Chronic and Acute Leukemias in Adults

Cancer Treatment and Research

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Cancer Treatment and Research

Foreword

Where do you begin to look for a recent, authoritative article on the diagnosis or management of a particular malignancy? The few general oncology textbooks are generally out of date. Single papers in specialized journals are informative but seldom comprehensive; these are more often preliminary reports on a very limited number of patients. Certain general journals frequently publish good indepth reviews of cancer topics, and published symposium lectures are often the best overviews available. Unfortunately, these reviews and supplements appear sporadically, and the reader can never be sure when a topic of special interest will be covered.

Cancer Treatment and Research is a series of authoritative volumes which aim to meet this need. It is an attempt to establish a critical mass of oncology literature covering virtually all oncology topics, revised frequently to keep the coverage up to date, easily available on a single library shelf or by a single personal subscription.

We have approached the problem in the following fashion. First, by dividing the oncology literature into specific subdivisions such as lung cancer, genitourinary cancer, pediatric oncology, etc. Second, by asking eminent authorities in each of these areas to edit a volume on the specific topic on an annual or biannual basis. Each topic and tumor type is covered in a volume appearing frequently and predictably, discussing current diagnosis, staging, markers, all forms of treatment modalities, basic biology, and more.

In Cancer Treatment and Research, we have an outstanding group of editors, each having made a major commitment to bring to this new series the very best literature in his or her field. Martinus Nijhoff Publishers has made an equally major commitment to the rapid publication of high quality books, and world-wide distribution.

Where can you go to find quickly a recent authoritative article on any major oncology problem? We hope that Cancer Treatment and Research provides an answer.

WILLIAM L. MCGUIRE
Series Editor

Preface

In adult leukemia rapid advances have been made in recent years. Major strides have occurred in our understanding of the etiology of human leukemia and the nature of the malignant cell. At the same time our ability to successfully treat, and not infrequently cure, patients with these diseases has greatly increased. With the recent rapid expansion of knowledge even the specialist has difficulty remaining current. The purpose of the 'Cancer Treatment and Research' series books on adult leukemia is to provide a regularly published single volume where important active areas of basic or clinical research on human leukemia are authoritatively and comprehensively summarized and interpreted, with an emphasis on the application of the new knowledge to control of the disease. It is hoped that these volumes will help bridge the gap between basic and clinical science and treatment of the patient.

The first volume in this series focussed on acute leukemia. In this second volume the scope has been broadened to include the chronic leukemias as well. The different topics, ranging from the viral induction of leukemia to its cure by intensive combination chemotherapy and bone marrow transplantation are lucidly discussed by internationally recognized experts in their respective fields. Most of the chapters consist of comprehensive state-of-the-art reviews and are accompanied by extensive bibliographies and detailed summary tabulations of data. Several articles include previously unpublished data. Brief outlines at the front of each chapter provide the reader with a rapid review of each chapter's contents and assist in locating specific information.

The scope of this volume is indicated by scanning the table of contents. The first four chapters consider various aspects of leukemogenesis and the nature of the malignant cell in different types of leukemia. In the first chapter, Gallo and his colleagues lucidly summarize current concepts regarding the role of retroviruses and oncogenes in the origin and pathogene-

sis of leukemia in man and describe in some detail their pioneering work on HTLV – the first virus to be clearly shown to cause a human leukemia. In the second chapter, Koeffler reviews our present understanding of differentiation and proliferation of myeloid leukemia which has resulted from his and others' abilities to establish human myeloid leukemia cell lines. In the third chapter, Catovsky and his colleagues provide a comprehensive and up-to-date review of the various B and T cell chronic lymphoproliferative disorders with an emphasis on their distinguishing immunologic markers and chromosome findings. In Chapter 4, Griffin provides one of the first detailed reviews of surface antigens in acute myeloblastic leukemia as defined by monoclonal antibodies.

Chapters 5 and 6 consider the clinical utility of two types of laboratory examinations for classifying and treating patients with acute myelogenous leukemia (AML). Brunning and his colleagues present new data from a very large series of patients on the relevance of the FAB morphologic and cytochemical classification of AML. Hutton summarizes and critically analyzes the current status and clinical use of *in vitro* chemosensitivity assays based on colony formation by tumor cells.

The last four chapters consider various aspects of therapy. In Chapter 7, Vogler critically evaluates various approaches to the major current-day problem in the treatment of AML – the prolongation of remission. In the following chapter Appelbaum and Thomas discuss the role of bone marrow transplantation in AML, acute lymphoblastic leukemia and chronic granulocytic leukemia based primarily on their excellent long-term results at the University of Washington which are well presented in a series of life tables. In Chapter 9, Cuttner and her colleagues consider the management of one of the unusual, and relatively infrequently discussed, problems in leukemia – hyperleukocytosis. Finally, in the last chapter, Hiemenz and Pizzo provide a remarkably comprehensive and clinically useful review of the many new developments in the management of infectious complications in patients with leukemia.

Overall, these 10 chapters provide a comprehensive picture of what we now know about a number of aspects of, and what we need to learn to better understand and treat, adult leukemia. It is hoped that this volume will be a valuable reference for all who study and treat leukemia and provocative and exciting reading for the generalist in medicine and science.

CLARA D. BLOOMFIELD

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1. Retroviruses and *Onc* Genes in Human Leukemias and Lymphomas

ERIC H. WESTIN, FLOSSIE WONG-STAAL, and ROBERT C. GALLO

1. INTRODUCTION

Retroviruses are of special importance in the understanding of the origin and pathogenesis of leukemias and lymphomas of man. Not only are they a major cause of naturally occurring leukemias and lymphomas in several animal species, thus providing useful model systems relating to the cause and development of neoplasias in man, but they also provide a means to identify and study some cellular genes (so-called *onc* genes) which are important in cell growth and differentiation and which have been transduced by the acutely transforming retroviruses. Many people believe that abnormal expression and/or qualitative modification of the c-*onc* genes are involved in the molecular pathogenesis of most or even all cancers no matter what the cause.

Our laboratory has been interested in retroviruses as they relate to human leukemias in both capacities, i.e., as etiological agents of some human leukemias-lymphomas and as a means of studying cellular genes (*onc* genes) that may be involved in human hematopoietic neoplasia. In the first part of this chapter we will describe studies on the structures and modes of expression of different human c-*onc* genes, and in particular, how structural and subsequently functional, alterations of these genes may play a role in certain human cancers. In addition, a brief review on *onc* genes as defined by their ability to transform mouse fibroblasts *in vitro* will be given. The second part of this chapter will present descriptions of the properties of a human retrovirus (HTLV), first isolated in our laboratory, and epidemiological and molecular studies that established HTLV as the etiological agent of the associated disease, adult T-cell leukemia-lymphoma (ATL). Emphasis will be placed on those studies that originated from our laboratory.

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2. *Onc* GENES IN LEUKEMOGENESIS

2.1. *Introduction*

Though the precise etiology of the majority of the leukemias remains unknown, recent advances in the molecular genetics of oncogenesis using a combination of retroviral derived transforming genes (*onc* genes) and transfection of total tumor cell DNA into NIH 3T3 cells provide the hope that at least a partial explanation may be forthcoming. Although not proven, it appears likely that mutation and altered expression of specific genes cause human leukemias. Several animal models of retrovirus induced neoplasia suggest these mechanisms are operative in several experimental systems [1]. In addition, the human counterparts of the *onc* genes implicated in some animal neoplasias have been found amplified or involved in specific translocations in some cases of human leukemias and lymphomas. A second group of transforming genes, many of which represent the cellular counterparts of retroviral derived *onc* genes, have also been recently isolated by their ability to transform cells after DNA mediated gene transfer [2]. Through examination of the effects of these various *onc* genes on different cellular systems and their role, if present, in the induction of ATL by HTLV, it would be hoped that at least a partial explanation of the molecular-genetic events involved in transformation of hematopoietic cells can be achieved.

2.2. *Retroviral Derived Cellular Onc Genes*

2.2.1. *Genetic Structure of Defective, Acutely Transforming Viruses.* Numerous retroviruses defective in replication and requiring a non-defective helper virus have been described. An important group of these defective retroviruses is the acutely transforming viruses which acquired specific *onc* genes required for transformation usually at the price of deletion of one or more viral structural genes. These have been isolated in association with non-defective helper viruses from a variety of sarcomas, carcinomas, leukemias and lymphomas in most major animal species except man.

2.2.2. *Cellular Origin of the Retroviral Transforming Genes.* The earliest characterization of an *onc* gene was that of Rous sarcoma virus (RSV) with its *src* gene. Of interest, this virus is helper independent and thus contains all necessary replicative genes in addition to its *onc* gene. Spontaneous, transformation defective mutants of RSV (td-RSV) later became available. By comparison to the wild type, these mutants were found to be identical to RSV except for deletion of the *src* gene. Transformation specific sequences were obtained by recycling RSV cDNA against td-RSV RNA to remove the sequences of the viral structural genes. Hybridization of these sequences to

normal chicken DNA showed that these sequences are homologous, though not necessarily identical, to a set of host cell DNA sequences [3]. A similar approach was taken to examine *onc* specific sequences in a variety of other acutely transforming defective retroviruses, and in each case a homologous cellular derived *onc* gene has been defined. These cellular *onc* genes have been found to be conserved among all vertebrates [4]. However, the role of these genes in normal cellular function and development remains to be elucidated. Considering the property of acute transformation potential conferred on the retroviruses that contain these genes, an important role in normal growth and development seems likely. The precise mechanism by which these cellular genes, when incorporated into a defective retrovirus, can lead to direct transformation has not been completely defined. However, some evidence indicates that viral induced transformation may be correlated with enhanced levels of expression of these genes [5–7]. However, other more subtle mechanisms for transformational activation of these genes, either by point mutation events or by deletion of or additions to the encoded proteins thus removing or blocking regulatory sites, could also be operative but remain to be proven.

2.2.3. *Cloning and Properties of the Human Homologues of Retroviral Onc Genes.* Human cellular homologues of several of the defective acutely transforming retroviruses including avian myeloblastosis virus (c-*myb*), avian myelocytomatosis virus (c-*myc*), S.T. feline sarcoma virus (c-*fes*), and simian sarcoma virus (c-*sis*) in addition to others have been cloned [8–11]. When these genes have been compared with their cellular counterparts several points are apparent. First, the cellular homologues are all much larger than the viral derived gene. This appears to be due to the presence of intervening sequences relative to the viral derived gene which may represent areas spliced out of the normal cellular RNA's. Many of these apparent intervening sequences also contain human repetitive sequences of the Alu I family. Second, in all cases, the mRNA species detected in the human cell is invariably larger than the amount of genetic material defined by the viral gene, indicating that in some cases, at least, the viral gene may represent only a portion of the total cellular gene.

