 30-41% [11-14].

In clinical trials especially the most frequently used drugs for AML, Ara-C and anthracyclines, have shown a close dose-response-relationship concerning CR-rates and DFS. However, the repetitive application of regimens including Ara-C and anthracyclines, as it is scheduled in most AML trials, results in a progressive decrease of the hematopoietic stem cell pool [23] and increases the risk of prolonged pancytopenia following postremission treatment (Table 3).

Myeloablative Therapy of AML

For many years myeloablative chemotherapy has been successfully used in postremission treatment including drugs with dose-limiting myelotoxic side effects, such as busulfan, cyclophosphamide, etoposide plus/minus total body irradiation. Despite an irreversible bone marrow damage auto-

logous hematopoietic stem cells may reconstitute hematopoiesis in a significantly shorter time interval than reconstitution is possible, even with G-CSF, after submyeloablative chemotherapy including high-dose Ara-C and mitoxantrone during postremission therapy (Table 3). A prerequisite of the autologous trans-plantation seems to be the eradication of leukemia by the preceding antileukemic therapy and a sufficient stem cell yield from bone marrow or peripheral blood. Therefore, results concerning autologous bone marrow transplantation (ABMT) have to be interpreted on the background of both the selection of antileukemic regimens reducing leukemic burden prior to autologous ABMT or peripheral blood stem cell transplantation (aPBSCT) and the conditioning regimen.

The most powerful effects of myeloablative chemotherapy combined with ABMT may be demonstrated in second CR: DFS is significantly superior to conventional dose-intensive chemotherapy when ABMT is performed in second remission [33]. ABMT in first CR may be superior to conventional treatment as shown in pilot studies as well as in large randomised trials [22, 34, 35]. It is remarkable that trials using HD-Ara-C during induction or postremission chemotherapy produce similar results concerning DFS as ABMT [11-14]. In five pilot studies using aPBSCT as postremission treatment an estimated 2-year DFS rate of 35-65% was reported [29, 31, 34, 35]. Further follow-up is required to confirm improved leukemia-free survival. The European Bone Marrow Registry reported an estimated 3-year DFS rate of 39% for AML patients autografted in first remission with aPBSC [36]. There is the need for randomised trials comparing standard chemotherapy with aPBSCT and the need to study minimal residual disease because of the great variability of disease-free survival following aPBSCT in the single pilot studies.

Peripheral blood stem cell transplantation is available since the introduction of human recombinant G-CSF in 1986. Randomised studies using different autologous stem cell sources, collected from peripheral blood vs bone marrow as well as case-matched historical comparison of autologous transplantation in AML, demonstrate significant advantages for aPBSCT following dose-intensive or myeloablative postremission chemotherapy: aPBSC contribute to a significantly shortened duration of neutropenia and thrombocytopenia (Table 3) and maintain long term hematopoiesis.

APBSC can be frequently collected in about 80% of AML patients in CR 1 and CR 2 (n=30) as shown by our own data. However, stem cell kinetics in peripheral blood significantly differ from that of lymphomas and solid tumors. Thus stem cell collection has to be adapted to these typical circumstances in AML [23, 31].

The early leucocyte recovery following aPBSCT about 10 days post transplantation, offers several advantages:

1 reduced morbidity and mortality,
2 keeping the time schedule of therapy thereby decreasing the risk of relapse and
3 overall improved applicability of high-dose chemotherapy also in elderly patients.

Additionally, stem cell processing offers multifold therapeutic options.

The leukemia-free survival of aPBSC-supported myeloablative post-remission chemotherapy compares to ABMT [36]. Therefore, independently of the autologous transplantation procedure the central question still is: what does make the qualitative difference in the probability of relapse between autologous and allogeneic transplantation in AML and how can chemotherapy be improved to compare to the results of allogeneic transplantation [37, 38]?

One possibility to explain the higher relapse rate in the autologous setting (Table 4), frequently discussed, could be the retransfusion of clonogenic leukemic cells. This was supported by the gene marking studies of Brenner et al which substantiate that retransfused leukemic cells may contribute to leukemia relapse, presumably additionally to a relapse in the host due to insufficient leukemia control by chemotherapy [39, 40]. Many pilot studies dealing with purging procedures assume purging to be superior to non-purging in the autologous setting [38, 40]. To date a definite answer cannot be given because of the multifold variables influencing outcome in these studies, such as

Table 4. Autologous BMT in AML (CR1): therapy-associated risk factors

Reference	n	Purging	Relapse rate (%)	Transplantation-associated deaths (%)
Cahn et al. (EBMT) [32]	786	±	50	10.5
Zittoun et a.l (GIMEMA) [22]	128	–	40.6	9.4
Stein et al. [25]	44	–	39	11
McMillan et al. [24]	76	–	47	6
Cassileth et al. [27]	35	+	35	6
Löwenberg et a.l [28]	32	–	60	9
Linker et al. [26]	32	–	22	5
	15	–	0 (M3, Me Eo)	0

purging technique, standardisation of these techniques, purging efficacy by the detection of minimal residual disease (MRD) and the significance of MRD as well as pretransplant dose-intensity of induction and consolidation regimens.

To assess the potential significance of purging procedures historical data about treatment outcome in AML are helpful:

1. Myeloablative chemotherapy in CR 2 supported with autologous bone marrow collected in CR 1 shows long-term results significantly superior to conventional chemotherapy. Therefore, the risk of reinfusion of residual leukemic cells seems to be of minor relevance to autologous transplantation than control of the disease in the host.
2. Results of allogeneic BMT in syngeneic twins during CR1 compares with ABMT or aPBSCT indicating that the difference in the probability of relapse between autologous and allogeneic transplantation is mainly due to the graft versus leukemia (GvL) effect [42]. The importance of the GvL effect is underlined by the fact that retransfusion of buffy coat of the donor may induce complete remission during relapse following allogeneic transplantation [43].
3. DFS of patients treated with myeloablative postremission chemotherapy and ABMT is superior or at least equivalent when compared to highly dose-intensive standard treatment schedules. Therefore, a potential reinfusion of leukemic cells does not contribute further to risk of relapse. If myeloablative chemotherapy combined with aPBSCT would only compare to dose-intensive standard treatment, the advantage of aPBSCT would be the generally improved tolerance of PBSC-supported high-dose chemotherapy which may be additionally extended to elderly patients [34]. (4) ABMT or aPBSCT in AML does not change pretreatment prognostic factors (Table 5) [25, 32, 38, 44-46, 51].

Table 5. Prognostic factors in AML

	Standard chemotherapy	Autologous transplantation	Allogeneic transplantation
No. of courses of induction chemotherapy to achieve CR	+°	+°	+°
LDH	+°	n.a.	n.a.
WBC	+°	n.s.	+°
Cytogenetic abnormalities	+°	(+)	(+)
Age	+°	+	(+)

Concerning disease-free survival
Concerning LFS, treatment related mortality
(°) trend, n.s. = not significant, n.a. = not available

Table 6. Autologous transplantation in AML (CR1): relapse behaviour

Reference study group	Patients	Purging	Median time to relapse	% Patients relapsing within first year (months)	Latest reported relapse (months)
Cahn et al. (EBMT) [32]	786	±	–	75	70
Zittoun et al. (GIMEMA) [22]	128	–	8	–	30
McMillan et al. [24]	76	–	8	82	26
Laporte et al. [41]	64	+	–	100	11
Stein et al. [25]	44	–	9.3	88	24.8
Yaeger et al. [46]	48	+	6	–	18
Carella et al. [47]	55	–	–	80	26
Burnett et al. [48]	54	–	7	–	27
Beelen et al. [49]	20	–	8	–	11
Löwenberg et al. [28]	32	–	–	80	16
Körbling et al. [50]	22	+	3.5	–	9
Sanz et al. [29]	24	–	–	100	–
Levy et al. [30]	15	–	7	100	11
Reichle et al. [31]	16	–	7	80	14

Especially the number of cycles to achieve CR is shown to be the most powerful prognostic factor for disease-free interval concerning standard chemotherapy, autologous and allogeneic transplantation, respectively. This is supported by the patients' characteristics relapsing in the Regensburg pilot study [31]. Thus, cytogenetically characterized subgroups in AML have still to be analyzed, e.g., by detection of MRD in bone marrow and apheresis product to result in the definition of distinct subgroups which would benefit from purging procedures. These procedures could be specifically developed for cytogenetically and immunologically defined subgroups and therefore, would be much better to standardize.

Presumably there might be an important biological difference between myeloablative and conventional postremission treatment. It is a consistent finding that relapses take place with a relatively constant ratio over several years following standard postremission treatment of de novo AML (Büchner et al., unpublished data). About 40 to 50% of these relapses are found during the first year. Following autologous transplantation most relapses are seen during the first year (> 80%) and the probability of relapse decreases after the second year to about 1% (Table 6).

Thus, myeloablative chemotherapy may results in a different relapse behaviour as compared to standard chemotherapy probably indicating better disease control in standard risk groups according to Keating et al [51]. This observation is supported by the analysis of risk factors in relapsing patients after myeloablative chemotherapy supported with aPBSC in our own series of patients [31].

Conclusion and Perspectives

Autologous PBSCT may further improve tolerance of high-dose postremission chemotherapy in de novo AML. Especially the often undertreated groups of elderly patients could benefit from stem cell-supported chemotherapy.

On the other hand, the idea of further dose-escalation derived from the results of recent multicenter studies, may be realized by aPBSC support. Mc Millan already performed double-transplantation with bone marrow derived stem cells in CR 1 and achieved in these selected patients a 5 year DFS of 64% [34]. A similar study design using aPBSC in one arm will be randomly compared with dose-intensive standard chemotherapy in the German AML Cooperative Group (AMLCG, see Fig. 1). Accompanying studies on MRD will offer new insights into the biological behaviour of cytogenetically defined AML subgroups.

An additional attempt to achieve clinical results similar to allogeneic transplantation could be the use of dendritic cells cultured from CD34-positive cells pulsed with in vitro leukemia-associated antigens which may

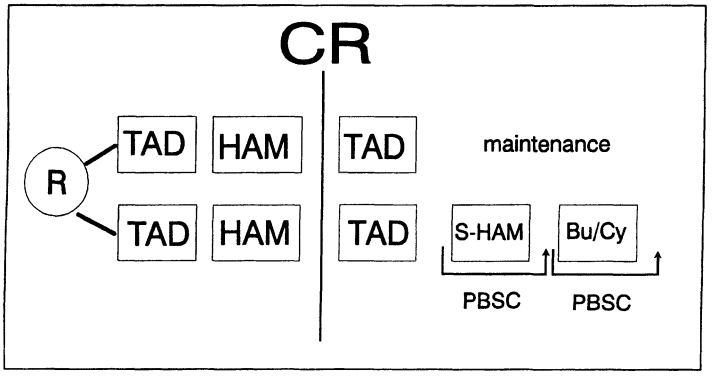

Fig. 1. AML Cooperative Group: current treatment strategy

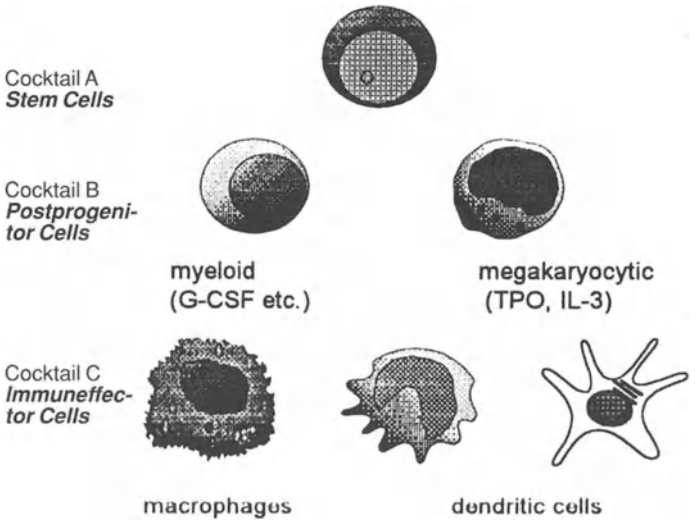

Fig. 2. Expansion (A) and differentiation of CD 34-positive cells (B,C) with specific cytokine cocktails

be retransfused to control MRD during the postremission phase (Fig. 2).

Other procedures for stem cell processing are in progress such as in vitro stem cell expansion either to purge or to improve low stem cell yield. Differentiation of stem cells into myeloid postprogenitor cells to shorten duration of aplasia are being used in ongoing clinical trials.

Taken together all the treatment options beeing developed in context with aPBST,

1. improvement of tolerance of dose-intensive chemotherapy in elderly patients,
2. further dose-escalation and by sequential myeloablative therapy
3. new techniques for stem cell processing, postremission treatment in AML may be significantly improved in the future.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Treatment of Relapsed and Refractory Acute Myeloid Leukemias – Strategies of the German AML Cooperative Group (AMLCG)

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Abstract. The trials of the AMLCG for the treatment of relapsed and refractory AML are directed towards the development and assessment of more effective antileukemic drug combinations and attempts to reduce the mortality rate through the application of hematopoietic growth factors and strategies for infection prevention. In a first study the S-HAM regimen was used to compare the efficacy of high dose (HD)(3 g/m^2) versus intermediate dose (ID)(1g/m^2) AraC and revealed a higher antileukemic activity of HD AraC in 186 randomized patients. This beneficial effect was, however, compensated by a higher rate of early deaths mainly from uncontrolled infections. In a subsequent study 68 patients were therefore randomized to the prophylactic application of fluconazol in addition to standard antimicrobial prophylaxis versus standard prophylaxis alone. In addition, all patients reveived G-CSF after the completion of S-HAM chemotherapy. While no differences in remission or early death rates were observed between patients with or without fluconazol prophylaxis the period of severe granulocytopenia was significantly shortened by G-CSF. This effect translated into a reduction of early deaths (21 vs. 30%) and an increase in remission rates (56 vs. 47%). In patients <60 years of age a significant prolongation of time to treatment failure (159 vs. 93 days, p=0.038) as well as of disease free survival was noted (209 vs. 97 days, p=0.003). These data indicate a beneficial effect of G-CSF during S-HAM therapy for relapsed and refractory AML both on early response and long term outcome. A current study has been initiated to further improve the S-HAM regimen through modulation of AraC metabolism by fludarabine.

Introduction

In an attempt to assess new therapeutic modalities the German AML Cooperative Group (AMLCG) has performed a sequence of studies in patients with relapsed and refractory acute myeloid leukemia (AML) which were designed to develop more effective antileukemic regimens and to also reduce the toxicity and treatment related mortality. These studies included the evaluation of intermediate dose cytosinarabinoside (AraC) versus high-dose AraC, the introduction of G-CSF to shorten the period of treatment associated neutropenia and the assessment of antifungal prophylaxis by oral fluconazole.

The basis for these studies were the standardized first-line regimens according to the protocols of the AMLCG and the development of criteria for the definition of refractoriness to conventional treatment [1-4].

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Prospective Randomized Comparison of Intermediate Dose AraC Versus High-Dose AraC

Patients with refractory and relapsed AML were entered in AMLCG Study R-02/89 and received the sequential HD-AraC mitoxantrone (S-HAM) combination comprizing AraC as three-hour-infusion twice daily on days 1 and 2 and mitoxantrone by 1-hour-infusion on day 3 and 4. After a three-day treatment free intervall the same sequence was repeated on days 8 and 9 and 10 and 11, respectively. Patients below the age of 60 were randomized between AraC doses of 3.0 g/m^2 versus 1.0 g/m^2 while patients of older age received either 1.0 g/m^2 or 0.5 g/m^2 AraC [5, 6]. From 212 patients that were randomized into this study, 186 are currently evaluable for response and toxicity. Table 1 depicts their entry criteria according to the disease stage and the respective response rates. Analysis of HD-AraC versus ID-AraC in patients under the age of 60 revealed comparable overall remission rates of 52 versus 45% respectively. Reasons for treatment failure, however, differed significantly in that patients with ID-AraC had significantly more treatment failures from persistant leukemia while a significantly higher early death rate was encountered in the HD-AraC group (Table 2). The higher early death rate was not due to differences in the recovery time from critical neutropenia (< 500/µl) nor from striking differences in non-hematologic toxicities. Except for severe CNS-toxicity which occurred only in the HD-AraC arm in 10% of cases, no major differences between both treatment arms were observed.

A more detailed analysis in relation to disease status revealed that patients with refractory AML experienced a significantly higher CR rate of 42% after 3.0 g AraC as compared to a CR rate of 33% in patients being treated with 1.0 g/m^2 AraC. In patients with good risk relapses, i.e. patients with relapses after preceding remissions of more than 6 months duration had a comparable response rate of 60% after 3.0 g/m^2 AraC and 54% after 1.0 g/m^2 AraC.

Hence, from this trial it could be concluded that increasing the dose of AraC exerts a higher antileukemic activity which does not translate into a higher response rate, however, due to an increase in treatment related mortality.

Table 1. AML-CG study R-02/89: Patient characteristics and response

	n	CR	ED	NR + PR
Primary NR	19	4 (21%)	5 (26%)	10 (53%)
CR < 6 mths	30	14 (47%)	6 (20%)	10 (33%)
CR > 6 < 18 mths	52	29 (56%)	13 (26%)	10 (18%)
CR > 18 mths	25	15 (60%)	6 (24%)	4 (16%)
≥ 2nd relapse	12	5 (42%)	4 (33%)	(25%)

Table 2. AML-CG study R-02/89: Patients < 60 years (n = 138)

AraC dose	CR	ED	NR + PR
3.0	38 (52%)	23 (32%)	12 (16%)
1.0	29 (45%)	11 (17%)	25 (39%)
	67 (49%)	34 (25%)	37 (26%)
		$p = 0.047$	$p = 0.008$

Prospective Randomized Evaluation of Antifungal Prophylaxis with Fluconazole

Based on the experiences of the AMLCG Study R-02/89 is was the major aim of the AMLCG Study R-03/92 to assess approaches to reduce the treatment related mortality. Since most treatment associated deaths are due to uncontrollable infections a considerable proportion of which is due to fungal infections, this study comprized the prospective randomized evaluation of systemic antifungal prophylaxis with oral fluconazole versus standard antimicrobial prophylaxis alone. From 86 patients that entered this trial 68 cases are currently evaluable for response and toxicity. The antileukemic regimen again comprized the S-HAM combination with an age and disease oriented adjustment of AraC dose. Hence, patients below the age of 60 with refractory disease were treated with single doses of 3.0 g/m^2 AraC while elderly patients and patients with re-

lapsed AML obtained AraC at a dose of 1.0 g/m^2. Patient characteristics are depicted in Table 3 together with the overall response rates of the respective subcatagories.

The primary endpoints of study R-03/92 were the rate of infections and particularly documented fungal infections as well as the requirement to initiate a systemic antifungal treatment by amphotericin B. As depicted by Table 4 no major differences were encountered in the frequency and type of infections between the control and patients receiving antifungal prophylaxis by fluconazole. In contrast, a significantly higher number of patients on the fluconanzole prophylaxis arm required systemic antifungal salvage treatment by amphotericin B. Since this parameter comprized a major endpoint of this study randomization was closed at this point.

As depicted by Table 5 no significant differences in antileukemic efficacy were observed between both treatment arms although a slightly higher CR rate of 61 versus 50% was achieved in patients receiving fluconazole prophylaxis.

The results of this trial indicate that systemic antifungal prophylaxis by fluconazole cannot be recommended in this subgroup of relapsed and refractory AML patients.

G-CSF for Shortening of Treatment Associated Neutropenia

The uniform antileukemic therapy in studies AMLCG R-02/89 and R-03/92 provided the means to assess the impact of post-treatment application of G-CSF which aimed at shortening the period of treatment associated neutropenia. This comparison was restricted to patients receiving comparable doses of AraC and thus comprized all patients entered in study R-03/92 but only 91 patients of study R-02/89. As depicted by table 6 both groups of patients were comparable in terms of disease stage and age distribution. As could be anticipated patients receiving G-CSF after the end of S-HAM therapy had a significantly shorter duration of critical neutropenia to a recovery to leukocyte counts > 1000/mm^3. This effect transla-

Table 3. AML-CG study R03/92: Patient characteristics and response

	n	CR	ED	NR + PR
Primary NR	5	1 (20%)	1 (20%)	3 (60%)
CR < 6 mths	17	5 (29%)	4 (24%)	8 (53%)
CR > 6 < 18 mths	31	22 (71%)	7 (22%)	2 (7%)
CR > 18 mths	14	9 (64%)	4 (28%)	1 (8%)
≥ 2nd relapse	1	1 (100%)	–	–

Table 4. AML-CG study R-03/92: Infectious complications

	Fluconazole	Control
No. infections	3%	6%
No. of febrile days	9	6
FUO	61%	50%
CDI	56%	50%
MDI	50%	31%

Table 5. AML-CG study R-03/92: Anti-leukemic efficacy

	Fluconazole	Control
CR	61%	50%
ED	22%	19%
PR + NR	17%	31%
DSF (mths)	7	5
Survival (mths)	7	7

Table 6. S-HAM with or without G-CSF support: Patient characteristics

	Study R-02/89	Study R-03/92
n	91	68
Age	50	48
Primary NR	16%	7%
CR < 6 mths	25%	25%
CR > 6 < 18 mths	36%	46%
CR ≥ 18 mths	15%	21%
≥ 2nd relapse	7%	1%

Table 7. S-HAM with or without G-CSF support: Anti-leukemic efficacy

	Study R-02/89	Study R-03/92
CR	47%	56%
ED	30%	21%
PR + NR	23%	24%
DSF (mths)	3.6	5.6
Survival (mths)	5.3	7.3

ted into an increase in the rate of complete remissions and a reduction in the frequency of early death (Table 7). Surprisingly a beneficial effect was also observed on the disease- free and overall survival which was most pronounced in patients below the age of 60 years.

Conclusions

In conclusion the sequence of the most recent trials of the AMLCG indicates that S-HAM is a highly effective regimen for relapsed and refractory disease. The dose of AraC can be adjusted to the disease stage in that patients with refractory disease benefit from the high-dose AraC application of 3.0 g/m^2 whereas patients with non refractory, relapsed AML are adequately treated by single doses of 1.0 g/m^2. The post-treatment application of G-CSF reduced the period of critical neutropenia and translated into a higher rate of complete remissions, a lower frequency of early deaths and potentially also a longer disease free and overall survival.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Intermediate Dose Cytarabine and Idarubicin for Salvage Therapy of Acute Myeloid Leukemia

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Abstract. Between September 1991 and May 1996, 56 patients with refractory or relapsed acute myeloid leukemia (AML) were treated with one cycle of timed sequential chemotherapy (TSC) consisting of intermediate-dose (ID) ara-C (1.0 g/m^2 every 12 h) on days 1, 2, 8, and 9, and idarubicin (IDA, 10 mg/m^2) on days 3, 4, 10, and 11. The study population included 22 patients with refractory AML, 33 patients with late first relapse occurring after a first complete remission of ≥ 6 months, and 1 patient for whom the clinical course before salvage therapy was unknown. Of the 56 patients entered onto the study, 25 (45%) achieved a complete remission (CR), 8 (14%) a partial remission (PR), 11 patients (20%) were classified as treatment failure due to resistant disease, and 12 patients (21%) died within the first 42 days after the initiation of salvage therapy, mainly due to infections. Response to salvage therapy was strongly influenced by disease status with 55% of patients with late first relapse achieving a CR as compared with 32% of patients with refractory AML. Severe myelosuppression was observed in most patients resulting in fever or documented infections in 70–90% of them whereas nonhematologic toxicity was tolerable. Among the 25 patients who achieved a CR, 6 patients received no postremission therapy, 10 patients were treated with 1 or 2 cycles of IDA (8 mg/m^2) and etoposide (150 mg/m^2) on days 1 to 3 as consolidation, 8 patients underwent an allogeneic or autologous bone marrow transplantation (BMT), and 1 patient was lost for follow-up. Overall, after a median follow-up of 44 months, the median remission duration is 9 months, and the median survival 9 months as well. We conclude that this chemotherapy regimen has significant antileukemic efficacy, especially in AML patients who relapsed after a durable first CR (≥ 6 months). The short duration of remission in patients not eligible for BMT, however, emphasizes the need for optimizing early postinduction therapy.

Introduction

Although advances in chemotherapy and supportive care have improved the prognosis of AML patients within the last 10–15 years, long-term disease free survival is still achieved in only 20 to 30% of cases [1, 2]. Most patients relapse with their disease and ultimately die of resistant disease.

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In order to improve the results of salvage therapy in AML, several approaches have been developed or are at present under investigation. These include new chemotherapeutic agents (e.g., nucleoside analogs, topoisomerase I inhibitors, platinum/alkylating analogs), pharmacologically optimized treatment with established cytotoxic drugs (e.g., ara-C), leukemia sensitization by the use of hematopoietic growth factors or TSC, and attempts at reversing leukemic cell resistance by blockers of multidrug resistance [1–6].

Most regimen currently used for salvage therapy of AML include ara-C, the most active single drug available for the treatment of AML, frequently in combination with topoisomerase II reactive agents, including anthracyclines, mitoxantrone, amsacrine, and etoposide (reviewed in [1, 6, 7]). In relapsed and refractory AML, higher than conventional doses of ara-C, i.e. ID or high dose (HD) ara-C, have been usually administered due to their enhanced efficacy [4, 5].

Based on the following evidence, our protocol for the treatment of patients with refractory or relapsed AML was designed: first, a prospective randomized trial of the German AML Cooperative Group (AMLCG) in patients with relapsed or refractory AML demonstrated that ID-ara-C currently represents the optimal dose for the treatment of advanced AML, while the use of HD-ara-C, although associated with higher antileukemic efficacy, did not translate into a higher response rate due to an increased treatment-associated toxicity [8]; second, preclinical studies with IDA, its pharmacological characteristics, as well as several randomized clinical trials comparing IDA with daunorubicin were suggestive of greater antileukemic activity of this new anthracycline analog (reviewed in [9]); third, several studies have shown that the concept of TSC, which postulated a benefit for a second sequence of chemotherapy with cycle-active drugs at the time of presumed peak cell recruitment induced by the first sequence, might be clinically useful both as first-line and as salvage therapy in AML patients [3, 8, 10, 11].

Based on these results, we began a prospective phase II clinical trial of TSC with ID-ara-C and IDA in patients with refractory or relapsed AML in 1991. The objectives of this study were to compare the antileukemic efficacy and toxicity of this regimen with a historical control group who had been treated with ID-ara-C and mitoxantrone [8]. As a secondary objective, we assessed the feasibility and toxicity of a consolidation chemotherapy consisting of 2 cycles IDA and etoposide for those patients who were not eligible for bone marrow transplantation (BMT).

We summarize here our results of this clinical trial.

Material and Methods

Patients

From September 1991 to May 1996, 56 patients from 8 participating hematologic centers in Berlin and Brandenburg were entered onto the study, after informed consent was obtained. Eligible patients included only those considered to have refractory or relapsed AML. The criteria for refractory AML were as follows [12]: nonresponse to first-line chemotherapy, including at least two conventional induction courses; early relapse within 6 months of first remission; second and subsequent relapses.

Further pretreatment characteristics are shown in Table 1. Diagnosis and classification of AML were made according to the French-American-British (FAB) criteria [13].

Treatment Regimens

Induction therapy consisted of ID-ara-C (1 g/m^2 every 12 h as a 3-h-infusion) on days 1, 2, 8, and 9, and IDA (10 mg/m^2 as short infusion over 30 min) on days 3, 4, 10, and 11. All patients received the full dose of ID-ara-C and IDA, except for 3 patients in whom the second part of the cycle (i.e., ID-ara-C on days 8, 9, and IDA on days 10, 11) was not administered because of infectious complications. The use of hematopoietic growth factors (HGF) after chemotherapy was optional, and HGF were administered at the discretion of the participating centers. All pa-

Table 1. Patient characteristics

No. of patients	56
Age	18–76 years, median 49 years
≤ 60 years	47
> 60 years	9
Sex	
Female	26
Male	30
FAB subtypes	
M1	16
M2	19
M3	1
M4	11
M5	6
M6	0
M7	1
Unclassified	2

tients received glucocorticoid eye drops during ara-C administration for the prophylaxis of photophobia and conjunctivitis as well as oral antimicrobial prophylaxis.

Therapeutic options offered to patients with CR included allogeneic BMT for those with a major histocompatible complex identical sibling, autologous BMT for those lacking a suitable donor, or consolidation chemotherapy. The latter should be administered within 3 to 6 weeks after CR, and consisted of 2 courses of IDA (8 mg/m^2 as a short infusion over 30 min), and etoposide (150 mg/m^2 as a short infusion over 1 h) on days 1 to 3.

Evaluation of Therapy and Toxicity

Antileukemic response and early death (ED) was judged according to criteria proposed by a National Cancer Institute-sponsored workshop [14]. The interval between the initiation of salvage chemotherapy and the posttreatment achievement of neutrophils >500/mm^3 and platelets >20 000/mm^3 was defined as time to recovery. Treatment toxicity was assessed by WHO criteria [15].

Statistical Analysis

Survival was calculated from the beginning of salvage therapy to the date of last follow-up or death. Remission duration was measured from the time of achievement of CR to the date of most recent follow-up or documented relapse at any site or death in remission. Survival and remission duration were plotted by the method of Kaplan and Meier [16].

Results

Efficacy of Therapy

According to the definition of the disease status (see above), the study population included 22 patients with refractory AML (nonresponse to first-line chemotherapy n = 2, early first relapse occurring after a first complete remission of less than 6-month duration n = 14, second and subsequent relapses n = 6), and 33 patients with late first relapse occurring after a first CR of a 6-month duration or more. The exact clinical course of 1 patient before salvage therapy was unknown.

Most patients (N=36) were recruited from the AMLCG trials, and had received a standardized first-line treatment consisting of thioguanine, conventional dose ara-C, daunorubicin (TAD) and HD-ara-C, mitoxantrone (HAM) as induction, TAD and sequential (S)-HAM as intensive consolidation, or TAD and monthly maintenance therapy over several years, as reported elsewhere [17]. Twenty patients received other induction and postremission treatments, mainly within the framework of the East German Acute Leukemia Study Group.

Response to salvage therapy in these 56 patients according to the disease status is shown in Table 2. Overall, 25 patients (45%) achieved a CR and 8 (14%) a PR. Eleven patients (20%) were classified as treatment failure due to resistant disease, and 12 patients (21%) died within the first 42 days after the start of treatment (ED). As shown in Table 2, response to salvage therapy was strongly influenced by the disease status. Of the 22 patients with refractory AML, 7 (32%) achieved a CR, while 18 of 33 (55%) patients with late first relapse entered CR. This difference was mainly due to the higher rate of resistant disease in patients with refractory

Table 2. Treatment results of induction therapy according to the status of AML

	n	Treatment result			
		CR n (%)	PR n (%)	NR n (%)	ED n (%)
All patients	56	25 (45)	8 (14)	11 (20)	12 (21)
History[a]					
– Nonresponse to first line chemotherapy	2	1 (50)		1 (50)	
– Early first relapse	14	4 (29)	3 (21)	3 (21)	4 (29)
– Second and subsequent relapse	6	2 (33)	2 (33)	1 (17)	1 (17)
– Relapse with remission duration > 6 < 18 months	24	13 (55)	2 (8)	4 (17)	5 (20)
– Relapse with remission duration > 18 months	9	5 (56)	1 (11)	1 (11)	2 (22)

[a] Unknown n = 1.

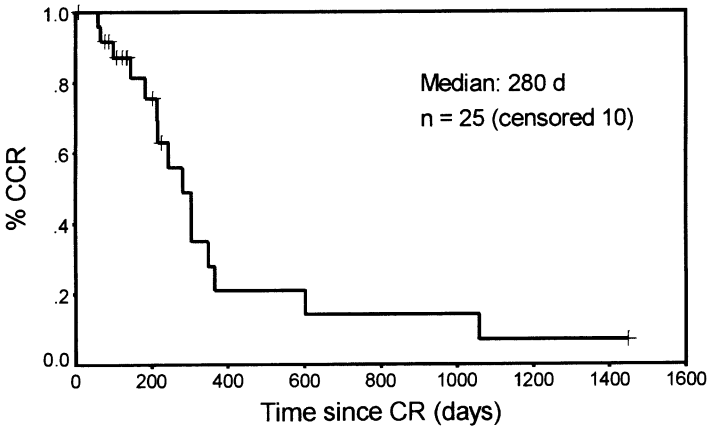

Fig. 1. Remission duration of 25 patients achieving CR. Patients treated with allogeneic or autologous BMT in CR were censored at the time of BMT

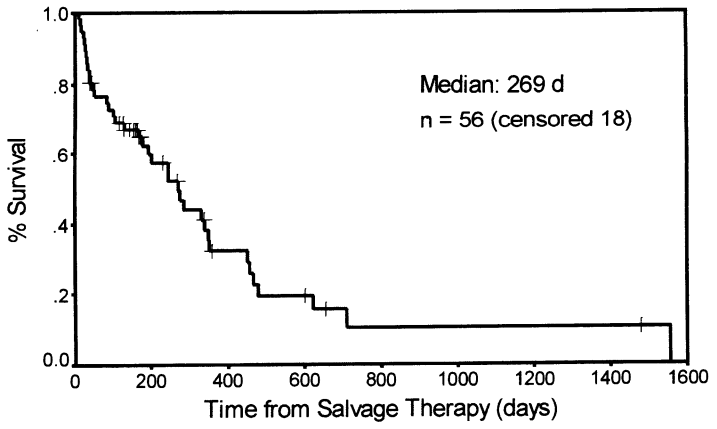

Fig. 2. Overall survival of all 56 patients. Patients treated with allogeneic or autologous BMT in CR were censored at the time of BMT

AML than in those with late relapse (6/22, 28% vs. 5/33, 16%, respectively) whereas the ED rate was similar in both groups (5/22, 22% vs. 7/33, 21%).

Among the 25 patients achieving a CR, 10 patients were treated with one (n = 3) or two cycles (n = 7) of IDA and etoposide. Eight patients underwent BMT (allogenic n=4, autologous n=4), 4 of them after one cycle of consolidation therapy. Other postinduction chemotherapy (e.g., ID-ara-C and IDA at reduced dosages) was administered in 3 pa-

Table 3. Nonhematologic toxicity

Toxicity	0		1		2		3		4	
(WHO Grade)	n	(%)	n	(%)	n	(%)	n	(%)	n	(%)
Documented infections	17	(31)	4	(7)	12	(21)	17	(31)	6	(10)
Fever	4	(7)	2	(4)	40	(71)	8	(15)	2	(4)
Clinical bleeding	34	(60)	14	(25)	7	(13)	–	–	1	(2)
Liver toxicity	35	(63)	11	(19)	6	(10)	2	(4)	2	(4)
Nausea/ Vomiting	29	(52)	8	(15)	8	(15)	10	(18)	1	(2)
Mucositis	35	(63)	5	(8)	8	(15)	7	(13)	1	(2)
Diarrhea	40	(71)	–	–	12	(21)	4	(7)	–	–
Obstipation	53	(94)	2	(4)	–	–	–	–	1	(2)
Creatinine elevation	36	(65)	16	(29)	3	(6)	1	(2)	–	–
Lung toxicity	40	(71)	1	(2)	8	(15)	2	(4)	5	(8)
Fever	4	(7)	2	(4)	40	(71)	8	(15)	2	(4)
Allergic reactions	53	(94)	1	(2)	1	(2)	1	(2)	-	–
Cardiac toxicity	47	(83)	4	(7)	1	(2)	2	(4)	2	(4)
CNS toxicity	51	(92)	2	(4)	1	(2)	1	(2)	1	(2)
Conjunctivitis	54	(96)	–	–	1	(2)	1	(2)	–	–

tients, whereas 6 did not receive further therapy, and one patient was lost for follow-up. Overall, the remissions after induction therapy lasted from 2 to 48+ months with a median remission duration of 9 months (10 patients censored, 8 of them because of BMT). The survival ranged from 11 days to 52 months with a median of 9 months. The overall survival and duration of CR are shown in Figs. 1 and 2.

Toxicity

The regimen was severely myelosuppressive with all patients developing neutropenia ($<500/mm^3$) and thrombocytopenia ($<20\,000/mm^3$). In patients achieving a CR, median time to recovery of neutrophils and platelets was 35 days (range: 28–62 days). HGF were not routinely administered, and, in fact, only 4 patients received G-CSF after chemotherapy. The neutropenia was associated with fever in 93% and documented infections in 70% of patients. Seven patients died of uncontrollable infections. While being thrombocytopenic, 40% of patients developed bleeding complications, which were fatal in one patient.

Serious nonhematologic toxicity (WHO grade 3 or 4) consisted mainly of nausea and vomiting (20% of patients), mucositis (15%), as well as lung (12%), liver (8%) and cardiac toxicity (8%). Serious central nervous system symptoms and conjunctivitis were observed in only one patient each. The nonhematologic toxicity is detailed in Table 3.

The consolidation therapy with IDA and etoposide was generally well tolerated. During the first cycle, vomiting was the only severe (WHO grade 3) toxicity occurring in one patient. The second cycle was complicated by cardiac toxicity, bleeding, and CNS toxicity in one patient each.

Discussion

The administration of TSC with ID-ara-C and IDA resulted in a CR rate of 45% in 56 patients who had refractory or relapsed AML.

Although the results of trials in refractory or relapsed AML are hardly comparable because of the substantial heterogeneity of patient populations as well as the limited information provided in most studies as to prior treatment and disease status, our results are in line with other recent studies combining ID-ara-C or HD-ara-C with different topoisomerase II reactive agents, including IDA, mitoxantrone, etoposide, and amsacrine as one continuous course or as TSC [10, 11, 18–22]. More important, our results are comparable to those obtained with TSC consisting of ID-ara-C and mitoxantrone as salvage therapy in a patient population with the same inclusion criteria and similar pretreat-

ment characteristics (e.g., median age, disease status), suggesting that both regimens have equivalent antileukemic efficacy in advanced AML ([8] Hiddemann et al., this Vol.).

Although the prognostic influence of a variety of clinical and laboratory parameters has been postulated in refractory and relapsed AML, most studies indicated that the response to first-line treatment, the duration of first CR, and the response to prior salvage regimens are the principal variables influencing the outcome of salvage therapy as well as survival [7, 12, 23, 24]. Accordingly, we observed a clear correlation between CR rate and the disease status at the onset of salvage chemotherapy. Patients with late relapse achieved a CR in 55% of cases as compared with 32% in patients with refractory AML. Since the incidence of ED was similar in both groups, the different CR rates were mainly due to a higher percentage of resistant disease in patients with refractory AML versus those with late relapse (26 vs. 15%, respectively).

As expected, the most severe side effect of TSC with ID-ara-C and IDA was profound myelosuppression, occurring in all patients. The duration of severe myelosuppression, as defined by the interval between the onset of salvage therapy and recovery of neutrophils and platelets, lasted from 28 to 62 days with a median duration of 35 days. Neutropenia was associated with a high frequency of infections (70% of patients) which were fatal (ED) in 7 patients. Comparable recovery times and frequencies of infectious complications have been reported in previous studies administering TSC with etoposide, mitoxantrone, and ID-ara-C [10, 11], or HD-ara-C and mitoxantrone ([8] Hiddemann et al., this Vol.) as salvage therapy in advanced AML. In view of this severe hematologic toxicity, the use of HGF after chemotherapy (e.g., G-CSF) might be recommendable for all patients (Hiddemann et al., this Vol.). Other side effects, usually observed after HD-ara-C based protocols, such as diarrhea, hepatic toxicity, conjunctivitis, and neurotoxicity occurred rarely, and, in general, the nonhematologic toxicity was tolerable in most patients.

Unfortunately, corresponding with other recent studies, the remission duration and overall survival of our patients was relatively short, and could not be substantially prolonged by the administration of consolidation therapy with IDA and etoposide. Interestingly, the only patient who achieved a long-lasting second CR after conventional consolidation chemotherapy was treated based on an individual policy with repetitive cycles of ID-ara-C and IDA at reduced dosages. At present, intensive consolidation by allogeneic BMT, autologous BMT, or peripheral blood stem-cell transplantation, performed early during remission, represents the only available therapeutic modality associated with a long-term disease-free survival in these poor-risk AML patients. In support of this view, 3 of 8 patients who underwent BMT in our study, are still in second CR.

In conclusion, the results of this study indicate that TSC with ID-ara-C and IDA is an effective regimen with tolerable toxicity for salvage therapy of AML.

Based on previous studies of the AMLCG in relapsed or refractory AML patients ([8, 18] Hiddemann et al., this Vol.) as well as data obtained in this study, the German AMLCG has initiated a prospective randomized multicenter trial in order to test whether the addition of fludarabine to ID- or HD-ara-C and IDA will improve the remission rate and disease-free survival. It has been recently shown that infusion of fludarabine after ara-C can increase ara-CTP accumulation by biochemical modulation in circulating leukemia blasts from patients with AML in relapse [25], and, thus, may lead to an enhanced antileukemic efficacy.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Superiority of High-Dose over Intermediate-Dose Cytosine Arabinoside (AraC) in the Treatment of Patients with High-Risk Acute Myeloid Leukemia (AML)

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Abstract. High-dose AraC is the most effective single agent for the treatment of high-risk AML. However, its optimal dose has not been determined yet. Therefore, a prospective randomized study was performed comparing two dose levels of AraC in patients receiving the sequential high-dose AraC and mitoxantrone regimen (S-HAM) for refractory and relapsed AML. AraC was given as a 3-h infusion q 12 h on days 1, 2, 8, and 9. Patients younger and older than 60 years were randomized to AraC dose levels of 3 vs. 1 g/m^2 and 1 vs. 0.5 g/m^2 per single dose, respectively. Randomization was stratified for primary refractoriness against induction therapy and length of first remission in relapsed patients. Mitoxantrone 10 mg/m^2 was given to all patients on days 3, 4, 10, and 11. Overall, from 186 evaluable patients 88 (47%) and 10 (5%) achieved a CR and PR, respectively, 39 (21%) had persisting leukemia, and 49 (26%) suffered from ED. For patients younger than 60 years the higher dose level resulted in a significant reduction of cases with persisting leukemia (12 vs. 31%, p=0.008) and a higher rate of ED (32 vs. 17%, p=0.047). These differences were not based on a prolongation of critical neutropenia (41 vs. 39 days). Within the subgroup of patients with refractory AML a trend towards a higher CR rate occurred in the group receiving 3 g/m^2 AraC (46 vs. 26%, p=0.12). For patients older than 60 years the analyses of treatment failures demonstrated a higher rate of persisting leukemia in the lower dose group (26 vs. 16%) with ED also occuring more frequently in the higher dose group (36 vs. 26%). Overall, there were no significant differences in total survival. These data indicate a dose-response relation of the antileukemic activity of AraC which does not fully translate into an increase in remission rates due to a higher incidence of early deaths after high doses of AraC predominantly from uncontrollable infections. In order to take full advantage of the higher antileukemic activity of high-dose AraC an optimization of supportive care is warranted.

Introduction

Management of acute myeloid leukemia (AML) essentially is based on the application of cytosine arabinoside (AraC) which is the most active single drug in the treatment of this disease [1]. Based on insights into the AraC pharmacology revealing a correlation

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between the extent of the accumulation of phosphorylated AraC (AraCTP) in leukemic blasts with the response to AraC therapy [2, 3] new strategies have been developed evaluating the application of higher doses of AraC. High-dose AraC alone or in combination with other drugs was shown to be effective in patients with AML resistant to standard dose AraC [4-6] and currently is integrated into first line therapy of AML [7-11]. However, taking into account the increase of severe toxicity encountered during high-dose regimens debate continues about the most useful dose of AraC. Most early studies analysed high-dose AraC at a level of three grams given twice daily for 3 to 5 days. Conversely, pharmacokinetically based support for the application of lower doses came from the observation that the intracellular enzyme deoxycytidine kinase which katalyses the formation of AraCTP is already saturated when AraC is administered at one gram as a 2-h infusion [12, 13]. However, interference of other mechanisms such as a decreased transmembrane transport into the cell and an increased intracellular deamination of AraCTP [14, 15] may counteract the AraCTP formation and its impact on cytotoxicity but could be overcome by higher doses of AraC. Therefore, the current study was designed to evaluate the antileukemic efficacy of the previously established sequential high-dose cytosine arabinoside and mitoxantrone (S-HAM) regimen [16] at two different dose levels in patients with relapsed or refractory AML.

Patients and Methods

Patients

Consecutive patients with relapsed and refractory acute myeloid leukemias who were admitted at the participating centers were eligible for the study. The diagnosis of AML was based on the revised French-American-British (FAB) Group criteria [17]. Refractoriness against standard chemotherapy was defined according to previously established criteria [18]: these included

a) primary resistance against two cycles of induction therapy;
b) first early relapse with a remission duration of less than 6 months;
c) second and subsequent relapse. Patients with first relapses after six months remission duration were not considered refractory to standard therapy and were included as relapsed AML.

All patients were recruited from the first line trials of the German AML Cooperative Group and had thus received a standardized first line treatment. In patients less than 60 years of age first line therapy consisted in double induction therapy with either the repetitive application of the 9 day regimen of thioguanine, cytosine arabinoside, daunorubicin (TAD-9/TAD-9) or the sequential application of TAD-9 followed by high-dose cytosine arabinoside and mitoxantrone (HAM). Older patients all received one course of TAD-9 and were treated by a second TAD-9 course only upon inadequate response to the first TAD-9 cycle. Patients of all ages who achieved a complete remission subsequently received TAD-9 for consolidation and monthly maintenance therapy for 3 years [19, 20].

Patients with antecedent hematologic disorders, secondary leukemias, and a preceding autologous or allogeneic bone marrow transplantation were excluded from the study. Further exclusion criteria comprized coronary heart disease; heart failure; cardiomyopathy; severe arterial hypertension; abnormal liver function tests (aspartate aminotransferase (AST), alanine aminotransferase (ALT), or alkaline phosphatase (AP) more than three times the upper normal limits; total bilirubin > 2.0 mg/dl); impaired renal function (serum creatinine > 2.0 mg/dl); severe infections; or pregnancy.

Antileukemic Therapy

Patients meeting the entry criteria were enrolled into the current study and were treated by S-HAM [16] comprising high-dose or intermediate-dose cytosine arabinoside (AraC), respectively, every 12 h by a 3-h infusion on days 1, 2, 8, and 9 and mitoxantrone

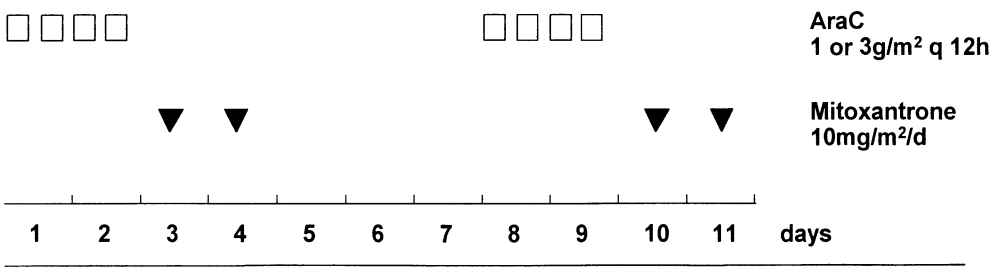

Fig. 1. Schedule of the sequential high-dose AraC and mitoxantrone protocol

10 mg/m^2/day as a 30-min infusion on days 3, 4, 10, and 11, respectively (Fig. 1). Patients younger than 60 years were randomly assigned to receive AraC at a dose levels of either 3 g/m^2 or 1 g/m^2 per application while older patients were randomized to either 1 g/m^2 or 0.5 g/m^2 AraC per single dose. To avoid imbalances in the patients' risk profile randomization was stratified for the following criteria:

1. Primary resistance against two cycles of induction therapy.
2. First early relapse with a remission duration of less than 6 months.
3. First relapse with a remission duration of more than 6 months and less than 18 months.
4. First relapse with a remission duration of more than 18 months.
5. Second and subsequent relapse.

To prevent high-dose AraC induced photophobia and conjunctivitis all patients received glucocorticoid eye drops every 6 h starting before the first dose and continuing for 24 h after the last dose of high-dose AraC. Antimicrobial prophylaxis consisted of cotrimoxazol 960 mg po three times daily, colistine sulphate two million units po four times daily, and amphotericin B suspension 40 mg po six times daily.

Study Parameters

Response to therapy was assessed according to CALGB criteria [21]. Complete remission status was defined as a normal cellular marrow with normal erythroid and myeloid elements and with myeloblasts, promyelocytes, and other leukemic cells totaling less than 5%, and with normal peripheral blood platelet and white blood cell counts for at least four weeks. Patients having more than 5% myeloblasts but fewer than 25%, with otherwise normal bone marrow, were considered to be in partial remission, as were patients fulfilling criteria of complete remission except for full recovery of peripheral blood platelet and/or white blood cell counts. Patients with persisting leukemic blasts in the bone marrow or blood or with leukemic regrowth within 4 weeks after initial response were considered as non-responders. Patients dying within 6 weeks after completion of antileukemic therapy without evidence of leukemic regrowth were classified as early deaths.

The duration of critical cytopenia was evaluated by the time for leukocyte recovery to more than 1000/µl from the onset of S-HAM treatment. The time to complete remission was measured from the onset of treatment to the date of documented complete remission and disease free survival from the date of documented complete remission to relapse or death during remission. Survival and time to treatment failure were measured by the time from the beginning of treatment to death and death without evidence of leukemia, documentation of persisting leukemia, or relapse, respectively.

Toxicity was evaluated according to the World Health Organization (WHO) grading system [22].

Statistics

The primary end point of the present study was the impact of high-dose AraC on the complete remission rate and the disease free

survival as compared to a randomly assigned control group receiving intermediate-dose AraC. The secondary end point was the incidence of hematologic and non-hematologic side effects. Numerical values were compared by the χ^2-test, by the Fisher's-exacttest, and by the student's t-test. Remission duration and survival was calculated according to Kaplan Meier estimates. Comparisons were carried out using the log-rank test.

Study Conduct

Prior to therapy all patients gave their informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study as well as of potential risks. The study design adheres to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.

Results

Patient Characteristics

Two hundred and twelve patients have been entered into the study from 28 centers in Germany and Austria, 186 of whom were fully evaluable. Causes for exclusion from analysis were death before start of therapy (1); secondary leukemia (3); treatment not in accordance with the result of the randomization (4); discontinuation of therapy on day five (9); and incomplete documentation (9). Seventy-three and 65 patients younger than 60 years were randomized to 3 g/m^2 and 1 g/m^2 AraC, respectively, while 25 and 23 older patients were randomized to 1 g/m^2 and 0.5 g/m^2 AraC, respectively. The patients' ages ranged from 17 to 75 years (median 50 years) and did not differ between the respective groups. All patients had received prior chemotherapy for their disease as indicated above. Twenty-one patients younger than 60 years had been treated with the HAM regimen as part of the induction therapy in each group. Overall, 27 (15%) patients had refractory disease and 43 (23%) had relapsed after a first complete remission of less than six months duration. In 71 (38%) and 30 (16%) cases the relapses occured after a complete remission of more than 6 but less than 18 months and of more than 18 months duration, respectively, while 15 (8%) patients suffered from second or subsequent relapses. The comparison of the profile of disease status revealed a similarity for all four study groups. AML subtypes were predominantly M1, M2, M4, and M5. All 186 patients received one course of S-HAM therapy.

Antileukemic Activity

Overall, 88 (47%) and 10 (5%) of the 186 evaluable patients achieved a complete and partial remission, respectively, while 39 (21%) patients were non-responders. Forty-nine (26%) patients suffered from early deaths (Table 1).

Within the group of patients younger than 60 years the application of high-dose AraC proved to be superior to intermediate-dose AraC with more patients achieving a complete remission (52 vs. 45%) and statistically significant less cases having persisting leukemia (12 vs. 31%; p=0.008). Also, a strong trend toward a higher efficacy in adequate clearance of bone marrow blasts seven days after completion of chemotherapy was observed (96 vs. 84%; p=0.053). However, more early deaths occurred in the high-dose group (32 vs. 17%; p=0.047) which in most cases were due to severe infections (27 vs. 11%; p=0.01). Specifically, the subgroup analysis in patients with refractory disease or relapses after a first complete remission of less than six months duration (Table 2) revealed more pronounced differences in the CR rates (46 vs. 26%; p=0.12) and a less obvious excess of early deaths (42 vs. 17%; p=0.13) while still significantly less patients had persisting leukemia (19 vs. 61%; p=0.02).

There were no significant differences between the two groups with regard to time to treatment failure (median 2.9 vs. 2.4 months), disease free survival (median 5.3 vs 3.3 months), or overall survival (median 4.2 vs. 5.3 months; Figs. 2-4).

Table 1. Antileukemic activity

	Patients < 60 years		Patients ≥ 60 years		Total
AraC dose	3 g/m²n=73	1 g/m²n=65	1 g/m²n=25	0.5 g/m²n=23	n=186
Complete remission	38 (52%)	29 (45%)	11 (44%)	10 (43%)	88 (47%)
Partial remission	3 (4%)	5 (8%)	1 (4%)	1 (4%)	10 (5%)
Persisting leukemia	9 (12%)[a]	20 (31%)[a]	4 (16%)	6 (26%)	39 (21%)
Early death	23 (32%)[b]	11 (17%)[b]	9 (36%)	6 (26%)	49 (26%)

[a] $p = 0.008$. [b] $p = 0.047$.

Table 2. Antileukemic activity in subgroups of patients younger than 60 years

Disease status	CR		PR		PL		ED	
AraC dose	3 g/m²	1 g/m²	3 g/m²	1 g/m²	3 g/m²	1 g/m²	3 g/m²	1 g/m²
Refractory disease	3	1	1	2	3	4	3	2
CR1 duration < 6 months	9	5	–	–	2	8	5	1
CR1 duration ≥ 6 < 18 months	16	13	1	2	3	4	9	4
CR1 duration ≥ 18	9	6	–	1	1	2	3	3
≥ 2nd relapse	1	4	1	–	–	2	3	1
High-risk[a]	13[b]	10[b]	2	2	5[c]	14[c]	11[d]	4[d]
	(42%)	(33%)	(6%)	(7%)	(16%)	(47%)	(35%)	(13%)

[a] High-risk comprises cases with refractory disease and relapses after CR1 < 6 months. [b] p=0.12. [c] p=0.02. [d] p=0.13.

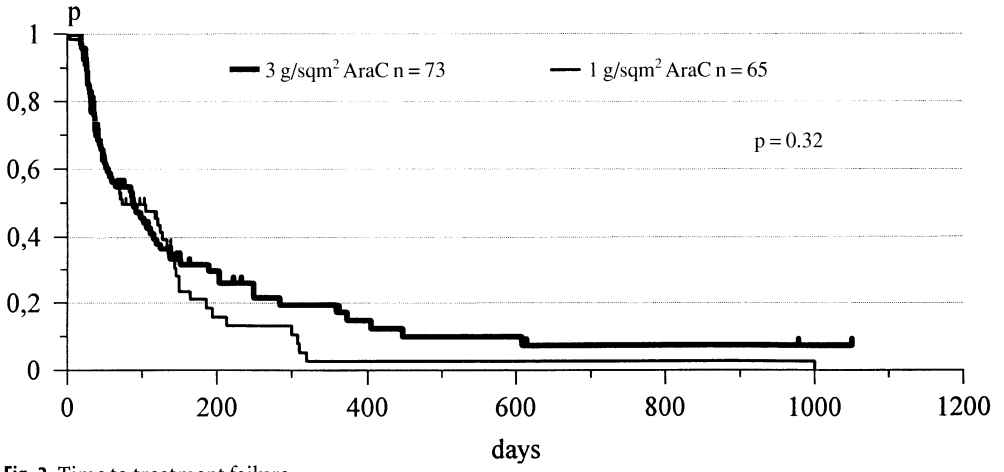

Fig. 2. Time to treatment failure

In patients older than 60 years the complete remission rate was the same for both groups. As in the younger patient groups, the more intensive therapy resulted in fewer cases with persisting leukemia and more early deaths (Table 1). However, due to the lower number of evaluable patients these differences were not statistically significant.

Side Effects

The dose-intensity of AraC had no influence on the recovery time of leukocytes to more than 1000/µl, which amounted to a median of 41 vs. 39 days in patients younger than 60 years and of 38 vs. 35 days in older patients (n.s.; Fig. 5).

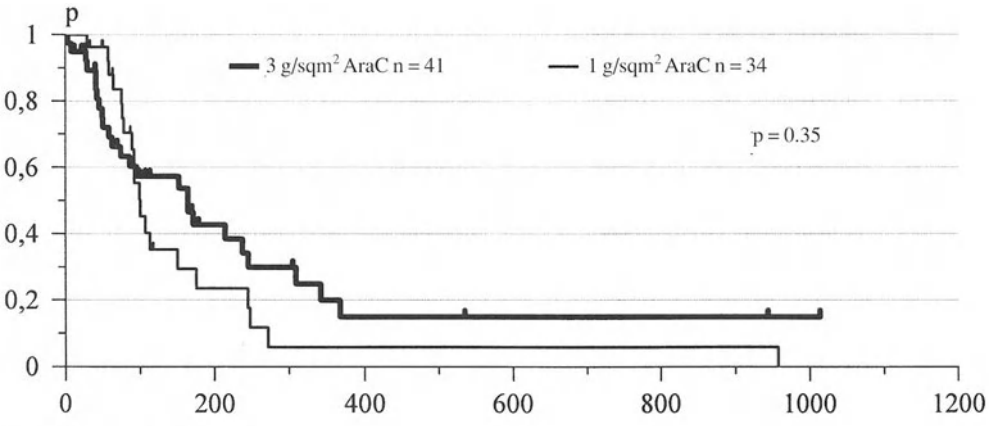

Fig. 3. Disease-free survival

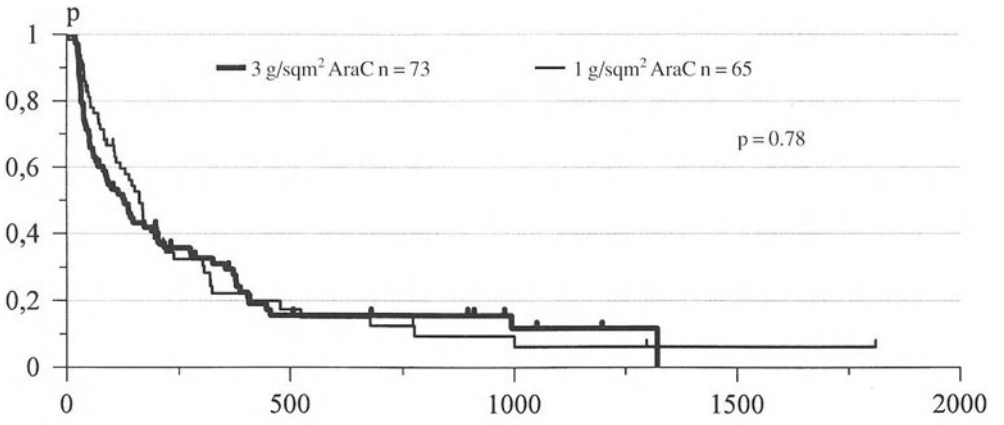

Fig. 4. Survival

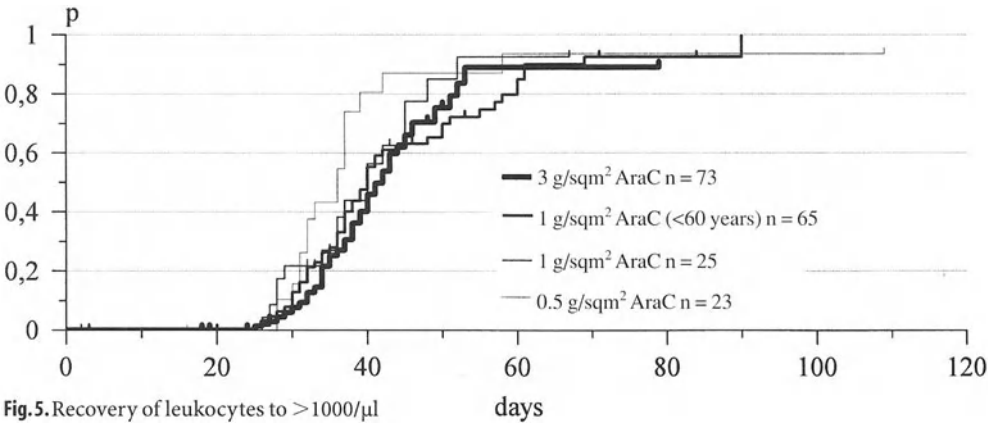

Fig. 5. Recovery of leukocytes to >1000/µl

Table 3. Non-hematologic side effects in patients younger than 60 years

AraC dose	3 g/m² n=73		1 g/m² n=65	
Toxicity	I°/II°	III° /IV°	I°/II °	III° /IV °
Infection	14 (19%)	43 (59%)	9 (14%)	32 (49%)
Nausea/vomiting	35 (48%)	19 (26%)	36 (55%)	11 (17%)
Diarrhea	16 (22%)	15 (21%)	19 (29%)	8 (12%)
Stomatitis[a]	25 (34%)	9 (12%)	12 (18%)	6 (9%)
Bleeding	26 (36%)	7 (10%)	20 (31%)	8 (12%)
Consciousness	7 (10%)	7 (10%)[b]	3 (5%)	–[b]
Bilirubin	29 (40%)	4 (5%)	24 (37%)	3 (5%)
AST/ALT	24 (33%)	3 (4%)	17 (26%)	5 (8%)
AP	19 (26%)	2 (3%)	20 (31%)	1 (2%)
Creatinine	14 (19%)	1 (1%)	11 (17%)	1 (2%)
Allergy	5 (7%)	2 (3%)	4 (6%)	–
Skin	23 (32%)	1 (1%)	19 (29%)	–
Heart function	3 (4%)	4 (5%)	2 (3%)	2 (3%)
Heart rhythm	14 (19%)	–	6 (9%)	–

[a] p=0.02. [b] p=0.01.

Table 4. Non-hematologic side effects in older patients

AraC dose	1 g/m² n=25		0.5 g/m² n=23	
Toxicity	I°/II°	III° /IV°	I°/II °	III° /IV°
Infection	3 (12%)	13 (52%)	3 (13%)	14 (61%)
Nausea/vomiting	15 (60%)	3 (12%)	13 (57%)	6 (26%)
Diarrhea	9 (36%)	4 (16%)	7 (30%)	7 (30%)
Stomatitis	10 (40%)	1 (4%)	4 (17%)	1 (4%)
Bleeding	7 (28%)	4 (16%)	5 (22%)	3 (13%)
Consciousness	2 (8%)	2 (8%)	4 (17%)	3 (13%)
Bilirubin	5 (20%)	2 (8%)	8 (35%)	2 (9%)
AST/ALT	8 (32%)	2 (8%)	6 (26%)	3 (13%)
AP	9 (36%)	–	6 (26%)	–
Creatinine	6 (24%)	2 (8%)	6 (26%)	1 (4%)
Allergy	2 (8%)	–	1 (4%)	1 (4%)
Skin	4 (16%)	–	9 (39%)	1 (4%)
Heart function	2 (8%)	2 (8%)	4 (17%)	2 (9%)
Heart rhythm	6 (24%)	–	5 (22%)	–

Also, in patients achieving a complete remission the time till CR did not differ significantly between the respective groups. CR was verified after 54 vs. 61 days in younger patients and after 48 vs. 46 days in older cases, respectively.

The non-hematologic side effects that were encountered during S-HAM therapy are summarized in Tables 3 and 4. Overall, the most frequent side effects were infections, nausea/vomiting, diarrhea, stomatitis, and bleeding. In patients younger than 60 years stomatitis was significantly more often recorded in the high-dose group (47 vs. 28%; p=0.02) but there were no differences in severe stomatitis according to WHO grade III/IV (12 vs. 9%). Severe infections, nausea/vomiting, and diarrhea (WHO grade III/IV) occurred slightly more frequent in the high-dose group. Only patients having received 3 g/m² AraC had severe disturbances of consciousness (10 vs. 0%; p=0.01). The incidences of bleeding as well as of the remaining side effects were the same for boths groups (Table 3). In older patients there were no significant differences in the side effects with the profile being similar to that observed in younger patients (Table 4).

Discussion

Addressing the still pending question of the optimal dose of AraC in patients with AML the current randomized study aimed at analysing possible benefits for patients with refractory and relapsed AML receiving high-dose AraC at a dose level of 3 g/m^2 rather than 1 g/m^2. Evidence for 1 g/m^2 of AraC being sufficient for obtaining the maximum antileukemic efficacy was based on insights into the AraC pharmacology revealing a saturation of the intracellular AraCTP formation at this dose level [12, 13]. Furthermore, there is a good correlation between the dose of AraC and the resulting toxicity [23, 24] also favoring the administration of intermediate-dose AraC. However, intermediate-dose AraC has never been directly compared to high-dose AraC leaving open the question of a possible overall benefit of higher doses of AraC due to a better antileukemic efficacy outweighing the increased toxicity. Therefore, the German AML Cooperative Group initiated the current study evaluating the antileukemic activity and the toxicity of high-dose versus intermediate dose AraC in patients with relapsed and refractory AML.

There were no major differences between the respective study groups in patients younger than 60 years (3 vs. 1 g/m^2) and in older patients (1 vs. 0.5 g/m^2) with regard to age, AML-subgroups, disease status, and prior therapy with high-dose AraC (Table 1). Overall, complete and partial remissions were obtained in 47 and 5% of the patients while 21% had persisting leukemia and 26% suffered from early deaths. These results confirm the high antileukemic efficacy of the S-HAM regimen as observed during a previous phase II study [16]. The analysis of the results obtained at the two different dose levels in patients younger than 60 years reveals a higher antileukemic efficacy of high-dose AraC as compared to intermediate-dose AraC with more patients achieving a complete remission (52 vs. 45%) and significantly less patients having persisting leukemia (12 vs. 31%; p=0.008). However, as could have been anticipated more patients succumbed to severe toxicity and died during neutropenia (32 vs. 17%) with infection related deaths occuring in 27 vs. 11% (p=0.01).

Considering possible differences in the superiority of high-dose over intermediate-dose AraC with regard to the patients' disease status, 3 g/m^2 AraC is confirmed to serve a significantly higher antileukemic potential in high-risk patients comprising those with refractory disease and relapses after a first complete remission of less than 6 months. Within this group of patients carrying a high risk of treatment failure [18] a higher rate of complete remissions was achieved (42 vs. 33%; p=0.12) and persisting leukemia occured significantly less frequent (16 vs. 47%; p=0.02) in patients receiving high-dose AraC. However, as observed for the whole study group, there were more early deaths (35 vs. 13%; p=0.13). In contrast, intermediate-dose AraC seems to be less toxic and equal effective as compared to high-dose AraC in other patients.

Major differences in the side effects were observed for stomatitis and severe (WHO III°/IV°) deterioration of consciousness which occurred significantly more frequent in the high-dose arm. Interestingly, the higher rate of early deaths and infection related deaths in the high-dose group was not due to a longer duration of critical neutropenia (41 vs. 39 days). Possibly, these differences are rather based on a higher incidence of epithelial damage in the gut as observed earlier [25].

While there are numerous studies comparing high-dose to conventional dose AraC in the treatment of patients with AML, unfortunately, no further trials have been performed yet analysing the antileukemic efficacy of high-dose AraC as compared to intermediate dose AraC. However, many single arm studies have been published reporting on either dose of AraC solely – in most case in combination with anthracyclines – with the resulting response rates in patients with refractory and relapsed disease essentially reflecting the observations of the present study (Table 5). Complete remission rates ranged from 34 to 58% vs. 30 to 66% for high-dose versus intermediate-dose AraC and persisting leukemia was diagnosed in 13 to 39% vs. 17 to 55% while early deaths occured in 7% to 33 vs. 9% to 17% [5, 26-41]. More importantly, the only trial of high-dose

Table 5. Trials of high-dose and intermediate-dose AraC in patients with refractory and relapsed AML

Reference	Regimen	Total					Refractory/CR1 < 6 months			
		n	CR	PR	PL	ED	CR	PR	PL	ED
Visani 94	AraC 2000; Flud; G-CSF	28	58		39	7				
Vogler 94	AraC 3000; VP16	44	42				28			
	AraC 3000	47	34				34			
Estey 93	AraC 2000/3000	19	47							
Martiat 90	AraC 3000; Mitox / AMSA	52	50				44			
Walters 88	AraC 3000; Mitox	44	36		37	27				
Hiddemann 87	AraC 3000; Mitox	40	53	3	13	33	43		17	35
Herzig 85	AraC 3000; Doxo / DNR	78	53							
Range	AraC 2000-3000		34-58	-	13-39	7-33	28-44	–	–	–
Amadori 96	AraC 500; VP16; Carbo	18	50			10				
Archimbaud 95	AraC 500; Mitox; VP16	133	60		29	11	44		46	10
Archimbaud 93	AraC 500; Mitox; VP16; GM-CSF	20	30		55	15	24		59	18
Carella 93	AraC 600; Ida; VP16	97	43		45	11	29		59	12
Estey 93	AraC 1000	40	30							
Spadea 93	AraC 1000; Mitox; VP16	74	55		35	10	42		45	12
Dekker 90	AraC 500; AMSA	32	44	9	38	9	25	5	60	10
Harousseau 90	AraC 1000; Mitox	30	57		27	17	50			
Paciucci 90	AraC 500; Mitox	44	31	9	49	11				
Amadori 89	AraC 1000; Mitox	47	66	2	17	15	31		38	31
Harousseau 89	AraC 1000; Ida	35	60	12	17	12				
Range	AraC 500-1000		30-66	2-12	17-55	9-17	24-50	–	38-60	10-31

AraC providing data on causes of treatment failure in high-risk patients with refractory disease or relapses after a first complete remission of less than 6 months duration [5] reported on only 17% cases having persisting leukemia which is substantially less than the 38 to 60% observed during intermediate-dose AraC. Early deaths occurred in 35 vs. 10 to 31% in this group of patients.

As for the present study, the hematologic toxicities encountered during the above mentioned trials did not depend on the dose level of AraC (neutropenia 25 to 28 vs. 19 to 33 days). Mucositis and diarrhea were reported to occur slightly less frequent during intermediate-dose AraC while central nervous system toxicity was limited to patients receiving high-dose AraC.

In summary, the results of the present study strongly suggest a higher antileukemic efficacy of high-dose AraC as compared to intermediate-dose AraC in patients with refractory AML or with AML relapsing after a first complete remission duration of less than 6 months. The pharmacologically based attempt to limit the application of AraC to intermediate-dose levels can not be supported and raises the question of mechanisms of resistance beyond the metabolism of AraC [1]. These may be limited to cases with advanced disease but possibly affect patients during first line therapy questioning the reduction of the dose of AraC solely to diminish the rates of early deaths as reported earlier [11]. Instead, to take full advantage of the potential of high-dose AraC the optimization of supportive care should be addressed in further trials aiming at a reduction of infectious complications by hematopoetic growth factors and developing antimicrobial strategies of effective porphylaxis and early intensive intervention upon the occurrence of infections.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Granulocyte Colony-Stimulating Factor Improves the Antileukemic Efficacy of the Sequential High-Dose Cytosine Arabinoside and Mitoxantrone Regimen (S-HAM) in Patients Younger than 60 Years with High-Risk Acute Myeloid Leukemia

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Abstract. Patients receiving intensive chemotherapy for advanced acute myeloid leukemia carry a high risk of treatment failure due to infectious complications and early relapses. In this regard, granulocyte colony-stimulating factor (G-CSF) provides an option for optimization of supportive therapy. In a prospective study, 68 evaluable patients with relapsed and refractory acute myeloid leukemia received G-CSF 5 µg/kg/day subcutaneously starting two days after the completion of treatment with the S-HAM regimen consisting of high-dose cytonsine arabinoside twice daily on days 1, 2, 8, and 9 and mitoxantrone on days 3, 4, 10, and 11. Ninety-one evaluable patients receiving S-HAM for refractory and relapsed acute myeloid leukemia without G-CSF support during a preceeding study served as control. The application of the growth factor resulted in a trend towards a lower early death rate (21 vs. 30%). More importantly, a marginally significant increase of the CR rate was achieved (56 vs. 47%, p=0.11). Partial remission occurred in 0% and 7% while persisting leukemias were documented in 24 and 16%, respectively. Probably, these differences are based at least in part on a significant reduction of the duration of critical neutropenia of less than 500/µl (36 vs. 40 days, p=0.008). This difference remained significant for the analyses of all subgroups as defined by refractoriness to induction therapy and duration of preceeding remissions. Only within patients older than 60 years the reduction of the duration of neutropenia was not significant. Further underlining the beneficial effect of G-CSF application, patients younger than 60 years receiving the growth factor had a significant improvement of time to treament failure (159 vs. 93 days, p=0.038) as well as of duration of disease free survival (203 vs. 97 days, p=0.003). These results indicate an improvement of the antileukemic efficacy of the S-HAM regimen by the administration of G-CSF.

Introduction

Patients receiving intensive chemotherapy for refractory and relapsed acute myeloid leukemia (AML) carry a high risk of treatment failure mainly due to severe infectious complications leading to early deaths rates of 15 to 30%. Furthermore, the duration of a second complete remission usually amounts to only a few months unless a bone marrow transplantation is performed [1-3]. Strategies to improve the outcome of patients with

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AML include antimicrobial prophylaxis and early antimicrobial intervention to lower the early mortality [4-7] as well as the intensification of the dose of cytosine arabinoside (AraC) which provides an increased antileukemic activity [8-10]. Hematopoietic growth factors may be used to lower the chemotherapy related toxicity and to further improve the efficacy of antileukemic regimens. In patients with solid tumors and lymphoid malignancies they have been shown to reduce the duration and the severity of neutropenia as well as the incidence of infectious complications associated with neutropenia [11]. As a result, the duration of the hospitalization for the treatment of these diseases could be reduced. However, the use of cytokines in patients receiving chemotherapy for AML remains controversial [12-16]. In these patients, in addition to the analyses of the above mentioned criteria, the evaluation of the application of growth factors also has to take into account a possible stimulation of leukemic regrowth on the one hand but – on the other hand – offers the opportunity to pursue strategies to extend the antileukemic efficacy of chemotherapeutic regimens. Laboratory evidence for a potential of G-CSF to recruit quiescent leukemic cells into the cell cycle provides the basis for cytokine priming in order to enhance the susceptibility of the cells to chemotherpy [12] and preliminary clinical data indicate the possibility of an improvement of the overall outcome of patients with AML by the use of G-CSF as supportive therapy [17-21]. To further define the role of G-CSF in the treatment of patients with high-risk AML, the German AML Cooperative Group evaluated the potential of its administration following intensive chemotherapy with the S-HAM regimen for refractory disease to reduce the treatment related toxicity and to improve the antileukemic efficacy.

Materials and Methods

Patients and Antileukemic Therapy

Consecutive patients with relapsed and refractory acute myeloid leukemias who were admitted at the participating centers were eligible for the study. The diagnosis of AML was based on the revised French-American-British (FAB) Group criteria [22]. Refractoriness against standard chemotherapy was defined according to previously established criteria [23]: These included

a) primary resistance against two cycles of induction therapy;
b) first early relapse with a remission duration of less than 6 months;
c) second and subsequent relapse. Patients with first relapses after six months remission duration were not considered refractory to standard therapy and were included as relapsed AML.

All patients were recruited from the first line trials of the German AML Cooperative Group and had thus received a standardized first line treatment. In patients less than 60 years of age first line therapy consisted in double induction therapy with either the repetitive application of the 9 day regimen of thioguanine, cytosine arabinoside, daunorubicin (TAD-9/TAD-9) or the sequential application of TAD-9 followed by high-dose cytosine arabinoside and mitoxantrone (HAM). Older patients all received one course of TAD-9 and were treated by a second TAD-9 course only upon inadequate response to the first TAD-9 cycle. Patients of all ages who achieved a complete remission subsequently received TAD-9 for consolidation and monthly maintenance therapy for 3 years [24, 25].

Patients with antecedent hematologic disorders, secondary leukemias, and a preceding autologous or allogeneic bone marrow transplantation were excluded from the study. Further exclusion criteria comprized coronary heart disease; heart failure; cardiomyopathy; severe arterial hypertension; abnormal liver function tests [aspartate aminotransferase (AST), alanine aminotransferase (ALT), or alkaline phosphatase (AP) more than three times the upper normal limits; total bilirubin > 2.0 mg/dl]; impaired renal function (serum creatinine >2.0 mg/dl); severe infections; or pregnancy.

Patients meeting the entry criteria were enrolled into the current study and were treated by S-HAM [3] comprising high-dose cytosine arabinoside (AraC) 3 g/m^2 or 1

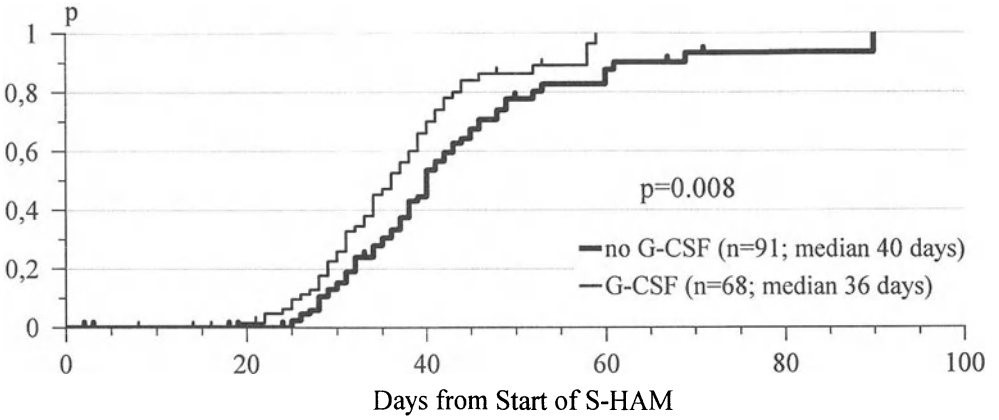

Fig. 1. Recovery of leukocytes to >1000/µl

g/m^2 every 12 h by a 3-h infusion on days 1, 2, 8, and 9 and mitoxantrone 10mg/m^2/day as a 30-min infusion on days 3, 4, 10, and 11, respectively (Fig. 1). The dose of AraC was adjusted to the disease status according to the results of a preceding trial comparing 3 g/m^2 vs. 1 g/m^2 AraC in patients with refractory AML [9]. Patients with refractory disease and younger than 60 years of age received 3 g/m^2 AraC while all other cases were treated with 1 g/m^2 AraC.

To prevent high-dose AraC induced photophobia and conjunctivitis all patients received glucocorticoid eye drops every 6 h starting before the first dose and continuing for 24 h after the last dose of high-dose AraC. Antimicrobial prophylaxis consisted of cotrimoxazol 960 mg po three times daily, colistine sulphate two million units po four times daily, and amphotericin B suspension 40 mg po six times daily.

Study Design

All patients received G-CSF 5 µg/kg/day subcutaneously starting on day 12 after the beginning of therapy. G-CSF was discontinued if a bone marrow examination on day 18, i.e., 1 week after completion of S-HAM, revealed more than 5% residual leukemic blasts. In patients with adequate blast cell clearance at day 18 G-CSF was continued until the neutrophil count reached a value of more than 1500/mm^3 for 3 consecutive days.

Ninety-one patients having received chemotherapy according to the S-HAM protocol during a previous trial in which no G-CSF support was applied served as historical control. For these patients the dose level of AraC and the first line therapy as well as the exclusion criteria were identical to those outlined above.

Study Parameters

Response to therapy was assessed according to CALGB criteria [26]. Complete remission status was defined as a normal cellular marrow with normal erythroid and myeloid elements and with myeloblasts, promyelocytes, and other leukemic cells totaling less than 5%, and with normal peripheral blood platelet and white blood cell counts for at least four weeks. Patients having more than 5% myeloblasts but fewer than 25%, with otherwise normal bone marrow, were considered to be in partial remission, as were patients fulfilling criteria of complete remission except for full recovery of peripheral blood platelet and/or white blood cell counts. Patients with persisting leukemic blasts in the bone marrow or blood or with leukemic regrowth within four weeks after initial response were considered as non-responders. Patients dying within 6 weeks after completion of antileukemic therapy without evi-

dence of leukemic regrowth were classified as early deaths.

The duration of critical cytopenia was evaluated by the time for leukocyte recovery to more than 1000/μl from the onset of S-HAM treatment. The time to complete remission was measured from the onset of treatment to the date of documented complete remission and disease free survival from the date of documented complete remission to relapse or death during remission. Survival and time to treatment failure were measured by the time from the beginning of treatment to death and death without evidence of leukemia, documentation of persisting leukemia, or relapse, respectively.

Toxicity was evaluated according to the World Health Organization (WHO) grading system [27].

Statistics

The aim of the current study was to assess the impact of the application of G-CSF on the early death rate during S-HAM therapy as compared to a historical control receiving S-HAM alone. Furthermore, comparisons of the rates of complete remission and of the disease free survival were planned to analyse a possible improvement of the antileukemic efficacy of S-HAM. Numerical values were compared by the χ^2-test, by the Fisher's-exact-test, and by the student's t-test. Remission duration and survival was calculated according to Kaplan Meier estimates. Comparisons were carried out using the log-rank test.

Study Conduct

Prior to therapy all patients gave their informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study as well as of potential risks. The study design adheres to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.

Results

Patient Characteristics

Sixty-eight patients were fully evaluable and were compared to 91 historical controls. Both groups were similar with regard to sex, age, white blood count, FAB subgroup, and disease status (Table 1).

Table 1. Patient characteristics

	No G-CSF n=91	G-CSF n=68
Sex (male/female)	49/43	37/31
Age (years, median/range)	50/17-75	47.5/17-73
WBC (median/range)	3400/600-212000	3950/400-82800
FAB subgroup		
M0	–	6 (9%)
M1	21 (23%)	5 (7%)
M2	24 (26%)	20 (29%)
M3	5 (5%)	1 (1%)
M4	19 (21%)	8 (12%)
M5	14 (15%)	8 (12%)
M6	1 (1%)	3 (4%)
M7	1 (1%)	1 (1%)
n.d.	6 (7%)	16 (24%)
Disease status		
refractory	15 (16%)	5 (7%)
CR1 duration <6 months	23 (25%)	17 (25%)
CR1 duration ≥6<18 months	33 (36%)	31 (46%)
CR1 duration ≥18 months	14 (15%)	14 (21%)
≥2nd relapse	6 (7%)	1 (1%)

Non-hematologic Side Effects

There were no differences between the two groups with regard to non-hematologic side effects (Table 2).