2.2.4. *Studies of Expression of the Human Homologues of the Viral Onc Genes in Leukemias and Lymphomas.* To study expression of the cellular *onc* genes in a variety of fresh and cultured human leukemias and lymphomas, the various human *onc* clones in addition to other viral derived *onc* clones have been utilized in conjunction with Northern blotting techniques of poly A selected mRNA [12, 13]. These have included evaluation of the genes homologous to the *onc* genes of Abelson murine leukemia virus (*abl*),

Table 1. Expression of *onc* genes in human hematopoietic cells

Cell type	Source	mRNA species detected with					
		v-*abl* Kb 7.2, 6.4 3.8 & 2.0	v-*myc* Kb 2.7	v-*myb* Kb 4.5	v-Ha-*ras* Kb 6.5 5.8 & 1.5	v-*sis* Kb 4.3	v-*fes* unknown
Myeloid	KG-1	++	++	++	+	−	−
	HL60	++	++++	++	+	−	−
	HL60+DMSO, RA	++	±	−	+	−	−
	Fresh AML cells (4 patients)	++	++	++	+	−	−
Erythroid	K562	++	++	++	+	−	−
Lymphoid							
T-cells:	CEM	++	++	+++	+	−	−
	MOLT4	++	++	+++	+	−	−
	HUT78	++	++	−	+	−	−
	HUT102	++	++	−	+	+	−
B-cells:	Raji	++	++	−	+	−	−
	Daudi	++	++	−	+	−	−
	NC37	++	++	−	+	−	−
Normal peripheral lymphocytes		NT	++	−	NT	NT	NT
Normal peripheral lymphocytes+PHA		NT	++	−	NT	NT	NT

NT = not tested.

avian myelocytomatosis virus (*myc*), avian myeloblastosis virus (*myb*), Harvey murine sarcoma virus (Ha-*ras*), simian sarcoma virus (*sis*), and feline sarcoma virus (*fes*). The expression of these cellular *onc* genes is summarized in Table 1. Briefly, the c-*abl* gene is expressed as multiple transcripts with little apparent variation in the number of copies per cell of the amount of each message present. In contrast, the c-*fes* and c-*sis* genes do not appear to be expressed in any of the hematopoietic cells examined with the exception of c-*sis* (mRNA size 4.2 kb) in several, but not all, of the HTLV positive mature T-cell malignancies. It is of interest, however, that the c-*sis* gene is expressed with high incidence in a variety of glioblastoma and sarcoma cell lines [14] but not in other cultured normal fibroblasts or solid tumor types.

Unlike c-*abl,* c-*fes* and c-*sis,* the expression of the c-*myb* and c-*myc* genes follows a more complex pattern. The c-*myb* gene is expressed as a 4.5 kb mRNA species and is found primarily in immature myeloid, erythroid and

lymphoid cells with no detectable expression in more mature T-cell and B-cell lines. Unlike *myb,* the c·*myc* gene (also expressed as a single mRNA of 2.4 kb) is expressed in all hematopoietic cell types examined including normal peripheral blood lymphocytes either at rest or after PHA stimulation. The level of expression, however, is quite variable with the highest level of expression seen in the acute promyelocytic leukemia cell line HL60. With induction of differentiation in HL60 with either retinoic acid or DMSO the level of expression of c-*myc* is markedly reduced and the c-*myb* expression becomes virtually undetectable. Data of this nature would lend support to the view that expression of these cellular *onc* genes is required at particular points in hematopoietic cell differentiation and that proper restriction of the activity of these genes might be required to permit normal phases of differentiation to occur. Thus, genetic alterations that might lead to the abnormal control of these genes such as alteration in or changes of a particular gene's promotor as might occur during amplification or chromosomal translocation may play an important role in leukemogenesis and carcinogenesis. It should be noted that, though alterations in a promotor might lead to enhanced levels of expression, an enhanced level of expression might not be required for the transformation events to take place but rather simply an inability to turn on or off a gene at a particular point in time might be all that is required.

2.2.5. *Amplification as a Mechanism of Activation of Cellular Onc Genes.* To investigate the origin of the unusually high level of c-*myc* expression in HL60 (approximately 10–20 fold above average), a more detailed study of the structure of this gene in HL60 was undertaken. It was found that the enhanced expression could be correlated with a similar level of amplification of the c-*myc* gene [15]. The entire gene, including 5′ and 3′ flanking sequences was found to be amplified without evidence for gross structural alteration of the gene itself or the nearby flanking sequences. Moreover, the gene is amplified in the DNA of the original uncultured cells obtained from the patient. Gene amplifications can be found to occur either in the form of double minute chromosomes which are unstable and variable in amount from cell to cell in addition to a form that is stably integrated into a chromosome which results in a cytogenetic marker known as a homogeneous staining region (HSR). Either form can be found when drug resistance gene amplification such as that of dihydrofolate reductase is studied. In the case of HL60 the amplification appears to be present in the stably integrated HSR form. This type of alteration of expression of a cellular *onc* gene by amplification may represent one mechanism by which these genes as a group function in leukemogenesis. However, if this is the case, at present this would appear to be a rare event as further surveys of other leukemias

and lymphomas have not shown a significant incidence of this, at least in the case of c-*myc*.

The only other currently known case of *onc* gene amplification in hematopoietic cells is that of the *abl onc* gene in K562 [16]. With these few examples currently available in hematopoietic neoplasias, it is thus difficult to establish any link between *onc* gene amplification and leukemogenesis. Perhaps the amplifications in these instances occur *in vivo* during progression of the leukemic state rather than at the time of origin and that this event permitted these cells to be more easily established in culture which is uncommon for leukemic cells.

2.2.6. *Correlation of Onc Gene Localization with Chromosomal Translocations.* Recently, attempts have been made to correlate localization of human *onc* genes with specific chromosomal aberrations in hematopoietic neoplasms (see Yunis and Jerge [17] for an excellent recent review of this). Many of the cellular *onc* genes have been localized to specific chromosomal sites. C-*sis* has been mapped to the long arm of chromosome 22 and the c-*fes* gene to chromosome 15 band q25–26 [18, 19]. The c-*myb* gene has been localized to chromosome 6 subregion q22–24, the c-*myc* was localized on chromosome 8 (q24) [20] and c-*mos* to chromosome 8 q22 [21]. Finally, c-*abl* has been localized to chromosome 9 (q34) [22]. Many of these genes have therefore been located in chromosome regions frequently involved in specific deletions and translocations that have been associated with a variety of human leukemias and solid tumors. For example, the 6q- chromosomal marker (c-*myb*) with the break point localized to band q22 has been detected in several cases in lymphomas and leukemias. Perhaps the now classic example, however, is the involvement of the c-*myc* gene in the chromosomal abnormalities associated with Burkitt's lymphoma.

The *myc* gene has been localized to the break point of chromosome 8 in the specific translocation associated with Burkitt's lymphoma. These translocations invariably include the fragment from the long arm of chromosome 8 to recipients of most commonly chromosome 14 but also 2 and 22. The latter chromosomes have been shown to carry genes for immunoglobulin heavy chains (μ), K light chains and λ light chains respectively [23–26]. In several Burkitt lymphoma cell lines, the *myc* gene has been shown to be translocated to a region of chromosome that encoded the μ chain of the immunoglobulins [27]. This was done utilizing somatic cell hybrids carrying the normal chromosome 14 and others carring the Burkitt lymphoma chromosome marker 14q+ derived from the Daudi lymphoma cell line and showing that the normal chromosome 14 did not contain *myc* sequences whereas the 14q+ marker chromosome did. In some cases it was possible to show that the entire *myc* gene was translocated at a point immediately

adjacent to the immunoglobulin heavy chain locus [27, 28]. Although the breakpoint was variable, it was present in these cases either in the J region or in the μ/a switch region of the immunoglobulin heavy chain locus. Of interest, the orientation of the two joined genes was, unexpectedly, head to head. The genes, joined at their 5′ ends, if transcribed, would have to be read in opposite directions, away from one another. That this translocation can lead to greatly enhanced levels of expression of c-*myc* [29] is in some controversy [30]. This could indicate that the association of the *myc* gene with transformation in B-cells may be a result of improper control of *myc* expression during normal B-cell differentiation rather than greatly increased absolute levels of c-*myc* mRNA.

Of further interest is that the human c-*mos* gene was mapped to chromosome 8 position 8q22. Although the state of c-*mos* has not been evaluated as extensively as c-*myc*, murine c-*mos* was linked to an immune cell neoplasia in which c-*mos* was rearranged and thereby activated in a murine myeloma [31]. No association for c-*mos* has thus far been shown in human disease although other studies are needed.

With other *onc* genes, the data to date that they may be involved in specific translocations remains sketchy. The c-*fes* gene may be involved in the specific translocation t (15; 17) seen in acute promyelocytic leukemia. However, uncertainty remains because of incomplete data regarding the actual break point. Finally, the c-*cis* and c-*abl* genes appear to be involved in the t (9; 22) Philadelphia chromosomal abnormality seen in the majority of chronic myelogenous leukemia patients [32]. However, to date there has been no proven consistent elevation or alteration of expression of c-*abl* in CML and no documented expression of c-*sis* in these cells. Thus, if these translocations play a role in the development of these malignancies, the mechanism under which they operate remains undefined.

The association of specific *onc* genes with chromosomal rearrangements characteristic of various neoplasias provides a compelling argument for *onc* gene involvement in neoplasia. If these are indeed involved in neoplastic transformation it is likely that their role represents one of several steps required for cellular transformation.

2.3. *Potential Transformation Genes Detected by Transfection Techniques*

2.3.1. *Use of Transfection Techniques to Detect Potential Activated Transforming Genes.* The observation that tumor cells maintain a stably transformed phenotype in a variety of *in vitro* and *in vivo* systems has led to the hypothesis that basic genetic alterations are required to preserve this characteristic during tumor cell growth. This then led several investigators to undertake experiments that would assay for dominantly acting transforming genes that would be active in confering the transformed phenotype on cells

in culture by transfer of tumor cell DNA. The technique utilized in these experiments was that of DNA mediated gene transfer or transfection [33]. Experiments of this nature are dependent on sensitive indicator cells which can take up DNA at high efficiency and whose transformation by loss of contact inhibition can be readily detected. The cell line most widely utilized to the present time has been the immortalized through contact inhibited NIH Swiss mouse fibroblast line, NIH 3T3. Detailed analysis of the transfection process has shown that only a small fraction of the cultured cells exposed to the foreign DNA take it up [34]. However, cells that do incorporate DNA into their genome do so by splicing several fragments of DNA collinearly at the site of incorporation [35]. This observation can be utilized in some transfection experiments in which phenotypic transformation can not be monitored by cotransfection of the DNA of interest with specific cloned genes which carry drug resistant selectable markers [36]. The recipient cells which after DNA transfection will grow in the presence of the appropriate selecting drug will have incorporated foreign DNA and can be checked for the presence of specific sequences of interest.

Integration of large quantities of foreign DNA during the course of a transfection experiment means that isolation of tumor genes based on the altered phenotype of NIH 3T3 cells required multiple rounds of DNA isolation from transformants and retransfection to purify the transforming genes from the extraneous sequences incorporated next to these sequences during the first transfer. To detect the presence of human tumor DNA workers have taken advantage of the Alu family of repeats [37]. These represent 300 base pairs of repeated sequences present in approximately 300,000 copies distributed throughout the human genome. These sequences are not homologous by hybridization to their murine counterparts and so can be used as a hybridization probe to detect human sequences incorporated into the mouse genome. This human repeated sequence can also be utilized in subsequent experiments to molecularly clone the transforming genes of interest.

2.3.2. *Detection of Activated Ras Onc Gene Family Members by Use of the NIH 3T3 Transfection System.* One group of well characterized tumor genes belongs to the *ras* family of *onc* genes. The members of this family are characterized by their homology to the Harvey murine sarcoma virus and Kirsten murine sarcoma virus which have closely related v-*onc* genes [38–40]. The *ras onc* genes code for 21 Kd phosphoproteins [41]. The human genome contains four sets of sequences closely related to these genes, c-Ha-*ras*-1 and -2 [42] mapped to chromosomes 4 and X respectively [43] and c-Ki-*ras*-1 and -2 [42] (mapped to chromosomes 6 and 12 respectively [43]). A *ras* related sequence which has distant homology to the other c-*ras onc* genes is n-*ras* [44] which is located on chromosome 1 [45].

To date, using transfection techniques a variety of *ras onc* gene family members have been isolated from carcinomas and sarcomas, in addition to some leukemias and lymphomas. These have included Ha-*ras*-1 from the bladder carcinoma cell line EJ [46–49], and the Ha-*ras*-2 gene from several colon carcinomas and lung carcinoma cell lines [44, 50], n-*ras* has been isolated from the neuroblastoma cell line SK-N-SH [44, 45] in addition to fibrosarcoma and rhabdomyosarcoma cell lines [45] and from the promyelocytic cell line HL60 [44]. In addition, *n-ras* has been found activated in a Burkitt lymphoma (A. W. Ramos, R. Weinberg, personal communication) and fresh acute myelogenous leukemic cells (C. Moroni, personal communication).

Since all three genes are present as normal loci, it was inferred that the normal genes must be altered in some way to have attained transforming properties. The EJ bladder carcinoma gene could readily transform NIH 3T3 fibroblasts when transfected whereas the same Ha-*ras*-1 clone obtained from a normal cell genome could not. Subsequent detailed analysis has shown that this difference in transforming potential is due to a substitution of a single amino acid glycine (present at position 12 in the normal Ha-*ras*-1 protein product) with valine [51–53]. The contribution that this point mutation made toward the development of the original cancer from which the T24 cell line was derived could not be assessed because there was no normal tissue available from the patient. In the case of the distantly related n-*ras* gene from tumors and its normal counterpart, nucleic acid sequencing experiments have indicated that a single basepair mutation leading to substitution of glutamine by lysine was critical (M. Wigler, personal communication).

Despite this elegant work, it should be noted that many tumor cells and cell lines do not score at all in the NIH 3T3 assay system indicating that alterations in members of the *ras* gene family may not be all that common. Since the indicator NIH 3T3 fibroblasts were originally selected for susceptibility to transformation by murine viruses and since *ras* was originally identified as the transforming gene in this system, this assay system may be selecting for modified *ras* genes and missing other important genetic alterations.

2.3.3. *Detection of an Activated Gene Related to Transferrin in Avian Bursal Lymphomas and Human Burkitt's Lymphomas.* Recent data have indicated that transformation in human tumors may represent a multistep phenomena as opposed to a single step as was initially implied in the early NIH 3T3 transfection experiments. In the case of ALV induced B-cell lymphomas, the virus has been shown to integrate near the chicken c-*myc* oncogene and to cause activation of this gene with an increased level of

expression. DNA from these same ALV induced lymphomas can also be used in the NIH 3T3 transfection system to obtain transformed foci. However, when the transforming gene of interest was cloned, it was found to be not related to the c-*myc* gene or any other known *onc* gene including those of the *ras* family, and to not be associated with ALV integration [54]. This gene, designated Blym-1, is a small gene of approximately 1 Kb which by nucleic acid sequence analysis encodes a 65 amino acid protein. This protein shows significant homology to the amino-terminal region of the transferrin family of proteins and might represent a type of growth factor.

In Burkitt's lymphoma, the involvement of the c-*myc* gene in the 8; 14 translocation has been discussed previously. Using a variety of Burkitt's lymphoma cell line, Diamond *et al.* [55] have shown using the NIH 3T3 transfection system that an activated transforming gene can be transferred to the NIH 3T3 cells and that this gene, designated HuBlym-1, is the human homologue of the same gene detected in ALV induced bursal lymphomas of chickens. Again it was shown that this gene was related to transferrin and was not related to any known *onc* gene including those of the *ras* family. In addition, analysis of transformants of NIH 3T3 cells showed no detectable EBV sequences indicating that the effect of EBV on normal B lymphocytes can be separated from the activation of the Blym transforming gene.

2.3.4. *Multiple Genetic Alterations are Required to Transform Normal Diploid Cell Lines that are not Immortalized.* Work in the avian and human B cell lymphoma systems have underscored the necessity of considering multi-hit models for transformation of normal cells. Other data, including work with the Abelson leukemia virus have provided further indications for this type of model. The Abelson leukemia virus which has the v-*abl onc* gene, causes tumors rapidly *in vivo* and efficiently transforms cells in culture. *In vitro* AbMuLV infection of bone marrow cells rapidly stimulates blastogenesis but causes transformation only after several weeks in culture [56] suggesting that time is required for a second step in transformation. This is further suggested by the finding that some AbMuLV induced tumors lose the viral genome after prolonged *in vivo* passage [57] and, in fact, suggest that a sequential rather than simple two hit model might be operative. Furthermore, transfection of NIH 3T3 cells with DNA from tumor cell lines which contained integrated v-*abl* gave transformants, but *abl* sequences were not present in the transfected cells [58]. Similar experiments have been performed with human tumor cells which have an abnormal expression of a c-*onc* gene. DNA from the HL60 human promyelocytic leukemia cell line, which contains an amplified *myc* gene, was active in the NIH 3T3 system. However, this system detected another activated *onc* gene,

the n-*ras* gene [45]. A similar finding was noted in the human leukemia cell line, SMS-SB, which has enhanced c-*abl* expression but also possesses a transfectable transforming gene not related to c-*abl* [59].

Recently, more direct evidence has been provided to support the concept of involvement of multiple *onc* genes acting simultaneously in order for transformation of a normal cell to occur. Land *et al.* [60] have shown that transfection of embryo fibroblasts by a human *ras* oncogene does not convert them into tumor cells unless the fibroblasts are established and immortalized prior to transfection. However, these embryo fibroblasts can be made tumorigenic if a second oncogene such as a viral or cellular *myc* gene or the gene for the polyoma large T antigen is introduced with the *ras* gene. Ruley [61] has also shown that polyoma virus middle T and the T24 Harvey *ras*-1 genes are individually unable to transform primary baby rat kidney cells. However, the adenovirus early region 1A provides functions which permit transformation of these primary cell lines following DNA-mediated gene transfer. This type of result provides direct evidence for at least a two function process required for transformation but does not rule out the requirement of even further steps that might be needed *in vivo.*

2.3.5. *Future Directions in Use of Transfection Systems for Detection of Activated Transforming Genes.* The findings indicating a multi-step process in transformation underscores the limitation of use of a single system such as NIH 3T3 in scoring for potential activated *onc* genes. However, in the context of looking at a single step in a multi-step process, the NIH 3T3 system remains useful and provides a system in which at least one aspect of transformed cell behavior can be studied.

Future work, however, will have to address the question of precisely where in the transformation process the NIH 3T3 active genes play a role and whether this is related to a primary event or to tumor progression such as multiple relapses in the leukemias and lymphomas and metastasis in the solid tumors. In addition, other systems need to be developed that will 1) detect genes other than those of the *ras* family and 2) allow transfection type assays to be performed in alternative cell types such as total bone marrow or T or B cells. In the latter case, in order to evaluate more completely the multistage phenomena associated with transformation it may be necessary to 'preprogram' cells with one '*onc*' gene and then use this as a target for other transfection experiments. Only with these types of approaches can the many steps required in transformation be sorted out and evaluated individually.

3. RETROVIRUSES IN HUMAN LEUKEMIA-LYMPHOMA

3.1. *Background Considerations*

There is abundant evidence implicating retroviruses in the etiology of naturally occurring leukemias and lymphomas of many animals (see Gross' book [ref. 62] for a historical account and ref. 63 for more recent developments). Evidence for a human leukemia virus, on the other hand, was slow to develop. The earliest studies were generally confined to a search of leukemic cells for recognizable retroviruses by electron microscopy. These studies provided equivocal results, but clearly proved that substantial virus replication, as occurs in viral caused leukemias of mice and in most feline leukemias, did not occur in humans. Immunological and molecular 'probing' for retroviral information was limited by the lack of a prototype human retrovirus. Only if the human virus had substantial homology with the available animal viruses could a clearcut positive result emerge, and such has not been the case. The bovine leukemia system served as an important model for human leukemias. Although epidemiological studies had long indicated that leukemias/lymphomas of cattle might be due to a transmissible virus, viremia and visible virus particles were not detected in the tumor cells in most cattle. Uncultured fresh bovine leukemia cells do not even express viral antigens or mRNA [64]. It was not until the cells were transiently grown in liquid suspension culture that bovine leukemia virus (BLV) was identified and later isolated by transmission into cells of a permanently established line [65]. Furthermore, once isolated it became clear that BLV was a unique mammalian retrovirus [66] and did not have substantial homology with the then known animal retroviruses. Therefore, prior to the physical isolation of BLV, evidence for a virus or integrated viral DNA sequences could not have been detected using probes derived from previously isolated retroviruses. These observations underscore the importance of long-term *in vitro* culture of appropriate target cells for isolation of a human retrovirus so that reagents prepared against a prototype human retrovirus can then be used for subsequent detection of related human retroviruses.