Table 2. Non-hematologic side effects

Toxicity	No G-CSF (n=91) I°/II°	No G-CSF (n=91) III°/IV°	G-CSF (n=68) I°/II°	G-CSF (n=68) III°/IV°
Nausea	44 (48%)	17 (19%)	15 (22%)	21 (31%)
Diarrhea	22 (24%)	14 (15%)	13 (19%)	11 (16%)
Mucositis	27 (30%)	8 (9%)	23 (34%)	10 (15%)
Bleeding	25 (27%)	15 (16%)	13 (19%)	2 (3%)
CNS	4 (4%)	6 (7%)	1 (1%)	2 (3%)
Bilirubin	31 (34%)	4 (4%)	14 (21%)	4 (6%)
AST/ALT	22 (24%)	6 (7%)	8 (12%)	3 (4%)
AP	28 (31%)	1 (1%)	9 (13%)	–
Creatinine	14 (15%)	4 (4%)	8 (12%)	–

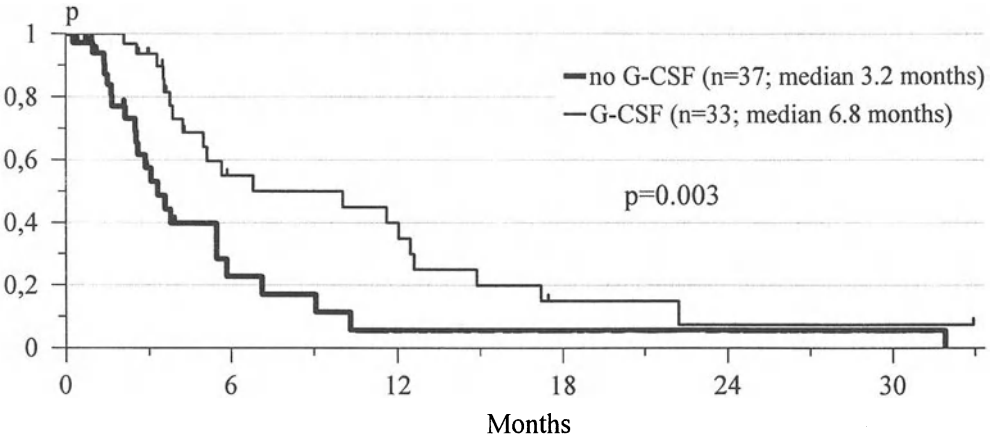

Fig. 2. Disease-free survival, patients younger than 60 years in complete remission

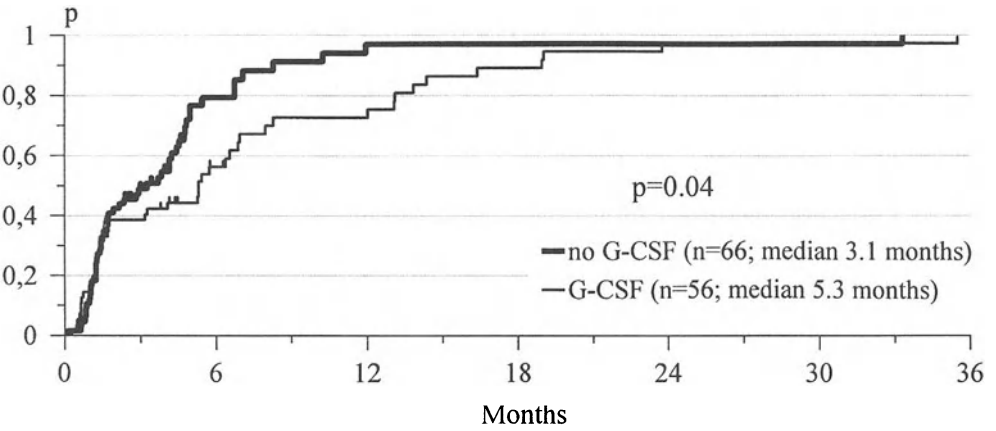

Fig. 3. Time to treatment failure, patients younger than 60 years

Hematologic Toxicity

The time of critical neutropenia was reduced significantly by the application of the growth factor (36 vs. 40 days for all patients, p=0.008, Fig. 1). The time to recovery of both neutrophiles to more than 1500/µl and thrombocytes to more than 100 000/µl, i.e. the time to complete remission, was similar for both groups (52 vs. 51 days).

Antileukemic Efficacy

The application of G-CSF resulted in a reduction of early deaths (21 vs. 30%) which was paralleled by an increase in the rate of complete remissions (56 vs. 47%). However, these differences did not reach statistical significance (Table 3).

A comparison of the remission qualities revealed a significant extension of the disease free survival in patients with G-CSF

Table 3. Antileukemic efficacy of S-HAM

n	No G-CSF (n=91)	G-CSF (n=68)
CR[a]	43 (47%)	38 (56%)
PR	6 (7%)	–
PL	15 (16%)	16 (24%)
ED[b]	27 (30%)	14 (21%)

[a] p=0.11. [b] p=0.13.

(median 5.6 vs. 3.6 months; p=0.046). This difference was more pronounced in patients younger than 60 years (median 6.8 vs. 3.2 months; p=0.003; Fig. 2) and was not observed in older patients.

Also, the time to treatment failure was prolonged in the G-CSF group (median 4.6 vs. 3.0 months; p=0.20) with the difference being statistically significant for younger patients (median 5.3 vs. 3.1 months; p=0.04; Fig. 3). The differences in the disease free survival and in the time to treatment failure were not based on differences in the time to complete remission which was similar in both groups (52 vs. 51 days for all patients, p=0.44; 52 vs. 52 days for patients younger than 60 years, p=0.54). In patients younger than 60 years the overall survival was slightly longer for those receiving G-CSF (median 7.8 vs. 5.3 months; p=0.27).

Discussion

The German AML Cooperative Group performed the current study with the aim to reduce the treatment related toxicity and to improve the antileukemic efficacy in patients receiving intensive S-HAM chemotherapy for high-risk AML. In these patients, in which the duration of critical neutropenia amounts to more than five weeks, severe infectious complications are frequently encountered and cause a high early death rate of more than 25%. Furthermore, within a substantial proportion of these patients treatment failure is due to the persistance of leukemia and early second relapses.

Besides the now well described potential of G-CSF to significantly reduce the duration of chemotherapy induced neutropenia the analyses of the current trial also revealed a benefit of the application of the growth factor with regard to the early mortality in that the early death rate could be reduced from 30 to 21%. Due to the relatively small number of patients this difference did not reach statistical significance (p=0.13). This reduction in early mortality translated into an overall improvement of the antileukemic efficacy of S-HAM with a strong trend towards a higher CR rate in the group receiving G-CSF (56 vs. 47%; p=0.11). Furthermore, in younger patients a better remission quality was observed for those within the G-CSF group in which the median disease free survival could be doubled (6.8 vs. 3.2 months; p=0.003).

Whereas the use of GM-CSF in patients with AML has been extensively studied, there is only a limited number of trials published so far analysing the effects of the application of G-CSF following chemotherapy in patients with AML. The detailed results of randomized studies in homogenous populations of patients are available in one case only, in which elderly patients with newly diagnosed AML received the growth factor after completion of induction chemotherapy with daunorubicin and standard-dose AraC [28]. A significant increase of the CR rate was observed in the G-CSF group (70 vs. 47%; p=0.002) which was more pronounced in patients with residual bone marrow blasts after chemotherapy (67 vs. 35%; p=0.01). Stressing the question of an antlileukemic role of the growth factor in these patients, there was also a statistically significant decrease in the number of patients having persisting leukemia (15 vs. 33%; p=0.007). However, the application of G-CSF had no influence on the remission quality in this study with both groups not differing in remission duration and event-free survival, respectively. Two further randomized trials reported yet were carried out by a Japanese group applying a combination chemotherapy with mitoxantrone, etoposide, and standard-dose behenoyl cytosine arabinoside. In the first study a heterogenous group of patients with relapsed and refractory acute myeloid leukemia, acute lymphoblastic leukemia, blastic crisis of chronic myelogenous leukemia, and myelodysplasia were treated with G-CSF after completion of the chemotherapy [29]. As in the current study, for patients with AML a trend towards more complete remissions (57 vs. 39%; p=0.16) as well as an improved remission duration (p=0.08) was observed in the G-CSF group. In the second trial the application of G-CSF was begun 2 days before the start of chemotherapy in patients with AML and MDS [30]. Only slightly more complete remissions were achieved in the G-CSF group (54 vs. 42%) while there were no differences in early death, event-free

survival, and disease-free survival. However, due to the small number of patients with AML (n=50) the results of this trial – as far as concerning the antileukemic efficacy of the treatment regimen and the overall outcome – are of limited significance.

Three non-randomized trials have been published so far evaluating the use of G-CSF in patients with AML in comparison with historical controls. The first study comprised 112 patients with newly diagnosed AML and MDS undergoing chemotherapy with fludarabine and intermediate-dose AraC [31]. The application of G-CSF was begun one day before the start of chemotherapy. Among the 69 patients with AML 65% achieved a complete remission as compared with 52% controls (p=0.09). The median survival was similar for both groups (9.0 vs. 6.7 months; p=0.24). In the second trial, 28 patients older than 60 years with de novo or secondary AML received idarubicine and standard-dose AraC induction therapy and consoldation with mitoxantrone, etoposide, and standard-dose AraC, each followed by G-CSF [32]. As compared with a historical control having received similar induction and consolidation therapies without growth factors a higher CR rate was achieved (58 vs. 46%; p=0.04) and the early death rate could be reduced (8 vs. 32%; p=0.04). There were no differences in disease-free survival and overall survival. The third study analysed the effects of G-CSF in a cohort of patients with AML in first CR receiving an intensive triple consolidation therapy [33]. Of 123 patients being treated with the third consolidation course comprizing mitoxantrone and diaziquone the first group of 62 patients was treated without growth factors and, due to the encountered substantial myelosuppression, the second group received G-CSF support following chemotherapy. The overall outcome was not influenced by the application of G-CSF with both groups not differing in remission duration (1.2 vs. 1.4 years; p=0.54) or survival (2.4 vs. 3.4 years; p=0.99).

As in the current trial, none of the published studies on the use of G-CSF in patients with AML reported an inferior overall outcome or a stimulation of leukemic regrowth in patients receiving the growth factor, confirming initial observations made by the Japanese group [34]. These studies rather are in favour of the use of G-CSF with regard to the antileukemic efficacy of the chemotherapeutic regimens applied and support the findings of the current study of an increased CR rate and a prolonged disease-free survival. However, the analyses presented here are the first ones reporting on the use of G-CSF following treatment with high-dose AraC, possibly explaining the more obvious improvement of the disease-free survival as compared to previous trials. As reported earlier, this more intensive regimen, with a more profound suppression of the bone marrow, may demonstrate a greater potential for a beneficial result from cytokine use [15]. Furthermore, there is laboratory [35-39] and clinical data [17-21] on an antileukemic potential of G-CSF warranting its further evaluation in patients with AML.

The results of the present study support the use of G-CSF following treatment with S-HAM in patients with advanced AML in order to reduce the chemoptherapy-induced toxicity and to possibly improve the antileukemic efficacy. However, besides two recently reported studies which will be published in detail shortly [40, 41], further analyses have to be performed in a randomized prospective manner to confirm the improvement of the antileukemic efficacy of high-dose AraC by the application of G-CSF.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Therapy of Advanced MDS, AML Evolving from MDS, or Secondary AML with Idarubicin Ara-C, VP-16, Followed by G-CSF-Priming Exhibits High Remission Rate

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Abstract. In a previous study for patients with advanced MDS, AML after MDS, and secondary AML, a remission rate about 45% was obtained after intensified chemotherapy. In order to increase the remission rate, a prospective trial with three cycles of idarubicin 10 mg/m^2 x 3d (2d in cycle 2 + 3), continuous infusion of ara-C 100 mg/m^2 x 7d (5d in cycle 2 + 3), etoposide 100 mg/m^2 x 5d (cycle 1-3), followed by amsacrine 60 mg/m^2 x 5d and ara-C 100 mg/m^2 x 5d in cycle 4, with G-CSF priming in the first two cycles was initiated. From February 1994 until October 1996, a total of 67 patients (34 male, 33 female) with RAEB-t (n = 11), secondary AML (n = 8), or AML following MDS (n = 48) were entered. 44 patients (age 33-75 years, median 58 years; 9 RAEB-t, 32 AML following MDS, 3 secondary AML) have finished induction therapy. After the first course of chemotherapy, 27 patients had a complete remission (CR 61.4%), 7 patients showed a partial remission (PR 15.9%), and 10 patients were non-responders (NR 22.7%). After cycle 2, 4 additional patients entered CR resulting in an overall CR rate of 70.5%. Overall, 4 patients died from early death, 4 from progression or relapse. Seven patients underwent stem cell transplantation. In course I, neutropenia (< 500/µl) lasted 15 (range 1-40) days, while the median number of days with febrile neutropenia was 13 (range 1-34). Thrombocytopenia (< 20 000/µl) lasted for a median of 9 (range 8-58) days. In conclusion, the intensive chemotherapy with idarubicin/ara-C/VP-16 combined with G-CSF priming results in a promising rate of remissions and is well tolerated.

Introduction

Ten percent to 40% of the patients with myelodysplastic syndromes (MDS) develop acute leukemia, and approximately 20 to 40% will die from infection and/or bleeding [1-8]. The use of multi-agent chemotherapy has generally been less effective for patients with MDS in transformation to acute myeloid leukemia (AML), or with AML that has evolved from MDS, or has occurred after previous cytotoxic chemotherapy than for patients with de novo AML [9-11]. Treatment-related deaths, especially from infections during prolonged neutropenia, are more frequent and complete remission (CR) rates are less frequent and shorter than in patients with primary AML [12].

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Additionally, recent randomized trials in elderly patients with high-risk AML have demonstrated that the rate of infection during induction therapy and partially of infection-related deaths during induction therapy can be reduced by using G-CSF [13, 14] or glycosilated GM-CSF [15, 16]. This use of growth factors could improve the complete remission rate [13, 15].

In a previous study, intensive chemotherapy with idarubicin, cytarabine (Ara-C), and etoposide (VP-16) induced CR in 45% of the patients with advanced MDS and high risk AML [17]. In an attempt to further ameliorate these results, the treatment was modified by application of G-CSF priming during the first two cycles of chemotherapy. The rationale was that G-CSF applicated during and after induction chemotherapy may increase leukemic cell kill and reduce the risk of infection and of early death.

Patients/Treatment Protocol

The chemotherapy regimen is outlined in Table 1. During the first and second course, the patients received 5 μg/kg G-CSF sc. from day -1 when leukocytes were lower than 50 000/μl or from day 1 when leukocytes were higher than 5000/μl until neutrophil counts exceeded 1000/μl. During the courses 3 and 4, G-CSF could be given facultatively from the first day after the end of chemotherapy until neutrophil counts higher than 1000/μl (Table 1).

From February 1994 until October 1996, 67 patients, being 18 years or older, with a WHO performance status 1 or 2, with refractory anemia in transformation to acute leukemia (RAEB-t), AML evolving from MDS, or AML secondary to previous cytotoxic chemotherapy were entered into this study after informed consent. The patients characteristics are summarized in Table 2. The diagnosis was established by examination of blood and bone marrow aspirate. By the of October 1996, 44 patients had finished induction chemotherapy and were therefore included in the analysis.

Patients were excluded from the study when they had previous chemotherapy for MDS or AML or a cytokine treatment within the preceeding 4 weeks. Further exclusion criteria were a secondary uncontrolled malignancy, severe uncontrolled infections, refractoriness to platelet transfusions, and severe disorders of the heart, the lungs, the liver, the kidneys, and the central nervous system. Patients who are older than 60 years did not receive more than two cycles of chemotherapy.

Table 1. Chemotherapy and priming

Cycle 1:	Idarubicin 10 mg/m^2 i.v,	Days 3-5
	Cytarabin 100 mg/m^2 i.v.[a]	Days 1-7
	Etoposide 100 mg/m^2 i.v.	Days 3-7
	G-CSF 5 μg/kg/day s.c.	Day -1/l[b]
		Until ANC 1000/μl
	[a] Continuous infusion.	
	[b] Leukocyte count > 50 000/μl from day 1. Leukocyte count < 50 000/μl from day 1.	
Cycle 2:	Idarubicin 10 mg/m^2 i.v.	Days 1-2
	Cytarabin 100 mg/m^2 i.v.[a]	Days 1-5
	Etoposide 100 mg/m^2 i.v.	Days 1-5
	G-CSF 5 μg/kg/day s.c.	Day -1[b]
		Until ANC 1000/μl
	[a] Continuous infusion.	
	[b] Leukocyte count > 50 000/μl from day 1. Leukocyte count < 50 000/μl from day 1.	
Early consolidation:	Idarubicin 10 mg/m^2 i.v.	Days 1-2
	Cytarabin 100 mg/m^2 i.v.[a]	Days 1-5
	Etoposide 100 mg/m^2 i.v.	Days 1-5
	[a] Continuous infusion. (facultatively G-CSF 5 μg/kg/day s.c. day 7 until ANC 1000/μl).	
Late consolidation:	Amsacrine 60 mg/m^2 i.v.	Days 1-5
	Cytarabin 600 mg/m^2 i.v.[a]	Days 1-5
	[a] Twice per day (facultatively G-CSF 5 μg/kg/day s.c. day 6 until ANC 1000/μl).	

Table 2. Patients characteristics

No. of patients	67
Male/female	34/33
Age (years)	
Median	58
Range	28-75
Diagnoses	
AML after MDS	48
RAEB-t	11
Secondary AML	8

Table 3. Results

	Evaluable patients	< 60 years	> 60 years
No. of patients	44	23	21
Complete remission	31 (70.5%)	14 (61%)	17 (81%)
Failure	13 (29.5%)	9 (39%)	4 (19%)
Early death	4 (9%)	0 (0%)	4 (19%)

Results

By October 1996, 44 patients have finished two courses of induction therapy and are actually evaluable for response (Table 3). After the first course of chemotherapy, 27 patients had a complete remission (CR 61.4%), 7 patients showed a partial remission (PR 15.9%), and 10 patients were non-responders (NR 22.7%). After cycle 2, 4 additional patients entered CR resulting in an overall CR rate of 70.5% (31 patients), while 13 patients (29.5%) had a treatment failure. In the patients older than 60 years 81% obtained a CR. Overall four patients, all older than 60 years, died from early death (9%). Seven patients underwent stem cell transplantation.

The median duration of neutropenia (< 500/μl) lasted 15 days (range 1-40 days) and 10 days (range 4-18 days) for induction 1 and 2, respectively. G-CSF was given for a median of 20 days (range 11-70 days) and a median of 20 days (range 9-47 days) for the two induction courses, respectively. After the first chemotherapy the median of low platelet count (< 20 000/μl) lasted 9 days (range 8-58 days), while a median of 9 platelet concentrates (range 3-33) were transfused.

Discussion

The trial presented here was prompted by a report that the use of G-CSF for acceleration of neutrophil recovery will reduce the rate of severe infections and early death [14]. It was suggested that G-CSF priming might improve the response ratres. The interim results in fourty-four patients illustrate that the combination of idarubicin, Ara-C, and VP-16 is highly effective for remission induction being in the upper range with regard to the rate of complete remission (70.5%). The median duration of continuous complete remission or relapse free survival is not yet evaluable.

In previous studies of aggressive chemotherapy in a comparable patient population, the rate of early death usually ranged between 20 and 45% [9, 17]. Due to this high rate of death during induction therapy, many centers hesitate to treat these patients aggressively, but rather give low-dose chemotherapy or supportive care only. Our former study with identical induction chemotherapy, but without G-CSF priming, exhibited a CR rate of 45%. In comparison, the good response rate and the decreased numbers of early death in our patients in the actual trial which were not higher than in younger patients treated for de novo AML, might be due to the use of G-CSF after each chemotherapy cycle which by accelerating neutrophil recovery would prevent or ameliorate the course of infections, as previously shown by other authors [14]. Thus, the low rate of early death makes our protocol a suitable treatment regimen for the mainly elderly patients with high-risk AML.

In conclusion, the early data of this treatment schedule suggest that improved treatment results can be obtained in patients with advanced MDS and high-risk AML. Further follow-up data are needed to evaluate the remission duration and the overall survival.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Immunomodulation and Therapeutic Effects of a Monotherapy with Interleukin-2 as an Induction Therapy in Patients with Poor-Prognosis Acute Myeloid Leukemia

D. L. Voliotis, S. Schmitz, P. Staib, H. Tesch, and V. Diehl

Abstract. IL-2 has been shown to induce several mechanisms of MHC-restricted and MHC-unrestricted cytotoxicity against leukemic blasts in vitro and in vivo. Also it is known that patients with AML have a reduced or defective NK-cell function. Based on these findings we designed a phase I/II trial with intermediate to high-dose IL-2 to study the feasibility, immunomodulation and therapeutic efficacy of a continuous escalating IL-2 regimen in AML patients with partial remission or refractory or relapsed disease who were not suitable for further chemotherapy. Patients were planned to receive 4 cycles of recombinant IL-2 (Proleukin, Chiron GmbH) from d1-d5 with escalating doses from 4.5 x 10^6 IU/m^2 to 18 x 10^6 IU/m^2. Therapy was given civ with a resting period of at least 48 hours between each cycle. As of March 1997 five patients were treated. Highest dose level was applicable in 2/5 patients. In 3/5 patients the continuation of therapy required dose reduction after initial escalation. With this individual dose-modification the therapy was applicable in all 5 patients over 4 cycles with tolerable toxicity. Immunological monitoring (FACS) showed a marked lymphocytic stimulation (up to > 70% of PBMNC) with induction of CD3+/CD8+/TCR$\alpha\beta$+ cytotoxic T-cells up to 50% of all PBMNC. Lymphocyte stimulation was detected even with low-dose IL-2 (4.5 x 10^6 IU/m^2) and persisted after the end of IL-2 application (up to 2 weeks). In 3/5 patients a reduction of the leukemic blasts in the bone marrow was observed. One of these responders (1/5 study patients) with AML after myelodysplastic syndrome showed a blast remission < 5% in the BM. This patient was further treated with a maintenance low-dose IL-2 therapy s.c. on an ambulatory basis and showed a continuous increase of hemoglobin and platelet levels over 7 months requiring no more transfusions and being in a very good condition without showing any signs of leukemic activity.

Conclusions. Immunotherapy with IL-2 in patients with refractory/relapsed AML is feasible and tolerable with an individual dose adaptation. A marked and sustained immunostimulation is induced. This seems to result in an antileukemic activity at least in a part of these patients. Further investigation is necessary to determine the possible clinical and immunological effects of IL-2 in AML. As the trial is ongoing, data presented here show an interim analysis.

Introduction

Interleukin-2 and Antitumoral Activity

Interleukin-2 (IL-2) is produced by T-helpers cells after macrophage activation by

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IL-1 and antigen stimulation. IL-2 effects result in an upregulation of MHC restricted cellular cytotoxicity by T-helper cells, cytotoxic and suppressor T-lymphocytes as well as in induction of MHC not-restricted cytotoxicity by Natural Killer (NK) cells, Lymphokine Activated Killer (LAK) cells and cytotoxic macrophages. IL-2 does induce a cytokine cascade with increased production of Tumor Necrosis Factors, Interferons and other Interleukins [31, 34, 37, 45].

In vivo, IL-2 leads to activation of NK-cells, LAK-cells and T-cells. Serum levels of different cytokines are increased (Interleukins 3, -4, -5, -6, -8, Tumor Necrosis Factor alpha, -beta, Interferon-Gamma, GM-CSF). Changes in the cell mediated immune response (delayed type hypersensitivity reaction) are observed [31, 32, 35-37, 44, 45].

A direct cytotoxic effect on the tumor cells seems to be secondary. The antitumoral activity of IL-2 is the result of an indirect immunological effect with three different underlying mechanisms:

On the one hand activation of non-specific cellular cytotoxicity (i.e. not MHC-restricted) is induced. This effect is mediated by NK- and LAK cells.

At the opposite there is a clonal expansion of cytotoxic T-cells (in the majority CD8+ cells) which is strictly regulated by stimulation of the T-cell receptors. This means that there is also a specific (i.e. MHC restricted) cellular response mechanism induced.

The third effector mechanism which is not primarily related to cellular cytotoxicity is the activation of the B-cell system. IL-2 can induce direct B-cell proliferation and immunoglobulin-secretion but it can also result in indirect B-cell stimulation via T-cell secreted secondary cytokines [30-33, 36, 38-43, 45, 46].

Interleukin-2 in the Treatment of AML

In numerous preclinical investigations there is evidence for the mechanism of IL-2 efficacy in AML: IL-2 leads among others to an activation of NK and LAK cells. Both cell populations have an antileukemic effect in vivo and in vitro. It is known that patients with overt leukemias have a reduced or deficient NK-cell activity [25, 35, 51-54]. Unlike this, patients with complete hematological remission after standard cytotoxic chemotherapy a functional and effective NK- and LAK-cell function can be demonstrated. From the blood and the bone marrow of leukemia patients it is possible to generate LAK cells with activity against AML-blasts after stimulation with IL-2 [2-6, 9, 10, 14-16, 23-28]. Furthermore there is convincing evidence for a stimulation of the T-cell system with induction of MHC restricted cellular cytotoxicity against autologous and allogeneic leukemic blasts.

Proceeding on the assumption that there is an effectiveness at low blast cell counts (i.e. "minimal-" or "limited-disease-AML") IL-2 is also been used after autologous bone marrow transplantation (ABMT) or peripheral autologous stem cell transplantation [7-9, 12] as well as in the maintenance therapy after induction of complete hematological remission with chemotherapy [1, 29]. The effectiveness of IL-2 in ABMT seems to be among others the result of a biological process very similar to the graft-versus-leukemia effect (GVL) which is well known in the setting of allogeneic bone marrow transplantation, but without the occurrence of a graft-versus-host effect (GVH) [11, 15-22].

In the murine model there is a strong correlation between resistance to a challenge with leukemic blasts and a previously performed NK-cell transfer in the same animals. In other experiments a specific dissociation of the graft-versus-leukemia effect and the graft-versus-host effect is possible with application of IL-2 after bone marrow transplantation in mice [13, 15-17].

IL-2 is effective according to an immune modulation. Effectivity in terms of leukemic cell reduction seems to appear at low tumor load particularly. There are clues about the fact that the effect (complete or partial remission) as well as the remission duration of IL-2 therapy depend on the tumor load. Some studies seem to refer to a limit of about 20-30% leukemic cells in the bone marrow above which the probability for disease reduction diminishes [47-50].

Patients and Methods

Patients

As of March 1997 a total of five patients, all with advanced disease, have been enrolled in this study. Characteristics of these patients are shown in Table 1. Median age was 59 years (range 49 to 67). 2 patients were females, 3 patients were males. According to French-American-British (FAB) classification 1 patients was AML M2 and one was AML M4. The other 3 patients had secondary AML or AML after myelodysplastic syndrome. The mean percentage of blasts in the bone marrow was 56% (range 30 to 90%). Inclusion criteria were cytologically diagnosed AML confirmed by cytochemistry in bone marrow aspiration in the following patient groups:

1. second or following early relapse (< 1 year),
2. second or following incipient relapse with an amount of 5-30% blasts in the bone marrow,
3. incomplete (partial) remission (5-30% blasts in the bone marrow) following second-line induction therapy,
4. refractory AML after inductive therapy as well as after second-line chemotherapy,
5. early relapse (< 1 year) following second-line induction therapy,
6. secondary AML in partial remission, refractory disease after first-line chemotherapy and early relapse (< 1 year).

Further inclusion criteria were ineligibility for intensive chemotherapy, absence of central nervous system involvement, any major organ failure or any active infectious process and written informed consent.

Standard chemotherapy had been stopped for at least 4 weeks and steroid treatment for at least 1 week prior to starting IL-2.

All patients had relapse after previous induction regimens. During the course of their disease, all patients had received intensive chemotherapeutic regimens with high dose ARA-C.

Aim of the Study

The principal aim of the study was to study the feasibility of an immunotherapy with intermediate to high-dose single agent Interleukin-2 given as a continuous intravenous infusion in patients with second or subsequent relapse of AML, refractory AML and secondary or MDS-AML after first-line therapy. Secondary study criteria were immunomodulation and therapeutic efficacy.

Study Design and Treatment Plan

Open, prospective, monocentric, non-randomized phase I-study for treatment of AML patients with Interleukin-2.

The preparation used was human recombinant Interleukin-2 (Proleukin, Chiron GmbH). The treatment protocol provides an individual and limited dose escalation. For each patient, four therapy cycles are planned. One cycle consists of five days, the application modus is carried out as a continuous intravenous regimen. The fol-

Table 1. Patient characteristics

Patient	Age	Diagnosis	Status at IL-2	BM blasts before IL-2	Outcome with IL-2 treatment
#1 R. H.	49 y.	de novo AML	2nd relapse	80%	Progressive disease
#2 M. B.	58 y.	de novo AML	2nd relapse	90%	Progressive disease
#3 G. F.	59 y.	MDS-AML	1st relapse	30%	Remission (10 months)
#4 H. B.	67 y.	MDS-AML	1st relapse	32%	Blast reduction
#5 R. M.	62 y.	MDS-AML	1st relapse	50%	Stable disease

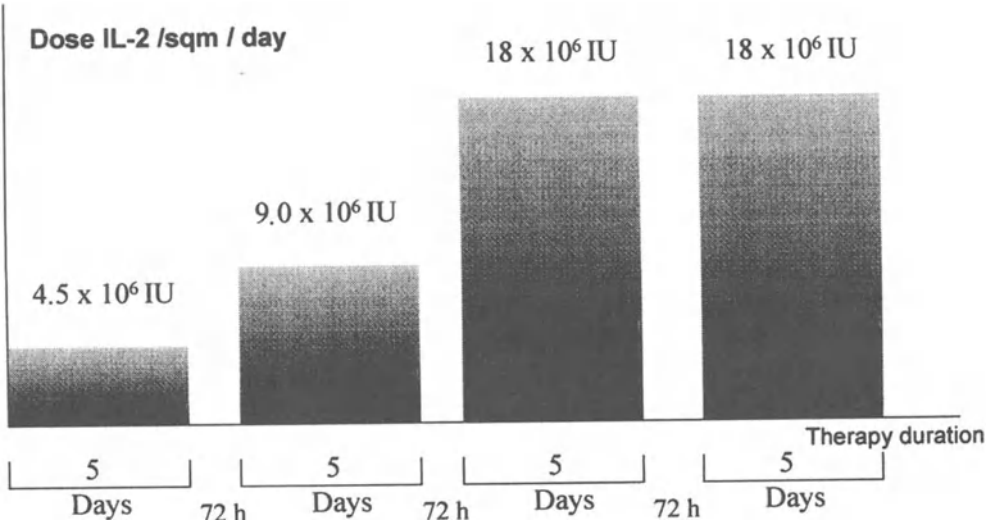

Fig. 1. Overview treatment schedule. Phase I study with IL-2 in acute myeloid leukemia

lowing dose escalation regimen is scheduled: 1st cycle: Day 01-05: 4.5 x 10^6 IU IL-2/m^2/24 h, 2nd cycle: Day 09-13: 9.0 x 10^6 IU IL-2/m^2/24 h, 3rd cycle: Day 17-21: 18.0 x 10^6 IU IL-2/m^2/24 h, 4th cycle: Day 25-29: 18.0 x 10^6 IU IL-2/m^2/24 h. After each cycle there is a resting period of 72 h until the start of the next cycle (Fig. 1). Patients who respond to IL-2 are scheduled to subcutaneous low-dose maintenance therapy with application of 3 Mill IU/day 3x/week on an outpatient basis.

Supportive Care and Monitoring

All patients were treated as inpatients in double rooms. All patients had double lumen central venous catheter and received prophylactic antipyretic therapy with acetaminophen 500 mg every 6 h. Standard selective oral antibiotic decontamination therapy was applied. Other antipyretic treatment was given only in febrile episodes > 38.5 °C, intravenous broad-spectrum antibiotics only in the presence of documented infection. Platelet transfusions were given with platelet count < 10 000/µl or fever > 38.5 °C or when bleeding complications occurred. Packed red blood cells (RBC) were transfused when hemoglobin was < 9.0 g/dl. Standard laboratory procedures including hematologic counts, liver and renal function tests, and electrolyte tests were performed daily. Bone marrow evaluations were performed in all patients before starting of IL-2 and after the fourth induction cycle.

Standard supportive care including furosemide for fluid retention, human albumin and colloid infusions for hypotension were used as needed.

Toxicity was defined according to the World Health Organization (WHO) grading system. Doses of IL-2 were not increased when simultaneous toxicities ≥ grade 2 were present. If toxicity ≥ grade 3 occurred, treatment was paused and after values returned to baseline continued with 50% dose reduction. During treatment with IL-2, no other chemotherapy or immunotherapy was performed.

Immunomodulation

In order to evaluate the immunological effects of treatment with IL-2, phenotypic changes in lymphocyte subpopulations were determined by flow cytometry before treatment was started, during treatment and

after termination. Briefly, heparinized mononuclear peripheral blood and bone marrow cells were obtained freshly after density gradient centrifugation (Ficoll, Sigma Chem.). Cells were selected from interphase, washed twice in phosphate-buffered saline, resuspended in PBS and double stained the same day with a panel of conjugated monoclonal antibodies against the following surface antigens: CD3, CD19, CD16/CD56, CD25, CD4, CD8, T-cell-receptors α/β, T-cell receptors γ/δ, CD45, CD14, CD45RO, CD45RA (all antibodies from Becton Dickinson, San José, CA). Measurement was performed on FACScan (Becton Dickinson).

Response Evaluation

Response to the treatment with IL-2 was evaluated with bone marrow aspiration biopsy after the last cycle. CALGB criteria for definition of hematologic response were used.

Results

Toxicity

Fever was observed in all patients. Other well-known IL-2-related side effects (i.e., flu-like symptoms, fluid retention, peripheral edema, metabolic toxicities) were observed in 4 out of 5 patients. In 1 patient, fever and general symptoms occurred only at the highest dose level. In 3 patients hypotension and tachycardia was observed. In 1 of 5 patients temporarily infusion with low-dose dopamine was necessary. One patient acquired pneumonia during therapy documented by chest X-ray, after which IL-2 treatment was paused and continued at the same dose level only after pulmonary infiltrate disappeared completely with antibacterial antibiosis and patient was without fever for 3 days. Three of 5 patients had a cutaneous rash resembling cutaneous graft-versus-host reaction. One of 5 patients showed furthermore pruritus requiring treatment with antihistaminic drugs. In 1 of 5 patients, treatment had to be stopped after application of 1.5 cycles because of patient's wish. Anemia requiring transfusion of blood (RBC) occurred in all patients and all patients required prophylactic platelet transfusion during the treatment, except petechiae without presentation of any serious bleeding complications. Despite these side-effects, no IL-2-related deaths occurred during the induction period. Maintenance therapy was performed in 2 patients without any side-effects except mild temperature ($< 38\,^{\circ}C$).

Clinical Results

By the end of induction cycles a blast remission to < 5% in the bone marrow was achieved in 1 of 5 patients with MDS-AML. This remission was not described as complete hematological remission because hemoglobin and platelet levels were not at normal values at the time of bone marrow aspiration biopsy. In this patient, low-dose subcutaneous maintenance therapy was initiated after dismission from hospital on an outpatient basis and the drug was self-administered. Under maintenance therapy, the levels for hemoglobin and platelets raised up to a maximum of 90 000/µl (platelets) and 12 g/dl (hemoglobin) requiring no transfusions of platelets and blood for a time period of 8 months up to December 1996. In the second patient, also with an MDS-AML, who showed a response to IL-2, a reduction of bone marrow blast proportion was observed from 32% (pre-treatment values) to 10% after the 2nd cycle. Unfortunately, this patient refused further continuation of treatment and subsequently developed disease progression. In the 3rd patient with MDS-AML, after application of four cycles of IL-2 as scheduled, a stable disease with no change of blast proportion in the bone marrow was observed. Two of the 5 patients, both with refractory/relapsed and rapid progressive de novo AML and a blast proportion of 80% and respectively 90% in the bone marrow showed a disease progression after application of full scheduled therapy and died of complications due to hematopoetic insufficiency (Table 1).

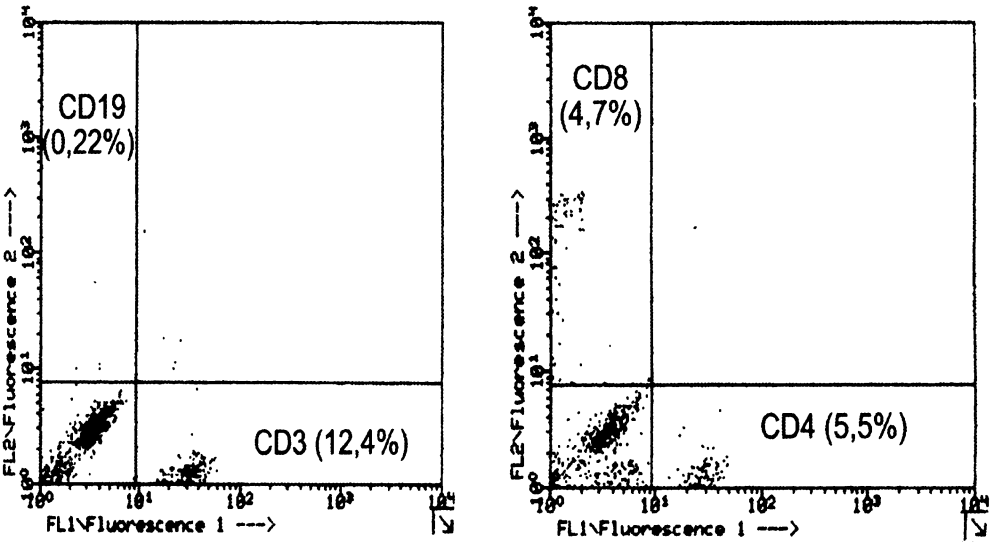

Fig. 2. T-cells (CD3+), CD4+ and cytotoxic T-cells before treatment (Pat. H. B.)

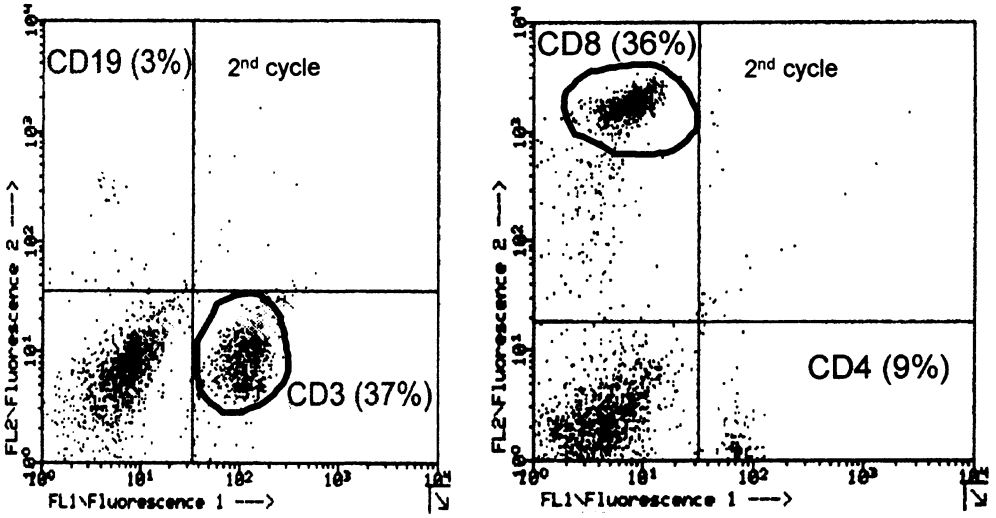

Fig. 3. T-cells (CD3+), CD4+ and cytotoxic T-cells during treatment (2nd cycle, Pat. H. B.)

Immunological Effects

Evaluation of immunological status with respect to surface immunophenotyping of mononuclear peripheral and bone marrow cells showed distinct changes in lymphocyte subsets. In 3 of 5 patients a marked lymphocytic stimulation was achieved. The amount of peripheral blood lymphocytes increased up to > 70% of all peripheral mononuclear cells. The IL-2 treatment schedule used in this study seems to induce a distinct subset of activated lymphocytes in the presence of leukemic blasts: i.e. T-lymphocytes with a specific phenotype: cytotoxic T-cells with expression of IL-2 re-

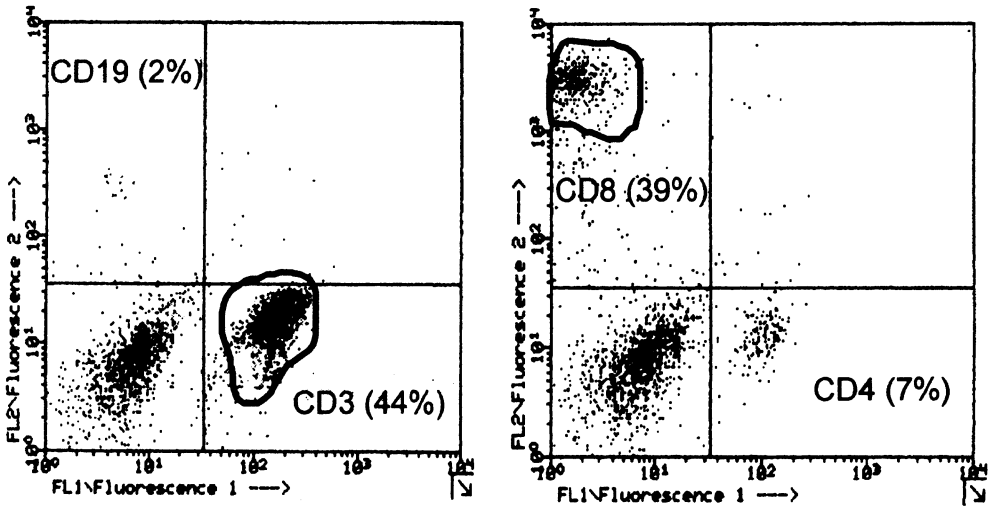

Fig. 4. T-cells (CD3+), CD4+ and cytotoxic T-cells 1 week after treatment (Pat. H. B.)

ceptor a-chain and T-cell receptors class αβ (CD3+/CD8+/ CD25+/TCR αβ+). The proportion of induced cytotoxic T-cells increased up to some 50% in one of the responding patients (see Figs. 2-4). This specific type of immunomodulation was observed in the 3 of 5 patients with MDS-AML who showed response to therapy. The immunomodulation was similar in all of these patients, and showed only quantitative differences. The activation which was achieved could be demonstrated even with low-dose IL-2 therapy (4.5 Mill Units/24 h). The immunostimulation was persistent for some time even after treatment was terminated, with a maximum immune response observed 10 days after treatment was stopped (Figs. 2-4). Two further observations are worth mentioning: The major part of lymphocyte activation of the lymphocytes could be demonstrated in the peripheral blood, the percentage of cytotoxic T-cells induced with this therapy in the bone marrow was to a much lesser extent. Furthermore, in this study and with the treatment schedule used, we did not observe any significant unspecific (i.e., HLA-unrestricted) cellular cytotoxicity with respect to induction of natural-killer-cells or lymphokine-activated-cells.

Discussion

There are several possible mechanisms for the effectivity of immunotherapy with Interleukin-2 in AML patients.

1. IL-2 can reduce relapses in a murine model of syngeneic BMT for AML,
2. LAK cells and NK cells, both of which are induced by IL-2, can lyse human hematologic malignancies, including myeloid leukemia cells,
3. IL-2 can reverse the deficient NK cell activity which has been shown in leukemia patients,
4. IL-2 can induce T-lymphocytes with specific cytotoxic activity against autologous and allogeneic human AML blasts which show a restricted i.e. specific T-cell receptor repertoire,
5. a graft-versus-leukemia (GVL) effect mediated by IL-2-induced cytotoxic cells with specific or non-specific ant-leukemia reactivity can be induced or augmented by exogenously administered IL-2.

There are already some studies reported showing the feasibility of different IL-2 schedules in AML patients both with advanced leukemia and after autologous bone marrow transplantation with well docu-

mented immunomodulatory effects. Some encouraging results have been obtained especially in patients with a low proportion of blasts in the bone marrow.

With regard to feasibility and toxicity, the results of this first analysis of the study performed in our institution show that an immunotherapy with continuous i.v. single-agent Interleukin-2 in patients with relapsed or refractory AML is feasible with an individually performed dose adaptation. The escalating dose protocol allows the doses of IL-2 to be modulated following individual tolerance. This regimen makes it possible for each patient to receive the highest tolerated dose without the risk of overtreatment and the appearance of severe side-effects. With the schedule used in our study toxicities did not exceed WHO grade III. No treatment related deaths occurred. The highest dose level was achieved in 2 of 5 patients and in 3 of 5 patient a dose-reduction after initial escalation was necessary. With this individual dose modification the therapy was completed in all five patients.

The immunologic studies performed showed that moderate to high-dose IL-2 administration leads to a marked lymphocytic stimulation, especially with induction of cytotoxic T-cells in patients with presence of leukemic blast cells in the bone marrow. The immunostimulation is persistent even after cessation of therapy (up to a maximum of 10 days). Interestingly, and in contrast to previous studies published, no significant induction of natural-killer cells with the schedule we used in our institution could be observed.

Concerning the antileukemic efficacy of IL-2 in AML patients, in 2 of 5 patients a reduction of blast counts was seen. In 1 of 5 patients a blast remission < 5% in the bone marrow could be achieved and after the end of induction therapy this patient was treated with low-dose s.c. IL-2 on an outpatient basis showing continuously rising peripheral blood cell counts during maintenance therapy. Both patients who showed response to induction IL-2 treatment had a relatively small proportion of blast cell counts in the bone marrow (below 30%). All 3 patients with high blast cell counts (> 80%) did not show any response to immunotherapy. This is concordant with observations in other studies and the biological assumption that an immunotherapy may be advantageous in the case of a low tumor burden.

In conclusion, the first results of this study indicate that

1. moderate to high doses of Interleukin-2 can be safely administered as an induction treatment to poor prognosis AML patients if an individual dose adaptation is performed with clinical and laboratory monitoring,
2. a marked and sustained immunostimulation with induction of specific cytotoxic lymphocytes can be induced using Interleukin-2 in patients with AML in second or subsequent relapse,
3. induction of hematological remissions to immunotherapy alone is possible especially in AML patients with a relatively low tumor burden,
4. maintenance therapy with low-dose subcutaneous Interleukin-2 can safely be administered on an outpatient basis after induction treatment.

Taken together, these data seem to confirm the possible antileukemic activity of Interleukin-2 in patients with acute myeloid leukemia. Further prospective clinical and laboratory investigation is needed to study the basis of this immunotherapeutic approach especially with regard to the biological mechanisms involved. This may be helpful to identify those patients who have a high probability for achieving remission after immunotherapy and who may benefit from such an approach.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Carboplatin-Based High-Dose Therapy for Refractory Acute Myeloid Leukaemia

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Abstract. A combination of carboplatin plus high-dose ara-C and either mitoxantrone or idarubicin (crossover) was used in 26 adults with refractory or relapsed AML and the following high-risk features: primary refractory 38%, > 2nd relapse 23%, 1st CR < 12 mos. 11%, age > 60 years 19%, FAB M5/secondary AML 23%, prior high-dose ara-C 35%, prior bone marrow transplant 27%. The first regimen (12 patients) with carboplatin 300 mg/m^2/d (24-hour infusion) on days 1-4, high-dose ara-C 1 g/m^2/bd on days 1-5, mitoxantrone (idarubicin) 12 (6) mg/m^2/d on days 1-3 was highly toxic resulting in the early death of 7 patients. With a reduced intensity regimen (14 patients) consisting of carboplatin as above, high-dose ara-C on alternate days (1, 3, 5), and mitoxantrone (idarubicin) reduced to 8 (5) mg/m^2/dose, the response rate was 64% (7 complete and 2 partial remissions). Complete remissions were consolidated with lower-intensity chemotherapy and autologous (n = 5) or allogeneic (n = 2) bone marrow/peripheral blood cell transplants. Overall survival was 2.1 months, responders living longer (median 11 months, 2-year probability 0.34) than nonresponders ($p < 0.001$). The second carboplatin-based regimen was effective in the management of patients with advanced-stage AML and ≤ 2 high-risk features. Mobilization of CD34+ circulating blood cells for autografting was possible and a prolonged remission was obtained in some of these patients.

Introduction

A recent definition of refractory acute myelogenous leukaemia (AML) includes patients failing primary induction chemotherapy or suffering from relapse within 2 years from first complete remission (CR) [1]. Previously, refractory AML was defined as resistance to induction therapy, relapse within 6-12 months from first CR, or second and subsequent relapse [2]. It is possible that the same terminology might apply, regardless time to recurrence, to cases relapsing after initial consolidation with high-dose cytosine arabinoside (ara-C) courses and/or autologous or allogeneic haematopoietic cell transplants. Refractory AML is an extremely difficult condition because the most effective treatment strategy, normally exploited upfront, is demonstrably failing in front of adverse biological determinants such as chromosomal translocations [3, 4] and multidrug resistance (MDR) mechanisms [5, 6]. The management of refractory AML has mainly focused on the achievement of a temporary response with alternative drugs or with investigational agents [7], in order to proceed expeditiously to a bone marrow transplant, preferably an allogeneic one in

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those patients with a compatible sibling donor. The efficacy of mitoxantrone, high-dose ara-C, and etoposide combinations was underscored [1, 2]. Recently, carboplatin (CBDCA) given by continuous infusion showed effective, although CR rates varied widely depending on patient selection, actual CBDCA dosage, schedule and association with other drugs [8-11]. CBDCA might play a role in MDR type-1 (P-170) positive AML, because invulnerable to P-170-mediated transport [12], and perhaps in recurrent rather than primary resistant disease. We used a combination of CBDCA, mitoxantrone and high-dose ara-C in a group of adults with refractory AML, first to obtain a response and secondly to consolidate it with aggressive therapy and autologous or allogeneic haematopoietic cell support. The chosen drugs have different mechanisms of action and are therefore expected to be non-cross-resistant. Etoposide was not included because a topoisomerase II inhibitor - such as mitoxantrone - and a substrate to the MDR type-1 drug efflux mechanism, and because already administered to most patients.

Materials and Methods

CBDCA-Based Regimens

Regimen 1 (R-1) consisted of CBDCA 300 mg/m^2/d as 24-h continuous infusion on days 1-4, ara-C 1 g/m^2/12-h on days 1-5, mitoxantrone (idarubicin) 12 (6) mg/m^2/d on days 1-3. Patients previously treated with adriamycin or idarubicin received the mitoxantrone schedule, and vice versa. Patients already exposed to both drug types received mitoxantrone once more. R-1 was followed by subcutaneous 5 μg/kg/d granulocyte colony-stimulating factor (G-CSF, from Hoffman-LA Roche or Dompe-Biotec, Italy), starting from day 6 and until the peripheral absolute neutrophil count exceeded 1.5 x 10^9/l after the leucocytic nadir. The reduced intensity regimen (R-2) was introduced after the evaluation of treatment results and toxicities observed with R-1 in the first patient cohort (see below for details). R-2 consisted of unmodified CBDCA, high-dose ara-C on alternate days (1, 3, 5), mitoxantrone (idarubicin) reduced to 8 (5) mg/m^2/dose, and unmodified G-CSF. The alternate-day ara-C schedule was derived from a Cancer and Leukemia Group B study [13]. In patients achieving a response, a consolidation course was planned with reduced drug dosages. Responders were considered eligible to high-dose consolidation with autologous or allogeneic haematopoietic cell support. Collection of peripheral blood CD34+ cells for autograft was attempted after the first consolidation course. The whole R-2 sequence is depicted in Fig. 1.

Prophylactic measures were hyperhydration to maintain an adequate urine output,

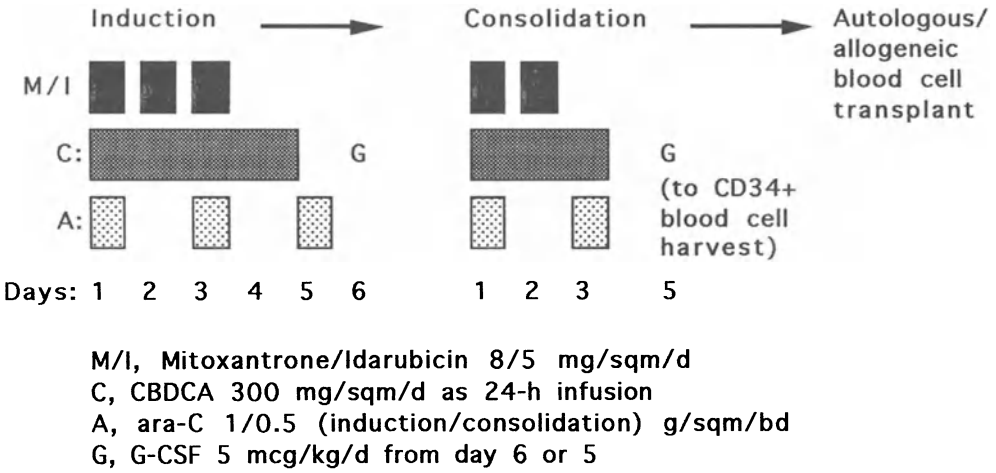

Fig. 1. CBDA-containing regimen 2 for refractory adult AML

oral ciprofloxacin 500 mg/bd, and transfusions with packed red cells and multiple donor platelets for haemoglobin < 8 g/dl and thrombocytopenia < 20 x 10^9/l, respectively. All intravenous medications were administered through a tunneled central venous access.

Definitions and Statistics

Refractory AML was defined as primary resistance to induction course(s), relapse within 12 months from first CR, and any subsequent relapse [1,2]. Additional high-risk factors in this series were age > 60 years, monoblastic/FAB M5 AML, secondary and myelodysplasia-related AML, and recurrence after 12 months from first CR if prior treatment included high-dose ara-C or autologous/allogeneic bone marrow/peripheral blood cell transplantation. Achievement of CR, in patients who reached a neutrophil count > 1.5 x 10^9/l and were transfusion-free, required the disappearance from the bone marrow of recognizable leukaemic cells and the simultaneous evidence of normal trilineage haematopoiesis. A partial response (PR) was defined as the persistence of 25% or less marrow blast cells, and no response > 25% blast cells. A subsequent relapse was diagnosed when marrow blast cells were 5% or greater. Comparisons between different prognostic and treatment groups were by means of the chi-squared test with Yate's correction, the Student's t test, and the log-rank test. Survival estimates and plots were produced by Kaplan-Meier analysis.

Results

Patients

The 26 consecutive patients entered on trial were all those relapsed after or primary resistant to front-line or other salvage regimens for adult and elderly AML between June 1994-July 1996, for whom intensive nonpalliative retreatment was felt appropriate. Adult AML regimens were with adriamycin-ara-C-thioguanine or idarubicin-ara-C-etoposide (induction) plus either high-dose ara-C or autologous bone marrow/blood cell transplantation after high-dose ara-C plus fractionated total body irradiation (postinduction). Elderly AML patients and adult relapse phases before the present study were managed with a mitoxantrone-ara-C-etoposide combination followed by high-dose consolidation if possible. One patient had an acute myeloblastic transformation of a Philadelphia-positive chronic myelogenous leukaemia, refractory to four courses with daunorubicin-thioguanine-ara-C. As of January 1997, the median observation time since diagnosis of refractory AML was 22 months, and range 6-30 months. Nineteen patients had refractory AML according to the proposed criteria [1,2] and seven additional patients at first bone marrow relapse met our inclusion criteria because of advanced age, adverse diagnostic features or prior treatments including high-dose ara-C and transplants. All patients had received prior treatments with one or more anthracycline-type drug and 81% with etoposide. Patient main characteristics, prior treatments and distribution of high-risk features by treatment protocol are illustrated in Table 1.

Treatment Outcome and Toxicity

The first group of 12 patients received R-1, a highly toxic schedule that was abandoned in favour of R-2. Two R-1 patients died of septic complications caused by ciprofloxacin-resistant strains of *Pseudomonas* spp. and *Enterococcus,* respectively. A third patient who died suddenly after the first day of therapy is nevertheless included into analysis. With R-2 the incidence of toxic side effects and early deaths was reduced substantially, leading to an overall response rate of 64%. Overall and comparative treatment outcome according to high-risk factors is summarized in Table 2. All CR were obtained with a single chemotherapy course.

Notably response rates declined as the cumulative incidence of high-risk indicators increased, from 64% (CR) with any which one to 50% (25% CR and 25% PR) with any two to none with three or more. In particular, six of 7 total CR obtained in cases with a single high-risk feature were from the group

Table 1. Patients with refractory AML at time of CBDCA-based therapy

Parameters	Total (n = 26)	R-1 (n = 12)	R-2 (n = 14)
Age (yr), median (range)	54 (26-64)	46 (26-67)	56 (31-64) $p = 0.06$
Gender, M:F	11:15	4:8	7/7
FABclass, M0:1:2:4:5	2:10:7:3:4	2:5:2:2:1	0:5:5:1:3
Prior anthracycline[a], MIT:IDA:DNR:ADR	11:10:2:7	6:2:2:6	5:8:0:1
Prior etoposide	21	8	13
High-risk features:			
Primary refractory	10 (38%)	3	7
1st relapse within 12 mos.	3 (11%)	1	2
> 2nd relapse	6 (23%)	4	2
Age > 60 years	5 (19%)	1	4
Secondary/M5 AML	6 (23%)	3	3
Prior high-dose ara-C	9 (35%)	6	3
Prior transplant	7[b] (27%)	4	3

[a] MIT, mitoxantrone; IDA, idarubicin; DNR, daunorubicin; ADR, adriamycin.
[b] 6 autologous, 1 allogeneic.

Table 2. Treatment outcome by CBDCA-based regimen

	Total (n = 26)	R-1 (n = 12)	R-2 (n = 14)
Early deaths	9 (35%)	7 (58%)	2 (14%) $p = 0.059$
AML regrowth	4 (15%)	1 (8%)	3 (21%)
CR + PR	10 + 3 (50%)	3 + 1 (33%)	7 + 2 (64%), $p = 0.2$
CR + PR[a]/by high-risk features:			
Primary refractory (n = 10)	3 + 2 (30% + 20%)	0/3	3 + 2/7
1st relapse within 12 mos. (n = 3)	1 (33%)	0/1	1/2
> 2nd relapse (n = 6)	2 + 1 (33% + 17%)	1 + 1/4	1/2
Age > 60 years (n = 5)	3 (60%)	0/1	3/4
Secondary/M5 AML (n = 6)	1 + 1 (17% + 17%)	0/3	1 + 1/3
Prior high-dose ara-C (n = 9)	2 + 1 (22% + 11%)	2/6	1 PR/3
Prior transplant (n = 7)	1 + 2 (14% + 28%)	1 + 1/4	1 PR/3

[a] CR figures unless otherwise indicated.

of 8 R-2 patients within this category (75% CR), this being the best CR figure from subgroup analysis (Table 3).

The likelihood of CR and PR was similar in patients treated with either mitoxantrone (7/17, 41%) or idarubicin (4/9, 44%). CR was the first in 3 patients with primary resistant disease, the second in 5 patients at first relapse, and the third in 2 more cases at second relapse.

Myelotoxicity was marked with both R-1 and R-2, with a median absolute neutropenic period in excess of 2 weeks despite additional G-CSF (Table 4). In contrast, regimen-associated gastrointestinal mucositis was generally mild with R-2, so that these patients were less prone to contract infectious complications (28 vs. 100%). Severe metabolic, renal, and auditory toxicities were not observed.

Table 3. Treatment outcome by cumulative incidence of high-risk features

No. high-risk features	Total[a] (n = 26)	R-1 (n = 12)	R-2 (n = 14)
1 (n = 11)	7 (64%)	1/2	6/8
2 (n = 12)	3 + 3 PR (25% + 25%)	2 + 1/8	1 + 2/4
> 2 (n = 3)	0	0/1	0/2

[a] CR figures unless otherwise indicated.

Table 4. Comparison of toxicities and complications

Parameters	R-1 (n = 12)	R-2 (n = 14)	
Neutrophils < 0.5 x 109/l, days	18 (9-36+)	18 (10-38)	ns
Platelets < 20 x 109/l, days	19 (9-36+)	21 (9-38)	ns
GI toxicity[a], no.	7 (58%)	2 (14%)	$p = 0.052$
Fever > 38 °C, days	6 (0-10)	4 (0-12)	ns
Cumulative infections, no.	17	4	$p = 0.0009$
Pneumonia, no.	8 (66%)	3 (21%)	$p = 0.053$
Sepsis, no.	9 (75%)	1 (7%)	$p = 0.0017$

[a] Grade III-IV gastrointestinal toxicity according to WHO scale.

Postremission Therapy and Survival

Of 3 PR patients, two received no further treatment because of prolonged peripheral pancytopenia and rapidly progressive disease, respectively. The third PR patient had a histocompatible sibling donor and underwent a second allogeneic peripheral blood transplantation after high-dose busulphan-melphalan conditioning. A durable remission status was achieved until the patient died 27 months later of sepsis and severe chronic graft-versus-host disease. Of 10 CR patients, one died soon of disseminated fungal infection and one in third CR was given no further therapy because aged > 60. Eight patients received the consolidation course with the subsequent aim to proceed to autologous or allogeneic transplants, and 6 actually underwent the procedure (1 allogeneic, 5 autologous) while 2 did not because of poor performance status and advanced age, respectively. The allogeneic blood cell transplant was complicated by a delayed platelet recovery that eventually caused a fatal cerebral haemorrhage. Autologous peripheral CD34+ blood cells were collected in 7 patients, ranging from 1-4.3 x 10^9/kg CD34+ cells. Five patients were autografted following high-dose BCNU-etoposide-ara-C-melphalan (n = 3) or high-dose ara-C-total body irradiation (n = 2) combinations. Time from CR to autograft was 1.5-5.8 months (median 3.3). The neutrophil count reached > 0.5 x 10^9/l after 8-20 days (median 12) from transplant. The time needed to reconstitute a spontaneous count > 20 x 10^9/l ranged from 18-91 days (median 30).

Median CR duration was 8 months and 2-year probability 0.34. Results were slightly better if the PR case achieving CR following an allograft is included. The length of disease-free interval was apparently related to the intensity of postremission therapy including transplants, since patients excluded from this treatment had shorter remissions (Table 5).

Table 5. Length of CR by postremission treatment intensity

Postremission therapy	No. of patients	CR duration (mos.)	Notes
None	2	0.5, 6	One early death by infection
Consolidation	2	3, 10	-
Consolidation, + allograft	1	14	Died in CR (haemorrhage)
Consolidation + autograft	5	5, 6+, 11, 22, 28+	
Allograft[a]	1	27	Died in CR (Sepsis, GVHD)

[a] PR patient achieving CR after allogenic blood cell transplant.

The patient with longer survival after autograft remained persistingly pancytopenic with a dysplastic bone marrow without blast excess. He responded well to intermittent G-CSF and danazol treatments. No patient was retreated intensively at recurrence. Median overall survival from entry into study was 2.1 months but responders lived significantly longer (median 11 mos., 2-year probability 0.40) than nonresponders, whose median survival was less than one month ($p < 0.001$ by the log-rank test) (Fig. 2).

Discussion

In this single-centre study we tested a CBDCA-containing high-dose regimen for adult patients with refractory AML. Essentially the results point to the importance of drug dosage and scheduling of CBDCA as

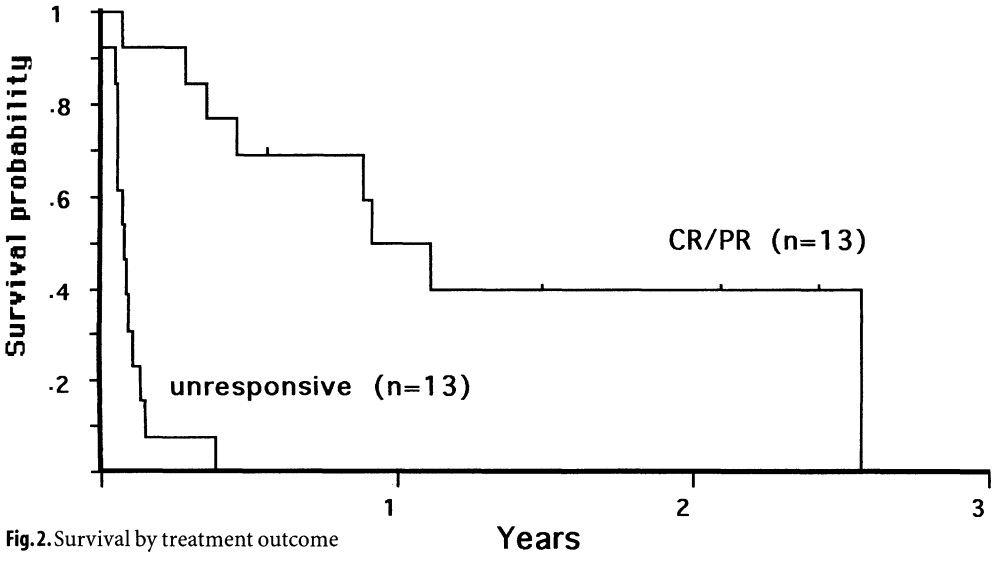

Fig. 2. Survival by treatment outcome

well as associated drugs, in order to keep toxicity within acceptable ranges and optimize clinical results. Cumulative CBDCA not exceeding 1500 mg/m^2 over 5 consecutive days was shown by phase I/II clinical studies to be both safe and relatively effective, whereas higher concentrations were associated with proportionally worse toxicities and those < 1000 mg/m^2 or < 300 mg/m^2/d with decreasing response rates [14, 15]. Therefore CBDCA 1200 mg/m^2 over 4 days was adopted for R-1 and maintained in R-2 even if toxicity problems eventually dictated for a general reduction of treatment intensity. When high-dose ara-C and mitoxantrone (or idarubicin) concentrations were reduced, untowards side effects and especially gastrointestinal toxicity became minimal but, in view of the good response rates to R-2, efficacy was preserved. The decreased mucosal toxicity and strictly related lower infectious complication rate were the result of the alternate-day instead of daily high-dose ara-C schedule, in accordance with the Cancer and Leukemia Group B consolidation study in adult AML [13].

One reason for using CBDCA was the reported invulnerability of this agent to MDR type-1-mediated drug transport. This drug resistance mechanism is often responsible for treatment failure in AML [5, 6]. Thus, despite blast cells can resist to platinum analogs by means of other drug transporters, DNA repair and glutathione-S-transferase [12], use of CBDCA in cases at great risk of overexpressing MDR type-1 seemed a logical stop, given the current shortage of alternative drugs and even if MDR status of single cases was unknown.

In refractory AML CBDCA alone was moderately effective, but a synergistic effect observed in vitro with some other drugs, including ara-C and mitoxantrone [16], prompted the evaluation of combination chemotherapy schedules. Single-agent CBDCA studies reported variable CR rates: < 10% [8, 17], 10-20% [18], > 20% [14, 15]. The highest figure was from a study where 9 (81%) of 11 patients unresponsive to standard front-line chemotherapy entered CR following CBDCA [19]. The association with etoposide gave no CR in one study [8] and 40% in another [11]. Other associations were with anthracyclines (CR 26-33%) [20, 21] and, more recently, with ara-C at standard or intermediate-dose, resulting in 27-50% CR rates [10, 22-24]. The low response rates evidentiated in some studies with CBDCA or CBDCA-etoposide might reflect, in our opinion, the selection of more heavily pretreated subjects who have already developed truly pleiotropic drug resistance, and certainly resistance to CBDCA. This is in keeping with the worse results we observed

in patients progressing after high-dose ara-C and/or total body irradiation and in general in those with 2 or more high-risk features. These data suggest that CBDCA should not be used alone in refractory AML, whereas associations with other drugs are not only possible but obviously indicated. There remains to consider that, when the CR rates approach 50%, none of the several regimens employed in refractory AML seems better than others nor activity of CBDCA can be easily demonstrated within multidrug combinations.

Eventually, results with R-2 were similar to the best reported recently with other intensive schedules, including or not CBDCA, and so was the final outcome of responders, meaning that, excluding patients with no additional high-risk feature in late first relapse, who can still respond quite well to chemotherapy [1], a significant prolongation of disease-free survival in refractory AML is only possible by performing an allogeneic/autologous bone marrow/peripheral blood transplant [1, 25].

The fact that peripheral blood CD34+ cells could be successfully mobilized for autografting purposes by R-2 consolidation, even in such heavily pretreated subjects, adds consistence to our experience and suggests further use of this procedure in refractory AML entering a remission. Eventually, to establish what is better, results obtained with CBDCA-based chemotherapy and autologous blood cell transplants should be compared with those from mafosfamide-purged autologous bone marrow transplants performed in comparable patients and risk setting [25]. Until that time, refractory AML patients responsive to salvage regimens and without a bone marrow donor should not be denied, when possible, an autologous peripheral blood transplant, because the faster haematologic recovery may reduce the risks of pancytopenic procedural deaths and because the results being reported in first CR phase do not show an increased recurrence rate compared with autologous bone marrow transplant [26].

Lastly, our report indicates that the different risk factors present in patients with refractory or recurrent disease can confer a varying likelihood of response to salvage therapy, which makes it mandatory their identification and a unified terminology to allow comparability between different studies and study arms.

In conclusion CBDCA with alternate-day high-dose ara-C and either mitoxantrone or idarubicin exerted an appreciable activity in refractory AML of adults, for both remission reinduction, consolidation and the mobilization of CD34+ circulating cells for autotransplants. The heterogeneous pattern of response and the distribution of high-risk features within patient subsets indicate that further progress may be possible by identifying which specific drug resistance mechanisms affect single cases and then, in this direction, by shaping highly individualized, risk-adapted drug and/or resistance modifier schedules. In 1997, there are no reasons to exclude CBDCA from these studies.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Phase I-II Study of Carboplatin with VP16 and Aracytin for Poor-Risk-Acute Leukemia

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Abstract. We conducted a phase I-II trial of escalating dose of carboplatin (CB) 0.750, 0.875, 1000 g/m^2 (level 1, 2, 3 respectively) given over 5 days as continuous infusion (CI) in association with ARAC 5 g/m^2 and VP16 0.5 g/m^2, to assess the limiting toxicity and the response rate of this combination. Twenty eight patients entered the study (19 phase I, 9 phase II) median age 42 years (22-55), 14 males, 13 females, 9 ALL, 19 AML (16 without antecedent of MDS or CML, 1 acutisation of CML, 2 acutisation of MDS). 3 patients received the therapy as primary treatment for AML. Four patients were refractory, 21 were in relapse.

For phase I study, 15/19 were evaluable for toxicity, 2/15 (level 1 and 2) developed grade 3/4 renal toxicity, both patients died at day 12 and 21 with persistent renal toxicity. No patient developed grade 3/4 toxicity at level 3 which was chosen for phase II study. Median CB area under curve (AUC) was 13 mg/ml × min (9.5-20) without difference between CB level 0.75, 0.875 and 1 g/m^2. Twenty one % (6/28) of the patients died in aplasia. Durations of grade 4 neutropenia and thrombopenia – evaluable in 19/22 patients – were 24 days (13-45) and 25 days (8-45), respectively, without influence of the CB AUC. Grade 3/4 extrahematological toxicity occurred in 37%, 45%, and 66% of the patients with low ($\leq$ 12), intermediate (12-15) and high ($\geq$ 15) AUC respectively. One patient with AUC 20 mg/ml × min cumulated grade 3/4 renal, mucosal and hepatic toxicities.

Overall response rate included 14% CR, 14% regeneration failure and 10% PR.

These data suggest the importance of CB dose optimization and recommend AUC 16 to 18 mg/ml × min for future trials.

Introduction

Treatment for relapsed or refractory acute leukemia is increasingly problematic since most patients had received high dose therapy – high dose ARAC, VP16, most efficient anthracyclines (Idarubicin, Miloxantron) – and bone marrow transplantation as first line strategy. Thus, the research for a new effective antileukemic agent is a major stake to overcome resistance in high dose acute leukemia.

Salvage therapy offer from 7 to 40% complete response (CR) rate according risk factors including initial karyotype, first CR duration, response to prior salvage [1-4] Carboplatin (CB) is a second generation platinum drug which, unlike Cisplatin, has prominent myelotoxicity and considerably less

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nephrotoxicity. Phase I study of Carboplatin given as single agent in poor risk, refractory or relapsed acute leukemia demonstrated that myelotoxicity was dose limiting and that the maximum tolerated dose (MTD) was 1.5 g/m^2 given as continuous infusion (CI) over 5 days. CR rate ranged from 12 to 28% [5-8].

We report here the result of escalating dose of CB (0.75 to 1 g/m^2) with fixed dose ARAC (5 g/m^2) and VP16 (0.5 g/m^2) given over 5 days in 28 patients with poor risk acute leukemia.

In the light of recent data reporting that Carboplatin AUC (mg/ml × min) is associated with hematological, extrahematological toxicity and efficacy, AUC were calculated retrospectively and data will be presented according AUC range.

Treatment Protocol

Induction course included VP16 0.1 g/m^2/day during 5 days (1-h infusion), ARAC 1 g/m^2/day during 5 days (6-h infusion) and CB at 0.150-0.175 or 0.2 g/m^2/day during 5 days (CI), G-CSF was given from day 10 (day 1 = first day of therapy) to day 20. If bone marrow aspiration showed blastic involvement at day 20, G-CSF was stopped. If aspiration showed hypoplasia without blastic cells, G-CSF was continued until neutrophil recovery ($\geq 0.5 \times 10e9/l$).

Response was evaluated with Preisler criteria: CR, absolute resistance, relative resistance (PR), regeneration failure (hypoplasia without blast), death in aplasia [9].

Dose escalation interpatient was conducted if 3/5 patients without protocol violation did not show grade 3/4 toxicities or related procedure death defined as prohibitive toxicities. Phase II trial included additional patients at the MTD level. Consolidation course included the same drug combination given for 3 days during for patients in CR or PR.

Results

Patients

Twenty eight patients entered the study. Median age was 42 years (22-58), 15 females, 13 males. There were 19 acute myeloid leukemia (AML) (16 AML without antecedent of CGL or MDS phase, 1 CGL blastic phase, 2 MDS blastic phase, M0, 1, 2 = 10 patients; M4, 5 = 8; M7 = 1), 9 acute lymphoid leukemia (ALL).

Three patients were untreated (3 blastic phase of MDS or CGL); 4 patients were refractory to 2 induction courses; 21 patients were in relapse, 12 had received bone marrow transplantation previously (2 allogeneic; 10 autologous). Only 5 patients had CR1 longer than 1 year.

Karyotype was evaluable in 25 patients: 10 had unfavorable karyotype [t(9;22) = 6; t(4;11) = 1; -5 = 1; -7 = 2]. 9 had normal karyotype, 6 had undetermined prognostic abnormality [t(14;19) = 1; t(3;12) = 1; +13 = 1; (-11;+21) = 1; -15 = 1; +1 = 1].

Phase I included 19 patients (7 level 0.75, 6 level 0.875, 6 level 1g/m^2). Four patients had protocol violation but received at least 80% of the planed dose and are kept for analysis. Prohibitive toxicities occurred in 1/5 patients without protocol violation at level 0.75 and 0.875 respectively but in 0/6 patients at level 1 g/m^2 which was chosen for the phase II trial and included 9 patients.

Carboplatin area under curve (CB AUC) (mg/ml × min) was evaluated retrospectively using the Calvert formula (10).

CB dose = AUC × (GFR × 25)

GFR = Glomerular filtration estimated by the creatinine clearance at day 1

25 = average non renal clearance for adult.

Median AUC was 13 mg/ml × min (9.5-20) for the whole cohort of patients. Because there were no discernable difference between the different levels, hematological and extrahematological toxicities are presented according AUC defined arbitrarily as low ≤ 12 (n = 8); intermediate > 12-> 15 (n = 11) or high ≥ 15 (n = 9) (Table 1).

Toxicity

Hematological Toxicity. Six patients (21%) died in aplasia - median Day 14 (8-26); 1 sudden death, 4 sepsis-associated deaths (*E. coli, Streptococcus, Candida, Aspergillosus*), 1 diffuse intravascular coagulopathy syndrom. Hematological recovery is obviously associated with leukemic response,

Table 1. CB AUC according to the different CB Level

UPN	CB dose g/m^2	Mean (± SEM)	Med. (Range)	AUC Low ≤ 12	AUC Intermediate > 12–>15	AUC High ≥ 15
pt 1 to 7 n = 7	0.750	12.2 ± 1.6	12 (10–15)	5 (71%)	1 (14%)	1 (14%)
pt 8 to 13 n = 6	0.875	12.9 ± 3.5	10 (9-5–20)	3 (50%)	1 (16%)	2 (34%)
pt 14 to 28 n = 15	1	15 ± 2.5	13 (12.5–20)	0 (60%)	9 (40%)	4
Total n = 28		14 ± 3	13 (9.5–20)	n = 8	n = 11	n = 9

Table 2. Neutropenia duration

	AUC Low ≤ 12	AUC Inter > 12–< 15	AUC High ≥ 15
< 0.1 × 10e9/l n = 22/22	n = 5 10 (4–24)	n = 11 17 (10–45)	n = 6 16 (14-77)
< 0.5 × 10e9/l n = 19/22	n = 5 22 (16–26)	n = 9 24 (13–45)	n = 5 18 (18-31)

Thrombopenia duration

	AUC Low ≤ 12	AUC Intermediate > 12–< 15	AUC High ≥ 15
< 20 × 10e9/l n = 22/22	20 (15–26)	26 (8–45)	20 (9–35)

however, 86% (19/22) of the patients had some degree of reconstitution (PN ≥ 0.5 × 10e9/l – Plt ≥ 20 × 10e9/l). Duration of grade 4 neutropenia (< 0.5 × 10e9/l) and grade 4 thrombopenia (< 20 × 10e9/l) was 24 (13/45) and 25 (8-45) days respectively without difference between patient AUC (Table 1).

Documented infection occurred in 18 patients with 8 septicemia (3 *Streptococcus*, 3 *Staphylococcus*, *E. coli*, 1 *candida*) and 18 major local infections (9 pulmonary infections – 3 aspergillosis, 6 interstitial pneumopathy – 6 stomatitis/gingivitis, 1 perineal infection, 1 conjunctivitis).

Extrahematological Toxicity. Fourteen patients presented grade 3/4 extra-hematological toxicities: 3/8 (37%) at the low AUC, 5/11 (45%) at the intermediate AUC, 6/9 (66%) at the high AUC (Table 3).

Three patients (10%) had major renal failure. One patient (AUC: 13) had grade 3 toxicity in the context of septic shock. In the two other patients, creatinine increased at day 8, one patient (AUC: 15.5) died anuric at day 12 of *E. coli* septicemia with grade 3 diarrhea, one patient (AUC: 20), required dialysis at day 19, died in aplasia with *Candida* septicemia at day 21. This patient cumulated also grade 3/4 diarrhea, mucositis, hepatitis. In the etiology of this major renal failure, it should be noted that the patient had received aminoglycoside and Vancomycin during Carboplatin administration.

Major grade 3/4 neurological toxicities occurred in 5 patients (17%), 3 were related to sepsis (2 septic shock; aspergillosis), 1 to haemorrhage, 1 patient developed transient monoparesia (< 8 h).

Grade 3/4 diarrhea and mucositis occurred in 21 and 28% of the patients respec-

Table 3. Extrahematological toxicity

Grade 3/4	AUC Low ≤ 12 n = 8	AUC Inter > 12–< 15 n = 11	AUC High ≥ 15 n = 9
Renal	0/0	1/0	0/2
Hepatic	2/0	1/1	2/0
Mucositis	2/0	2/1	1/2
Neurotox.	0/1	1/0	0/3
Total 6/1	8/2	5/8	
Patients with grade 3/4 toxicities	3/8 (39%)	5/11 (45%)	6/9 (66%)

Table 4. Responses

	Death in aplasia	Absolute failure	PR	Regeneration failure	CR
ALL-n = 9	3	2	2	1[b] (11%)	1 (11%)
AML-n = 19	3	9	1	3	3
wo atcd MDS-CML - n = 16	3	6	1	3 (18%)	3 (18%)
MDS-CML BP - n = 3	0	3	0	0	0
Total n = 28	6	11	3[a] (10%)	4 (14%)	4 (14%)

[a] None of the PR was maintained at day 28 of consolidation course.
[b] This patient was aplastic (0.5 × 10e9/l WBC) at D45. Progressive aspergillosis led to infusion of peripheral blood stem cell collected during CR1. He died at day 90 without leukemic progression.

tively for a median of 9 days [8-15]. Hepatic toxicity was modest.

Responses. Complete responses occurred in 4 patients (14%) (1/9 ALL = 11%; 3/19 AML = 16%). None of the 3 partial responses were maintained at day 28 of the consolidation course. Regeneration failure occurred in 4 patients (1/9 ALL = 11%; 3/19 AML = 16%, Table 4).

No patient with refractory or untreated poor risk leukemia obtained any degree response.

Median time to progression for the 8 patients in CR or regeneration failure was 2 months (2-6 months). Six patients had not received any other therapy, 1 ALL [t(9;22)] had received alpha Interferon for 5 months, 1 AML received autologous bone marrow transplantation after consolidation.

No correlation between AUC of the responders (CR or regeneration failure) or non-responders were discernible.

Discussion

Prolonged aplasia had been regularly reported for patients - including acute leukemia - receiving Carboplatin at dose ≥ 1.5 g/m^2 [5-8, 11]. On the contrary, several authors emphasized the scarce extra-medullary toxicities [5-8], although increased gastrointestinal toxicities had been reported with the association to VP16 [12-17].

At conventional dose (0.3 g/m^2), a wide range Carboplatin AUC are reported among patients and AUC is correlated with myelotoxicity. Recommended AUC for standard therapy for solid tumor is 5 mg/ml × min. Without stem cell support, Carboplatin has been escalated up to 800 mg/m^2 [11]. High dose Carboplatin 1.2 to 2 g/m^2 can also be given safely alone or in combination with the support of stem cells and optimal efficacy is defined for AUC 18 mg/ml × min [11, 18]. Dose above AUC 18 were associated with excessive peripheral neuropathy.

Median AUC for the present study was 13 mg/ml × min, not surprisingly with a wide range from 9 to 20 without significant difference between CB level 0.5, 0.875 or 1 g/m^2. If dose optimization is critical for carboplatin activity and toxicity in leukemia as it is in solid tumor, data should be reviewed according delivered CB AUC.

Hematological toxicity is difficult to assess because most of the patients were heavily preatreated (43% bone marrow transplantation) and that recovery is associated with leukemia response. Most of the patients had anyway some degree of neutrophil and platelet reconstitution. Duration of grade 4 neutropenia and thrombopenia was prolonged [24 days (8-45)] without any difference between the three AUC categories (low = $\leq$ 12 mg/ml × min; intermediate = > 12 $\ll$ 15 and high $\geq$ 15) probably because substantial high CB dose and other drugs were given at any levels. Grade 3/4 extrahematological toxicities occurred in 37, 45 and 66% of patients with low, intermediate and high AUC, respectively. It is difficult to assess for hepatic or mucosal and gastrointestinal toxicities the respective responsabilities of either CB or AraC and VP16. Grade 3/4 mucositis occurrence is interestingly low, with 28% incidence, when compared to salvage therapy combining anthracyclines to VP16 and ARAC (40-60%).

Grade 3/4 renal toxicity occurred in 3 patients (0, 10, 22% of patients with low, intermediate and high AUC respectively). The two patients who died anuric or required dialysis had 15.5 and 20 mg/ml × min AUC values respectively. Both patients were septic but creatinine increase preceeded diagnostic of documented infection. The patient with the higher AUC – level 0.875 – cumulated also grade 3/4 hepatic, mucosal and gastrointestinal toxicies. This patient received antibiotics including aminoglycosides and Vancomycine during the administration for CB which probably participated to the renal toxicity. The last patient developed grade 3 toxicity after diagnosis of *Streptococcus* infection in parallel with hemorrhagic cystitis and recovered completely within 8 days. Pilot trial of CB for acute leukemia had regularly pointed out the scanty extra-hematological toxicities. However, Vogler had already reported 17% of grade 3/4 renal toxicity in 46 patients treated with 1575 g/m^2 [8].