3.2. *Growth of Mature Human T-Cells with T-Cell Growth Factor (TCGF) and the Isolation of the First Human Retroviruses (HTLV)*

The growth of mature human T-cells in long-term suspension culture was made possible by the discovery of a protein called T-cell growth factor (TCGF) [67]. Using highly purified TCGF, T-cells from normal individuals can be grown only after lectin or antigen stimulation for induction of TCGF receptors [68]. In contrast, some neoplastic mature T-cells (later learned to be those infected by human retrovirus) respond to TCGF directly, appar-

ently due to constitutive expression of the receptors for TCGF [69], and they become established T-cell lines [68]. It was from some of these cultured lines that the human type-C retrovirus known as human T-cell leukemia/lymphoma virus or HTLV was first isolated [70, 71]. Morphologically, HTLV is a typical type-C retrovirus as seen by electron microscopy. Like other retroviruses, HTLV contains a reverse transcriptase and a high molecular weight RNA genome of approximately 9 Kb or 70 S. Three viral core proteins, also referred to as *gag* proteins, have been identified and consist of: p24, the major core protein; p19, the amino terminal *gag* protein; and p15, a nucleic acid binding protein located at the carboxy terminus of the *gag* gene. These correspond to the p30, p15 and p10 *gag* proteins of the murine retroviruses. HTLV is unrelated to known animal retroviruses by standard nucleic acid hybridization [72] and immunologic cross reactivity studies [73]. However, the amino acid sequence of HTLV p24 shows distant but significant homology with BLV p24 suggesting a common ancestral origin of BLV and HTLV [74].

HTLV has been clearly shown to be an exogenous human virus since HTLV related sequences are not present in the DNA of normal uninfected human cells, but are readily detected in DNA or RNA from HTLV positive tumor cells. In addition, HTLV sequences were not found in the normal Epstein-Barr virus infected B cells of the HTLV positive patient CR [75]. Only his neoplastic T cells contained HTLV specific sequences.

Since the first isolations of HTLV reported in 1980, many additional isolates have been obtained from cell lines established from patients with mature T cell malignancies and some normal family members from different parts of the world.

In addition to new isolates obtained in Gallo's laboratory [76, 77], investigators in several laboratories in Japan have shown the presence of retrovirus particles by electron microscopy in cultured T-lymphocytes of ATL patients [78]. In some cases, a virus was isolated from the cell lines. Furthermore, investigators in London [79], Amsterdam [80] and at Duke University in the U.S. [81] have also obtained HTLV isolates. Comparison of the restriction enzyme sites of different HTLV proviruses as present in leukemic patients from the U.S., Japan, the Caribbean and elsewhere showed that the initial virus isolates in the U.S. serve as the prototype of a well conserved virus subgroup (HTLV-I) which consists of the majority of HTLV isolates [82]. However, a virus obtained from a leukemic cell line of a patient with a T cell variant of Hairy cell leukemia, is only distantly related to HTLV-I isolates even though it also infects mature T cells and its core antigens cross react with HTLV-I proteins [77, 83]. Therefore, this virus forms a distinct subgroup (HTLV-II). In time, other members of subgroup II or more subgroups of HTLV may be identified.

3.3. *Seroepidemiology of HTLV*

3.3.1. *Characteristics of HTLV Associated Diseases.* Natural virus infection frequently elicits serum antibodies to the viral antigens. Extensive seroepidemiological surveys of T cell leukemia patients and normal donors for antibodies to HTLV have been conducted to determine the relationship of HTLV with particular human malignancies (Table 2). Since the virus appeared from the initial series of results to be T cell-tropic, a large number of patients with a variety of T cell malignancies were screened for evidence of HTLV infection [84]. In the U.S., HTLV-positive T cell malignancies occur sporadically. There is a form of adult T cell leukemia (ATL) more common in Japan. Clinical epidemiology of Japanese ATL had indicated a geographic clustering in the southwestern islands of Kyushu and Shikoku and the involvement of an infectious vector, possibly a virus in its etiology [85]. Our studies have shown that nearly 90% of all Japanese ATL patients have serum antibodies to HTLV, obviously indicating that HTLV is associated with this disease [86, 87].

The Caribbean basin has been found to be another area where HTLV-associated malignancies appear to be endemic. Antibodies to HTLV were found in all West Indian patients with T cell lymphosarcoma cell leukemia, an aggressive malignant disease similar to Japanese ATL [88]. Other cases of T cell leukemia-lymphomas associated with HTLV have also been found in the Boston and Seattle areas, Alaska, the southeastern U.S., Central and South America, Africa and Israel (84, C. Saxinger *et al.*, unpublished). It has

Table 2. Prevalence of natural antibodies to HTLV in sera of patients with malignancies of mature T-cells, their healthy relatives, and random normal donors

Serum donors	Antibodies to HTLV[a]	
	# Positive/# Tested	% Positive
Japanese ATL patients	40/46	87
Healthy relatives of ATL patients	19/40	48
Random healthy donors, non-endemic area	9/600	2
Random healthy donors, endemic area	50/419	12
Healthy relatives of U.S. patients with HTLV-associated malignancy	2/12	17
Unrelated healthy donors, Washington, D.C.	1/185	<1
Unrelated healthy donors, Georgia	3/158	2
Caribbean T-LCL patients	11/11	100
Healthy relatives of Caribbean patients	3/16	19
Random healthy donors, Caribbean	12/337	4

[a] Antibodies were detected by RIP of HTLV p24 or by the solid-phase RIA.

become apparent that most HTLV positive lymphomas and leukemias fall into an unusual clinical syndrome consisting of adult onset, usually with a rapid disease course, often associated with lymphadenopathy and hepatosplenomegaly, circulating large and usually pleomorphic lymphocytes with lobulated nuclei, mature T cell surface phenotypic markers (usually $OKT4^+$), and frequent hypercalcemia and skin manifestations [84]. Despite the $OKT4^+$ phenotype, the T cells from this disease often have a suppressor function. For consistency, this aggressive disease entity associated with HTLV is now uniformly named ATL for adult T-cell leukemia-lymphoma. It should be emphasized, however, that HTLV antigen or antibody has been identified in a few instances in the more typical, less aggressive mycosis fungoides and Sézary syndrome patients. Whether HTLV or a related virus is involved in this disease commonly requires further studies.

3.3.2. *Antibodies to HTLV in the Normal Population and Healthy Relatives of ATL Patients.* Sera of random normal donors in the U.S. and Europe generally do not show antibodies to HTLV. In Japan where HTLV infection is endemic in certain parts, the prevalence of serum antibodies in normal populations varied considerably and correlated closely with the geographic distribution of ATL (Fig. 1). Thus, the incidence was 15–16% in the Nagasaki and Kagoshima areas of Kyushu Island, about 9% in the Uwajima area of Shikoku Island but was only about 2% in Honshu Island and even lower in Hokkaido Island [85, 86]. It is known that once an individual acquires serum antibodies to HTLV, these individuals become antibody carriers for long periods even without chances of frequent re-exposure to the virus. Unlike in Japan, clustering of ATL in specific regions of the Caribbean basin has not been studied. About 4% of the normal Caribbean population screened showed antibodies to HTLV indicating that HTLV infection may be generally widespread in these regions (Table 2). HTLV endemic regions have now been identified in several areas of the world, including parts of the Africa continent (C. Saxinger *et al.*, in preparation).

If HTLV is an infectious virus, close family members of virus positive patients will be the most likely group to have a high incidence of serum antibodies to HTLV proteins. This indeed turns out to be the case. The single significant group that is positive for HTLV-specific antibodies in the U.S. is the family members of HTLV-positive patients (Table 2). Four relatives each of patients CR, MJ, and WA, all of whom were sources of separate HTLV isolates were studied. The wife of CR and the mother of WA were found to be positive for antibodies to HTLV p24 and p19. Similarly, four Caribbean families were screened and 3 of 16 members were antibody-positive. By far the highest incidence noticed was among relatives of Japanese ATL patients. Nineteen individuals out of 40 members studied belong-

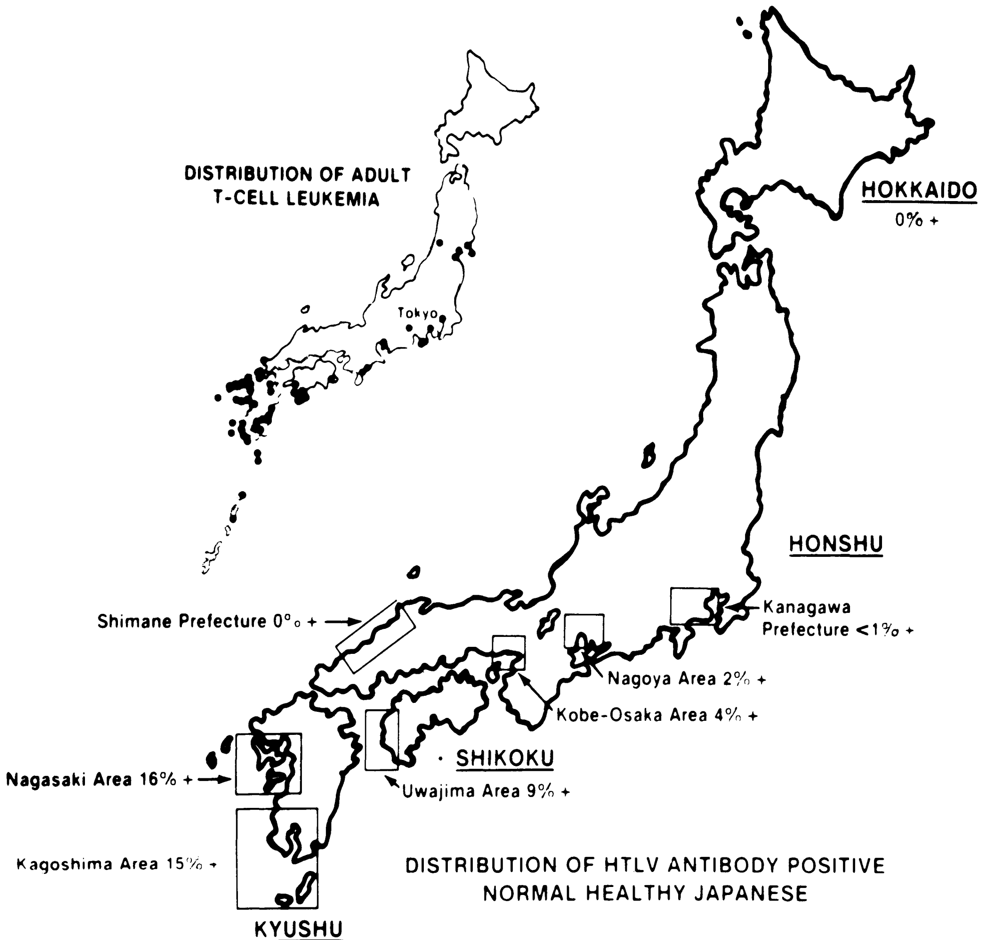

Figure 1. Comparison of the distribution of Japanese adult T-cell leukemia patients (pleomorphic type) with the distribution of HTLV antibody positive healthy Japanese.

ing to 12 families had antibodies to HTLV proteins. In one of the Japanese families, four members including one patient, his brother and both his parents had antibodies against HTLV p24 and p19.

In summary, in certain geographic locations where the virus is endemic, for example in Southern Japan and in the Caribbean basin, a significant percentage of the normal population have antibodies against HTLV. In addition, in any given area, family members of virus positive patients exhibit a much higher incidence of infection than healthy donors not known to be related to HTLV-positive patients. This is a further confirmation of the transmission of HTLV by horizontal infection.

3.4. *Molecular Epidemiology of HTLV-Related Diseases*

Molecular cloning of HTLV genomic sequences has provided probes to

search for homologous viral sequences in fresh tissues, even in the absence of viral antigens, particles or antiviral antibodies [83, 89, 90]. In addition, by utilizing restriction endonucleases that cleave specific viral fragments, a careful comparison can be made between infected tissues from different individuals to compare internal viral sequences. Studies have been carried out in a wide variety of hematologic malignancies [82, 89]. Several conclusions can be made from these studies: i) Cells from some patients with mature T cell malignancies, including all patients with ATL, contain one or a few copies of HTLV-I provirus. Cells from other types of malignancies involving immature T cells, B cells or myeloid cells are by and large negative. ii) The correlation of the surveys by molecular hybridization and by serology is not 100%. For example, two patients who had no circulating antibodies against HTLV antigens are positive for HTLV provirus. On the other hand, a patient with T-ALL has antibodies even though his leukemic cells are immature T cells, normally not the target for HTLV infection. By hybridization, his leukemic cells indeed lacked HTLV DNA sequences. Therefore, the discrepancies between serology and molecular hybridization could be due to patients who are HTLV positive but do not express virus and/or antiviral antibodies, or patients who have been exposed to HTLV and therefore are positive for anti-HTLV antibodies, but whose disease is not linked to HTLV. iii) The tumor cells are clonal expansions of single infected cells. Monoclonality is a common feature of tumor induced by the chronic leukemia viruses. This finding will have bearing in the possible mechanism of neoplastic transformation as discussed later.

3.5. *In Vitro Transformation of Normal Cord Blood T Cells by HTLV*

An unusual property of HTLV is its ability to directly immortalize normal human cord blood and bone marrow T cells *in vitro*. HTLV was transmitted into cord blood or bone marrow cells from HTLV positive cell lines by cocultivation [78, 91, 92] and in a few instances by addition of cell free virus particles (unpublished data with P. Markham and Z. Salahuddin). For the cocultivation experiments, the donor cells were inactivated by exposure to X-rays or mitomycin-C treatment. The recipient cells that grew out were typed by karyotype, HLA patterns and other morphologic characteristics as well as for the expression of HTLV p24, p19 and reverse transcriptase. The cells were clearly derived from the normal recipient blood cells as determined by karyotype and HLA analysis. The infected cells resemble neoplastic T cells in many respects. First, they develop lobulated nuclei and some grow as multinucleated giant cells, common morphological features of many HTLV-associated primary malignant cells. Second, infected T cells have the potential for indefinite growth. In contrast, mitogen stimulated cord blood T cells from the same patients consistently exhibited growth 'crises'

after one month in culture, even in the continued presence of TCGF. Third, infected cord blood cells have a decreased requirement for TCGF for growth. Normal cord blood T cells require 10–12% (v/v) TCGF for growth whereas the HTLV infected cord blood T cells can grow in 0–5% (v/v) TCGF. Fourth, like almost all HTLV associated primary neoplastic cells, the infected cells express high levels of TCGF receptors. A comparison of these and additional properties of the HTLV infected and uninfected human cord blood T cells with HTLV-positive human neoplastic T cells is summarized in Table 3. The data indicate that HTLV is capable of transformation of cord blood T cells *in vitro*.

Table 3. Comparison of properties of HTLV positive human neoplastic T cells with normal uninfected and HTLV infected human cord blood T cells

Property	HTLV positive neoplastic T cell lines	Cord blood T cells	
		HTLV infected	Mitogen stimulated
1. *In vitro* growth	>180 days (immortal)	>180 days (immortal)	<50 days (temporary)
2. Requirement for exogenous TCGF (v/v)	0–5%	0–5%	10–12%
3. TCGF receptors (TAC)[a]	+++	+++	+
4. Transferrin receptor[a]	+++	+++	+
5. E-Rosette	+++	+++	+++
6. S-IgH[b], EBNA[c], TdT[d]	–	–	–
7. Cell phenotype:			
(a) Inducer/helper (OKT4, Leu 3)[a]	Most or all	Most or all	Most or all
(b) Suppressor/cytotoxic (OKT8, Leu 2A)[a]	Few or none	Few or none	Few or none
8. Cell morphology:			
(a) Presence of multinucleated giant cells	+	+	–
(b) Presence of lobulated nuclei	+	+	–
9. HLA 'modification':			
(a) Expression of additional HLA antigens	+	+	–
(b) Expression of HLA-Dr[a]	+	+	–
10. HTLV p19, p24, and RT expression	+	+	–
11. Type C virus particles (EM)	+	+	–

[a] Determined by cell sorter using monoclonal antibodies.
[b] S-IgG, cell surface immunoglobulins
[c] EBNA, Epstein-Barr nuclear antigen
[d] TdT, terminal deoxynucleotidyl transferase

3.6. *Possible Mechanisms for HTLV Transformation*

Analyses of the cloned complete HTLV genomes indicate that HTLV does not contain a cell derived *onc* gene [93]. This fact coupled with the clonality of HTLV associated tumors suggest that HTLV is a chronic leukemia virus in spite of its capacity to transform T cells efficiently *in vitro*. In addition to the genes coding for *gag, pol* and *env* proteins, the nucleotide sequence of HTLV-I genomes reveals a potential coding region between the *env* gene and 3′ LTR. This region, named pX, contains overlapping open reading frames for four small peptides of 10,000 to 27,000 daltons [93]. Although there is as yet no evidence that any of these peptides is actually made in the infected cells, it has been speculated that these proteins may play a role in transformation of the leukemic cells. Fresh leukemic cells and established cell lines of ATL patients have been examined for expression of viral mRNA [94]. While all the tissue culture cell lines express multiple mRNA species including a 9.0 Kb *gag-pol* mRNA, a 4.2 Kb *env* mRNA and a 2–2.5 Kb species containing only pX and LTR sequences, not all fresh leukemic cells express viral mRNA. This result is reminiscent of the bovine leukemia system where no bovine leukemia virus (BLV) mRNA is detected in tumors that have integrated BLV DNA [64]. Therefore, expression of a viral gene product, including pX, does not seem to be necessary for the maintenance of the leukemic state. However, it is still possible that expression of a viral protein is necessary early in the disease, resulting in active proliferation of the infected cells, and a secondary event, for example, a gene mutation can cause leukemic conversion of one cell which then expands into a clonal tumor.

Expression of some cellular genes of possible relevance in HTLV leukemogenesis has also been studied [94]. Although all HTLV infected cells express high levels of TCGF receptor [95], only rarely do they express TCGF at the RNA [94, 96] or protein level [97]. Thus, a simple autostimulation model is unlikely. However, the TCGF receptor expressed in the infected cells may have been altered so that it can recognize a different growth factor or so that it behaves as if it is bound to TCGF. Expression of cellular genes homologous of known viral *onc* genes including *myc, myb, sis, fes, abl, ras* and *src* has also been examined [94]. There is no evidence of activation of these *onc* genes with the possible exception of *sis* which is expressed in a high percentage of HTLV primary and *in vitro* transformed cell lines (our unpublished data). However, *sis* expression must not be a requisite for maintaining the leukemic state since some HTLV leukemic cells do not synthesize detectable levels of this protein.

In summary, consistent expression of viral genes, the gene for TCGF and many of the human homologues of known viral *onc* genes does not appear necessary for maintenance of the leukemic state, although any of these may

be required early in the disease. Alternatively, HTLV may activate other cellular genes. Examination of the sites of integration using flanking sequences from cloned proviruses ruled out a single conserved site, but not a multiple but limited number of sites. More studies will be required to determine if gene activation by specific integration of HTLV on the chromosome is involved in the mechanism of leukemogenesis by HTLV.

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2. Study of Differentiation and Proliferation of Leukemic Cells Using Myeloid Leukemia Cell Lines

H. PHILLIP KOEFFLER

1. INTRODUCTION: ESTABLISHMENT OF HUMAN MYELOID LEUKEMIA CELL LINES

The study of proliferation and differentiation of acute myelogenous leukemia cells is greatly aided by the establishment of human myeloid leukemia cell lines [1–8], (Table 1 and Figure 1). The leukemic lines are blocked at different stages of maturation: The KG-1a cells are very young myeloblasts; KG-1 are myeloblasts; HL-60 are promyelocytes; ML-1 and 3 are myelomonoblasts; U937 and THP-1 are monocytoid lines; K562 and HEL are early myeloid blasts and/or erythroblasts. Cells of each of the lines except THP-1 have prominent chromosomal abnormalities and these same changes were also present in the blast cells of the patients. The KG-1, ML-1 and 3, HL-60, THP-1 and U937 cells can be triggered to differentiate to macrophage-like cells. The HL-60 cells can differentiate to granulocytes. The K562 and HEL cells can differentiate to more mature red cell precursors after exposure to several agents.

A variety of distinct morphological, antigenic, histochemical and functional markers of maturation appear as the leukemia cells undergo granulocyte or macrophage-like differentiation (Table 2). The leukemic lines provide, therefore, not only the opportunity to study induction of myeloid leukemic cell differentiation but also provide the opportunity to study the characteristics of a homogeneous population of cells during the different stages of myeloid maturation.

Cells from early passages of KG-1 and, less so, from HL-60 and THP-1 are stimulated to proliferate by the granulopoietin known as colony-stimulating factor (CSF). The clonal growth of the K562 cells is slightly enhanced by erythropoietin-potentiating factor (EPA), a lymphokine that enhances early red cell precursor growth.