Complete response (CR), partial response (PR) and regeneration failure (RF) were obtained in 14, 10 and 14% of the patients. All the partial responders progressed during the consolidation course. Duration of response for CR and patients with regeneration failure was 2 months (2-6 months). CR rate for patients with durable first CR $\geq$ 12 months was 40% (2/5) and opposed to 12% (2/16) for patient with first CR < 12 months. These results are superposable to the literature data for other salvage regimens.

Conflicting studies of association with VP16 have already reported CR rates ranging from 0 to 40% [13-15]. One study had reported interesting 40% CR rates of CB and AraC combination [16].

In conclusion, CB AUC in this study vas not correlated to leukemic response but was associated to grade 3/4 renal, gastrointestinal and hepatic toxicities in 1 patient with AUC value above 18 mg/ml × min.

These data provide argument to deliver optimized dose of CBC according AUC value around 18 to 16 mg/ml × min as already performed in solid tumors and to recommend exclusion of nephrotoxic drugs concurrent to CB administration in future trials.

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Minimal Residual Disease

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Detection and Quantification of Minimal Residual Disease in Childhood B-Precursor Acute Lymphoblastic Leukemia by Limiting Dilution and PCR Application

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Abstract. Polymerase Chain Reaction (PCR) on Immunoglobine heavy chain and T-cell-receptor can be applied for the detection of Minimal Residual Disease (MRD) in children with B-precursor Acute Lymphoblastic Leukemia (ALL). We have previously shown that the presence of PCR detectable malignant cells at the end of induction therapy is strongly correlated with occurrence of a relapse. We currently investigate whether the predictive value of PCR can be increased by quantification of MRD during induction therapy.

A patient-specific nested PCR for tumor-specific Immunoglobine (IgH) and T-cell receptor gene (TCR) on two-fold diluted DNA samples (20 replicate experiments) is used in limiting dilution to detect leukemic cells. As this PCR can detect a single cell, the number of positive PCR-reactions at a certain dilution endpoint measures the number of residual leukemic cells using Poisson statistics. The applicability of this approach was evaluated on bone marrow samples taken at diagnosis and during remission of a child with oligoclonal (on Southern Blot analysis) B-precursor ALL.

At first remission the bone marrow contained residual ALL-cells between 4×10^{-3} and 5×10^{-4} cells. As multiple rearrangements were found in this oligoclonal patient, we tried to quantify each clone separately. We were able to demonstrate the different behaviour in reduction during therapy of each clone. The clone which grew out during relapse, showed the slowest response during induction therapy.

We conclude that precise quantification of MRD during induction therapy can be investigated by application of a tumor-specific-IgH/TCRδ-PCR on serially diluted DNA samples. Based on the results of MRD detection for the various clones in this oligoclonal patient, we conclude it may be necessary to use multiple markers for MRD, also during induction therapy.

Introduction

Although progress in the therapy of children with ALL has resulted in an improved survival up to 70-80%, still 20-30% relapse despite the current risk-adapted ALL treatment protocols. Standard treatment of childhood ALL consists mainly of a course of intensive (remission induction) chemotherapy and central nervous system (CNS) prophylaxis, followed by low dose maintenance therapy for two years. Sometimes a consolidation course is included in the protocol. More intensive treatment is given to patients identified as high risk patients, e.g. children with high initial leukocyte count,

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young age, particular chromosomal aberrations.

Haematologic remission in ALL is defined as fewer then 5% lymphoblasts in bone marrow, examined on standard morphology. However, these patients may still harbour as many as 10^{10} neoplastic cells. The detection of cytomorphological techniques is 1 to 5%, which implies that these techniques can provide only limited information about the effectiveness of therapy. Other techniques i.e. morphology, immunology, cytogenetics and Southern blotting also have a relatively low sensitivity. PCR enables the detection at lower levels, down to 10^{-3} to 10^{-6} (1000 to 1 leukemic cell in 10^6), if targeted at leukemia-specific sequences such as junctional regions of rearranged immunoglobin (IgH) and T-cell receptor (TCR) genes or breakpoint fusion regions of chromosome aberrations. PCR analysis of junctional regions of IgH and TCR genes is informative in 90% of B-precursor ALL and virtually all T-ALL . It was demonstrated that presence of MRD at the end of induction therapy predicts long-term outcome in ALL [1,2,3]. Precise quantification of cell reduction after initiation of therapy to document the speed in reduction of leukemic mass, probably give more specific information about the effectiveness of therapy given and as a result on the predictive value of MRD testing. Also other studies indicate the relation between the level of MRD and the probability of a relapse-free survival. It is even suggested that a level of MRD $> 10^{-3}$ at diagnosis may correspond with poor prognosis and occurrence of relapse [4].

Although leukemia is a monoclonal proliferation, Southern blot analysis of leukemic cells at diagnosis shows that a considerable number of patients (30-40%) present with a oligoclonal leukemia in respect to the IgH gene configuration [5, 6] and even 85% of the patients is oligoclonal at PCR level [7]. Oligoclonality is the major drawback of MRD detection using antigen receptor clone-specific oligonucleotides. However, it is generally assumed that clonal evolution and outgrowth of new clones occurs in the follow-up of the disease and that oligoclonality is not hampering the PCR approach in the beginning of the disease. This would imply that the response on induction therapy could be measured using a single marker. However it has never been investigated whether the different subclones at diagnosis, respond equally on induction therapy. Selection of a relatively resistant clone already during induction therapy might be of importance for this. Performing PCR analysis the several clones can be analysed separately in order to detect such a mechanism.

In the present study, we demonstrate in one patient with oligoclonal ALL that the different clones did not behave similar in respect to reduction during therapy. We conclude that it is important to follow the various clones, by the use of multiple markers, specific for each clone.

Methods

Patients and Materials

The bone marrow samples from a child with B-precursor ALL treated in the Emma Kinderziekenhuis/AMC were obtained at diagnosis, at the end of induction therapy (28 days of treatment) and at relapse. Immunophenotypic and cytogentic analysis was routinely performed at diagnosis or relapse using standard techniques.

This patient was selected on basis of oligoclonality as identified by means of Southern blot analysis. Oligoclonality was diagnosed in case the restriction fragment length pattern either the number of bands exceeded the number of chromosome 14 copies per cell or in case there were extreme differences in intensity of the bands, indicative of the existence of subpopulations. The first was the case in this selected patient.

The patient was treated according to the BFM strategy based protocol ALL-VIII from the Dutch Childhood Leukemia Study Group (DCLSG) [8]. Induction therapy (28 days) included prednisolone, vincristine, daunorubicine and asparaginase, additionally methotrexate, prednisolone and cytarabine were administered intrathecally. CNS prophylaxis and consolidation treatment contained high dose methotrexate, adriamycin, asparaginase, cyclophosphamid, cytarabine, 6-thioguanine and intrathecal medica-

tion as previously described. Maintenance therapy included 6-mercaptopurin and methotrexate. Total duration of treatment was 2 years.

DNA Isolation

The mononuclear cell fraction was isolated by Ficoll-Hypaque density gradient centrifugation. DNA isolation was performed using the QIAamp Blood Kit.

Digestion was performed with QIAGEN Protease, used in conjunction with lysis buffers provided by QIAamp Blood Kit. DNA was purified on QIAamp Spin Columns and adsorbed onto the QIAamp silica membrane in a brief centrifugation step. Purified DNA is eluted from the QIAamp Spin column in a low salt buffer AE. The highly concentrated DNA was stored at –20 °C [9].

Southern Blot Analysis

High molecular weight DNA was isolated according to standard procedures [10]. Ten microgram of DNA was digested with the appropriate restriction endonucleases (Promega, Madison, WI) and transferred onto nitrocellulose filters (Schleicher en Schuell, Dassel, Germany). Hybridization and washing conditions were as described previously according to standard procedures [11,1]. The H24 probe is a 2.8-kb EcoRI/BglII restriction fragment [12]. The Jδ1 probe is a 1.5-kb SacI/SacI restriction fragment [13] and was kindly provided by J.J.M. van Dongen (Erasmus University, Rotterdam, The Netherlands).

Prevention of Cross Contamination

In order to prevent cross contaminations, all pre-PCR procedures (DNA isolation, preparation of PCR stock solutions) were performed in specially assigned rooms, spatially separated from the post-PCR laboratory. Addition of the DNA template to the (mineral oil sealed) PCR mixes was performed in a laminar flow hood, using aerosol resistant filter tips.

PCR Reactions

All primers were synthesized on a DNA synthesizer (Applied Biosystems model 392; Palo Alto, CA, USA) and purified with oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA, USA). Immunoglobin heavy chain genes were amplified by PCR with consensus primers for the framewotk region 3 (Fr3) and a consensus joining primer (Jh21).

Reactions were performed in mixtures as previously described [1]. The input of genomic DNA was 1 μg per reaction. The PCR reaction protocol was done in a thermal cycler (Perkin Elmer Cetus Model 480; Norwik, CT, USA) and consisted of 10 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 45 s at 55 °C and finally 5 min at 72 °C.

Direct Sequence Analysis of VhDJh Junctional Regions

PCR product was size separated on a 20 x 20 cm 8% acrylamidegel. Positive bands were excised from the gel. The product was eluted from gel slices for 1 h at 55 °C in 50 μl water. One microliter of the elution volume was used as a template for 30 cycles of reamplification with the original primers. Two microliters of second-round PCR product were directly used as a template in the sequencing reaction. Twenty cycles of 10 s at 95 °C and 10 s at 65 °C were performed with the Brl sequencing kit (BRL, Gaithersburg, MD, USA), using one of the PCR primers, end labeled with ^{32}P (Amersham, UK). Products were always sequenced on both strands in order to eliminate sequencing artefacts.

Development of Allele-Specific Primers and Detection of MRD

The sequence of oligonucleotides of the junctional regions of rearranged Ig and TCR genes are assumed to be different in each lymphocyte and thus also in each leukemia. By sequence analysis, it is possible to determine each sequence and to develop allele-specific primers as a tumor marker for each clone. Initially, a two-round seminested al-

lele-specific PCR was done. In the first round, IgH and TCR junctional regions were amplified with the consensus primers Fr3 and Jh21. In the second round, a semi-nested PCR was performed, using the allele-specific primer in combination with the consensus primer Jh21. PCR mixtures and the PCR protocol of the first PCR reaction were as described above.

The second semi-nested PCR had an input of 2 µl of PCR product of the first PCR reaction. PCR mixtures were the same. After testing the allele-specific primers at different temperatures, we concluded that 60 °C was the ideal temperature for these primers. For this reason, the temperature in this PCR protocol had slightly changed. The reaction protocol consisted of 10′ 95 °C, followed by 35 cycles of 30 s at 95 °C and 45s at 60 °C, and finally 10 min at 72 °C.

Ten microliters of PCR products of the second PCR reaction were size separated by electrophoresis over 10% acrylamide mini gels (Hoefer Scientific Instruments, San Francisco, CA, USA) and were made visible by ethidium bromide staining.

Liquid Hybridization

Hybridization was as previously described [13]. Oligonucleotides, complementary to IgH-DJh and TCR Vδ2Dδ3 junctional regions were synthesized on a DNA synthesizer (Applied Biosystems model 392). Hybridization mixtures were prepared in 0.5 ml Eppendorf Tubes and contained 5 µl PCR product, 4 µl 20 x SSC (standard saline citrate) and 30 µl water. Unpurified oligonucleotide (80 ng) was end-labeled with 40 µl Ci ^{32}P-gamma dATP and non-incorporated nucleotides were removed with a sephadex G-50 column. Approximately, 1 ng oligonucleotide (1 µl) was added to the hybridization mixtures, which were subsequently covered with mineral oil. The hybridization reactions were performed in a thermal cycler (Perkin Elmer Cetus model 480). The protocol was identical for all oligonucleotides and consisted of 10 min at 95 °C followed by 15 min at 60 °C and cooling to 20 °C. Ten microliters of hybridization mixture were size separated by electrophoresis through 10% acrylamide mini-gels in order to separate free oligonucleotides from the oligonucleotide/PCR product complexes. Gels were dried and films exposed at −70 °C with intensifying screens for 24-48 h.

Quantification of MRD Levels by Limiting Dilution

Limiting dilution assays were performed with a two-round-patient-specific PCR on two fold diluted DNA samples to quantify leukemic residual cells.

Since this PCR is able to detect a single cell, the number of positive PCR reactions at a certain dilution is distributed according to a Poisson distribution and as such quantification of the malignant cells is possible. Preliminary experiments with five fold dilutions of DNA-samples in water were performed in five fold to find out at which dilution the PCRs become negative. Subsequently, two fold serial dilutions are made to examine at which dilution the PCR actually becomes negative. These two fold dilutions range from the highest preliminary dilution showing amplification to the lowest preliminary dilution not showing amplification in the five fold diluted samples [15]. The semi-nested patient-specific-PCR was performed in 20 replicate experiments per dilution. Each PCR-product was made visible after gel electrophoresis and ethidium bromide staining.

Statistical analysis was performed using Poisson Statistics [16, 17].

Results

The results of one oligoclonal patient who was diagnosed at the age of 1.2 y and experienced a relapse 11 months after initiation of therapy. Karyotyping was as followed: 46XY, t(x;12)(p11;q12-13)del(1)(q31;q44)t(7;9)(q21;p21)[24]. Immunophenotype analysis ($CD10^+$, $CD19^+$, TdT^+ and $cIgM^+$) showed a precursor-B-ALL.

Southern blot analysis with the IgH probe (H24) on high molecular DNA isolated from diagnosis bone marrow samples showed the presence of a weak germline and three rearrangements. These three rearrangements

Table 1. IgH rearrangements as determined by PCR

Primer	p125-1	p125-2	p125-3	p125-4	p125-R
IgH at diagnosis	+++ [a]	+++	+++	+++	+
IgH at relapse	-	-	+++	-	+++

[a] +++ major clone, + minor clone, - no clone detected.

Tabel 2. Results of limiting dilution experiments

Patient at diagnosis										
Input no of cells[a]	160	80	40	20	10	5	2.5	1.25	0.625	0.312
Primer:										
p125-1	16[b]	10	4	5	2	-	-	-	-	-
p125-2	-	-	-	18	15	13	8	3	2	-
p125-3	-	-	-	-	19	18	16	14	6	2
p125-4	-	-	-	20	18	9	10	6	1	-
p125-R	-	17	17	7	6	2	-	-	-	-

Patient during remission								
Input no of cells[a]	Undiluted 160 000	1280	640	320	160	80	40	20
Primer:								
p125-1	0[b]	0	0	0	0	-	-	-
p125-2	-	-	-	17	13	10	3	-
p125-3	-	-	-	20	19	10	6	2
p125-4	-	-	1	2	2	0	-	-
p125-R	-	-	-	20	18	15	6	3

[a] The number of cells were estimated assuming that 1 μg of DNA was isolated from 160 000 cells.
[b] Number of positive experiments out of 20 experiments, as performed for each dilution.

were of different intensity: one major rearrangement (R3) and two minor rearrangements (r1, r2), seen as faint bands. The Jδ1 probe did not reveal any rearrangements. Since this patient had only two chromosomes 14, oligoclonality was proven, with only rearrangements of the IgH-gene. At relapse Southern blot analysis revealed a germline pattern but a different pattern of rearrangements. The major rearrangement R3 was still present as a major band. In contrast, the minor rearrangements r1,r2 were not detected and a new rearrangement (R), of the same intensity as the rearrangement R3, was seen.

To identify small subclones below detection level for Southern blothing and also to determine the IgH rearrangements of the clones as seen on the blothing of samples taken at diagnosis and at relapse, we PCR amplified and sequenced Vh-D-Jh and D-Jh rearrangements with a panel of Vh-family-specific Fr1-primers (Vh1-6), D-family specific-primers (DLR, DXP, DN, DK) and a consensusVh-Fr3 primer in combination with Jh primers. Results are shown in Table 1.

PCR analysis at diagnosis resulted in 7 rearrangements, consisting of 4 major (named p 125-1/2/3/4) and 3 minor clonal rearrangements (p125-R/5/6). Sequential analysis of p125-4/R/5/6 showed related sequences resulting from Vh-replacements with identical D-J joinings. The clonal rearrangements p125-1/2/3 were unrelated. The precise nucleotide sequence of each junctional region of the IgH gene was determined by sequence analysis. Subsequently allele-specific primers, complementary to the CDR3 region of IgH, were designed. With these primers, we amplified all leukemic rearrangements in

Table 3. Statistical analysis of results of limiting dilution

Primer	% incidence at diagnosis	Standard deviation	% incidence at remission	Standard deviation	Reduction factor
p125-1	0.9	0.6-1.2	-	-	90 fold
p125-2	15.4	11.4-21.0	0.3	0.2-0.4	51 fold
p125-3	54.2	40.4-72.8	0.6	0.2-0.4	90 fold
p125-4	18.4	13.8-24.8	0.03	0.01-0.09	613 fold
p125-R	2.9	2.1-4.0	0.6	0.5-0.8	48 fold

DNA from bone marrow obtained from the patient at relapse. In relapse material, two major clonal rearrangements (p125-3/R) were present. The clonal rearrangement p125-3 was already present as a major clone at diagnosis. Rearrangement p125-R was a minor clone at diagnosis but presented as a major clone at relapse.

To quantify each clone and to follow the growth kinetics of each clone, we performed a two-round PCR with the specific primers in serial dilutions of samples taken at diagnosis and at the end of induction therapy. The patient was morphologically in complete remission at the end of induction therapy. Levels of MRD varied from 1×10^{-2} to 1×10^{-6}. Results in detail are shown in Table 2.

The statistical analysis of the results, as shown in Table 3, indicated that P125-3 was the most important and largest clonal rearrangement at diagnosis, i.e., 54%. Rearrangement p125-R was a small clone at diagnosis, with an appearance rate of 2.9%.

At the end of induction therapy, all rearrangements were detectable at a much lower level (10^{-2}-10^{-3}) than at diagnosis.(10^{-5}-10^{-6}). The rearrangements p125-1 could not be detected. Proportionally, rearrangement p125-3 was still the largest rearrangement with an appearance rate of 0.6%.

P125-R showed relatively the smallest reduction in response to therapy, as this rearrangement was present in 0.6% of the cases.

Discussion

Previous studies [1, 4, 18] show that evolution of MRD is an independent predictor of outcome. As sensitivity of PCR techniques varies between 1×10^{-4} to 1×10^{-6}, during the past years it has become clear that there is a need for more precise quantification of residual malignant cells. Assessment of the effectiveness of new treatment protocols can by this means be done more adequately and eventually allow a better stratification according to risk groups.

Several groups tried to develop PCR-based techniques to quantify MRD. Limiting dilution is one of the principles to reach this goal [15, 18]. It is based on all-or-none end-point results at higher dilutions and on the premise that one or more targets in the reaction mixture always give rise to a positive end-point. Accurate quantitation is achieved by performing multiple replicates at serial dilutions of the samples. In the present study we show that limiting dilution is a very accurate method to follow and quantify MRD in leukemic patients. The main limitation comes from its principle i.e. the high number of simultaneous tests that have to be performed, since the random distribution of targets follows Poisson's law at the dilution end-point. As a results this method is rather laborious and has to be adapted for routine testing.

Precursor-B-ALL was assumed to be a monoclonal proliferation. However, Southern blot analysis of leukemic blasts has shown that in 30-40% of patients the leukemia is oligoclonal in respect to the IgH/TCR configuration [5, 6].

In the present study, we analysed in detail one patient with precursor-B-ALL who showed to be oligoclonal on Southern blot analysis and by PCR analysis as multiple IgH rearrangements were detected. The 5 clonal rearrangements were different in occurrence and growth kinetics during therapy. Clonal rearrangements were detectable at MRD levels of 10^{-5}– 10^{-6} at diagnosis. In remission there was a reduction of approxi-

mately 3 log leading to detectable MRD levels of 10^{-2}– 10^{-3}. From the four major rearrangements at diagnosis only one major rearrangement (R3) was detected at remission/relapse. The now three rearrangements became detectable in very small amounts. A minor clone (barely detectable at diagnosis), became more predominant in remission samples and in relapse samples later.

These results illustrate that each clone has a different pattern of growth and reduction. It also shows the importance of following each leukemic rearrangement in oligoclonal patients, already during the disease.

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Monitoring of Minimal Residual Disease in Childhood Acute Lymphoblastic Leukemia – Preliminary Data from a Prospective Study

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Abstract. Minimal residual disease (MRD) was evaluated in bone marrow samples prospectively obtained from children with acute lymphoblastic leukemia (ALL) during complete remission (CR), using allele-specific markers based on junctional regions generated through the recombination of T-cell receptor δ (TCRδ), TCRγ, immunoglobulin κ (Igκ) chain or sil-tal genes. Suitable TCRδ, TCRγ, Igκ and sil-tal recombinations were identified by Southern blot analysis in 27/29 (93%) T-ALL and 152/174 (87%) precursor-B ALL patients. Two or more independent markers were available in 156/203 (77%) cases. Allele-specific oligonucleotides (ASO) were used either as clonospecific probes or primers and reached a detection level between 10^{-4} to 10^{-6} in 192/212 (91%) recombined alleles. Six hundred and forty-one remission samples from 112 cases (20 relapsed, 92 in continuous CR) have been analyzed so far. MRD was demonstrated during a period of 6 months after diagnosis in the vast majority of patients who relapsed during the course of treatment. In contrast, 88% of the patients who remained in continuous CR became PCR-negative within 3 months of treatment. Remarkably, 58% of them achieved PCR-negativity within 1 month. Our data suggest that the MRD status at 3 and 6 months after diagnosis may represent a clinically relevant parameter for a second stratification of childhood ALL.

Introduction

Due to recent advances in the treatment of acute lymphoblastic leukemia (ALL), a clinical complete remission (CR) can be induced in the vast majority of children. A cure rate of 75% is achieved by chemotherapy alone [1, 2]. However, relapse continues to be the major reason for treatment failure, and is caused by the proliferation of residual leukemia cells that are not eradicated by therapy. Of those patients eventually cured, there is a group, as yet insufficiently characterized, who may in fact receive overtreatment which could leave them to face long-term adverse effects. In this context PCR strategies utilizing V(D)J junctions of rearranged TCR or Ig loci as clonospecific markers have been introduced for the detection of minimal residual disease (MRD) in ALL [3-5]. Current PCR protocols allow reliable detection of one leukemia cell among 10^4 to 10^6 normal cells in more than 90% of ALL cases, and are expected to provide novel criteria for the individualization of treatment modalities. The majority of previous MRD studies were performed retrospectively [6-

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8]. Although some general conclusions have been drawn from these investigations, several of the shortcomings associated with a retrospective study, such as case selection, hamper a balanced interpretation of the data. Therefore, a prospective PCR trial was initiated by the German ALL-BFM Study Group as a joint effort of the European BFM Trial. Here we report on the current status of this MRD study.

Material and Methods

Cells

After informed consent bone marrow (BM) and peripheral blood (PB) samples were obtained from 203 children with ALL (174 precursor-B ALL and 29 T-ALL) who were enrolled in the multicenter ALL trial ALL-BFM 90 of the German Berlin-Frankfurt-Münster (BFM) study group between 1991-1993. These patients were selected based on the availability of cryopreserved cell samples from initial diagnosis and complete remission. Methods and criteria for the definition of immunophenotypes have been previously described [9, 10]. Leukemia cell samples contained more than 90% of blasts.

Southern Blot Analysis

High molecular weight genomic DNA was prepared from cryopreserved cells. TCRδ, TCRγ, Igκ and sil rearrangements were identified by Southern blot analysis as reported previously [11], using the TCRDJ1, Jγ1.3, Jγ2.1, κde, κJ5 and sil probe, respectively [12-16] (kindly provided by Prof. J.J.M. van Dongen; Erasmus University Rotterdam, The Netherlands).

Sequence Analysis of Junctional Regions

Sequences of the primers used for amplification or sequence analysis of junctional regions have been described elsewhere [11, 16-18] with the exception of the Igκ primers; Igκ-I5′ (5′-AGGAGACAGAGTCACCATCAC TTG-3′), Igκ-II5′ (5′-TGGAGAGCCGGCCT CCATCTC-3′), Igκ-III5′ (5′-GGGAAAGAG CCACCCTCTCCTG-3′), Igκ-IV5′ (5′-GGC GAGAGGGCCACCATCAAC-3′), Igκ-RS5′ (5′-GTTATTCCCAAAAGCTCAATCTCAA AG-3′), Igκ-DE3′ (5′-CCCTTCATAGACCC TTCAGGCAC-3′) Igκ-DEseq (5′-TTCCTA GGGAGGTCAGACTC-3′).

To isolate junctional regions for consecutive sequence analyses 3′-PCR primers were biotinylated. PCR, isolation of biotinylated amplification products and consecutive sequence analysis were performed as described elsewhere [19-20]. All junctions were sequenced in both directions. Rearrangements on both alleles of one patient were analyzed according to Breit et al. [18].

Detection of Residual Leukemia in Complete Remission Samples

The detection level was determined semiquantitatively by comparing the strength of the signals of the remission samples with serial dilutions of the initial leukemic materials by usage of either radiolabeled clonospecific probes generated via PCR, or allele-specific oligonucleotides (ASO) derived from the junctional region as previously described [19]. ASO were used either as clonospecific probes or clonospecific primers in a second round of amplification as previously reported (19).

Results

Southern Blot Analysis

Southern blot analysis revealed a TCRγ rearrangement in 98 of 174 precursor-B ALL and 26 of 29 T-ALL. A TCRδ rearrangement was identified in 110 precursor-B ALL and 19 T-ALL cases. Eighty-eight precursor-B ALL cases exhibited Igκ rearrangements. One T-ALL showed a sil-tal recombination. Thus, at least one recombination event at the four marker loci was identified in 152 of 174 precursor-B ALL (87%) and 27 of 29 T-ALL (93%) cases. Two or more independent rearrangements were observed in 156 of the 203 ALL cases.

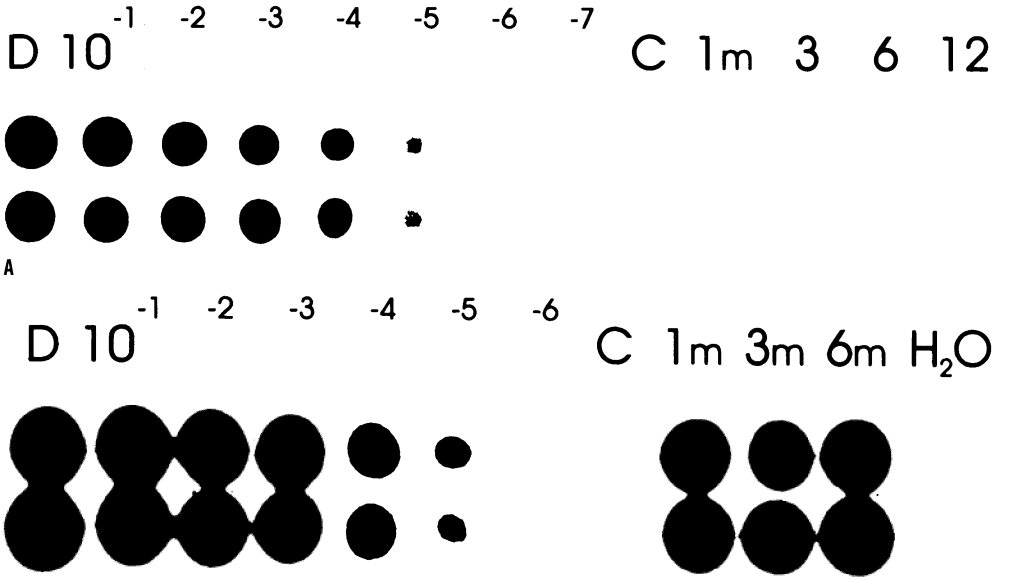

Fig. 1 A–B. Autoradiograph showing remission status with clonospecific Vδ2Dδ3 probes in two ALL patients with relapse *(A)* and in continuous complete remission *(B)*. Leukemia cell DNA obtained at initial diagnosis *(D)* was serially diluted (10^{-1}-10^{-7}) into peripheral blood cell DNA of healthy individuals *(C)*. A constantly high amount of residual blasts persists in remission samples from case A obtained 1, 3 and 6 months after diagnosis (1-6 m). Case B became PCR-negative within 1 month, and showed no evidence of MRD over a 3-year period (1 m-36). H_2O, negative control reaction without DNA

Characterization of Clonospecific Probes and Allele-Specific Oligonucleotides

In line with previous reports, common patterns of TCRγ, TCRδ and Igκ rearrangements were selected for PCR analysis of the junctional regions. Forty-four TCRγ and 16 TCRδ junctions were amplified and sequenced in 27 T-ALL cases. In 152 precursor-B ALL cases 134 TCRγ, 132 TCRδ and 109 Igκ rearrangements were confirmed by PCR and analyzed by direct sequencing.

The detection limit of allele-specific probes or oligonucleotides was tested for 212 alleles (70 TCRγ, 105 TCRδ, and 36 Igκ and 1 sil-tal recombinations). One hundred and ninety-two of the 212 tested alleles (91%) exhibited an adequate detection level between 10^{-4} to 10^{-6}. A relatively limited sensitivity of 10-3 was observed more frequently for ASOs derived from Igk junctions (22%) than for those from TCRγ (10%) or TCRδ (3%) loci.

Detection of Minimal Residual Leukemia

Six hundred forty-one BM samples were obtained from 112 children with ALL in clinical complete remission, and analyzed for the detection of MRD with independent markers. This event could be predicted by PCR in 18 of 20 relapsed cases. Strikingly, 13 cases exhibited a constantly high amount of residual blasts (Fig. 1A). At the end of the 9-month period following diagnosis 5 patients showed PCR-negativity, but demonstrated a steady increase of leukemia cells at least 3 months before clinical manifestation. These 18 patients relapsed before the completion of chemotherapy. Two cases, however, showed a long-lasting PCR-negativity until late relapse 2 and 7 months after termination of treatment.

The vast majority (88%) of the 92 patients who were in continuous complete remission became PCR-negative during the first 3 months of treatment. In fact, 58% of these cases became PCR-negative very quickly, i.e., within 1 month after the initiation of

chemotherapy (Fig 1B). With the exception of 3 cases where there were fluctuating amounts of residual blasts around the detection level of PCR, these patients never showed PCR-positive results during the clinical follow-up.

Discussion

In this prospective study we have investigated the presence of MRD in 641 BM samples obtained from 112 ALL patients who were considered to be in CR according to morphological criteria. We were able to characterize 178 rearranged TCRγ, 148 TCRδ, 109 Igκ and 1 sil-tal alleles by PCR amplification and consecutive direct sequence analyses. To avoid false negative results due to changes in TCR and Ig recombination patterns during follow-up, at least two independent markers should be used to confirm results from a single PCR approach in each ALL case. Although the frequency of sil-tal recombination in this study was much lower (1/29 T-ALL) than reported previously [16], our combination of target genes appears to be quite useful, since 77% of our ALL cases exhibited two or more independent targets for the detection of MRD.

The sensitivity of the clonospecific probes and primers was examined in 212 alleles, and 91% of them achieved a detection level of at least 10^{-4}. A relatively low detection level of 10^{-3} was shown in 22% of the allele-specific oligonucleotides derived from Igk junctions. Since the Igk marker was introduced quite recently [15], its overall relevance for the detection of MRD in B-lineage leukemias remains to be defined.

In this study, the vast majority of patients in continuous CR became PCR-negative within 6 months after diagnosis. This contrasts with results obtained in retrospective analyses from several laboratories, including ours, that indicated a rather prolonged decline of residual leukemia cells during maintenance therapy in about one third of the continuous CR patients. After termination of treatment patients are generally PCR-negative. This contrasts with a recent study [21], showing frequently persistent leukemia cell populations in children with ALL in remission after the cessation of chemotherapy. This discrepancy might be due to the sensitivity of the detection system used in the latter study, but is not readily explained.

Another interesting observation is that most of the relapsed cases showed constantly positive PCR results, while none of the patients who became PCR-negative during the first month of therapy showed disease recurrence. This disparity in MRD status between patients with relapse or in continuous CR became pronounced at 3 and 6 months after diagnosis, suggesting that results obtained at these 2 stages might serve as a stratification parameter. The PCR status determined as early as 1 month after diagnosis [22] appears to be a less suited discriminator according to our data.

Based on the data obtained in the framework of the European BFM, it appears reasonable to assume that novel criteria will become available for 2 groups of patients – those who achieve lasting PCR – negativity within 4 weeks of remission induction and those who exhibit a constantly high leukemia load during the initial months of treatment. For the first group, we may now have a rationale for individualized reduction/shortening of treatment, while for the second one intensified strategies may be considered.

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Clonal Chromosomal Abnormalities in the Stem Cell Compartment of a Patient with Acute Myeloid Leukemia in Hematological Complete Remission

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Abstract. In 70-80% of the patients with AML complete remission can be achieved by intensive chemotherapy. However 50-70% of adults with AML will ultimately relapse due to regrowth of residual leukemic cells below the level of hematological detectability. We now were able to examine the bone marrow of a patient with de novo AML M2 add(2)(q37) in hematological complete remission. The CD34+/CD38- and CD34+/CD38+ subpopulations were isolated by FACS (purity >98%) and analyzed by conventional cytogenetic analysis. In 1 of 4 metaphases the clonal chromosomal abnormality was detected in the CD34+/CD38- stem cell compartment. One of 17 analyzed metaphases showed the additional material in the CD34+/CD38+ subpopulation. This observation shows that cells with an immature stem cell-like immunophenotype and leukemia-specific clonal chromosomal abnormalities can survive high-dose chemotherapy in the niche of the stem cell compartment. Further studies have to be initiated to evaluate the leukemogenic potency of these cells.

Introduction

Acute myeloid leukemia arises from the clonal expansion of a malignant transformed progenitor cell [1-7]. In 70-80% of the patients complete remission can be achieved by intensive chemotherapy. However 50-70% of adults will ultimately relapse [8-9]. This suggests, that residual disease must have been present in these patients below the level of morphological detectability. Several techniques have been developed to detect minimal residual disease, such as immunophenotyping, molecular biology, conventional cytogenetic analysis, fluorescence in situ hybridization [10, 11, 13, 14, 15, 16, 21]. However the mere detection of a leukemia-specific or -associated genetic marker does not necessarily indicate minimal residual disease. Using qualitative reverse transcriptase-polymerase chain reaction (RT-PCR) a persistence of detectable levels of AML1-ETO transcripts in patients in long-term remission of AML with t(8;21) was shown [21]. On the other hand, leukemic progenitor cells may be indistinguishable from normal hematopoietic progenitors. They express CD34 and lack CD38 [18-20]. Recent studies showed an involvement of early progenitors characterized by a CD34+/CD38- immunophenotype in the leukemic process [2, 3, 4, 5, 7]. So far there are no experimental data whether cells with an immature stem cell-like immunophenotype and leukemia-specific clonal chromosomal abnormalities can survive intensive chemotherapy in the niche of the stem cell compartment.

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Patient and Methods

Patient

The bone marrow aspirate of a 46 year old male patient with de novo AML M2, 46, XY, add (2) (q37) in hematological complete remission was selected for sorting procedures. He had no history of prior hematological disorders and had not taken any regular medication nor chemotherapy. The patient was diagnosed as a de novo AML 31 months before and is still in continuous complete hematological remission. Diagnosis and classification of AML were based on light microscopy of Pappenheim stained slides, and on cytochemical reaction with periodic acid schiff (PAS), myeloperoxidase, and esterase. Slides were reviewed by two independant hematologists according to the criteria of the French-American-British (FAB) classification [17]. Treatment was initiated according to the protocol of the German AML Cooperative Group [8, 9] and consisted of a double induction course with TAD 9 followed by HAM and a consolidation course with TAD 9. Cytogenetic analysis of sorted subpopulations were performed at diagnosis and in complete remission after the consolidation chemotherapy. The patient's characteristics are shown in Table 1.

Immunophenotyping

Immunophenotyping was performed at diagnosis by multiparameter flow cytometry using a whole blood lysis method and a set of monoclonal antibodies against myeloid and lymphoid lineage-associated antigens [16].

Fluorescence Activated Cell Sorting

One to two $\times 10^7$ mononuclear cells were isolated by Ficoll gradient centrifugation (Seromed, Germany), washed twice in RPMI 1640 (GIBCO, Germany) and doublestained with anti-CD34 FITC and anti-CD38 PE (Becton Dickinson, San Jose, CA, USA). The $CD34^+/CD38^-$ and $CD34^+/CD38^+$ population were sorted according to their light scatter properties and fluorescence intensity using a FACS Vantage (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm Argon laser, Lysis software. Sort purity was >98%. Figure 1 shows the four dimensional flow cytometric analysis of the patient at diagnosis and in CR.

In Vitro Culturing and Cytogenetic Analysis

The FACS isolated cells were incubated at 37 °C for 72 h in RPMI 1640 (GIBCO, Karlsruhe, Germany) supplemented with 20% FCS (HyClone, Logan, USA), 100 U/ml recombinant human (rh) granulocyte colony-stimulating factor (Amgen, Thousand Oaks, CA), 100 U/ml glycosylated rh granulocyte-macrophage colony-stimulating factor (Behringwerke, Marburg, Germany), 100 U/ml rh interleukin-3 (Behringwerke), 1 U/ml rh erythropoietin (Boehringer, Mannheim, Germany), and 50 ng/ml rh stem cell factor (Genzyme, Boston, MA). Cells were incubated with 0.3 µg/ml colcemide (GIBCO, Germany) for 12 h. Chromosome preparation and staining by a modified GAG-banding technique have been described previously. The karyotypes were classified according to the International System of Chromosome Nomenclature (ISCN) [22]. Figure 2 shows the metaphase of the CD34+/CD38- subpopulation of the patient in CR.

Results

We analyzed the bone marrow from a patient with AML M2 46 XY add(2)(q37) at the time of initial diagnosis and in hematological complete remission. Immunophenotyping at diagnosis showed a dominant blast population with expression of CD11b, CD11c, CD13, CD33 and HLA-DR. An aberrant expression of CD7 was detected, as well as a high expression of CD34 with a significant proportion of CD34+/CD38-/HLA-DR- blasts. Immunophenotyping during complete remission revealed a persistence of a very small proportion of aberrant CD7-positive cells. Three samples were analyzed at both timepoints: one unsorted sample, the CD34+/CD38- and the CD34+/CD38+ subpopulations. The percentage of CD34+/

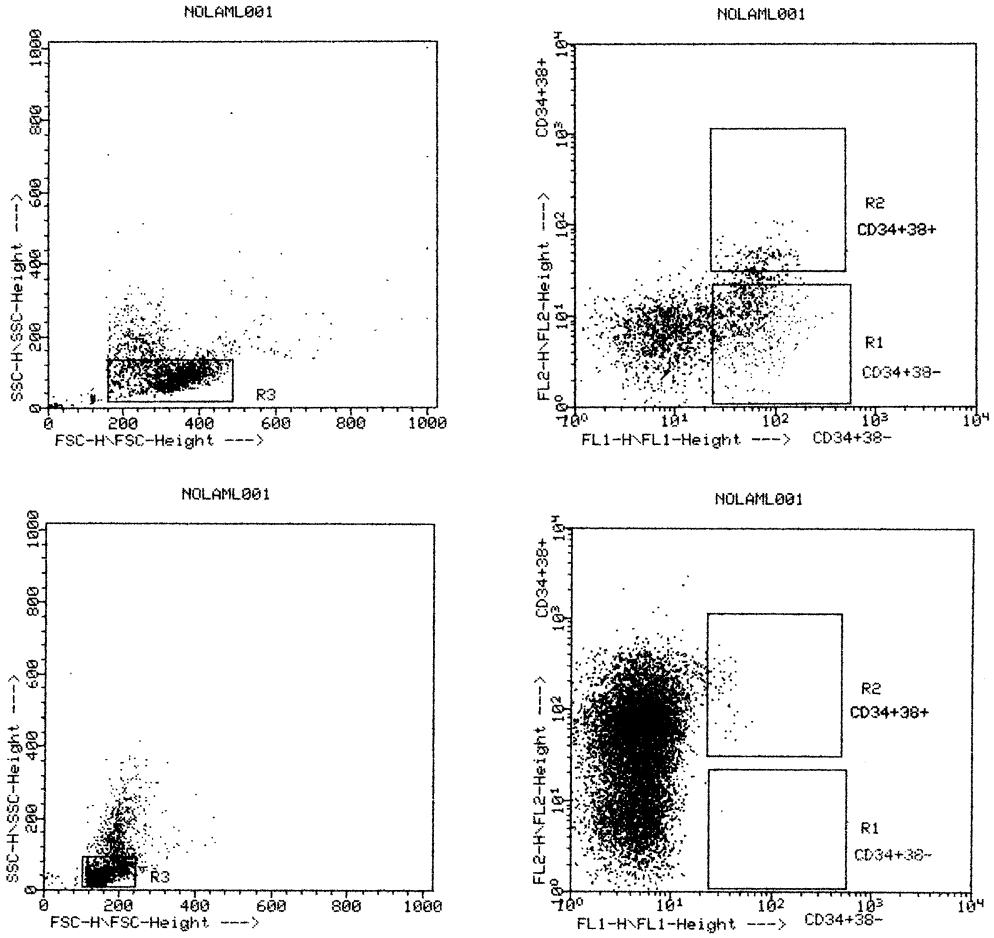

Fig. 1. a Diagnosis. b Complete Remsission. Four dimensional flow cytometric analysis of the bone marrow from the patient *a* at primary diagnosis and *b* in hematological CR; 10 000 cells were acquired in listmode on a FACScan with two gates showing the two different maturational subpopulations (R1:CD34+/CD38-; R2:CD34+/CD38+) that were sorted in concordance with their light scatter characteristics (R3)

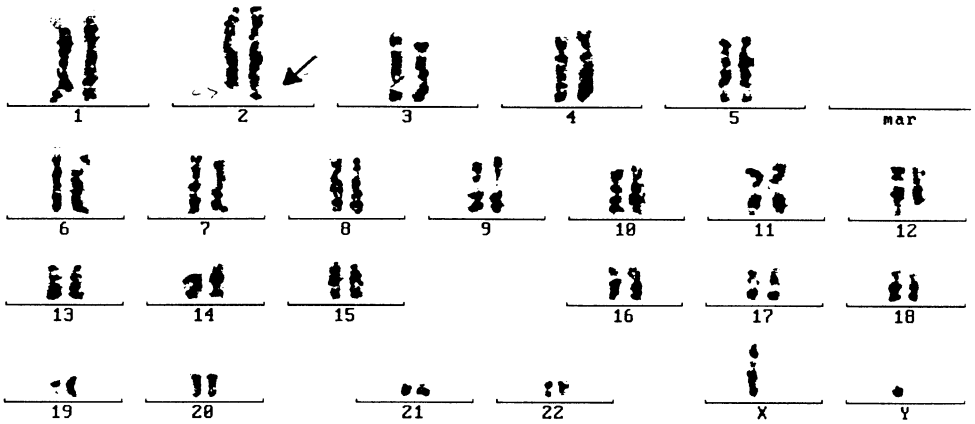

Fig. 2. Giemsa-banded karyotype of a flow-sorted CD34+/CD38- bone marrow cell from the patient with AML M2 in CR, showing 46, XY, add (2)(q37)

Table 1. Cytogenetic analysis of a patient with AML M2 at diagnosis and in CR, showing the patient's characteristics, the sorted subfractions with their percentage and the metaphases analyzed.

Time of analysis	Karyotype unsorted	Metaphases analyzed	Sorted sub-populations	Cells after sort. ($\times 10^3$)	Percentage of sorted sub-populations	Karyotype of sorted cells	Metaphases analyzed
Diagnosis	Normal	16	CD34+/38-	55	30.4	Normal	0
	add(2)(q37)	18				add (2q)	2
			CD34+/38+	60	7.7	Normal	3
						add (2q)	8
CR	Normal	19	CD34+/38-	4.65	< 0.1	Normal	4
	add(2)(q37)	7				add (2q)	1
			CD34+/38+	6.0	0.3	Normal	17
						add (2q)	

CD38- cells was 30.4% at diagnosis and < 0.1% in CR showing a high expression of CD34 at diagnosis and a normal distribution in CR. The cellular yield of sorted cells ranged from 55 000 and 60 000 at diagnosis and 4650 and 6000 in CR. At diagnosis we found a mosaic with 16 normal and 18 abnormal metaphases in the unsorted sample, whereas in the CD34+/CD38- stem cell compartment only two abnormal metaphases were detected. The CD34+/CD38+ subpopulation revealed again a mosaic with 3 normal and 8 abnormal metaphases. In complete remission we found 19 normal and 7 abnormal metaphases in the unsorted sample, 4 normal and 1 abnormal metaphases in the CD34+/CD38-, 17 normal and 1 abnormal metaphases in the CD34+/CD38+ subpopulation. The results are summarized in Table 1.

Discussion

Acute myeloid leukemia arises from the clonal expansion of a malignant transformed progenitor cell. In 70-80% of the patients complete remission can be achieved by intensive chemotherapy. However 50-70% of adults will ultimately relapse. First clinical data of autologous transplantation of progenitor cells isolated from the bone marrow or peripheral blood were not indicative for a significant improvement over conventional therapy [23]. These clinical data were supported by gene marker studies demonstrationg residual leukemic cells in the retransfused stem cell autograft [11]. We analyzed the FACS sorted stem cell population of a patient with AML M2 46, XY, add (2)(q37) in hematological remission to look for a persistance of the leukemia-specific aberration and for the stage of maturation in which residual leukemic cells may be found. Our findings show that the analysis of highly purified early progenitor cells in this patient is possible even if the number of sorted cells is very small. The leukemia-specific clonal chromosomal abnormality was found in the stem cell compartment at diagnosis as well as in hematological complete remission. This result may have substantial implication for the monitoring of residual disease in acute leukemia by multiparameter flow-cytometry. Stem cells which seem normal according to their immunophenotype might harbour a reservoir for relapse-inducing leukemic cells. Further analysis have to be made to eluminate the clinical relevance of these persistent leukemic cells as well as their importance for future clinical treatment especially autologous peripheral stem cell transplantation.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Detection of Minimal Residual Disease in Patients with Acute Myeloid Leukemia

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Abstract. Patients (pts) with acute myeloid leukemia (AML) relapse at a significant rate and disease recurrence cannot be predicted accurately with current laboratory methods. Multiparameter flow cytometric analysis permits sensitive detection of leukemic cells in a heterogeneous bone marrow (BM). The aim of our study was the detection of minimal residual disease (MRD) in AML, correlation of MRD with subsequent relapse and duration of the 1st complete remission (CR). We investigated samples of BM from 37 pts with AML before treatment using two-color flow cytometric analysis and panel of monoclonal antibodies for the detection of cell-surface leucocyte antigens (T-cell, B-cell, and myeloid associated antigens). An aberrant leukemic immunophenotype (lymphoid and myeloid antigen coexpression, asynchronous expression of early and mature myeloid antigens) was defined in 78% of AML pts. Twenty pts were followed serially during induction/consolidation and during continuous CR. BM samples were obtained after peripheral blood recovery. Leukemia samples were followed based on abnormal antigen combinations present at diagnosis on the blasts cells and we counted the percentage of aberrant cells present during CR and established a threshold level for the presence of AML cells. Eight pts with MRD (> 0.12% cells positive for abnormal antigen expression) had short CR (median 4.7, range 3-6 months). Twelve pts below the MRD threshold (≤ 0.12% residual AML) achieved longer periods of CR (median 11.7, range 8-16 months). Six pts remain alive in continuous 1st CR. The threshold level of MRD (0.12%) was based on clinical observation and confirmed by statistical analysis using 3 parameter probability modeling. These parameters included the threshold level of residual AML cells in BM samples and the mean CR duration in patients above and below the threshold level. We confirm the method to detect residual AML cells during 1st CR and have developed a model to predict the length of CR. Long-term followup of a large cohort of AML pts will be necessary to confirm the utility of this model.

Introduction

Patients with acute myeloid leukemia (AML) relapse at a significant rate and disease recurrence cannot be accurately predicted with current laboratory techniques. Recurrent disease is presumably due to residual leukemic cells that have resisted induction chemotherapy (Drach et al. 1992). With the application of multiparameter flow cytometry, polymerase chain reaction, fluorescence in-situ hybridization analysis, the identification of small amounts of leukemic cells below the level of detectability by the routine methods (morphologic examination) became possible. Multiparameters flow cytometric analysis permits a sensitive detection of leukemic cells within a heterogeneous

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bone marrow cell population. In two or three color immunofluorescence analysis the leukemic cells demonstrate multiple differences from their presumed physiological counterparts due to different light scattering properties and an aberrant antigen expression (Terstappen et al. 1991). These differences allow to distinguish the leukemic cells from normal and to detect in remission bone marrow the residual tumor cells with an aberrant immunophenotype revealed at diagnosis. Recently, it was proved that the presence of these cells in complete remission (CR) correlates with a high probability of relapse.

The aim of the present study was to detect in CR bone marrow by means of flow cytometry the leukemia cells that survived the chemotherapy for AML (minimal residual disease) and to find the correlation of MRD with a probability of relapse and duration of the 1st CR.

Material and Methods

Patients

Immunophenotyping of de novo AML blasts was performed before any treatment in 37 AML patients. The diagnosis of AML was established according to FAB classification. There were 14 patients with M2, 6 with M3, 15 with M4, 2 with M5; 12 of them were males and 25 females. The median age was 37 years (range: 16-61). All patients were included in the Russian AML multicenter trial and randomized at diagnosis to 7+3 protocol (daunorubicin 45 mg/m^2 i.v. on days 1-3 and Ara-C 100 mg/m^2 i.v. bid 1-7 days; 4 courses), or to 7+3+VP-16 (VP-16 120 mg/m^2 i.v. was added to 7+3 on day 17 through 21 of the course). After the completion of 4 induction/consolidation courses maintenance treatment was started and conducted for 3 years of CR.

The samples of 20 AML patients were studied at diagnosis, in a morphologic remission after induction/consolidation and then during continuous CR. Complete remission was defined at less than 5% blasts in the normocellular bone marrow, normal peripheral blood count, the absence of extramedullary leukemia with the persistance of these characteristics for more than 1 month.

Preparation and Staining of AML Cells at Leukemia Presentation

Heparinized marrow aspirate samples taken at diagnosis were diluted in phosphate-buffered saline (PBS) and mononuclear cells were isolated by Ficoll separation technique, following by two washes with PBS, lysis of red blood cells 0.83% ammonium chloride. Washed mononuclear cells were incubated for 30 min with an array of directly fluoresceinisothiocyanate (FITC) and phycoerythrin (PE) labeled monoclonal antibodies (Mo Abs) against myeloid, lymphoid, progenitor cells antigens with appropriate isotype-matched negative controls. After incubation cells were washed twice, and analysed by two-color flow cytometry.

Preparation and Staining of the Patients' CR Bone Marrows and Bone Marrow Cells from Healthy Donors

Heparinized bone marrow aspirates were obtained from CR patients after consolidation (2-3 courses) and during continuous remission at time of peripheral blood recovery after chemotherapy. Control bone marrow aspirates were obtained from three allogenic bone marrow transplant donors. Mononuclear cells were isolated by Ficoll separation technique. Remission AML samples were analysed basing on abnormal antigen combinations on the blast cells revealed at diagnosis. Aberrant leukemic immunophenotype included: the coexpression of lymphoid and myeloid antigenes, the asynchronic expression of early and mature myeloid antigens. Appropriate pairs of directly labeled MoAbs were used against atypical marker combinations.

Flow Cytometric Analysis of Bone Marrow Samples

This was performed on EPICS-C flow cytometer (Coulter Corp.). The 488 nm line of

Table1. Antibodies used for immunophenotyping

Antibody	FITC/PE	CD	Ig class	Company
Birma-K3	FITC	CD34	G1	DAKO
HPCA-2	PE	CD34	G1	Becton Dickinson
Bra-55	FITC	CD45	G1	Sigma
DU-HL60-3	PE	CD15	M	Sigma
Leu-M7	PE	CD13	G1	Becton Dickinson
Leu-M3	FITC	CD14	G1	Becton Dickinson
Leu-M9	PE	CD33	G1	Becton Dickinson
Leu-9	FITC	CD7	G1	Becton Dickinson
UCHT-2	FITC	CD5	G1	Sigma
G-11	PE	CD2	G1	Caltag
My-7	PE	CD13	G1	Coulter
SJ5-1B4	PE	CD10	G1	Caltag
MT310	PE	CD4	G1	DAKO
HD-37	PE	CD19	G1	DAKO
VIM-2		CDw65	M	Behring
JC159	FITC	Glycophorine A	G1	DAKO
Mouse control	FITC		G1	DAKO
Mouse control	PE		G1	DAKO
Mouse control	FITC		G2	Becton Dickinson
Mouse control	FITC		M	Sigma

the standard water-cooled argon laser was used for exitation, at 500 mV power output. FITC and PE fluorescence emission were collected through 530 nm SP, 590 nm LP filters and beam splitter 550 nm. Compensation for fluorescence emission overlap of each fluorochrome into inappropriate channels was set using single-labeled samples. The leukemic blast population from the diagnosis sample was characterized by forward and side light-scatter properties and gated. Remission samples gating on forward versus side light-scatter was used to enrich for the lymphocyte+blast populations; 10 000 events were counted in each sample.

Statistical Analysis

The remission duration in patients with greater than the 0.12% positive for aberrant immunophenotype cells in remission were compared with those with ≤ 0.12% positive cells in remission by the method of Kaplan and Meier. The differences were analyzed by the logrank chi-squared test. The threshold level of MRD (0.12%) was confirmed by statistical analysis using 3 parameters probability modeling. These parameters included the threshold level of residual AML cells in bone marrow samples and the mean CR duration in patients with residual cells above and below the threshold level.

Results

Aberrant Leukemic Immunophenotype was Defined in 78% (29 of 37) of all Cases of AML

Aberrant expression of antigens included following combinations: coexpression of lymphoid CD2, CD5, CD7, CD19 with myeloid antigens; asynchronous expression of early CD34 and mature CD13, CD14, CD15, CDw65 antigens. Coexpression of T lym-

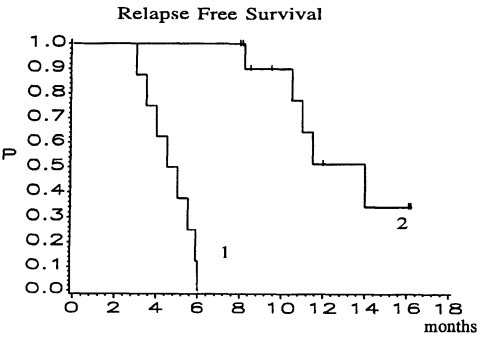

Fig 1. 1 Residual leukemic cells, N = 8 (level > 0.12%); 2 no residual leukemic cells, N = 12 (level ≤ 0,12%); $p = 0.001$

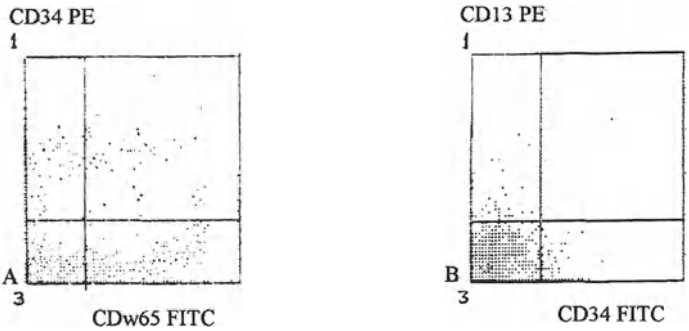

Fig 2 A-B. Bone marrow cells with atypical antigen expression in CR patients with AML determined by double color flow cytometry. Cells positive for both antigens are shown in quadrant 2. *(A).* 0.69% of the residual cells coexpress CD34 and CDw65 in patient with short remission duration. *(B).* 0.01% of the residual cells coexpress CD34 and CD13 in patient with long-term remission.

phoid (CD2, CD5, CD7) with myeloid markers occurred in 7 of 20 patients (35%), followed during remission. Coexpression of B lymphoid marker (CD19) with myeloid markers occured in 3 (15%) patients. Asynchronous expression of myeloid antigens was found in 14 of 20 patients (70%).

Remission AML marrow samples were analysed by two-color flow cytometry for detection of residual leukemic cells expressing the presentation aberrant immunophenotypes. The percentages of aberrant cells in the gated region were counted. Afterwards the threshold level for the presence of MRD was established and it appeared to be 0.12% of positive for aberrant expression tumor cells in CR. Eight patients with MRD had >0.12% cells positive for abnormal antigen expression and CR duration was short in them – median 4.7 months (range 3 to 6 months). Twelve patients with ≤ 0.12% residual AML cells (below the MRD threshold), achieved long CR duration with median of 11.7 months (range 8 to 16 months). Six of them remain alive in the first continuous CR. The data were analyzed by the method of Kaplan and Meier and results shown in Fig 1.

Two-color flow cytometry scans of two patients in remission are presented in Fig 2.

The analysis of three normal bone marrow samples by means of two-color flow cytometry did not show any aberrant immunophenotype.

Statistical Model

The threshold level of MRD (0.12%) was established basing on clinical observation and confirmed by statistical analysis.

Let t be survival time and x be a prognostic variable. In our case t is remission duration, x is number of residual cells.

Suppose that there is a threshold level q. If $x \le g$ for some individuals then the predicted mean survival time is $\mu 1$ and if $x > g$ then the predicted mean survival time is μ_2. Also suppose that survival function is exponent in both cases.

That is we have following 3 parametric model for survival function:

$$S(t) = \begin{cases} e^{-t/\mu_1}, x \le q \\ e^{-t/\mu_2}, x > q \end{cases}, \quad (1)$$

Let $\{(x_i,t_i,c_i), i = 1,...,n\}$- be the sample of n observation, where x_i is the number of cells, t_i is the survival time (exact or survival), and c_i is the censor indicator (c = 0 for uncensored survival times, c = 1 for censored times).

The likelihood function for (1) will be following [1]:

$$L(\mu_1,\mu_2,q) = \prod_{i:c_i=0, x_i \le q} \frac{1}{\mu_1} e^{-t_i/\mu_1} \prod_{i:x_i=1, x_i \le q} \frac{1}{\mu_1} e^{-t_i/\mu_1} \prod_{i:x_i=0, x_i > q} \frac{1}{\mu_2} e^{-t_i/\mu_2} \prod_{i:c_i=1, x_i > q} \frac{1}{\mu_2} e^{-t_i/\mu_2}, \quad (2)$$

With fixed parameter q, the maximum likelihood estimations of μ_1, μ_2 are

$$\hat{\mu}_k = \frac{T_k}{r_k}, (k = 1,2), \quad (3)$$

where

$$T_1 = \sum_{i:x_i \le q} t_i, \quad \text{is the sum of all times for individuals with } x \le q, \tag{4}$$

$$r_1 = \sum_{i:x_i \le q} (1 - c_i), \quad \text{is the number of uncensored times for individuals with } x \le q, \tag{5}$$

$$T_2 = \sum_{i:x_i > q} t_i, \quad \text{is the sum of all times for individuals with } x > q, \tag{6}$$

$$r_2 = \sum_{i:x_i > q} (1 - c_i) \quad \text{is the number of uncensored times for individuals with } x \le q. \tag{7}$$

So the maximum of likelihood function by two parameters (μ1, μ2) will be function of parameter q:

$$\max_{\mu_1,\mu_2}(L) = \left(\frac{r_1}{T_1}\right)^{r_1}\left(\frac{r_2}{T_2}\right)^{r_2} e^{-(r_1+r_2)}, \tag{8}$$

where $(r_1+r_2) = r$ is the total number of uncensored observations is constant by q.

To estimate the value q it needs to maximize the independent by q part of (8):

$$l(q) = \left(\frac{r_1}{T_1}\right)^{r_1}\left(\frac{r_2}{T_2}\right)^{r_2}. \tag{9}$$

Function (9) is step wise function, it changes in the points $q=t_1,t_2,...,tn$.

If the sequence (t1,..,tn) is sorted by its values then (9) could be rewritten as:

$$lm = \left(\frac{m - \sum_{i=1}^{m} c_i}{\sum_{i=1}^{m} t_i}\right)^{(m - \sum_{i=1}^{m} c_i)} \left(\frac{n - m - \sum_{i=m+1}^{n} c_i}{\sum_{i=m+1}^{n} t_i}\right)^{(n - m - \sum_{i=1}^{m} c_i)}, (m = 1,2...n). \tag{10}$$

Maximum likelihood estimation of thresh level is the point where (10) reaches the maximum value. That is

$$\hat{q} = t_M: M = \arg\max(l_m). \tag{11}$$

Algorithm.The values (10) can be easily programmed. It can be realized as EXELL table and likelihood maximum will be estimated graphically as it will be shown below. It is more convenient to use the following function as a criteria of minimizing:

$$Q = -Ln(l_m). \tag{12}$$

Data and Results

The results of observations of 20 patients are presented in the Table 2. The row corresponding to maximum likelihood estimation is expressed by color. So the maximum likelihood estimation of threshold level of residual number cells is:

$\hat{q} = 12 \cdot 10^{-5}$

Corresponding estimations of mean remission duration in two groups of patients are

$\mu_1 =$ 1.7 months for group with $q > 12$

$\mu_2 =$ 25.7 months for group with $q \le 12$

Discussion

CR in acute leukemia is established when bone marrow contains <5% morphologically identifiable leukemic blasts with normal peripheral blood count and abcense of extramedullary leukemia. The morphological method has its limitation as 1-5% blasts in the bone marrow may correspond to a total

Table 2. Remission durations of 20 patients

No. of patient	No. of residual cells by 10000	Remission durations	Censor indicator	Criteria value
1	1	8	1	66.22
2	1	8.5	1	65.52
3	1	12	1	64.47
4	3	8.1	1	63.71
5	4	9.5	1	62.76
6	5	11	0	60.87
7	6	14	0	59.17
8	7	8.2	0	58.30
9	9	16.2	1	56.92
10	11	11.5	0	56.12
11	12	16.1	1	54.07
12	12	10.5	0	53.45
13	14	5.9	0	53.78
14	18	3	0	54.81
15	24	3.5	0	55.96
16	26	4	0	57.27
17	34	6	0	58.62
18	50	4.5	0	60.56
19	69	5.5	0	62.95
20	220	5	0	

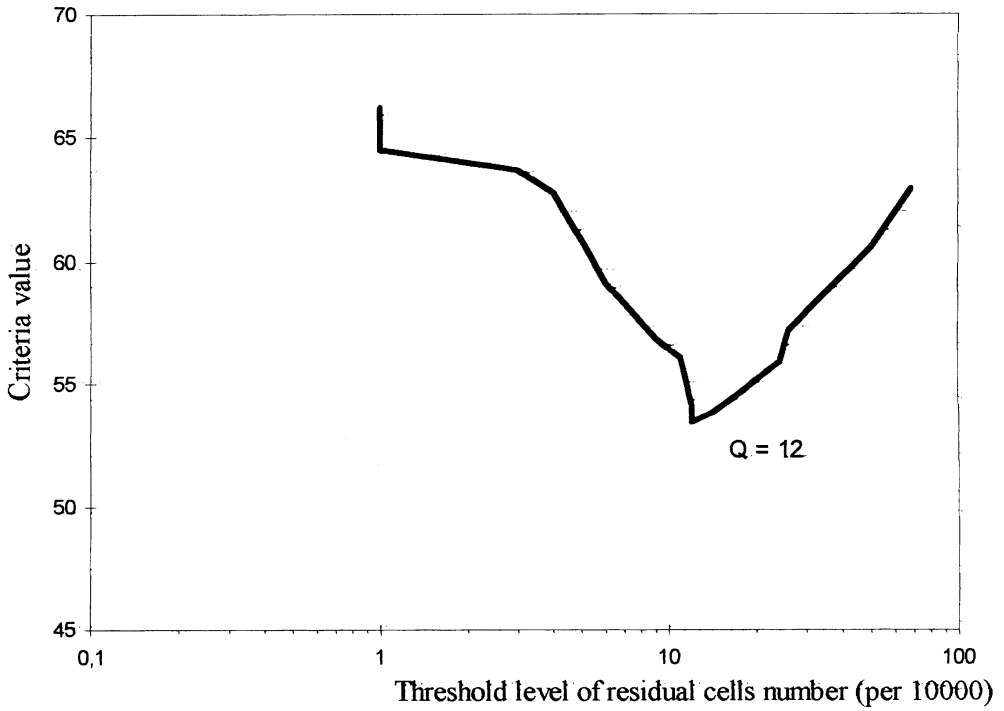

Fig. 3. Maximum likelihood estimation of threshold level of residual number cells

of 10^{10}-10^{11} neoplastic cells (Van Bekkum 1984, Ryan and Van Dongen 1988) so the sensitivity of the standard microscopy is 10^{-1}-10^{-2}. Several approaches have been devised for detecting small numbers of neoplastic cells in blood and bone marrow. Advantages of the immunologic techniques over the other available methods (cytoge-

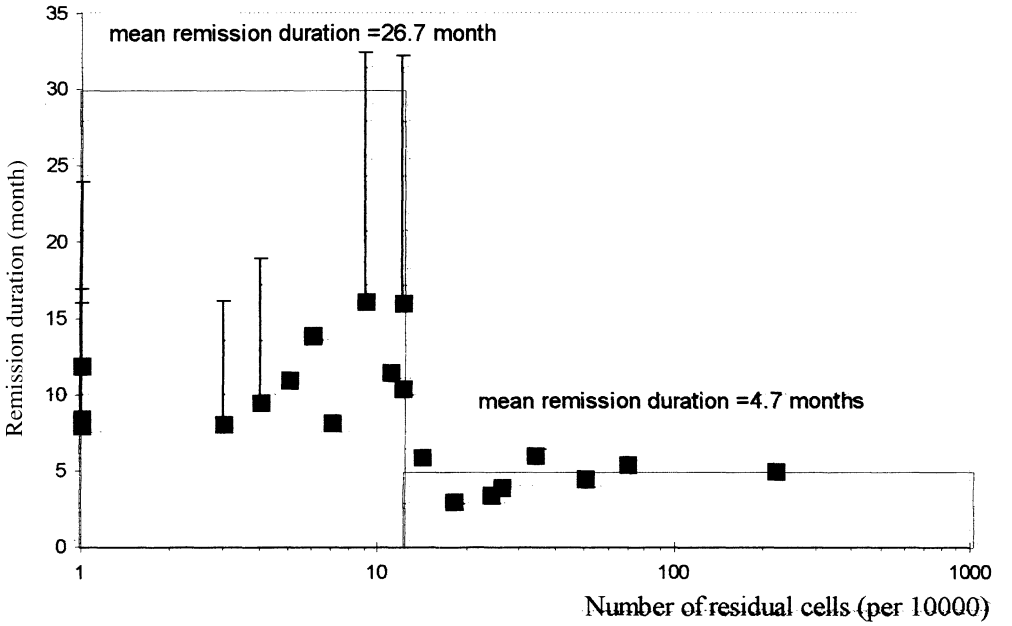

Fig. 4. Remission duration as a function of residual cells number.

netics, FISH, PCR) include speed of testing, ability to quantitate precisely the number of residual leukemic cells and to assess their viability. Aberrant expression of surface antigens allows to follow residual leukemic cells during remission. The limitation of this approach is the aberrant leukemic immunophenotype is detected not in all de novo AML cases. Only 78% of patients expressed the aberrant immunophenotype in this study. The most frequent abnormal immunophenotype found was – asynchronous myeloid antigen expression. And the most frequent lymphoid markers coexpressed with myeloid antigens were T-cell associated antigens and especially CD7.

The correlation was established between percentage of the aberrant cells and duration of the 1 st complete remission. Eight patients with abnormal cells > 0.12% had CR ≤ 6 months, twelve patients with residual cells ≤ 0.12% had CR > 6 months. The threshold level of MRD (0.12%) revealed by clinical observation was confirmed by statistical analysis. Results from various studies indicated the same correlation between the amount of residual cells and the remission duration with/without subsequent relapse. The possible occurrence of phenotypic switches during relapse may lead to false negative results (Campana 1993). In order to avoid such mistakes few abnormal combinations have to be followed simultaneously.

In conclusion, we have to confirm that the two-colour flow cytometry is a very practical and useful method for detection of small cell populations and allows to follow residual AML cells during CR. MRD predicted CR duration and probability of relapse. And the threshold level of residual tumor cells in CR was confirmed by a novel statistical model, developed in the study. Long term follow-up of a larger cohort of AML patients is necessary to confirm the utility of this model.

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RT-PCR for the Detection of Minimal Residual Disease in Acute Myeloblastic Leukemia with t(8;21) and t(15;17)

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Abstract. The reciprocal translocations t(8;21) and t(15;17) are among the most common chromosomal aberrations in patients (pts.) with acute myeloblastic leukemia (AML). Both translocations result in chimeric fusion mRNAs [AML1/ETO for t(8;21) and PML/RARa for t(15;17)] which can be detected by specific amplification with reverse transcriptase polymerase chain reaction (RT-PCR). In addition to primary diagnosis, RT-PCR can be used in these pts. for the sensitive monitoring of minimal residual disease (MRD) in complete remission (CR) after intensive polychemotherapy. In this study, we analysed 9 pts. with t(8;21) and 16 pts. with t(15;17) in CR for MRD using nested RT-PCR for AML1/ETO and PML/RARa. In the t(8;21) group 4/9 pts. became PCR-negative after induction and/or consolidation therapy; 3 of these pts. are in ongoing CR after 12 to 72 months (mo). 1 pt. relapsed after 24 mo. 5/9 pts. remained PCR-positive after chemotherapy; 3 of these pts. are in ongoing CR after 2 to 48 mo, 1 pt. relapsed after 12 mo and 1 pt. died in CR. In the t(15;17) group 15/16 pts. became PCR-negative after 1 or 2 cycles of induction chemotherapy. 13 of these pts. are in ongoing CR after 3 to 78 mo. 2 pts relapsed after 21 and 22 mo. and in both pts. clinical relapse was preceeded by the recurrence of PML/RARα fusion transcripts in morphological CR. 1/16 pts. remained PCR-positive after induction and consolidation therapy but became negative after allogeneic bone marrow transplantation and is in CR after 15 mo. Taken together, in most of the t(15;17) positive AML pts. the leukemic clone can be reduced below the detection level by aggressive chemotherapy. Because the recurrence of PML/RARa transcripts in CR is correlated with later clinical relapse, serial PCR-analyses should be performed in these pts. In contrast, a considerable proportion of AML-pts. with t(8;21) remain PCR-positive after chemotherapy apparently without correlation to clinical outcome. Further prospective studies are necessary to determine whether PCR-analyses are of clinical relevance in these pts.

Introduction

The t(8;21)(q22;q22) and t(15;17)(q22;q12) are balanced reciprocal chromosomal translocations which occur frequently in patients with de novo acute myeloblastic leukemia [1-3]. Both chromosomal aberrations result in characteristic fusion genes: The t(8;21) fuses the 5-part of the AML1-gene on chromosome 21 to the almost complete open reading frame of the ETO gene on chromo-

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some 8 and results in the transcription of a chimeric AML1/ETO fusion mRNA from the der(8) chromosome [4]. The AML1 gene encodes for a transcription factor essential for normal hematopoiesis [5]. The function of ETO is still unknown. In all AML1/ETO positive patients the breakpoints for the t(8;21) cluster within a single intron of AML1, resulting in a constant AML1/MTG8 fusion mRNA [4,6]. In the t(15;17), the gene for the retinoic acid receptor-α (RARα) from chromosome 17 is fused to the gene for PML, a putative transcription factor, on chromosome 15 resulting in a PML/RARα-fusion mRNA [7]. In contrast to AML1/ETO, three different breakpoint cluster regions within the PML-gene (bcr1, bcr2, bcr3) have been described for PML/RARα [2] while a possible correlation of these molecular differences with clinical characteristics of the patients remains controversial [8-10]. Since the genes involved in both translocations have been cloned and sequenced, the t(8;21) and t(15;17) can not only be detected by conventional cytogenetics but also by reverse transcriptase polymerase chain reaction (RT-PCR) with specific primer pairs for AML1/ETO and PML/RARα [11,12]. Using this technique, the fusion transcripts can be detected in all patients with t(8;21) and t(15;17) at initial diagnosis. Moreover, RT-PCR can be used as a sensitive tool for the detection of residual clonal cells in complete remission after polychemotherapy [11,13]. However, since the role of the chimeric genes in leukemogenesis has still to be definitely determined, the clinical significance of PCR-analyses in clinical remission is still under investigation. We studied 9 patients with t(8;21) and 16 patients with t(15;17) at various time points in hematological remission by RT-PCR and correlated the results with the clinical outcome of the patients.

Material and Methods

Leukemic Blasts and Remission Bone Marrow Samples

Bone marrow samples were obtained from 9 AML-patients with t(8;21) and 16 patients with t(15;17) at initial diagnosis and at various time points during/after chemotherapy. The leukemic blasts/bone marrow mononuclear cells were enriched by centrifugation over a Ficoll-Isopaque gradient (1.077 g/ml). The cells were used either freshly or after storage in liquid nitrogen (−196° C).

PCR Analysis

Total cellular RNA was extracted from 10^7 cells using the Trizol-method (GibcoBRL) according to the manufacturers guidelines. cDNA was synthesized for 1 h at 37 °C using 2 μg RNA directly after isolation, random primers at a concentration of 1 μmol/l and murine Moloney virus reverse transcriptase in a total volume of 20 μl. The primers for the amplification of the PML/RARα and AML1/ETO fusion transcripts [11,12] are shown in Tables 1 and 2. PCR-amplification was carried out with 1 μl cDNA in a total volume of 50 μl PCR-buffer with 0.5 μmol/l primers, nucleotides 100 nmol/l and Taq-polymerase (Perkin-Elmer) 0.02 U/μl. Thermal cycling was: 94 °C for 60 s, 57 °C for 60 s and 72 °C for 120 s with cycles repeated 35 times. After the first 35 cycles with the outer primers, 2 μl of the reaction product was further amplified for another 35 cycles with the nested primers under the same conditions. Amplification of the c-abl transcript was used as a positive control. In negative controls, the RNA template was omitted. Five μl of the reaction products were migrated on 1.5% agarose gels, stained with ethidium bromide and analysed.

Table 1. Primers for the AML1/ETO-PCR

Outer AML1-Primer	5'-AGCCATGAAGAACCAGG-3'
Outer ETO-Primer	5'-AGGCTGTAGGAGAATGG-3'
Nested AML1-Primer	5'-TACCACAGAGCCATCAAA-3'
Nested ETO-Primer	5'-GTTGTCGGTGTAAATGAA-3'

Table 2. Primers for the PML/RARα-PCR

Outer PML-Primer	5'-AGCGCGACTACGAGGAGATG-3'
Outer RARα-Primer	5'-CATGTTCTTCTGGATGCTGC-3'
Nested PML-Primer	5'-CTGGTGCAGAGGATGAAGTG-3'
Nested RARα-Primer	5'-CCATAGTGGTAGCCTGAGGA-3'

Results

PML/RARα-Positive Patients

Sixteen patients with t(15;17) were analysed for residual disease after chemotherapy (Fig. 1). 9 of these patients had a bcr3-breakpoint and 7 a bcr1-breakpoint.

Thirteen patients were studied in 1st CR (Fig. 1a). 12 of these patients (nos. 1-10, 12 and 13) became PCR negative in 1st CR after induction or consolidation therapy.

Nine of these patients are in ongoing CR after 3 to 78 months. One patient (no. 10) died in CR after allogeneic stem cell transplantation. Two patients (nos. 12 and 13) relapsed and in both clinical relapse was preceded by the recurrence of PML/RARα transcripts. One patient (no. 11) remained PCR positive in CR after consolidation chemo-

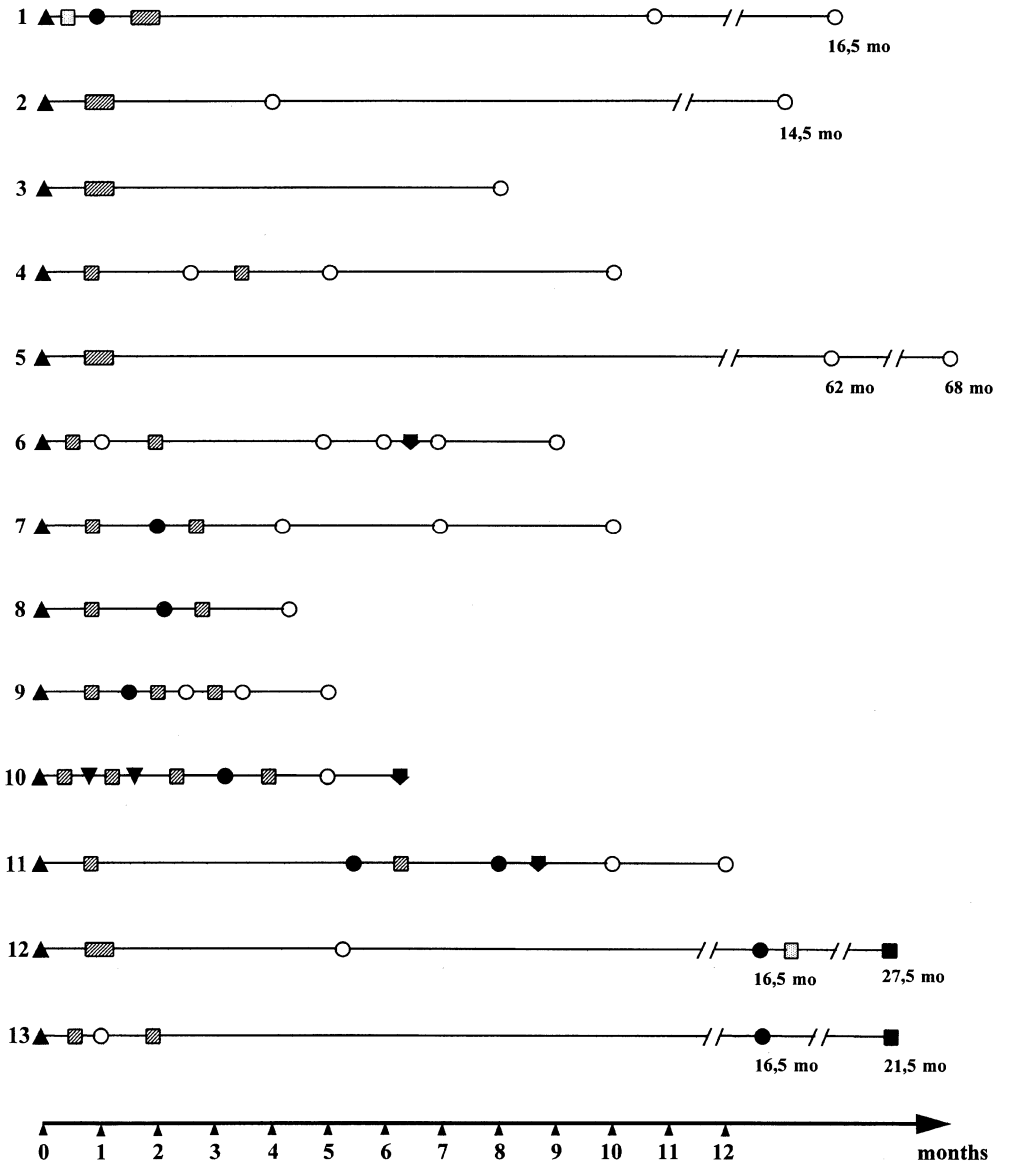

Fig. 1a. t(15;17) positive patients studied for residual disease in 1st CR

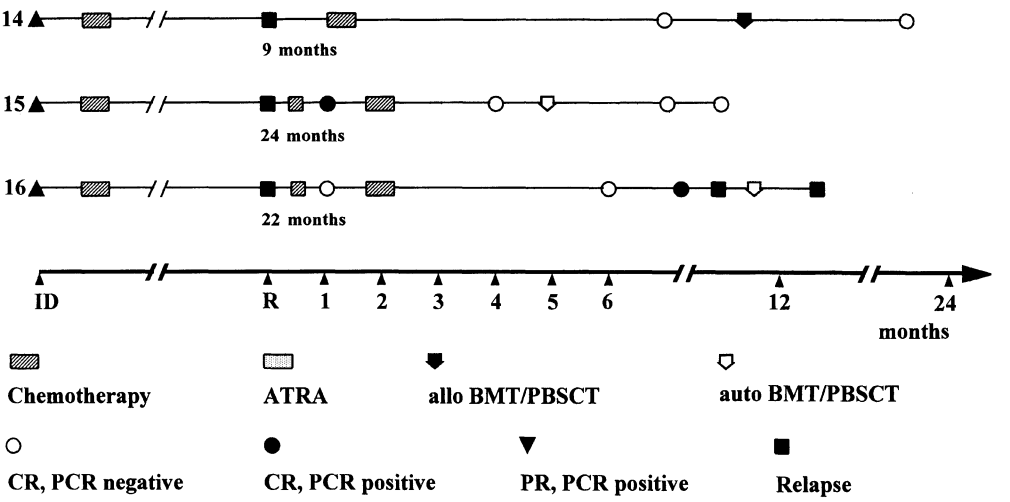

Fig. 1b. t(15;17) positive patients studied for residual disease in 2nd CR

therapy. He was allografted and is currently in ongoing CR and PCR-negative.

Three patients (nos. 14-16) were studied in relapse and 2nd CR (Fig. 1b). All 3 became PCR-negative in 2nd CR after chemotherapy. Patient 14 was allografted and is currently in ongoing 2nd CR. Patient 15 was transplanted with PML/RARα-negative autologous peripheral stem cells. He is in CR and PCR-negative 6 months after transplantation. Patient 16 became again PCR-positive and suffered subsequently from 2nd relapse.

AML1/ETO-Positive Patients

Nine patients with t(8;21) were studied for residual disease after chemotherapy (Fig. 2); 4/9 patients (nos. 1-4) became PCR-negative after induction or consolidation therapy. Three of these patients (nos. 1-3) are in ongoing 1st CR after 12 to 72 months. Patient 4 relapsed and was again PCR-negative after chemotherapy for relapse; subsequently he died in CR after allogeneic BMT; 5/9 patients (nos. 5-9) remained PCR-positive in 1st CR. Two of these patients (nos. 5, 7) are in ongoing 1st CR; 2/5 patients (nos. 8 and 9) relapsed. Patient no. 6 died in CR.