A number of EBV-transformed B-lymphocyte lines have been established

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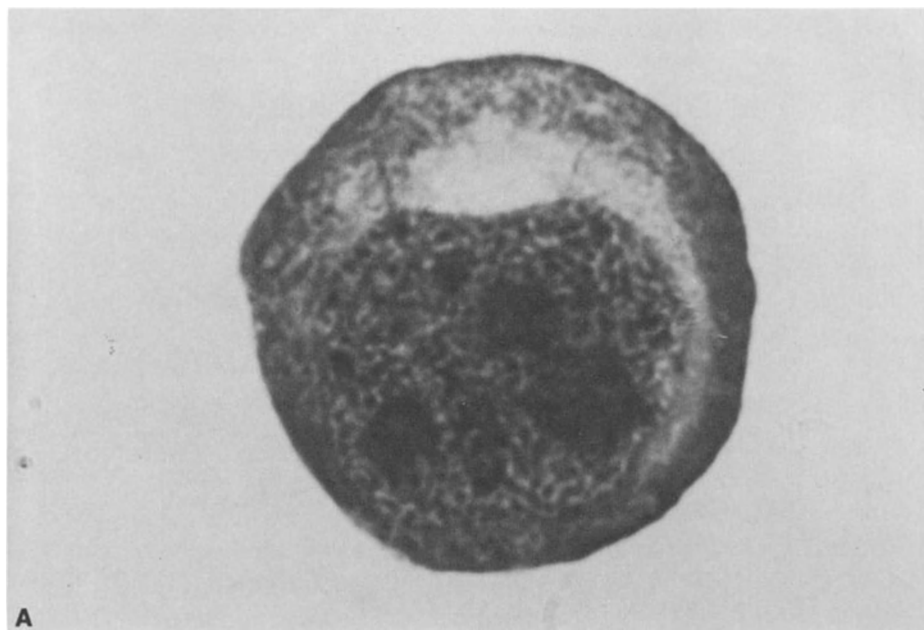
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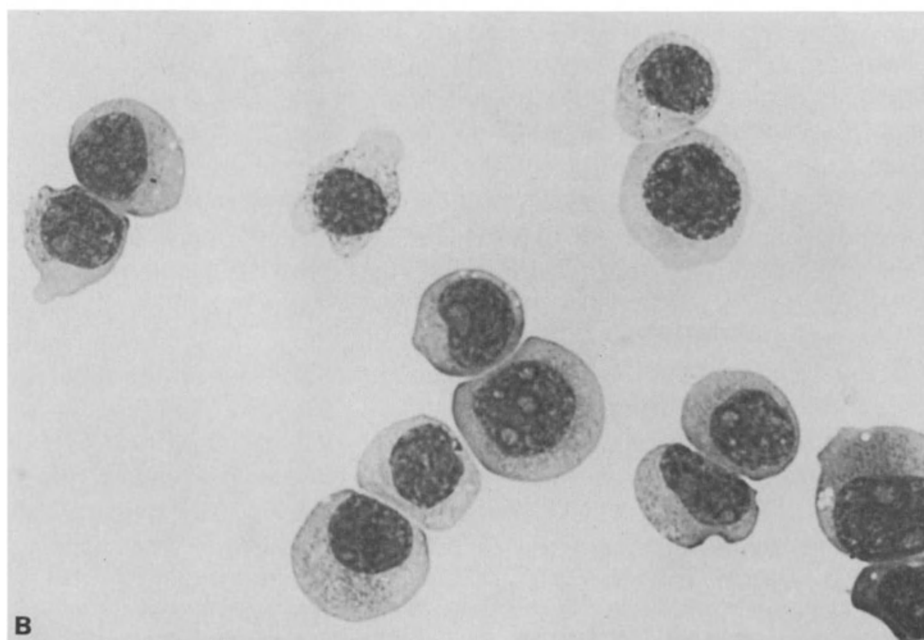
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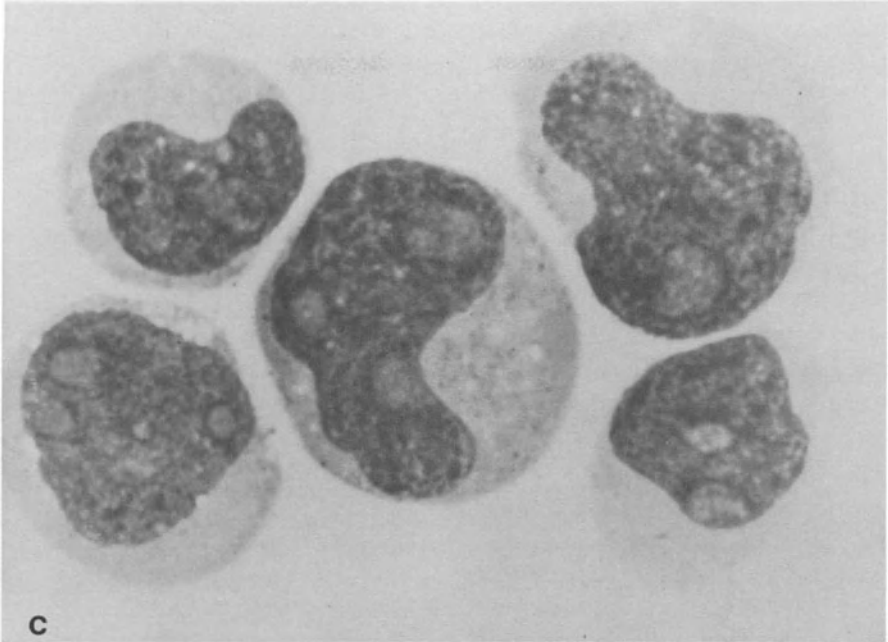


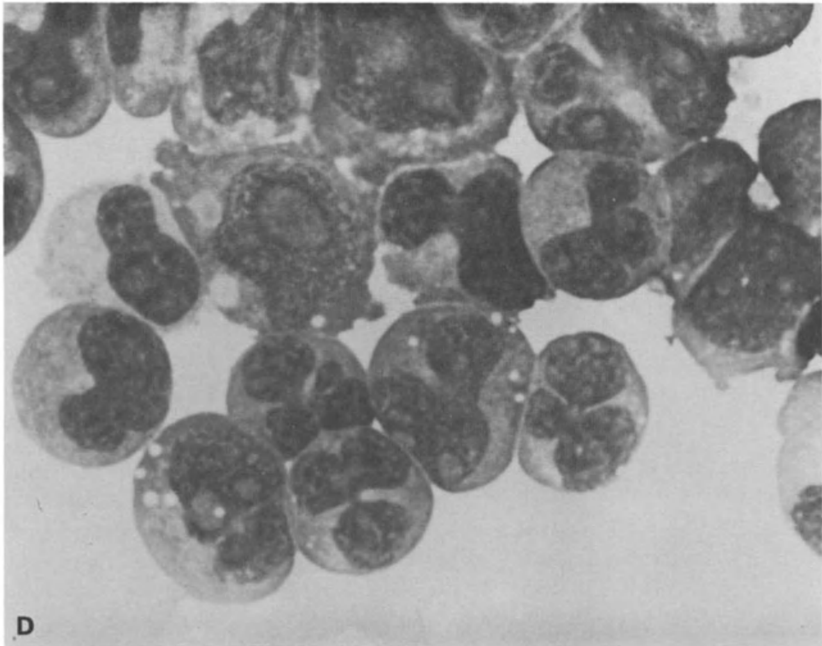


Figure 1. Microphotographs of cells from human myeloid leukemia cell lines after Giesma staining: 1a.) K562 erythroblasts, 1b.) HL-60 promyelocytes, 1c.) KG-1 myeloblasts, 1d.) ML-3 myelomonoblasts.

from patients with myeloid hematopoietic malignancies. Expression of gene products commonly associated with normal monocytes or granulocytes, including lysozyme, leucocyte alkaline phosphatase and a myeloid specific esterase (AS-D-chloroacetate), have been rarely detected in several EBV-positive B-lymphocyte lines [9]. Synthesis of myeloid specific gene products in cell lines with mixed lymphoid and myeloid phenotype occurs very rarely, and the cells probably represent aberrant activation of several myeloid genes rather than a malignant counterpart of a normal hematopoietic precursor blocked at a specific state of maturation.

The development of myeloid cell lines is difficult. Numerous attempts to established myeloid lines have been made and thus far eight well-characterized lines have been developed (Table 1) and several myeloid lines have been established but not well described. Examination of the patients or the methods used to establish the eight myeloid lines provides little insight on how to establish a myeloid line (Table 2). Cell lines were established from five leukemic subtypes. Two of the patients had erythroleukemia. The immortalized leukemic cells of 6 of 7 patients have prominent karyotype abnormalities, but no common chromosomal change is evident. The lines were established from pleural fluid on two occasions, bone marrow once,

Table 1. Human myeloid leukemia cell lines

Cell line	Stage of differentiation	Triggered to differentiation	Inducers of differentiation	Responsive to growth factor	Ref.
KG-la	Early myeloblast	No	No	No	5
KG-1	Myeloblast	Macrophages	Phorbol diesters; Teleocidins	CSF*	4
HL-60	Promyelocyte	Granulocytes; macrophages	Numerous	Slightly to CSF	3
ML-1 & 3	Myelomonoblast	Macrophages occasional granulocytes	Phorbol diesters; Teleocidins	No	4
U937	Monocyte-like	Macrophages	Phorbol diesters; Teleocidins	No	2
THP-1	Monoblast	Macrophages	Phorbol diesters	No	8
K562	Early blast and/or erythroblast	Early erythroblast	Hemin; butyrate	EPA**	1
HEL	Early blast and/or erythroblast	Early erythroblast	Hemin		7

* CSF, colony stimulating factor
** EPA, erythropoietin potentiating factor

Table 2. Growth conditions used to establish human myeloid leukema cell lines

Cell line	Concentration of cells in initial culture (per ml)	Culture medium	Feeder layer	Serum supplement	Other additives	Time before emergence of cell line	Source of cell	Prominent chromosomal abnormality	Patient [1] disease
KG-1	1.0×10^6	Alpha (Flow)	No	20% FCS	10^{-4}M alpha-thioglycerol	3 wks	Bone marrow	Yes	EL
HL-60	0.25×10^6	RPMI-1640 (GIBCO)	No	15% FCS [2]	Conditioned medium from human embryonic fibroblasts	3 wks	PB [3]	Yes	APL
ML-1 & 3		RPMI-1640	No	20% FCS	No		PB	?	AML
U937		RPMI-1640	Glia cells or fibroblasts	10% NBCS [4]	No	7 wks	Pleural fluid	Yes	Histiocytic lymphoma
THP-1	3×10^6	RPMI-1640	No	20% FCS		2–3 wks	PB	Normal	Monocytic
K562		MEM [5]	No	15%FCS	No	2–3 wks	Pleural fluid	Yes	CML-BC
HEL		RPMI-1640	No	12%	1 mM pyruvate	4 wks	PB	Yes	EL

[1] EL, erythroleukemia; APL, acute promyelocytic leukemia; CML-BC, chronic myelogenous leukemia, blast crisis; AML, acute myelogenous leukemia.
[2] FCS, fetal calf serum.
[3] PB, peripheral blood.
[4] NBCS, newborn calf serum.
[5] MEM, minimal essential medium (Eagle's).

and peripheral blood in four of the patients. Most of the cell lines were established in a rich culture medium (usually RPMI-1640) containing usually 15-20% fetal serum. A feeder layer of fibroblasts was used once [2] and conditioned medium from a human embryonic fibroblast culture was felt to be indispensable on another occasion [3]. The HL-60 cells have the proto-oncogene known as *myc* amplified at least 16- to 32-fold with parallel increased expression of *myc*, but this does not account for immortalization of at least the KG-1 and K562 cells which have normal expression of the oncogene. The difficulty in establishing myeloid lines probably is intrinsic to the cell and not an environmental problem such as absence of a 'leukemic growth factor' or presence of another cell type such as a cytotoxic T lymphocyte.