Discussion

It was the aim of this study to further analyse the prognostic value of RT-PCR-analyses in patients with AML and t(8;21) or t(15;17). All patients with t(15;17) under study were treated with intensive polychemotherapy +/- all-trans-retinoic-acid (ATRA). In contrast to patients treated with ATRA alone described by other investigators [11], in the vast majority (15/16) of the patients treated with chemotherapy the leukemic clone could be reduced below the level detectable by a two step nested RT-PCR. This was also true for 3 patients in 2nd complete remission indicating that the blasts at relapse are again sensitive to chemotherapy. All long term disease free patients under study were negative for PML/RARα. This indicates that the absence of detectable residual t(15;17) positive cells is a prerequisite for long term remission. However, 3 patients with a previously negative PCR result ultimately relapsed and in all cases relapse was preceded by a positive PCR result. In all cases the recurring PML/RARα fusions transcripts displayed the same breakpoint as at initial diagnosis strongly indicating that the relapse had emerged from residual cells from the original clone. One in 16 patients remained PCR-positive after induction and consolidation therapy but became negative after allogeneic BMT. This confirms that patients who

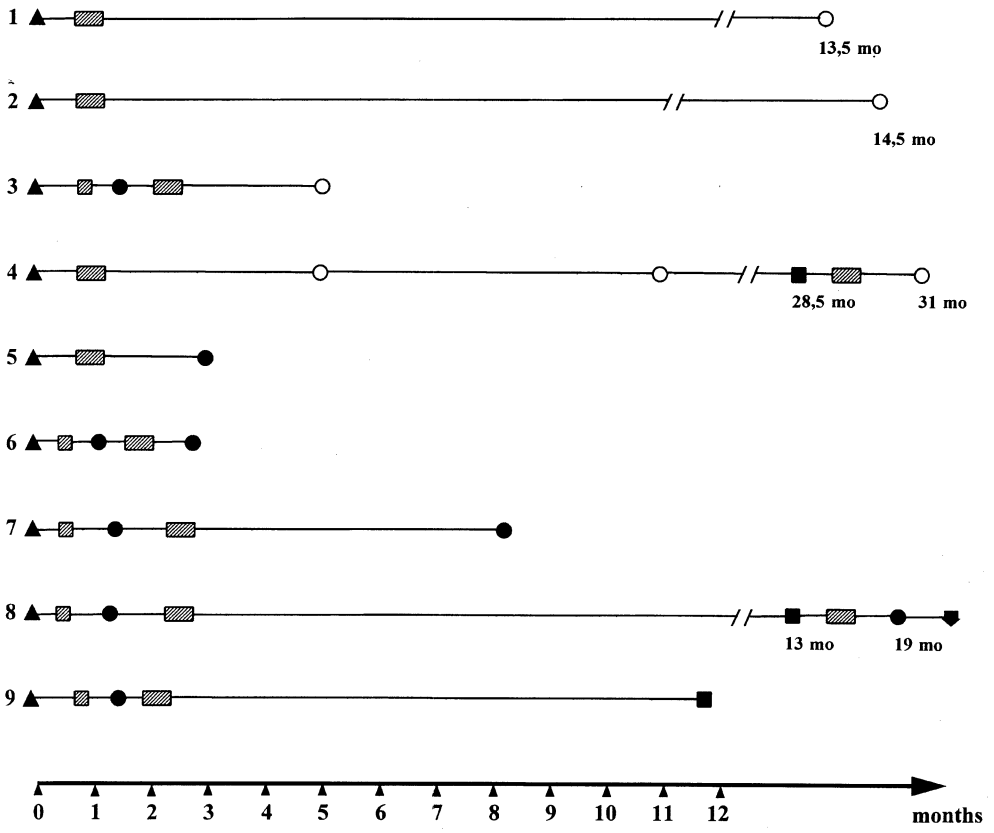

Fig. 2. t(8;21) positive patients studied for residual disease

achieve no molecular complete remission by chemotherapy might be salvaged by a further escalation of therapy.

In contrast, in the t(8;21) positive patients no clear correlation between PCR result and clinical course could be detected. Although all patients displayed identical AML1/ETO fusion transcripts at initial diagnosis, PCR-analysis revealed a heterogenous response to chemotherapy: as previously described, a considerable proportion of the patients remained PCR-positive in complete remission even after high-dose AraC chemotherapy [13]. On the other hand we and other investigators also found patients who became AML1/ETO negative after induction and/or consolidation chemotherapy [14,15]. However neither a positive nor a negative PCR result was predictive for the clinical outcome. Taken together, in t(15;17) positive patients serial RT-PCR analyses in complete remission are a valuable diagnostic tool to detect patients at high risk for relapse who might probably benefit from further intensification of therapy. As a clinical consequence, serial PCR-analyses should be performed in all t(15;17) positive patients. In t(8;21) positive patients however it is not yet clear if the heterogenous response to chemotherapy on the molecular level has clinical significance. Therefore, further prospective investigations are necessary to define the prognostic value of standard RT-PCR and other methods such as quantitative PCR [16] in t(8;21) positive patients.

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RT-PCR for the Diagnosis of Acute Myeloblastic Leukemia with Inv(16) and Detection of Minimal Residual Disease

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Abstract. One of the most common structural chromosomal aberrations in acute myeloblastic leukemia (AML) is the pericentric inversion of chromosome 16, inv(16) (p13q22) which results in the rearrangement of the CBFβ- and the MYH11-gene and the transcription of a chimeric CBFβ/MYH11 fusion mRNA. In a retrospective analysis, 329 patients (pts.) with de novo AML were evaluated for CBFβ/MYH11 fusion transcripts using reverse transcriptase polymerase chain reaction (RT-PCR). In 14/329 pts. (4%) CBFβ/MYH11 fusion transcripts could be detected. All pts. displayed the same breakpoint at bp 1921 of the MYH11-gene. In 4 pts. the inv(16) could not be detected by conventional cytogenetical analysis: 2 pts. showed a normal chromosome 16, in another 2 pts. a deletion del(16q22) was detected. Combined cytogenetical analysis and fluorescence in situ hybridization (FISH) with a-satellite probes for the chromosomes 7, 8, X and Y revealed additional numerical chromosomal aberrations in 4/14 pts. (+8, n=2; +8 and +21, n=1; +9 and +21, n=1). Five pts. were analysed for CBFβ/MYH11 fusion transcripts in complete remission (CR) after polychemotherapy. 4/5 pts. became PCR-negative after induction and/or consolidation therapy; 1 of these pts. relapsed after 10 months (mo) and was again CBFβ/MYH11 positive. After additional therapy this pt. achieved a 2nd CR but remained PCR-positive. The other 3 pts. are in ongoing CR after 10 to 94 mo. 1/5 pts. remained PCR-positive even after high-dose AraC consolidation and relapsed after 8 mo. Taken together, RT-PCR is a rapid and sensitive tool for diagnosis of inv(16) positive AML which might be more sensitive than conventional cytogenetical analysis. The clinical relevance of MRD detection in clinical CR has to be determined in prospective studies.

Introduction

The inversion 16 inv(16)(p13;q22) is a recurring structural chromosomal aberration in patients with acute myeloblastic leukemia (AML) [1]. This abnormality is highly correlated but not uniformely restricted to the FAB subtype M4eo [2]. Patients with inv(16) have been found to be associated with a relatively good prognosis especially after high-dose AraC consolidation [3,4]. This indicates that the detection of an inv(16) is important for an optimal treatment of AML patients. However, conventional cytogenetic analysis of AML blasts is hampered by the

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fact that not in all patients sufficient metaphases can be obtained and that gene rearrangements can also occur in chromosomes appearing normal by conventional banding techniques [2]. The inv(16) results in the fusion of the CBFβ-gene encoding for the β-subunit of the CBFα/CBFβ-transcription factor complex and the gene for a smooth muscle myosin protein MYH11 [5]. The cloning and sequencing of these genes allowed the detection of inv(16) by reverse transcriptase polymerase chain reaction (RT-PCR) for the chimeric CBFβ/MYH11 fusion mRNA (6). Moreover, RT-PCR can be used for the sensitive detection of residual clonal cells after chemotherapy [7]. In this study, we used RT-PCR for CBFβ/MYH11 fusion transcripts for the diagnosis of inv(16) in a series of 329 unselected AML patients. In addition, the CBFβ/MYH11 positive patients were investigated for further numerical chromosomal aberrations and the clinical relevance of PCR analyses in complete remission was analysed.

Material and Methods

Leukemic Blasts and Remission Bone Marrow Samples

Bone marrow samples were obtained from patients with de novo AML at initial diagnosis and at various time points during/after chemotherapy. The leukemic blasts/bone marrow mononuclear cells were enriched by centrifugation over a Ficoll-Isopaque gradient (1.077 g/ml). The cells were used either freshly or after storage in liquid nitrogen (-196 °C).

PCR Analysis

Total cellular RNA was extracted from 10^7 cells using the Trizol-method (GibcoBRL) according to the manufacturers' guidelines. cDNA was synthesized for 1 h at 37 °C using 2 μg RNA directly after isolation, random primers at a concentration of 1 μmol/l and murine Moloney virus reverse transcriptase in a total volume of 20 μl. The primers for the amplification of the CBFβ/MYH11 fusion transcripts [6] are shown in Table 1. PCR-amplification was carried out with 1 μl cDNA in a total volume of 50 μl PCR-buffer with 0.5 μmol/l primers, nucleotides 100 nmol/l and Taq-polymerase (Perkin-Elmer) 0.02 U/μl. Thermal cycling was: 94 °C for 60 s, 58 °C for 60 s and 72 °C for 120 s with cycles repeated 40 times. Amplification of the c-abl transcript was used as a positive control. In negative controls, the RNA template was omitted. 5μl of the reaction products were migrated on 1.5% agarose gels, stained with ethidium bromide and analysed.

Table 1. Primers for the CBFβ/MYH11-PCR

CBFβ-Primer	5-CAGGCAAGGTATATTTGAAGG-3
MYH11-Primer	5-CTCCTCTTCTCCTCATTCTGCTC-3

FISH Analysis

The enriched leukemic blasts from initial diagnosis were resuspended at a concentration of 5×10^6 cells/ml in PBS and subsequently sedimented onto adhesive glass slides covered with poly-lysine (Biorad). After that, the cells were fixed in methanol/acetic acid (3:1) and air dried. Thereafter the cells were dehydrated by a graded series of ethanol. DNA was denaturated by incubation for 2 min in 70% formamide, 2×SSC pH 7 at 70 °C. Denaturation was stopped by incubation in ethanol (-20 °C). Therafter the cells were hybridized overnight at 37 °C with a digoxigenin or biotin labelled α-satellite probe specific for the centromeric region of chromosome 7, 8, X or Y (Oncor, Braunschweig) in a humidified atmosphere. The cells were washed after hybridization in 0.25×SSC, pH 7 at 72 °C for 5 min. After that, the cells were washed in PBD at room temperature. Thereafter the cells were incubated with 50μl of a FITC-labelled anti-digoxigenin antibody or streptavidin for 5 min at 37 °C. After washing in 1×PBD the nuclear DNA was counterstained with propidium-iodide/antifade (1:1). The slides were analysed with a fluorescence microscope.

Table 2. Characteristics of the CBFβ/MYH11 positive patients

No. of patients	14/329 (4%)
m:f	13:1
Median age (range)	37 (20-71)
Cytogenetical analysis and FISH	- Trisomy 8 (n=2) - Trisomy 8 and 21 (n=1) - Trisomy 9 and 21 - Del16(q22) (n=2) - Normal chromosome 16 (n=3)

Results

RT-PCR for CBFβ/MYH11 Fusion Transcripts

Fourteen of 329 patients (4%) were CBFβ/MYH11 positive. All patients showed the same breakpoint at nucleotide 1921 of the MYH11-gene "type A", [6] resulting in an identical 414bp product. The characteristics of these patients are outlined in Table 2.

FISH Analysis and Conventional Cytogenetics (Table 2)

In 4/14 patients the inv(16) could not be detected by conventional cytogenetics: 2 patients showed a normal chromosome 16 and 2 patients displayed a deletion del(16q22).

Additional FISH analysis showed a trisomy 8 in 3/14 patients. Moreover, by conventional cytogenetics in 1 patient with trisomy 8 an additional trisomy 21 was found; 1 patient had a trisomy 9 and a trisomy 21.

Detection of Residual Disease by RT-PCR

In 5 patients bone marrow samples could be analysed by RT-PCR in complete remission (CR) and/or relapse of the disease. These data are outlined in Fig. 1. Four of 5 patients (Nos. 1-4) were PCR-negative after induction- and/or consolidation-therapy. 3 of these patients (No. 1-3) are in ongoing CR af-

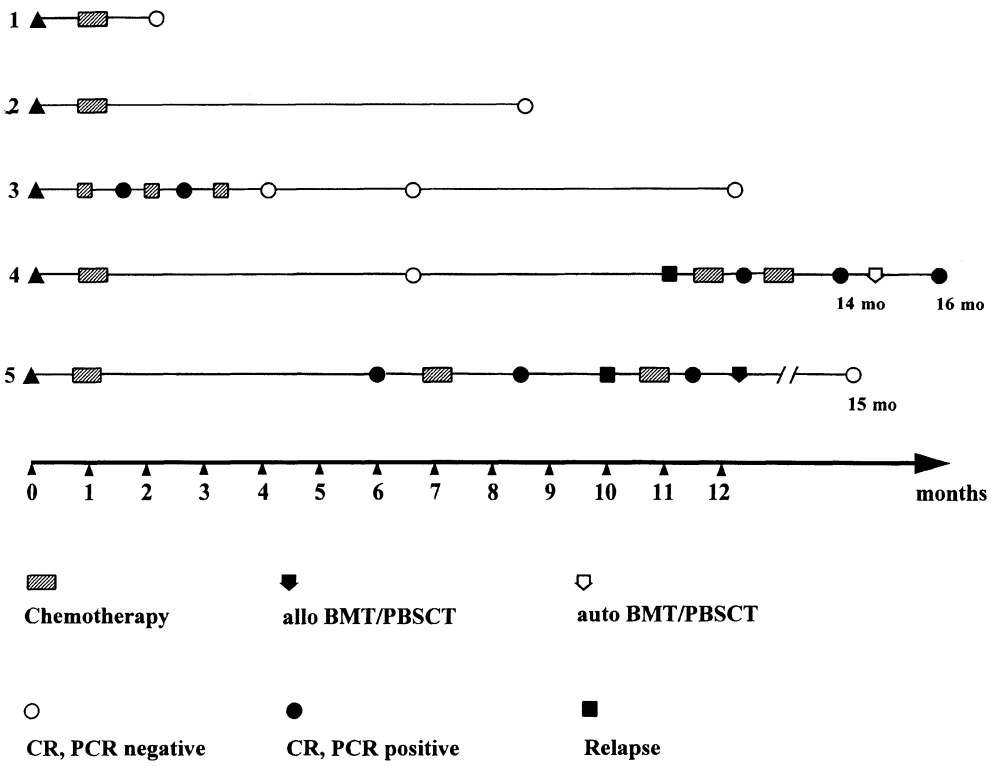

Fig. 1. Detection of residual disease in inv(16) positive patients

ter 10-94 months. The fourth patient relapsed and was again CBFβ/MYH11-positive. He remained PCR-positive in second CR after therapy for relapse including autologous peripheral stem cell transplantation. 1/5 patients (No. 5) remained PCR-positive in first CR after chemotherapy. He relapsed after high-dose-AraC consolidation, achieved a second CR but remained PCR-positive. After allogeneic peripheral stem cell transplantation he became PCR-negative but died in CR.

Discussion

We report here the use of routine RT-PCR for the detection of CBFβ/MYH11 fusion transcripts, the molecular hallmark of the inv(16) in a series of 329 unselected patients with de novo AML. Moreover, the CBFβ/MYH11 positive patients were further characterised by additional FISH and cytogenetic analysis for numerical chromosomal aberrations and in five patients RT-PCR was used for the detection of residual disease after chemotherapy. In all patients under study a sufficient amount of RNA could be obtained for molecular analysis. CBFβ/MYH11 fusion transcripts were detected in 14/329 patients (4%). As previously shown, the "type A" breakpoint at nucleotide 1921 of the MYH11 gene was also predominant in our series of patients (6). The comparison with conventional cytogenetic analysis revealed a superior sensitivity of the PCR-technique: comparable to previous studies [2,8], in 4/14 patients (29%) the inv(16) had not been detected by banding analysis but had been diagnosed as del(16)(q22) or normal chromosome 16. In 4/14 patients additional numerical chromosomal aberrations were detected by FISH and cytogenetic analysis. These aberrations involved chromosomes 8, 9 and 21. In contrast, no loss of an X- or Y-chromosome, which is frequently associated with another recurrent structural chromosomal abnormality, the t(8;21)(q22;q22) [1] and no monosomy 7 was found in the CBFβ/MYH11-positive patients suggesting a non-random pattern of the association between structural and numerical chromosomal aberrations. RT-PCR analysis in 5 patients in complete hematological remission revealed a heterogeneity between these patients with regard to response to the chemotherapy on the molecular level: Whereas in some patients the CBFβ/MYH11 positive clone could be reduced below the detection level by standard induction chemotherapy, in others, as previosly described [7,9] a positive PCR result was obtained even after high-dose AraC consolidation or autologous peripheral stem cell transplantation. However, one of these patients became PCR-negative after additional allogeneic bone marrow transplantation indicating that the leukemic clone might be eradicated by further escalation of the therapy. PCR analysis in clinical complete remission to detect patients at high risk for relapse has been used especially in patients with translocation t(15;17) where the recurrence of the characteristic PML/RARα fusion transcripts precedes clinical relapse by several months [10]. In contrast to that, in our five CBFβ/MYH11 positive patients studied for residual disease, no clear correlation between PCR results and clinical outcome could be observed in that the two relapses occurred regardless of a previous positive or negative PCR result comparable to patients with t(8;21), where AML1/ETO transcripts are frequently expressed by patients in remission [11]. Taken together, PCR-analysis for CBFβ/MYH11 fusion transcripts should be performed in all AML patients at diagnosis to allow an optimal sensitivity for the detection of this prognostically important cytogenetic aberration. Because of the high frequency of additional numerical chromosomal changes PCR-analysis should be accompanied by FISH and banding analysis. The clinical relevance of the detection of residual clonal cells in clinical complete remission as a basis for a response-adapted chemotherapy however has to be determined in prospective studies.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Monitoring of PML-RAR alpha in Patients with Acute Promyelocytic Leukemia (APL) by RT-PCR

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Abstract. Detection of the PML-RAR alpha messenger RNA in patients in clinical remission is an indicator of relapse in APL. Early salvage therapy is considered in this situation. Therefore, the PML-RAR alpha transcript status is an useful tool in monitoring the clinical course of APL.

Patients. Transcription of PML-RAR alpha was analyzed in 39 patients. 26/39 have been treated with anthracyclin/ara-C containing regimens, with or without ATRA. In 20 patients, sequential blood and/or bone marrow specimens were available in the course of the disease. In 11 patients only initial diagnostic material, and in 8 patients, material in clinical remission was available.

Methods. A set of 6 primers was used to amplify chimeric PML-RAR alpha transcripts of different genomic breakpoints by two step RT-PCR. cDNA synthesis was performed using random primed total RNA. The sensitivity of PML-RAR alpha detection was 10^{-4}. For validation, PCR products were sequenced. cDNA quality was shown by quantification of *abl*-transcripts in the same samples.

Results. L-type transcripts were amplified in 16 cases, whereas S-type transcripts were found in 9 patients. Of 17 patients who have been monitored during the course of the disease, one was treated with ATRA alone because of concomitant diseases. In this patient the PML-RAR alpha was still detectable after 6 months and 8 months after diagnosis. All other patients (n = 20) in clinical remission after chemotherapy alone or in combination with ATRA remained PCR negative 3.3-48 months after diagnosis (median, 14.5 months).

Conclusion. In all chemotherapy treated patients no molecular relapse has been observed so far. This result correlates with our clinical data.

Introduction

The acute promyelocytic leukemia (AML) is characterized by the occurrence of t(15;17), a reciprocal chromosomal translocation which fuses genes encoding the PML on chromosome 15 and the nuclear retinoic acid receptor alpha on chromosome 17 [1, 2]. This genomic rearrangement leads to the formation of two functional chimeric genes PML-RAR alpha and RAR alpha-PML [3, 4]. Transcription of these chimeric genes provides potential molecular markers that can

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be detected in leukemic cells of almost 100% of APL patients by reverse transcription polymerase chain reaction (RT-PCR) reaching a considerably greater sensitivity than conventional cytogenetics (70-80%) [5-7]. PML/RAR alpha gene rearrangement leads to two different RNA isoforms (short and long form, respectively) depending on the location of the breakpoints on chromosome 15. Furthermore one splicing variant of the L-form has been identified [3, 7]. For monitoring of the minimal residual disease detection of PML/RAR alpha fusion transcripts can be routinely used. Persistence or reappearance of a positive PCR for PML/RAR alpha after a negative period is correlated with a subsequent relapse [8]. However, a few of PCR negative patients show relapse.

This highly sensitive method is being used with the following objectives.

In supplement to cytology and cytogenetics the detection of PML/RAR alpha supports the diagnosis of the AML subtype M3. The monitoring of minimal residual disease (MRD) by the evaluation of PCR status in the course of the disease serves as prediction parameter for relapse. Furthermore, investigation of bone marrow and PBSC for PML/RAR alpha fusion transcripts can be used to assess quality of autologous transplants.

Material and Methods

Patients

Specimens (either bone marrow or peripheral blood samples) of 39 patients with APL have been investigated. These specimen included samples of 11 patients at the time of initial diagnosis, of 19 patients at the time of initial diagnosis and at least once in the course of treatment. Of 9 patients, samples were available in the course of treatment only. Twenty six of 38 patients have been treated with Anthracyclin/AraC containing regimens with or without all-*trans* retinoic acid (ATRA). Twenty two of these patients were treated with ATRA combined with a double induction strategy according to the protocol established by the cooperative AML study group (Prof. Dr. Büchner, Münster) [9]. One patient was treated with ATRA alone because of concomitant diseases.

RNA Preparation and cDNA Synthesis

Total leukocyte RNA was extracted from 10 to 20 ml of peripheral blood and/or from 1 to 5 ml bone marrow aspirate after lysis of red blood cells [10]. Samples were received either locally or by mail and spent between 1 to 4 days in transit. The extracted RNA was reverse transcribed by random priming using Stratagene's first strand cDNA synthesis kit according to the manual of the manufacturer.

Synthetic Oligonucleotides

Primers were deduced using PML (Acc: X63131, M73778, S50913), RAR alpha (Acc: X56057, X56058, X58685) cDNA sequences obtained from EMBL/GB databases and synthesized with an Applied Biosystems oligonucleotide synthesizer. RAR alpha specific primers: GTGGTAGCCTGAGGACTTGTCC TGACAGAC (p1, corresponding to RAR alpha Exon 3 outside), GGCTGGGCAC TATCTCTTCAGAACTGCTGC (p2, corresponding to RAR alpha Exon 3 inside); PML specific primers: GGATGCTGTGCTG CAGCGCATCCGCACGGG (p4, corresponding to PML Exon A outside), TGGTGCA GAGGATGAAGTGCTACGCCTCGG (p5, corresponding to PML Exon A inside), ACAACGACAGCCCAGAAGAGGAAGTGC AGC (p6, PML Exon D), GCACACCC CGTGCCAGTGTACGCCTTCTCC (p7, PML Exon C).

Nested PCR and Controls

One tenth of the random primed cDNA was added to the first step PCR reaction mix (total volume: 50 µl) containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatine, 0.25 mM each dNTP, 1.5 U Taq DNA-polymerase (Boehringer, Mannheim), 25 pmol of each oligomere primer set (for detection of L-transcripts: p1+p7, for S-tran-

scripts: p1+p4). Using a Biometra Trioblock DNA thermal cycler PCR cycle parameters were: hot start at 94 °C for 5 min, 16 cycles: 1 min 94 °C, 2 min 60 °C, 2 min 72 °C, final extension step: 7 min at 72 °C. 3 µl of product of the first round PCR were added into the second round of PCR using primers sets p2+p6 and p2+p5 for amplification of PML/RAR alpha L- and S-transcript, respectively, under the same reaction conditions except a cycle number of 32. To detect possible traces of genomic DNA in the RNA preparations PCR with an *abl* specific primer set as described by Cross et al. [11] was performed. Negative controls containing water instead of template were also included in each experiment.

Generally, 1/5 of the PCR reactions was separated by agarose gel electrophoresis (3% NuSieve (BioRad), 1% agarose), and amplification products were visualized under UV light after ethidium bromide staining.

As an internal standard for cDNA quality abl was quantified in all samples using a competitor plasmid construct and a set of different oligonucleotide primers [11, 12]. RNA quality was expressed as the ratio between the PCR products obtained with wild type *abl* and synthetic *abl* competitor (absolute number of competitor molecules added to the PCR reaction: 10^4 to 10^6). The lower limit for RNA samples to be used in PML/RAR alpha PCR was set to a log equivalence point of 3.5. Strict precautions were taken to prevent contamination of samples, and all experiments included negative controls from all stages of the reactions.

Results

A two-step RT-PCR was used for detection of PML/RAR alpha fusion transcripts in RNA from peripheral blood cells/bone marrow of 39 patients with APL. For differentiation of the two common types of fusion transcripts (L-form and S-form) specific primer sets were employed in each step of the nested PCR. Figure 1 shows the PCR products of the second PCR step demonstrating two specimen with S-type and 3 samples with L-type

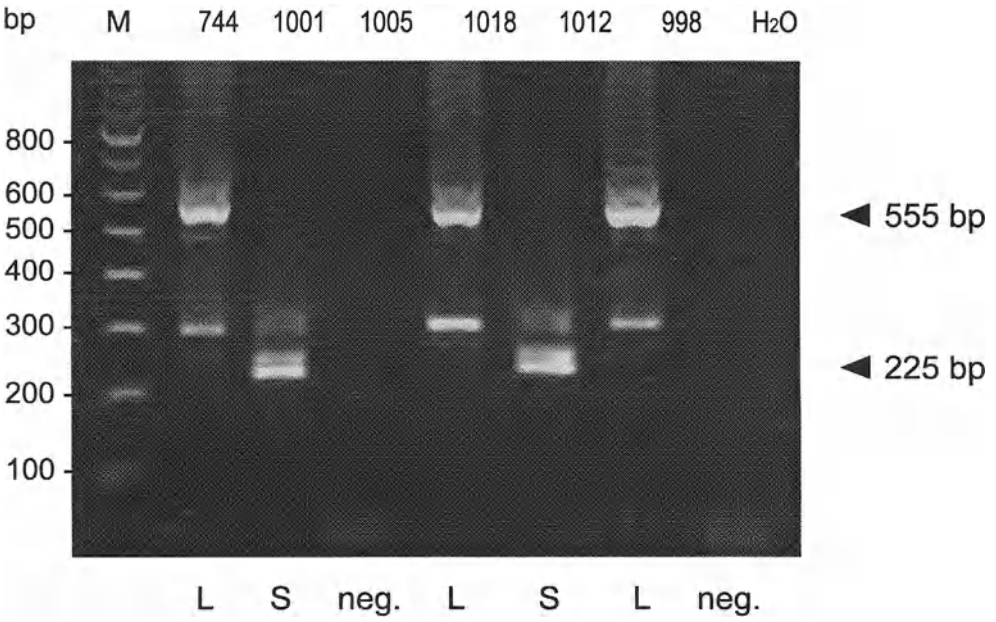

Fig. 1. Detection of PML/RAR alpha fusion transcripts by nested RT-PCR. 1/10 of the amplification products obtained in the second round of PCR was separated by gel electrophoresis (3% NuSieve/1% Agarose) and visualized by ethidiumbromide staining. Sizes: transcript type L: 225 bp; S: 555 bp. No products were detected in RT-PCR using RNA of a healthy individual (sample 1005) and water as template (neg. control)

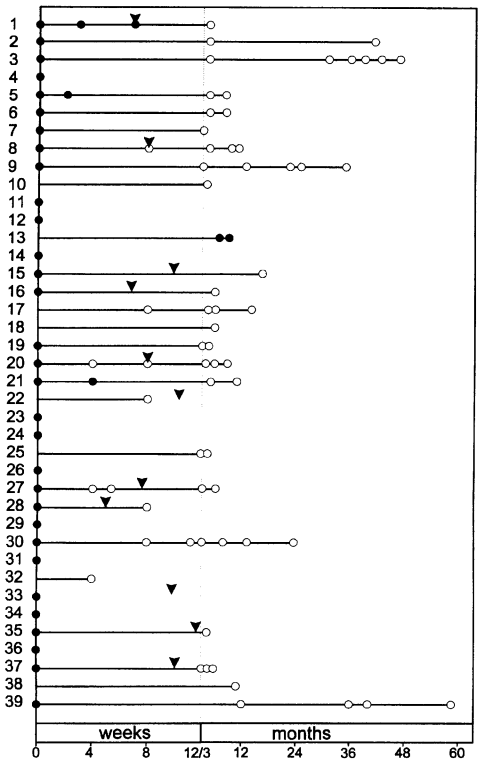

Fig. 2. Monitoring of minimal residual disease by RT-PCR of PML/RAR alpha fusion transcripts. 101 RNA samples of 39 patients in the course of their disease were investigated. Abbreviations: ○ PCR negative, ● PCR positive, ▼ date of hematologic complete remission

fusion transcripts. The amplification products resembling the S-type display the expected size of 225 bp, those resembling the L-type match 555 bp in length. So far, 101 samples derived from 39 patients have been investigated (Fig. 2). Transcripts of L type were found in 71% of cases (22/31) whereas S type transcripts were detected in 29% of patients (9/31). Of 8 patients samples only taken in status of complete hematological remission were available and therefore, the type of PML/RAR alpha fusion transcript could not be analyzed.

All patients (n = 20) in clinical remission after chemotherapy alone or in combination with ATRA remained PCR negativ 3.3-48 months after diagnosis (median, 14.5 months) as shown in Fig. 2. No molecular relapse has been observed so far. This result correlates with our clinical data as no patient under chemotherapy regimen with and without ATRA showed a clinical relapse so far [9]. One patient was treated with ATRA alone because of concomitant diseases. In this patient the PML/RAR alpha transcript was detectable 6 and 8 months after diagnosis.

A sensitivity of 10^{-4} in the nested PCR was reached as calculated from dilution series mixing RNA from APL blasts with normal (negative) RNA. In the first step of the nested PCR the sensitivity was only 10^{-1}.

Discussion

One objective of our study is to improve diagnosis of patients with APL and to investigate the influence of PML/RAR alpha fusion type (S, L type) on prognosis and clinical outcome. A second goal is the monitoring of minimal residual disease during therapy. As shown by Fenaux and Chomienne, the recurrence of PML/RAR alpha positivity preceedes the clinical relapse [8]. In our collective, all patients who reached PCR negativity for PML/RAR alpha remained negative so far. These findings match our clinical data as no patient so far investigated showed a clinical hematological relapse. Nevertheless, the period of observation has to be prolonged.

Previously, it has been described [6] that sporadically, PCR negative patients showed clinical relapse. This could be due to a long interval between the last sample collection and the observed relapse. To overcome this problem, sample collections in closer intervals can improve monitoring of APL patients.

Another explanation is the still low sensitivity (10^{-4}) of PML/RAR alpha detection by RT-PCR which is caused by the very low transcription rate of PML/RAR alpha in malignant cells of APL patients [12]. The stimulation of PML/RAR alpha transcription can be attained by in vitro cultivation of mononuclear cells isolated from APL patients in medium containing alpha interferon. By increase of the PML/RAR alpha transcription level in cells of the malignant clone, an earlier detection of the recurrent disease might be possible in APL patients.

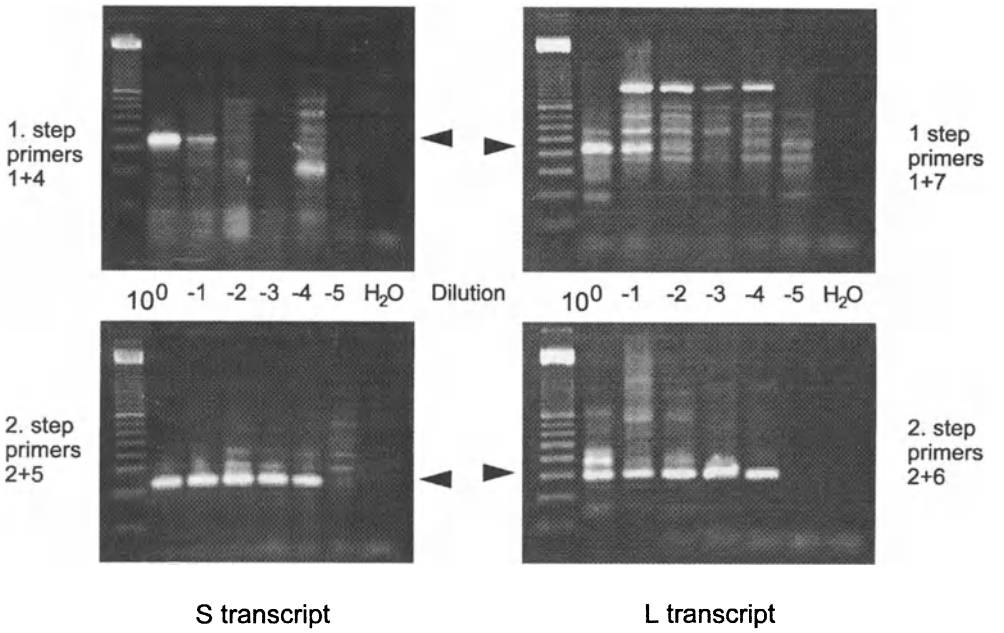

Fig. 3. Sensitivity of nested PCR for detection of PML/RAR alpha S- and L-fusion transcripts: 1/10 of the amplification products obtained in the first *(upper panel)* and second round *(lower panel)* of PCR was separated by gel electrophoresis (3% NuSieve/1% Agarose) and visualized by ethidiumbromid staining. Amplified products are indicated by *arrowheads*: S-type, length: 555 bp; L-type, length: 225 bp). Corresponding primer sets used in first and second round of amplification were: S-type: 1+4, 2+5: L-type: 1+7, 2+6. *Marker* 100-base pair ladder

Taken together, an improved detection of PML/RAR alpha by RT-PCR could lead to an earlier detection of the recurrent disease and therefore, could improve the prognosis by an early salvage therapy in APL patients.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Detection Limits of Malignant Cells with Leukemia-Associated Phenotype (LAP) in Acute Myeloid Leukemia

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Abstracts. Leukemic blasts in AML show aberrations in the expression of cytoplasmic and cell surface antigens from normal hematopoiesis in about 80% of all cases. Multiparameter flow cytometry with double and triple combinations of monoclonal, directly fluorescence-conjugated antibodies may be used for detection of Minimal Residual Disease. The level of sensitivity of this method for the different marker combinations has not been unequivocally established.

Bone marrow aspirates of 22 patients with de novo and secondary AML were investigated at diagnosis for identification of the individual LAP. Patients had one, two or more informative LAPs. To determine detection limits, aspirates of 18 patients with morphologically normal bone marrow served as controls. Leukemic samples were diluted into normal bone marrow aspirates at concentrations from 0.01% up to 10.0% and analysed in the informative scatter gate. Leukemia associated phenotypes were classified as asynchronous in 7 cases, aberrant in 14 cases and combined asynchronous and aberrant in 6 cases. The lowest detection limit was 0.01% in 4 cases, 0.05% in 2 cases, 0.1% in 2 cases, 0.5% in 6 cases, 1% in 5 cases, 5% in 11 cases and 10% in 2 cases. The highest sensitivity was achieved in combinations of CD34 with CD7, CD14 or CD15.

Our data show that the limit of the immunophenotypic detection of persistent leukemic cells varies between different patients. The sensitivity has to be determined individually in prospective studies of Minimal Residual Disease.

Introduction

Leukemic blasts in AML show aberrations in the expression of cell surface and cytoplasmic antigens from normal hematopoiesis in about 80% of all cases, called leukemic associated phenotype (LAP). LAPs are classified as aberrant, asynchronous and combined asynchronous-aberrant. Multiple combinations of monoclonal antibodies against cell surface antigens are used to discriminate normal and hematopoietic progenitor cells. This offers the possibility to detect persistent leukemic cells (MRD) during chemotherapy with significantly higher sensitivity than light microscopy. Prospective studies show a correlation of detection of MRD in hematologic complete remission and a significantly worse long term prognosis for these patients. MRD monitoring could be a basis of patient selection for more intensive treatment strategies, which are associated with high morbidity and mortality. However, the detection limits for cells with aberrant or asynchronous antigen expression have not been formally established for the multiple antigen combinations.

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Material and Methods

Patients

Bone marrow aspirates of 22 patients with de novo and secondary AML were investigated at diagnosis. Diagnosis and FAB-classification were based on light microscopy and cytochemical staining (PAS, POX, Esterase). Aspirates from 18 adults with hematologically normal bone marrow served as controls; 17 of all 22 patients included in this study were adults, 14 with de novo, 3 with secondary AML. Five cases of childhood de novo AML were investigated. The subtypes according to FAB criteria are presented in Table 1. Aspirates from 18 adults with hematologically normal bone marrow served as controls.

Cell Preparation

The preparation of leukemic as well as normal bone marrow aspirates for FACS analysis started with erythrocyte lysis using ammoniumchloride solution in a concentration of 10%. Aspirates and ammoniumchloride solution were incubated in a proportion of 1:13 for 8 minutes at 36 °C and centrifugated at 330 *g* for 7 min at room temperature. The pellet was resuspended in 2 ml PBS solution and washed twice by centrifugation at 330 *g* for 5 min. The cells were resuspended in 1-2 ml PBS solution and adjusted to a concentration of 5 x 10^6 cells/ml. 100 µl of the suspension was incubated with 5-10 µl monoclonal directly fluorescence-conjugated antibodies at 4 °C for 20 min in triple combinations. Undiluted leukemic and normal bone marrow aspirates served as positive and negative controls. Leukemic cells with the informative LAP were diluted into normal cells at concentrations from 0.01, 0.05, 0.1, 0.5, 1, 5 to 10%. After a final centrifugation at 330 *g* for 5 min the cells were prepared with 0.5 ml PFA (paraformaldehyde 0.5%).

Flow Cytometry (FCM) and Data Analysis

FCM analysis was performed on a FACScan (BDIS) using the FACScan Research Software (BDIS). A standardized instrument setup was achieved by adjusting the light scattering detectors with lymphocytes binding CD8, CD4 and CD20 in a standard position of forward light scatter (FSC) and orthogonal light scatter (SSC). FSC and SSC signals and two fluorescence signals were determined for each cell and data of 20 000 events were stored in list mode data files. The analysis of the four dimensional data was performed with PAINT-A-GATE^{Plus} software (BDIS). The program transforms the SSC parameter according to a polynomial function by which the resolution between cell population in SSC increases and permits the identification of multiple cell populations in the multidimensional data space.

Table 1. FAB classification of patients included in this study

	M1	M2	M3	M4	M4Eo	M5	Unclass.
n	3	6	1	5	1	1	5

Table 2. Triple antibody combinations (Biomed protocol 1995) for the initial characterisation of the individual LAP

CD15 (MMA, FITC)	CD34 (QBEND1, PE)	HLA-DR (L243, PERCP)
CD15 (MMA, FITC)	CD117 (95C3, PE)	CD14 (TöK4, TRIC)
CD2 (S5.2, FITC)	CD56 (MY31, PE)	CD33 (4D3, TRIC)
CD7 (8H8.1, FITC)	CD 13 (L 138 PE)	CD19 (4G7, PERCP)
CDw65 (88H7, FITC)	CD11b (D12, PE)	CD4 (SK3, PERCP)
CD61 (RKK-PL, FITC)	Glyc.A (D2.10, PE)	CD45 (2D1, PERCP)
CD13 (SJ1D1, FITC)	CD33 (P67.6, PE)	CD34 (QBEND1, PE-CY5)

Antibodies and LAP

At diagnosis we investigated the leukemic bone marrow aspirates with a standardized AML protocol (Table 2) for identification of the individual LAP. We determined an individual blast gate for every patient and analyzed every step of dilution as well as the normal bone marrow within this gate.

Results

Informative Antibody Combinations

The immunophenotypic staining of each leukemic aspirate with triple colour immunofluorescence lead to a high number of possibilities of double and triple antibody combinations which can individually used as LAP regarding the phenotypic heterogeneity of AML. Some combinations were identified in more than one patient (Table 3).

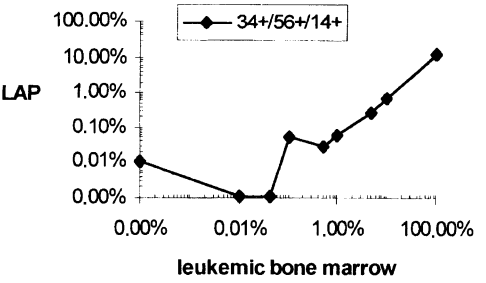

Fig. 1. Dilution experiment with leukemic bone marrow from patient K O. (adult) with CD7 FTT/56 PE/14 TRIC positive cells with normal bone marrow

Table 3. Informative antibody combinations

LAP	n
CD2/33/34	
CD15/56/34	2
CD34/14	
CD2/34	
CD7/33/34	3
CD56/34	4
CD7/33	
CD7/34	5
CD15/34	7

Only combinations which are informative in more than one patient are listed.

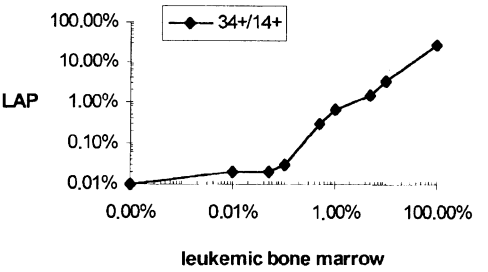

Fig. 2. Dilution experiment with leukemic bone marrow from patient N.A. (child) with CD34 FITC/14 TRIC positive cells with normal bone marrow

Table 4. Incidence of LAP positive cells in normal bone marrow

Aberrant	%	Asynchr.	%	Combined	%
CD2/33	0.05	CD14/34	0.01	CD2/15/34	0.05
CD2/34	0.01	CD15/34	0.21	CD7/34/14	0
CD2/33/34	0.01	CD15/117	0	CD7/15/34	0.04
CD7/13	0.71	CD15/34/DR	0.07	CD15/56/34	0
CD7/33	0.93	CD15/117/34	0.01	CD34/19/14	0
CD7/34	0.04	CD33/117	0	CD34/56/14	0.01
CD7/13/14	0	CD33/117/14	0.02		
CD7/13/33	0.15				
CD7/33/14	0.01				
CD7/33/34	0.19				
CD19/34	0				
CD56/33	0.17				
CD56/34	0.15				
CD34/56/33	0.05				

LAP-Positive Cells in Normal Bone Marrow

LAPs were expressed in normal bone marrow in rates between 0% and 0.93% (Table 4). In 20 of the 27 antibody combinations the expression of LAP was below 0.1%. Triple combinations varied between 0% and 0.19% which is lower than in the double patterns. CD7/13/14, CD19/34, CD15/117, CD33/117, CD7/34/14, CD15/56/34 and CD34/19/14 were not expressed in normal bone marrow samples; 99% quantiles were set as limit of negative control.

Detection Limits of Leukemic Cells with LAP

In the 22 patients 27 different double or triple leukemic associated marker combinations were identified. The LAPs were classified as aberrant in 14 cases, asynchronous in 7 cases, combined asynchronous and aberrant in 6 cases. The lowest detection limit was 0.01% in 4 cases, 0.05% in 2 cases, 0.1% in 2 cases, 0.5% in 6 cases, 1% in 5 cases, 5% in 11 cases and 10% in 2 cases. The highest sensitivity was achieved in double combinations of CD34 with CD7, CD14 or CD15 (Tables 5-7). These combinations do not have necessarily the lowest expression rates in normal bone marrow. The correlation of the number of positive cells in pure leukemia and the respective detection limit are shown in Fig. 3.

Patient H. A. (child) had 58% CD7/34 positive blasts in 100% leukemic bone marrow, the detection limit was 0.01%. There was an inverse correlation between the number of CD7/34 positive cells and the detection limit: A patient with 22% CD7/34 positive cells had a detection limit of 0.5%, a patient with 2% had a limit of 5%, while patients with even lower numbers of 0.1% and 0.03% had no positive blasts in the respective gate (limit at 100%).

Comparison of Double with Triple Antibody Combinations

Eight cases with similar expression rates in pure leukemia were compared according to their double and tripe antibody combina-

Table 5. Detection limits and expression in pure leukemic suspensions of asynchronous LAPs

Asynchronous	% in pure leukemia	Detection limit %
CD14/34 (Fig. 2)	26	0.01
	44	0.01
	29	0.1
CD15/34	45	0.01
	1.2	1
	17	1
	8.4	5
0.31	100	
1.1	100	
0.17	100	
CD15/117	1	100
CD15/34/DR	6.7	1
CD15/117/34	0.7	100
CD33/117	1.1	10
CD33/117/14	0.27	100

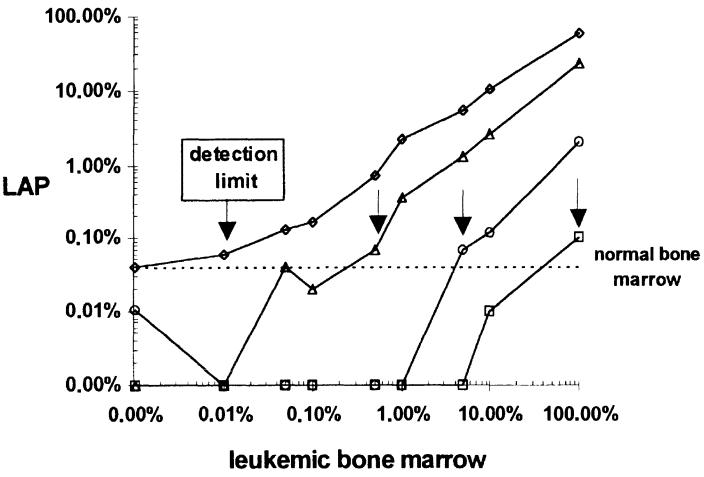

Fig. 3. Comparison of sensitivity in 4 patients with identical LAP.
Legend: Four patients H.A. (-child), B.A. (adult, □), R.I. (-adult, △), F.R. (adult, ○) with CD7/34 positive cells in dilution experiment with normal bone marrow. The curves show individual detection limits of the LAP. Normal bone marrow expression is marked as limit of negative control

Table 6. Detection limits and expression in pure leukemic suspensions of aberrant LAPs

Aberrant leukemia	% in pure limit %	Detection
CD2/33	32	0.5
CD2/34	0.38	5
	1.1	5
	1	100
CD2/33/34	7.4	1
	1.4	5
CD7/13	0.5	100
CD7/33	2.8	100
	0.11	100
	0.21	100
	0.44	100
	0.71	100
CD7/34 (Fig. 3)	58	0.01
(Fig. 3)	22	0.5
(Fig. 3)	2.0	5
(Fig. 3)	0.1	100
CD7/13/14	0.6	5
CD7/13/33	10	5
CD7/33/14	15	100
CD7/33/34	83	5
	2.4	10
	0.14	100
CD19/34	6.8	0.5
CD56/33	0.04	100
CD56/34	17	0.5
	1.8	5
	16	5
	17	5
CD34/56/33	0.02	100

Table 7. Detection limits and expression in pure leukemic suspensions of combined aberrant-asynchron LAPs

Combined	% in pure leukemia	detection limit %
CD2/15/34	0.08	100
CD7/34/14	4.4	1
CD7/15/34	8.8	0.5
CD15/56/34	12	0.05
	1.7	0.5
CD34/19/14	17	0.05
CD34/56/14 (Fig.1)	12.7	0.1

tion (Table 8). Five triple combinations showed a higher sensitivity, one double combination was more sensitive than triple and two comparisons were equivalent.

Discussion

Methods for detection of MRD include PCR for analysis of fusion gene transcripts or

Table 8. Comparison of double with triple antibody combinations

LAP	% in pure leukemia	Detection limit %	Sensitivity
CD7/34	2.0	5	
CD7/33/34	2.4	10	Double higher
CD2/34	1.1	5	
CD2/33/34	1.4	5	Equivalent
CD15/34	8.4	5	
CD15/34/DR	6.7	1	Triple higher
CD7/15/34	8.8	0.5	Triple higher
CD15/34	17.1	1	
CD15/56/34	12.5	0.05	Triple higher
CD56/34	1.8	5	
CD15/56/34	1.7	0.5	Triple higher
CD56/34	17.1	0.5	
CD56/34	17.3	5	
CD34/56/14	12.7	0.1	Triple higher
CD34/14	26.4	0.01	
CD34/56/14	12.7	0.1	
CD34/19/14	17.1	0.05	Equivalent

clonal gene rearrangements, clonogenic growth of leucemic cells in vitro and multiparameter flow cytometry. Due to the identification of individual LAPs in about 80% of all cases [8] and the prognostic impact [4,5], multiparameter flow cytometry is suited for the detection of MRD. The purpose of this study was to identify detection limits of leukemic blasts with aberrant, asynchronous or combined aberrant-asynchronous antigen combinations in AML. 22 patients were investigated at diagnosis for the individual LAP using triple-color immunofluorescence. Several other studies have defined positivity by a limit of 20% LAP expressing cells [4]. In our study also low numbers of positive cells of different antigen combinations in pure leukemic bone marrow were regarded as a leukemia associated immunophenotype. As previously reported [7] our data show heterogeneity of AML in the aberrant expression of antigens, but also in the antigen density of the respective cell surface markers. In addition, acute myeloid leukemia may consist of several subpopulations. Sensitivity can be increased by gating on a defined population. Depending on that blast gates, different levels of sensitivity for detection of cells with leukemia-associated phenotype in normal bone marrow may result. Thus, the LAPs CD7/CD13 and CD7/CD33 have not been detected in up to 106 cells of

normal bone marrow aspirates [1,3]. In contrast to these studies, in 20 of all 27 antigen combinations the expression varied between 0.01 and 0.93% in normal bone marrow after aquiring 20 000 cells. The 99% quantile of expression in normal aspirates was 0.93% for CD7/33 and 0.71% for CD7/13. The comparatively high expression of leukemia associated phenotypes in normal bone marrow aspirates may be explained by the use of different blast gates in light scatter profiles. Especially the exclusion of unspecific antibody binding monocytes has not been solved. The scatter profiles for blasts in our set of patients with AML included the monocytic scatter gate. In order to standardize detection of minimal residual disease, more studies on expression of cells with a leukemia associated phenotype in normal bone marrow are necessary to avoid false positive results. Unequivocal limits were set in 7 antigen combinations, where no blasts expressing respective combinations in normal bone marrow were found. It is remarkable that five of these cases were triple combinations. The question of distinction in antigen expression in normal and regenerating bone marrow has to be considered. We suggest that the process of cell maturation of regenerating bone marrow could produce lineage restricted failures which could be identified as asynchronous LAPs. In account to the identification of triple combinations at diagnosis and analyzing triple and respective double combinations at least two LAPs were attached to every patient. In other studies [3] more than one atypical antigen combination was present in only 10% of all investigated AML cases. The higher proportion of several LAPs per patient may be explained by the acceptance of low numbers of positive blasts for the LAP. In a comparison of staining techniques, triple combinations were more sensitive than double combinations in 5 of 8 samples with similar expression rates in pure leukemic aspirates in both groups. The highest sensitivity was achieved in patients with CD7/34, CD14/34, CD15/34 positive blasts. The detection limit was 0.01% for these cases, the expression in pure leukemic aspirates varied between 26 and 58%. The highest number of positive cells in pure leukemic bone marrow for triple combinations was 17%, which may underline the role of initial expression of LAP for the sensitivity. The results show the high sensitivity of FCM for detection of MRD in AML but the relative sensitivity has to be determined for each individual sample. Our data suggest the possibility to set an initial LAP with the highest sensitivity in double or triple combination for different patients prior to prospective trials of MRD monitoring.

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Supportive Therapy

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Interventional Antimicrobial Therapy in Febrile Neutropenic Patients (PEG Study II)

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Abstract. The aim of this study was to evaluate the efficacy and safety of an empiric therapy escalation scheme comprising the early institution of antifungals. The study was conducted at more than 20 German hospitals over a 5-year-period beginning in 1991. Patients with a documented infection or fever of unknown origin (FUO) were randomized to receive empiric therapy Step I: either an extended spectrum penicillin (Pen) or cephalosporin (Ceph), each in combination with an aminoglycoside (Amg).

Those patients who did not defervesce after 72 h of therapy were randomized to receive Step II: imipenem-cilastatin (Imi) and a glycopeptide (Glp) or Imi + Glp + fluconazole (Fluc) or Imi + Glp + amphotericin B (AmB) + 5-flucytosin (5FC).

After further 72 h of unsuccessful treatment patients were randomized to receive Step III: Pen + Ceph + Amg + AmB + 5FC or quinolone + Amg + AmB + 5FC.

In case of pneumonia AmB with or without 5FC was added; 1041 patients were randomised; 934 (89.7%) patients were evaluable for the intention-to-treat analysis. Underlying diseases were: 54.5% AML, 19.1% NHL, 13.6% ALL/AUL, 7.3% M.Hodgkin, 5.6% others. Initial diagnoses were 82.2% FUO, 9.1% pneumonia, 8.8% other documented infections.

FUO responded to therapy in Step I in 51.8%, in Step II in 59.1% and in Step III in 12/23 patients. The response rates as treated in Step II were 55.6% (Imi + Glp) vs 77.8% (Imi + Glp + AmB + 5FC), and 62.5% (Imi + Glp + Fluc).

The overall response including all modifications of therapy was 97.7% (FUO), 94.5% (bacteremia), 78.2% (pneumonia) and 88.8% (other documented infections); 6.5% patients died within the study period.

We conclude that the supplementation of amphotericin B after 72 h of unsuccessful empiric therapy improves the response rate in neutropenic fever of unknown origin.

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Introduction

Intensified chemotherapy leads to longer and more severe periods of immunosuppression. Therefore the advent of potent cytostatic regimens is accompanied by an increased risk of infections during the time of low leukocyte counts [1]. Approximately 50-60% of neutropenic patients defervesce under the initial antibiotic regimen. There is a lack of data evaluating second-line antibiotic regimens in those patients who do not defervesce. The Paul Ehrlich Gesellschaft (PEG) focused on this problem in treatment protocols for patients with fever and infection during neutropenia.

PEG Study I

The first PEG protocol (1985-1990) implemented a sequential therapeutic strategy devided into three steps. While the initial antibiotic combination therapy (ß-lactam plus aminoglycoside or double ß-lactam) was successful in 68.4%, the Step II escalation regimens supplementing a double ß-lactam by either vancomycin or an aminoglycoside resulted in a mere 50.3% response [2]. The Step III escalation consisted of changing the antibiotic regimen to double ß-lactam or imipenem-cilastatin and/or adding rifampicin plus amphotericin B plus 5-flucytosin. The response rate was 72.7%. As a consequence antifungal therapy was introduced into Step II in the PEG Study II.

Material and Methods

Inclusion Criteria

Patients were included into the study if they developed fever above 38.4 °C during absolute neutrophil count <1000/µl. For eligibility patients had to be diagnosed one of the following underlying diseases: acute leukemia, chronic myelogenous leukemia in acceleration, advanced myelodysplastic syndrome, high grade non-Hodgkin´s lymphoma or Hodgkin´s disease stages IIB - IV according to the Ann Arbor system.

Exclusion Criteria

The main exclusion criteria were impaired liver or kidney function, antibiotic therapy within the preceding 7 days and HIV infection.

For antibiotic escalation scheme in neutropenic patients with fever of unknown origin see flowchart (Fig. 1).

Results

Patient Population

During a 5-year-period from 1991 to 1996 1041 patients were included into the protocol. Of these 107 (10.3%) eluded evaluation. 934 (89.7) patients were analysed according to intention-to-treat. In terms of efficacy 74 (7.1%) patients were not assessable, so that 860 (82.6%) patients were evaluable for efficacy.

Age ranged from 17 to 92 years (median 45.3). 551 (59.4%) patients were males and 376 (40.6%) females. The underlying diseases (Fig. 2) were 54.5% acute myelogenous leukemia, 13.6% acute lymphocytic leukemia, 1.7% chronic myelogenous leukemia, 1.0% advanced myelodysplastic syndrome, 19.1% high grade non-Hodgkin´s lymphoma and 7.3% Hodgkin´s disease.

Infections at Entry

On entering the study 767 (82.2%) patients had fever of unknown origin. 85 (9.1%) had pulmonary infiltrates and 82 (8.8%) had other clinically documented infection.

Response in Patients with FUO

In the intention-to-treat analysis 397 (51.8%) patients responded to the initial antibiotic therapy. There was no significant difference between both groups. Modification of therapy and protocol violations were considered as failures. In 304 (39.6%) patients initial therapy failed and 16 (2.1%) patients died under the initial therapy. 50 (6.5%) episodes were not assessable.

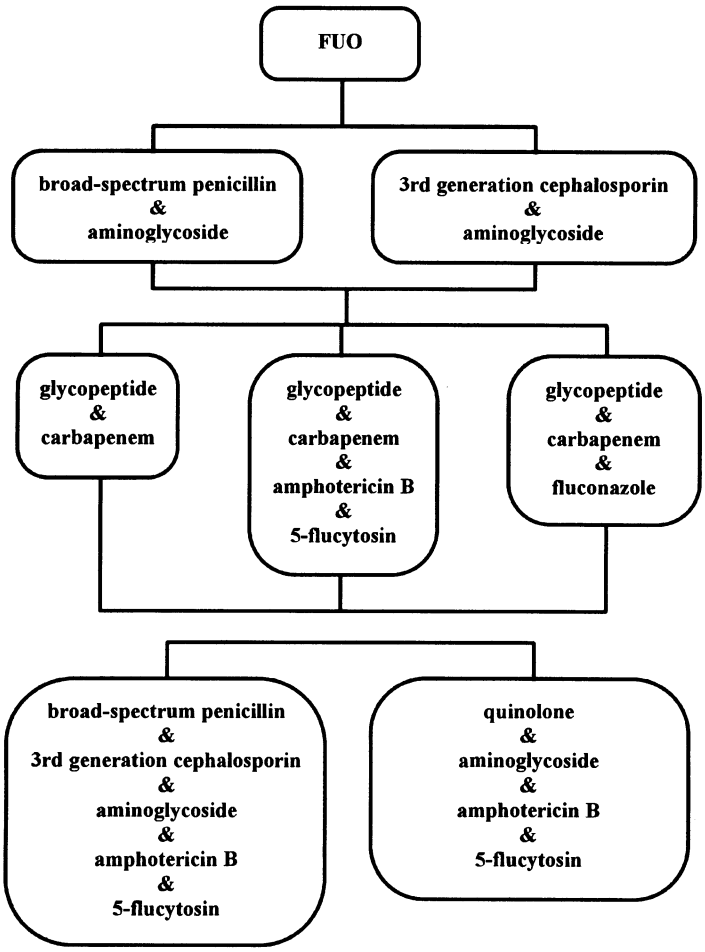

Fig. 1. Antibiotic escalation scheme in neutropenic patients with fever of unknown origin

Of the 304 non-responders to Step I, 171 were escalated to Step II therapy according to the protocol. In Step II 101 (59.1%) patients responded and 53 (31.0%) patients failed. 2 (1.2%) patients died and 15 (8.8%) were not assessable.

Twenty three patients were escalated to Step III. 12 (52.2%) responded to therapy, while 4 failed. One (4.3%) patient died and 6 (26.1%) patients were not assessable in this step.

Clinical response was achieved in 55.6% of patients treated with imipenem-cilastatin plus vancomycin; those patients treated additionally with amphotericin B & 5-flucytosin responded in 77.8%. Patients under treatment with imipenem-cilastatin plus vancomycin plus fluconazole defervesced in 62.5%. Overall response for Step II therapy of fever of unknown origin was 64.5%

At end of study the overall response including all modifications of therapy was 97.7% in patients with fever of unknown origin.

Discussion

We report on an escalation scheme in the antibiotic therapy of fever in neutropenic hosts that comprises the early supplementation of antifungals. The Infectious Diseases Society of America proposed the intention-to-treat analysis for the evaluation of anti-infective drugs for the treatment of febrile episodes in neutropenic hosts [3]. Accord-

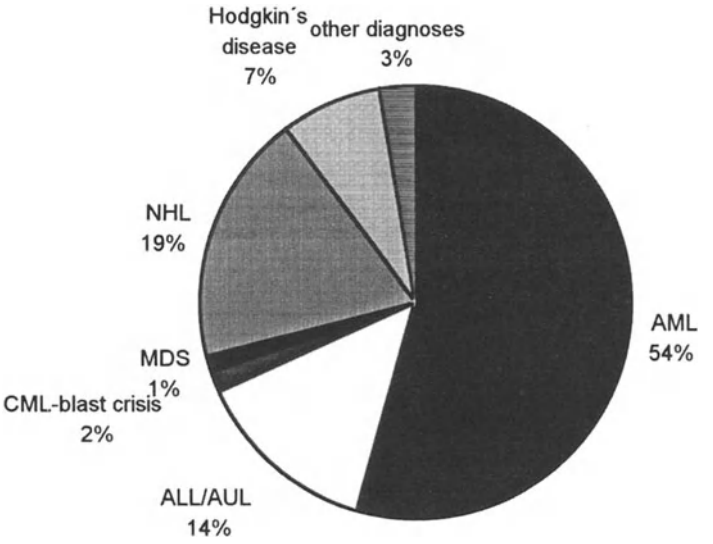

Fig. 2. Underlying diseases in 934 evaluable patients

ing to this guideline the primary response rate of 51.8% is comparable to the results of several large studies recently published [4, 5]. Besides the intention-to-treat analysis, the overall response describing the clinical outcome is of paramount importance. Including all modifications of antibiotic therapy the overall response in this clinical trial was 97.7% in patients with FUO. In our patients with fever of unknown origin the overall mortality was 1.6%. These data are close to those reported from other groups [6, 7, 8]. The efficacy of a sequential strategy with imipenem-cilastatine alone after failure of different initial regimens has been found to be 60% in episodes of fever of unknown origin [9]. The addition of glycopeptides as second-line empiric therapy resulted in a response rate of 70% [10]. In this study the overall response to the second-line therapy was 64.5%. In comparison to the PEG Study I the response rate of Step II therapy was improved from 50.3% to 64.5% [11].

In conclusion the sequential anti-infective strategy described is safe and effective, and encourages the early administration of antifungals in the treatment of neutropenic fever of unknown origin.

PEG Study III

As a consequence, the PEG Study III, which started in early 1997, will maintain the second-line administration of a quinolone plus a glycopeptide. This combination therapy will be supplemented randomly by either amphotericin B or fluconazole. Several clinical trials suggested, that monotherapy is as safe and effective as combination therapy [12, 13, 14]. For that reason the initial therapies compared in Study III are the combination of a ß-lactam plus an aminoglycoside versus carbapenem and cefepime monotherapy.

Acknowledgments. We are indebted to the study patients for their willingness to volunteer for the trial; to the many physicians who referred patients, for their cooperation; and to Ms. A. Körner, M.Sc. and K. Fehnle, Ph.D. from the PEG study office.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Management of Infections During Intensified Therapy of Acute Leukemias

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Abstract. In febrile neutropenic patients with acutes, empirical antimicrobial intervention is mandatory. Large randomized clinical trials have elucidated the benefit of broad-spectrum beta lactam antibiotics used as single drugs or in combination with aminoglycosides in order to provide activity against gram-negative aerobes as well as against streptococci and Staphylococcus aureus. As a result, infection-related mortality could be reduced to less than 10 per cent also in patients undergoing intensified remission induction or consolidation therapy. Distinct subgroups of patients have been identified who need an empirical modification of antimicrobial treatment, i.e. patients with catheter-related infections, patients with pulmonary infiltrates and patients with unexplained fever not responding to first-line antibiotics. In two consecutive, prospectively randomized trials conducted by the Paul Ehrlich Society it was demonstrated that empiric antifungal therapy is beneficial for second-line treatment in patients with persistent unexplained fever and should be part of first-line approach in patients with lung infiltrates. The empirical addition of glycopeptides, however, should be restricted to patients with catheter-related infections due to coagulase-negative staphylococci. In patients with life-threatening infections such as sepsis or documented fungal pneumonia, the adjunctive treatment with recombinant hematopoietic growth factors must be considered, even though the empirical addition of these factors is not justified on a routine basis.

Infections in Neutropenic Patients

Patients with profound neutropenia induced by intensive myeloablative chemotherapy have an approximately 90% risk of acquiring an infectious complication. The causative microorganism remains unknown in 70% of all febrile episodes (fever of unknown origin, FUO). Since the majority of these cases can be successfully treated with antimicrobial agents even in the absence of sufficient numbers of neutrophils, it can be assumed that they are caused by infectious agents [1, 2].

In microbiologically documented infections, the spectrum of microorganisms involved has change markedly over the past two decades. Before the introduction of antimicrobials with high activity against gram-negative aerobes including Pseudomonas species, these pathogens were by far dominating [3]. Due to the use of oral antimicrobial prophylaxis targeting these gramnegative organisms as well as of long-term indwelling venous catheters, gram-positive

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Table 1. Typical pathogens associated with characteristic clinical symptoms

Clinical symptoms	*Typical pathogens*
Erythema/pain at venous access	Coagulase negative staphylococci
Mucosal ulcers	Alpha hemolytic streptococci, Candida spp.
Single point-like erythemas	Gram-positive cocci, Candida spp.
Necrotising skin lesions	Pseudomonas aeruginosa, Aspergillus spp.
Retinal infiltrates	Candida spp.
Diarrhea, meteorism	Clostridium difficile
Enterocolitis, perianal lesion	Polymicrobial incl. anaerobes
Lung infiltrates ± sinusitis	Aspergillus spp., mucoraceae
Interstitial lung infiltrates + retinal hemorrhage	Cytomegalovisus

cocci have become the dominating pathogens isolated in these patients. With the prolongation of drug-induced neutropenia, the frequency of secondary or superinfections caused by pathogens resistant to the established broad-spectrum antimicrobial regimens, first of all pathogenic fungi, increases markedly.

In 30% of infectious episodes, a clinically identified focus may provide useful evidence with respect to the causative microorganisms (Table 1).

Beyond this typical association of clinical findings with causative microorganisms, the spectrum of involved pathogens changes according to the time of detection after the onset of fever. In early microbiologically documented infections, the proportion of gram-positive cocci is approximately 50% and of gram-negative aerobes about 40%, whereas fungi, mostly Candida and Aspergillus spp., are isolated infrequently. In infections with delayed microbiological documentation, i.e., samples positive later than 5 days after first fever, fungi can be isolated in more than 50% of cases, whereas the proportion of both gram-negative as well as gram-positive bacteria decline to about 25% each [2]. In patients with pulmonary infiltrates, however, this pattern of isolated microorganisms is markedly different with (mostly filamentous) fungi dominating also in early microbiologically documented cases [4, 5].

Empirical Antimicrobial Approach to Febrile Neutropenic Patients

Since it could be demonstrated that infections in neutropenic patients can be associated with 50% mortality when not appropriately treated [6], numerous clinical studies during the past 30 years have been conducted to elucidate the use of empirical broad-spectrum antimicrobial treatment. Discrepancies between results reported from these studies were caused by the heterogeneity of criteria for patient inclusion and for response assessment. Therefore, recommendations for the design and reporting of results of clinical trial have been elaborated by consensus conferences of the Immunocompromised Host Society as well as of the Infectious Disease Society of America in the early 1990s [7-9]. Since then, the majority of large scale clinical studies have been conducted in accordance with these recommendations and shown less heterogenous results [10-15].

Criteria for the institution of empirical antimicrobial treatment in neutropenic patients are clearly defined (Table 2).

A careful physical and radiological examination is mandatory to discriminate between cases of unexplained fever (FUO) and those with a clinically documented focus of infection (CDI). Standard procedures for microbiological analysis, particularly repeated venous blood cultures, help to iden-

Table 2. Criteria for the institution of empirical antimicrobial therapy in neutropenic patients

- Granulocyte count $< 1.0 \times 10^9$/l
- Oral temperature ≥ 38.5 °C or at least twice ≥ 38.0 °C within 12 h
- No evidence of non-infectious cause of fever
  - Underlying malignancy
  - Transfusion of blood products
  - Drug reaction (cytokines, anthracyclines, antimicrobial agents)

tify microbiologically defined infections (CMDI), the latter being differentiated into those with and without bacteremia.

Fever of Unknown Origin (FUO)

Standard regimens for empirical first-line treatment in patients with FUO are based upon beta-lactam antibacterials with certain activity against gram-negative aerobes, particularly enterobacteriaceae and Pseudomonas aeruginosa, streptococci and methicillin-susceptible Staphylococcus aureus in combination with aminoglycoside antibiotics. Intent-to-treat analyses demonstrate complete response rates of 50 to 60% in these patients. Significant differences between third generation cephalosporins, acylaminopenicillins or carbapenems for the beta-lactam compound or different available aminoglycosides have not been detected [2, 10-15].

Very few data, however, are reported on escalating antimicrobial treatment regimens in patients not responding to first-line therapy. Thus, little is known about the character of these non-responding cases of FUO. The only study group that included second- and third-line randomized treatment strategies is the Interventional Antimicrobial Strategy Study Group of the German Paul Ehrlich Society. This study group could demonstrate that supplementation of two-drug first-line combinations by a third antibiotic in order to achieve a maximum antibacterial spectrum including multi-resistant pathogens, i.e., double beta-lactam plus an aminoglycoside or plus vancomycin, only results in 50% response rates, whereas 75 to 80% response can be achieved by the addition of antifungals ([2], PEG Study II unpublished. Therefore, early supplementation of broad-spectrum antibacterials by a parenteral antifungal agent appears to be appropriate, because a substantial proportion of persisting FUOs might represent occult fungal infections. The implementation of an azole antifungal already into the empirical first-line approach in FUO patients, that has been reported as beneficial in defined subgroups of neutropenic cancer patients [16], might be an overtreatment of 50 to 60% of these patients because they can be expected to completely respond to standard antibiotics.

The criteria for response assessment in FUO patients as defined by consensus papers have been challenged with regard to the prognostic impact of persisting febrility in the absence of any other clinical sign of infection [17]. In a recently published trial, the EORTC-IATCG allowed to continue a randomly assigned treatment regimen also in case of persisting fever unless patients were clinically instable, thereby demonstrating that the median time to defervescence in FUO patients may exceed 96 h even if the allocated treatment is effective [11]. Data from a prospectively randomized trial comparing treatment modification with a continuation of the established regimen in patients with persisting FUO without any other critical sign of infection are not available as yet.

Overall, a complete response, defined as a stable defervescence without the need for any further antimicrobial treatment, can be achieved by systematically escalating antibacterial and antifungal therapy in approximately 95% of patients with FUO [2, 12].

Once patients being treated empirically for FUO have responded to antimicrobial treatment, the established regimen should be continued for at least 7 days in patients being persistently neutropenic, whereas in patients with neutrophil counts increasing to $> 1.0 \times 10^9$/l treatment can be discontinued after two days of stable defervescence. A follow-up, however, of at least 7 days after treatment termination, should be mandatory in order to record eventual secondary treatment failure.

Clinically Documented Infections

The identification of a focus of infection by physical examination or imaging procedures can be used for a more sophisticated selection of antimicrobial drugs for empirical therapy. As outlined in Table 1, typical patterns of microorganisms are found in association with distinct clinical symptoms of infection. Although this pattern does not allow a highly specified therapy, it gives reason

for the recommendation that

- In patients with abdominal and/or perianal signs of infection, anaerobe pathogens should be included in the spectrum of antimicrobial activity.
- In patients with skin or venous access infections, antibiotics with sufficient activity against multi-resistant gram-positive cocci should be administered.
- In patients with pulmonary infiltrates, early parenteral antifungal treatment directed against filamentous fungi must be considered
- In patients with single point-like erythemas, antimicrobial agents active against gram-positive cocci should be part of the empirical treatment regimen.
- In patients with symptoms of a severe enterocolitis, empirical administration of oral metronidazole or even an oral glycopeptide should be implemented at least until the results of microbiological analyses are available.

With respect to the increasing frequency of vancomycin-resistant enterococci being selected in association with the widespread use of glycopeptide antibiotics, as well as to the high treatment costs and the potential for adverse events under vancomycin treatment, it must be pointed out that also in cases with evidence of skin and/or venous access infection, empirical supplementation of standard antimicrobial treatment by vancomycin or teicoplanin right from the start should be handled with caution. Numerous studies have demonstrated that delayed supplementation of these agents restricted to patients not responding to standard first-line regimens such as beta-lactam plus aminoglycoside as well as to patients with multiply resistant staphylococci isolated from blood cultures provides an overall efficacy equivalent to the first-line empirical addition of glycopeptides [18-21].

It must be underlined, however, that patients with pulmonary infiltrates, documented by conventional chest radiography or CT scan, have an extraordinarily high chance of having invasive fungal infections. Conventional microbiological analyses of bronchial secretions or bronchoalveolar lavage samples usually fail to detect these fungi. The same must be stated for serological procedures. The strictly repeated application of molecular diagnostics using polymerase chain reaction in bronchoalveolar lavage fluid samples has provided a high proportion of cases with suspect for a fungal pulmonary infection, however, this procedure needs to demonstrate its benefit in sufficiently large prospectively randomized clinical trials [22]. Considering the prognostic significance of early antifungal intervention in these cases, it must be recommended that the empirical first-line approach should include amphotericin B, whereas fluconazole has no proven benefit in patients with pulmonary infiltrates not responding to a standard antibacterial first-line regimen [23]. The spectrum of microorganisms others than fungi detected in neutropenic patients with pulmonary infiltrates includes gram-negative aerobic rods as well as streptococci and Staphylococcus aureus, and in rare cases also Pneumocystis carinii and Legionella pneumophila [4]. Since the latter two pathogens can be easily detected in bronchioalveolar lavage (BAL) samples by immunological methods, it is recommended to perform fiberoptic bronchoscopy and BAL in patients not responding to an empirical antimicrobial first-line therapy. The implementation of drugs active against these two pathogens, i.e., high-dose trimethoprim-sulfamethoxazole and a macrolide antibacterial, into the empirical first-line treatment of patients with lung infiltrates is not encouraged.

Two major problems must be considered in febrile neutropenic patients with unexplained lung infiltrates:

- Microorganisms cultured from samples such as "sputum", saliva, nasal or oropharyngeal swabs, removed venous catheter material or even from blood cultures must be interpretated cautiously with respect to their etiologic significance. In particular, coagulase-negative staphylococci or Corynebacterium spp. isolated from blood cultures as well as selected bacteria such as enterococci or Candida spp. cultured from samples of the oropharynx or the upper airways can not be regarded as causative pathogens in patients with lung infiltrates. They may,

however, indicate other infections in addition to pneumonia.
- Non-infectious causes such as diffuse alveolar hemorrhage, adult respiratory distress syndrome, radiation-induced pneumonitis, drug toxicity or lung involvement by the underlying malignancy may be present in approximately 20% of cases with unexplained lung infiltrates refractory to antimicrobial agents. In these cases, which may also present or persist beyond periods of neutropenia, transbronchial or open lung biopsy is recommended.

Microbiologically Documented Infections

Microbiological findings may be helpful for treatment modification in order to target antimicrobial activity and to avoid unnecessary toxicity. Beyond this, the pattern of microorganisms as well as their susceptibility profile provides important guidelines for the selection of empirical first-line antibiotics in each institution. Therefore, microbiological diagnostics are mandatory in all cases of febrile episodes in neutropenic patients.

It must be emphasized, however, that the interpretation of microbiological findings should address the questions of the etiologic relevance in relation to the clinical presentation of an infection and of the possible involvement of additional pathogens not detectable by the applied diagnostic method. The false interpretation of microbiological findings associated with lung infiltrates has been discussed above. In patients with enterocolitis, the isolation of pathogens from fecal samples may miss important other microorganisms involved in the pathogenesis. Also in patients with venous catheter-associated infections, pathogens other than coagulase-negative staphylococci isolated from blood cultures may be involved but not detected [24-26]. Considering these diagnostic pitfalls, it is not surprising that in prospective clinical trials on infections in neutropenic patients, microbiologically documented infections treated with "targeted" antimicrobial drugs have shown no higher response rates than clinically documented infections treated empirically [2, 4].

In cases of consistent microbiological findings allowing a more specific antimicrobial therapy, the selection of appropriate drugs should be based upon the following criteria:
- In vitro susceptibility profile of isolated pathogens.
- Pharmacokinetic aspects (sufficient penetration to the focus of infection).
- Toxicity profile.
- Patient-related contraindications.
- Personal experience with standard regimens.
- Pharmaco-economic factors.

Overall, a close interdisciplinary co-operation of clinicians, microbiologists, radiologists and clinical pharmacologists should be encouraged in order to improve the management of febrile neutropenic cancer patients. With respects to pharmaco-economic aspects, however, the preference of presumably more favorable substances must be carefully balanced with regard to markedly higher treatment costs of multiple-drug "salvage" regimens and prolonged time required for stable clinical response.

Hematopoietic Growth Factors

The prophylactic use of recombinant hematopoietic growth factors such as granulocyte or granulocyte-macrophage colony-stimulating factor results in a significant reduction of neutropenia and febrile episodes in patients undergoing myelosuppressive chemotherapy. Beyond this, also a functional activation of mature granulocytes is induced by these cytokines. Therefore, also their interventional administration in neutropenic patients with severe infections has been subject to randomized clinical trials comparing their efficacy in comparison with placebo. The majority of these trials has not shown a significant benefit, yet a higher incidence of adverse effects and a significant increase of treatment costs [27, 28]. However, single studies have indicated a significant improvement of treatment outcome as well as a reduction of treatment costs [29-31], so that no definitive recommendation

for the clinical management can be given. Optimization of drug selection, timing and dosing schedules might help to find a more appropriate place of growth factors in the setting of febrile complications in neutropenic patients. Therefore, further well-designed, randomized clinical studies on the efficacy of interventional growth factors are encouraged.

Future Perspective

The efficacy of antimicrobial treatment strategies demonstrated by large clinical studies is excellent in neutropenic patients with unexplained fever (FUO) as well as in patients with clinically and/or microbiologically documented infections apart from pulmonary infiltrates. The early detection of lung infiltrates and the prompt initiation of antifungal treatment in these patients has resulted in a significant improvement of treatment outcome. However, invasive fungal infections, particularly those caused by filamentous fungi, remain a major challenge for the management of infectious complications in patients with high-grade hematological malignancies undergoing intensive myelosuppressive chemotherapy.

Major improvements may be achieved by:

- Minimally toxic yet highly effective beta-lactam antibiotics suitable for monotherapy.
- Highly effective oral antimicrobial drug regimens allowing the avoidance or abbreviation of hospitalization for parenteral antimicrobial treatment.
- New antifungal agents with superior efficacy against Aspergillus infections.
- Effective approaches for chemo- and immunoprophylaxis of infection.
- Identification of significant prognostic factors allowing prospective stratification between high- and low-risk patients.
- Valid immunological techniques for non-invasive identification of infectious pathogens and for early detection of life-threatening septic infections as well as for the reliable assessment of treatment response.
- More appropriate and more effective administration of recombinant hematopoietic growth factors.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Management of Fever in Adult Hematological Neutropenic Patients: Comparison of Tazocillin + Aminoside versus Ceftazidime + Aminoside in 466 Febrile Episodes

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Abstract. Two successive studies comparing tazocilline (12 g piperacillin + 1.5 g tazobactam/ day) + aminoside (15 mg/k/day amikacin in the first trial, 3 mg/kg/d tobramycin in the second) to ceftazidime (3 g/day) + aminoside included 466 febrile episodes in profoundly neutropenic patients after chemotherapy (or chemo-radiotherapy) for hematological malignancies. Patients were treated for acute leukemia (72%), or autografted for non-Hodgkin lymphoma or myeloma (25%), few patients for solid tumors. The mean duration of neutropenia was 21 days ± 11 (92% > 7 days); 466 febrile episodes were analysed for intention-to-treat, and were attributed to bacteremia in 119 cases, to local infection in 178 cases, and were of unknown origin (FUO) in 169 cases. A majority of Gram positive strains (55.5%) was isolated at day 0. The 72 h apyrexia was obtained more often (51.6% of the cases) with Tazocillin + aminoside than with ceftazidime + aminoside (33.6%, $p < 0.0001$). This results was due to a better control of bacteremia ($p = 0.03$) and local infection ($p = 0.006$) with tazocillin + aminoside, but not FUO ($p = 0.19$). Identical results of both antibiotic associations were observed during the first course of antitumoral chemotherapy, but tazocillin was significantly superior for 72 h apyrexia when fever occurred during consolidation therapy ($p < 0.0001$) or relapse treatment ($p = 0.006$). Superinfections due to Gram positive strains and major infectious events (infectious deaths and retardations of underlying disease treatment due to infection) were less frequent when patients received tazocillin + aminoside (respectively $p = 0.002$ and $p = 0.02$). The initial empirical antibiotic association remained unchanged and lead to a persisting apyrexia in only 20.3% of the cases treated with tazocillin + aminoside, but this was significantly better than with the other association (9.5%, $p = 0.02$).

Introduction

During the past 20 years, the treatment of leukemia (Table 1), non-Hodgkin lymphoma (NHL) and multiple myeloma has become progressively more and more aggressive, and led to an increasing duration of neutropenia and to a dramatic deterioration of digestive mucosa. Despite this fact, the proportion of infectious death during neutropenia did not change dramatically, and represented around 10% of the chemotherapy-induced neutropenia episodes in acute myelogenous leukemia (AML). This was due to the progress in the management of neu-

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Table 1. AML EORTC protocols (< 60 years old): 1978-1993

Years	Protocol	Induction	Consolidation 1	Conso 2/Maintenance	Infectious deaths
1978-1982	AML5	Dox 50 x 1 + Ara-C 160 x 7	Dox x 1 + Ara-C x 7	6 MP + MTX	?
1983-1986	AML6	DNR 30 x 3 + Ara-C 200 x 7 VCR x 1	DNR30 x 1 + Ara-C200 x 7 VCR x 1	DNR x 1 (6) Ara-C x 5	13%
1987-1992	AML8	DNR 45 x 3 +	Amsa x 3 +	DNR x 3 + HD Ara-C or ABMT	8%
1993-	AML10	Ara-C 200 x 7 DNR50 x 3 + Ara-C 100 x 10 + VP 16 x 3	HDAra-C HD Ara-C + Anthrac.	Hemat stem cell transplantation	11%

tropenic febrile patients, and to the increasing efficacy of antibiotics. During the last 10 years, the incidence of Gram positive strains (streptococci, staphylococci) became predominent in bacteremia, and necessitated to revise the empirical antibiotic therapy [1]. One of the reference associations ceftazidime and aminoside, did not sufficiently cover these Gram positive strains, which explained the extensive use of glycopeptides, essentially vancomycin. Another strategy was to use an association of ureidopenicilline and inhibitor of beta-lactamase, like tazocillin (piperacillin + tazobactam) [2], instead of ceftazidime. Tazocillin is not as potent as ceftazidime against pseudomonas, but this family of Gram negative bacilli is now rarely responsible for bacteremia, for an unexplained reason. In the other hand, tazocillin is a powerful antibiotic against a vast majority of Gram negative and positive strains (streptococci, but not staphylococci resistant to methicillin), including anaerobies.

For these reasons, we tested an association of tazocillin and aminoside in severely neutropenic patients with fever, compared to the reference association [3], ceftazidime and aminoside.

Patients and Methods

Two successive trials using the same dose of tazocillin (4 g piperacillin/0.5 g of tazobactam/8 h) and ceftazidime (1 g/8 h) and aminoside (15 mg/kg/d of amikacin in the first trial, 3 mg/kg/d of tobramycin in the second study) were analyzed together. The first study was multicentric (8 French centers participated, including 219 patients), the second was monocentric (Hôtel-Dieu, Paris, including 247 febrile episodes), but both included the same kind of adult patients (expected neutropenia above 7 days): a majority of AML (57%), 15%, of ALL, 16% of lymphoma and 12% of multiple myeloma undergoing autograft. Seventy % of the patients were treated with heavy chemotherapy, the others by chemotherapy or chemo/radiotherapy followed by circulating stem cell or bone marrow autograft (23%) or allograft (4%), leading to a 20.9 $\pm$ 11 days of neutropenia ($< 0.5 \times 10^9$ PMN/l), with a duration above 7 days in 90% of the cases. Forty seven % of the patients were in the first phase of their disease, 33% in complete remission, and 20% in relapse. Oral decontamination (non-absorbable, except in a few cases) was given in 48.7% of the cases.

The inclusion criteria were the same: neutropenia below 0.5×10^9 PMN/l (or expected to be so within 48 h), fever above 38.5 °C during 3 h, and no isolation of strains resistant to ceftazidime or tazocillin before inclusion.

Vancomycin was added at 72 h in the case of persistent fever in both branches in the first trial, but only in the case of microbiologically documented resistance in the tazocillin branch in the second trial. Amphotericin B was added at day 5 of fever in both protocols.

We pooled the 2 trials, and check for the major endpoints retained for analysis of the 466 febrile episodes ("intend to treat"): apyrexia at 72 h without antibiotic modifications, frequency of superinfections and major infectious events (MIE: infectious deaths, infectious events leading to a retardation of the cancer treatment), and use of vancomycin.

The Chi2 test was used for discontinuous variables, and Student's t test for continuous variables.

Results

Table 2 shows the characteristics of the 466 febrile episodes receiving one of the two antibiotic associations. The only borderline significant difference concerned the type of patients treated, with a higher number of patients with acute leukemia in the ceftazidime group ($p = 0.053$).

One hundred and eighty one micro-organisms were isolated at day 0 from blood and sites of infection (Table 3). Three Gram positive strains represented half of the bacteria: methicillin resistant (20%) and sensitive (15%) staphylococci, and streptococci (17%). Gram negative bacilli still represented 44.7% of the micro-organisms, with a predominance of *Escherichia coli* (23.7%).

The results of both regimens are presented in Table 4. The apyrexia without antibiotic change was more frequently obtained in the tazocillin branch (51.4% vs. 33.6%, $p < 0.0001$), but was limited to fever related to bacteremia and local infections. The results on septicemia was rather poor (46% apyrexia after Gram (–) bacteremia, 29% after Gram (+) bacteremia for the best association, i.e., tazocillin + aminoside), but corresponded to the apyrexia at 72 h: the negativity of blood cultures were certainly satisfactory, but were not systematically noted. No difference between antibiotics was observed for the FUO group.

The patients treated for the first times did not show any difference for antibiotic response (45.6% apyrexia with tazocillin vs. 40% with ceftazidim, $p = 0.7$), but the patients receiving subsequent antitumoral chemotherapy had a better control of their

Table 2. Comparison of both cohorts at inclusion (day 0)

	Tazo. + Amino-side	Cefta. + Amino-side	*p* value
Number of febrile episodes	221	245	
Underlying disease			
Acute leukemia	68%	76%	$p = 0.053$
Non Hodgkin lymphoma/ Multiple myeloma	32%	24%	
Phase of the disease			
First phase	48%	46%	$p = 0.9$
Complete remission	32%	35%	
Relapse	20%	19%	
Type of treatment			
Induction chemotherapy	70%	73%	$p = 0.18$
Autograft	27%	22%	
Allograft	3%	5%	
Oral decontamination:	51%	47%	$p = 0.36$
Type of infection			
Bacteremia	24%	28%	$p = 0.46$
Local infection	41%	37%	
FUO	36%	35%	
Gram (–) strains D0	30	44	$p = 0.2$
Gram (+) strains D0	44	47	
Number of days with $< 0.5 \times 10^9$ PMN/l	20.3 ± 10 d	21.4 ± 12 d	$p = 0.3$

Table 3. Micro-organisms responsible for infections at D0

Bacteremia and strains from local infections:

- Gram (–) bacilli: 81 strains (44.7%)
  - *E. coli* :43 (23.7%)
  - *Pseudemonas aeruginosa* :11
  - *Klebsiella* :9
  - Other Gram (–) B :18

  (4 Fusobacter, 3 Enterobacter cloacae, 2 Citrobacter, 2 Morganella morgani, 2 Proteus mirabilis, 1 Bacteroides fragilis, 1 Acinetobacter baumani, 1 Leptotrichia bucchalis, 1 Captocytophaga sputigena, 1 Hemophilus parainfluenza)

- Gram (+) strains: 100 strains (55.2%)
  - Staph Methi R :36 (20%)
  - Streptococci :31 (17%)
  - Staph Methi S :27
  - Other Gram (+) :6

  (1 Pneumococcus, 2 Clostridium, 1 Bacillus, 1 Stomatococcus, 1 Enterococcus)

Table 4. Results of both antibiotic regimens

	Tazo. + Aminoside	Cefta. + Aminoside	*p* value
Apyrexia at 72 h, no AB change	51.4%	33.6%	$p < 0.0001$
In patients with:			
Bacteremia (119 fe)	39%	22%	$p = 0.04$
Gram (–) bacteremia	46%	39%	$p = 0.3$
Gram(+) bacteremia	29%	12.5%	$p = 0.06$
Local infections (178 fe)	52%	32%	$p = 0.005$
FUO (158 fe)	58%	48%	$p = 0.19$
Oral decontamination (225 fe)	50.5%	27.7%	$p < 0.0001$
No oral decontamination (237 fe)	52%	39.5%	$p = 0.05$
In patients during:			
Induction treatment (217 fe)	45.6%	40%	$p = 0.7$
Consolidation treatment (153 fe)	59%	31%	$p < 0.0001$
Relapse treatment (89 fe)	54.5%	24.4%	$p = 0.003$
After 72 h: occurrence of:			
Superinfections	16%	25%	$p = 0.01$
Major infectious event	8%	15%	$p = 0.02$
Including infectious deaths	5.4%	7.2%	$p = 0.6$
"Complete" AB success (persisting apyrexia without AB change)	20.3%	9.5%	$p = 0.001$
Number of days with fever during neutropenia	6.5 ± 6 d	7.5 ± 7 d	$p = 0.1$

first febrile episode when treated empirically with tazocillin and aminoside (59 vs. 31% for consolidation, 54.5 vs. 24.4% for relapse, $p < 0.0001$ and $p = 0.003$, respectively). This could be explained by the increasing occurrence of Gram positive strains during consolidation (Fig. 1).

The occurrence of superinfections (bacteremia or local infections) were more frequent in the tazocillin group (16 vs. 25%, $p = 0.01$), and these superinfections were less severe under tazocillin (8% MIE vs. 15%, $p = 0.02$). The frequency of infectious deaths is the same in the two branches (5.4% in the tazocillin branch, 7.2% in the ceftazidime branch).

The duration of neutropenia could explain the very low rate of "complete success", i.e., when the first antibiotic association was maintained unchanged until PMN recovery, with a persisting apyrexia: only 20.3% of the febrile episodes were treated with tazocillin and aminoside alone, versus 9.5% with ceftazidime and aminoside ($p = 0.001$). This is largely explained by the possibility of using vancomycin in case of persisting fever in the patients treated with ceftazidime, because of the risk of streptococci bacteremia [4]. This explains also the higher frequency of use of vancomycin in the ceftazidime-treated patients (83.5 vs. 64.5% in the tazocillin group, $p < 0.0001$).

A documented fungal infection (septicemia with candida in 8 cases, 2 organ candida abcesses, pulmonary aspergillosis in 10 cas-

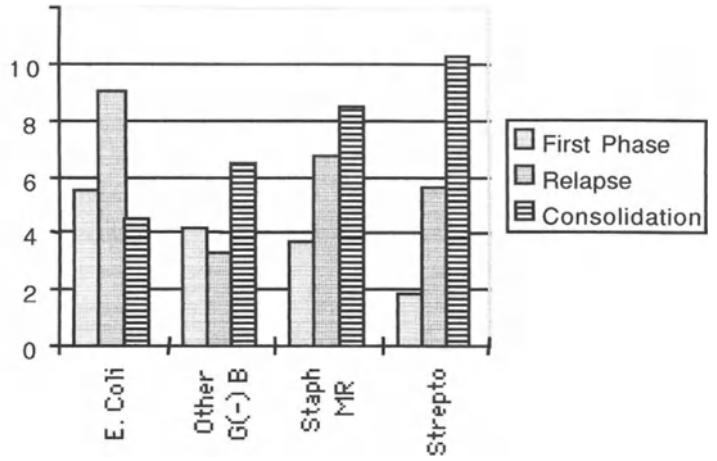

Fig. 1. Occurrence of microorganisms responsible for septicemia according to the phase of the underlying disease

Table 5. Risk factors for fungal infections

	Fungal infection 20/466	*p* value
Antibiotic regimen:		
Tazo + aminoside (221 fe)	3.2%	$p = 0.25$
Cefta + aminoside (245 fe)	5.3%	
Underlying disease:		
Acute leukemia (336 fe)	5.1%	$p = 0.18$
Other (130 fe)	2.3%	
1st phase of the hematological disease (217 fe)	4.6%	$p = 0.97$
Complete remission (155 fe)	3.9%	
Relapse (89 fe)	4.5%	
Cancer Treatment:		
Induction treatment (326 fe)	5.2%	$p = 0.39$
Autograft (107 fe)	1.9%	
Allograft (16 fe)	6.2%	
Oral antibacterial decontamination (225 fe)	4.9%	$p = 0.56$
No decontamination (237 fe)	3.8%	
Type of first infection (D0)		
Septicemia (119 fe)	2.5%	$p = 0.6$
Local infection (178 fe)	5.6%	
FUO (161 fe)	4.3%	
Apyrexia at 72 h		
Yes (195 fe)	2.1%	$p = 0.04$
No (253 fe)	5.9%	
Major infectious event		
Yes (54 fe)	25.9%	$p < 0.0001$
No (412 fe)	1.5%	–

	Fungal infection	No fungal infection	*p* value
Duration of neutropenia	25 ± 11 days	21 ± 11 days	$p = 0.11$
Number of days with fever	15 ± 9 days	6.7 ± 6 days	$p < 0.0001$
Duration of antimicrobial treatment	30 ± 15 days	21 ± 12 days	$p = 0.002$

es) was diagnosed in 20 patients (4.3%), and was highly correlated with the M I E ($p < 0.0001$). We try to identify the predisposing factors for these fungal infections (Table 5), and showed that neither the antibiotic given, nor the phase of the disease were linked to systemic fungal infection in our study. The higher amount of total antibiotics received could only be due to the persisting fever observed in these patients (15 ± 9 days versus 6.7 ± 6 days in patients with no fungal infections, $p < 0.0001$). When neutropenia duration was analyzed according to the occurrence of candida or aspergillosis documentation, a trend was observed for a longer neutropenia in the aspergillosis group (20.7 ± 11 d in patients without fungal infections, 23.3 ± 9 d in patients with candidosis, 26.7 ± 13 d in patients with aspergillosis, $p = 0.2$).

Discussion

We conducted two successive studies comparing the association of an ureidopenicillin (piperacillin), an inhibitor of betalactamase (tazobactam) and an aminoside, to the association of reference ceftazidime and aminoside as empirical treatment in neutropenic patients. The results of the first trial showed an advantage for tazocillin, with a higher incidence of 72 h apyrexia, a lower frequency of superinfections and less days with fever [5, 6]. These results are unusual for these kind of trials, comparing empirical antibiotic associations: an "absence of difference" is generally observed [7]. Cometta et al. reported the results of an EORTC protocole using the same empirical antibiotic combination at higher dosages – piperacillin/tazobactam 16/2 g daily plus amikacin 20 mg/kg daily vs. ceftazidime 6 g daily plus amikacin – tested in 706 febrile and neutropenic cancer patients. The result of 72 h apyrexia with the Tazocillin plus amikacin combination was statistically of borderline significance (61% response for tazocillin versus 54% response for ceftazidime plus amikacin, $p = 0.05$). This trial, as others conducted by EORTC, included all kinds of cancer patients, including those with very short neutropenia.

To confirm the efficacy of tazocillin in hematological patients, we decided to compare in our center the same β-lactamins at the same dosage, but associated with tobramycin, according to the absence of resistant strains to aminosides observed locally. The cumulated number of febrile episodes analyzed was high for the category of patients treated, with a mean duration of neutropenia above 20 days, and permitted an analysis of circumstances where tazocillin + aminoside association was interesting to use em-

pirically. When a patient received oral decontamination and was treated for consolidation or relapse (usually with high doses of cytosine arabinoside), empirical tazocillin + aminoside gave more often apyrexia than ceftazidime + aminoside. The frequent occurrence of streptococci during consolidation treatments and, at a less extend during relapse treatment, as observed in our study, could explain the advantage to use tazocillin. On the other hand, no differences were observed between the 2 associations in the patients treated for induction of acute leukemia, and a different strategy, using less expensive antibiotics, could probably be tested.

No differences were observed in the infectious mortality between the 2 antibiotic regimens, and the incidence of deaths due to micro-organisms was rather low, according to the severity of the neutropenia episodes. The major cause of death were systemic fungal infections (documented or suspected), as now in patients with prolonged neutropenia [8], and we tried to check for variables predictive for occurrence of such infections. It was surprising to note that the duration of neutropenia was not found as a major predictive factor for fungal infection. A more important parameter could be the cumulative duration of the previous neutropenia episodes, but these data are lacking in our study. A relationship was already described between the doses of vancomycin received and the occurrence of candidosis [9]. We observed a higher consumption of vancomycin and other antibiotics when a fungal infection occurred, but it was probably only due to the persisting fever, leading to the use of empirical antibiotics.

It is probably possible, with an extended analysis of risk factors for Gram(–), Gram(+) micro-organisms and fungal infections in neutropenic patients, to propose a "targeted" empirical antibiotherapy in such patients.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Management of Febrile Neutropenia in 396 Episodes in Acute Leukemia Patients with Once-Daily Administration of Ceftriaxone

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Abstract. Introduction. Purpose of this study was to evaluate the efficacy of ceftriaxone in a multicenter non randomized trial as first-line treatment in acute leukemia patients with febrile neutropenia under chemotherapy. Additional antibiotics were added as clinically indicated.

Material and Methods. Patients with a neutrophil count <1000/µl, fever >38.5 °C and/or C-reactive-protein (CRP)> 1.0 mg/dl were included. 396 evaluable neutropenic febrile episodes were documented from 38 centers from February 1992 to January 1996. Patients ≤ 15 years of age received 80 mg/kg BW ceftriaxone (max. 4 g/d), > 15 years 2 g per day. Age distribution was as follows: ≤ 15 years n=158, >15 years n=238. Mean CRP was 5.1 mg% (SD ± 6.0), mean maximum temperature 39.0 °C (SD ± 0.7), median Karnofsky-performance score 6.0, mean neutrophil count 368/µl (SD ± 313). In 264 of 396 evaluable episodes (64.9%) FUO was diagnosed. The mean treatment duration was 7.9 days. In 94 episodes initial treatment was with ceftriaxone alone, and combination therapy was given in 302 cases (aminoglycosides+/-glycopeptides).

Results. Response to initial treatment was observed in 205 episodes(51.8%). Non-responders (n=191) to initial treatment responded to an escalated or alternate antibiotic regimen in 80.6% (n=154). A total of 359 (90.7%) documented responses were obtained.

Conclusion. Ceftriaxone can be considered as a safe and adequate first-line treatment in febrile neutropenia. The addition of glycopeptides or aminoglycosides should be considered in non response or suspected non-sensitive microorganism.

Introduction

Standard management of febrile neutropenia consists of the administration of broad-spectrum antimicrobial agents after appropriate evaluation. This therapeutic concept has led to a significant improvement in the treatment and outcome for immunocompromised und immunosuppressed patients. The antibiotic regimens being proposed for the treatment of infections in febrile granulocytopenic patients with acute leukemia include combination therapy of various antibiotics in order to achieve broad gram-positive and gram-negative antibacterial coverage and also to minimize the development of resistance [1, 2, 3]. Not all febrile neutropenic patients have the

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same risk for developing complications. Recently developed risk assessment strategies can help to identify low-risk patients at the onset of febrile episodes.

These low-risk patients might be treated with once daily parenteral administration of an antibiotic resulting in a better utilization of resources, and a greater patient compliance as a result of an enhanced quality of life, possibly in an outpatient setting. The pharmacokinetic and antibacterial profile of ceftriaxone allows to consider this drug attractive for single-dose therapy, alone or in combination. Ceftriaxone has a long half-live, and its spectrum of activities includes most gram-positive cocci, which are the predominant pathogens in patients with cancer and neutropenia, and gram-negative rods (with the exception of *Pseudomonas* species). However, *Pseudomonas* species are now less common isolated in patients with cancer and granulocytopenia, as shown in other studies [4] . Thus, the need for aminoglycoside-containing combination must be reassessed in view of this striking epidemiological change [5].

The convenience and potential cost-effectiveness of once daily administration of antibiotics in patients with leukemia prompted this large non-randomized trial, in order to determine, whether ceftriaxone alone or in combination is a safe and adaequate first-line therapy in patients with acute leukemia and whether an outpatient treatment might be possible in clinically stable patients.

Material and Methods

From February 1992 to January 1996, 396 neutropenic febrile epsiodes were documented in 355 patients from 38 centers in a multicenter non-randomized trial. Patients were included if they had fever ≥ 38.5 °C, a neutrophil count ≤ 1000/µl, and/or a C-reactive-protein(CRP) > 1.0 mg/dl, and a presumed infection (i.e., fever not likely to be due to a noninfectious cause such as drug or blood product administration, etc.). The trial had no age limitation. Age distribution is shown in Fig 1. Ceftriaxone was adminis-

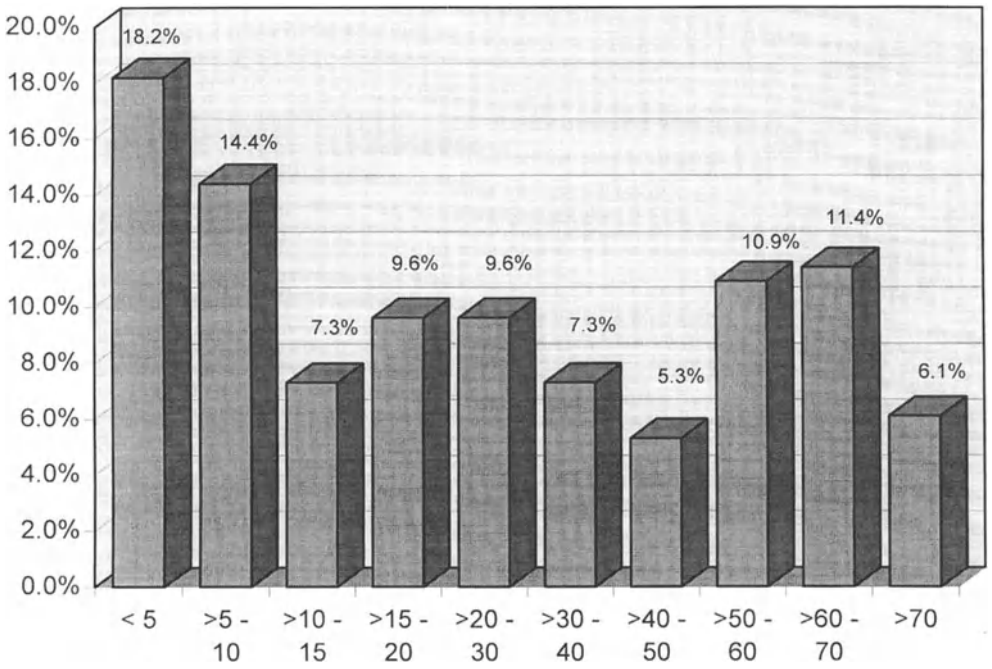

Fig. 1. Age distribution

tered once daily at a dose of 2 g i.v. (patients > 15 years) or 80 mg/kg i.v. in patients ≤ 15 years. Additional antibiotics were allowed and added as clinically indicated.

The primary endpoint of the trial was the success rate of the first line treatment and the need for antibiotic modification. Minor endpoints were culture results, feasibility in an outpatient treatment, response in an outpatient management, and adverse events.

Recommended pretreatment investigations included medical history, physical examination, documentation of clinical infection sites, blood cultures, midstream specimen of urine, swabs from any other sites of infection, C-reactive protein, and chest X-ray.

Febrile episodes were classified as documented infection without bacteremia (FUO), cinically documented infections, and microbiological documented infections with bacteremia. Patients were evaluated in the beginning of therapy and the completetion of the therapeutic trial (i.e. overall evaluation), additional examination was recommended as clinically indicated.

Treatment was regarded to be successful, if fever and clinical signs of infection (whenever present) resolved and if the infecting microorganisms (whenever isolated) were eradicated without change of allocated antibacterial therapy and the temperature declined to < 38.5 °C.

Therapy was considered as a failure when the patient died of the primary infection during the observation period, when a breakthrough bacteremia was documented, and when the documented pathogen was resistant to the ceftriaxone medication and no response was seen, which usually prompted modification of or addition to the protocol antibacterial therapy in an attempt to eradicate the primary infection. However, modification of the concomitant ceftriaxone antibiotic was allowed but was considered as a failure of the first line treatment.

Patients were excluded, if they had a neutrophil count > 1000/µl , fever < 38.5 °C, known or suspected hypersensitivity to ceftriaxone, known resistance of the causative organism to ceftriaxone, i.v. pretreatment with cephalosporines.

Results

In 355 patients 421 neutropenic febrile episodes were documented; 25 episodes were withdrawn from analysis because they did not meet the inclusion criteria, however, these patients were included in the safety analysis. Clinical characteristics of the remaining 355 evaluable patients are illustrated in Table 1.

Thus 396 episodes were evaluable in the intent-to-treat analysis for response to antibacterial therapy. Age distribution: ≤ 15 years n=158, >15 years n=238. Initial treatment was ceftriaxone alone in 94 episodes, and combination therapy in 302 cases, which consisted mostly in aminoglycosides (n=214) +/-glycopeptides (n=71). Antibiotic treatment was started in 34 patients in an outpatient setting (< 15 years: n=26 / > 15 years: n=8), and in 28 patients (< 15 years: n=23/ > 15 years: n=5) on an inpatient basis and continue later on to a outpatient.

The median duration of neutropenia was 14 days in patients ≤ 15 years and 12 days in patients >15 years. The mean CRP was 5.1 mg% (SD± 6.0), the mean maximum temperature was 39.0 °C (SD ± 0.7). The median Karnofsky-performance score was 6.0, and mean neutrophil count 368/µl (SD ± 313). A

Table 1. Clinical characteristics of 355 evaluable patients

	Age ≤ 15 years		Age > 15 years		Total	
	N	%	N	%	N	%
Episodes	158	46.3%	238	42.5%	396	44.0%
• AML	44	12.9%	150	26.8%	194	21.5%
• ALL	106	31.1%	55	9.8%	161	17.9%
• AUL	0	0.0%	5	0.9%	5	0.6%
• Blast crisis in CML	0	0.0%	18	3.2%	18	2.0%
• Other	8	2.3%	9	1.6%	17	1.9%

Table 2. Infectious manifestations

	Age ≤ 15 years		Age > 15 years		Total	
	N	%	N	%	N	%
FUO	110	69.6%	154	64.7%	264	66.7%
Defined infection	48	30.4%	84	35.3%	132	33.3%
• Pneumonia	6	3.8%	37	15.5%	43	10.9%
• Sepsis	12	7.6%	16	6.7%	28	7.1%
• Skin/soft tissue infections	5	3.2%	17	7.1%	22	5.6%
• Respiratory infections	10	6.3%	7	2.9%	17	4.3%
• Stomatitis	9	5.7%	3	1.3%	12	3.0%
• Urinary tract infection	2	1.3%	5	2.1%	7	1.8%
• Other site of infection	10	6.3%	9	3.8%	19	4.8%
Total episodes	158	100.0%	238	100.0%	396	100.0%

Table 3. Response rates at the end of the initial antibiotic therapy

	Age ≤ 15 years		Age > 15 years		Total	
	N	%	N	%	N	%
• Response	97	61.4%	108	45.4%	205	51.8%
• Non-response	61	38.6%	130	54.6%	191	48.2%
Total episodes	158	100.0%	238	100.0%	396	100.0%

wide range of infectious manifestations was demonstrated in patients enrolled in the study (Table 2). In 264 episodes (64.9%) of 396 evaluable episodes, FUO was diagnosed. In 132 episodes, non-FUO infections were observed which consisted in pneumonia in 43 cases (10.9%), sepsis in 28 episodes (7.1%), skin/soft-tissue infections 22 episodes (7.1%), respiratory-infections in 17 episodes (4.3%), urinary tract infections in 7 episodes (1.8%), and other sites of infections in 19 episodes (4.8%). The mean treatment duration was 7.9 days.

Five patients died during the observation period due to pneumonia (n=1), sepsis (n=1), cardiac failure (n=2), or progression of AML (n=1). In one of these patients coagulase-negative staphylococci were cultured from the blood, in an other case Pseudomonas was found in the sputum, however no Pseudomonas aeruginosa was isolated from the blood. The infection was bacteriologically documented in 102 (25.7%) episodes, 57 (14.4%) of whom had causative microorganisms cultured from their blood. Coagulase-negative staphylococci were isolated most frequently (6.31%) from the blood, which was followed in frequency by viridans group streptococci (2.27%), *Pseudomonas* species (1.26%), and Enterobacteriacae (1.01%).

In 205 patients (51.8%), the infection resolved without modification of the initial antibiotic therapy (Table 3).

After addition of further antibiotics to ceftriaxone, mainly aminoglycosides (n=22) and/or glycopeptides (n=47), the infection resolved in another 56 patients, leading to a cumulative response rate of 65.9% at the end of the ceftriaxone treatment period (Fig.2). Nonresponders (n=135) to initial ceftriaxone treatment responded to an alternate antibiotic regimen in 72.6% (n=98), mostly imipenem (n=64), ceftazidime (n=45), and teicoplanin (n=20). In total 359 (90.7%) documented responses were obtained. A summary of the clinical response rate is presented in Fig. 3.

Patients treated in an outpatient setting had a better response (91.2 vs. 50%) primar-

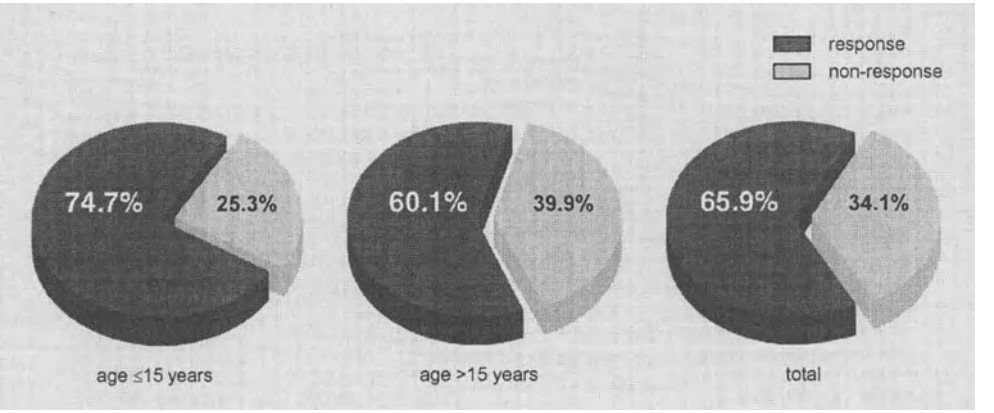

Fig. 2. Response rates at the end of therapy with ceftriaxone

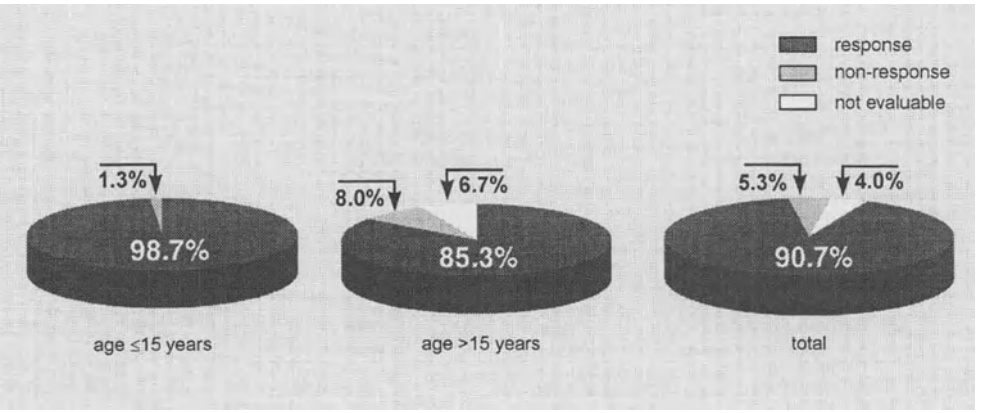

Fig. 3. Response rates at the end of the study

Table 4. Response rates for low risk[a] and high risk[b] patients

	Low risk[a]		High risk[b]		Total	
	N	%	N	%	N	%
At the end of initial antibiotic therapy						
• Response	27	67.5%	100	49.8%	127	52.7%
• Non-Response	13	32.5%	101	50.2%	114	47.3%
Total episodes	40	100.0%	201	100.0%	241	100.0%
At the end of the study						
• Response	37	92.5%	181	90.0%	218	90.5%
• Non-Response	1	2.5%	12	6.0%	13	5.4%
• Not evaluable	2	5.0%	8	4.0%	10	4.1%
Total episodes	40	100.0%	201	100.0%	241	100.0%

[a] Low risk: ≥ 500 granulocytes/mm^3 before therapy and duration of neutropenia < 7 days.
[b] High risk: < 500 granulocytes/mm^3 before therapy and duration of neutropenia ≥ 7 days.

ily due to a more stable clinical condition at inclusion. Outpatient treatment failed in 9 episodes (5 patients < 15 years of age/4 patients > 15 years of age) and administration to the hospital was necessary.

Patients with FUO responsed in 55.3% compared to those with defined infections in 40.0%. An unfavorable outcome was associated with the diagnosis of pneumonia (34.9%). Although treatment was changed, the cumulative response rate reached only 48.8% in episodes with pneumonia. Systemic antifungal therapy was initiated as concomitant first treatment step in 19 episodes, and in 23 episodes antimycotic treatment was initiated at the end of ceftriaxone treatment. Patients with a neutrophil count $\geq 500/mm^3$ and a duration of neutropenia <7 days were identified as low risk patients, with a response rate of 67.5% (Table 4). The outcome for high risk patients to first line treatment was 49.8%; however, overall response was obtained in 90.0% at the end of the study.

Adverse events were documented in 15 patients, including skin reactions in 9 patients, gastrointestinal irritation in 4 patients, and liver enzyme elevation in 2 patients.

Discussion

The data of this study support the concept that once-daily administration of ceftriaxone alone or in combination with aminoglycosides and/or glycopetides can be considered as an effective and safe treatment in febrile neutropenic patients. The response rates to once-daily administration of ceftriaxone are comparable to reported data in the literature from other antibiotic regimens [5–8]. The response rate for the first line-treatment was improved by addition of further antibiotics (aminoglycosides and/or glycopetides). However, the failure rate was not associated with high mortality, since only five deaths were reported in this trial. Two of these patients died from infections, one due to sepsis and other one due to pneumonia. Antibiotic therapy was well tolerated with minimal side effects. The incidence of drug related toxicity was low. There was no need to measure serum levels when the patients were treated with ceftriaxone alone.

Patients enrolled in this study were treated for a variety of severe bacterial infections caused by a wide spectrum of microorganisms. The percentage of positive microbiological documented infections (25.8%) is comparable to other studies [5, 9], however bacteremia was isolated only in 14.4%. As reported previously [4, 14], the incidence of gram-positive infections, i.e staphylococci species, was much higher than the incidence of gram-negative infections, especially *Pseudomonas aeruginosa*. The decreased frequency of *Pseudomonas aeruginosa* has permitted greater flexibility in the selection of antibiotics for empirical therapy in febrile neutropenia [14].

Only a few clinical studies in febrile neutropenia using once-daily dosing of ceftriaxone alone or in combination therapy have been published to date, however, they support the once-daily dosing concept [6–9]. The used single-daily-doses of ceftriaxone alone or in combination have been shown to be safe and effective in this setting. Finally, once-daily administration schedules are less labor-intensive and more cost effective than multiple-daily-dosing regimens. In addition, once-daily dosing is easier to use in an outpatient setting. Several clinical trials have demonstrated the efficacy of an outpatient therapy in febrile neutropenic patients [6, 10, 11]. As reported by other authors, outpatient management can be a safe and adaequate therapy, especially in children [13].

When patients are treated in febrile neutropenia in an outpatient setting it is important to identify the low-risk patients. CRP-levels alone failed to discriminate patients with high- and low-risk. Combination of neutrophil counts (< 500/ vs. > 500/µl) and days of neutropenia(<7 vs. >7 days) identified low-risk patients with a high response rate in the first line treatment (67.5 vs. 49.8%). Apparently the response rates of patients with FUO were higher than in cases with clinically defined infections as reported before [4, 5, 7-10, 12].

Patients with pneumonia had a lower response rate, even when switched to an alternate antibiotic regimen (overall response rate 81.4%). Fungal infections which often

are responsible for lung infiltrates might be the reason for this poor outcome [12]. As recommended by other study groups, early therapy with amphotericin B might be the treatment of choice.

We conclude that ceftriaxone is a safe and adequate first-line treatment in febrile neutropenia in patients with acute leukemia. Outpatient management with once-daily antibiotic treatment during consolidation courses might reduce costs and is able to improve quality of live in a special selected group of patients. From our experience, however, we recommend such a treatment modality only in clinically stable patients with a low-risk profil and a good compliance. In all other cases inpatient treatment should be the approach of choice to be used. The addition of aminoglycosides with a high anti-*Pseudomonas* activity should be recommended in high-risk patients. Initial treatment with glycopeptides is necessary when clinical or laboratory signs of infections with staphylococcus species are observed. With the results of the present study, an improved convenience for the patients and the cost reduction in health care should be considered as important advances of once-daily antibiotic treatment.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Failure of Fluconazole Prophylaxis to Reduce Mortality During Treatment for Refractory Acute Myeloid Leukemia: Results of a Phase III Multicenter Study

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Abstract. The current randomized study was initiated to assess the efficacy of fluconazole as systemic antifungal prophylaxis in high-risk patients with relapsed acute myeloid leukemia (AML) undergoing intensive re-induction therapy. From 68 fully evaluable patients 36 were randomized for fluconazole and 32 to the control group. No major differences were observed in the number of episodes of fever of unknown origin (61 vs. 50%) or clinically defined infections (56 vs. 50%). Microbiologically defined infections were more frequent in the fluconazole group (50 vs. 31%; $p = 0.09$) mainly due to a higher incidence of bacteremias (42 vs. 22%; $p = 0.07$). There were two cases of proven invasive fungal infections in each group. Systemic amphotericin B was applied more frequently to patients receiving fluconazole prophylaxis (56 vs. 28%; $p = 0.02$). There was no impact of fluconazole prophylaxis on the rate of early death or on overall survival. In patients with high-risk relapsed AML undergoing intensive salvage therapy the general use of fluconazole as antifungal prophylaxis can therefore not be supported.

Introduction

Invasive fungal infections (IFI) have increasingly become a matter of concern in patients receiving intensive myelosuppressive therapy for hematologic malignancies [1-4]. Especially, in cases with prolonged neutropenia [5] systemic fungal infections may contribute substantially to infectious complications and early death. Measures for early detection and effective prophylactic strategies using active and non-toxic antifungal agents are therefore urgently needed. Until now, oral application of amphotericin B suspension comprises the most frequently applied approach. This treatment has been found to reduce the rate of IFI during chemotherapy induced neutropenia [6] but is hampered by a moderate to poor patient compliance and the lack of systemic activity. As a member of the new azole antifungal agents, fluconazole features a broad range of antifungal activity and has an excellent oral bioavailability [7]. It causes few side effects and thereby offers a promising option for systemic antifungal prophylaxis in immunocompromised patients with severe and prolonged granulocytopenia. Therefore, the German AML Cooperative Group initiated a prospective randomized multicenter study to evaluate the impact of fluconazole prophylaxis on the incidence of proven or suspected systemic fungal infections and the need for systemic antifungal therapy with amphotericin B in high risk patients with relapsed and refractory acute myeloid leuk-

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emia (AML). In preceding phase II studies patients undergoing intensive salvage therapy with sequential high-dose cytosine arabinoside and mitoxantrone (S-HAM) for refractory or relapsed AML were found to experience a median duration of critical neutropenia of more than 5 weeks and an early death rate of 28% [8]. These patients were therefore selected as main target group in whom a beneficial effect of antifungal prophylaxis could be expected.

Materials and Methods

Patients and Antileukemic Therapy

Consecutive patients with relapsed and refractory acute myeloid leukemias who were admitted at the participating centers were eligible for the study. Refractoriness against standard chemotherapy was defined according to previously established criteria [9]: These included

a) primary resistance against two cycles of induction therapy;
b) first early relapse with a remission duration of less than 6 months;
c) second and subsequent relapse.

Patients with first relapses after 6 months remission duration were not considered refractory to standard therapy and were included as relapsed AML.

All patients were recruited from the first line trials of the German AML Cooperative Group and had thus received a standardized first line treatment. In patients less than 60 years of age first line therapy consisted in double induction therapy with either the repetitive application of the 9 day regimen of thioguanine, cytosine arabinoside, daunorubicin (TAD-9/TAD-9) or the sequential application of TAD-9 followed by high-dose cytosine arabinoside and mitoxantrone (HAM). Older patients all received one course of TAD-9 and were treated by a second TAD-9 course only upon inadequate response to the first TAD-9 cycle. Patients of all ages who achieved a complete remission subsequently received TAD-9 for consolidation and monthly maintenance therapy for 3 years [10, 11].

Patients meeting the entry criteria were enrolled into the current study and were treated by S-HAM [12] comprizing high-dose cytosine arabinoside (AraC) 3 g/m^2 or 1 g/m^2 every 12 h by a 3-h infusion on days 1, 2, 8, and 9 and mitoxantrone 10 mg/m^2/day as a 30-min infusion on days 3, 4, 10, and 11, respectively. The dose of AraC was adjusted to the disease status according to the results of a preceeding trial comparing 3 g/m^2 vs. 1 g/m^2 AraC in patients with refractory AML [8]. Patients with refractory disease and younger than 60 years of age received 3 g/m^2 AraC while all other cases were treated with 1 g/m^2 AraC.

To prevent high-dose AraC induced photophobia and conjunctivitis all patients received glucocorticoid eye drops every 6 h starting before the first dose and continuing for 24 h after the last dose of high-dose Ara-C. All patients also received G-CSF 5 µg/kg/day subcutaneously starting on day 12 after the beginning of therapy. G-CSF was discontinued if a bone marrow examination on day 18, i.e., 1 week after completion of S-HAM revealed more than 5% residual leukemic blasts. In patients with adequate blast cell clearance at day 18 G-CSF was continued until the neutrophil count reached a value of more than 1500/mm^3 for the 3 consecutive days.

Patients with antecedent hematologic disorders, secondary leukemias, and a preceding autologous or allogeneic bone marrow transplantation were excluded from the study. Further exclusion criteria comprized abnormal liver function tests (aspartate aminotransferase (AST), alanine aminotransferase (ALT), or alkaline phosphatase (AP) more than three times the upper normal limits; total bilirubin > 2.0 mg/dl); impaired renal function (serum creatinine > 2.0 mg/dl); severe infections or pregnancy.

Study Design and Antimicrobial Strategies

On the basis of the above mentioned antileukemic strategy the current study aimed to assess the efficacy of fluconazole in addition to standard antimicrobial prophylaxis for the prevention of IFI and the need for empiric systemic antifungal therapy with

amphotericin B. The study conduct was designed in way of a prospective randomized multicenter analysis. Prior to the start of chemotherapy patients were randomly assigned to either (a) systemic antifungal prophylaxis with fluconazole 400 mg po daily in addition to standard antimicrobial prophylaxis consisting of co-trimoxazol 960 mg po three times daily, colistine sulphate two million units po four times daily, and amphotericin B suspension 40 mg po six times daily or to (b) standard antimicrobial prophylaxis only. To avoid imbalances in the risk profile patients were stratified for age ($<$ and $\geq$ 60 years) and disease status (primary refractoriness; relapse after $\leq$ 6 months of first remission; relapse after $>$ 6 months and $\leq$ 18 months of first remission; relapse after $>$ 18 months of first remission; second and subsequent relapse).

Antimicrobial prophylaxis was continued until parenteral antibiotic and antifungal therapy was required or until a leukocyte count of more than 1000/μl was reached. Parenteral antimicrobial therapy was initiated upon the occurrence of fever of unknown origin (FUO) or fever with clinical and/or microbiologically verified infections. Antimicrobial treatment for FUO comprized two drug combinations of an aminoglycoside with either a third generation cephalosporin or ureidopenicillin. Upon persistence or recurrence of fever a combination of carbapenems with glycopeptides was initiated combined with systemic antifungal therapy with either amphotericin B in cases with fluconazole prophylaxis or fluconazole in patients without prior systemic antifungal treatment. In all cases, treatment of pneumonia consisted in systemic amphotericin B in addition to antibiotic therapy.

Study Parameters

Infectious complications were classified according to the Consensus Report of the Immunocompromised Host Society [13]: (a) fever of unknown origin (FUO) not accompanied by either clinical or microbiologic evidence of infection; (b) clinically defined infections refering to the diagnosis of a site of infection without determination of the infectious agent; (c) microbiologically defined infections consisting of bacteremia, fungemia, a microbiologically defined site of infection or a combination of the three. Additionally, IFI were classified according to criteria suggested by Behre and coworkers [14].

Toxicity was evaluated according to the World Health Organization (WHO) grading system [15].

Response to therapy was assessed according to CALGB criteria [16]. Complete remission status was defined as a normal cellular marrow with normal erythroid and myeloid elements and with myeloblasts, promyelocytes, and other leukemic cells totaling less than 5%, and with normal peripheral blood platelet and white blood cell counts for at least four weeks. Patients having more than 5% myeloblasts but fewer than 25% blasts, with otherwise normal bone marrow, were considered to be in partial remission, as were patients fulfilling the criteria of complete remission except for full recovery of peripheral blood platelet and/or white blood cell counts. Patients with persisting leukemic blasts in the bone marrow or blood or with leukemic regrowth within 4 weeks after initial response were considered as non-responders. Patients dying within 6 weeks after completion of antileukemic therapy without evidence of leukemic regrowth were classified as early deaths.

The duration of critical cytopenia was evaluated by the time for leukocyte recovery to more than 1000/μl from the onset of S-HAM treatment. The time to complete remission was measured from the onset of treatment to the date of documented complete remission and disease free survival from the date of documented complete remission to relapse or death during remission. Survival and time to treatment failure were measured by the time from the beginning of treatment to death and death without evidence of leukemia, documentation of persisting leukemia, or relapse, respectively.

Statistics

The primary end point of the present study was the impact of fluconazole prophylaxis in addition to standard antimicrobial prophy-

laxis on the incidence of IFI and the requirement of additional systemic antifungal therapy with amphotericin B as compared to a randomly assigned control group receiving standard antimicrobial prophylaxis alone. The secondary end point was the rate of early death. Randomization was to be terminated if a statistically significant difference in the incidence of IFI and/or the requirement of additional systemic antifungal therapy with amphotericin B emerged with $\alpha = 0.05$ and $\beta = 0.20$. Assuming a reduction of 20% in the incidence of IFI or the requirement of systemic antifungal therapy by fluconazole prophylaxis, 73 patients were anticipated to be enrolled into each treatment arm. Numerical values were compared by the χ^2-test, by the Fisher's-exact-test, and by the Student's t-test. Remission duration and survival was calculated according to Kaplan-Meier estimates. Comparisons were carried out using the log-rank test.

Study Conduct

Prior to therapy all patients gave their informed consent for participation in the current evaluation after having been advised about the purpose and investigational nature of the study as well as of potential risks. The study design adheres to the declaration of Helsinki and was approved by the ethics committees of the participating institutions prior to its initiation.

Results

Termination of Patient Recruitment

The recruitment of patients into the current study was terminated upon achievement of a statistically significant difference in the frequency of the initiation of systemic antifungal therapy with amphotericin B between the fluconazole group and the control group.

Patient Characteristics

Between May 1992 and January 1996, 84 patients entered the study 68 of whom were fully evaluable. Causes for exclusion from analysis were secondary leukemia (3 patients); application of the study drug before randomization (2); treatment not in accordance with the result of the randomization (1); discontinuation of the S-HAM chemotherapy after the first four days (2); and incomplete documentation (8). There were 36 and 32 evaluable patients randomized to the fluconazole arm and to the control arm, respectively. Both groups were comparable in patient characteristics and risk profiles. The patients' ages ranged from 21 to 73 and 17 to 72 years (median 52 and 45 years) for the fluconazole prophylaxis and control groups ($p > 0.05$), respectively. All patients had received prior chemotherapy for their disease

Table 1. Patient characteristics and risk factors

	Fluconazole (n = 36)	Control (n = 32)
Age (years; median/range)	52/21-73	45/17-72
Sex (male/female)	20/16	17/15
Disease status		
< 60 years + refractory AML	2 (6%)	2 (6%)
< 60 years + CR duration ≤ 6 months	7 (19%)	7 (22%)
< 60 years + CR duration > 6 months	19 (53%)	18 (56%)
< 60 years + 2nd relapse	–	1 (3%)
> 60 years	8 (22%)	4 (13%)
Antifungal treatment during preceding neutropenias	30 (83%)	24 (75%)
For invasive pulmonary aspergillosis	2	1
For invasive candidiasis	3	3
For suspected IFI	15	14
For FUO	10	6
Infections at study entry	7 (19%)	2 (6%)
FUO	6	1
Gastrointestinal tract infection	1	–
Soft tissue infection	–	1
Recovery of leukocytes to > 1000/μl (days after start of chemotherapy; median/range)	35/22–58	34/19–59
Time to complete remission (days after start of chemotherapy; median/range)	51/30–71	57/37–93
Duration of fluconazole prophylaxis (days; median/range)	23/2–56	–

as indicated above. Two (6%) patients in each group were younger than 60 years and had AML refractory to first line therapy. Early and late relapses following a first complete remission of ≤ and > 6 months duration occurred in 7 (19%) and 19 (53%) vs. 7 (22%) and 18 (56%) patients younger than 60 years, respectively. The patients' age was more than 60 years in 8 (22%) vs. 4 (13%) cases ($p > 0.05$) (Table 1). All evaluable patients received one course of S-HAM therapy. During preceding neutropenias systemic antifungal therapy was given to 30 (83%) vs. 24 (75%) patients, in 9 of which IFI had been documented (*Aspergillus* ssp. 2 vs. 1; *Candida* ssp. 3 vs. 3). Antifungal therapy had been initiated for suspected IFI and for FUO resistant to antibiotic treatment in 15 and 10 vs. 14 and 6 cases, respectively. Infections at study entry were more frequent within the fluconazole prophylaxis group [7 patients (19%) vs. 2 patients (6%)], mainly due to a higher prevalence of FUO [6 (17%) vs. 1 (3%); $p = 0.07$]. The median duration of critical cytopenia was similar in both groups with the recovery of leukocytes to more than 1000/μl occurring at a median of 35 vs. 34 days after the start of therapy (Table 1). Consequently, there was no significant difference in the median time to complete remission (51 vs. 57 days after the start of therapy; $p = 0.06$). Fluconazole prophylaxis was carried out for a median of 23 days (range 2 to 56 days).

Table 2. Infectious complications

	Fluconazole (n = 36)	Control (n = 32)
Number of febrile episodes (median/range)	1/0-3	1/0-3
Number of febrile days (median/range)	9/0-29	6/0-60
Time to first infection (days; median/range)	10/0-33	15/0-22
No infection	1 (3%)	2 (6%)
FUO	22 (61%)	16 (50%)
Clinically defined infections	20 (56%)	16 (50%)
Pneumonia	6 (17%)	6 (19%)
Sepsis syndrom	–	–
Septic shock	2 (6%)	1 (3%)
Gastrointestinal tract infection	9 (25%)	3 (9%)
Perianal infection	–	3 (9%)
Katheter related infection	1 (3%)	2 (6%)
Other	2 (6%)	1 (3%)
Microbiologically defined infections	18 (50%)	10 (31%)
Bacteriemia	15 (42%)	7 (22%)
Gram-positive	12 (33%)	4 (13%)
Gram-negative	3 (8%)	3 (9%)
Fungemia	2 (6%)	1 (3%)
Pneumonia	6 (17%)	3 (9%)
Sepsis syndrom	2 (6%)	2 (6%)
Septic shock	2 (6%)	2 (6%)
Gastrointestinal tract infection	1 (3%)	2 (6%)
Perianal infection	–	–
Katheter related infection	1 (3%)	2 (6%)
Other	2 (6%)	1 (3%)

Toxicity of Antileukemic Therapy

Severe non-hematologic toxicities (WHO grade III/IV) during the S-HAM therapy were not dependent on the use of fluconazole prophylaxis and consisted mainly of nausea/vomiting, stomatitis, and diarrhea. Furthermore, mild or severe elevation of liver enzymes and parameters for cholestasis were not different between the fluconazole prophylaxis group and the control group.

Infectious Episodes

The median number of febrile episodes was 1 in both groups (range 0 to 3), whereas the total number of febrile days was higher in the group receiving fluconazole prophylaxis (median 9 vs. 6 days; range 0 to 29 vs. 0 to 60 days; $p > 0.05$; Table 2). The median time to the first febrile episode was 10 vs. 15 days after the start of therapy ($p > 0.05$).

The infectious complications that were encountered during S-HAM therapy are summarized in Table 2. Three patients remained free from infections throughout the whole study period. No major differences between both study groups were observed for the incidence of FUO [22 episodes (61%) vs. 16 episodes (50%)] or clinically defined infections [20 (56%) vs. 16 (50%)]. There was a not statistically significant trend towards more infections of the gastrointestinal tract in patients receiving fluconazole prophylaxis (9 vs. 3; $p = 0.08$).

Marked differences were seen in the incidence of microbiologically defined infections [18 (50%) vs. 10 (31%); $p = 0.09$] mainly due to a strong trend towards more bacteremias in the fluconazole group [15 (42%) vs. 7 (22%); $p = 0.07$] which reached significance for gram-positive bacteremias (33 vs. 13%; $p = 0.04$). Bacteremias also occurred earlier in this group. The frequencies of all other categories of infections were similar for both groups. Overall, 12 (33%) vs. 9 (28%) cases with pneumonia were encountered which occurred earlier in patients receiving fluconazole prophylaxis.

In total, causative organisms were predominantly gram-positive and gram-negative bacteria which were identified in 26 and 14 cases, respectively. Fungal infections were documented in only 4 patients. There were 2 vs. 2 cases with proven invasive candidiasis while invasive pulmonary aspergillosis was not observed. Possible invasive fungal infections occurred in 19 (53%) vs. 11 (34%) patients ($p = 0.10$).

Antimicrobial Therapy

Antifungal therapy was initiated in 22 (61%) vs. 18 (56%) patients (Table 3). Systemic amphotericin B was given to significantly more patients of the fluconazole prophylaxis group [20 (56%) vs. 9 (28%); $p = 0.02$]. Seven patients within the control group received fluconazole intravenously as initial antifungal therapy, non of which required subsequent parenteral amphotericin B. The median number of antibiotic regimens was 3 vs. 2 (range 0 to 7 vs. 0 to 6).

The antimicrobial therapy was successful

Table 3. Antimicrobial therapy

	Fluconazole (n = 36)	Control (n = 32)
Systemic antifungal therapy	22 (61%)	18 (56%)
Amphotericin B p = 0.02	20 (56%)	9 (28%)
Fluconazole	–	7 (22%)
5-Flucytosine	15 (42%)	7 (22%)
Itraconazole	1 (3%)	5 (16%)
Ambisome	1 (3%)	1 (3%)
Number of antibiotic regimens (median/range)	3/0-7	2/0-6

Table 4. Efficacy of antimicrobial therapy

	Fluconazole (n = 35)	Control (n = 30)
Complete remission	25 (71%)	20 (70%)
Improvement	3 (9%)	3 (10%)
Death due to infection	7 (20%)	6 (20%)
Septic shock	5	3
Candida krusei	1	–
Candida tropicalis	–	1
Streptococcus viridans	–	1
Enterococcus spp.	1	–
Pneumonia	2	3

in 25 (71%) vs. 21 (70%) of 35 vs. 30 patients with infections (Table 4), whereas in 3 (9%) vs. 3 (10%) cases only an incomplete control of the infections was obtained. Fatal infectious complications occurred in 7 (19%) vs. 6 (19%) patients in four of whom fungi or bacteria were documented to be the causative organism.

Overall Response and Antileukemic Activity of S-HAM

Corresponding to the similarity of results on the frequency and severity of infectious complications no significant differences were observed in overall response, disease free and overall survival. The rates of early death were 22 vs. 19%, respectively, 61 vs. 50% of patients achieved a complete remission and 17 vs. 31% were non-responders. Median time to treatment failure was 5.2 vs. 3.2 months, median disease free survival for patients in complete remission was 6.7 vs. 5.1 months, and median total survival was 7.3 vs. 6.8 months.

Discussion

The German AML Cooperative Group performed the current study with the aim to reduce the incidence of IFI and the requirement of systemic antifungal therapy with amphotericin B by antifungal prophylaxis with fluconazole in addition to standard antimicrobial therapy in patients receiving intensive chemotherapy for high-risk AML. In these patients infectious complications are frequently encountered a considerable

proportion of which are proven or suspected to be of fungal origin. Furthermore, the high early death rate of more than 25% demands an improved antimicrobial management. Among potential candidate drugs fluconazole appeared most attractive because of its pharmacokinetic profile, its broad range of antifungal activity, and its good tolerability [17-20].

Fluconazole prophylaxis has been assessed already in randomized studies in patients receiving intensive chemotherapy or bone marrow transplantation mainly for acute leukemias. The majority of these trials revealed that fluconazole reduced the incidence of candida colonisation significantly but that it was not superior in the prevention of IFI in any analysis [21-29].

There are only few placebo controlled studies evaluating the prophylactic antifungal efficacy of fluconazole. Two early trials were performed in heterogenous collectives of oncologic patients receiving chemotherapy, radiotherapy, antibiotic, or glucocorticoid therapy [30, 31]. Fluconazole significantly reduced the incidence of oropharyngeal candidiasis but had no effect on the occurrence of IFI. This negative result was thought to emerge from the application of low doses of 50 mg fluconazole only which is well below currently used doses.

In fact in two subsequent trials 400 mg fluconazole daily not only reduced the incidence of superficial fungal infections but also of IFI in patients undergoing bone marrow transplantation. In one of these studies even a reduced overall mortality until day 110 posttransplantation was observed in the fluconazole arm [32, 33].

Winston et al. were the first to provide data about a prophylaxis with fluconazole 400 mg daily in patients receiving intensive chemotherapy mainly for acute leukemias [34]. In a randomized multicenter study a significant decrease of fungal infections was shown in the fluconazole arm (9 vs. 21%; $p = 0.02$), mainly due to a reduction of superficial fungal infections. Also, at the end of the study period the colonisation of patients with candida albicans was reduced significantly. However, neither the incidence of IFI (4 vs. 8%; $p = 0.30$) nor the frequency of the application of systemic amphotericin B (64 vs. 74%; $p = 0.10$) were influenced by the fluconazole prophylaxis. As a result, the mortality was the same for both groups (21 vs. 18%).

Another analysis was performed by Schaffner et al. in a randomized single-center fashion evaluating the antifungal prophylaxis with fluconazole 400 mg daily in patients receiving chemotherapy for acute leukemias and high-grade lymphomas [35]. These authors also found a significant reduction of the incidence of oropharyngeal candidiasis (1 vs. 12%; $p = 0.01$) as well as of the colonisation with candida spp. (8 vs. 37%; $p < 0.0001$) in patients with fluconazole prophylaxis. In contrast to the results obtained by Winston et al. a trend towards a reduced need for systemic amphotericin B was reported (33 vs. 47%; $p = 0.08$) which reached statistical significance in patients with FUO (16 vs. 30%; $p = 0.04$). However, as in the above mentioned study the incidence of IFI (11 vs. 11%) as well as the mortality rate (5 vs. 7%) could not be diminished by fluconazole prophylaxis. Also, the significance of this analysis is restricted by the relatively short duration of neutropenia which amounted to a median of 22 days thereby raising the question if immunosuppression was severe enough to allow fluconazole prophylaxis to be effective.

Further evidence on the efficacy of fluconazole prophylaxis came from a placebo controlled randomized single-center study [36] in a series of patients undergoing allogeneic or autologous bone marrow transplantation or intensive chemotherapy for acute leukemia or blast crisis of chronic myelogenous leukemia. This study revealed a significant reduction in the need for systemic amphotericin B therapy (22 vs. 58%; $p < 0.01$). In addition, the number of days on fever was significantly diminished (5 vs. 9 days; $p < 0.05$). Besides the heterogeneity of the study population the results of this study are compromised by a relatively short duration of critical neutropenia of only 16 days. As could have been anticipated, no differences were observed in neither the incidence of IFI nor in the mortality rate.

Taking the short-comings of the above mentioned analyses into account the current study was carried out in a homogenous

group of patients all suffering from advanced acute myeloid leukemia and all being treated by an identical type of intensive antileukemic therapy and a standardized antimicrobial prophylaxis and intervention. These patients were chosen for the current approach because they face a prolonged period of severe neutropenia regularly exceeding 30-35 days and a high risk of lethal complications from severe infections being in the range of 25-30%.

The randomization resulted in a similarity of the patients' risk profile in both group excluding potential biases by differences in disease status, prior infections, infections at study entry, or duration of critical neutropenia. All patients randomized to receive fluconazole tolerated the drug very well for a median duration of 23 days. Whereas the time to the occurrence of infectious episodes and their duration as well as the incidences of FUO and of clinically defined infections did not differ between the two groups an excess of bacteremias led to a higher frequency of microbiologically defined infections in patients receiving fluconazole prophylaxis. Systemic antifungal therapy with amphotericin B was initiated significantly more frequent in the group with fluconazole prophylaxis while fluconazole was used successfully as interventional therapy in seven patients of the control group. There were seven and six cases suffering from fatal infections, respectively. As a result, no differences in the antileukemic activity of the S-HAM regimen were observed between the two groups.

Overall, the infectious complications manifested themselves similarly in both study arms. Especially, pneumonias – which cause a major threat to patients with prolonged severe immunosuppression [37] – were not less frequent in patients with fluconazole prophylaxis (33 vs. 28%). More episodes of bacteremia were encountered in the fluconazole prophylaxis group (42 vs. 22%; $p = 0.07$), mainly due to gram-positive bacteria (33 vs. 13%; $p = 0.04$). Similar observations were made in two previous studies already [19, 35]. Schaffner et al. also detected more bacteremias during fluconazole prophylaxis (36 vs. 21%; $p = 0.04$) the difference being mainly due to gram-negative bacteria (15 vs. 5%; $p = 0.05$). In a placebo controlled trial Palmblad et al. observed more bacteremias during ketoconazole prophylaxis (74 vs. 37%; $p = 0.0001$). However, it remains unclear if there was a causal connection between prophylaxis and bacteremia or if there were differences between the study and control groups with regard to patient conditions.

The current study failed to detect a difference in the incidence of IFI between study group and control and is thus in accordance to previous analyses [34-36]. This is based in part on the overall low rate of proven IFI and reflects the difficulties of this diagnosis when using the rigid criteria suggested by Behre et al. [14].

A major and yet not previously reported finding of the current study is an excess use of systemic amphotericin B therapy in patients receiving fluconazole prophylaxis. This finding probably results from the general strategy of antifungal therapy that was followed in this study. In most other trials, patients with fever or infections and non-response to antibiotics for three to six days usually receive amphotericin B as antifungal therapy [32-36]. In contrast, patients within the control group of the current study were scheduled to receive intravenous fluconazole for systemic antifungal treatment when FUO persisted during 3 days of antibiotic therapy and amphotericin B was initiated only upon persistence of FUO for a further 3 days. As a result, seven patients within the control group received antifungal therapy with fluconazole none of which had to proceed to systemic amphotericin B. This finding suggests that at least in FUO fluconazole may be effective and may thus replace the more toxic treatment with systemic amphotericin B. These results furthermore suggest that the need for systemic amphotericin B therapy may have been exaggerated in preceding investigations. In fact, a most recent trial by the Paul Ehrlich Society for Chemotherapy emphasized that fluconazole serves as a valuable therapeutic alternative to intravenous amphotericin B for interventional therapy in patients with FUO resistant to antibiotic regimens [38]. This finding stimulates to reconsider the most appropriate place of fluconazole treatment and favors its

application for intervention rather than for antifungal prophylaxis. This conclusion is supported by the potential risks of fluconazole prophylaxis including breaktrough candidemias and an increasing incidence of infections due to candida non-albicans species [39-43].

Also taking into consideration the secondary end point of the current trial with fluconazole failing to reduce the mortality rate within the study group the general use of the drug as antifungal prophylaxis can therefore not be supported. Fluconazole as a potent antifungal agent rather should be preserved as an option for interventional therapy in patients with suspected or proven IFI. This strategy seems appropriate at least in non-neutropenic patients for which in a randomized study fluconazole was shown to be equally effective to intravenous amphotericin B in the treatment of candidemia [44]. Further evidence for the efficacy of fluconazole as interventional antifungal therapy also in neutropenic patients comes from a matched cohort study in which cancer patients with candidemia treated with fluconazole obtained the same response rate as those who received amphotericin B therapy [45]. The validity of these results for neutropenic patients in general is hampered by a proportion of only 24% of the patients being neutropenic at enrolment into the study. Therefore, further assessment of the applicability of fluconazole to severely immunocompromised neutropenic patients with suspected or proven IFI should focus on randomized comparisons with intravenous amphotericin B hitherto comprising the standard interventional antifungal therapy.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Aspergillus terreus Necrotic Chest Skin Lesion in a Case of Acute Myeloid Leukemia

P. Ryšková[1], P. Lemež[1], V. Rosová[2], B. Laštʼovičková[3], and A. Jirásek[1]

Abstract. Isolated primary cutaneous aspergillosis is a rare complication of severe immunocompromised states. A case of *Aspergillus terreus* necrotic chest skin lesion in a patient with acute myeloid leukemia is reported. This lesion occurred following the use of plastic adhesive tape. The patient was succesfully treated with a regimen consisting of amphotericin B, granulocyte-colony stimulating factor and local antimycotic therapy. Factors predisposing to this infection included local skin trauma, profound granulocytopenia and antibiotic usage.

Introduction

Fungal infections complicating antileukemic chemotherapy are becoming more common with the intensification of chemotherapy accompanied by longer and more pronounced granulocytopenia [1]. *Aspergillus* species are ubiquitous saprophytes rarely causing invasive disease in healthy individuals. Invasive aspergillosis is, however, frequently diagnosed in immunocompromised hosts [2, 3]. Aspergillosis is second only to candidiasis in frequency of mycoses among cancer patients [3], affecting all the tissues, including the skin [3-7]. Isolated primary cutaneous aspergillosis without other organ involvement has rarely been reported. When this entity does occur it is usually localized at the site of an indwelling venous catheter [3,4,6].

In this report we describe the successful treatment of a neutropenic female with isolated primary *Aspergillus terreus* infection of chest skin resulting from a local skin trauma caused by the use of adhesive tape.

Case Report

A 59-year-old woman was admitted to the Institute of Hematology and Blood Transfusion in March 1994 for newly diagnosed acute promyelocytic leukemia. The patient underwent induction therapy with *trans*-retinoic acid (ATRA), achieving complete remission. Ketoconazol 200 mg/d p.o. or fluconazol 200 mg/d for antimycotic prophylaxis or amphotericin B (AmB) 1 mg/kg/d as empiric therapy of FUO (fever of unknown origin) were used in three consolidation courses of chemotherapy. No signs of a mycotic infection were observed.

Neutropenia (less than 0.5×10^9/l) developed since the day 6 of the 4th consolidation course consisting of 10 high doses of cytosine arabinoside 2000 mg/m^2/3 h infusion every 12 h and daunorubicin 45 mg/m^2/day on days 4 and 5 (HDAC/DNR). The patient was given prophylactic co-trimoxazol, ampicillin, ceftriaxon and fluconazole 400

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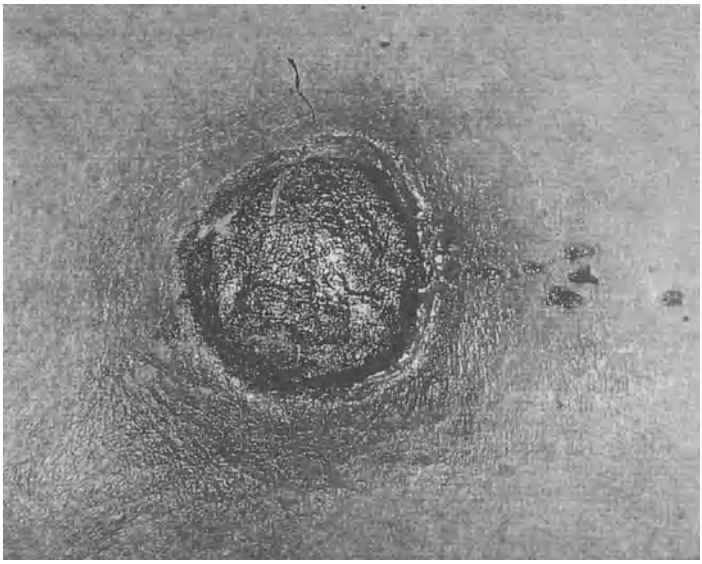

Fig. 1. Cutaneous lesion of 5 cm in diameter with demarcating central necrosis on chest (on the day 25).

mg/d p.o. as a prevention of infections. On day 11 a painful erythema developed on her chest in the place of the central venous catheter plaster fixation. Oxacillin was added on the next day, metronidazol and amilocin on day 11, but the erythema spread up to 18 cm in diameter and central necrosis of 3.5 cm across developed on day 19. Ampicilin and oxacillin were discontinued and teicoplanin added on day 18. An *Aspergillus* skin infection was suspected and thus AmB B 0.6 mg/kg treatment was initiated on day 19. The next day the patient became febrile (38.2 °C) and the dose of amphotericin B was increased to 1.1 mg/kg/d. Granulocyte-colony stimulating factor (Neupogen) 480 ug/d s.c. was started on day 22 to shorten the period of granulocytopenia, and fluconazole was discontinued. Mupirocin ointment, sulphadiazine and clotrimazol creams were used locally. On day 25 the neutrophil count was above $1x10^9$/l. Skin lesion began to improve and the central necrosis to demarcate (Fig. 1). The patient was only subfebrile and after day 28 afebrile. The treatment with Neupogen was stopped on day 29 when absolute neutrophil count reached $9.2x10^9$/l. On day 30 the central necrotic tissue of 5 cm in diameter spontaneously segregated. Repeated surface bacterial cultures of the lesion were negative. *Aspergillus terreus* Thom was found by mycologic cultivation (Fig. 2). Septate hyphae and vascular thrombosis was observed on histological examination of the segregated necrotic tissue (Figs. 3 and 4). The *Aspergillus* strain was sensitive to amphotericin B and resistant to fluconazole in vitro. On day 45 AmB administration was stopped. The complete dose of AmB was 28 mg/kg. On day 67 the skin defect had healed. Repeated serological tests for *Aspergillus* antigen (Pastorex Aspergillus, Sanofi Diagnostics Pasteur, France) and antibodies (Itest, Hradec Králové, Czech Republic) were negative throughout the course of treatment. The patient remains clinically well, in complete remission, without any evidence of aspergillosis. She did not undergo any other cytotoxic therapy.

Discussion

Fungal infections occur in immunocompromised patients. Risk factors for invasive fungal infections include prolonged granulocytopenia, corticosteroid therapy, broad-spectrum antibiotic or immunosuppresive therapy, diabetes, total parenteral nutrition, and local tissue injury [1-3, 5, 7-10]. The main predisposing factor in patients with acute leukemia is chemotherapy-induced

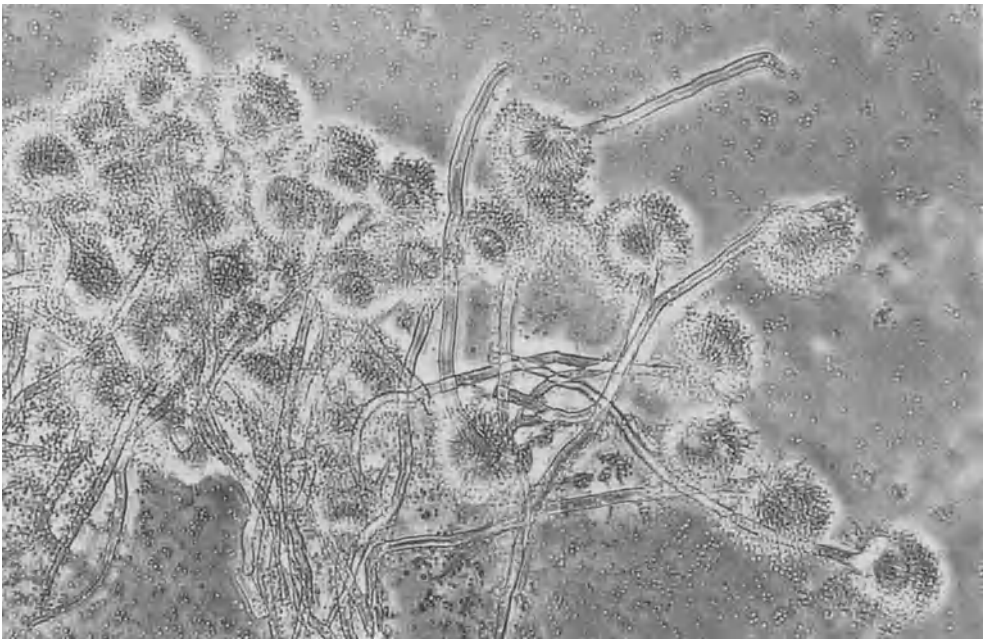

Fig. 2. Aspergillus terreus hyphae with konidiophores x300

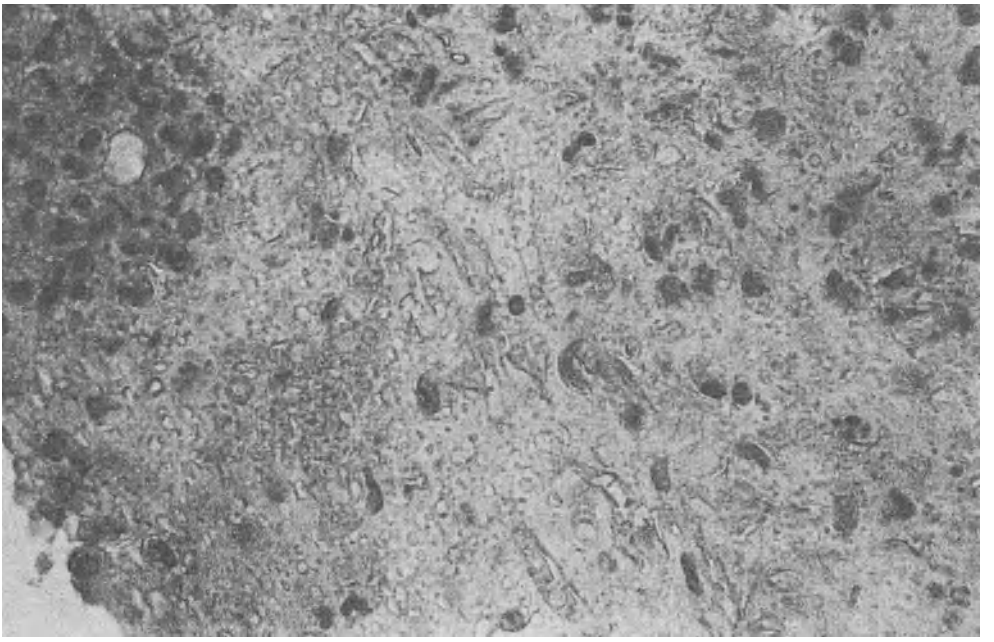

Fig. 3. Aspergillus hyphae septate in segregated cutaneous necrosis. Hematoxylin and eosin x400

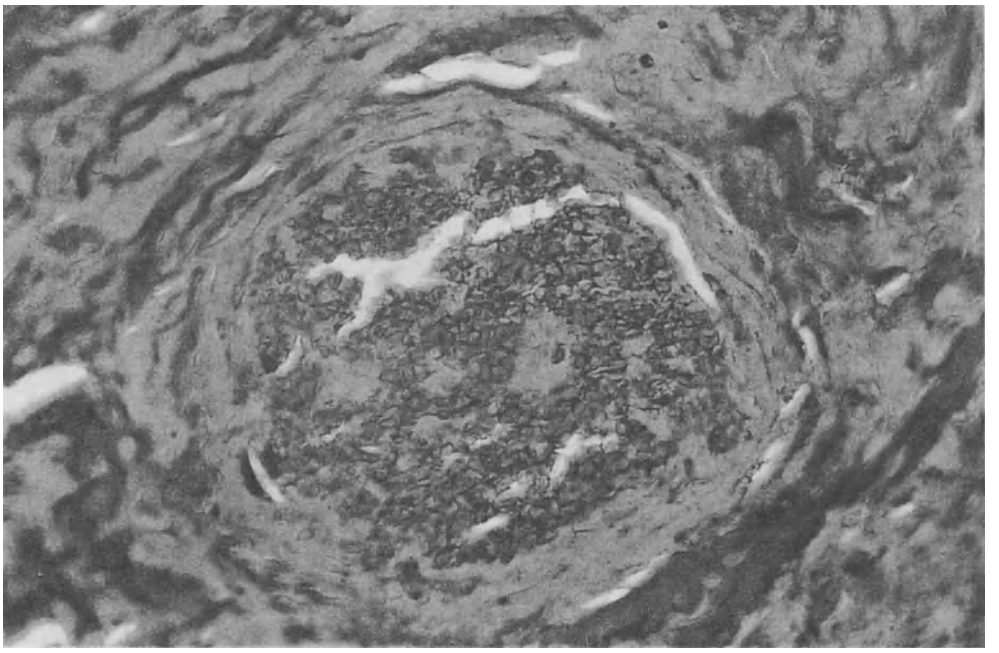

Fig. 4. Vascular invasion by Aspergillus with secondary thrombosis in segregated cutaneous necrosis. Warthin-Starry x400

prolonged granulocytopenia [10]. The respiratory tract is the usual entry for Aspergillus. Cutaneous aspergillosis is an unusual variant of invasive aspergillosis and may represent either hematogenous dissemination or a primary lesion caused by local trauma.

Our patient gradually developed necrotic cutaneous lesion on her chest since day 6 of granulocytopenia. This lesion was not related to the central venous catheter. A break in the skin resulting from the use of adhesive tape probably led to establishment of cutaneous aspergillosis. There have been reports of cutaneous fungal infections at the site of peripheral intravenous catheter [3, 6] or Hickman central venous catheters [1, 4, 9]. Less common as entry for fungal infection is a local trauma associated with maceration of skin affected by rash [6]. The skin defect did not improve after changing antibiotics. Therefore we started an empiric antifungal therapy with amphotericin B. Mycologic cultivation techniques are too slow to detect fungal infections. Serological methods for the detection of *Aspergillus* antibodies are often falsely negative because of immunoalteration of the patients. In the presented case serological detections of *Aspergillus* antigen were negative using a test that is highly specific but only moderately sensitive [11].

Primary cutaneous aspergillosis is often followed by pulmonary involvement [1,4] or disseminated infection [4, 6]. Duration of antifungal therapy should continue until the granulocyte recovery [1,2,5,6,8].

Conclusion

Fungal infections occur in patients undergoing intensive antileukemic chemotherapy. *Aspergillus* can invade virtually any tissue. We report an unusual manifestation of isolated cutaneous aspergillosis associated with skin trauma not related to the indwelling venous catheter. First signs of infection developed on day 6 of granulocytopenia. This case aims to alert physicians to the possibility of onset of rare cutaneous *Aspergillus* infection even after a short period of granulocytopenia.

Diagnostic procedures for mycologic assessment are time consuming and have lim-

ited sensitivity, early empiric aggressive antifungal therapy is therefore necessary.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Local Hydrotherapy as a Treatment Method of Oral Cavity Infections in Children with Acute Leukemias and Lymphomas

T. Urasiński, Z. Hamerlak, J. Fydryk, and J. Peregud-Pogorzelski

Introduction

Mucosal infections, inflammations and ulceration, known as stomatitis are among the most common complications in children with cancer. Granulocytopenia, decreased rate of mucosal regeneration and saliva excretion are factors predisposing to these entities. It has been also shown that cytotoxic therapy results in an impaired salivary peroxidase system, which may contribute to some of the oral complications seen in leukemia patients. Children with acute leukemias and lymphomas on intensive chemotherapy are particularly susceptible due to profound granulocytopenia. Episodes of stomatitis decrease the child's quality of life causing chronic pain, contribute to a poor nutrition state and may serve as a nidus for systemic dissemination [1,2,3,9].

Oral cavity infections are not easy to prevent and control. Relatively good results can be obtained by the use of isodine, chlorhexidine or meridol gargle. Some studies indicate that mouth-washing with sucralfate suspension is save and effective in reducing oral mucositis induced by chemotherapy [4, 5,8].

One of us (H. Z.) proposed his own method of low-pressure oral cavity hydrotherapy with the use of machines of own construction. Preliminary observations on the group of adult patients have been previously reported [6]. This report is to assess the efficacy and safety of this method in children with acute leukemias and lymphomas receiving intensive chemotherapy.

Patients and Method

The study comprised 107 children (61 boys, 46 girls) aged 2.5–17 years with acute leukemias (84 patients) or malignant lymphomas (23 patients) treated in our Department, who – while on intensive chemotherapy – developed at least one episode of stomatitis. The severity of oral lesions before hydrotherapy, classified according to WHO staging is presented in Table 1 [7].

Hydrotherapy is a form of a mouthwash with large amounts of therapeutic fluid. In

Table 1. Severity of hydral lesions before hydrotherapy

Stage (WHO classification)		No. of episodes
I.	Mucosal edema and redness, slight pain	107
II.	Mucosal edema and redness with superficial eruptions, solid food can be consumed	107
III.	Mucositis of the whole oral cavity, only liquids can be consumed	55
IV.	Enteral nutrition not possible	38

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small children it is performed manually, in cooperating patients (usually children older than 3 years) stationary or portable machines are applied (Figs. 1, 2). The construction of machines has been described elsewere [6].

During a single mouthwash, lasting 7–15 min, the oral cavity is rinsed with 10–80 l of fluid at a temperature of 20–30 °C. It is given under the pressure of 0.2–2.5 atm., removing mechanically necrotic tissues, debris and pathogens. The procedure is performed several times a day and the amount of fluid used per patient per day can be as high as 200 l.

There are two standard forms of therapeutic fluid. For patients with grade I or II stomatitis 200 ml of 3% solution of H_2O_2 is added to 10 l, of water. For patients with grade III or IV stomatitis, instead of H_2O_2, 50 ml of 44% of magnesium sulfur is dissolved in the same amount of water. To control local pain both fluids contain 50 ml of 2% xylocain per 10 l. The fluid's pH is chosen to neutralize the pH of the oral cavity. In most cases acidic fluids obtained by the addition of lactic acid were applied. In patients with acidic pH of the oral cavity alkaline fluids, obtained by the addition of dolomite were used. If mucosal bleeding occurred, 150 g of EACA were added to 10 l of fluid.

During the years 1991–1995 we performed over 4000 procedures in our department, including 1450 with stationary and 2550 with portable machines. The number of procedures performed daily on the ward reaches 20–30.

In children with grade I or II stomatitis, pain relief was seen within the first 24 h after the beginning of hydrotherapy and the time for resolution of the oral lesion did not exceed 4 days. In patients with grade III or IV stomatitis pain relief occurred within the first 1–2 days and the time to local recovery was not longer than 7 days.

In children who had suffered from stomatitis before, prophylactic hydrotherapy during intensive treatment was introduced. Episodes of grade III or IV stomatitis did not occur in these cases; only lesions of grade I or II were seen (patients were able to consume solid food).

Fig. 1. The stationary machine for hydrotherapy

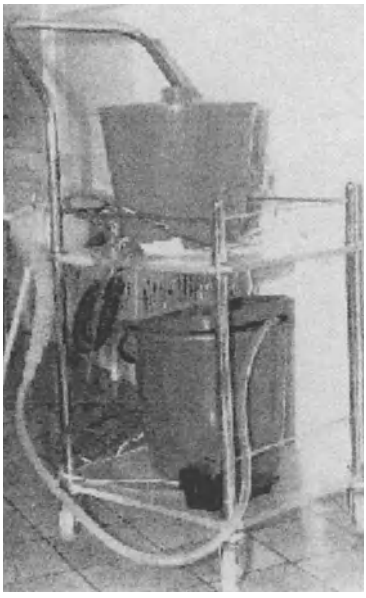

Fig. 2. The portable machine for hydrotherapy

Discussion

This report is not a controlled study, but rather a subjective comment.From the very beginning we were delighted by the fact that the method was easily and quickly accepted by all children, who were asking for subse-

quent procedures. All patients reported that hydrotherapy brought immediate, but transient pain relief. Thus we decided not to form a control group.

We do not know any reports concerning mechanical mouthwash as the treatment method of stomatitis. In our opinion the advantage of hydrotherapy relies on the mechanical cleaning of the oral cavity. Large amounts of fluid remove necrotic tissue, debris and pathogens, thus forming better conditions for mucosal repair. This effect is amplified by the change in the oral cavity's pH and by the addition of disinfectants. Pain relief obtained by the addition of local anesthetics is also of great value, improving the child's life standard.

The method sesems to be safe; except for one case, we did not see any complications. This only one adverse event was fluid aspiration in the case of 2.5 years byo with Down's syndrome and acute lymphoblastic leukemia. It is clear that younger children must be assisted by the medical staff or parents; older patients are able to perform hydrotherapy by themselves. Trained parents can proceed with hydrotherapy even at the patient's home; this offers a chance to shorten the length of hospitalisation. The number of home procedures in our experience exceeded 1800.

Our preliminary observations seem to indicate that oral hydrotherapy is a good prophylactic and therapeutic measure in oral cavity complications in children with acute leukemias and lymphomas on intensive chemotherapy.