Myeloid cell lines have not been established from normal individuals. One cell line with myeloid characteristics was established from a patient with congenital hypoplastic (Diamond-Blackfan) anemia [10]. Myeloid cells can be cultured for extended periods (≤ 20 weeks) by using a long-term bone marrow culture system that relies on a marrow adherent feeder layer which includes fibroblasts, endothelial cells, and macrophages [11–13]. The number of mature granulocytes present in the cultures after 3 to 5 weeks is small, but the cells are functionally normal [14]. Vitamin D has been reported to permit the prolonged proliferation of normal human cord blood myeloid cells in liquid culture [15].

2. HUMAN ERYTHROLEUKEMIA CELL LINES

The K562 cell line was established from a patient with blast crisis of chronic myeloid leukemia (CML) [1]. The cells were initially felt to be blocked at the very early myeloid blast stage; however, sublines of K562 contain erythroleukemic cells. The cells synthesize glycophorin which is a specific red blood cell membrane protein [16]. The cells constitutively produce fetal and embryonic hemoglobin and in the presence of hemin (approximately 50 µm), or sodium butyrate (about 1.4 mM) the cells increase globin mRNA production 3–4 fold [17-20]. This represents 10–20% of the level achieved in normal marrow erythroblasts. Near normal levels of hemoglobin (26–34 pg Hgb/cell) are obtained if the K562 cells are exposed simultaneously to hemin and an inhibitor of cell division [20]. The K562 cells display a differential expression of the globin genes after differentiation is triggered by hemin or butyric acid [18]. Butyric acid exposed K562 cells contain mostly Gower I ($\zeta_2\varepsilon_2$) and Portland ($\delta_2\zeta_2$) globin. After hemin-treatment of K562, the most abundant hemoglobin synthesized is HB $\times$ ($\varepsilon_2\gamma_2$) and the second most abundant is Bart's (γ_4). The cells produce

constitutively fetal hemoglobin (Hgb F) but do not produce adult hemoglobin (β, δ, α). Sublines of K562 contain trisomy of chromosome 11 which contain the beta-like globin genes and tetrasomy of chromosome 16 which contain the alpha-like globin genes [18]. These findings might play a role in the amount of globin transcription in the cells.

Although hemin and butyrate induce increased synthesis of fetal and embryonic hemoglobin in K562 cells, little evidence exists that other phenotypic changes of red cell maturation can be induced in the cells. The K562 cells probably have a restricted expression of erythroid specific genes either because of incomplete expression of the normal genetic program for red blood cell differentiation or because of deranged gene expression in neoplastic cells.

Another human erythroleukemia cell line has been established and is known as HEL [7]. The uninduced HEL cells produced predominantly $G\gamma A\gamma$ chains and a very small amount of the embryonic (Σ, ζ) $\alpha\alpha$ chains. Similar to the K562 cells the synthesis of the globin proteins by HEL increased about 10-fold after 3–4 days exposure to 10–100 μM hemin. The erythroleukemia cell lines may provide a model to help understand the molecular basis of thalassemia and globin gene switching.

3. STUDY OF GRANULOCYTE DIFFERENTIATION USING MYELOID LEUKEMIC CELL LINES

Human acute myelogenous leukemia (AML) often arises from neoplastic transformation at the pluripotent stem cell level leading to a block in cell maturation at the recognizable myeloblast or promyelocyte stage. The leukemic patient often dies of infection because the blast cells cannot mature to functional end cells. Instead, many of the leukemic cells remain in the proliferative pool and rapidly accumulate. A central question in the study of AML is whether the leukemic cells are capable of maturation under certain environmental circumstances or whether the defect is complete and unchangeable by external conditions. This question has profound therapeutic importance. Authors have claimed that normally differentiated cells recovered in semi-solid gel or liquid culture from leukemic patients represented leukemic cell maturation [21–25]. Other investigators, however, reported that leukemic cells showed little evidence of cellular differentiation or showed incomplete cellular maturation [26–29]. A difficulty in interpreting reports of leukemic cell maturation relate to the possibility that the normally differentiated cells in the colonies arose from residual nonleukemic precursors. A similar problem exists in interpreting the conflicting data on

human leukemic cell differentiation both in diffusion chambers implanted into the peritoneal cavity of mice and *in vivo* data [30–32]. The origin of the mature cell cannot be determined conclusively without an easily measured cytogenetic or enzymatic marker of the neoplastic clone. Leukemic cell maturation has been confirmed in several patients by the presence of Auer rods in the granulocytes.

Leukemic cell lines have been developed to simplify the study of leukemic cell differentiation. Leukemic lines allow the convenient investigation of a homogeneous population of neoplastic cells. The study of leukemia using cell lines derived from lower animals allows experimental investigations that are not possible in man. The first extensively studied myeloid leukemic line was the murine myeloid leukemic cell line known as M-1 [33, 34]. A variety of clones of M-1 with various potentials to differentiate have been established.

Exposure with various inducers *in vitro* causes the differentiation sensitive M-1 clones to mature into macrophages and granulocytes. Syngeneic mice inoculated with M-1 cells die of leukemia but exposure of the M-1 cells *in vitro* to an inducer of differentiation causes the cells to lose their leukemogenicity *in vivo* [35]. Differentiation resistant M-1 clones often cannot be triggered to mature *in vitro*, and the cells continue to be leukemogenic when injected into mice. Development of leukemia can be inhibited or the survival time of mice prolonged after differentiation sensitive M-1 cells are injected into syngeneic mice and the mice are treated with inducing agents of myeloid differentiation [36, 37]. Inducers of myeloid cell differentiation often have little effect on the survival times of mice inoculated with differentiation resistant leukemic cells [36, 37]. Recently, investigators showed that the injection of M-1 cells into embryos at 10 days of gestation can result in adult mice whose granulocytes are partially derived from M-1 leukemic clones [38]. No cells with the leukemic morphology could be identified. The results suggest that malignant cells may respond to the controls that regulate normal growth and differentiation in the embryo. The murine M-1 experiments suggest that certain leukemias may respond to inducers of differentiation.

Induction of granulocyte differentiation of human leukemic cells can be studied using the promyelocytic leukemia cell line HL-60 [3].

3.1. *Nonphysiological Inducers of Granulocyte Differentiation*

A variety of agents induce HL-60 promyelocytes to become intermediately or fully mature granulocytes (Fig. 2). The potency of the various agents can be evaluated both by the percentage of cells that mature and the molar concentrations at which various agents cause differentiation. Many of the nonphysiological inducers of differentiation are compounds capable of in-

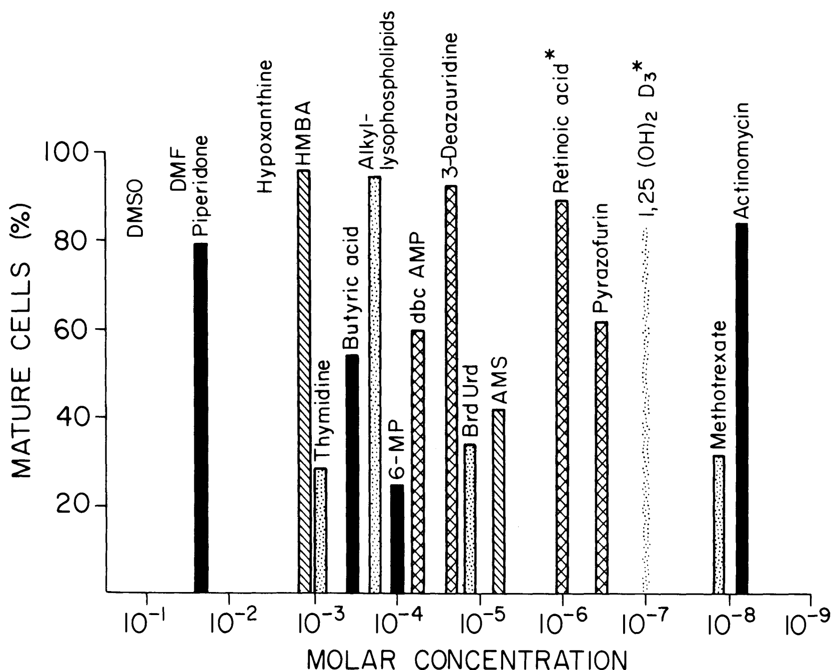


Figure 2. Induction of HL-60 maturation was examined in most studies after approximately 6 days of exposure to the compound and the concentration of the compound usually represents that amount which produced 50% reduction in growth of HL-60 cells compared to control culture. Reduction in cell number is probably due both to formation of mature nondividing cells and to cytotoxicity of the inducing agent.

* Physiological substance

Solid bars ■ represent compounds that also trigger maturation of HL-60 and both murine M-1 myeloid and Friend cells; ▧ compounds that trigger differentiation of HL-60 and Friend cells; ▦ compounds that induce differentiation of HL-60 and M-1 cells; ⊠ compounds that only trigger maturation of human HL-60 cells.

DMSO: dimethyl sulfoxide / DMF: dimethyl formamide / HMBA: hexamethylene bisacetamide / 6-MP: 6 mercaptopurine / BrdUrd: Bromodeoxyuridine / $1{,}25(OH)_2$ vitamin D_3.

ducing maturation of 80% of the HL-60 cells by 6 days of culture. The polar-planar drugs (DMSO, HMBA, acetamide, piperidone, triethylene glycol) induce a large percent of the cells to differentiate, but high concentrations ($10^{-1}-10^{-2}$ M) of these compounds are required for induction of differentiation [39-42].

A variety of purines and pyrimidines and their analogs can induce granulocyte differentiation of HL-60 cells. In this category, 3-deazauridine (2.5×10^{-5} M) and hypoxanthine (5×10^{-3} M) induce differentiation of the greatest percent (85%) of HL-60 cells. The 3-deazauridine is a competitive inhibitor of the conversion of UTP to CTP and is not incorporated into nucleic acid [43]. Hypoxanthine is part of the purine salvage pathway. Most

of the purine and pyrimidine analogs produce maturation at comparable molar concentrations, 10^{-5} to 10^{-4} M. Thymidine at a high concentration (10^{-3} M) can trigger about 1/3 of the cells to mature [40].

Several chemotherapeutic agents are potent triggers of myeloid differentiation. Actinomycin-D (8×10^{-9} M) and methotrexate (1.4×10^{-8} M) induce maturation of 85% and 35% of the HL-60 cells, respectively [39–41]. Bromodeoxyuridine [40, 41, 44] and 5-azacytidine [40], at about 3×10^{-5} M induce approximately 1/3 of the HL-60 cells to mature; 6-thioguanine, daunomycin, cytosine arabinoside, and vincristine are weak inducers of HL-60 differentiation. Irradiation has no effect on maturation of HL-60 [39].

Alkyl-lysophospholipids are synthetic analogs of lysophospholipids and several of these analogs can induce differentiation and inhibit proliferation of HL-60 and M-1 cells [45]. These compounds probably interfere with phospholipid metabolism of leukemic cells. Preliminary evidence suggest that normal hematopoietic cells can metabolize these compounds and proliferation of the normal hematopoietic stem cells may not be inhibited by the analogs [45].