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Transplantation

Acute Leukemias VII
Experimental Approaches and Novel Therapies
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The Role of BMT in AML in Relation to Prognostic Characteristics

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Introduction

The majority of young patients (<60 years) with AML will enter complete remission with intensive treatment. How much and the nature of treatment necessary to prevent relapse in individual patients is unclear. Several robust characteristics such as age, karyotype, presenting WBC and, to a lesser extent, sex, FAB category and phenotype, have all been associated with increased or decreased risk of relapse. In considering strategies of achieving cure for young patients there have been three approaches used to consolidate CR1, chemotherapy, allo or auto BMT. Allogeneic has been accepted as the treatment of choice for all patients if a matched sibling is available, generally resulting in a probability of cure of around 50%. Broadly similar results have been reported for autologous BMT. During the 1980s these were considered to be superior to what chemotherapy was capable of achieving. However there are several examples of improved survival being achieved as more intensive chemotherapy is given in induction and consolidation. There has been suspicion that a number of selection biases were working in favour of transplanted patients, such as an inherent reduced relapse risk because of the time interval which elapses between CR attainment and delivering the BMT, during which time some patients will relapse. An unknown number of patients who would be eligible for BMT may not receive it for reasons of clinical and haematological toxicity or other social or financial reasons.

There is also the question of timing of transplant. Are there some patients who could safely be left, and salvaged if and when they relapse? If such a strategy is adopted should the transplant be used as the initial treatment of relapse as suggested by Seattle experience or if a second remission is achieved? It is correct that transplant is the best form of treatment in second remission? Several substantial issues therefore remain to be resolved, some of which have been addressed by a number of large prospective trials. The UK MRC 10 study was conducted between 1998-1994 with the major aim of evaluating the additional role of BMT (allo or auto) when given to patients who had already received 4 courses of intensive chemotherapy.

Patients and Treatment Protocol

In all, 1966 patients were recruited, of which 20% were children under 15 years. There was an induction randomisation to a DAT (3 + 10) (Daunorubicin d1-3, Cytosine Arabinoside 12 hourly d 1-10 Thioguanine d 1-10) versus ADE in which Etoposide was substituted for Thioguanine. Course 2 was limited to 8 days of Cytosine and/or Thioguanine(DAT or ADE 8+3+5). All patients then received MACE (Amsacrine, Cytosine, Etoposide) as course 3 and MidAC (Mitozan-

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trine, AraC) as course 4. Patients who did not have a matched sibling donor were scheduled to have bone marrow harvested and cryopreserved unpurged, following which they were randomised to receive an autoBMT as a 5th course or no further treatment (STOP) with the option of autograft in CR2 if they relapsed. All recipients of transplants were to be prepared with cyclo/TBI. No reinduction chemotherapy was specified for relapse, but preparation with Bu/Cy was recommended for autoBMT in CR2.

Important parameters were identified for relapse risk. The most powerful of which were karyotype, extent of clearance of blasts after course 1, and patient age.

Karyotype

A favourable group was identified which had a survival of 74% from the start of course 2. These comprised t8:21, t15:17 and inversion 16 either as single lesions or with other abnormalities. Patients designated unfavourable risk comprised those with abnormalities of chromosome 5 or 7 or complex lesions and had survival of 19% at 7 years. All other patients, including those with a normal karyotype, were designated as standard risk with an intermediate survival (43%) (p=<0.0001).

Blasts in Bone Marrow

A bone marrow was undertaken 18-21 days after the end of course 1, to assess response to therapy. Patients were divided into three categories based on morphology, <5% blasts, >5-20% blasts or >20% blasts. This assessment had an influence on the risk of relapse for patients who were then or subsequently confirmed to be in CR (<5% blasts in a regenerating marrow). The survivals were respectively 53, 44 and 22%. It is of interest to note that the identification of patients with >20% blasts at this stage – even although they subsequently entered CR, was highly predictive of a short remission duration and survival.

When karyotype and blast cell status were combined a simple risk score could be allocated for more than 90% of patients. Good risk patients were defined only on the basis of a favourable karyotype although patients with an M3 morphology – which had a high concordance with t15:17 – are regarded as good risk. Poor risk comprises unfavourable karyotype or >20% blasts in the post-course 1 marrow. Standard risk comprises of all other patients. The 7 year survival of these three groups were 74, 45, 15%, which was highly significant (p=<0.0001) and was explained by different respective relapse risks of 34, 50 and 74%.

In order to evaluate alloBMT, patients were analysed on donor versus no donor basis as a surrogate for an intention to treat analysis, and also taking into account the risk groups.

Results

Two hundred and thirty three patients received allogeneic BMT. The risk group as described above influenced the survival after alloBMT: 70% Good, 62% Standard, 38% Poor, and to the 223 patients who receive an autoBMT – good 73%, standard 55%, and poor 40%. However, evaluation of BMT was analysed on an intention to treat basis.

Donor vs. No. Donor Analysis

Three hundred and forty nine patients had a donor available of whom 56% went on to receive the alloBMT. Overall, there was a significantly lower risk of relapse in the donor available arm (Table 1) but this was in part counterbalanced by more deaths, thus overall survival was not significantly different from the no-donor arm. When the arms are compared within the risk categories there is a similar proportionate reduction in relapse risk in each category. There is no evidence of a survival benefit in good risk patients partly because of a small excess of deaths but also because it was possible to salvage patients with good risk disease who relapsed. The patient numbers in the poor risk category are too small for a definitive conclusion but no benefit was observed. There was a survival benefit in the donor arm for standard risk

Table 1. Donor versus no donor comparisons by risk group

	Number		Received BMT	Relapse[a]		DFS[a]		Mortality[a]		Survival[a]	
	D	ND	(%)	D	ND	D	ND	D	ND	D	ND
All patients	349	750	56	32	48	56	46	19	12	59	52
Good risk	114	223	50	24	36	61	59	20	7	68	77
Standard risk	183	423	61	34	52	54	42	18	12	59	47
Poor risk	52	104	54	60	77	31	21	21	8	24	23

[a] At 5 years. D = Donor Available ND = No Donor.

patients which, although not substantial (59 vs. 47%) was significant ($p = 0.01$). This benefit was achieved even though only 111/183 in the donor arm received the BMT.

Autologous BMT

Only a minority (38%) of patients who were eligible for randomisation were randomised. Of the 190 allocated to autoBMT 126 (66%) actually received it. The results overall and by risk category are shown in Table 2. There was no evidence to suggest that the patients who were randomised were a selected subpopulation with an inherently better prognosis. First there was no significant change in the ratio of risk groups at the time of CR achievement and those randomised. In addition there was no significant difference in survival between those who elected or wen randomised to autoBMT or stopped treatment.

On an intention to treat analysis it was clear that the relapse risk was substantially reduced in the autograft arm despite the fact that only 66% actually received the autoBMT. This translated into a significantly better disease free survival but the overall survival benefit has only become significantly apparent on longer (>2 year) follow-up patients. There was however an excess of deaths (12%) following the autograft, thereby abrogating the potential antileukaemic benefit. These overall effects were also generally seen within the risk subgroups. All enjoyed a similar proportionate reduction in relapse risk. In good risk patients – probably by chance – there was no excess mortality but salvage in the chemotherapy arm was superior, i.e., a high second remission rate and better survival from relapse.

Discussion

This large trial exemplifies the difficulty which studies of this design have with compliance with randomisation and delivering the allocated treatment. It has long been suspected that patients who came to transplantation were already selected because some patients may already have relapsed. In this experience, 40% of those with donors were not allografted and only 37% of those considered fit for randomisation were randomised, of whom only two-thirds received the autograft.

On an intention-to-treat analysis (i.e., donor vs. no donor) survival benefit was ap-

Table 2. Autologous BMT versus no further treatment (STOP) by risk group

	Number		Autograft received	Relapse[a]		DFS[a]		Survival from relapse[d]		Survival[a]	
	A	S	(%)	A	S	A	S	A	S	A	S
All patients	190	191	66	37	58[b]	54	40[c]	15	18	57	45
Good risk	51	44	61	25	49[c]	70	48[c]	38	38	74	61
Standard risk	97	112	69	40	59[c]	49	39	12	9	52	40
Poor risk	20	15	85	56	73	44	27	0	15	49	39
Unknown	22	20	50	33	58	44	42	17	9	49	44

[a] At 5 years A = AutoBMT S = STOP. [b] $p = 0.0007$. [c] $p = 0.04$. [d] At 3 years.

parent only in patients with standard risk disease. This perhaps underestimates the value of transplant in this subgroup because the benefit was only derived from the 111 patients actually transplanted, which was 61% of those with a donor. On a similar basis, no benefit was seen for patients with good risk disease, in whom 50% of those with a donor received the transplant. The problem was that the procedural mortality (20%), together with the improved rate of salvage of those who relapsed, counterbalanced the reduced relapse risk. It is conceivable that, had more patients received the allograft, transplant would have reduced survival.

Autologous BMT very substantially reduced relapse risk, but it had an associated procedural mortality of 12%. This, together with a meaningful salvage level for children and good risk patients, meant that it has taken longer follow-up to show a survival benefit. If patients who are 2 years or more out from randomisation are compared then a significant difference in survival at 7 years (p=0.006) emerges.

Transplantation is an expensive, toxic therapy. Even in this trial, where the overall survival in non-transplanted patients from diagnosis is a creditable 40% at 7 years, there is strong evidence that additional treatment to four courses (in this case transplantation), substantially further reduced the risk of relapse. The current prospective MRC Trial (MRC 12) is addressing the question of whether more treatment, i.e., with a fifth chemotherapy course, can achieve this result at less cost in terms of toxicity.

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Early Allogeneic Transplantation Favorably Influences the Outcome of Adult Patients Suffering from Acute Myeloid Leukemia

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Abstract. Allogeneic BMT for patients with acute myeloid leukemia (AML) is presently a reference therapy. The indications for this therapy may likely rely upon prognostic factors, and their importance are constantly investigated. To examine the impact of time from diagnosis to transplant on survival and Leukemia Free Survival (LFS), we analyzed 109 patients from the data base of the SFGM, patients who all had received an HLA-identical allogeneic BMT for a diagnosis of AML in First Complete Remission (CR1) between January 1987 and December 1992. All patients were prepared with cyclophosphamide (Cy) and Total Body Irradiation (TBI) (Cy - TBI), and Methotrexate (MTX) + Cyclosporine A (CSA) was used as Graft-vs.-Host Disease (GVHD) prophylaxis. Eleven patients needed 2 courses of induction to achieve CR. Time between diagnosis and BMT was 120 (64-287) days. Forty nine patients developed a grade 2 acute GVHD (Actuarial probability = 46%). With a median follow-up of 52 months (30-100), the 5 year probabilities for transplant related mortality (TRM), relapse, overall survival and LFS are respectively 25, 26, 59%, and 55%. A multivariate analysis showed that survival is adversely influenced by 3 independent factors : time to transplant (>120 days vs. ≤120 days), acute GVHD (grade 2-4 vs. grade 0-1) and age (>33 vs. ≤33). LFS is influenced by only the first two of these factors. The favorable impact of less time from diagnosis to transplant should lead to proceeding to the transplant as soon as possible. Practically speaking, this means that when such therapy is chosen for a patient with CR1 AML, the search for an allogeneic donor should be immediately investigated and transplant performed as soon as possible.

Introduction

The place of allogeneic bone marrow transplantation (BMT) as post remission treatment of acute myeloid leukemia (AML) is still discussed because of improvement of chemotherapic consolidation and best definition of transplantation indications. Indeed, for about 10 years continuous efforts have been made to ameliorate treatment of AML. After reaching remission, consolidation is now systematically given and is based, for most of the patients, on high dose chemotherapy especially including cytarabine [1-3]. With this strategy, overall survival is not significantly different between allogeneic BMT and chemotherapy, because of transplant related mortality (TRM). Moreover, development of cytogenetic technics allow definition of new prognostic groups of patients [4]. These open wider the debate on the indication of transplantation which we

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do not want to treat here. However, we must keep in mind that cases where Allogeneic BMT is feasible are few (less than 10% of all AML) and that in no published comparative study was the transplant arm worse than chemotherapy. Thus the following question arises : when a patient suffering from AML reaches remission and has the possibility of performing a transplant, what should the doctor do? We showed that a highly selected group of patients, whose graft is carried out within 100 days after diagnosis, had a good long term outcome [5]. Early transplant possibly explains these results and, in case of confirmation, that means that all should be done to perform BMT as soon as possible after complete remission. However, before making such conclusions, an investigation of the SFGM database was necessary to explore the relationship with others factors.

Patients and Methods

Patients and Donors

In order to study the outcome of patients transplanted according to standard procedures for AML in first complete remission (CR 1), we selected in the SFGM registry a population according to the following criteria : BMT performed after January, 01 1987 (n = 347) ; graft from an HLA-A, -B, -DR, identical and mixed lymphocyte culture (MLR) negative sibling (n = 331) ; conditioning regimen only with cyclophosphamide and total body irradiation (TBI) (n = 142) ; graft versus host disease prophylaxis only with Methotrexate and Cyclosporine A (n = 122).

Finally, combining all these criteria we came out with a subgroup of 109 patients, characteristics of whom are presented in Table 1. Data from these patients who were transplanted in 25 centers were reviewed. Forty two of these patients have been previously reported and correspond to the patients transplanted less than 100 days after diagnosis [5].

The donor population consisted of 59 males and 50 females. Graft was sex mismatch in 47 cases, and ABO mismatch in 40 cases.

Table 1. Patients, disease, transplant and outcome characteristics

Characteristics at diagnosis	
Age (yr) : median (range)	33 (16-50)
M/F	64/45
WBC ($\times 10^9$/l)	
median (range)	12 (1-250)6
patients with WBC > 100 : N	
FAB	
M 1 - M 2 : N (%)	55 (50)
M 3 : N (%)	15 (14)
M 4 - M 5 : N (%)	33 (30)
M 0 - M 6 - M 7 : N (%)	6 (6)
Karyotypes done: N (%)	64/109(58)
good prognosis karyotypes[a] : N (%)	16 (25)
normal karyotypes : N (%)	32 (50)
others karyotypes : N (%)	16 (25)
No. patients requiring 2 induction courses to achieve remission : N (%)	11 (10)
Time between diagnosis and BMT (days) : median (range)	120 (64-287)
	52 (30-100)
Follow up (months) : median (range)	52 (30-100)
Graft versus host disease	
aGVHD : N	70
Grade >= 2 aGVHD : N (KM probability)	49 (46 %)
Chronic GVHD : N/evaluable	31/94
Relapse : N : 5-year KM probability	20:26% (17-38)
Transplantation-related mortality : N : 5-year KM probality	27:25% (17-34)
Causes of death : N	
Relapse : N	16
GVHD ± interstitial pneumonitis : N	13
Interstitial pneumonitis : N	5
Multi-organ failure and various	9
Survival : N : 5-year KM probability	66:59% (4868)
LFS : N : 5-year KM probability	62:55% (45-65)

WBC : white blood cell[a]. Good prognosis karyotypes : t(8 ; 21) ; t(15 ; 17) ; inv16. aGVHD : acute graft versus host disease ; KM : Kaplan-Meier.
aGVHD : acute graft versus host disease ; KM : Kaplan-Meier.

Preparative Regimen

All patients received 60 mg/kg cyclophosphamide intravenously (IV) on each of two successive days followed by TBI.

Graft Versus Host Disease (GVHD) Prophylaxis

All patients received a short course of Methotrexate (MTX) and Cyclosporine A (CSA) [6] as GVHD prophylaxis. None of them received a T-cell depleted graft nor prophylaxis with monoclonal antibodies.

Statistical Analysis

The analysis was conducted in July 1995 after a minimum follow-up of 30 months. Survival was calculated from the date of BMT to the time of leukemia or transplant-related death with all other patients being censored at the time of last follow-up. LFS was measured from day 0 to the occurrence of relapse or death. When death occurred it was attributed to relapse when indicated or to transplant related causes if not (transplantation-related mortality = TRM). The probability of relapse was calculated from day 0 until relapse, censored by death or end of follow-up. Probabilities of survival, LFS, TRM and relapse were calculated using the Kaplan Meier method [7] and compared by the Log-rank test [8]. Variables potentially affecting survival and LFS were assessed in a multivariate analysis by the Cox proportional hazard model in a stepwise regression [9]. Mean comparison were performed using the Student's t-test or the Mann and Withney U-test. The χ^2 test or the Fisher's exact test were used to test for differences among groups.

Results

Engraftment and Graft Versus Host Disease

Four patients died prior to engraftment and 105 reached hematological recovery. Seventy patients developed an acute GVHD which was severe (grade 3-4) for 49 patients (Kaplan-Meier probability = 46%). Out of 94 patients who were evaluable for chronic graft versus host disease (cGVHD) 31 (36%) developed a clinical cGVHD needing immunosuppressive therapy.

Survival, Relapse and Disease-Free Survival (Table 1)

This resulted in an overall 5 year-TRM of 25% (range 17-34) which, in univariate analysis, was influenced by age [≤33 years : 13% vs > 33 years: 36% ($p<0.05$)] and moderate to severe acute GVHD [grade 0-1 : 14% vs grade 2: 38% ($p<0.05$)].

Variable eventually influencing outcome are presented in Table 2.

Table 2. Multivariate analysis of survival and LFS

Survival			
Log of likelihood = -344759	Score = 15712		$p = 0.0013$ (DF = 3)
	RR	95 % C I	p
Age	1.95	1.03 - 3.69	0.04
Time diagnosis/BMT	2.29	1.21 - 4.34	0.01
Acute GVHD	2.00	1.07 - 3.77	0.03
Leukemia Free Survival			
Log of likelihood = 383601	Score = 10911		p = 0.0043 - (DF = 2)
	RR	95 % C I	p
Time diagnosis/BMT	2.10	1.15 - 3.87	0.02
Acute GVHD	2.09	1,15 - 3.78	0.02

Time Elapsed Between Diagnosis and BMT

We further analyzed 2 groups of patients according to the time elapsed between diagnosis and BMT (≤120 days and >120 days). Patient, disease, and transplant characteristics do not differ notably between the two groups (data not shown). Survival and LFS are higher for the patients transplanted more quickly ($p<0.04$) (Table 3). These differences seem to result from an increase in the rate of relapse and transplant mortality in the group of patients transplanted after

Table 3. Patient, disease, transplant characteristics and outcome of patients with time diagnosis - BMT <= or > 120 days

	<= 120 days (N = 55)	> 120 days (N = 54)	
Time between diagnosis and BMT (days) : median (range)	92 (64-120)	164 (122-287)	$p<0.001$
Follow up (months) : median (range)	49 (27-94)	56 (27-100)	
Graft versus host disease			
aGVHD : N	35	35	
Grade >= 2 aGVHD: N(KM probability)	27 (49.4 %)	22(41.7 %)	
	19/49	12/45	
Chronic GVHD : N/evaluable			
Relapse : N (5-year KM probability)	8[18% (9-31)]	12[36% (20-55)]	NS
Transplantation-related mortality : N(5-year KM probability)	10[17%(9-29)]	17(33% (22-47)]	NS
Survival : N (5-year KM probability)	39[71(57-82)]	27(46% (32-61)]	p<0,04
LFS : N (5-year KM probability)	37[69(55-79)]	25(42% (28-57)]	p<0,04

WBC : white blood cell. Good prognosis karyotypes : t(8 ; 21) ; t(15 ; 17) ; inv16.
aGVHD : acute graft versus host disease ; KM : Kaplan-Meier.

120 days (relapse : 18% vs. 36% p = NS ; transplant related mortality : 17% vs. 33% p = NS), still these differences do not reach significance.

Discussion

For more than a decade, allogeneic BMT has been, available is a therapy for a large number of teams. Recent contributions like better standardization of conditioning regimen [10], GVHD prevention [11], management of viral diseases [12] have lead to a consistent decrease of TRM. Thus, the first goal of this study was to describe the present reality of allogeneic BMT in the situation of consolidation for AML in CR 1. We describ a group of 109 patients registered, between 1987 and 1992, in 25 French BMT units belonging to the SFGM. All patients received cyclophosphamide and TBI as the conditioning regimen. GVHD prophylaxis was performed by a combination of Cyclosporine and Methotrexate because of the superior efficacy of this regimen compared with single agent immunosuppression [13]. No patient received a T-cell depleted graft since it has been demonstrated that this technique increases the incidence of graft rejection and relapse [14, 15]. Patients who received a monoclonal antibody as GVHD prophylaxis were also excluded because this technique was not yet widely disturbed [16]. With a Kaplan-Meier probabilities for TRM, relapse, survival and LFS of, respectively, 25, 26, 59 and 55%, the outcome of these 109 patients is quite identical to those published in the recent literature [1, 3, 17-20]. Among variables associated with the outcome, acute GVHD appears as a relevant factor for TRM and survival as it has been found in number of others studies [21, 22]. Grade 3 –4 acute GVHD was furthermore associated with a decreased LFS. There was a trend for a protective effect of chronic GVHD against relapse but it did not reach significance.

Time elapsed between diagnosis and BMT is not a useful prognosis factor of the outcome but others studies showed that it may be of interest [5, 10, 23, 24]. In our analysis patients transplanted prior to day 120 had superior LFS and survival probabilities (69 vs. 42% and 71 vs. 46%, respectively). A lower TRM in this group is generally thought to explain these differences [24]; in our study TRM was decreased [17 vs. 33%) but it did not reach significance. We had think that these patients might have a better performance status at the time of grafting especially because they did not show evidence of major chemotherapy toxicity. Outcome analysis did not show a greater relapse rate for patients grafted within 120 days. Thus, long term re-

missions following allogeneic BMT did not seem to have been selected from patients already cured by chemotherapy, probably because GVL effect is the most important mechanism in eradicating leukemia [25].

In patients treated by allogeneic BMT for AML the impact of presentation features is low but transplant-related variables appear to have a major impact on the outcome. Thus, the therapeutic strategy should take into account these parameters which are under control of physicians. The possibility of using allogeneic peripheral stem cell transplantation, for example, may reduce TRM and is currently under investigation. If confirmed, the impact of time to transplant could also be an important factor and it may speed the process of transplant. Indeed, the sequential strategy that considers transplantation after relapse suffers from major drawbacks : transplant at the due time is often not possible for various reasons (patient status, organizational problems, disease progression); immunological control delivered by allogeneic BMT seems less efficient with more advanced diseases [26]. Thus, we think that when transplantation is indicated, it should be performed as soon as possible. Practically, this means that a search for an allogeneic donor should be started without delay.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Antithymocyte Globulin as Part of the Pretransplant Conditioning Regimen May Reduce Transplant-Related Morbidity and Mortality in Chronic Myelogenous Leukemia Patients Undergoing Allogeneic Bone Marrow Transplantation

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Abstract. Antithymocyte globulin (ATG) as part of the pretransplant conditioning regimen has been shown to reduce graft rejection and acute graft versus host disease (GvHD) after related allogeneic bone marrow transplantation (BMT) for severe aplastic anemia (SAA) and after unrelated BMT for hematological malignancies. Here, we report our initial experience with the pretransplant application of ATG in 16 patients undergoing allogeneic BMT for chronic myeloid leukemia (CML) between 1993 and 1996. ATG (30 mg/kg body weight) was given as 12-h continuous infusion (CI) on 3 successive days during the conditioning therapy. Outcome was compared with that of 30 CML patients transplanted between 1990 and 1996 who did not receive ATG. Ten patients (63%) in the ATG-group vs. 2 patients (7%) in the non-ATG group received bone marrow from a matched unrelated donor. Myeloid engraftment (day of ANC > 0.5 x 10^9/l) did not differ significantly between ATG (median: 17.5; range: 13–24 days) and control patients (median: 19; range: 11–28 days). Day-100 survival was 69 and 74%, respectively. Acute graft versus host disease (GvHD) ≥ grade II developed in 44% of the ATG-patients (grade II: 21%; grade III: 0; grade IV: 13%) and in 76% of the patients not receiving ATG (grade II: 17%; grade III: 38%; grade IV: 21%). Significantly more patients not receiving ATG developed severe acute GvHD (grade III and IV)(p = 0.002). Extensive chronic GvHD occured in none of the ATG patients vs. 19% in the non-ATG patients (p = 0.015). Overall survival is 69% for the ATG-group (median follow-up: 371; range 240–1310 days) vs. 43% for the control group (median follow-up: 1498; range; 772–2303 days). These preliminary results suggest that ATG as part of the pretransplant conditioning might reduce severe acute GvHD and extensive chronic GvHD and improve overall survival by reducing transplant-related mortality.

Introduction

Acute graft versus host disease (GvHD) remains one of the major problems after allogeneic bone marrow transplantation (BMT) leading to substantial posttransplant morbidity and mortality [1-3]. Despite prophylaxis with a combination of cyclosporine A (CsA) and four doses of intravenous methotrexate (Mtx) [4], 30–60% of patients undergoing allogeneic BMT experience acute GvHD grade ≥ II. One of the risk factors for acute and chronic GvHD is the underlying disease. Studies have shown a more than 2.5-fold higher cumulative incidence for patients with chronic myeloid leu-

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kemia (CML) as opposed to other disease categories [5, 6]. In an attempt at reducing the high incidence of graft rejection after allogeneic BMT from HLA-identical siblings for severe aplastic anemia (SAA), antithymocyte globulin (ATG) has been included in the preparative chemotherapy leading to a significant reduction of graft rejection. Interestingly, acute GvHD grade $\geq$ II in these patients was reduced as well ranging from 0–15% [7-9]. To decrease the incidence of graft rejection [10, 11] and graft-versus-host disease [12, 13] after BMT from unrelated donors, ATG has been used as part of the conditioning regimen. Kolb et al. [12] reported that ATG not only modified acute GvHD but also improved survival of patients with unrelated donors.

Here, we report our preliminary results of 16 CML patients who received ATG during the preparative chemo/radiotherapy (ATG-group) and compare the results with 30 CML patients undergoing allogeneic BMT without the pretransplant administration of ATG (non-ATG-group).

Materials and Methods

Patients

The records of 16 CML patients who underwent allogeneic BMT with ATG (ATG group) from March 1993 until February 1996 were reviewed and compared with those of 30 CML patients who underwent allogeneic BMT without ATG (non-ATG group) from February 1990 until September 1995 at the University Hospital Eppendorf, Hamburg. Patient characteristics are shown in Table 1. The preparative regimen for the CML consisted of fractionated total body irradiation (fTBI; 2 x 2 Gy/day for 3 days) or busulfan (Bu; 4 mg/kg for 4 days), cyclophosphamide (CTX; 60 mg/kg for 2 days) with or without etoposide (VP16; 15–45 mg/kg). Rabbit antithymocyte globulin (ATG; 30 mg/kg; Fresenius AG, Germany) was administered by slow intravenous infusion on days -3, -2, and -1 before transplantation. All patients received 250 mg of methylprednisolone and 2 mg of the antihistamine Clemastine prior to the ATG infusion.

Table 1. Details of CML patients receiving ATG and CML patients not receiving ATG

	ATG (N=16)	Non-ATG (N=30)
Diagnosis:		
CML	16	30
1. CP	14	25
> 1. CP	0	2
AP 2	3	
Age (years):		
Median	36.5	31.5
Range	23–58	16–52
Donor:		
Related	6	28
Unrelated	10	2
Conditioning regimens:		
TBI	10	4
Busulfan	6	26
VP16	1	10
CTX	16	16

Except for eight non-ATG patients receiving prednisone and cyclosporine A (CsA), prophylaxis of acute GvHD of all other patients consisted of intravenous CsA starting on day -1 plus four doses of intravenous methotrexate (MTX) posttransplant. Two patients in the non-ATG group who were transplanted from HLA-identical unrelated donors participated in a randomized study comparing a humanized anti-Il-2 receptor monoclonal antibody versus placebo as additional GvHD prophylaxis. Acute GvHD was diagnosed and graded according to the Glucksberg criteria [14] and was treated in both groups with methylprednisolon and/or antithymocyte globulin (ATG). The maximum grade of acute GvHD achieved by day 100 posttransplant was recorded. On admission, all patients prophylactically received a chinolone antibiotic (ofloxacin or ciprofloxacin) and fluconazole. Beginning in January 1995, all patients received prophylactic metronidazole (MET) [5, 15, 16] for gastrointestinal decontamination (ATG-group N = 10; non-ATG-group N = 1) and the IgM-enriched intravenous immunoglobulin (IVIG) Pentaglobin [17, 18] on days 1, 3, 7, 14, 21, and 28 (ATG-group N = 11; non-ATG-group N = 1) or non-enriched IVIG (0.5 gm/kg) on day 1, 14, 28 (ATG-group N = 6; non-ATG-group N = 29). All patients received IVIG on days 56, 84, and 120 after transplantation. Grading of chronic GvHD has been described before

[19]. All patients received methylprednisolone and an antihistamine prior to marrow infusion to prevent untoward effects. Further posttransplant supportive care has been described before [20].

Chimerism

Hematopoietic chimerism was documented by the determination of donor-type DNA by PCR amplification of 6 different polymorphic loci: HLA-DP (chromosome 6), LDL-receptor (chromosome 19), glycophorin A (chromosome 4), hemoglobin g-globin (chromosome 11), D7S8-VNTR (chromosome 7) and group specific component (chromosome 4)(Ampli Type PM, Perkin Elmer – Roche Mol. Sys., Branchburg, USA). The percentage of mixed hematopoetic chimerism was estimated from the staining intensity after reverse hybridisation to allele specific oligonucleotide probes [21].

Statistical Analysis

The statistical significance was analyzed using the Student's t test for the median (range) values and the Fisher's Exact test for the percentage values.

Results

Patient Characteristics, Engraftment, and Chimerism

The characteristics of the 16 patients who received ATG and the 30 patients who did not receive ATG are depicted in Table 1. Administration of ATG was associated with fever in most and chills in some patients, but was otherwise well tolerated. Fourteen patients developed fever on one of the three days, 5 patients on 2 of the 3 days of the ATG infusion. Four patients developed chills. Both reactions were readily controlled by temporary discontinuation of the infusion and antipyretics. One patient developed serum sickness with facial, manual, and pedal edema, and athralgia which was treated with steroids.

Table 2. Hematological engraftment of CML patients receiving ATG and CML patients not receiving ATG

	ATG (N=16)	non-ATG (N=29)	p^a
ANC > 0.5×10^9/l (median)	17.5	19	NS[a]
Range (days)	13–24	11–28	
Platelets > 20×10^9/l (median)	24	19	NS[a]
Range (days)	13–85	11–38	

[a] Student's t test.

Ten of 16 patients (63%) in the ATG group vs. 2 of 30 patients (7%) in the non-ATG group were transplanted with bone marrow from an HLA-identical unrelated donor. Since all recipients of an unrelated donor bone marrow were conditioned with TBI, 10 of 16 patients in the ATG-group vs. 4 (2 patients with unrelated donors and 2 patients in accelerated phase) of 30 patients in the non-ATG group received total body irradiation as part of the pretransplant conditioning (Table 1).

Time to myeloid engraftment defined as the number of days from marrow transplantation until the absolute neutrophil count was sustained above 0.5 x 10^9/l was not signficantly different between both groups (Table 2). One patient in the non-ATG group died too early to be evaluable for myeloid engraftment. Time to platelet engraftment defined as the number of days from transplantation until the platelet count was sustained above 20 x 10^9/l was neither significantly different between the two groups (Table 2).

Hematopoietic chimersim could be evaluated for 12 of 16 patients in the ATG group. All 12 patients had completely donor-derived hematopoiesis analyzed at different time points ranging from 3 to 58 weeks after BMT (median 12.5 weeks). All evaluable patients (N = 5) in the non-ATG group showed full donor hematopoiesis 6 – 105 weeks after transplantation.

Acute and Chronic GvHD, Day-100, Overall Survival, and Cause of Death

There was no statistically significant difference in the incidence of acute GvHD (grade

Table 3. GvHD and survival of CML patients receiving ATG and CML patients not receiving ATG

	ATG (N=16)	(N=10)	Non-ATG (N=30)	p
Acute GvHD:				
GvHD	63%	(40%)	79%	NS[a] (0.04)
GvHD grade II	44%	(10%)	72%	NS[a] (0.0007)
GvHD grade III–IV	13%	(0)	59%	0.0028[a] (0.0008)
Chronic GvHD:				
Chronic GvHD	45%	(62%)	62%	NS[a] (NS)
Limited	45%	(62%)	43%	NS[a] (NS)
Extensive	0	(0)	19%	0.015[a] (0.015)
Survival:				
Day-100 survival	69%	(80%)	74%	NS[a] (NS)

[a] Fisher's Exact test for the percentage values (ATG patients vs non-ATG patients). Values in parentheses for the ATG patients represent the values for the ATG patients receiving bone marrow from an unrelated donor. *p* values in parentheses = ATG patients receiving bone marrow from an unrelated donor vs. all non-ATG-patients.

I – IV) or GVHD grade ≥ II: ATG-group 63 and 44% vs. non-ATG group 79 and 72%, respectively. Considering only the ATG patients receiving marrow from an unrelated donor (N = 10) and comparing them with the non-ATG group, there was a significant difference in the overall incidence of acute GvHD, GvHD grade ≥ II, and GvHD grade III-IV (Table 3). Furthermore, there was a significant difference in the incidence of severe acute GVHD (grade III–IV) between the entire ATG-group (13%) and the non-ATG group (59%)(p = 0.028; Table 3). Excluding the patients in the non-ATG group who received CsA and steroids (N = 8) instead of CsA and methotrexate (N = 22) did not change the difference in the incidence of severe acute GvHD. Chronic GvHD occurred in 45% of the evaluable patients in the ATG-group and 62% in the non-ATG group (not significant; Table 3). While there was no difference in the incidence of limited GvHD, extensive chronic GvHD occurred in a significantly higher proportion of patients not receiving ATG (19%) vs patients receiving ATG (none; p = 0.015; Table 3). Excluding the patients in the non-ATG group who received CsA and steroids (N = 8) instead of CsA and methotrexate (N = 22) did not change the difference in the incidence of extensive chronic GvHD.

Within the first 100 days posttransplant, 5 patients (31%) who received ATG and 8 patients (26%) who did not receive ATG died, giving a day-100 survival of 69 and 74%, respectively. Kaplan-Meier analysis (Fig. 1) did not reveal a statistically significant difference in long-term survival between the two groups (p = 0.39). With a median follow-up of 371 days (240–1310 days) and 1498 days (772–2303 days), 11 of the ATG patients (69%) and 13 of the non-ATG patients (43%) are alive, repectively. Acute and chronic GvHD were the main reasons for mortality in both groups: ATG: 3 of 5 patients (60%) vs. non-ATG: 11 of 17 patients (64%). Patients receiving ATG as part of the conditioning did not experience more opportunistic infections. EBV-induced lymphoma did not occur in either group.

Discussion

This retrospective analysis was aimed at defining the contribution of ATG on the risk of acute GvHD after allogeneic marrow transplantation (BMT). Our results confirm earlier reports [12, 13] which showed a beneficial effect of pretransplant administration of antithymocyte globulin (ATG) on the incidence and severity of graft-versus-host disease and survival after allogeneic bone marrow transplantation (BMT). ATG administration did not delay engraftment, did not result in detectable mixed chimerism, and did not lead to more infectious complications. Because of the small sample size and relatively short follow-up, our data must be viewed as preliminary. Nevertheless, the results are encouraging.

ATG has been used to decrease the incidence of graft rejection after allogeneic bone marrow transplantation (BMT) for severe aplastic anemia (SAA) [7-9] and after BMT with unrelated donors for hematological malignancies [10]. Based on encouraging results from conditioning regimens containing antithymocyte globulin combined with

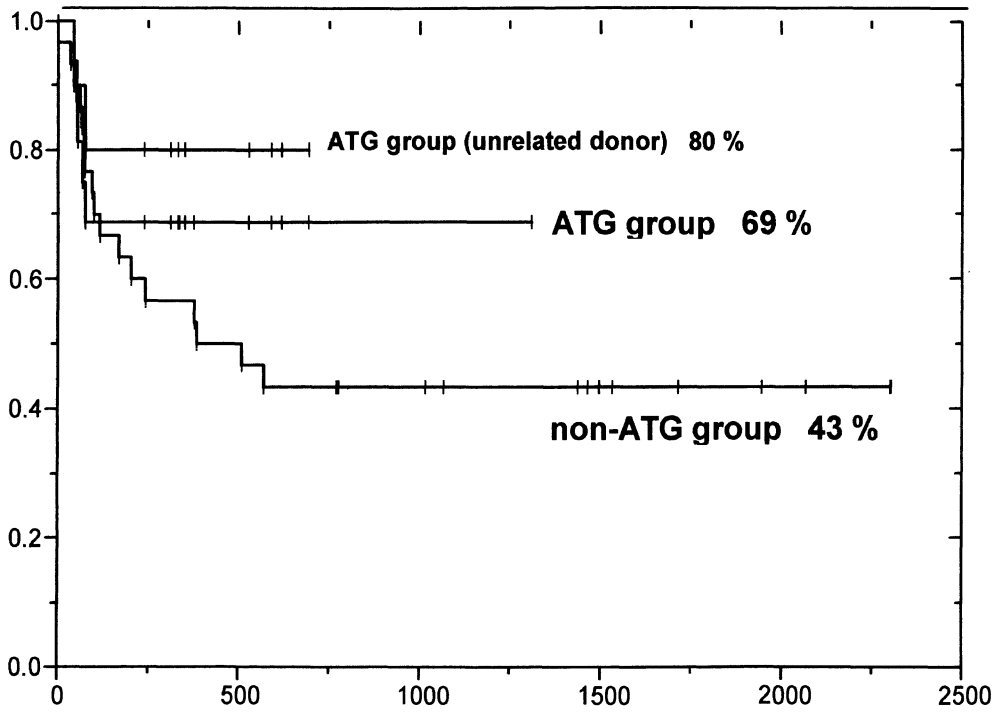

Fig. 1. Posttransplant survival of all ATG patients (N = 16) vs. ATG patients receiving marrow from unrelated donors (N = 10) vs. non-ATG patients

alkylating agents, Storb et al. [22] used cyclophosphamide plus ATG to prepare patients with severe aplastic anemia (SAA) who had rejected the first transplant. Since this regimen proved to be very effective, it was also used for patients with SAA receiving their first transplant [7] leading to an excellent 92% 3-year actuarial survival. Only two of 39 patients rejected their grafts. Interestingly, acute GvHD occurred in only 15% and chronic GvHD in 34% of patients. In another report, eight of nine patients transplanted for SAA survived without graft rejection or development of acute or chronic GvHD [8]. In an attempt at reducing the higher incidence of graft rejection after allogeneic BMT from unrelated donors, many transplant centers have included ATG in the pretransplant conditioning.

ATG has also been used with varying success as prophylaxis and treatment of graft-versus-host disease after allogeneic bone marrow transplantation. In general, it can be effective if given to recipients before or shortly after transplantation or if the hematopoietic graft is incubated with ATG before infusion [23-29]. Two early prospective studies randomly assigned HLA-identical allogeneic marrow graft recipients to receive or not receive prophylactic ATG in addition to regular methotrexate. Neither study demonstrated a lessened severity or incidence of GvHD or an improvement in survival compared to the control group [31, 32]. In another study [33], however, a combination of methotrexate, ATG, and prednisone significantly decreased the incidence of acute graft-versus-host disease (Mtx: 48% vs. Mtx + prednisone + ATG: 21%), but there was no significant difference in survival or the incidence of chronic GvHD between the two groups.

Earlier studies have shown that the incidence of GvHD after marrow transplantation from HLA-identical siblings for chronic myelogenous leukemia is similar when methotrexate (Mtx) or cyclosporine A (CsA) alone is used [34]. However, a significant re-

duction in the cumulative incidence of grade II to IV acute GvHD was observed in patients who received a combination of CsA and short-course Mtx (33%) as compared to those receiving CsA alone (54%) ($p = 0.014$). The actuarial survival rates in the two groups at 1.5 years were 80 and 50%, respectively ($p = 0.062$) [35]. Thus, the failure of earlier studies to show a beneficial effect of prophylactic ATG on the incidence and severity of acute and chronic GvHD and survival might be attributable to the suboptimal prophylaxis of GvHD (Mtx without CsA).

Recently, two groups [12, 13] reported that ATG not only modified acute GvHD but also improved survival of patients who received marrow from unrelated donors. As suggested by earlier studies in dogs [36], the effect of ATG seemed to be dose dependent since acute GvHD developed in three patients not given ATG, in 21 of 23 patients given 5 mg/kg ATG on 4 successive days, 14 of 21 patients given 10 mg/kg ATG on 4 successive days, and five of ten patients given 20 mg/kg ATG on 4 succesive days ($p = 0.04$). The cumulative ATG dose which seemed to be most effective in that series (80 mg/kg) is similar to the cumulative ATG dose (90 mg/kg) in our patients.

Considering the higher incidence of acute GvHD after BMT from unrelated in comparison to related HLA-identical marrow donors and the much higher proportion of unrelated donors for patients who received ATG, one would assume that the patients who received ATG were at an increased risk of developing acute GvHD in comparison to the non-ATG group. The opposite result in our patients further supports the effectiveness of ATG.

A retrospective analysis from Essen involving 194 patients showed that complete and sustained growth suppression of intestinal anaerobic bacteria independently reduced the risk of grades II – IV acute GvHD after HLA-identical sibling BMT [5]. In an open prospective randomized trial, the same group was able to demonstrate that intestinal bacterial decontamination by a combination of ciprofloxacin plus metronidazole reduced the initial and maximum degree of severity of acute GvHD when compared to patients only receiving ciprofloxacin [15, 16]. All of our patients received a prophylactic chinolone antibiotic. Starting at the beginning in 1995, all patients received metronidazole for anaerobic decontamination. Patients also received regular intravenous immunoglobulin which has been shown to reduce the rate of GvHD and postengraftment septicemia [37-42]. Also starting at the beginning of 1995, patients received IgM-enriched intravenous immunoglobulin (Pentaglobin) on days 1, 3, 7, 14, 21, and 28 after transplantation instead of regular immunoglobulin on days 1, 14, and 28. IgM-enriched immunoglobulins have been shown to reduce peak endotoxaemia significantly after bone marrow transplantation and to protect patients from dying from infection in the first 100 days after transplantation [17, 18]. Furthermore, Pentaglobin has been shown to be effective in the treatment of mild and moderate GvHD [43]. Whether ATG by itself as suggested by other studies [12, 13] or in combination with Pentaglobin and/or metronidazole leads to less severe acute and chronic GvHD and improved survival can not be determined by the results of the present report since most of the ATG patients, (N = 10) received Pentaglobin and metronidazole (compared to only one patient in the non-ATG group).

Two randomized studies have shown that the incidence or severity of bacterial, fungal, and nonpulmonary viral infections does not appear to be increased in patients receiving ATG as GvHD prophylaxis [32] or treatment [44]. We found no increased incidence of infection in patients who received ATG. Enhanced immunosuppression by the administration of ATG has to be weighed against the severe imunosuppression by acute and chronic GvHD and their treatment.

To further define the role of prophylactic administration of ATG, one has to consider some key variables such as the timing of ATG administration [36], the particular lot of ATG used, the species in which the ATG was produced (horse vs. rabbit), the dose administered, or the metabolism of ATG in individual patients [32].

We conclude that ATG (30 mg/kg on 3 days) as part of the pretransplant conditioning regimen for patients with chronic myelogenous leukemia undergoing related or

unrelated bone marrow transplantation might lead to improved disease-free survival by reducing transplant-related mortality from severe acute and extensive chronic GvHD. ATG does not appear to abrogate the graft-versus leukemia effect in CML patients.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Marrow Transplants from Unrelated Donors for the Treatment of High-Risk Acute Leukemia

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Introduction

Transplantation of allogeneic stem cells has become a well established treatment for a variety of hematological malignancies including poor-risk acute leukemias [1]. This procedure has the highest probability of success if donor and recipient are closely HLA-matched. For patients without a suitable relative, one option is to identify an HLA-identical unrelated volunteer donor [2]. Despite the enormous polymorphism of HLA genes, the probability of finding a compatible unrelated donor is increasing since the international registries contain now more than 3.5 million HLA-typed volunteers. Shortening the time needed for the donor search has made unrelated donor bone marrow transplants (URD-BMT) a realistic alternative for the treatment of patients with acute leukemia. Five-hundred ninety-five URD-BMT were facilitated by the U.S. National Marrow Donor Program (NMDP) for the treatment of acute leukemias between 1989 and 1993 [3]. Ninety-four acute leukemia patients receiving marrow transplants from unrelated donors were reported to the European Group for Blood and Marrow Transplantation during 1992 [4], 165 in 1993, [5], 240 in 1994 [6], and 349 in 1995 [7]. Here, we report the results of URD-BMT performed in Seattle for the treatment of acute leukemia, during the last 16 years. Here we focus on detailed analysis of the factors associated with the outcome in 174 patients with primary acute leukemia receiving URD-BMT and on description of the outcome in a cohort of 18 patients with Philadelphia-positive (Ph^+) acute lymphoblastic leukemia (ALL) as an example of a very high risk disease that should be treated by transplantation early during first remission.

Prognostic Factors in Patients with Primary Acute Leukemia

Patients and Methods

One-hundred seventy-four patients with primary acute leukemia received unrelated donor marrow transplants at the Fred Hutchinson Cancer Research Center (FHCRC) or the Seattle Veterans Affairs Medical Center, between September 1979 and June 1994. Other patients with acute leukemia transplanted from unrelated donors during the same period not included in this report are those with preceding myelodysplasia, leukemia secondary to chemotherapy or radiation, leukemia relapsing after autologous transplant, and patients transplanted with T-cell depleted marrow. Characteristics of the patients are summarized in Table 1. All patients transplanted during first complete remission had poor prognosis fea-

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Table 1. Characteristics of 174 patients receiving unrelated donor marrow transplants for acute leukemia (After Sierra J, Storer B, Hansen JA et al. Transplantation of marrow cells from unrelated donors for treatment of high-risk acute leukemia: The effect of leukemic burden, donor HLA-matching and marrow cell dose. Blood 1997, Vol 89: pp 4226-4235)

Characteristic	Remission n= 66	Relapse n= 94	PIF n= 14	Total n= 174
Age (years)				
<18/≥18	33/33	33/59	5/9	71/103
Median (range)	17 (1-47)	21 (.5-51)	31 (7-54)	20 (.5-54)
Sex (male/female)	34/32	53/41	11/3	98/76
AML/ALL/Hybrid AL	21/41/4	40/49/5	13/1/0	74/91/9
WBC at diagnosis ($x10^9$/l)				
Median (range)	10 (.7-195)	15 (.7-650)	17 (1.3-288)	13 (.7-650)
Patient CMV seropositive pretransplant	27	44	11	82
Donor HLA-minor mismatched	22	34	6	62

Abbreviations: AL, acute leukemia; ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; CMV, cytomegalovirus; PIF, primary induction failure; WBC, white blood cells.

tures. Duration of the first remission was less than one year in 35 (83%) of the 42 patients transplanted in first relapse, and in 16 (46%) of the 35 patients transplanted in second remission.

The interval time between initiation of the donor search and transplant ranged between 1 and 68 months (median 5.3 months). The FHCRC Clinical Immunogenetics Laboratory confirmed the HLA typing for all patients. Histocompatibility criteria for donor selection have been previously published [8]. HLA-DRB1 allele compatibility was assessed by hybridization of amplified DNA with sequence-specific oligonucleotide probes (SSOP) in 151 (87%) pairs [9]. In the remaining cases (13%), HLA-DR typing was performed using nylon-wool purified B cells in a modified microtoxicity crossmatch assay [10]. "Dw" assignments were made by stimulation with HLA-D homozygous typing cells in a standard HLA-D typing assay [11]. The number of patients receiving marrow from donors with minor HLA-disparity is shown in Table 1.

One-hundred sixty-eight patients (96%) received cyclophosphamide, 60 mg per kg recipient body weight, intravenously on each of 2 successive days and total body irradiation (TBI) as conditioning regimen for transplantation. TBI was hyperfractionated and administered to a total exposure of 12 Gy in 4 (2%) cases, 13.2 Gy in 94 (53%), 14.4 Gy in 64 (37%), and 15.75 Gy in 6 (4%). Chemotherapy alone was the preparative regimen in 6 (4%) cases.

Aspirated marrow collected at the harvest center was generally transported at room temperature and was administered intravenously after completion of the preparative regimen. The median number of nucleated cells infused, not corrected for potential blood contamination, was 3.65×10^8/kg (range 0.7 to 46.1). The median (range) values in children and adults were 4.8 (1.4-46.1) $\times 10^8$/kg and 3.3 (0.7-9.9) $\times 10^8$/kg, respectively.

To prevent bacterial infections intravenous antibiotics were administered while the absolute neutrophil count (ANC) was less than 0.5×10^9/l. Only 8% of patients (n=13) received granulocyte-macrophage colony stimulating factor (GM-CSF) with the intention of accelerating neutrophil recovery. Cyclosporine and methotrexate, administered according with the schedule reported elsewhere (12), was the GVHD prophylaxis in 148 (85%) cases.

The first of three consecutive days when the absolute neutrophil count (ANC) surpassed 0.5×10^9/l was considered the day of myeloid engraftment. Primary failure to engraft was defined as the absence of myeloid engraftment in patients who survived in remission for at least 28 days after transplantation. ANC recovery above $0.5x10^9$/l followed

by a sustained decrease of counts to below $0.10x10^9$/l was defined as secondary graft failure. The first of seven consecutive days with a platelet count exceeding $50x10^9$/l, in absence of transfusion support, was considered the day of platelet engraftment.

Consensus criteria were used to diagnose and grade acute and chronic GVHD [13]. Primary treatment for acute GVHD was glucocorticoids, and secondary treatment included anti-T cell antibodies in some cases [14-16]. Patients who survived in remission at least 100 days after transplantation were evaluable for the occurrence of chronic GVHD. In absence of extensive chronic GVHD, immunosuppressive therapy was tapered and discontinuation by day 180 if the patients remained free of chronic GVHD. Patients with clinical extensive chronic GVHD were continued on immunosuppressive therapy for at least an additional 9 months [17-18].

Results of the study were analyzed as of June 30, 1995. Cumulative incidences of granulocyte recovery, platelet recovery, acute GVHD, chronic GVHD, non-leukemic death, and relapse were calculated. Leukemia-free survival (LFS) was estimated according the Kaplan-Meier method [19]. Donor and transplant characteristics were evaluated in a univariable analysis. P-values appearing in the figures represent the results of log-rank tests [20]. Parameters influencing LFS, non-leukemic death, and relapse were analyzed in the entire group of 174 cases, and separately in the 66 patients transplanted in remission versus the 94 patients transplanted in relapse. Variables significant at a level ≤ 0.1 in the log-rank test were evaluated in a multivariable analysis using the Cox regression method [21]. A multivariable analysis of parameters predicting granulocyte and platelet engraftment, development of grades III-IV acute GVHD and extensive chronic GVHD was carried out in the entire group of 174 patients.

Results

Engraftment

Ten of 16 patients who died during the first 28 days after transplantation never achieved a neutrophil count above 0.5×10^9/l. One hundred fifty-six (98%) of the remaining 158 patients achieved sustained donor engraftment. One patient surviving more than 28 days failed to engraft and another had secondary graft failure. Median time to an ANC greater than 0.5×10^9/l was 21 days. Administration of a marrow cell dose above the median of 3.65×10^8 nucleated cells/kg and transplantation in remission were associated with faster neutrophil recovery (RR: 1.5, C.I.: 1.1-2.0, p=0.01, and RR: 1.5, C.I.: 1.1-2.1, p=0.01 respectively). Episodes of neutropenia below 0.5×10^9/l occurred between day 42 and 84 after transplantation in 2 (2%) patients in the high cell dose group and in 13 (15%) in the low cell dose group. Self-sustained platelet counts above 50×10^9/l by 100 days occurred in 48% of patients. Patients receiving $>3.65\times10^8$ marrow nucleated cells/kg and those transplanted in remission had a faster platelet recovery (RR: 4.5, C.I.: 2.7-7.5, p<0.001 and RR: 2.6, C.I.: 1.7-4.2, p<0.001, respectively).

GVHD

The cumulative incidence of grades II-IV acute GVHD was 82% and of grades III-IV GVHD was 47%. A marrow cell dose above the median value of 3.65×10^8/kg was the only factor associated with a lower incidence of grade III-IV acute GVHD (RR: 0.6, C.I.: 0.4-0.9, p=0.01). Ninety-two (53%) of the patients survived in remission at least 100 days after transplantation and were at risk for chronic GVHD. Clinical extensive chronic GVHD developed in 52% of evaluable patients with a median onset of 206 days after transplantation. Older patient age (RR: 1.03, C.I.: 1.01-1.05, p=0.01) and previous grades III-IV acute GVHD (RR: 1.8, C.I.: 1.04-3.3, p=0.04) were associated with an increased incidence of clinical extensive chronic GVHD. Marrow cell dose did not affect the development of extensive chronic GVHD.

Non-Leukemic Death

Cumulative mortality from non-leukemic death was 39% at 5 years, with 73% of these events occurring within the first 100 days. Marrow cell dose above the median was the

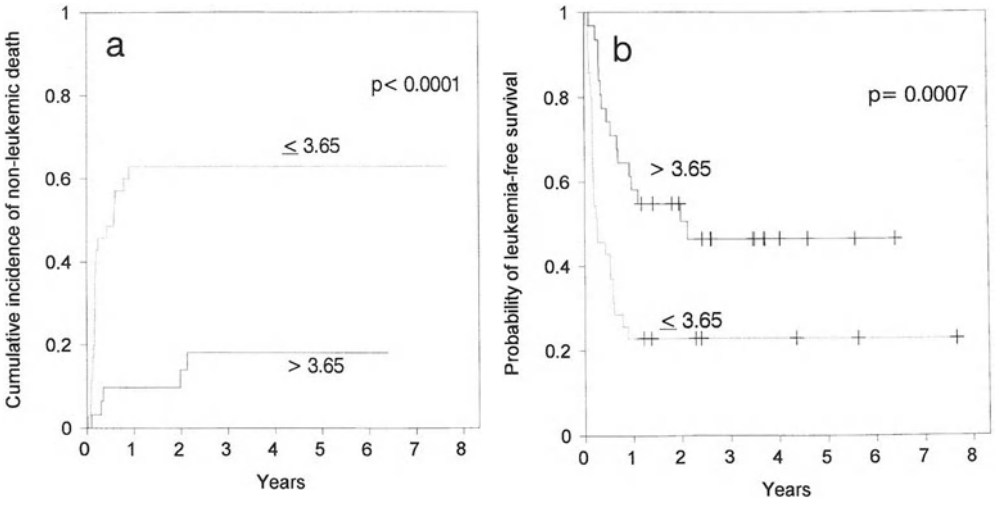

Fig.1a,b. Effect of marrow cell dose (nucleated cells 3 108/kg of recipient body weight) on: *a* cumulative incidence of non-leukemic death after transplant in remission, *b* leukemia-free survival after transplant in remission

only significant factor associated with a lower risk of non-leukemic death in the entire group of 174 patients (RR: 0.5, C.I.: 0.3-0.8, p=0.003). The effect of marrow cell dose was even more prominent in the 66 patients transplanted in remission (RR: 0.2, C.I.: 0.1-0.4, p=0.0001, Fig.1a). For the latter group, the favorable effect of a high marrow cell dose on non-leukemic death remained significant when considering marrow cell dose as a continuous variable in the models. Among patients transplanted during remission, a higher marrow cell dose was associated with a lower cumulative incidence of non-leukemic death both in the 33 patients <18 years of age (73 vs. 15%) and in the 33 patients ≥18 years of age (61 vs. 28%). In addition, patient age was not a significant factor in any of the multivariable analyses that considered the effect of marrow cell dose. Fatal bacterial or fungal infections were more frequent in patients in remission transplanted with low cell doses than in those transplanted with a higher cell dose (32 vs. 3%, p=0.006). There was no significant association between marrow cell dose and non-leukemic death among patients transplanted during relapse. The absence of blasts in the peripheral blood before starting the conditioning regimen was the single factor associated with a decreased risk of non-leukemic death in this group (RR: 0.3, C.I.: 0.1-0.7, p=0.002).

Relapse

The cumulative incidence of leukemic relapse among the 174 patients was 41%. This complication appeared between 0.7 months and 5.5 years after transplantation with a median onset at 9 months. Marrow cell dose had no influence on the risk of relapse after transplantation. Transplantation during remission was associated with a significantly lower risk of leukemic relapse compared to transplantation in relapse or after primary induction failure (RR: 0.3, C.I.: 0.2-0.5, p=0.0001; Fig. 2a, 2b). Among patients in relapse, the presence of <30% leukemic blasts in the marrow before starting the conditioning regimen was associated with a decreased risk of post-transplant leukemia recurrence (RR: 0.4, C.I.: 0.2-0.8, p=0.01, Fig. 3a). The incidence of relapse was lower in the group of patients transplanted from HLA-mismatched donors compared to matched donors (RR: 0.5, C.I.: 0.3-0.9, p=0.03). The use of an HLA-mismatched donor was the single factor associated with a decreased risk of recurrent malignancy after transplantation (RR: 0.2, C.I. 0.05-1.1, p=0.06) among the 66 patients transplanted during remission.

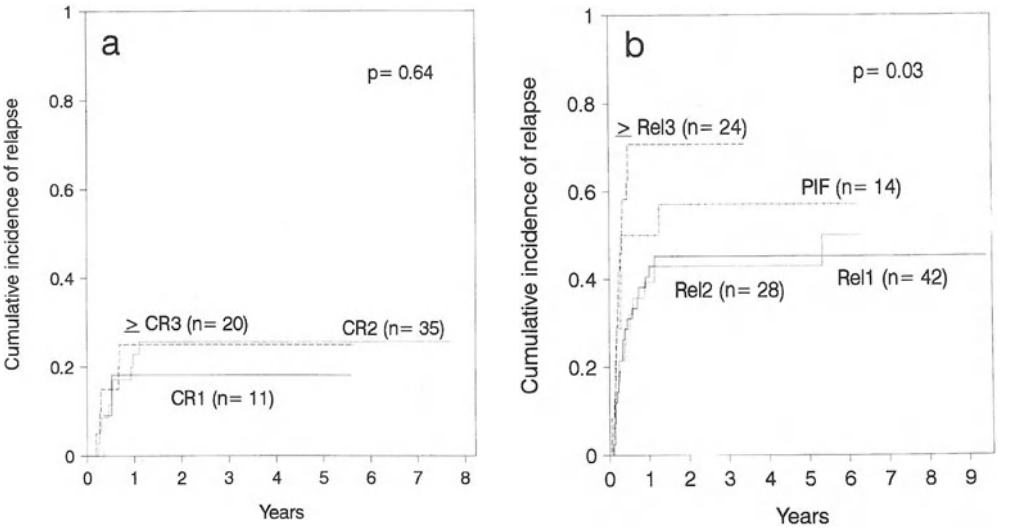

Fig. 2 a, b. Cumulative incidence of relapse of patients transplanted in remission. *b* Cumulative incidence of relapse of patients transplanted with active leukemia

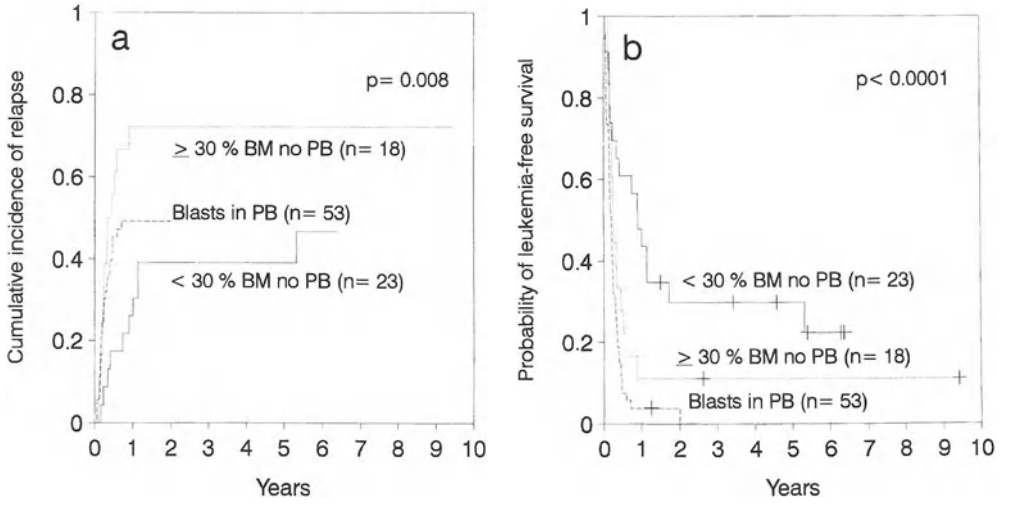

Fig. 3 a, b. Cumulative incidence of relapse b leukemia-free survival depending on the number of blasts in marrow *(BM)* or blood *(PB)*

Leukemia-Free Survival

After a median follow-up of 3.5 years (range 1.2 - 9.6 years), 34 (19%) of the 174 patients remain alive and disease-free. The Karnofsky score is 100% in 25 (73%) of these 34 patients, between 90 and 99% in 4 (12%) and below 90% in 5 (15%). Seven patients remain under treatment for chronic GVHD for periods ranging between 16 and 69 months, whereas the remaining 27 (79%) have discontinued immunosuppressive treatment.

LFS was not significantly different in ALL vs. AML or hybrid leukemia (p=0.21). In the group of 11 patients transplanted in first remission, 6 remain alive and disease-free. Fifteen patients with AML transplanted in second remission had a 27+11% probability of

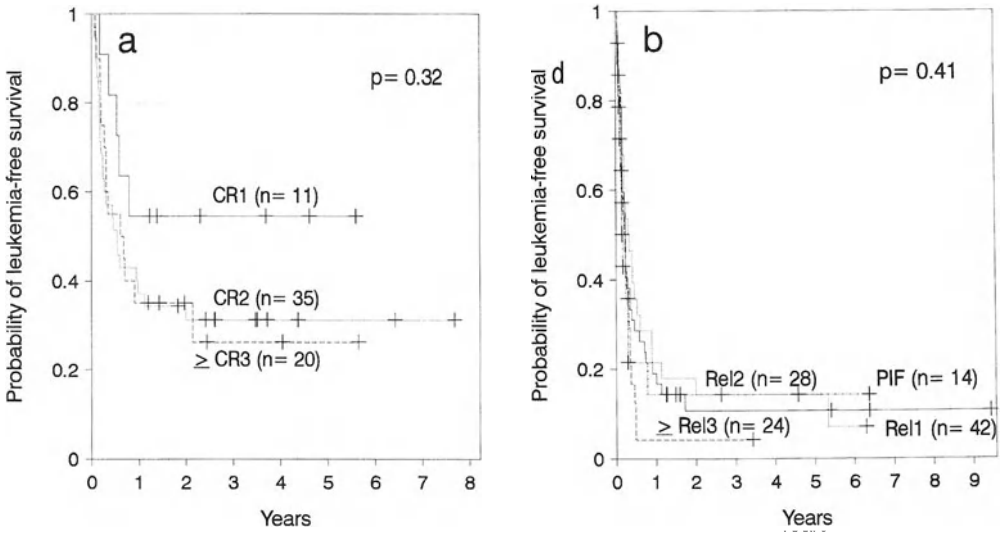

Fig. 4 a, b. Leukemia-free survival of patients transplanted in remission. *CR1* first remission; *CR2* second remission; ≥*CR3*, third or subsequent remission. *b* Leukemia-free survival of patients transplanted with active leukemia. *Rel1* first relapse; *Rel2* second relapse, ≥*Rel3*, third or subsequent relapse; *PIF* primary induction failure

Table 2. Multivariable analysis of factors associated with leukemia-free survival (After Sierra J, Storer B, Hansen JA et al. Transplantation of marrow cells from unrelated donors for treatment of high-risk acute leukemia: the effect of leukemic burden, donor HLA-matching and marrow cell dose. Blood 1997, vol 89: pp 4226-4235)

Group	Parameter	Favorable	Leukemia-free survival		
		Category	RR[a]	95% CI	*P* value
All patients (n= 174)	Status	Remission	0.5	0.3-0.7	0.0001
	Cell dose	> 3.65[b]	0.6	0.5-0.9	0.01
	Patient CMV status	Seronegative	0.7	0.5-1.0	0.05
Remission (n= 66)	Cell dose	> 3.65[b]	0.3	0.2-0.6	0.0009
Relapse (n= 94)	Blasts in PB	None	2.5	1.7-5.0	0.0001

Abbreviations: CI, confidence interval; CMV, cytomegalovirus; n, number; PB, peripheral blood; RR, relative risk.
[a] Relative risk refers to relapse or death; RR> 1 is unfavorable.
[b] Marrow nucleated cells $\times 10^8$/kg recipient body weight.

LFS at 5 years vs. 37+11% for 19 patients with ALL in second remission (p= 0.7). None of 3 patients with AML in subsequent remissions survives vs. a 25+12% LFS at 5 years for 16 patients with ALL. In patients transplanted during relapse, 40 with AML had a 12±5% LFS at 5 years and 49 patients with ALL had a 10+ 4% LFS (p= 0.7). LFS was significantly better in patients transplanted during remission than in those transplanted in relapse or after primary induction failure (Fig. 4a, b, Table 2). Patients transplanted during relapse with less than 30% blasts in the marrow and no blasts in the blood had a 5-year leukemia-free survival of 30±10%, those with ≥30% blasts in the marrow and no blasts in the blood had a 5-year LFS of 11±7%, and patients with blasts in the blood

had a LFS of 0% at 2 years (Fig. 3b, Table 2). In the multivariable analysis, higher marrow cell dose was significantly associated with improved LFS (Table 2), remaining the single favorable factor among patients transplanted during remission (Fig. 1b, Table 2). The effect of marrow cell dose was not dependent on patient age and obesity. As shown in Table 2, LFS was better for patients with negative CMV serology before transplant.

Transplation for Treatment of Philadelphia Chromosome-Positive ALL

Patients and Methods

Between November 1988 and December 1995, 18 patients with Ph chromosome-positive ALL received URD-BMT at the FHCRC. Thirteen of these patients have also been included in the previous section. The diagnosis had been established at other institutions, and initial treatment had consisted of intensive chemotherapy regimens for high-risk ALL. The Ph chromosome had been detected at diagnosis in 16 patients and was evident after relapse prior to transplant in one other case. In one additional patient in whom cytogenetic study had been unsuccessful, the bcr-abl rearrangement was present at diagnosis. Characteristics of the patients are summarized in Table 3. In 17 patients, leukemic cells had a B-lineage CD10+ phenotype, and one patient had a CD10- disease.

A single disparity at one HLA-A, B or DRB1 locus was present in 7 (39%) donor-recipient pairs (Table 3). The regimen used for conditioning was cyclophosphamide, 60 mg/kg, intravenously on each of 2 successive days, and TBI to a total exposure of 13.2 Gy (11 fractions) in patients $\geq$ 18 years of age and 14.4 Gy (12 fractions) in children. Males received 4 Gy of additional testicular radiation. All patients received 2 doses of intrathecal methotrexate before the transplant and 4-6 additional doses after engraftment. A median of 3.0×10^8/kg recipient body weight (range 1.2-9.9) nucleated marrow cells were harvested. Cytomegalovirus (CMV) serology pretransplant was positive in 7 patients. In the remaining 11 cases CMV serology was negative in both donor and recipient. Acute GVHD prophylaxis consisted of cyclosporine and methotrexate in 12 cases [12], methotrexate and FK506 in 3 [22], cyclosporine plus methotrexate and humanized monoclonal antibody against the interleukin-2 alpha chain receptor (anti-Tac) in 1 patient [23], and T-cell depletion of the marrow (total depletion of CD4+ cells and partial depletion of CD8+ cells) plus cyclosporine and methotrexate posttransplant in 2 patients who received marrow from a one-HLA-antigen major mismatched donor (study in progress). Definitions for engraftment, graft failure, acute GVHD, chronic GVHD, non-leukemic death, relapse, and LFS have been described above. Follow-up of the patients was updated as of July 1, 1996.

Results

None of the 17 patients surviving at least 28 days after transplantation experienced graft failure. Median time to recover 0.5×10^9/l ANC was 23 days (range 12-36 days). A platelet count above 50×10^9/l was achieved in 13 patients at a median time of 26 days (range 13-361 days) after transplant. Grade II-IV and grade III-IV acute GVHD appeared in 76 and 35% of patients, respectively. Eight (62%) of the 13 survivors in remission for more than 100 days developed chronic GVHD at a median of 130 days (range 100-285 days) and five continue on immunosuppressive treatment between 269 and 2191 days after transplant (median 377 days). Non-leukemic death occurred in four patients (22%) and resulted from cardiac failure in one case, idiopathic pneumonia syndrome in two, and pulmonary aspergillosis in one.

Two patients surviving in remission have chronic GVHD.

Leukemia recurred at a median of 107 days (range 56-135 days) after transplant in 5 of 10 (50%) patients transplanted with primary refractory or relapsed disease, and there were no recurrences among the 8 patients transplanted during remission. All patients who relapsed after transplantation

Table 3. Characteristics and outcome in 18 patients receiving marrow transplants from unrelated donors for treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia (After Sierra J, Radich J, Hansen JA, et al. Marrow transplants from unrelated donors for treatment of Philadelphia chromosome-positive acute lymphoblastic leukemia. Blood 1997, in press)

Age (yrs)	Gender (Pt/D)	Time interval search start to BMT (m)	Donor HLA-mismatching	Disease stage at BMT	GVHD Acute grade	GVHD Chronic	Relapse (m)	Survival (m)
25	M/M	8.9	None	1st Remission	III	Extensive	No	>73.0
47	F/F	3.6	None	1st Remission	II	Extensive	No	>41.5
26	M/F	2.5	None	1st Remission	II	No	No	>24.7
3	F/M	4.8	None	1st Remission	III	Extensive	No	>17.1
1.7	M/F	3.8	None	1st Remission	II	Extensive	No	>16.5
43	F/F	6.1	None	1st Remission	I	Extensive	No	9.6
51	M/M	4.5	None	1st Remission	I	No	No	>9.3
34	M/M	2.9	None	2nd Remission	I	Limited	No	>9.0
20	M/M	4.0	DRB1[a]	1st Relapse[c]	II	Extensive	No	>31.4
29	M/M	3.5	None	1st Relapse	II	Extensive	No	>12.6
43	M/M	2.6	None	1st Relapse	II	NA	1.9	4.0
47	F/F	5.9	A[b]	2nd Relapse	I	No	4.5	5.2
8	F/M	4.9	B[a]	2nd Relapse	IV	NA	No	2.0
20	M/M	4.1	DRB1[b]	2nd Relapse	NA	NA	No	0.1
19	F/F	3.0	DRB1[a]	3rd Relapse	III	NA	3.2	3.7
35	F/M	5.6	Ba	3rd Relapse	IV	NA	No	2.5
21	F/M	11.7	None	4th Relapse	III	No	3.9	4.2
25	M/M	8.3	DRB1[a]	Induction failure	II	No	3.6	4.9

Abbreviations: BMT, bone marrow transplant; D, donor; F, female; GVHD, graft-versus-host disease; M, male; m, months; NA, not applicable; Pt, patient; yrs, years. [a]Minor mismatch defined as a single disparity for HLA-A or HLA-B antigens belonging to the same crossreactive group, or a single DRB1 disparity for subtype alleles within the same DR specificity. [b]One-antigen major mismatch defined as a single disparity not fullfilling the criteria for minor mismatch. [c]Transplanted in CNS relapse and marrow remission. The dashed line separates patients in complete remission at transplantation from those with primary refractory or relapsed leukemia.

died shortly thereafter. Tests for the bcr-abl gene rearrangement were negative in 7 of the 9 survivors. The remaining 2 patients tested positive by PCR at 3 months after transplant, but remain in cytogenetic and hematological remission 10 and 38 months after the positive PCR test.

After a median follow-up of 17 months (-range 9 to 73 months), 9 of the 18 patients are alive in continuous remission. Kaplan-Meier probability of LFS is 49±12% at 2 years (Fig. 5). Six of the seven patients transplanted in first remission, 2 of 3 in first relapse, and the one in second remission are leukemia-free survivors. The Karnofsky scores of the 7 surviving adults are 100% in 3, 90% in 2, and 80% in 2 cases. One of the 2 children who survive has a Lansky play score of 100% and the other has a score of 60% due to extensive scleroderma.

Discussion

This report, studies from other institutions [24-26] and the data published by the NMDP [3,27] (Table 4) demonstrate that URD-BMT can be effective therapy in children and young adults with poor-risk acute leukemia when an HLA-compatible family donor is not available. As observed in matched sibling donor transplants, the outcome was strongly influenced by disease-stage, and was significantly better among patients transplanted during remission than among those in relapse. The results observed in patients with Ph-positive ALL, with 6 of 7 patients transplanted in first remission surviving leukemia-free, support continued investigations of the usefulness of URD-BMT in first remission for patients with high risk of leukemia recurrence after more conventional treatment. This group would include patients with ALL or AML who have difficulty

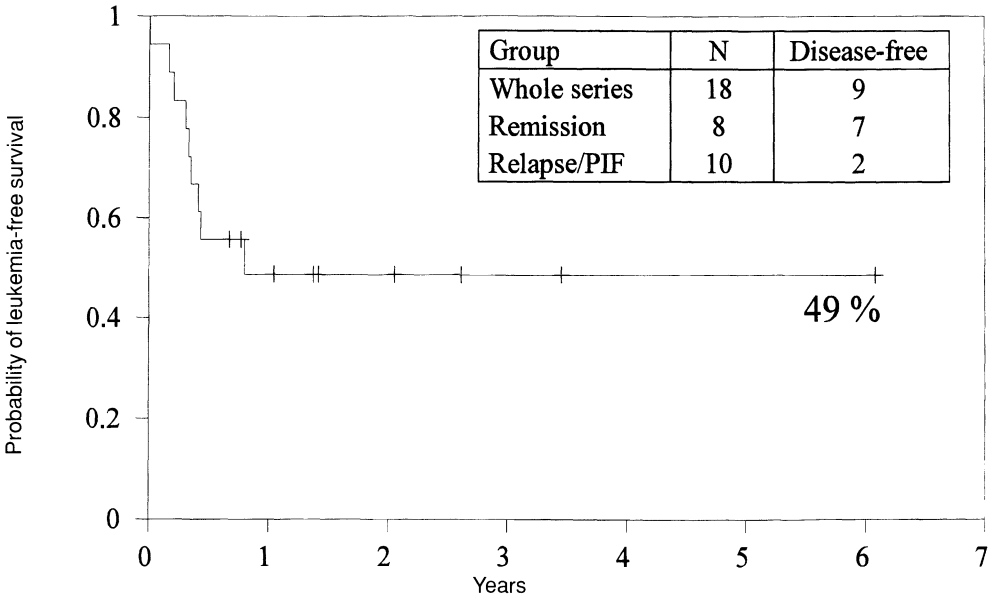

Fig 5. Leukemia-free survival after unrelated donor marrow transplantation in 18 patients with Philadelphia chromosome-positive acute lymphoid leukemia. *N* Number of patients; *PIF*, primary induction failure

Table 4. Results of unrelated-donor marrow transplants for acute leukemia facilitated by the US National Marrow Donor Program (After Beatty PG, Kollman C, Howe CWS In: Cecka and Terasaki, Eds., Clinical Transplants 1995, UCLA Tissue Typing Laboratory, Los Angeles, California, 1996, pp 271-277)

Disease	No. of patients	2-year survival
AML in first complete remission	39	39 %
AML in second complete remission	63	26 %
AML in ≥ third remission or relapse	149	13 %
ALL in first complete remission	57	38 %
ALL in second complete remission	113	45 %
ALL in third or greater complete remission	94	24 %
ALL in relapse	80	14%

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoid leukemia.

in achieving a complete remission (CR) and those with cytogenetic abnormalities indicating adverse prognosis, such as those involving chromosomes 5, 7, 11 or of multiple random abnormalities.

The low relapse rate observed after URD-BMT for Ph-positive ALL contrasts with the high frequency of leukemia recurrence observed when these patients are treated with chemotherapy [28], or with autologous [29-34] or allogeneic marrow transplantation from relatives [29, 31, 32, 33, 35, 36]. The low relapse incidence in the URD-BMT recipients could result from a powerful graft-versus-leukemia effect. This hypothesis is supported by the lower incidence of leukemia recurrence observed after URD-BMT as compared to autologous marrow transplantation in patients with acute leukemia [8] (Table 5) or compared to marrow transplantation from HLA-identical siblings in patients with chronic myeloid leukemia who receive T-cell depleted [37] or non-depleted marrow [2]. The graft-versus-leukemia effect might be amplified in presence of HLA-disparity, since we found a significant decrease in the relapse rate in the group of patients receiving marrow from HLA-mismatched donors.

Recent reports emphasize the survival advantage for patients receiving URD-BMT from donors matched for HLA-DRB1 by high resolution methods [39-40]. Here we show that in patients with acute leukemia, the use of marrow from donors with limited

Table 5. Probability of relapse and leukemia-free survival after autologous or unrelated donor marrow transplantation for acute leukemia. (Busca A, Anasetti C, Anderson G et al. Unrelated donor or autologous marrow transplantation for treatment of acute leukemia. Blood 1994; 83: 3077-3084)

Disease stage	BMT	Patients (n)	Relapse at 3 years	*P*-value	LFS at 3 years	*P*-value
Remission	Autologous	31	55%	0.08	25%	0.45
	Unrelated	18	27%		33%	
Relapse	Autologous	46	65%	0.72	5%	0.75
	Unrelated	25	48%		12%	

Abbreviations: LFS, leukemia-free survival.

HLA-A, B or D/DRB1-disparity is associated with higher morbidity from severe GVHD but no detrimental effect on survival [38]. The use of a partially mismatched donor could double the number of patients who can be transplanted [2].

Approximately 30% of patients with AML who received an URD-BMT in second remission became long-term leukemia-free survivors in this study. Survival was higher in patients with ALL presumably reflecting the inclusion of a high proportion of children. A report from the NMDP showed a 45% probability of survival at 2 years in patients transplanted for ALL in second CR [3]. We and others have observed remarkably good results after URD-BMT for ALL in children [25, 26, 41, 42]. In a previous report from our institution, leukemia free survival at 3 years was 47% in 15 children with ALL in first or second remission [42]. Another report from the UK in 50 children with ALL in second remission showed a 53% LFS at 2 years [26]. These data strongly support the consideration of URD-BMT in children with poor-risk ALL in second remission when no HLA-compatible family donor is available.

Patients in third or subsequent CR may also achieve sustained leukemia-free survival after URD-BMT. In our series, 5-year LFS in this group was 26%. In NMDP data the 2 year LFS was 22% in ALL patients in CR3 [3]. Since patients with this disease stage are rarely curable with chemotherapy or autologous marrow transplantation, URD-BMT is their best available treatment. The results of transplantation for patients in relapse with a low percentage of blasts in the marrow ($\leq$30%) and no blasts in the blood were similar to those observed in patients in second or third CR. Therefore, attempts to obtain a CR in patients in this situation should be avoided if timely URD-BMT is feasible. Other patients in relapse are not good candidates for transplantation from unrelated donors [3, 24]. This conclusion is emphasized in the international guidelines regarding indications and practice of URD-BMT [43-44]. New approaches to decrease leukemia recurrence and procedure-related mortality are required in this group of patients.

In contrast to previous studies [24, 27], we did not identify age as an independent prognostic factor for URD-BMT. In our series, the more favorable outcome in younger patients resulted from a higher marrow cell dose, and this factor remained significant when children and adults were analyzed separately. No data regarding marrow cell dose were available in previous reports identifying age as prognostic variable in acute leukemia patients receiving URD-BMT [24, 27]. A high marrow cell dose was associated with faster and more robust engraftment and less severe acute GVHD. In patients transplanted during remission, these benefits resulted in decreased transplant-related mortality due to infections and an improved survival. A high marrow cell dose did not protect patients transplanted during relapse from non-leukemic death. In this group non-leukemic death was usually due to causes other than infection. Patients transplanted in relapse who died from an infection were se-

verely neutropenic and febrile before starting the conditioning regimen. High marrow cell dose might not be effective in overcoming an infection ongoing before transplant.

A higher cell dose was associated with a lower incidence of graft rejection and a higher speed of engraftment in related or unrelated donor marrow recipients [45-50]. Two studies have also found associations between a higher cell dose and improved survival after related donor transplant for acute leukemia [51,52]. An increased dose of CD34-positive cells was also associated with a decrease of transplant related mortality in a recent report of patients receiving T-cell depleted marrow [53]. In an International Bone Marrow Transplant Registry study, patients receiving a higher cell dose had less severe GVHD [51]. This finding could be explained by a decreased incidence of early posttransplant infections that might amplify GVHD. The same argument has been used to explain why a higher dose of spleen cells is associated with lower GVHD and mortality in class II major histocompatibility complex-incompatible murine transplants [54].

Our data support the hypothesis that improved leukemia-free survival could be achieved by increasing the number of stem cells in the graft. Conversely, a randomized study of GM-CSF administered early after transplant did not show a survival improvement [55]. Whether the use of other hematopoietic growth factors could be beneficial remains to be explored. The number of harvestable marrow cells is limited, as reflected by the fact that in our series the 4×10^8 cells/kg requested from harvest centers was achieved only in 41% of cases. Blood could be considered as an additional source of hematopoietic stem cells. The yield of progenitors from peripheral blood can be greatly increased by the treatment of the donor with hematopoietic growth factors [56]. The use of growth factor-mobilized peripheral blood stem cells from HLA-identical siblings has been associated with rapid hematological recovery after transplantation [57, 59], and immune reconstitution might also be improved [60]. The published data indicate that there is no increase in the risk of acute GVHD in patients receiving mobilized peripheral blood stem cells from HLA-identical siblings [57-59, 61]. In a report describing 6 acute leukemia patients receiving peripheral blood stem cells from unrelated donors, none developed moderate or severe acute GVHD [62]. These encouraging preliminary results will need confirmation in other studies. The much larger number of donor T-cells compared to marrow administered when mobilized peripheral blood is used for transplantation raises the concern that increased incidence of chronic GVHD could result [63].

The data reported here indicate that URD-BMT is a useful treatment for high-risk acute leukemia patients when transplantation is carried out during remission or during relapse with low proportion of blasts in the marrow and no blasts in the blood. Results were especially remarkable in patients with Ph-positive ALL who received marrow from unrelated donors during remission. A higher marrow cell dose was strongly associated with improved outcome. This finding provides a rationale for testing the hypothesis that increasing the stem cell numbers by using mobilized peripheral blood progenitor cells from unrelated volunteer donors could improve survival by providing better reconstitution of hematopoietic and immune function.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Blood Progenitor Cells and Donor Lymphocyte Transfusion: the New Modes of Marrow Transplantation

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Traditionally, hematopoietic support for high dose chemotherapy and radiation therapy has been harvested from bone marrow from either the posterior iliac crests, sternum or tibia (in small children). With the clinical use of hematopoietic colony-stimulating factors, substantial increases in hematopoietic progenitors and stem cells capable of reestablishing hematopoiesis after myeloablative therapy can be obtained in peripheral circulation. By apheresis, these cells can be collected and use as a source of hematopoietic cellular reconstitution after autologous or allogeneic transplantation. Numerous studies have documented that autologous transplantation of peripheral blood progenitors results in rapid hematopoietic recovery leading to an almost preferential consideration of peripheral blood progenitor cells over bone marrow as a source of hematopoietic cells for autologous and allogeneic transplantation.

These hematopoietic stem cells are undifferentiated cells expressing a phenotype of CD34+, thy-1_{lo}, CD38- and not expressing mature lineage related antigens are capable of reconstituting myeloid and lymphoid cell lineages after transplantation [1]. Cells with the phenotype and function of stem cells are present in peripheral blood as summarized in Table 1. While there are no definitive assays for human stem cells, primitive progenitors derived from peripheral blood have the biologic effect of initiating growth of long term hematopoietic cultures and engraft in immunodeficient mice [2]. In vivo, blood derived hematopoietic cells can reconstitute hematopoiesis in animals and humans [3-8]. Complete myeloid and lymphoid chimerism after allogeneic peripheral blood progenitor cell transplants has been documented [9-11]. Thus, all the cells necessary for engraftment and durable reconstitution of hematopoiesis and immunity can be collected from peripheral blood.

While the initial successful use of peripheral blood progenitor cell transplants was in CML patients [12], subsequently, peripheral blood progenitors cell transplants were applied to patients with lymphoma [13], particularly in patients in whom marrow harvest was not possible because of prior radiotherapy or involvement by malignant cells [6, 14-16]. Patients with acute leukemia have had cells collected during recovery from chemotherapy used for transplantation [17, 18].

Hematopoietic progenitors are present in low frequency in the peripheral blood of normal individuals [19]. Systemic treatment with G-CSF increases circulating multipotent progenitors, exceeding pretreatment levels by approximately 10-fold [20]. Combinations of colony stimulating factors, such as interleukin-3, stem cell factor (kit ligand), flt-3 ligand, thrombopoietin, and GM-CSF

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or G-CSF may have synergistic effects in blood progenitor mobilisation [21-24]. Circulating progenitors increase markedly during the recovery phase after cyclophosphamide chemotherapy [25]. This mobilization is further increased by treatment with either G-CSF [26] or GM-CSF [27, 28]. Ideally, the mobilizing chemotherapy for autologous transplantation would employ agents active and indicated for treatment of the patients malignancy. The peripheral blood progenitor cell collection would then be integrated into the planned chemotherapy treatment. Allogeneic transplantation donors obviously cannot utilize chemotherapy and has generally utilized G-CSF alone [9-11].

Typically mobilized peripheral blood harvests have greater than 1×10^{10} mononuclear cells collected yielding a patient cell dose of $3\text{-}5 \times 10^8$ mononuclear cells/kg. The stem cells necessary for reconstitution of hematopoiesis are present within the CD34+/CD38/thy-1_{lo} subpopulation [29]. Most centers use the number of CD34-positive cells as an indicator of repopulating stem cell content [30-32]. There are two major conclusions which can be drawn from studies of cell dose. There is a threshold dose of progenitors (approximately $2\text{-}4 \times 10^6$ CD34+ cells/kg) to achieve reliable engraftment. Unfortunately even with large collection of stem cells, there does not appear anyway to avoid an obligate period of profound pancytopenia lasting approximately 8 days before hematopoietic recovery begins to occur. This probably reflects the time necessary for differentiation and maturation to occur.

Whether peripheral blood progenitor cells for transplantation has advantageous relative to bone marrow remains controversial. With optimal mobilization, one can collect a larger number of CD34+ cells from the blood. There is also a larger number of lymphocytes in PBPC collections which could have importance in immune recovery and immunologic antitumor effects [33]. It is feasible to use PBPC if the marrow is involved with tumor cells, although tumor cells usually circulate in higher frequency in these patients, and may contaminate PBPC as well as marrow collections.

The more rapid recovery of hematopoiesis after transplantation of mobilized peripheral blood progenitor cells compared to bone marrow has been often documented [20, 34-37]. It is not clear whether the more rapid hematologic recovery is due to qualitative differences between blood and marrow progenitors, the larger number of progenitors normally collected, or the priming effects of the mobilizing growth factor therapy. It is also unclear whether similar priming/mobilizing therapy would also increase bone marrow progenitors for collection.

There are disadvantages for using peripheral blood progenitors for transplantation. Occasional patients will not mobilize well, particularly those who are heavily pretreated with myelotoxic therapy. There is need for vascular access; subclavian central venous catheters are required for patients without large veins. Mobilizing regimens are highly myelosuppressive and have their own risks, cost and toxicity. Purging procedures, if performed, become costly and logistically difficult when applied to the to the log greater number of cells collected in after pheresis, particularly if multiple collections are required.

Early literature indicated peripheral blood progenitor cells had less tumor contamination. Because of the higher cell content in peripheral blood progenitor cell collection, it is more difficult to quantify the number of clonogenic cells in peripheral blood progenitor cell collections compared to marrow, but it is clear that malignant cells can be found in each source [38-41]. Whether the mobilization procedures involving hematopoietic growth factors utilized to increase the level of circulating normal progenitors also increases malignant cell contamination remains controversial. On the other hand, chronic myelogenous leukemia patients recovering from intensive chemotherapy preferentially mobilize nonmalignant diploid cells early in the course of recovery [40].

The use of mobilized peripheral blood progenitors has led to several improvements in the clinical use of autologous transplantation. The more rapid hematopoietic recovery results in shorter lengths of stay and fewer days at risk for infection, resulting in a reduction in the overall morbidity and cost of the procedure [36]. Depending on the pa-

tient population, mobilizing regimen, complications and the number of apheresis collections required, PBSC transplants can be more or less cost effective than bone marrow. Relative cost effectiveness depends on the costs of the mobilization process, the chemotherapy and growth factor regimen as well as management of complications must be considered. If a single apheresis is required for cell collection, this is less costly than bone marrow harvest which requires general anesthesia and use of operating room facilities. This may not be the case if multiple apheresis and cell storage procedures are required. In nearly every study, recovery post transplant is comparable or more rapid with peripheral blood progenitors [35-37]. This has allowed development of outpatient transplant programs in which patients are discharged promptly after high dose therapy, knowing that hematopoietic recovery will be prompt and there will only be a brief period at risk for infectious complications [42].

Allogeneic peripheral blood progenitor cell collection yields a 10 fold higher dose of T-lymphocytes in PBPC collections than in a typical bone marrow harvest, leading to concern that these transplants could produce more severe GVHD. On the other hand, this larger t-lymphocyte dose from an allogeneic donor could favorably affect immune reconstitution and the graft-versus malignancy effect. T-cell depleted transplants have been associated with a higher relapse rate similar to that observed in syngeneic transplant recipients. Donor t-lymphocyte infusions have been used to rescue grafts after relapse following allogeneic bone marrow transplants in CML patients These responses have also been noted in AML,CLL and myeloma patients. Initial clinical data with allogeneic peripheral blood progenitor cell transplants has indicated favorable results with rapid engraftment with no increase in the rate of acute GVHD [8, 11, 43], but some studies indicate a higher rate of chronic GVHD as was documented a decade ago in aplastic anemia patients receiving buffy coat infusions after bone marrow transplants [44]. Recently fludarabine chemotherapy has been shown to allow engraftment of allogeneic peripheral blood progenitor cell transplants and donor lymphocyte infusions [45-46]. This strategy of achieving initial partial engraftment with peripheral blood progenitor cell infusions followed by boosts of t-lymphocyte or of lymphocyte subsets are being explored by our group and other groups.

A number of important issues remain. How can the number of stem cells in mobilized peripheral blood progenitor cells be determined and what dose is required for transplantation. Do blood derived progenitor cells really have an advantage compared to marrow or would chemotherapy and growth factor stimulation primed marrow cells to respond in a similar fashion? An even greater issue is the clinical value and cost effectiveness of dose intensive therapies requiring cellular support for the treatment of hematologic malignancies and solid tumors. This is an area in which misuse or premature translation of technology to community centers is a major concern. The role of high dose chemotherapy and bone marrow or PBSC transplantation is still debated vs. standard chemotherapy for many indications. Use of mobilized peripheral blood progenitor cell transplants to support standard dose chemotherapy or only modest dose escalation is unlikely to be beneficial and will add considerable expense. Patients receiving high dose therapies should enter into clinical trials designed to evaluate their therapeutic role or into studies designed to further advance development of this technology.

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Allogeneic Transplantation of Purified Peripheral Blood Stem Cells from Unrelated Donors

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Abstract. Concerns in donor safety and the unknown incidence of acute and chronic GVHD caused by the large amount of T-cells have hampered the clinical introduction of allogeneic peripheral blood stem cell transplantation(allo-PBSCT) from unrelated donors. We performed allo-PBSCT from matched unrelated donors in 6 patients (1 AML 2. PR, 4 CML 1. CP, 1 PNH). For ex-vivo T-cell depletion we performed immunomagnetic CD34-positive selection on the pheresis products to reach a target of less than 2×10^5 CD3 positive NC/kg. The median content of CD34 positive NC in the post-selection product was 4.6×10^6/kg(r = 2.2-7.0). Enriched PBPC were cryopreserved and transfused 2 days after conditioning with Busulfan (16 mg/kg), Cyclophosphamide (200 mg/kg) and 2.5 mg/kg ATG i.v. from day -5 to -2. Further GVHD-prophylaxis was performed with CSA (5 mg/kg i.v./d) and short-course MTX. Five patients engrafted promptly (median days to > 500 PNC/µl, > 50 000 platelets/µl day +17, +25 respectively). In one case with a HLA DP mismatch the patient experienced primary graft-failure and had to be reinfused with autologous bone marrow. Until now, no patient has developed acute GvHD > grade I. By the time of writing, 4 patients are alive (day 265, 201, 77, 36). One patient died of CMV pneumonia on day 134. The patient with AML relapsed on day 245 whereas the other patients with allogeneic engraftment are disease-free. These first results suggest the feasibility of allogeneic PBSCT from unrelated donors. The role of T-cell depletion in this setting is not clear. Whether the higher number of transplanted progenitors can compensate for the draw-backs of T-cell reduction has to be investigated.

Introduction

Mobilised peripheral blood progenitors (PBSC) from allogeneic HLA-matched donors have become an alternative source of hematopoetic stem cells. As seen in the autologous setting, PBSC lead to a faster engraftment compared to bone marrow [1]. In the series published so far, no difference in terms of acute and chronic GvHD have been registered [2].

Ethical considerations and concerns in the safety of the growths factors used for mobilisation have limited the use of allogeneic PBSC to sibling transplants. Another risk, yet undefined for unrelated PBSCT, is the large amount of donor T-cells contained in the pheresis product. To reduce the risk of clinical significant acute GvHD and early morbidity, we performed a trial using CD 34 positive selected PBSC from unrelated donors for allogeneic transplantation. With the large amount of transplantable progenitors we hope to avoid the risk of graft-failure known after allogeneic transplantation

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of T-cell depleted bone marrow. CD34 positive selection allows to conserve the negative fraction with a content of 20-40% T-cells.

Material and Methods

After giving informed consent, healthy HLA class I serological and class II genotypically matched donors were stimulated with 10 µg/kg subcutaneous G-CSF for 5 consecutive days. During mobilisation hematological parameters were monitored intensively as well as side effects of the growth factor administration. On day 5 and 6 after the start of the G-CSF application 2 large-volume leukapheresis were performed using peripheral venous access. 3 times total blood volume were processed each day. If the platelet count before the second apheresis was below 100 000/µl, autologous platelets were retransfused to avoid clinically significant thrombocytopenia.

From the two collection days 7-8 $\times$ 10^{10} nucleated cells were pooled for CD34 positive enrichment. The residual cells were cryoconserved as back-up. CD34 positive selection was performed with the Isolex 300 SA device (Baxter Healthcare, Irvine, USA). Cell washes, antibody labeling and separation were performed according to the manufacturers guidelines. In brief, the pheresis product underwent 2 low-spin centrifugations to reduce the number of platelets. After resuspension, cells were incubated with a monoclonal mouse anti-human CD34 antibody for 15 min at room temperature. Prior to antibody incubation polyvalent IgG was added to block unspecific binding. After incubation the lableled cells were mixed with sheep-anti mouse antibodies conjugated with dynabeads and magnetic separation followed in a semi-automated fashion. After depletion of the CD34 -negative fraction the beads were detached by adding a release peptide supplied by Baxter. The positive fraction was washed twice in PBS/5% ACD-A/2% albumin. Cell counts, flow cytometry, viability testing and colony assays were performed with the cells from both positive and negative fraction. The positive fraction was cryoconserved in a volume of 20 ml using donor plasma as freezing solution.

All patients at our institution underwent the same conditioning protocol: After placement of a central venous-line, patients received 16 mg/kg Busulfan divided in 16 oral doses on 4 days (day -9 to -6). On day -5 to -2, 50 mg/kg Cyclophosphamide were infused giving a total dose of 200 mg/kg. Also on days -5 to -2 Anti-Thymocyte Globulin (ATG, Merieux) was infused in a daily dose of 2.5 mg/kg. Forced hydratation was started after admission and Mesna was infused during the days of cyclophosphamide administration. GvHD prophylaxis consisted of Cyclosporine A at a dose of 4 mg/kg starting one day before PBSCT and Methotrexate 10 mg/m2 on day 1, 3 and 6 with leukovorin rescue on day 7 and 8. The cryopreserevd were thawed and infused directly 48 h after the end of the conditioning therapy. Patients were treated in single rooms with high efficiency air-particle elimination. Supportive care contained intravenous piperacillin, acyclovir and fluconacole as well as platelet substitution as soon as less then 20 000/µl platelets were measured in the morning. Packed red blood cells were transfused when hemoglobin levels fell under 8 g/dl. All patients received 10 000 IE Heparin/day as prophylaxis against veno-occlusive liver disease.

Results

The results of the purification procedures and the clinical course of the first 6 patients are summarized in Table 1.

Purity, Yield and T-Cell Content after CD34 Positive Selection

The median purity of the CD34 positive fractions was 88% with a range from 64 to 96%. A median of 55%(r = 38-100) of the starting amount of CD34 positive NC could be recovered in the positive fraction. The transplanted dose of CD34 positive progenitors varied from 2.2 to 7.0 $\times$ 10^6/kg (median: 4.6). The yielded NC showed a median vitality of 98% (r = 97-100%). The enrichment

Table 1. Results of purification and clinical course in patients

Patient -ID	Disease -status	CD34. pos. NC/kg	CD3. pos. NC/kg	First day > 500 PMN/µl	First day > 50 000 platelets/µl	Grade of acute GvHD	Actual status
01	AML 2. PR	2.2	1.7	22	29	0	Relapse d 245
02	CML blast crisis	4.2	1.5	18	26	I (Skin)	Alive d 300 BM, BM Ph neg., PCR neg.
03	CML 1. CP	7.0	1.8	15	24	I (Liver)	Died d 120 CMV pneumonia
04	CML 1. CP	4.8	0.4	16	23	I (Skin)	Alive PCR neg d 156
05	CML 1. CP	3.8	1.2	Graft failure	-	0	Alive after autol. Rescue
06	PNH, Aplasia	4.0	1.8	17	25	0	Alive d 45

described above lead to a effective passive T-cell reduction. The unmanipulated pheresis products contained a median of 3.1×10^8 CD3 positive T cells/kg. A median 3.2 log (r = 2.9-3.4) T cell reduction following positive selction resulted in the transfusion of 1.6×10^5/ kg CD3 positive NC. Analysis of CD34 positive subpopulations revealed 2- 6.7% CD34+/38-, 0.8-2% CD34+/DR- and 1% CD34+/Thy 1+(dim) NC.

The median duration of each procedure was 6.25 h excluding cryopreservation.

Clinical Results

All patients transplanted at our institution so far, are summarized in Table 1.

The median time of neutrophil recovery (> 500 PMNC/µl) was 17 days (r = 14-22). Platelets regenerated early (> 50 000/µl day 25, r = 23-29), compared to BMT from unrelated donors. Only mild (Grade I) GvHD was seen in 3 patients. One patient with a minor mismatch did not engraft. Possibly a high dose cotrimoxacol treatment of pneumonia influenced the clinical course negatively in this patient, since a sufficient number of CD34+ NC had been transfused. Autologous bone marrow could be infused as rescue. Donor chimerism is documented in one patient 300 days after being transplanted in accelerated phase of CML. This patient is free of disease, even when tested by PCR for the bcr-abl rearrangement. The patient with relapsed AML suffered from a second relapse on day 245 after transplantation. Unfortunately, one patient was lost after day 100 due to CMV pneumonia.

Discussion

Graft engineering has emerged rapidly during the last years of blood stem transplantation. Since recipients of T-cell depleted bone marrow have experienced graft-failure in a considerable number of cases, this method has not become a routine approach [3]. On the other hand, the higher rate of relapse after T-cell depletion has discouraged most clinicians, who participated in those early trials. One report has shown a fixed number of T-cells to be one way to avoid the mentioned problems [4]. Even though these results are encouraging, the time to engraftment might be accelerated significantly by the use of G-CSF mobilised peripheral blood stem cells [5]. The addition of mobilised peripheral blood stem cells as graft facilitating cells enabled Aversa et al. to establish stable engraftment in recipients of haploidentical grafts [6]. We believe that allogeneic recipients of MUD (matched unrelated donor) transplants might experience a reduction of early mortality by the use of peripheral blood stem cells.

The large amount of T cells in the pheresis products is supposed to result in a higher incidence of acute GvHD [7, 8]. Strikingly, the

first clinical series of allo PBSCT from matched sibling donors have shown no significant incresae in the incidence of GvHD, compared to historical controls, transplanted with unmanipulated bone marrow [9].

The first results of our pilot study, even though not being conclusive, show the feasibility of the approach. The time to engraftment was short, and thrombopoesis recovered fast. Almost no GvHD was seen, which is unsusal after unrelated BMT. With this abbrogation of GvHD, one might try to further accelerate engraftment by omitting methotrexate.

The large amount of CD3 positive cells can be stored and might be a source for adoptive immunotherapy in case of CMV infection or relapse. If the reinfusion of T-cells at a special time point might be crucial, can not be said at the moment. Another, yet not answered question is the role of other accessory cells contained in the apheresis bag in terms of durable engraftment, immunologic recovery, graft-versus leukemia effects and even graft- versus host disease.

Nevertheless, we believe that large-scale graft-engineering of PBSC can broaden the range of effective prophylaxis strategies against GvHD and avoid the negative aspects of T-cell depletion by using larger number of progenitors. Additionally, it might enable physicians to introduce the results of basic science into clincial routine. The use of allogeneic PBSC, being discussed controversially at the moment, has to be studied more extensively in the unrelated setting to get the answers to these questions.

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Ex Vivo Manipulation of Peripheral Blood CD34$^+$ Cells

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Abstract. The success of autologous peripheral blood progenitor cell (PBPC) transplantation is challenged by relapse of malignant disease which might – at least in part – be mediated by graft contaminating tumor cells. Although the clinical role of tumor cell depletion still remains to be demonstrated in prospective, randomized trials, multiple purging strategies are currently pursued in the context of autologous stem cell transplantation. This report is discussing ex vivo manipulations of PBPC transplants with respect to purging of tumor cells, including the positive selection of CD34$^+$ cells with or without negative depletion as well as ex vivo expansion techniques. In addition, adoptive immunotherapy strategies using ex vivo generated autologous dendritic cells for the treatment of minimal residual disease after stem cell transplantation will be discussed.

Introduction

Gene marking studies and retrospective clinical trials have demonstrated that contaminating tumor cells can contribute to disease recurrence after high-dose chemotherapy and autologous stem cell transplantation [1–4]. Up to now, however, there are no randomized clinical trials available which indicate a clinical benefit of any purging strategy. Therefore, purging of tumor cells still remains an experimental approach which requires further investigation.

Unfortunately, currently performed purging strategies (e.g. immunomagnetic bead separation, 4-HC, or monoclonal antibodies and complement) are unsatisfactory and only result in a tumor cell reduction of only about 2–4 log, which may not be relevant clinically. Moreover, purging is particularly difficult in lympho-hematopoietic malignancies, e.g. acute or chronic myelogenous leukemia, since normal stem cells are currently phenotypically unseparable from their malignant counterpart. Therefore, it appears almost impossible to selectively deplete malignant lympho-hematopoietic cells with immunological strategies on the basis of identified surface antigens. It appears more promising to separate normal and malignant hematopoietic stem cells based on functional differences, e.g. treatment with 5-FU [5] or vitamin D analogs [6, 7]. Alternatively, efforts are currently performed to identify new antigens which are exclusively expressed either on normal stem cells or on leukemic cells, such as the HER-2 receptor tyrosine kinase [8].

Positive Selection of CD34$^+$ PBPC

Contaminating tumor cells can be detected in both, autologous bone marrow and peripheral blood stem cell transplants by PCR

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or immunocytochemistry, particularly in patients with follicular lymphoma, multiple myeloma, acute leukemia, neuroblastoma, as well as breast and small cell lung carcinoma [9, 10]. In addition, we have shown that epithelial tumor cells can be mobilized concomitantly to normal $CD34^+$ progenitor cells upon chemotherapy plus growth factor induced mobilization of PBPC [11]. This observation has been recently confirmed by several investigators in other malignancies, including Non-Hodgkins lymphoma and multiple myeloma [12, 13]. The simultaneous mobilization of normal progenitor cells and tumor cells – at the time where PBPC are usually harvested by leukapheresis – clearly indicates the requirement for purging in order to provide a tumor cell free stem cell product.

One approach to separate normal progenitor cells from contaminating tumor cells constitutes in the positive selection of $CD34^+$ cells. This method, however, can only be applied in CD34-negative malignancies, like solid tumors, but not in patients with acute leukemia. In addition, it is not clear whether this technique might be helpful in lymphoid malignancies, e.g. follicular lymphoma or multiple myeloma, since the origin of the lymphoma or myeloma stem cell has not yet been definitively identified. Nevertheless, several studies are currently underway in which the efficacy of a CD34 cell selection in terms of tumor cell purging will be evaluated.

The efficacy of the avidin-biotin immunoaffinity column (Ceprate SC, CellPro, Bothell, WA, USA) separation of CD34 cells with respect to tumor cell purging is summarized in Table 1. The reduction of tumor cells using this method is in the range of 2 to 4 log, however, there are specimens which still contain tumor cells. For instance, in a myeloma study, only 62% of the preparations were purged below the level of detection whereas 38% of them were still PCR positive (Table 1). Similar results have been observed in breast cancer as well as in follicular lymphomas. Based on these studies, it is evident that CD34 cell selection alone with only a 2–4 log depletion of tumor cells is not sufficient for purging in most of these malignancies. Therefore, better selection strategies or a combined, positive/negative strategy need to be developed in order to improve current purging results.

Table 1. Purging of tumor cells upon positive selection of $CD34^+$ cells

	Disease	Depletion (log)	Preparations < detection level
Shpall 1994	Breast	1–>4	38–100%
Schiller 1995	Myeloma	2.7–4.5	62%
Lemoli 1996	Myeloma	2.5–3	n.d.
Gorin 1995	NHL	n.d.	89%

Negative Tumor Cell Depletion

One approach in this context is a negative depletion strategy which can be ideally performed after positive selection of $CD34^+$ cells [17, 18]. We have recently investigated a double purging strategy in collaboration with CellPro in five patients with stage IV breast cancer. The patients had advanced metastatic disease with immunocytochemical marrow involvement. Before CD34 cell selection, all unmanipulated PBPC preparations had evidence of micrometastatic disease. After CD34 cell selection (Ceprate SC system), the cells were labelled with a cocktail of anti-breast cancer monoclonal antibodies and then passed again over a second avidin-biotin immunoaffinity column. The overall cumulative depletion of tumor cells was about 5 logs, and the percentage of CD34 cells was 80–85% with a yield of approximately 50%. However, not all preparations were purged below the level of detection, indicating that even this combined strategy may not be sufficient to achieve a tumor cell reduction of at least 6–7 log.

Ex Vivo Expansion of $CD34^+$ PBPC

Another strategy for purging is the reduction of the blood volume to be processed for PBPC harvest, followed by ex vivo expan-

sion of positively selected $CD34^+$ cells in order to generate enough numbers of progenitor and stem cells for transplantation. This method requires only a small volume of the patient's blood for peripheral blood progenitor cell collection and thus may help to reduce the overall tumor cell load in the final autograft. Thus, only a blood sample of approximately 100–200 ml at the optimal time point of progenitor cell release would be required, processed and expanded ex vivo in the presence of appropriate hematopoietic growth factors. This method would thus obviate the need for leukaphereses processing 10–30 litres of blood for PBPC collection. By reducing the volume of blood to be processed, this strategy would result in a 1–2 log reduction of tumor cells (compared to a 10–20 litre apheresis) in the starting population of $CD34^+$ PBPC prior to ex vivo expansion.

Recently, we have shown that committed progenitor cells can be generated ex vivo in a stroma-free liquid culture system supplemented with various cytokines including stem cell factor (SCF), IL-1, IL-3, IL-6, and Epo [19]. Similar observations have been observed with slightly different cytokine combinations by other investigators as well [21–23]. Although we observed a high rate of progenitor call expansion, the number of long-term culture initiating cells (LTC-IC) under these culture conditions were not increased, but maintained [20].

On this background, we performed a clinical trial in 10 patients with advanced solid tumors undergoing high-dose chemotherapy and PBPC-transplantation [24]. The stem call grafts in these patients were generated ex vivo from only 10–15 x 10^6 positively selected $CD34^+$ cells, a number of $CD34^+$ cells which corresponds to only 10% of the $CD34^+$ cells present in a "standard" 6 liter apheresis at our institution. This number of $CD34^+$ cells contained 1.2–1.5 x 103 LTC-ICs/kg, and about 3 x 10^3 CFU-GM/kg. After expansion, the numbers of LTC-IC were in the same range, i.e. about 1.5 x 10^3/kg, while the numbers of CFU-GM increased 50–55 fold. After transplantation of these ex vivo generated cells, rapid reconstitution of both neutrophils and platelets was observed after a high-dose, non-myeloablative preparative regimen consisting of VP16, ifosfamide, carboplatin, and epirubicin (VIC-E). Since endogenous reconstitution was likely to occur after this regimen, this study did not provide an answer with respect to long-term capabilities of ex vivo generated PBPCs [24, 25]. Nevertheless, this study was the first proof of principle showing that ex vivo generated hematopoietic progenitor cells could successfully be used in a clinical setting after high-dose chemotherapy.

The most important question in the context of the clinical use of ex vivo expansion is whether primitive stem cells can also be expanded. There is evidence that the number of LTC-IC can indeed be expanded about 10–50 fold in the presence of FLT-3 ligand [26] Ziegler et al., own unpublished observation), however, no studies in humans with respect to the functional capabilities of ex vivo generated cells in the long-term have been reported. In animal models, the functional capacity of ex vivo generated stem and progenitor cells has been investigated, and several studies have shown that long-term hematopoiesis may be impaired, particularly when cytokine combinations including IL-1 and IL-3 were used [27–29]. On the other hand, there are reports which do not show functional deficits of ex vivo generated progenitor and stem cells with respect to long-term maintenance of hematopoiesis after TBI-containing preparative regimens [28, 30, 31].

There are several other critical issues concerning the clinical application of ex vivo expanded progenitor cells. Among them, one of the most relevant questions is whether this technique can also be applied in "poor mobilizers", and thus might be helpful in the accrual of heavily pretreated patients which otherwise would not be candidates for high-dose chemotherapy trials. Based on our own results, however, it is not possible to expand $CD34^+$ cells collected from heavily pretreated patients (unpublished observation). The $CD34^+$ cells from these patients have a severely impaired functional capacity and therefore, "poor mobilizers" cannot be considered as good candidates for successful ex vivo expansion strategies.

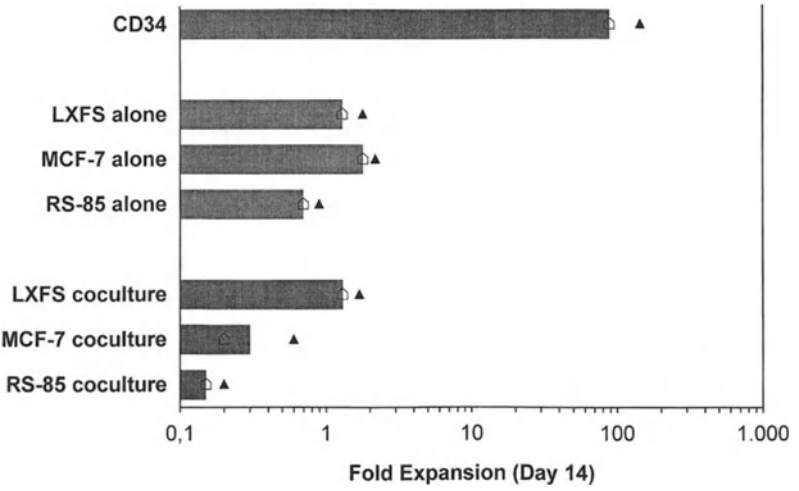

Fig. 1

Concomitant Expansion of Contaminating Tumor Cells?

The concept of ex vivo expansion will only be advantageous for the patient if contaminating tumor cells are not expanded concomitantly during cytokine-supported ex vivo expansion cultures. We addressed this question in stage III and stage IV primary breast cancer patients after 2 cycles of induction chemotherapy [32]. In this study, we analyzed unmanipulated PBPC together with $CD34^+$ selected cells and ex vivo expanded cells for the presence of contaminating epithelial tumor cells. We found that unmanipulated PBPC contained tumor cells in 3/24 preparations, while no tumor cells could be detected in CD34-selected preparations or ex vivo expanded cell preparations from these patients at a sensitivity of 1:4 x 10e5. Therefore, to principally answer the question whether contaminating tumor cells might be expanded concomitantly to normal $CD34^+$ cells, we cocultured defined numbers of solid tumor cell lines with $CD34^+$ cells from normal donors in a serum-free liquid culture system (Fig. 1). We found that the tumor cell lines did not expand significantly in number when compared to the normal $CD34^+$ cells, suggesting that cytokine-supported expansion of $CD34^+$ PBPC can confer a proliferative advantage of hematopoietic calls without an apparent risk of cocomitantly expanding residual epithelial tumor cells [32, 33]. However, none of the culture manipulations performed here resulted in a complete loss of tumor cells. Similar observations have now been seen in ex vivo expansion cultures with normal $CD34^+$ cells and primary follicular lymphoma cells (B. Meister, own unpublished observation).

Differential Expansion of $CD34^+$ Progenitor Cells for the Generation of Immune Effector Cells (Dendritic Cells)

Treatment of minimal residual disease after high-dose chemotherapy is one of the most important clinical issues in autologous stem cell transplantation. Since dendritic cells (DC) can be generated ex vivo from PB or BM $CD34^+$ cells under appropriate conditions [34–36], it might be possible to use tumor-antigen transfected or peptide pulsed DC for adoptive immunotherapy approaches after high-dose chemotherapy.

We and others have recently shown that FLT-3 ligand is one of the most important components for the generation of DC ex vivo [37, 38]. Over a 21-day culture period, we were able to expand the number of CD1a/CD86-positive DC approximately 20–40-fold [38]. Moreover, these cells were functionally fully active, as demonstrated in a MLR reaction as well as in the presentation of soluble proteins to autologous T-cells (e.g.

tetanus toxoid). In addition, these cells have been shown to be approximately 10–20 fold more active in antigen presentation compared to normal peripheral blood mononuclear cells [35]. Hopefully, these cells hold promise in the near future when used clinically for the treatment of minimal residual disease after autologous stem cell transplantation.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Cord Blood Transplantation in Hematopoietic Malignancies

E. GLUCKMAN, V. ROCHA, C. CHASTANG, for EUROCORD

Abstract. EUROCORD registry has collected more than 140 cases of cord blood transplant. Among the first 85 patients, 53 were transplanted for an hematologic malignancy including 36 acute leukemia, 21 in 1st and 2nd CR and 15 in > than CR2 or resistant relapse, 5 chronic myeloid leukemia in CP1, 1 in CP2, 1 in CP3 and 1 in BC, 5 myelodysplastic syndrome (1 transformed in AL), 2 non Hodgkin lymphoma and 2 neuroblastoma. The 1 year survival was 43% with a median follow-up time of 250 days; in related transplants, it was 48% and in unrelated 41% with a shorter follow-up. Good survival was associated with age < 6 years $p = 0.08$, weight < 20 kg $p = 0.03$, negative CMV serology $p = 0.009$, HLA identity $p = 0.026$, sex identity $p = 0.08$, number of nucleated cells infused > 3.7×10^7/kg $p = 0.009$ and standard risk leukemia. Patients transplanted for leukemia in CR1 and CR2 had a survival of 56% while patients transplanted in later phase had 8% survival. 82% of the patients reached > 500 PMN at day 45, 7 patients died earlier. Engraftment was associated with the number of cells infused, age and weight. GVH grade II-IV was observed in 52.5% of the cases, it was usually mild. Leukemic relapse was observed in 11 cases, 3 AML, 5 ALL, 2 MDS, and 1 CML. 8 patients transplanted in advanced disease relapsed early, 3 patients relapsed later and are still alive either after donor lymphocyte transfusions or a second BMT. The primary causes of death were rejection, leukemic relapse, or transplant related toxicity. This study shows that related and unrelated match or mismatched cord blood transplant is a good source of hematopoietic stem cells in patients with hematological malignancies.

Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) has been used to treat thousands of patients, adults and children, with life-threatening hematological diseases. The principal limitations of allogeneic bone marrow transplantation are, for the majority of the patients, the lack of suitable HLA-matched donors and the complications of graft versus host disease associated with HLA disparities. In the absence of a suitable HLA identical sibling donor, alternative donors such as mismatched related or matched unrelated donors are searched. In these transplants, major and minor histocompatibility differences are often unrecognized by current matching tests, explaining the relatively high frequency of post transplant complications, graft failure, graft versus host disease and delayed immune reconstitution. In the absence of a perfectly matched donor, several means of circumventing the HLA barrier have been investigated: the incidence and severity of graft versus host disease (GVHD) can be reduced

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by T cell depletion or CD34+ cell selection; rejection can be avoided by increasing the number of stem cells infused; growth-factor-mobilized peripheral blood stem cells contain ten times more HSC than bone marrow cells, thus improving the rate and speed of engraftment after autologous and allogeneic HSCT.

The lack of an HLA-identical sibling donor in 70% of the cases has been overcome through the establishment of large panels of potential marrow donors but these panels are restricted in term of HLA polymorphism and of ethnic diversity. This is most marked in Europe where each nation has its own ethnic diversity. Limitation is also related to the fact that results of allogeneic bone marrow transplantation from normal bone marrow donors are dependent of HLA-A, -B, -C, -DRB1, -DQB1 and -DPB1 identity between donor and recipient. The description of new more sensitive techniques of typing by molecular biology and sequencing and, the cloning of new minor histocompatibility antigens has decreased the probability of finding a fully matched donor. Functional tests MLC, CTLp and HTLp have given contradictory results and cannot be used routinely. Therefore, despite a bone marrow donor registry which contains more than 3.7 millions bone marrow donors worldwide, some patients cannot be transplanted because of the lack of an HLA identical donor. New approaches have been investigated including the use of HLA partially mismatched T-depleted mobilized peripheral blood hematopoietic stem cells, or on the other hand, the use of umbilical cord blood hematopoietic stem cells. During development, the hematological and immunological system of the fetus and the new-born exhibits different properties as compared to the adult system [1]. The expected advantages of umbilical cord hematopoietic stem cells are the enrichment of immature progenitors hematopoietic stem cells which should facilitate engraftment [2, 3] and the immune immaturity of the immune system at birth which should decrease the incidence and severity of graft versus host disease [4]. Cord blood stem cells have distinctive proliferative advantages which include increased cell cycle rate, autocrine growth factors production and increased telomere length [5, 6, 7]. The small number, and the relative immaturity and enrichment in naive T cells of cord blood lymphocytes, should reduce the risk and severity of GVHD [8, 9]. This feature may permit a greater degree of HLA disparity between donor and recipient than is usually accepted with adult blood or marrow hematopoietic stem cell transplants.

Since the first cord blood transplant performed in 1988 [10], cord blood transplantation is increasingly used as a new source of hematopoietic stem cells and more than 200 cases have been reported. In order to develop and evaluate cord blood transplant results, the European Blood and Marrow Transplantation group (EBMT) has organized a Concerted action the EUROCORD group.

The objectives of EUROCORD are:

1. to standardize the methods of collection, testing and cryopreservation of cord blood;
2. to study the properties of haematopoietic progenitors and gene transfer in cord blood;
3. to study the immune function of cord blood lymphocytes;
4. to coordinate and facilitate the exchange of sera and cells from donor and recipients of cord blood transplants;
5. to establish a European Registry of patients treated by cord blood transplants and design protocols comparing cord blood transplants with alternative conventional blood and bone marrow hematopoietic stem cell transplants.

Cord Blood Banking

Cord blood banking for allogeneic unrelated and related transplants has several advantages including the availability of this source of stem cells, the low viral infection rate at birth, the speed of the search and the possibility to collect cord blood in ethnic groups not represented in bone marrow donors registries. There are potential problems related to the cost and several legal and ethical issues.

In Europe, cord blood banking has been developed in several countries including

France, UK, Italy, Belgium, the Netherlands and Spain. Other local banks have been collecting cord blood mostly for related transplants. There is not in Europe, at this time, any reported private bank or bank which collects cord blood for autologous use. As of 29 November 1996, BMDW (Bone Marrow Donor Worldwide. Edition 31) offers a total of 7592 cord blood typed, 2724 are provided by Europe and 4868 by the New York cord blood bank.

Several meetings have been organized to address the issues of standardization of methods of quantification of hematopoietic stem cells, development of techniques for cell concentration and enrichment without substantial loss of hematopoietic progenitors, detection of transmissible infectious and genetic diseases, detection of maternal cell contamination and, legal and ethical aspects of cord blood banking and transplant.

Informed Consent

The issue of informed consent has been discussed as there is some variability in its purpose. It is generally agreed that cord and placental blood is a discarded product and can be used without asking permission but, informed consent must be asked for doing tests on mother and cord blood. In some countries, mother's informed consent is asked after delivery and, if it is obtained, a full medical history is taken, blood sample is drawn from the mother to test for infectious disease markers. In other countries, information on cord blood donation for allogeneic use is given long before delivery and the informed consent process engaged in advance. This gives the opportunity of excluding donors with high risk of transmitting any infectious or genetic disease through careful collection of the family and mother history.

There has been some concerns about the process of information of the family on the results of infectious and genetic disease screening. Another issue is the follow-up of the donor and, in case of need, the possibility for the family to retrieve the autologous cord blood.

Collection

Two techniques are used, one is to collect cord blood in the delivery room, while the placenta is still in utero, the other is to collect cord blood after delivery in an adjacent room[10, 11]. Both techniques have advantages and disadvantages. The first one should be made by an extra personnel usually a midwife in the delivery room. The advantage is that the volume of cells collected is higher if the cord is clamped early and the collection begun immediately but this can disrupt the normal process of delivery and is not always feasible. The other technique is easier, it can be performed by dedicated personnel, it might decrease the number of cells collected and perhaps increase the risk of bacterial contamination or clotting.

Infectious Disease Screening

Syphilis and viral tests including HIV, Hepatitis B and C, CMV are performed on mother blood. In some countries, HTLV-1 and toxo are also tested. Most of the time, virology tests are not performed directly on cord blood but a separate aliquot is kept to perform these tests before transplant. Considerable discussion has been engaged on the risk of collecting infected cord blood during the window period before seroconversion. When possible, cord blood is quarantined until a confirmatory test is performed on the mother 3 to 6 months after delivery. In the other cases, performance of very sensitive tests, including viral antigen or nucleic acid screening tests, before delivering the transplant, should considerably decrease the risk of viral transmission. The risk of transmitting viral infection from transfusion has been described recently [12]. It is estimated at 1/641.000 for HTLV1, 1/103.000 for HCV, 1/63.000 for HBV and 1/493.000 for HIV. The risk might be higher in cord blood donors because of the different recruitment of the donor population.

Bacterial infection is also a major issue but it seems that the frequency of bacterial contamination diminishes with the expertise of the staff in charge of the collection. In all cases, bacterial culture for anaerobic

and aerobic bacteria must be performed and results sent to the transplant physician when the cord blood is delivered for transplant.

Genetic Diseases

This should be directed by the familial history, the ethnic background and the follow-up of the donor. Tests on cord blood are expensive and there is no real consensus on the type and number of tests which should be performed. Also, there are some concerns about the notification of the results to the family.

Maternal Cell Contamination

Recent studies have shown that the presence of maternal cells in cord blood is very frequent, the detection depends on the sensitivity of the technique used [13, 14]. With very sensitive techniques, maternal cells are always detected in low number in cord blood. The problem is to know what is the clinical relevance of maternal cell contamination. Some fear that maternal cells might engraft resulting in graft versus host disease. So far, no case has been observed, to the contrary, there is one observation of a patient who received a heavily maternal cells contaminated cord blood without any evidence of GVH [15], for these reasons, screening for maternal cell contamination is not included in the general practice of cord blood banking.

HLA and Red Cell Typing

HLA typing is performed on an aliquot of cord blood. Usually, HLA-A and -B antigens are identified by serology and HLA-DRB1 by DNA amplification methods. In addition, Eurocord is collecting DNA from donor and recipient to perform molecular typing for HLA-C, -DQB1, -DPB1, some HLA-A and -B antigens and other markers. Some banks routinely type the mother for HLA in order to have information on the haplotype and control the accuracy of cord blood typing.

Cell Processing

Because of the small volume of cord blood ranging from 40 to 150 ml, there is some concern that any attempt at cell manipulation and concentration might result in a considerable cell loss which might impair engraftment [2]. Many banks freeze in a programmed cell freezer, whole cord blood in 10% DMSO; others use HES (hydroxy-ethyl starch) sedimentation for volume reduction and removal of red cells [11, 16]. This last technique, if proven safe, would have the considerable advantage to reduce the storage space in liquid nitrogen freezers. CD34+ cells selection and stem cell expansion are currently investigated mostly for gene transfer experiments or use of cord blood transplants in adults. Thawing technique is well established, it aims at removing red cells and DMSO [11].

Evaluation of stem cell content is a very important issue since several studies have shown that there is a correlation between the number of nucleated cells infused and engraftment. There is a correlation between placental weight, time of clamping, speed of processing, volume collected and stem cell content. Quantification of hematopoietic stem cells in cord blood is not always easy, most studies refer to nucleated or mononucleated cells infused per kg after thawing. Enumeration of CD34+ cells by Facs analysis has entered in routine practice in most laboratories but results are not always reproducible. Others count the number of CFU-GM in clonogenic assays. There is a lot of variation between laboratories which explain why quantification has been a problem and why, in the European study, a correlation was found between the number of nucleated cells and engraftment, this correlation was not found, anymore, when looking at the number of CD34+ cells and CFU-GM.

Ethical and Legal Aspects

Cord blood can be collected for an unrelated or a related transplant, and more hypothetically for autologous use. For unrelated cord blood banks, the mother must be aware that the donation is anonymous and free and

there is no guarantee to retrieve the infant cord blood in case of need for family or autologous use [17]. Private banks have been set up mostly in the USA for collecting cord blood for autologous use [18, 19, 20, 21]. Furthermore, the use of fetal and cord blood cells has raised ethical concerns related to the procedure of informed consent, the detection of infectious and genetic diseases and how to inform the donor, while preserving the anonymity and freedom of both donor and recipient.

Cord Blood Transplant Results in Europe

Patients' Characteristics

Eurocord Transplant has established a Registry for studying results of cord blood transplant (CBT) in Europe [22]. Between October 1988 and April 1996, 85 patients had received a CBT in 26 centers [1, 23-33] The mean follow-up time was 17 months (range 3 to 93 months). The median age was 6 years (0.2-39 y), median weight 20 kg (5-68 kg). Fifty three patients had a malignant disease: 36 acute leukemia (AL) with 25 acute lymphoblastic leukemia (ALL) and 11 acute myeloid leukemia(AML), 21(58%) were in 1st or 2nd complete remission (CR) and 15(42%) were in > CR2 or resistant relapse, 5 chronic myeloid leukemia (CML) in first chronic phase (CP1), 1 in 2nd CP, 1 in 3rd CP and 1 in blastic crisis (BC), 5 myelodysplastic syndrome (MDS) (1 transformed in AL), 2 non Hodgkin Lymphoma (NHL) and 2 neuroblastoma. Thirty two patients were transplanted for non malignant diseases. Ten patients had received a previous BMT(9 autologous and 1 allogeneic).

Donors' Characteristics

Cord blood was provided by the New-York Blood Center in 19 cases (Dr P. Rubinstein), Hopital Saint Louis in 11 cases (Dr M. Benbunan), Milano cord blood bank (Pr G. Sirchia) in 8 cases, Dusseldorf cord blood bank (Pr P. Wernet) in 2 cases and local banks in 45 cases.

The donor was an HLA identical sibling in 40 cases, a mismatched family donor in 17 cases, an HLA identical unrelated donor in 4 cases and a mismatched unrelated donor in 24 cases.

HLA compatibility was defined by an identity for HLA-A and B determined by serology and DRB1 typing by SSOP. According to this definition, 44 patients received an HLA identical CBT, 23 pts a 1 HLA antigen mismatched CBT, 8 pts a 2 antigens mismatched CBT and 10 pts a 3 antigens mismatched CBT. One difference for HLA-A was observed in 23 cases, for HLA-B in 28 cases and for HLA-DR in 16 cases, 2 patients received a 2 DRB1 disparate CBT.

Conditioning and GVH Prevention

Conditioning regimens varied markedly according to diagnosis, previous treatments and disease status. Overall, patients with an HLA identical sibling CBT received the same conditioning as they would have received for a bone marrow transplant, patients with a mismatched transplant received intensification with various chemotherapies and ATG. A total of 39 patients received total body irradiation based regimen at a mean dose of 11 grays (range 5-18 Gy); 38 patients received a busulfan (BU) containing regimen at the dose of 16 mg/kg, this dose was adjusted to the body surface in young children at the dose of 480 mg/m^2. Thirty one patients received anti thymocyte globulin (ATG) and 6 patients a monoclonal anti T antibody in the preparative regimen for a mismatched CBT. GVH prevention consisted of Cyclosporine A alone in 40 HLA identical sibling CBT; it was associated with Prednisone in 23 patients, at low dose < 2 mg/kg in 10 cases or > 2 mg/kg in 13 cases, 17 patients received cyclosporine A and short methotrexate, 2 patients received cyclosporine with methotrexate and prednisone, 4 patients received ATG or monoclonal anti T antibody in addition to cyclosporine and prednisone.

Characteristics of Cord Blood Infused

The median volume of cord blood collected was 105 ml (range 41-360 ml), the median

number of nucleated cells per bag was 11×10^8 (range 2-46.7), with a median number of CFU-GM 4.3×10^5 (range 0.01-298) and of CD34+ cells of 3.7×10^6 (range 0.5-17.6). The median number of nucleated cells (NC) infused was 3.7×10^7/kg, (range 1-18), of CFU-GM 1.8. 10^4/kg (range 0.1-220),of CD34+ cells 1.6×10^5/kg (range 0.5-20).

Survival

The overall of 1-year survival for 53 patients with hematological malignancies was 44% with a median follow-up time of 250 days (Table 1). It was 48% in related and 41% in unrelated CBT ($p = 0.0125$). In patients less than 6 years old, it was 52 and 31% in older patients ($p = 0.08$) The number of NC infused was a major factor affecting survival which was 62% in 21 patients receiving more than 3×10^7/kg NC compared to 33% in patients receiving less ($p = 0.0099$). Patients with CMV negative serology (25 pts) had a 1 year survival of 68% compared to 22% in 27 pts who were CMV positive ($p = 0.008$). Diagnosis and stage of the disease affected survival. Patients with leukemia transplanted in first or second complete remission (21 patients) had a survival of 56 and patients transplanted in later stage of the disease or resistant relapse (15 patients) had a survival of 8%. In HLA identical CBT, survival was 56 and 34 % in HLA mismatched CBT ($p = 0.3$).

In a multivariate analysis, good survival was associated with use of a related vs an unrelated donor. RR: 2.0, $p = 0.038$, HLA identity vs. 1, 2 or 3 HLA antigens differences. RR: 1.4, $p = 0.02$, number of NC infused $\geq 3.7\times10^7$/kg. RR: 2.9, $p = 0.004$ and pretransplant recipient CMV negative serology. RR: 2.7, $p = 0.008$.

Engraftment

In patients with engraftment, the median day to reach > 500 PMN/μl was 24 days (8-45 days), > 1000 PMN/μl 29 days (12-102 days), the median day to reach > 20 000/μl platelets was 37 days (14-387 days), and > 500 00/ml platelets was 54 days (16-387). In patients with malignancy, 82% had more than 500 PMN at day 45 with 7 patients not evaluable because they died before this date. Patients who received less than 3×10^7/kg NC had a 67% probability of engraftment, while patients receiving more than the median dose of NC had 100% probability of engraftment ($p = 0.0001$). There was a significant difference in the delay to reach 500/μl PMN according to the number of NC infused, it was 34 days in patients receiving less than 3.7×10^7/kg NC versus 24 days in patients receiving more than this dose ($p = 0.0005$).

In a Cox model, 4 factors were associated with engraftment. They were HLA identity (RR: 3, $p = 0.003$), origin of cord blood related vs. unrelated CB (RR 2.2, $p = 0.04$), $\geq 3.7\times10^7$/kg NC infused (RR: 2.6, $p = 0.001$), and diagnosis other than aplastic anemia or hemoglobinopathy (RR: 3.5, $p = 0.0005$).

Table 1. Factors associated with 1-year survival after cord blood transplant for hematological malignancies.

Variable		N	1 year survival%	P
Global	53	44		
Donor related		33	48	
	unrelated	20	41	0.3
Age <6	23	59		
	>6	30	31	0.08
Weight <20 kg>		21	60	
	20 kg	31	34	0.04
Nuc cells/kg>3.7.107		21	62	
	<3.7.107	32	33	0.0099
CMV negative		25	68	
	positive	27	22	0.009
HLA	0 diff	23	62	
	1 diff	16	36	
	2 diff	5	80	
	3 diff	9	22	0.002
Sex				
	Identical	29	31	
	Different	24	57	0.07
TBI				
	Yes	36	47	
	No	17	35	0.4

Graft Versus Host Disease

In the whole population analysis, it was scored grade 0 in 33 cases, grade I in 16 cases, grade II in 15 cases, grade III in 8 cases and

grade IV in 2 cases. It was related to the number of HLA disparities but not to the type of mismatch A, B or DR. In 64 patients with engraftment, the probability of GVH $\geq$ II was 21% in HLA identical CBT and 60% in HLA mismatched CBT ($p = 0.0008$). It was 21% in HLA identical CBT, 54% in 1 ag HLA mismatched CBT, 58% in 2 HLA antigens mismatched CBT and 75% in 3 HLA antigens mismatched CBT ($p = 0.02$). Severity of GVH was mild and 2 patients only died of GVH. GVH was observed in 52.5% malignant diseases, 20% bone marrow failure syndromes and 12% other inherited diseases ($p = 0.0195$). The number of NC infused influenced GVH only in mismatched transplants ($p = 0.0557$). Median time to GVH was identical in matched and mismatched transplants.

Chronic GVH was observed in 5 of 41 patients surviving with engraftment for more than 90 days, it was limited in 4 cases and extensive in 1 case. It was observed after an HLA identical CBT in 2 cases and after an HLA mismatched transplant in 3 cases.

Other Complications, Leukemic Relapse and Cause of Death

Most complications were observed before day 100. The frequency of interstitial pneumonitis (IP) (16 cases), veno occlusive liver disease (VOD) (7 cases) and acute respiratory distress syndrome (ARDS) (7 cases) was relatively high, as it was not associated with HLA disparity, it might be related to transplant related toxicities in patients transplanted with advanced diseases.

Leukemic relapse was observed in 11 cases, 3 AML, 5 ALL, 2 MDS and 1 CML. Eight patients relapsed early after CBT on days 19, 20, 48, 67, 68, 100, 125 and 328. This is due to the high number of patients transplanted in late phase of the disease. Three patients relapsed later and are still alive; one had CML with molecular and cytogenetic relapse on day 1102, she is in complete remission after treatment with donor lymphocyte transfusions and interferon. Another patient had ALL with t9;22, he had a molecular relapse on day 1008 without cytogenetic or bone marrow relapse and complete donor chimerism. He is now in complete remission of one donor lymphocyte transfusion. The third patient with AML relapsed on day 250, he is currently alive in remission after bone marrow transplantation with the cord blood donor.

The primary cause of death of 39 patients was rejection/non engraftment in 10 cases, leukemic relapse in 8 cases, disease progression in 1 MDS and 2 neuroblastoma, GVH in 2 cases, interstitial pneumonitis in 2 cases, VOD in 4 cases, acute respiratory distress syndrome in 1 case, viral infection in 3 cases, fungal infection in 3 cases, cardiac toxicity in 2 cases and unknown cause in 1 case.

Other Results

J. Kurtzberg et al. [34] reported, recently, a series of 25 patients who received an unrelated cord blood transplant provided by the New York cord blood bank. All except one were children, 23 had a malignancy, 12 were in first or second complete remission(standard risk), 7 were in relapse or in advanced stage of the disease, 2 had leukemia complicating Fanconi anemia and Kostmann's syndrome, 1 neuroblastoma and 1 common variable immunodeficiency disease-myelodysplastic syndrome. There were various degrees of HLA incompatibilities. The number of nucleated cells infused correlated with the rate of myeloid engraftment($p = 0.002$). GVH was observed but it was mild. Twelve of the 25 patients had an event free survival rate of 48%. Seven of the 19 patients undergoing transplantation for malignant conditions survived, 6 of 12 patients transplanted in first or second CR survived but most have a short follow-up of less than 1 year; only 1 of 8 patients who underwent CBT while in remission and survived for more than 100 days had a leukemic relapse.

J. Wagner et al. [35] reported 18 patients who received an unrelated CBT from the New York cord blood bank. Most were children, 13 had a malignant disease, 5 patients only were in 1st or 2nd remission, the other had more advanced disease. Among 5 good risk patients, all were alive with a very short follow-up ranging from 170 to 317 days.

Discussion

Cord blood transplant is now a well recognized source of hematopoietic stem cells for allogeneic transplant. Recently published results and EUROCORD study show that the number of cells present in a single cord blood is sufficient to engraft most children and some adults. Engraftment has been a concern because of the limited number of cells present in one cord blood. In our series, the number of cells present in one cord blood was 11×10^8 nucleated cells with a range of 2 to 47×10^8. The median number of CD34+$\times10^8$ cells was 3.7 with a range from 0.5-7.5 and of CFU-GM.10^5 4.3 with a range of 0.01 to 38. We found that the number of nucleated cells infused was a major factor for engraftment and survival. Due to the heterogeneity of the patients groups and of the methods of transplant, it is very difficult in absence of prospective studies to make recommendations on the optimum number of cells needed for long term engraftment. In vivo expansion with hematopoietic growth factors such as G-CSF and thrombopoietin, improvements of stem cell recovery from cord blood collection or ex-vivo HSC expansion must be investigated prospectively.

Graft versus host disease is a major complication of HLA incompatible bone marrow transplantation. The use of cord blood cells which are immunologically immature might diminish the incidence and severity of this complication. As in previously published series, GVH was present, it was correlated with the degree of HLA disparity, usually, it was not life-threatening and the incidence of chronic GVH was low. It is impossible from this and previously published series to conclude on the relative protection of cord blood lymphocytes against GVH because most of the patients were children and GVH is known to be decreased in this population of patients. More patients and more follow-up are needed to evaluate the modification of the risk of relapse and possible loss of the GVL effect with cord blood cells.

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Interferon in Hematologic Malignancies

Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Interferon Alpha and Cytosine Arabinoside in the Treatment of Chronic Myelogenous Leukemia

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Introduction

The effectivness of interferon alpha (IFN) for the treatment of chronic myelogenous leukemia (CML) during the chronic phase is now well established. Several studies have shown that IFN produces hematologic remission in 60% to 80% of patients [1]. In some studies the achievement of complete hematologic response within 3 months seems to represent a significant factor which correlates with a cytogenetic response [2]. Also, the treatment with IFN resulted in suppression of the Ph-positive cells population which was complete in 7% to 25% of the patients [3, 4]. A major cytogenetic response (i.e., less than 35% Ph-positive cells) was noted in 35 to 45% of the patients. Such cytogenetic response was not only complete but also prolonged for periods ranging between 2 and 8 years [5].

Several groups have also shown that survival was improved in patients without any cytogenetic response. This latest result was reported by the UK Medical Research Council trial [6]. Moreover, it would appear that IFN also delays the appearance of blast crisis. The incidence of major and complete cytogenetic responses fluctuates from studies to studies (for example 7 to 25% for the complete response). This discrepancy between trials may partially explain the differences in survival rates. However, the cytogenetic response rates obtained with IFN alone remain low. Accordingly, new strategies combining IFN and other cytotoxic agents in order to improve the cytogenetic response rates are warranted.

In leukemic patients the aim of cytotoxic therapy is eradication of the leukemic stem cells with relative spering of the normal bone marrow stem cells. Cytarabine (1-β-D-arabinofuranosylcytosine) (Ara-C) is a pyrimidine nucleoside analogue with a configuration similar to that of cytidine and deoxycytidine, but differing in that the sugar moiety is arabinose rather than ribose or deoxyribose. The cytotoxic effect of Ara-C is thought to be determined mainly by the intracellular synthesis and retention of Ara-C triphosphate (Ara-C-CTP), which interferes with DNA synthesis. For that reason Ara-C is preferentially cytotoxic to proliferating cells. In vivo, Ara-C is rapidly inactivated to uracil-arabinoside (Ara-U), so that Ara-C chemotherapy consisting of push injection or a few hour of infusion only results in short-term exposure of the cells to Ara-C. A comparison of pharmacokinetics of continuous intravenous and subcutaneous infusion of Ara-C demonstrated that steady-

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state serum Ara-C levels were similar with both routes of administration [7]. Because of its short half-life after a single intravenous or subcutaneous injection and its S-phase activity, prolonged intravenous infusion of Ara-C have been administered to patients with acute myelogenous leukemia. The administration of Ara-C by continuous IV infusion results in plasma Ara-C levels ranging between 10^{-8} and 10^{-7} mol/l. In contrast, peak plasma level is approximately 10^{-6} mol/l after a subcutaneous single injection. Furthermore, plasma Ara-C levels are undetectable 6 h after bolus injection. Thus using a dose schedule of daily injection of Ara-C could result in an 18 h interval with no detectable drug before the next injection [8].

Ara-C is an attractive chemotherapy for the treatment of CML. In vitro studies demonstrated a preferential inhibition by Ara-C of granulocytes-macrophage colony forming cells (CFU-GM) from patients with CML compared to CFU-GM from normal subjects. Two groups found that CFU-GM from patients in the chronic phase CML were inhibited by significantly lower concentration of Ara-C than were CFU-GM from normal subjects [9, 10]. Because of this in vitro activity, Ara-C has been tested in vivo for the treatment, as single agent, of patients with CML in chronic phase [11, 12]. Hematologic remission was consistantly observed and, in addition, several patients had a cytogenetic response. Although these results suggest that prolonged continuous administration of Ara-C can innibit Ph+ hematopoiesis, this inhibition was always transient and there is at the present time no evidence that such treatment could prolonged the survival.

One of the mechanisms by which IFN act as a antitumor agent is that IFN blocks cell cycle progression in the G0/G1 phase [13]. The effect of IFN alone and in combination with Ara-C on normal and leukemic human hematopoietic progenitor cells have been investigated in vitro. Mononuclear cells from normal bone marrow, peripheral blood of patients with chronic phase CML, or the cell line HL-60 were incubated with various type of interferons followed by the addition of Ara-C. The survival of normal CFU-GM was significantly increased if the cells were incubated with IFN 1 h before Ara-C exposure. These in vitro studies suggest that IFN and Ara-C act synergistically. It is assume that normal cells are blocked in phase G0/G1 by IFN and are, hence, protected from the cytotoxic effect of Ara-C which act in S-phase, whereas leukemic cells which are more autonomous in their division are destroyed by Ara-C [14]. Because of these mecanisms of action, one could suggest that the treatment of patients with CML CP using these two agents could result in more cytogenetic responses and then better survival.

Thus, we conducted 3 successive trials in order to assess the usefulness of a combination of Ara-C and IFN.

Patients and Treatment

The Pilot Study

In 1986, we initiated a first pilot study by using such a combined approach. Twenty four patients (12 previously treated by standard chemotherapy and 12 previously untreated) received hydroxyurea (HU) 50 mg/kg/day orally and IFN-α2a at a starting dose of 5×10^6 IU/m^2/d subcutaneously. Courses of low-dose Ara-C were given to 11 patients. Ara-C was administered once a day 10 to 15 days per month at a dose of 10 to 20 mg/m^2/d. Acomplete hematologic response (CHR) was obtained in 18 to 24 patients. Eight out of 24 patients obtained a major cytogenetic response. Furthermore, a rapid cytogenetic improvement was recorded in six out of 11 patients receiving courses of low dose Ara-C, with a complete Ph chromosome suppression in 4 [15]. Summarizing, IFN improves survival in that fraction of patients in which it induces a cytogenetic response. The combination of IFN and Ara-C should make it possible to increase the cytogenetic response rate and improve survival even further. The two French multicentric trials, the CML 88 and the CML 91 studies were initiated in order to explore further this hypothesis.

The CML 88 Trial

The CML 88 protocol was the first French multicenter randomized study.

The principal assessment criterion was the study of the cytogenetic response rate in the experimental group (IFN + Ara-C) compared with the cytogenetic response rate in the reference group (IFN alone). Secondary criteria consisted of patient tolerance of the treatment, and survival. Inclusion criteria were as follows: diagnosis of CML based on cytogenetic analysis (complex translocations and certain additional cytogenetic anomalies were not considered as exclusion criteria, but anomalies such as iso 17q, duplication of Ph1 or trisomy 8 were regarded as exclusion criteria since they indicated and accelerated phase of the disease); CML in the chronic phase, aged between 15 and 70 years, WHO score 0, 1, 2, without visceral impairement, renal, hepatocellular or heart failure; no treatment or conventional chemotherapy of less than 6 months duration following diagnosis. Patients were randomized by telephone (to the coordination center) and included in one of the 2 maintenance therapy arms. The maintenance therapy had to be initiated in the 3rd month of induction treatment in a patient in stable hematologic remission and no longer receiving HU.

In the induction phase, all patients received IFNα-2b starting at 5×10^6 IU subcutaneously plus HU 50 mg/kg/d orally until stable hematologic remission was achieved. The maintenance treatment was scheduled to begin during the third month in a patient in stable hematologic remission no longer receiving HU. Patients were randomized to receive either IFN-α-2b alone (5×10^6 IU/m ^{2}) or the same dose of IFN plus low-dose Ara-C 10 mg/m^2/d subcutaneously 10 days each month. The protocole was stopped in the following situations: if a related or unrelated compatible HLA donor was found, and if a bone marrow allograft appeared possible, given the patients's age and condition, or if severe toxicity required the discontinuation of treatment; if complete hematologic remission was not obtained after 3 months of induction treatment; if a hematologic relapse occurred, in spite of the correct administration of interferon; if a cytogenetic relapse occurred (Fig. 1).

237 patients were registered (centralized registration in Poitiers) over a 33 month period from April 1988 to January 1991. Patient registration was carried out by 36 French Hematology centers and was stopped on January, 16, 1991. An analysis of this trial was performed with 207 evaluable patients after a median follow-up of 50 months. In this trial, 30 patients are not available for the analysis. Of these, data are still expected for 5 patients, 2 patients refused treatment and follow-up, while 6 others were wrongly diagnosed. In addition, 17 patients were not evaluable per protocol because of exclusion criteria, i.e. the interval with respect to diagnosis was excessive in the case of two patients, blast crisis occurred right away in 3 patients, an allograft was directly performed in 4 patients; 7 patients had chronic myelocytic leukemia without Philadelphia chromosome but with a bcr/abl rearrangement, and 1 patient committed suicide a few days after randomization. The number and distribution of non analyzable patients is similar in both groups and does not impede the analysis.

The distribution of clinical and laboratory parameters was identical in the two

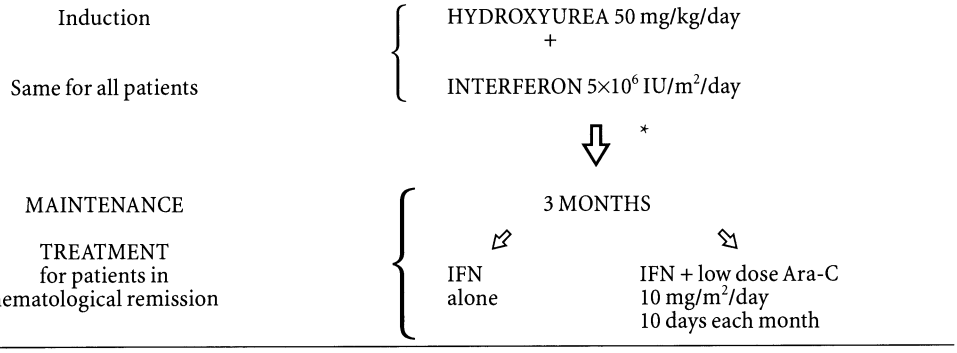

Fig. 1. CML 88: treatment plan

Table 1

Month	3	6	9	12	15	18	21	24	27
IFN + ARA-C									
No. in CHR	50	54	51	38	26	26	16	15	8
WBC									
min.-max.	2.1-9.8	2.5-8.3	2.5-9.8	2-7.6	1.9-8.8	2.4-8	2.3-8.2	2.5-8.7	2.7-6.8
mean	5	4.1	4.3	4.2	4.5	4.4	3.8	4.5	4.1
Platelets									
min.-max.	14-386	57-406	34-441	33-416	74-430	73-377	73-441	79-306	104-269
mean	166	167	176	176	189	184	184	183	201
IFN									
No. in CHR	64	60	45	40	19	18	13	11	3
WBC									
min.-max.	2-9.8	1.3-9.7	1.9-9.6	2.2-8.9	1.8-9.6	1.9-6.1	2.3-9.4	2-5.3	3.5-5.7
mean	4.8	4.7	4.5	4.9	4.1	4.3	4.7	3.8	4.3
Platelets									
min.-max.	16-425	59-398	87-368	27-441	100-313	83-320	87-345	62-269	171-341
mean	189	187	193	206	209	216	216	154	234

groups. Each risk category (low, intermediate or high), based on the Sokal score, was evenly distributed in the two groups. A CHR was observed in 83% of patients, without difference between the two arms. Statistical analysis of the cytogenetic response does not demonstrate a superiority of the IFN + Ara-C arm in terms of achieving major cytogenetic responses. However, in the IFN + Ara-C group 24 of 103 patients (23%) were in complete cytogenetic response (CCR), whereas in the IFN-alone group 16 of 104 (15%) patients obtained this result. Thus in the CML 88 study, the rate of CCR seems higher in the IFN + Ara-C arm, but this is only a trend.

In this protocol the side effects of IFN-α were important including fever, chills, malaise, headache, anorexia, joint pain, vomiting, low backache, myalgia, various type of neuropathy, changes in mood and concentration, psychiatric disorders, abnormalities of liver enzymes, leukopenia and thrombocytopenia. Sufficient dosage is important because it is necessary to induce leukopenia and thrombocytopenia in order to obtain cytogenetic responses. In some patients, serious side effects were dose limiting and for some of them treatment had to be interrupted. These included neurotoxicity with frontal lobe signs (depression) and immune mediated events. A triad of depression, fatigue and insomnia was frequently observed in some patients. A poor neuropsychiatric tolerance must be recognized early with the need to stop interferon treatment in case of neuropsychiatric symptoms and in particular in case of suicidal tendencies. The objective of CML 88 trial was to assess patients tolerance of IFN when administered during the chronic phase of chronic myelocytic leukemia. Also it sought to demonstrate the possible effect of adding courses of treatment with Ara-C to maintenance therapy with IFN in order to increase the cytogenetic response rate. The combination of HU-IFN given as induction treatment produced a satisfactory hematologic remission rate. Moreover the hematological toxicity of this new association was acceptable.

In patients maintained in CHR by the treatment, the evolution of the mean leukocyte and platelet counts was compared. Table 1 shows that there was no difference with regards to these parameters between the two groups for patients maintained in CHR.

The medullary cellularity was evaluated every 3 months by a marrow puncture and graded as: hypercellular (4), normal cellularity (3), hypocellularity (2), extremely low cellularity (1). Medullary cellularity was studied over a 2 year period. Table 2 shows that there were no significant differences in this parameter between the two treatment groups: the percentage of patients with hy-

Table 2. Evolution of medullary cellularity

Grade	Dg[a]	3	6	9	12	15	18	21	24 months
IFN + ARA C									
	0	12	12	14	10	13	7	4	0
1	0%	21%	29%	31%	26%	50%	33%	33%	0%
	1	7	9	10	9	4	4	2	7
2	1%	13%	21%	22%	23%	15%	19%	17%	29%
	11	33	20	19	19	8	9	5	8
3	15%	59%	48%	45%	49%	30%	43%	41%	33%
	59	4	1	2	1	1	1	1	0
4	89%	71%	2%	4%	3%	4%	5%	8%	0%
TOTAL	71	56	42	45	39	26	21	12	15
IFN									
	1	13	19	10	7	4	6	0	4
1	1%	20%	34%	24%	18%	21%	35%	0%	40%
	2	11	13	10	9	7	2	3	2
2	2%	17%	23%	24%	23%	37%	12%	43%	20%
	12	31	17	15	19	5	7	4	3
3	14%	48%	30%	36%	49%	26%	41%	57%	30%
	73	10	7	7	4	3	2	0	1
4	82%	15%	13%	17%	10%	16%	12%	0%	10%
TOTAL	88	65	56	42	39	19	17	7	10

[a] Dg : diagnosis.

pocellularity or very low cellularity is the same in both groups.

In this trial, patients who achieved a major or a complete cytogenetic response had a significantly better survival than those with minor or no response. The probability of obtaining complete cytogenetic remission was greater in the interferon-Ara-C group, but this was only a tendency.

The CML 91 Trial

The CML91 trial was partially based on the CML 88 study. One of the objectives of the CML 91 trial was to treat patients on the basis of prognostic scores derived from Sokal criteria. Also the patients were treated according to their age. Patients less than 35 years of age whatever their score with a family donor were allografted after a short period of hydroxyurea (usually within 12 months). The same procedure was required for patients between 35 and 50 years of age with a high score (i.e., > 1.2). All the other patients were included in the trial. Patients for induction were randomized between two regimens, HU + IFN (same doses as in the CML 88 study) or HU + IFN + courses of low-dose Ara-C, which was administered at a dose of 20 mg/m^2/10 days 2 weeks after the beginning of the induction regimen (Fig. 2).

The endpoints were overall survival, CHR at 6 months, major cytogenetic response (i.e., less than 35% Ph+ cells) at 12 months. In this second trial courses of low-dose Ara-C were administered at a dose of 20 mg/m^2/day 10 days per month which is a dose twice higher compare to that administered in the previous study. Also, the dose could be increase up to 40 mg for a period of 15 days per month. The first course of Ara-C was started earlier, 2 weeks after the beginning of the induction regimen. HU was discontinued if a stable hematologic remission was achieved. Patients who achieved hematologic response at 6 months (HU being discontinued) or major cytogenetic response at 12 months continued in each group. Patients who died not achieved either hematologic response at 6 months or major cytogenetic response at 12 months had several therapeutic options. An allogeneic related or unrelated bone marrow transplant was proposed for those with a suitable donor. Other patients could received high dose chemothera-

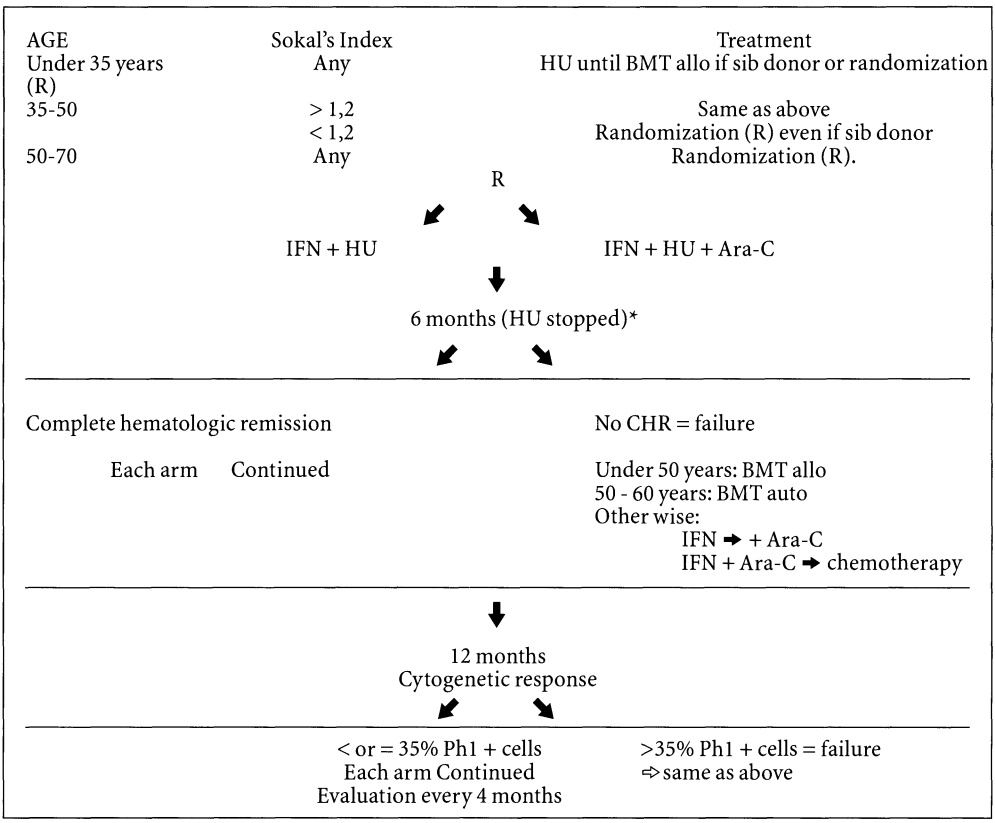

Fig. 2. CML 91 – design of the study

py followed by autologous stem cell reinfusion.

From January 1991 to May 1996, 745 pts (age $\leq$ 70 y) with a CML Ph+ chronic phase were included. All pts were given simultaneously hydroxyurea (HU) at a daily dose of 50 mg/kg and IFN α2b at a starting dose of 5×10^6 IU/m^2/day for induction. HU was discontinued when a CHR was achieved. Pts randomized in the IFN + Ara-C group received the same regimen plus monthly courses of Ara-C.

A preliminary analysis was performed on 646 randomized pts, 324 in the IFN group and 322 in the IFN + Ara-C group. Risk categories based on the Sokal score were evenly distributed in the two groups: 48% (low), 40% (intermediate), 12% (high) in the IFN + Ara-C group and 41, 42, 17% in the IFN group. At 6 months, the CHR rate was higher in the IFN + Ara-C group (67%) compared to IFN group (54%) ($p = 0.002$), but time to response was similar in both groups. At 12 months, a major cytogenetic response was obtained in 96 (39%) out of 248 pts with Ara-C and in 55 (22%) out of 249 pts without Ara-C ($p < 0.001$). Survival of pts in the IFN + Ara-C group was improved. During the first 12 months, the mean daily dose of IFN was 5×10^6/day in both groups, the median number of courses of Ara-C was 7 with a median daily dose of 31 mg. Adverse events were responsible for discontinuation of treatment in 71 pts included in the IFN-Ara-C group and in 75 pts in the IFN group. Main side effects with Ara-C were gastro-intestinal disorders and thrombocytopenia [16]. This preliminary analysis of this second trial shows that patients who received IFN and Ara-C had a better survival than those who received IFN alone. The cytogenetic response rate was also significantly improved with the combination of Ara-C and IFN.

Conclusion

The treatment of patients with chronic phase CML with a combination of IFN and Ara-C resulted in higher major and complete cytogenetic response rates compare to IFN alone. A relationship was found between the survival and the cytogenetic response. Thus IFN and Ara-C can be proposed to patients with CML as initial treatment.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Alpha-Interferon Role in the Management of Follicular Lymphoma Patients. The GELA Experience

B. COIFFIER

Follicular lymphomas (FL) represent between 20 and 25% of all lymphomas and are slightly more frequent in America than in Europe and less frequent in Asia [1]. They are composed of follicle center cells, a mixture of centrocytes (small to large cleaved follicle center cells) and centroblasts (large noncleaved follicle center cells). Usually, centrocytes are predominant and centroblasts are the minority. The pattern of proliferation is usually follicular but diffuse areas may be present and in some cases they may predominate. The proportion of centroblasts and the size of centrocytes vary in different lymph nodes from the same patient [2]. Histologic grades associated with different outcome have been described according to the percentage of each constituent but they have proved to be irrelevant because proliferation is a continuous graduation and this grading is difficult to reproduce among groups of pathologists [3,4]. Tumor cells are usually Slg^+, B-cell-associated antigen$^+$ (CD19, CD20, or CD22), $CD10^+$, $CD5^-$, and $CD23^-$. Translocation t(14;18) involving rearrangement of bcl-2 gene is present in 70 to 90% of the cases, resulting in abnormal expression of this anti-apoptotic gene.

Most patients have widespread disease at diagnosis and truly localized disease is rare [5]. Involved sites are predominantly lymph nodes, spleen, and bone marrow, and occasionally peripheral blood or extranodal sites. Extranodal site involvement, other than bone marrow or blood, is often associated with a more aggressive disease or an histologic progression. These lymphomas are characterized by a rather ineluctable evolution toward an histologic transformation into large cell or small non-cleaved cell lymphoma. During the first phase of the disease, lymphoma cells are sensitive to various treatment types but a true complete remission (CR) is hardly reached. With relapses, with or without histologic transformation, lymphoma cells become resistant to treatment. The most important prognostic factors for follicular lymphoma patients do not differ from those associated with survival in large cell lymphomas, i.e., age, performance status, stage, tumor burden, lactic dehydrogenase (LDH) and BBB2-microglobulin levels. The application of the International Prognostic Index allows the definition of four subgroups with a rather different outcome, patients with intermediate-high and high risk having a less than 30% 5-year survival [6]. These patients may be divided into three clinical subgroups defined by the tumor burden: patients with localized disease, patients with disseminated disease but low tumor burden, and those with high tumor burden. The criteria of high tumor burden are one or more tumor masses larger than 7 cm, poor performance status, above normal value LDH level, above 3 mg/l ß2-microglobuline level, any tumor mass responsible of a compressive syndrome, pleura or ascitis ef-

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fusion, blood involvement, and any cytopenia. Even if these criteria are not internationally accepted, most large groups use them [4].

Follicular lymphomas are considered as low-grade lymphomas because the initial clinical picture shows little aggressiveness with only nodal involvement and because the overall survival of these patients is long with a median survival ranging from 7 to 10 years, particularly for patients with a low tumor burden. However, the annual death rate is constant, around 6–7% a year. Freedom-from-progression (FFP) survival is much shorter than overall survival and the median FFP survival ranged from 2 to 3 years according to prognostic parameters. Reaching a CR or a very good partial response (persistance of only a small bone marrow involvement) has been associated with a better outcome and, thus, this has to be the aim of the treatment in young patients [5].

Treatment of a follicular lymphoma patient must be decided according to the stage of the disease, its tumor burden, and the presence of adverse prognostic factors. Localized disease, stage I and II without large tumor burden, is seen in 10 to 15% of the patients. Half of them have a true localized disease and may be cured by local radiation therapy. In these patients, chemotherapy must be delayed until recurrence.

Patients with disseminated disease must be treated according to the presence or absence of adverse prognostic factors. Those patients without adverse prognostic factors may be initially not treated until disease progression and the appearance of any criteria of high tumor burden. Spontaneous regressions (< 5%) may occur but most of the patients have stable disease for a median of 12 months (small and large cell FL) or 3 to 4 years (small cell FL). All patients eventually progressed and died from their disease, 40 to 70% after histologic transformation, with a median survival of 6 to 8 years [7]. This approach may be valid for elderly patients but must be reconsidered for younger patients according to results obtained for patients with more aggressive disease. However, no prospective trial have shown that treating these young patients earlier was associated with longer survival. Our present recommendation is to closely observed the disease progression during the first 6 months after diagnosis and to start chemotherapy that is used in patients with adverse prognostic factors in case of disease progression. Otherwise, the "watch and wait strategy" is currently the best bait for elderly patients with low tumor burden.

At least 50% of FL patients need to be treated because of the presence of adverse prognostic factors. Treatment choice has long been a matter a discussion mainly because randomized trials have been realized in all FL patients or by mixing these patients with other so-called "low-grade" lymphomas. FL patients have been considered as non curable and manageable with non-aggressive treatment, that is chlorambucil or CVP [cyclophosphamide, vincristine, and prednisone]. However, patients who reached CR at the end of first line treatment have a longer survival, a longer time-to-treatment failure (TTF), and a lower probability of histologic transformation at recurrence. CR should then be the goal of first-line treatment, particularly in young patients and, in this setting, more intensive therapy such a CHOP [cyclophosphamide, doxorubicin, vincristine, and prednisone], CHVP (Fig. 1), PmM (Prednimustine plus mitoxantrone), or any comparable anthracycline-containing regimen allowed better CR rate than non-doxorubicin containing regimens. Fludarabine allows a high response rate in relapsing patients and in first-line patients but TTF is not different from that obtained with other regimens [8]. Until results of current randomized trials comparing fludarabine with CVP or CHVP, it should be reserved for relapsing patients. Duration of treatment should be adapted to the response and PR patients at 12 months have to continue courses till reaching a clinical CR.

Patients with predominantly large cell FL have been considered in the Working Formulation as having a worse outcome. If most of these patients have adverse prognostic factors and need to be treated at diagnosis, response to treatment and survival are not different from other FL patients with the same poor prognosis parameters. Therapeutic strategies defined for other FL patients are recommended.

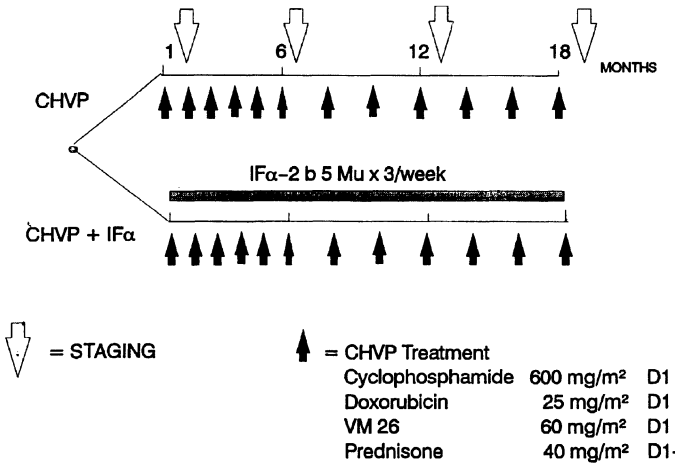

Fig. 1. Schema of the GELF-86 protocol

Table 1. Interferon trials in follicular lymphoma patients

Trial	Follicular Only	Induction	Maintenance	TTF	Survival
ECOG	No	Yes	No	$p < 0.05$	NS
GELA	Yes	Yes	Yes	$p < 0.001$	$p < 0.01$
St. Barts	Yes	Yes	Yes	$p < 0.05$	NS
EORTC	Yes	No	Yes	$p < 0.05$	NS
German	No	No	Yes	$p < 0.05$	Not analyzed

NS: Not significant.

Interferon AAA-recombinant has been used either in association to initial chemotherapy regimen or as maintenance in responding patients in order to increase the response rate, decrease the relapse rate, and increase TTF and overall survival (OS) [9]. Whatever the setting, addition of interferon increased significantly TTF or disease-free survival (DFS) but it increased OS in only the GELF-86 trial (Fig. 1) that used the longer duration of interferon treatment. If complementary studies have to be done to know the best interferon dose (currently, 3 to 5 MU three times a week), the duration of treatment (probably longer than 18 months), and the best setting (induction, maintenance, or both), interferon have to be included in the treatment of FL patients (Table 1, [4]).

The GELA study [10] randomized the m-CHVP regimen (cyclophosphamide 600 mg/m^2, doxorubicin 25 mg/m^2, VM-26 60 mg/m^2, and prednisone 60 mg/m^2 every 4 weeks for 12 courses) versus m-CHVP plus interferon 5 MU 3 times a week for 18 months. Patients treated with interferon have a significantly increased response rate, a significantly longer disease-free survival, and a significantly longer survival (Figs. 2, 3). This difference persists and increases with a longer follow-up in the group of patients analyzed in the initial paper or in a larger group of patients comprising all randomized patients.

In none of the trials incorporating interferon in combination with chemotherapy or after achieving a response, a severe detrimental effect was associated with interferon treatment but more pronounced cytopenia have been described in patients treated with the association of chemotherapy plus interferon without clinically significant adverse event.

In conclusion, the ideal treatment of follicular lymphoma patients is not yet determined and further studies are needed to define the role of new strategies such as early

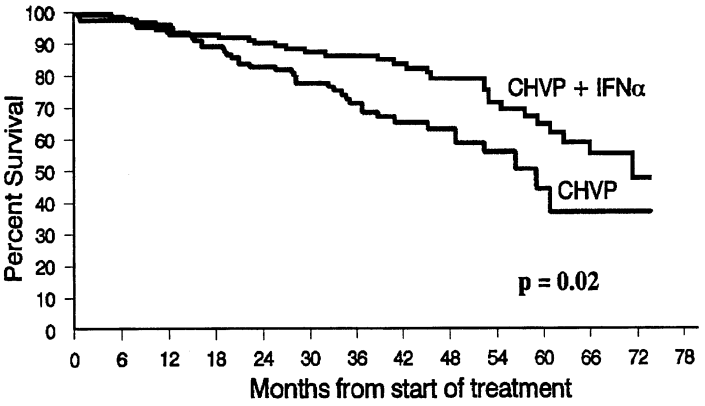

Fig. 2. Updated survival of the 242 patients included in the GELF-86 and in the paper published in the New England Journal of Medicine with a median follow-up of 40 months

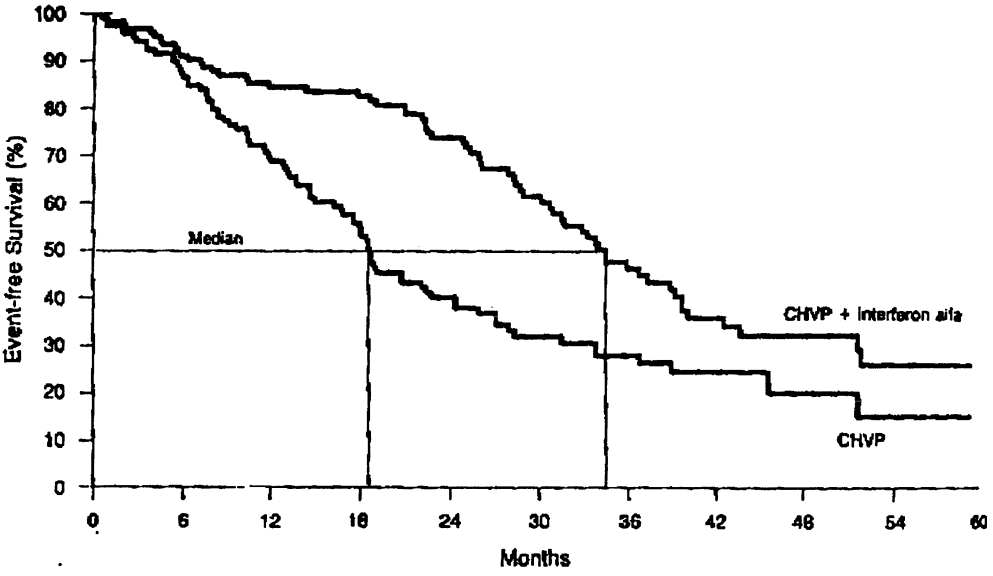

Fig. 3. Freedom-from-progression survival of the 242 patients included in the GELF-86 and in the paper published in the New England Journal of Medicine

intensification with stem cell support or anti-CD20 antibodies. However, there is now sufficient evidence that showed a benefit in duration of response or duration of survival for follicular lymphoma patients with a high tumor burden being treated with an anthracycline-containing regimen to include AAA-interferon in their treatment either in combination with chemotherapy or after achieving a response. Interferon must be used at the dose of 5 MU three times a week for at least 18 months.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Treatment of Centroblastic-Centrocytic and Centrocytic Lymphomas at Advanced Stages – Results of the German Low-Grade Lymphoma Study Group

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Abstract. The current multicenter study was initiated to address two major questions in the treatment of advanced stage non-Hodgkin lymphomas (NHL): (1) the activity of two different cytoreductive regimens and (2) the impact of interferon-α (IFN-α) maintenance.

Patients with advanced stage III and IV centroblastic-centrocytic (CB-CC) and centrocytic (CC) lymphomas were randomized to initial therapy with either prednimustine/mitoxantrone (PmM) or cyclophosphamide/vincristine/prednisone (COP). Responding cases underwent a second randomization for IFN-α or observation only. From the 525 evaluable patients 85% achieved a remission with similar overall response rates for PmM (85%) and COP (86%). Significantly more CR were obtained after PmM (35% versus 21% $p < 0.05$); 247 cases were subsequently randomized for IFN-α versus observation. As of November 1996 a significant advantage in favor of IFN-α was observed. In the IFN-α group the median DFS was 32 months as compared to 19 months in the control group ($p = 0.0033$). Analysis of IFN-α in association with initial cytoreductive treatment showed a more beneficial outcome for PmM plus IFN-α with an estimated median DFS of 43 months as compared to 27 months for COP plus IFN-α. These data indicate a significant prolongation of DFS by IFN-α maintenance and suggest that this effect is related to the efficacy of initial cytoreductive chemotherapy.

Introduction

Low-grade lymphomas comprize a heterogenous groupe of disorders that are characterized by a prolonged clinical course and a low proliferative activity. Recent molecular

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and cytogenetic techniques have provided new insights into the pathogenesis of these diseases. Hence, centroblastic-centrocytic (CB-CC) lymphomas frequently reveal a translocation between chromosomes 14 and 18 [t(14;18)] which leads to the association of the bcl-2 gene with the gene for the immunoglobuline heavy chain. This translocation induces an over expression of the bcl-2 product and a prolongation of apoptosis [1, 2, 3]. Centrocytic (CC) or mantle cell (MCL) lymphomas are associated with the translocation t(11;14) which leads to a deregulation of the cycline D1 gene and an expansion of cells from the lymph node mantle zone [4, 5, 6].

Despite of the increasing knowledge about the pathogenesis of malignant lymphomas, little progress has been achieved in their treatment. The prognosis of patients with low grade lymphoma has almost remained unchanged over the past decades. The median survival of patients with CB-CC lymphomas is in the range of 5-8 years while the median live expectancy for patients with CC or MCL lymphomas amounts to approximately 3 years [7, 8, 9, 10, 11].

In order to develop new therapeutic perspectives for patients with CB-CC and CC lymphomas the German Low Grade Lymphomas Study Group embarked on a prospective randomized study evaluating the efficacy of two different cytoreductive regimens followed by a second randomization two assess the impact of interferon alpha maintenance therapy in responding cases [12].

Patients, Treatment Protocol and Methods

Patients at ages 18-75 with previously untreated CB-CC and CC lymphomas of advanced stages were eligible for the current study. While patients with centrocytic lymphomas were entered on therapy immediately after diagnosis, cases with CB-CC lymphomas were eligible only under the following prerequisites:

- presence of B-symptoms and/or
- impairment of hematopoesis with $< 1\,500/mm^3$ granulocytes and/or
- $< 100.000/mm^3$ thrombocytes and/or
- < 10.0 g/l hemoglobin and/or
- progressive disease and/or
- bulky disorder

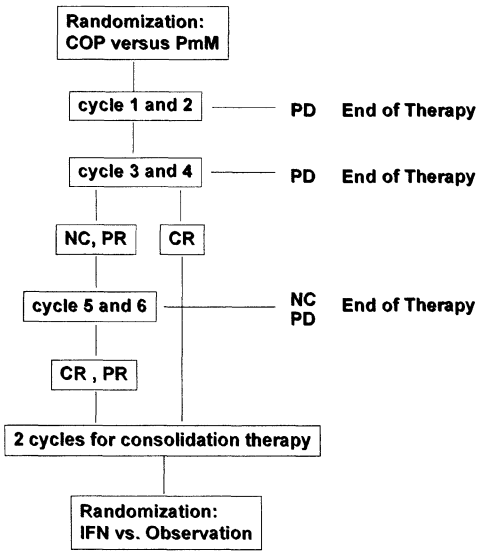

Fig. 1. Low-grade non-Hodgkin-lymphomas at advanced stages

The conduct of the study is depicted in Fig. 1. It thus comprises the prospective randomized comparison of the efficacy of standard cyclophosphamid, vincristine, prednisone (COP) with the anthrachinone containing combination of prednimustine, mitroxantrone (PmM). In patients responding to either one therapy a second randomization was performed to assess the impact of interferon alpha maintenance on disease free survival as compared to no further treatment.

The COP combination comprized cyclophosphamide 400 mg/m^2 per day, day 1-5, vincristine 1.4 mg/m^2 per day (max. 2.0 mg) day 1 and prednisone 100 mg/m^2 per day, days 1-5. This treatment was repeated every 3 weeks.

The PmM regimen consisted in prednimustine 100 mg/m^2/day, days 1-5 and mitroxantrone 8 mg/m^2/day, days 1 and 2. PmM cycles were repeated every 4 weeks.

Boths protocols were applied for a maximum of 6 courses. In patients achieving a complete or partial remission two further courses were applied for consolidation.

Patients who were subsequently randomized for interferon maintenance were given a dose of interferon of 5 Mill. U. 3 x per week. The dose of interferon was adjusted to side effects and was sequentially reduced to a minimal dose of 1 Mill U. 3 x per week.

Results

Between May 1989 and July 1996 525 patients entered the study; 412 suffered from CB-CC lymphomas while 86 patients were diagnosed as CC lymphomas. At the time of evaluation 352 cases were fully evaluable. As depicted in Table 1, both regimens achieved comparable overall response rates of 85% after COP and 86% after PmM. PmM, however, resulted in a significantly higher rate of complete remissions (35% versus 21%, $p < 0.05$).

From the 281 patients that achieved complete or partial remissions 247 patients underwent the second randomization for interferon versus observation only. Both study arms were well balanced for histologic subtype, age distribution, stage of disease, type of initial chemotherapy and remission rate (Table 2).

At the time of evaluation in November 1996 the median event free interval was 23 month for all patients with a projected disease free survival of 30% at 5 years. In patients receiving interferon maintenance the median event free interval was 32 months and hence significantly longer as in the control group with 19 months ($p = 0.0033$) (Fig. 2).

A separate analysis of CB-CC and CC lymphomas indicated a beneficial effect of interferon alpha maintenance for both subtypes. The difference to the observation only group was significant for CB-CC lymphomas and did not reach statistical significance in CC lymphomas due to the low number of patients entered.

Interferon alpha maintenance was well tolerated. Major side effects comprized granulocytopenia and flue like symptoms. These side effects were usually of mild to moderate degree and less than 30% of patients terminated treatment due to intolerable side effects within the first 3 years of application.

Table 1. Initial therapy with PmM versus COP followed by Interferon alpha versus observation

	COP	PmM
n	160 (100%)	183 (100%)
CR	34 (21%)	59 (35%)
PR	102 (64%)	86 (51%)
NC	7 (4%)	6 (4%)
PD	14 (9%)	17 (10%)
EX	2 (1%)	2 (1%)

Table 2. Interferon alpha versus observation stratification criteria

	IFN	Observation
n	119	128
Age < 60 years	73	81
Age ≥ 60 years	46	47
FCL	94	97
MCL	22	25
COP	52	59
PmP	57	60
CR	41	44
PR	65	69

Discussion

The present study of the German Low Grade Lymphoma Study Group addressed two major questions in the treatment of low grade lymphomas on the basis of prospective randomized comparisons. The first approach comprizes the cytoreductive efficacy of COP versus PmM which showed no differences in the overall response rates but a significantly higher rate of complete remissions after PmM. The second major question addressed the impact of interferon alph maintenance on the disease free survival. In this study interferon alpha maintenance was given without a timely limitation until relapse or untolerable toxicity. The obtained results indicate that interferon alpha maintenance is well tolerated and can be applied for a prolonged period of time exceeding 3-5 years. Interferon alpha maintenance resulted in a significant prolongation of the disease free intervall and reduced the risk of relapse to less than 50% as compared to a control group.

A beneficial effect of interferon alpha maintenance was also shown by other groups [13, 14, 15, 16]. In all other studies

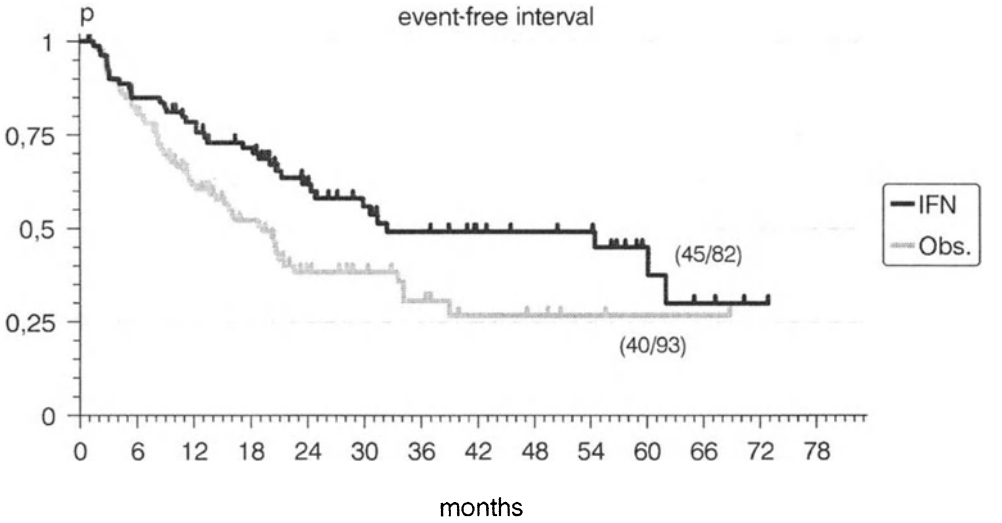

Fig. 2. German low grade NHL study group, Interferon-alpha maintenance vs. observation

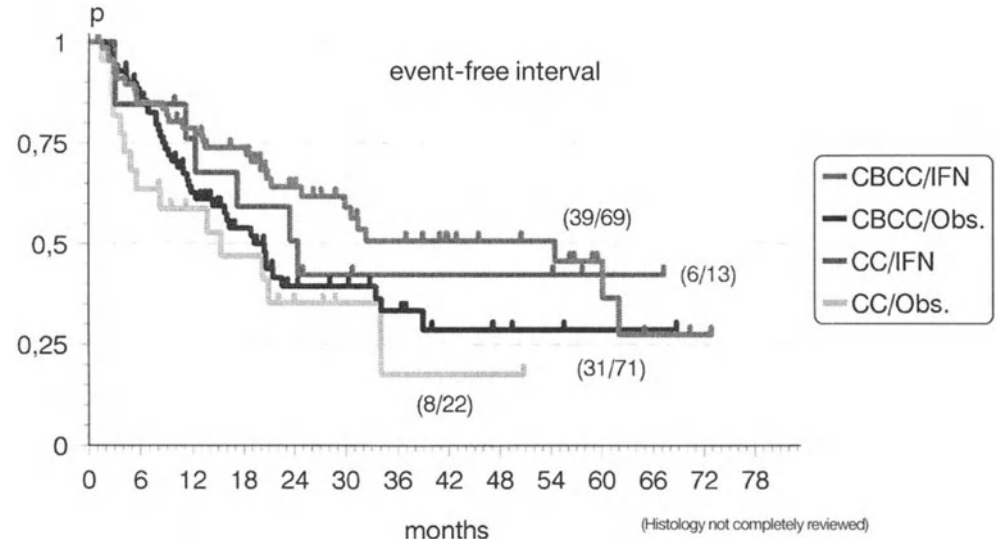

Fig. 3. German low grade NHL study group, Interferon-alpha maintenance vs. observation

interferon alpha maintenance was, however, terminated after a period of 12-18 months. This strategy led to an increased relapse rate after the termination of interferon alpha maintenance and a narrowing of the disease free survival curves after 3-5 years. In contrast to these results the disease free interval of the present study remained superior for interferon alpha maintenance at 4-5 years still. This result strongly suggests that interferon alpha maintenance allows a prolonged suppression of residual low grade lymphomas and inhibits the regrowth of residual tumor cells.

Based on the current results, initial cytoreductive chemotherapy with subsequent interferon alpha maintenance must be considered as standard therapy for patients with low grade lymphomas at advanced stages.

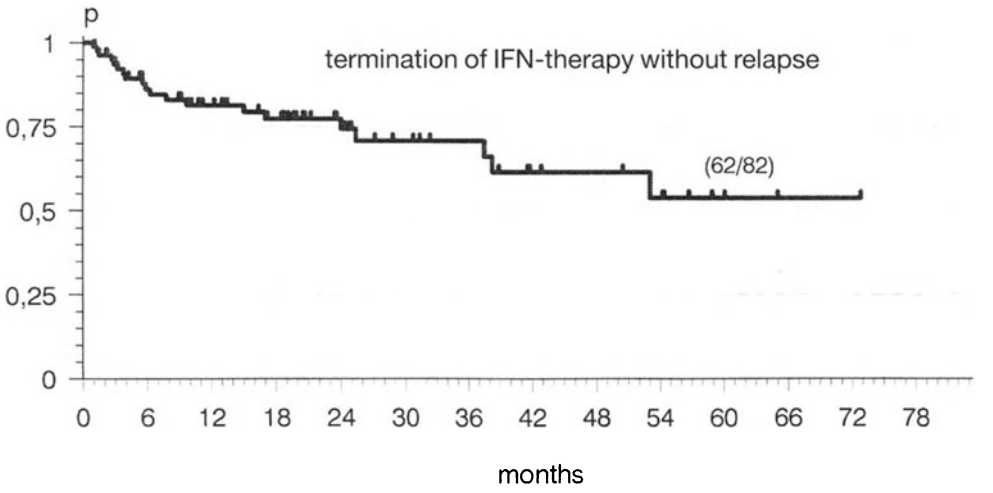

Fig. 4. German low grade NHL study group, Interferon-alpha maintenance vs. observation

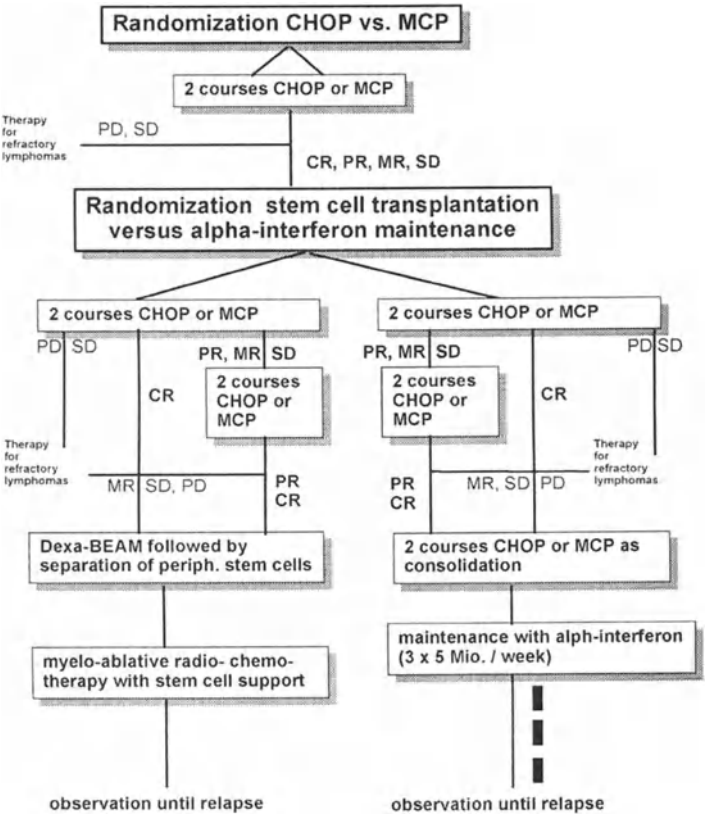

Fig. 5 Patients < 60 years and qualified for stem cell transplantation. Evaluation of curative potential of myelo-ablative radio-chemotherapy with peripheral stem cell transplantation in first remission

In spite of these encouraging results further improvements are required. These include the assessment of more effective initial cytoreductive regimens, an optimisation of interferon alpha maintenance and the evaluation of a potentially curative approach including myeloablative radio-chemotherapie with subsequent stem cell transplantation [17, 18, 19, 20, 21]. These questions are addressed by the most recent trials of the German Low Grade Lymphoma Study Group (Fig. 3)

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
Hiddemann et al. (eds)

Quantitative Assessment of the Impact of Interferon Alpha (IFN) vs. Busulfan vs. Hydroxyurea and of Risk Profile on Survival in Chronic Myelogenous Leukemia (CML)

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Abstract. All randomized studies have shown that IFN prolongs life as compared to conventional chemotherapies. The prolongation of survival varies between 11 and 21 months. As an example, the updated German CML Study I (IFN vs. hydroxyurea vs. busulfan, median observation time 70.4 months) is shown [significant survival advantage of IFN over busulfan ($p = 0.007$) and of hydroxyurea over busulfan ($p = 0.05$), but not of IFN over hydroxyurea ($p = 0.24$)]. The increment of survival by therapy in all studies is small. Overall survival differs between studies and depends on patient selection, phase of CML in which IFN therapy is started, strategy of IFN therapy (monotherapy or IFN combinations) and intensity of treatment with hydroxyurea. If studies are ranked according to risk profile, those with a higher proportion of good risk patients observe longer overall survival. If stratified for risk profile, survival differences, in analyzed instances, disappeared. Additional information gained from a comparison of the protocols of the German and Italian randomized studies shows that IFN is most effective in patient populations that

1. include early asymptomatic cases,
2. exclude cases with more than 10% circulating blasts or extramedullary manifestations and,
3. exclude patients older than 70 years.

The emerging evidence is that IFN is superior in early phase CML and less effective in later chronic phase whereas hydroxyurea is more effective in later chronic phase. The impact of risk profile on survival overrides that of drug therapy by a factor of about two. Depending on the composition of the patient population the life prolonging effect of IFN is therefore more or less visible or obscured. Optimal management of CML has to consider that

1. IFN prolongs survival in the majority of CML patients,
2. IFN and hydroxyurea are both superior to busulfan,
3. hematologic and cytogenetic remissions both are important time dependent predictors of survival,
4. an early start of IFN therapy is advantageous, and
5. efficient reduction of tumor burden correlates with survival.

This indicates that an intensification of treatment, e.g. as with idarubicin and ara C, or with high dose chemotherapy followed by autografting might achieve further prolongation of survival as studied by the ongoing German CML Study III.

Introduction

All randomized studies have shown that IFN alpha prolongs life as compared to conventional chemotherapies. The prolongation of

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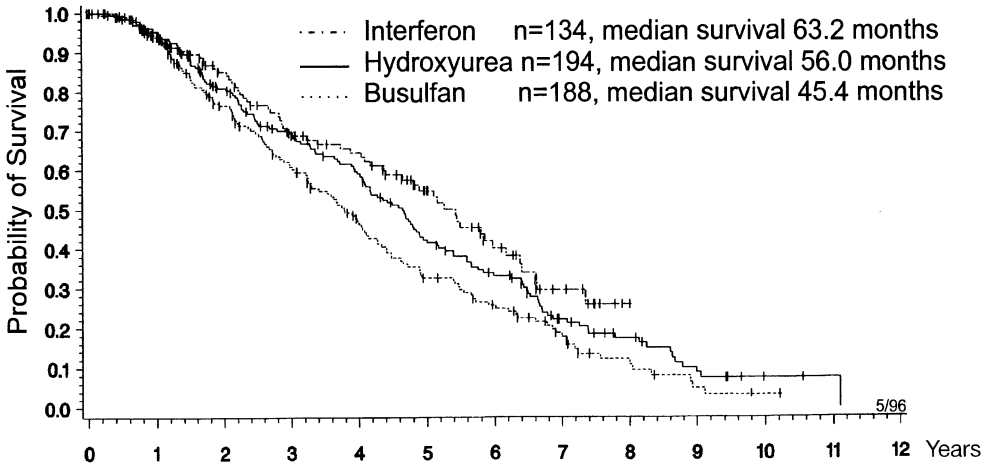

Fig. 1. Update of German CML-study I comparing interferon α, hydroxyurea and busulfan as of 5/96

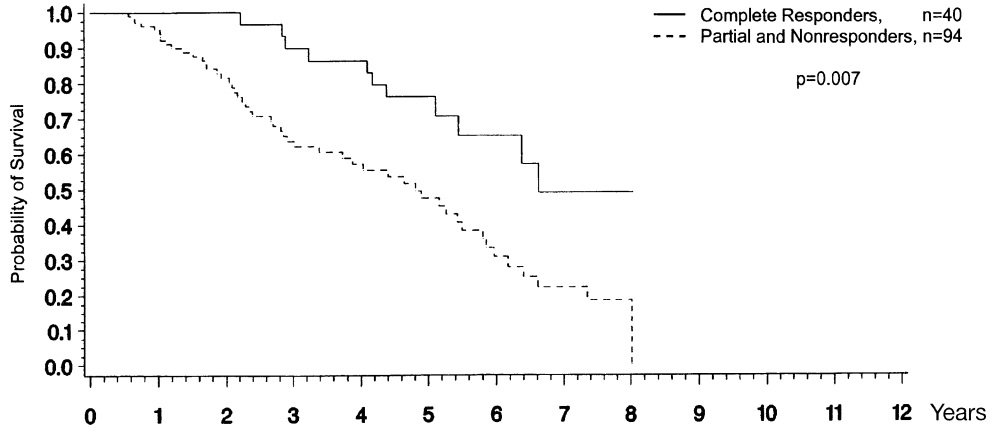

Fig. 2. Update of German CML-study I analyzing complete hematologic IFN responders vs. partial and non-responders as of 5/96. Time to response is corrected for according to the method of Simon and Makuch [11]

survival varies between 11 and 21 months [1-4]. As an example of an outcome of these studies, the updated survival curves of the German CML study I are shown. The increment of survival by therapy is small, but significant if IFN, or hydroxyurea, is compared with busulfan (Fig. 1). Complete hematological IFN-responders have a significant survival advantage over partial or non-responders (Fig. 2). This observation was also made by others [2,3] indicating that hematological response is a good, probably the best, time dependent prognostic factor. Also cytogenetic IFN-responders have a survival advantage over non-responders (Fig. 3). The difference is not yet significant in the German study but a trend is recognized in agreement with observations by others [2, 3]. Cytogenetic response was preceded by complete hematological remission in all cases. In agreement with earlier observations [5], these findings indicate that effective reduction to tumor load correlates with better disease control and longer survival.

Overall survival differs between studies and apparently depends on patients selection, phase of CML at which IFN therapy is started, strategy of IFN therapy (monother-

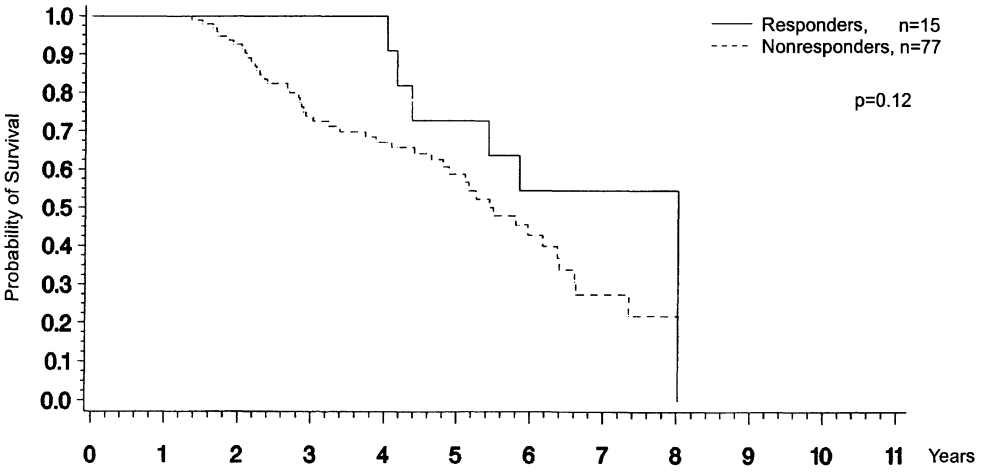

Fig. 3. Update of German CML-study I analyzing cytogenetic IFN-responders vs. non-responders as of 5/96. Time to response is corrected for according to Simon and Makuch [11]

Table 1. Risk profiles in CML patient populationsand correlation with survival.(Published studies on interferon-a-treated Philadelphia positive CML patients)

Study	Patients on IFN-α n	Median survival (months)	5-year-survival (%)	Risk profile (Sokal) Low n (%)	Intermediate n (%)	High n (%)	Risk index (n low risk / n high risk)
Mahon et al. 1996 [12]	81	n.a.	>77	39 (48%)	32 (40%)	10 (12%)	3.90
Kantarjian et al. 1995 [13]	274	89	63	124 (52%)	59 (25%)	54 (23%)	2.30
Italian group 1994 [2]	218	72	60	94 (43%)	72 (33%)	52 (24%)	1.81
Ohnishi et al. 1995 [4]	80	n.a.	54	29 (37%)	26 (33%)	23 (30%)	1.26
Hehlmann et al. 1994 [1]	133	66	59	36 (27%)	47 (35%)	50 (38%)	0.72
Shepherd et al. 1996 [14]	267	63	54	67 (25%)	89 (33%)	111 (42%)	0.60
Kloke et al. 1993 [8]	62	n.a.	n.a.	32 (52%)	18 (29%)	12 (19%)	2.67
Alimena et al. 1988 [6]	35	n.a.	n.a.	16 (46%)	12 (34%)	7 (20%)	2.29
Guilhot et al. 1991 [15]	24	n.a.	n.a.	8 (33%)	12 (50%)	4 (17%)	2.00
Thaler et al. 1991 [16]	44	n.a.	n.a.	19 (43%)	15 (34%)	10 (23%)	1.90
Benelux study 1996 [17]	97	n.a.	n.a.	29 (30%)	40 (41%)	28 (29%)	1.04

n.a. = not available

apy or IFN in combination) and the intensity of treatment in the hydroxyurea control group. If studies are ranked according to risk profile, studies with a higher proportion of good risk patients observe longer overall survival (Table 1). If stratified for risk profile, survival differences disappeared in analyzed instances. In a comparison of the protocols of the German and Italian randomized studies with exchange of data bases, different patient populations resulting from different entry criteria of protocols were identified as the major reason for differences in study outcome. If the patient populations of the Italian and German studies were adjusted according to the entry criteria of the protocols, survival differences in the IFN arm disappeared. Differences in therapy such as IFN dosage and intensity of HU treatment were of only minor relevance. The additional information obtained from the comparison of the Italian and German studies suggests that IFN is most effective in patient populations that include early asymptomatic cases, exclude patients with extramedullary manifestations or peripheral blast

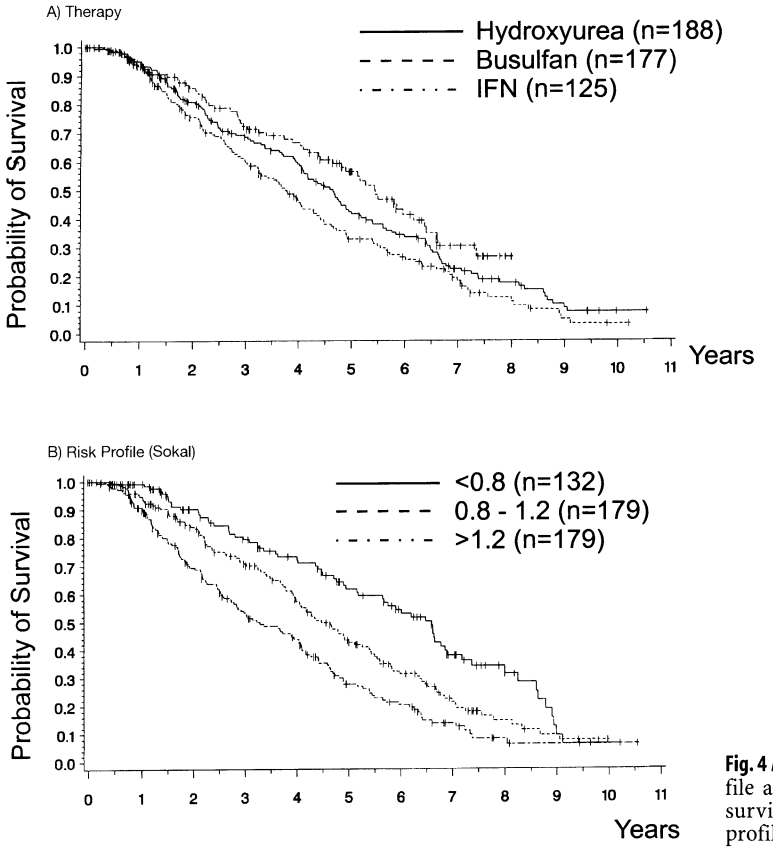

Fig. 4 A, B. Impact of risk profile and of drug therapy on survival. *A* Therapy, *B* Risk profile.

counts of more than 10%, and exclude patients older than 70 years. In agreement with earlier observations [6, 7] evolving evidence indicates that IFN is superior in early phase CML, whereas HU is more effective in later and accelerated phases.

Depending on the risk profile and on the composition of the patients population the life prolonging effect of IFN is more or less visible or obscured. Since the impact of risk profile on survival is frequently not, or not sufficiently, considered in reports on survival times in CML, it appeared of importance to quantify the relative impact of risk profile and of therapy on survival in CML.

Results

In order to quantify the relative impact of risk profile and of therapy on survival, the updated German CML Study I was used which compares IFN, hydroxyurea and busulfan in a randomized phase III trial. Median observation time was 70.4 months. For quantification of the impact of risk profile and therapy, the survival curves stratified for risk profile and for therapy were compared (Fig. 4). It is evident from the figures that the impact of risk profile at diagnosis is greater than that of therapy. The survival difference between high and low risk groups is with 40 months twice the maximum difference between therapy groups which is 20 months (Table 2). A similar result is found in all risk groups according to Sokal and in all therapy groups (Table 3). If the risk groups are stratified according to therapy and if the therapy groups are stratified according to risk profile, the maximal survival differences between risk groups are also about twice the maximal survival differences between therapy groups. This is illustrated in Fig. 5.

Table 2. Relative impact of risk profile and therapy on survival in CML

Risk group (Sokal)	Median survival (months)	Therapy	Median survival (months)
Low	79	IFN	65
Intermediate	54	HU	56
High	39	Bu	45
Max. survival difference	40		20

Table 3. Relative impact of risk profile and therapy on survival

	Median survival (months)			
Risk groups (Sokal)	Busulfan	Hydroxyurea	Interferon-α	Max. difference
Low	71	78	>96	>25
Intermediate	45	58	65	20
High	33	40	56	23
Difference low-high	38	38	>40	

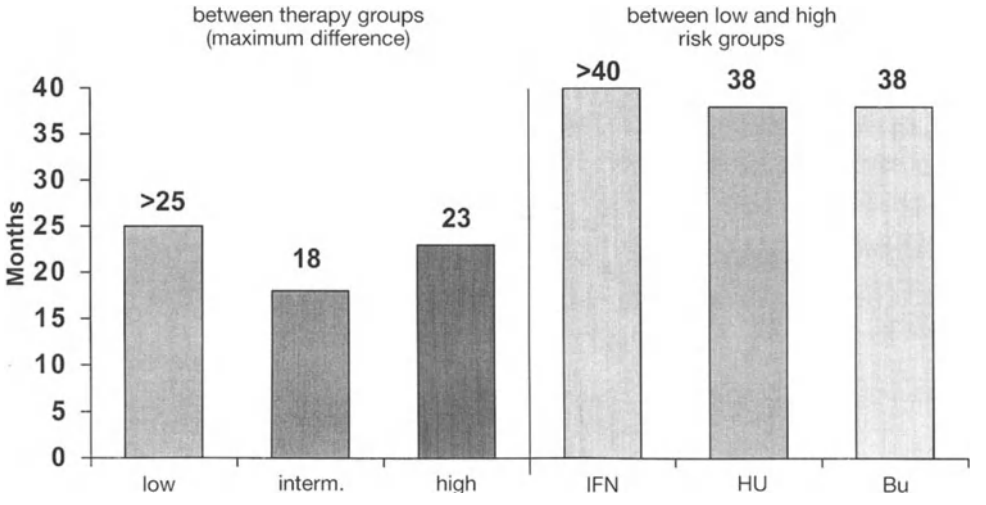

Fig. 5. Survival differences between risk and therapy groups according to Sokal's index

Several studies have reported that Sokal's index is less effective in IFN treated patients [1, 8, 9]. As an example the survival curves of the German IFN and chemotherapy treated patients classified according to Sokal's index are shown (Fig. 6). It is evident that the separation in the IFN group is not satisfactory. Therefore a new score that is more effective in IFN patients was also used. This new score uses age and spleen size which are used also by Sokal's index, and in addition sex, erythroblasts and circulating eosinophils [10]. The new score classifies IFN patients better than Sokal's index whereas chemotherapy patients are classified equally well (Fig. 7). Preliminary validation of the new score with an unpublished, ongoing study of our group also shows that the new score classifies IFN

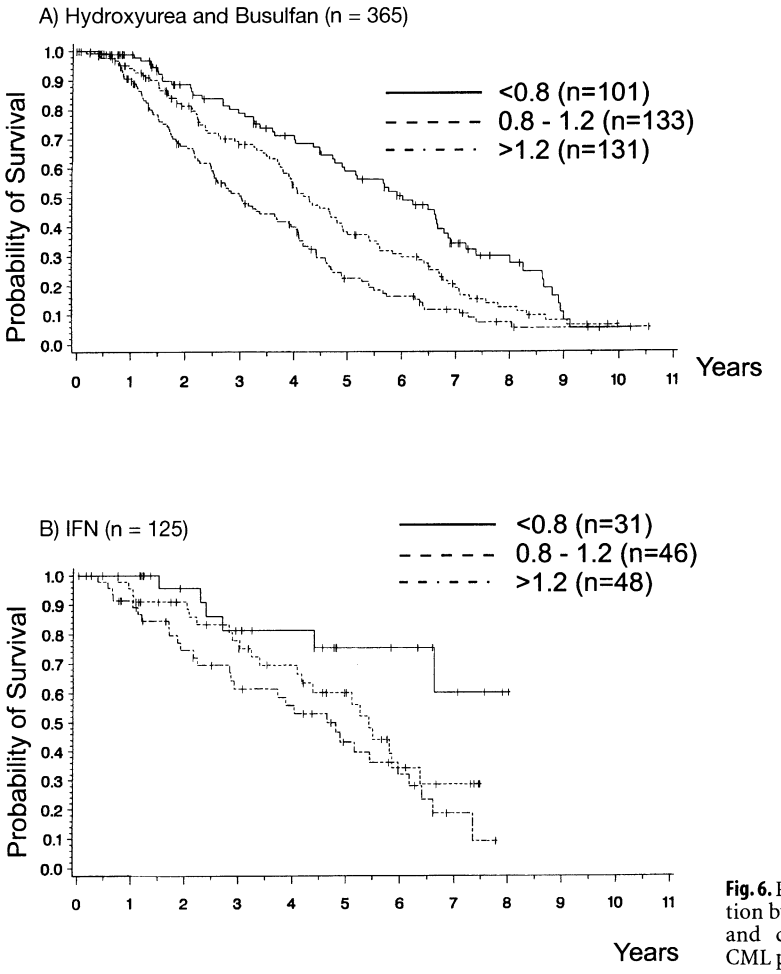

Fig. 6. Prognostic discrimination by Sokal's index of IFN- and chemotherapy treated CML patients

and chemotherapy-treated patients equally well. Application of the new score confirms that the impact of risk profile at diagnosis on survival overrides that of therapy by a factor of about 2. It is important to note that, in spite of the lower impact of therapy as compared to risk profile, survival is prolonged by therapy in each risk group as shown in Fig. 8.

Conclusions

We conclude that

1. Risk profile at diagnosis is still more important for survival than drug therapy in all risk groups.
2. Therapy prolongs survival in all risk groups.
3. IFN prolongs survival in the majority of patients with CML and is most effective early in the course of the disease.
4. IFN and HU are both superior to busulfan.
5. Hematologic and also cytogenetic remissions are both important time dependent predictors of survival under IFN-therapy.
6. IFN and HU appear most effective, if they aim at normal or low normal WBC counts.
7. Efficient reduction of tumor burden correlates with better disease control and with longer survival suggesting that an intensivation of therapy, e.g., by addition of intensive or high dose chemotherapy might further prolong survival in CML.

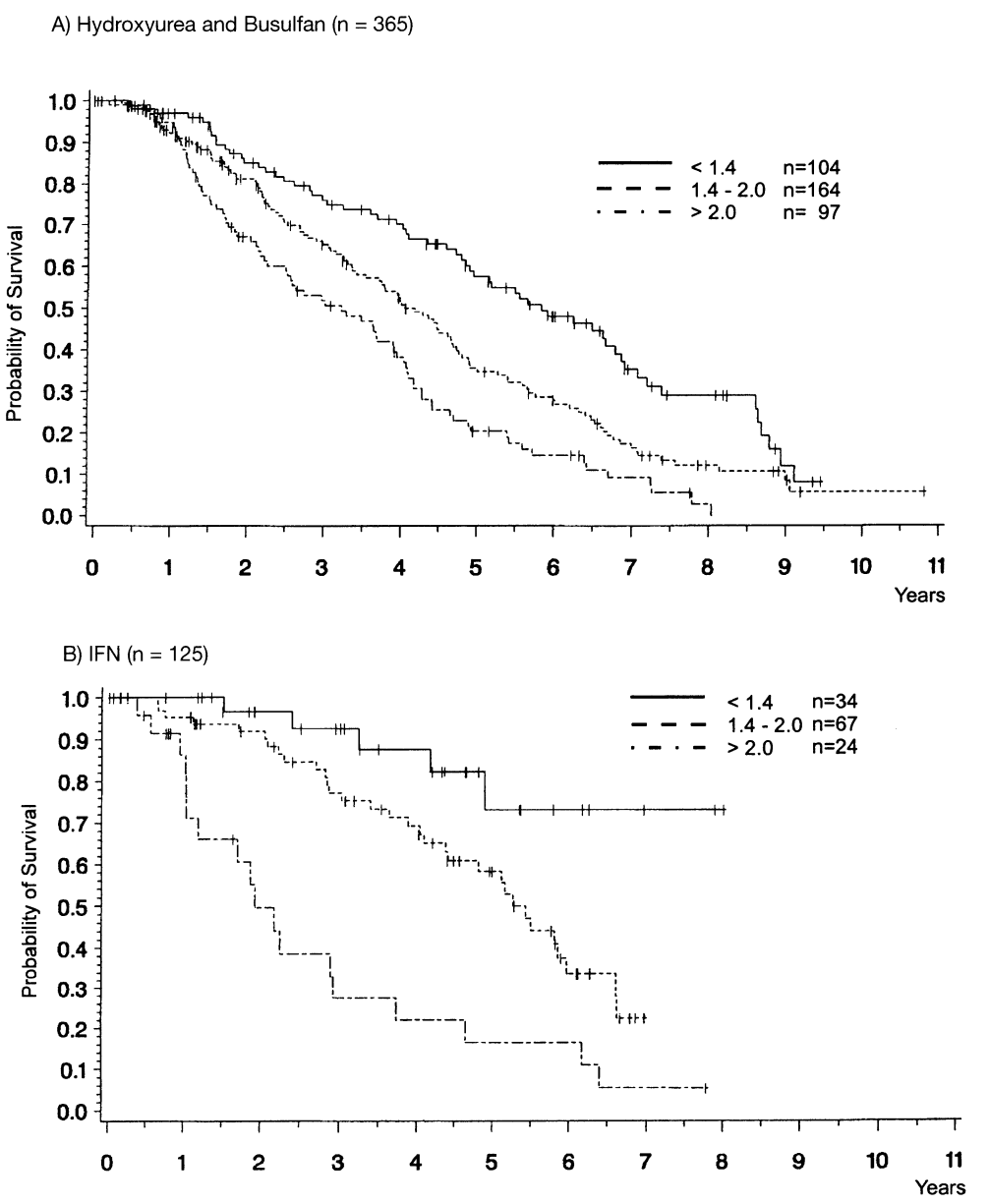

Fig. 7. Prognostic discrimination by the new score of IFN-and chemotherapy treated CML patients

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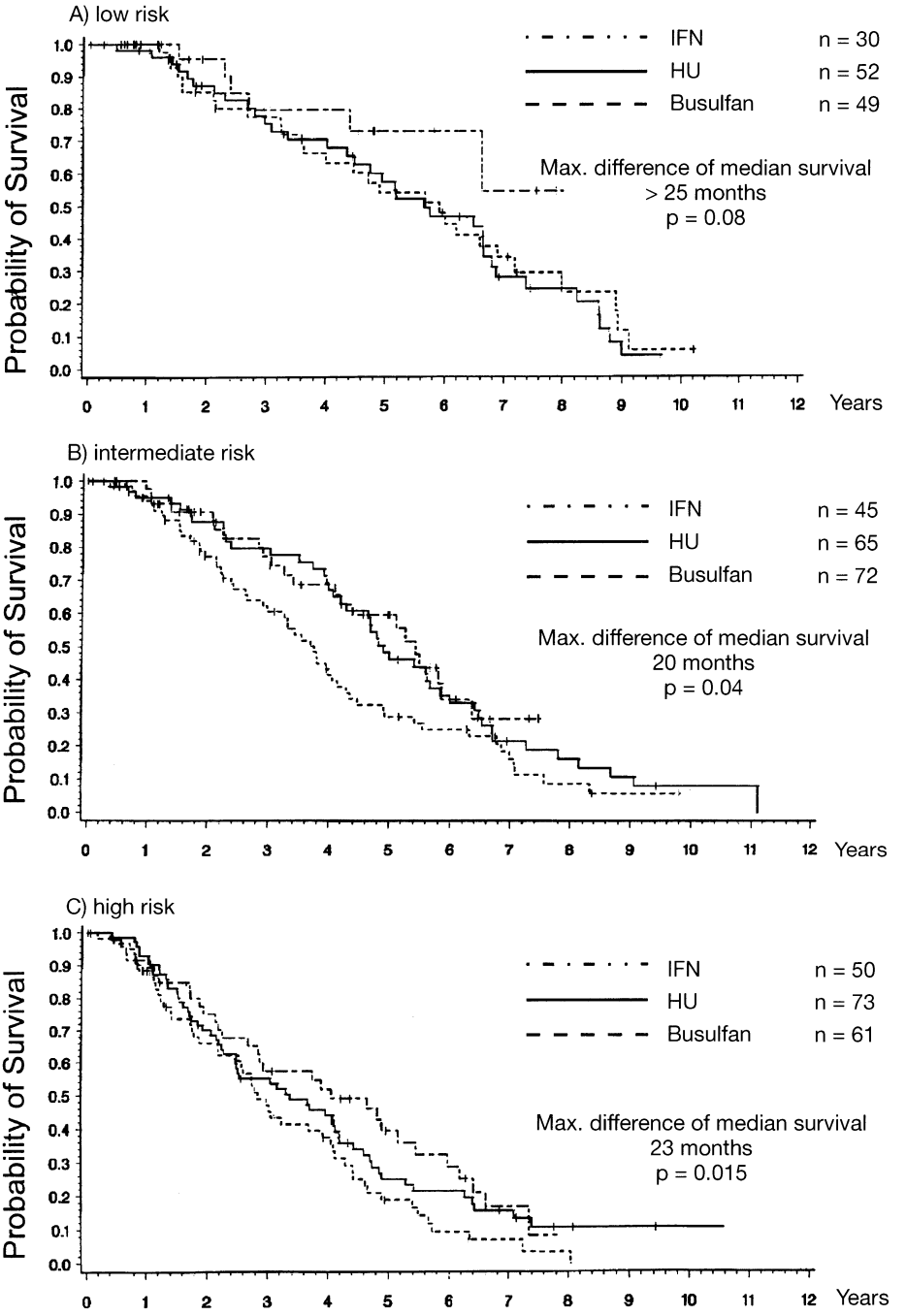

Fig. 8. Impact of drug treatment in CML according to Sokal's risk groups

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Interferon Alpha and Beta2 Microglobulin in Multiple Myeloma

G.W. Middleton and R. L. Powles

Introduction

Interferon has been used for many years in the treatment of multiple myeloma. This paper aims to provide an overview of clinical studies of Interferon (IFN) in multiple myeloma (MM), its biological activity in the disease and the impact of its use on known prognostic factors.

Clinical Studies of IFN Alpha in MM

In 1979 the first trial of IFN α as single agent in the treatment of MM was published [1]: all four patients responded. Response rates from subsequent trials enrolling more than 20 patients ranged from 13.5 to 50% [2-4] demonstrating that IFN α had definite single agent activity against MM, albeit less than that of conventional chemotherapy [5]. A recent meta-analysis of randomised studies of combined chemotherapy and IFN induction treatment has shown a statistical improvement in response rate, progression free and overall survival for the addition on IFN to chemotherapy [6].

Intuitively, it might be expected that biological response modifiers would exert their greatest effect where the tumour burden is relatively low – minimal residual disease and complete remission. Seven randomised studies of Interferon α as maintenance therapy following chemotherapy have been reported to date, 6 following conventional induction regimens [6, 11] and one following high dose Malphalan and autologous bone marrow rescue [12] and these results are summarised in Table 1. Two studies showed an overall survival advantage, whilst two showed neither survival nor remission prolongation benefits. The remainder showed improvements in progression free survival (PFS) but not overall survival (OS). We have previously reported the results of a randomisation of 84 patients to 3 mega units/m^2 Interferon α thrice weekly following high dose Melphalan and autologous bone marrow rescue and have shown a prolongation in the duration of remission for those randomised to IFN of 19 months when compared to that of controls who had a PFS of 27 months ($p = 0.03$). Initial analyses suggested a survival benefit but with longer follow up this has disappeared: patients who relapsed on the control arm have often subsequently received Interferon (17/31) whereas those relapsing on the active treatment have rarely received further IFN (4/24). This cross over may explain the lack of an overall survival benefit and the overall deterioration of the significance of the results in all aspects of this trial.

An important point was that the PFS advantage was confined to those achieving complete remission following high dose treatment: those achieving partial remission or no response, had no prolongation of re-

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Table 1. Summary of IFN maintenance studies

Study	Year	Number	Response criteria	Progression free survival	Overall Survival	Reference
Mandelli	1990	101	OR=>50% reduction	26/12 vs. 14/12 $p = 0.0002$	52/12 vs. 39/12 $p < 0.05$	[7]
Westin	1995	125	RMH	60/52 vs. 25/52 $p = 0.0001$ CR 88/52 PR 66/52	35/12 vs. 36/12 ns CR 43/12 PR 22/12	[8]
Ludwig	1996	100	SWOG	18/12 vs. 8/12 $p < 0.01$	51/12 vs. 34/12 $p < 0.05$	[6]
Browman	1995	176	>50% red.	17/12 vs. 12/12 $p = 0.003$	44/12 vs. 33/12 $p = 0.049$	[9]
SWOG	1994	193	SWOG	12/12 vs. 11/12 ns	32/12 vs. 38/12 ns	[10]
Peest	1995	117	>25% red.	13/12 vs. 13/12 ns	41/12 vs. 46/12 ns	[11]
Powles	1993	84	RMH	46/12 vs. 27/12 $p = 0.003$	Not reached	[12]

mission with maintenance Interferon. We define complete remission patients as those with no measurable para protein or Bence-Jones protein and with less than 5% marrow plasma cell involvement in the bone marrow and thus such patients have very low disease burdens. A proportionately greater benefit for those with relatively low disease burdens has been confirmed in other studies. Westin et al. using the same response criteria, showed that the magnitude of prolongation of plateau phase was greater for those achieving complete remission as opposed to those in partial remission [8]. Although they showed no overall survival advantage there was a clear trend towards a difference in survival in those with complete remissions when compared with those achieving partial response (43/12 vs. 22/12 respectively).

In the first published randomised study of maintenance Interferon (IFN) Mandelli et al. showed that the benefit of IFN in prolonging both PFS and OS was seen only in patients responding to induction therapy [7].

A recent report from the Nordic myeloma study group randomising patients to IFN or no IFN at the start of induction therapy with Melphalan and Prednisolone demonstrated an improvement in maintenance of response and prolongation of plateau phase duration only in patients achieving at least a partial response with no benefit in the minor responders [13]. Thus there is persuasive evidence that IFN α is of valve for those patients achieving significant reductions in myeloma cell mass and consequently low residual tumour burdens. Two studies however have failed to show any benefit from maintenance Interferon. In the trial of Peest et al. [11] only 31% of 52 patients treated with Interferon had a reduction of para protein by 75% and the negative results here may well have been due to the inclusion of a significant number of patients with relatively large residual tumour bulks. The negative results from a South Western Oncology Group Study [10] are more difficult to explain as the SWOG define responders as those with a greater than or equal to 75% reduction in tumour mass on two readings, one month apart. Although this would include patients achieving only a short lived partial remission as defined by the Royal Marsden Hospital criteria, other studies have shown that patients achieving partial remission do obtain benefit. There was no separate analysis of PFS or OS according to degree of response. Finally differences in dosing are unlikely to be of major importance as the two studies showing an overall survival advantage used relatively low doses of 3 Mμ and 2 Mμ/m^2 thrice weekly.

Due to the positive results of our randomised study we now routinely offer Interferon maintenance following induction and consolidation chemotherapy. An analyses of 195 newly diagnosed patients being treated at the Royal Marsden Hospital has shown that 141 patients received some form of high dose consolidation; 112 received high dose Melphalan with 90 of these receiving autologous bone marrow and 22 receiving peripheral blood stem cell rescue; 57/141 high dose patients received maintenance Interferon initially as part of the randomised study. Although not a randomised comparison, it is interesting to see that of those patients receiving Interferon following high dose treatment had significant improvements in progression free survival over those having no maintenance therapy (median PFS 44/12 as against 21/12). There was also a trend for improved survival which did not reach significance. At present we routinely use peripheral blood stem cell rescue following first high dose consolidation. A comparison of 37 patients receiving IFN following autologous bone marrow transplant with 39 patients undergoing peripheral blood stem cell transplant, demonstrated no difference in tolerability of Interferon with respect to dose modification, breaks in treatment or discontinuations with very similar toxicity profiles and almost identical median times to commencement of maintenance therapy following the high dose procedure [14].

The Biological Effects of IFN Alpha in MM

IFN α has diverse biological activities. Its effects on immune effector cells may underlie some of its anti-myeloma activity. It increases expression of class I MHC and this may be relevant to T cell mediated immune surveillance. IFN increases NK cell activity and causes differentiation of pre NK cells to mature functional cells [15]. Such immunomodulatory activities are crucially dependent upon the presence of accessory cells in the micro environment.

Einhorn et al. showed that IFN α had direct cytotoxicity against MM cells independent of these accessory cells and also that this was independent of simple inhibition of cellular multiplication as measured by tritiated thymidine incorporation (their cell populations had little proliferative activity) [16]. Although the molecular basis of this cytotoxicity is still not fully understood, there are two potential mechanisms.

The pathogenetic role of IL-6 in MM has long been an area of controversy. However, Hata et al. have demonstrated IL-6 production by less mature MM cells by examination of cell populations and individual cells [17]. Terminally differentiated cells lose IL-6 responsiveness via a loss of the IL-6 receptor and also no longer express IL-6. IL-6 lack leads to apoptosis of even cell lines independent of exogenous IL-6 [18]. Other cells in the marrow micro-environment, particularly monocytes and osteoblasts, produce IL-6 which may stimulate MM cell growth via a paracrine mechanism.

Epstein has proposed a model whereby immature proliferating MM cells respond to IL-6 via an autocrine loop and upon differentiation respond further to paracrine IL-6 as MM cell IL-6 expression is lost [19]. Finally fully mature MM cells may become independent of IL-6 upon loss of the IL-6 receptor and as suggested by in vitro experiments [20]. Anthes et al using the U266 cell line which is maintained in part by an autocrine IL 6 loop, showed that exposure to IFN α reduced tritiated thymidine incorporation whilst actually increasing IL-6 production [21]. There was a dose dependent reduction in IL-6 binding sites but maintenance of binding site affinity. Although effects on receptor shedding and internalisation were not investigated, reduction in IL-6 receptor number was suggested by a lack of competitive binding of IFN for the IL-6 receptor. This may be at the level of IL-6 receptor messenger mRNA translation: if anything there was a slight increase in IL-6 receptor message. An effect on E1F-2A transcription may play a part.

Although structural abnormalities of the c-myc are rare in MM [22], the gene product is expressed at high levels even in terminally differentiated plasmacytoid cells with a low mitotic rate (reviewed in [19]). Most transcription takes place off of the P1 or P2 promoter with only 5% arising from the 5′ P0 promoter. However in 70% of MM cells from

patients, transcription is initiated from P_0 and P_0 usage can be driven by a combination of IL-2 and Il-6. There are two extra open reading frames in P_0 transcripts and these may be of relevance in pathogenesis. Kimci et al. have demonstrated that Interferon causes down regulation of c-myc and such cells at rest in G_0 G_1. [23]. This is precisely the state of MM cells during plateau phase and may underlie the effect of Interferon in maintaining remissions [24]. c-myc is also crucially involved in apoptosis. In Burkitt lymphoma cells IFN inhibits ionomycin induced apoptosis by causing a marked decrease in c myc message [25]. Sangfelt et al. have shown that the MM cells of 8/9 responders to IFN α either had no or weak Bcl-2 immunoreactivity and that IFN responsiveness was correlated with low Bcl-2 expression [26]. Bcl-2 expression independent of major structural rearrangements at the gene locus, is seen in 75% of MM patients and may counteract any c-myc induced pro-apoptotic drift.

The Effect of IFN on β2m Estimations

Since the initial report of Norfolk et al. in 1979 [27] there have been numerous studies investigating the usefulness of β2m estimations in the management of patients with MM. Its use as a prognostic factor at presentation is well established. Bataille demonstrated a tight association between high presenting values and early death and also significant survival differences between both patients with normal renal function and those patients with the same Salmon and Durie stage when stratified for initial β2m [28]. Although some studies are negative [29] a number of recent analyses show that Beta 2m has the greatest prognostic power amongst several variables in multi variate analysis [30-32].

Beta 2m also correlates with initial Salmon Durie stage although it is difficult to predict stage from Beta 2m in any individual patient [29].

The value of serial Beta 2m estimations has been less fully evaluated. Garewal et al. suggested a tight correlation between serial Beta 2m and M protein changes. In two patients Beta 2m predicted for early rapid progression more accurately than the M-protein [33]. Patients with serial plateau phase estimations below 5.7 mg/l had a favourable outcome compared to early relapse in those failing to achieve stable plateau phase values of less than 5.7 mg/l. Furthermore, high β2m at relapse is strongly associated with a fulminant course. Batailles' study also demonstrated the association between values at relapse and subsequent clinical course and the enhanced accuracy over M-protein of detecting fast progressive disease early on in the course of treatment. There was a clear statistical difference in survival for 36 patients in plateau phase when grouped according to maximum β2 response (<2, >2 <3, >3). This study strongly suggested that the prognostic usefulness of beta 2m was not only to limited to presentation but extended to plateau phase and relapse. This conclusion was challenged by Boccadero et al. in an analyses of 21 patients [34]. In 11 there was a tight correlation between β2m and M-protein during remission and at relapse. In 6 the β2m was not raised at diagnosis and in 2 of these it subsequently accurately predicted for relapse. Only 2 patients had elevated β2m throughout remission with no further increase upon relapse. However an analyses of 512 patients reaching plateau phase in the MRC 4th and 5th myelomatosis trials, clearly showed that β2m during plateau was a strong predictor of subsequent outcome although only for the subsequent 2 years [35].

In summary, β2m estimation provide powerful prognostic information at presentation and correlates with initial stage are accurate predictors of early progression and have continued prognostic power during plateau phase, and values at relapse accurately predict the subsequent clinical course.

As an immunomodulator, IFN has effect on cytokines and acute phase protein expression. Only one study to date has investigated the effects of IFN on β2m levels[36]. Eight patients were given IFN for 1-2 weeks prior to chemotherapy and serial assays were formed. All patients demonstrated a rise in Beta 2m ranging from 29% to 185% (mean 109%) above pre treatment values with a peak in the first week in 6 and a fall back towards pre treatment values. Mem-

brane β2m levels increase in mononuclear cells exposed to Interferon [37] and IFN a enhances its release from myeloma cells [38].

We wished to assess the effect of IFN on β2M levels in a large cohort of patients and selected 143 patients either in CR at high dose of subsequently entering CR following high dose consolidation. Thus all patients had undergone a high dose procedure removing any bias that the procedure per se may have had on β2M levels. As mentioned above, complete remission patients are defined as having no detectable M-protein and Bence-Jones protein and less than 5% plasma cells in the bone marrow and thus are an ideal cohort to follow the effects of IFN relatively independently of significant variation in tumour burden-all patients had effectively minimal residual disease. As the β2m provides prognostic information at various points during the course of treatment β2m values for those on and those not on IFN were compared at attainment of remission, after 3, 6, 12 and 24 months of stable complete remission and at subsequent relapse

There were very significant differences in β2m values at all of these time points with IFN increasing β2m over values in the non IFN treated group. All of these differences were highly statistically significant.

High β2m at relapse may be due to an increased number of fulminant relapses with subsequent early death. Therefore, we looked at deaths within 3 months following relapse: there was one within each group. As plateau β2m of greater than 3 mg/l predicts for poor outcome [29] (all plateau values on Interferon were greater than 3 mg/l and all not on Interferon were less than 3 mg/l) we performed a life table survival analysis of the two groups – those receiving IFN (58 patients) at or after CR and those not receiving Interferon (85 patients) – to ensure that the higher β2m values were not indicating a worse outcome. There was clear separation in the survival curves in favour of the IFN treated group. Too much emphasis should not be placed on these survival differences as this was not a prospective randomised comparison and no attempt was made to stratify the prognostic variables at presentation. However all patients were in complete remission and clearly differences in the serial β2m estimations between the two groups was not due to the Interferon group having a worse overall outcome.

Thus, IFN has a significant impact on β2m levels at all stages where β2m has prognostic value thus making its use in those on IFN problematic. Coupled with the fact that Interferon is more efficient at inhibiting M protein excretion than the proliferation of MM cells[39], it suggests that different prognostic factors need to be identified for the optimal assessment of MM patients taking Interferon.

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Acute Leukemias VII
Experimental Approaches and Novel Therapies
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Maintenance with Interferon Alpha in Myeloma

H. Ludwig, and E. Fritz

Abstract. Although IFN maintenance treatment is routinely used in many myeloma treatment centers, its clinical efficacy is still a matter of controversy. IFN, a biologic response modifier, inhibits myeloma cell growth and stimulates important functions of the immune system. Randomized clinical trials have yielded ambiguous results regarding improvements of remission duration and survival times. However, a meta-analysis of all available randomized studies which compared IFN maintenance treatment with untreated controls (involving 1213 myeloma patients) showed small but significant gains in both relapse-free and overall survival. Several studies report beneficial effects of IFN maintenance treatment after high-dose therapy with autologous bone marrow or stem cell transplantation. The most frequently observed adverse effects of IFN maintenance therapy are fever and flu-like symptoms, which mainly occur during the early treatment phase, and fatigue. When patients are given the opportunity to state their personal preferences, the majority of them will opt for IFN maintenance treatment if the expected median gains in relapse-free or overall survival are 6 months or more. Patients who agree to the treatment tend to be younger, to have had personal experience with IFN, and to be in more advanced myeloma stages. We recommend IFN maintenance treatment in myeloma patients with low tumor burden, such as patients in complete remission after high-dose treatment and bone marrow or peripheral stem cell transplantation.

Introduction

Interferon-α (IFN) is the therapeutic agent which has most recently been added to the therapeutic armament against myeloma. After successful induction treatment of myeloma patients, the kinetics of myeloma growth show a plateau phase which should be retained as long as possible. It is known that IFN is able to arrest to some degree tumor cells in the resting phase of the cell cycle [1]. In addition, non-cycling tumor cells are considered particularly responsive to the antiproliferative activity of IFN [2]. For these reasons, IFN is expected to prolong the maintenance phase of multiple myeloma.

Although many treatment centers use IFN already routinely during the maintenance phase of myeloma treatment, opinions about its benefits are still controversial. In this present situation, a thorough analysis of all available results of clinical trials on the use of IFN in maintenance treatment of myeloma patients is needed to reveal its clinical efficacy.

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Biology of IFN Effects in Myeloma Patients

Effects on the Proliferative Activity of Myloma Cells

With respect to the treatment of myeloma patients, several biologic activities of IFN are relevant. Among these, the direct, dose-dependent [3] growth inhibitory effect of IFN on myeloma cell lines [4] and on in vitro myeloma colony formation [5] seems to be particularly important. These effects have been observed both in IL-6-dependent and IL-6-independent myeloma cell lines [6]. In vitro studies have also shown a direct cytotoxic effect of IFN on myeloma cells [7].

The expression of the IL-6 receptor is down-regulated by IFN, and the receptor's sensitivity to its ligand is reduced [8]. In a few selected cell lines, however, low concentrations of IFN induced autocrine production of IL-6, thus enhancing the proliferation of the myeloma cell clone [9]. In other cell system, IFN has been reported to down-regulate the expression of the oncogenes c-myc and N-ras [10, 11]. This suppression could also be induced by IFN in the fraction of myeloma patients, in which these oncogenes are over-expressed.

Effects on the Immune System of Myeloma Patients

IFN is a potent stimulator of several immune functions. Its stimulation of the cytolytic activity of natural killer (NK)-cells [12] and of macrophages [13] may be particularly important in myeloma, since NK-cell function is markedly reduced in patients with high tumor loads [14]. In vitro studies indicate the presence of cytolytic T cells in myeloma patients which react to autologous myeloma cells and possess the potential to lyse them [15]. IFN may enhance their activity. In addition, IFN increases the expression of the major histocompatibility antigens [16] and of tumor-associated antigens [15] which may facilitate the recognition of tumor cells by the immune system. These IFN-induced stimulations of immune functions may provide better defences against relapse from the plateau phase during the maintenance phase of the disease.

Effects on Paraprotein Synthesis

After binding of IFN to its receptor, the intracellular signal transduction activates a rather complex transscriptional pathway [16] which results in modulated expression of several genes. These gene modulations either enhance or suppress the synthesis of several proteins. Thus, IFN down-regulates monoclonal protein production of cultured [17] as well as in vivo myeloma cells during long-term treatment [18]. Theoretically, this effect could delay the recognition of relapse, if remission maintenance was solely judged on the basis of a plateau phase of the M-component. Clincal and laboratory data, however, usually show concordance between the M-component level, clinical symptoms, and the number of myeloma cells in the bone marrow of the patient.

Clinical Studies on IFN Maintenance Treatment

Randomized Trials Comparing IFN Maintenance Treatment to "Wait and See"

IFN maintenance treatment was first investigated by Mandelli et al. in a randomized trial involving 101 patients who had achieved PR, CR or stable disease after either MP or VMCP/VBAP induction therapy [19]. Patients were randomized to the IFN arm or to a control arm without any maintenance treatment. The results of the trial, a significantly prolonged median remission duration in the IFN arm (26 vs. 14 months in controls) and improved median survival in patients maintained with IFN (52 vs. 39 months in controls), were encouraging.

A number of clinical trials randomizing IFN maintenance treatment and untreated controls have subsequently been performed [20-26]. Table 1 shows the main characteristics of these studies, which partly involved relatively large patient populations. Their results did not consistently confirm the positive outcome of the first, Italian trial. Although in all studies which contained information on remission duration [20-22, 24, 25], this period of relapse-free survival was always longer in the IFN arm than in pa-

Table 1. Characteristics of randomized trials on interferon maintenance treatment

First Author	No. of Patients IFN	Controls	Induction regimen	Response to induction	IFN dose x 10^6 ($MU/m^2/w$)
MRC (unpublished)	143	141	Mostly ABCM	Plateau phase	5.6
Salmon	97	96	VMCP/VBAP or VAD or VMCPP/VBAPP	CR	5.6
Browman	85	91	MP	CR, PR	6.0
Westin	61	64	MP	CR, PR	9.4
Peest	52	65	MP or VBAMDex	CR, PR, SD[a]	9.4
Mandelli	50	51	MP or VMCP/VBAP	CR, PR, SD	14.0
Ludwig	46	54	VMCP or IFN+VMCP	CR, PR, SD	3.8
Powles	42	42	High-dose M and ABMT	CR, PR, SD	9.0
McSweeney	15	18	DHBI	CR, PR, SD	5.6
Total	591	622			

[a] SD: stable disease.

tients without maintenance treatment (Fig. 1), the observed differences reached statistical significance in only a few studies [22, 24, 25], and were minimal in others [20, 23]. Substantial increases in survival times were also observed in some trials [22, 24, 25] but not in others. Details of these outcomes will be presented below.

Table 1 also shows that Mandelli et al., who achieved one of the best outcomes, used

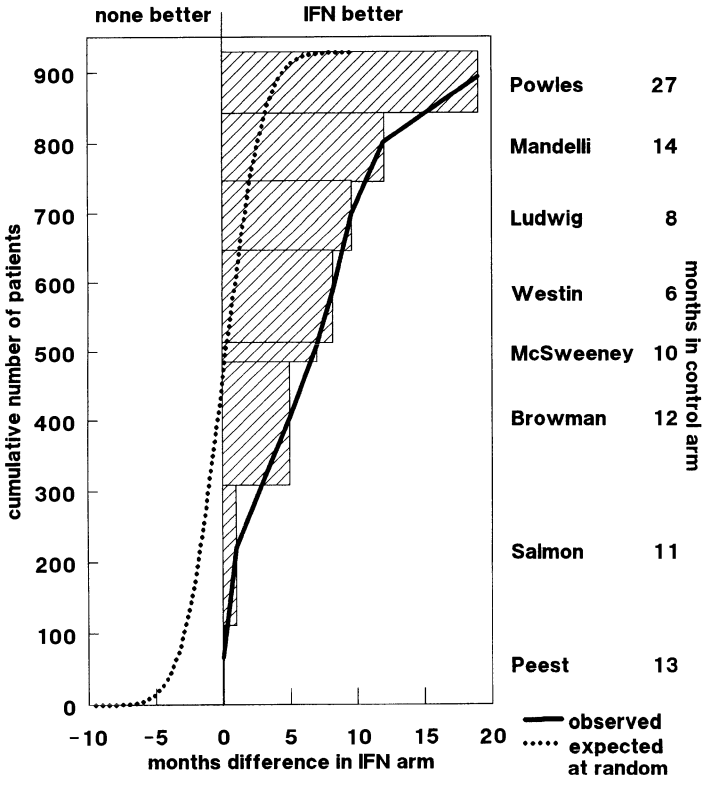

Fig. 1. Metaanalysis of randomized trials on IFN maintenance treatment vs. controls: relapse-free survival. *Lengths of shaded bars* represent the differences between the medians observed in the trial arms, heights the proportions of enrolled patients. The connecting solid line indicates significantly ($p < 0.01$) better results in the IFN arm than expected from random errors of measurement *(dotted line)*

the highest IFN dose. The second-highest dose, however, was applied by Peest et al., who reported negative results, and the lowest dose, employed by Ludwig et al., did achieve significant improvements. Systematic investigations of optimal IFN doses during maintenance treatment are urgently needed.

Another issue which should be systematically investigated, preferably within the framework of a metaanalysis, is whether there is a limit to IFN benefits during long-term treatment. Reports of patients successfully maintained in remission by IFN treatment for 1 or 2 years [24] are presently based on small patient samples only.

Studies on IFN Maintenance Treatment after High-Dose Therapy and BMT

Many clinical trials on high-dose treatment with autologous bone marrow or peripheral stem cell transplantation include IFN maintenance treatment in their regimens for all myeloma patients enrolled [27, 28]. Some of these studies investigated the efficacy of IFN treatment without randomization by univariate or multivariate regression analysis of prognostic factors. Such a retrospective analysis of predictive factors in a multicenter European study on 384 myeloma patients who had received autologous stem cell transplantation, found an association between posttransplant IFN treatment and improved survival in patients who had achieved CR or PR after transplantation [29].

Several studies have investigated the benefits of IFN maintenance treatment in prolonging survival. A randomized trial comparing IFN maintenance treatment with untreated controls was published by a British study group [25]. It found significant prolongations of survival in both complete and partial responders, but significant prolongation of remission duration only in patients who had achieved CR. It seems that IFN maintenance treatment is particularly beneficial in myeloma patients with low tumor burden. A French study group, however, which performed autologous stem cell transplantations in 133 myeloma patients [30], found no beneficial effect of IFN maintenance treatment with regard to survival.

A pilot study was undertaken in order to determine the feasibility and toxicity of IFN as maintenance therapy after allogeneic bone marrow transplantation for multiple myeloma [31]. The study incorporated planned dose escalation of IFN in successive patient cohorts from an initial dose of 1 MU three times weekly to a target dose of 3 MU three times weekly. It showed that the use of IFN in myeloma patients early after allogeneic BMT is associated with a significant risk of GVHD, which is dose-related, and that the maximum tolerated dose in the early post-transplant period is 1-2 MU three times weekly.

Combined IFN-Chemotherapy in Myeloma Maintenance Treatment

A prospective randomized multicenter trial was undertaken to evaluate the role of the combination of IFN-alpha with chemotherapy (alternating cycles of VAD, MP, and CP) in maintenance treatment of multiple myeloma [32]. Median survival as well as response duration of the two maintenance groups were similar (overall survival: 36 and 31 months in IFN and IFN-chemotherapy, respectively; response duration: 13 and 15 months, respectively), while toxicity was more pronounced in the combination maintenance arm.

IFN Combined with Corticosteroids

A recent randomized trial on 89 myeloma patients showed that the addition of prednisone (50 mg on alternate mornings) to IFN (3 MU TIW) may improve the outcome of maintenance treatment [33]. Median progression-free survival was significantly longer in patients undergoing combined IFN/P treatment than in those who received IFN alone (19 vs. 9 months). Overall survival was possibly also prolonged by the addition of prednisone to IFN (57 vs. 46 months).

Corticosteroids not only reduce the toxicity of IFN without ameliorating its treatment efficacy [34], but also participate in

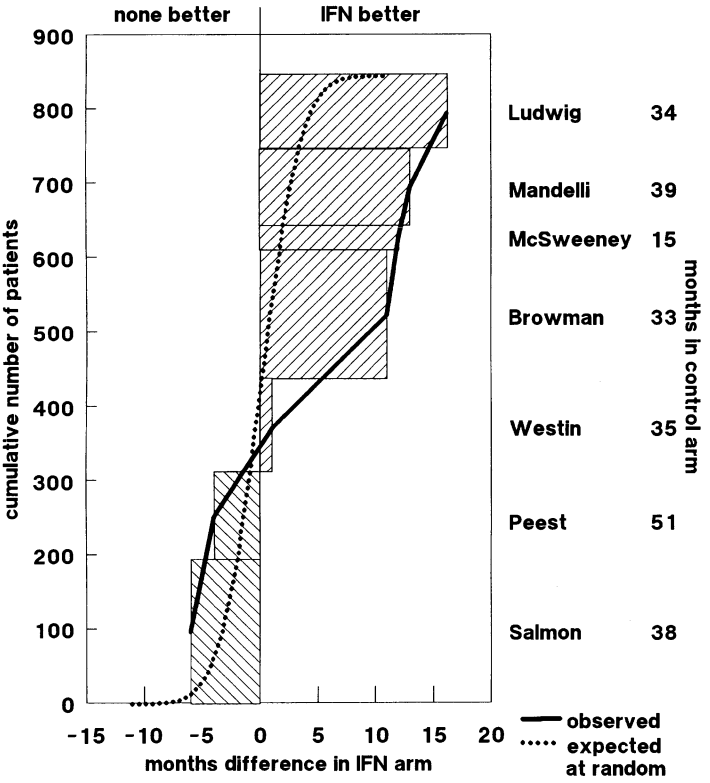

Fig 2. Metaanalysis of randomized trials on IFN maintenance treatment vs. controls: overall survival. *Shaded bars* represent the differences between the medians of the trial arms, the *dotted line* indicates the cumulative normal distribution which would have occurred if the differences had been mere random fluctuations. The observed differences *(solid line)* show significantly ($p < 0.02$) better results in the IFN arm.

suppressing the synthnesis of IL-6, a potent myeloma growth factor [35, 36]. A European multicenter randomized trial comparing IFN plus dexamethasone with IFN alone for maintenance treatment of myeloma patients is presently being conducted and should release first interim results in the near future.

Toxicity of IFN

IFN induces dose-dependent adverse effects which include, particularly during the early treatment phase, fever and flu-like symptoms. IFN-treated patients also suffer from fatigue, muscle pain, and, less frequently, from loss of appetite, nausea, vomiting, headaches, dizziness, and local erythema. If hemotoxicity occurs, it is usually mild. Long-term treatment may induce mild alopecia and neurological or psychiatric problems in a small fraction of cases. Table 2 lists estimated frequencies of IFN toxicities.

Long-term maintenance treatment with IFN is usually well tolerated by myeloma patients. However, this consistent report in the literature involves a bias. As only 60% of IFN-maintained myeloma patients remain on IFN after 24 months [37], patients suffering from adverse effects will have terminated the treatment by that time. Thus, a toxicity frequency of up to 40% has to be assumed for long-term IFN maintenance treatment, too.

Table 2. Estimated frequency of adverse effects during IFN treatment

Fever (2-3 weeks)	50-70%
Flu-like symptoms	20-30%
Fatigue	30-50%
Muscle pain	10-30%
Nausea	5-15%
Neurologic/psychological	5-15%

Metaanalysis of Randomized Trials Using IFN Maintenance Treatment

The controversy about the efficacy of IFN in maintenance treatment, particularly with

regard to survival benefits, can only be resolved by careful evaluation of all randomized trials, which compared patients receiving IFN maintenance treatment to untreated controls. Figures 1 and 2 reveal that the differences between the two subgroups were relatively small. In fact, unequivocal outcomes would require larger patient populations than investigated by any single study. Only metaanalysis of all data provides a sufficiently large number of patients.

We performed metaanalyses on all available randomized studies which met the following criteria:

1. application of IFN-α,
2. randomization to an IFN arm or a control arm without any maintenance treatment,
3. the previous induction treatment had to be well documented, and
4. information had to be available either on the median remission durations or the median survival times of both trial arms.

When 9 trials involving 1213 patients were analyzed, both remission duration and survival were significantly ($p < 0.01$ and $p < 0.02$, respectively) longer in the IFN arm than in the control arm (median gains 6 and 5 months, respectively) (Figs. 1, 2).

Patient Preferences with Respect to IFN Maintenance Treatment

Medical decision-making concerning the treatment of myeloma patients with IFN involves weighing the expected gains, namely, increased quantity of life and improved quality of life from prolonged relapse-free survival, against the risks of diminished quality of life from adverse treatment effects. In order to assess the patients' view on this topic, we interviewed 355 US myeloma patients by telephone asking their opinions about acceptance or rejection of a proposed treatment which matched the conditions of IFN therapy [38]. The interviewees were given information about frequencies and characteristics of IFN toxicity, financial cost, and the expected median gains in remission duration and survival as derived from the metaanalyses [24]. Then they were asked whether they would agree to the treatment if they could expect the following median gains:

1. 7 months in relapse-free survival and
2. 3 months in overall survival from IFN maintenance treatment (Table 3). Data on the patients' replies are shown in Table 4.

Table 3. Patient preference of interferon maintenance treatment

Imagine you are now in remission and your induction treatment has stopped today, would you agree to start this new treatment if the average remission duration was 7 months longer than without treatment?
yes ☐ possibly ☐ no ☐
☐ not at all
☐ if gain at least ..... months
Would you agree to this treatment if the average life expectancy was 3 months longer than without treatment?

Hypothetical IFN maintenance treatment was accepted by 58% when considering the 7-month prolongation of remission duration, while only 32% agreed to it when considering a median gain in survival time of only 3 months. Generally, the acceptance rate of the proposed treatment increased with increasing gains in treatment outcome, and rendered a break-even point between expected burdens and expected benefits at approximately 6 months' time gain (improved relapse-free or overall survival).

Patients who agreed to the proposed treatment tended to be younger (<60 years), to have had personal experience with IFN

Table 4. Patient preference of interferon maintenance treatment

Median gain: +7 months remission duration		
Choice:	Yes	57.7%
	Possibly	18.9%
	No	23.4%
	Not at all	75.9%
	If gain at least 21 (12-120) months	24.1%
Median gain: +3 months survival		
Choice:	Yes	32.1%
	Possibly	13.0%
	No	54.9%
	Not at all	47.2%
	If gain at least 12 (5-120) months	52.8%

Table 5. Influence of experience with interferon: refusal of unidentified maintenance treatment

	Previous or current IFN treatment Yes	No	*P*
7 months gain in remission duration	16.5%	27.5%	<0.02
3 months gain in median survival	47.4%	59.5%	<0.02

Table 6. Patients refuse proposed preatment significantly more often if:

• No prior experience with interferon	$p < 0.0001$
• >60 years of age	$p < 0.0001$
• feeling well	$p < 0.01$
• urban residence	$p < 0.05$
• education below college level	$p < 0.05$

(Table 5), and to suffer from more advanced myeloma stages as compared to patients who rejected the proposed treatment (Table 6). Knowledge of the 6-month risk/benefit trade-off preferred by the majority of the interviewed myeloma patients with regard to IFN treatment may facilitate decision-making in clinical oncology.

Conclusions

IFN maintenance treatment renders significant benefits to myeloma patients. However, the median magnitude of these gains, which may be achieved at the cost of reduced quality of life, is only marginal. In this difficult situation of medical decision-making regarding IFN treatment, the patient's personal opinion should be carefully considered. Myeloma patients do have individual preferences with respect to IFN maintenance treatment, and the majority of patients will communicate them, if asked. Some patients may need guidance and support to make their personal decision; a minority of patients will not be able to participate in medical decision-making. From the scientific point of view, we recommend maintenance IFN treatment of myeloma patients with low tumor burden, such as patients in complete remission following high-dose treatment and bone marrow or peripheral stem cell transplantation. Decision-making about IFN treatment would be greatly facilitated by knowledge of additional prognostic factors of IFN effectiveness, which should more precisely define subgroups of myeloma patients who benefit from IFN maintenance treatment.

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