How the various agents trigger differentiation of HL-60 promyelocytes to granulocytes is not clear. Common chemical features shared by a wide variety of inducers can be identified, but in general this approach is not particularly helpful. The polar-planar compounds are generally of low molecular weight (60–150 daltons), are often effective cryoprotective agents and are capable of acting as Lewis' bases. Studies in Friend cells have shown that the agents have a profound effect on the plasma membrane fluidity. Cooper and co-workers found that the HL-60 cells triggered to differentiate underwent a progressive decrease in membrane fluidity secondary to an increase in both the cholesterol/phospholipid molar ratio and the degree of fatty acyl chain saturation within the cell membrane [49]. The authors noted that high concentrations of DMSO could increase HL-60 membrane fluidity but the concentration of DMSO (1.25% v/v) normally used to trigger HL-60 differentiation was unable to alter membrane fluidity of HL-60 cells. The authors felt that the alteration of membrane fluidity was a consequence of the differentiation process [49]. The polar-planar compounds are also freely permeable throughout the cell and possess a free electron pair which could interact directly with hydrogen bonds within chromatin. The nucleosides and their analogs which induce HL-60 differentiation are capable of either increasing or decreasing cellular purine or pyrimidine nucleoside levels. No evidence exists that these natural compounds function physiologically. Data suggest that the purine derivative, hypoxanthine, can trigger differentiation without being incorporated into RNA or DNA [50, 51]. A similar finding has been noted with Friend murine erythroleukemia cells [52]. Studies from our laboratory with BrdUrd suggest that the agent must be incorporated into

the DNA of HL-60 to trigger differentiation [44]. Compounds such as aphidicolin inhibit DNA synthesis without being incorporated into DNA and can induce differentiation of HL-60 [53]. Marked increases of ornithine decarboxylase activity and polyamine biosynthesis are important to proliferation of HL-60 cells but do not appear to play a critical role in differentiation of HL-60 [54]. Actinomycin D is a potent inducer of human HL-60 and murine Friend and M-1 leukemia cells. Concentrations as low as 1.5 ng/ml can induce differentiation of greater than 80% of the blast cells. At that concentration approximately one out of 10^5 deoxyguanine-deoxycytosine base pairs is bound to actinomycin D [55]. It is not known if the DNA binding is critical to differentiation. As HL-60 cells differentiate to granulocytes, a histone polypeptide known as HP appears and probably represents a proteolytic product of histone H2A [56]. The HP protein is noted in normal mature granulocytes and may play a role in the maturation process.

Many of the compounds that induce the HL-60 cells to mature can also induce the cells from a murine myeloid leukemia line (M-1) to mature to granulocytes (Fig. 2). The findings suggest that both myeloid leukemia cell lines share common cellular target sites for the action of many of the inducing compounds. However, several inducers of murine M-1 cells cannot trigger maturation of HL-60, including dexamethasone and x-irradiation [34]. The unresponsiveness of HL-60 to glucocorticoids cannot be explained by lack of steroid receptors because the cells have a large number of high affinity glucocorticoid receptors. Evidence from other model systems have suggested that cyclic nucleotides may play an integral role as a secondary message to trigger differentiation [57]. Agents that increase intracellular concentration of adenosine 3′ 5′-cyclic monophosphate (cAMP) have very little effect on triggering maturation of the murine M-1 cell line but high concentrations of the agents can induce partial maturation of HL-60 cells [58, 59]. The HL-60 cells exposed to 500 µM N^6, O^2 dibutyryl adenosine 3′ 5′ cyclic monophosphate express formyl peptide and complement receptors, reduce NBT, adhere to substrate, initiate chemotaxis and morphologically mature to the myelocyte and metamyelocyte stage. Another study has found that HL-60 cells are able to undergo differentiation after initial exposure to 10 nM retinoic acid for one day and then subsequent culture with agents known to increase intracellular cAMP levels [60]. Neither the low concentration of retinoic acid nor the cAMP-inducing substances alone are able to induce differentiation.

Commitment is defined operationally as the capacity of cells that have been exposed to an inducer both to express differentiated characteristics in the absence of that inducer and to lose proliferative capacity. Exposure of HL-60 cells to DMSO for 12 hours is the minimal requirement to promote

Table 3. Characteristics of myeloid leukemic cells at various stages of differentiation

	Promyeloblast	Myeloblast	Promyelocyte	Myelocyte	Metamyelocyte	Macrophage
	KG-1A	KG-1	HL-60	HL-60	HL-60	KG-1 HL-60 ML-3
All surface antigens:	No Ia antigen	Ia	No Ia			Ia antigen
	i-antigen		I			No macrophage-specific antigen
	Gp*95		Gp130			
	Gp*105					
					granulocyte antigens	
Cell surface markers:			(±) **FLMP receptor	(+ + +) FLMP receptor		FLMP receptor
			(±) Fc receptor	(+ + +) Fc receptor		Fc receptor
			(+) ***C′ receptor	(+ + +) C′ receptor		Phorbol receptor
	Phorbol receptor					
Functional markers:		CSF responsive	Slight CSF responsive			
					Superoxide production	Slight superoxide
					Phagocytosis	Phagocytosis
					Chemotaxis	
					Bacterial killing	Bacterial killing

Histochemical markers: AS-D Chloroacetate Esterase; Myeloperoxidase; Sudan black; (+) Lysozyme——(+ +) Lysozyme — (+ + +) Nonspecific Acid Esterase + + +; (+ + +) Lysozyme

	Myeloblast	Promyelocyte	Myelocyte	Metamyelocyte	Macrophage
Cell lines	KG-1	HL-60	HL-60	HL-60	KG-1, HL-60, ML-3

Oncogene Transcription: ****	Myeloblast	Promyelocyte	Myelocyte	Metamyelocyte	Macrophage
myc	+ +	+ + + + +	+/−	−	−
myb	+ +	+ +	+/−	−	−
fes	+ +	+ +	+/−	−	−
abl	+	+	+	+	+
ras^H	+	+	+	+	+

* Gp: glycoprotein.

** FLMP: f-met-le-pheu chemotactic peptide.

*** C′: complement receptor.

**** +, gene is transcriptionally active; +/−, gene is transcriptionally less active or not detectably active. myc, cellular homolog of avian myeloblastosis virus; fes, homolog of Snyder-Theilin feline sarcoma virus; abl, homolog of Abelson virus; ras^H, homolog of Harvey murine sarcoma virus.

commitment to terminal myeloid differentiation [61]. Agents that elevate intracellular cAMP cause commitment of HL-60 within 24 to 48 hours of exposure to the compounds [59]. The inducer-mediated commitment of human promyelocytic cells to differentiation is stochastic and is not linked to cell division [61, 62].

Many of the inducers of myeloid differentiation also trigger erythroid differentiation of the murine Friend erythroblast cell line (Figure 2). However, several inducers of the Friend erythroblast line have no effect on the human or murine myeloblast cell lines. The erythroblast specific inducers of differentiation include hemin, prostaglandin E, and ouabain. Hemin also induces globin synthesis in the K562 and HEL human erythroblast cell lines.

Reduction of methylation of cytosine bases (MeC) in the DNA probably plays a regulatory role in differential gene expression in several tissues. We studied the changes of MeC during differentiation of promyelocytic HL-60 cells to granulocytes [63]. Genes that become transcriptionally more or less active with HL-60 differentiation were identified, cloned, and used as probes to examine changes in MeC as myeloid differentiation occurs. Over 10 genes were examined and the data suggest that methylation changes may not be necessary for differentiation of promyelocytes to granulocytes.

DNA sequences homologous to the oncogenes of RNA tumor viruses (known as cellular oncogenes, c-onc) possibly have a role in normal and leukemic myeloid cell differentiation and proliferation (Table 3). The c-onc genes are conserved in the genomes of the entire vertebrate kingdom and 23 different c-onc genes are known at this time.

The *c-myb* gene is the cellular homolog of the transforming gene of avian myeloblastosis virus (AMV) which causes acute myelogenous leukemia in chickens [64]. The gene is active in myeloid hematopoietic progenitor cells. The *myb* gene is transcriptionally active in myeloblasts (KG-1), promyelocytes (HL-60) and erythroblasts (K562) [65, 66] (Table 2). The transcription of *myb* is markedly decreased when HL-60 promyelocytes differentiate to granulocytes [65] and KG-1 myeloblasts and HL-60 cells differentiate to macrophages [66]. The *myc* gene is the cellular homolog of the avian myelocytomatosis virus strain MC29 which causes B-cell lymphomas in chickens [67]. The gene is actively transcribed in KG-1 and HL-60 cells [65, 68–70]. The transcription of *c-myc* is markedly decreased when HL-60 cells differentiate to mature granulocytes and moderately decreases when KG-1 differentiate to macrophages [66, 68]. The *c-myc* gene is amplified approximately 16 to 32-fold in the HL-60 cell line, as well as in the original uncultured leukemic leukocytes obtained from the peripheral blood of the patient from whom HL-60 was established [69, 70]. The *myc* onc gene amplification correlates with increased levels of expression of the gene and malignant transformation may have resulted from the increased expression of *myc*

gene. However, we examined *myc* gene copy number in KG-1, ML-3, and blast cells from 10 AML patients and found that the cells did not have *myc* gene amplification; therefore, *myc* gene amplification is clearly not a general mechanism of leukemogenesis [66]. The viral *fes* gene causes sarcomas in cats; the eukaryotic homolog is expressed in both HL-60 and KG-1, and the gene becomes transcriptionally less active as the cells differentiate to macrophage-like cells [66]. The combined results suggest that the *myb, myc* and *fes* genes are transcriptionally active during early stages of hematopoietic cell growth and become less active with differentiation. The cell-derived sequence homologous to the transforming genes of the Abelson murine leukemia virus (*abl*) and Harvey murine sarcoma virus (ras^{H}) are expressed at a relatively constant level in myeloblastic KG-1, KG-1 induced macrophages, promyelocytic HL-60 and HL-60 induced granulocytes and macrophages [66, 68]. These findings are consistent with the interpretation that *c-abl* and $c\text{-}ras^{H}$ genes may have a role in normal myeloid cellular functions at a variety of stages of maturation.

The role of oncogenes in leukemogenesis and normal myeloid differentiation should be defined in the next several years.

Dr. A. Lusis and myself have examined the polypeptide synthesis patterns of HL-60 cells before and after induction of the cells to granulocytes or macrophage-like cells [71]. The proteins of the leukemic cells were labelled with ^{35}S-methionine and electrophoresis of labeled samples in sodium dodecyl sulfate (SDS) was performed according to Laemmli [72], and two-dimension electrophoresis was carried out using the procedure of O'Farrell exept with a few modifications [73]. New protein synthesis was examined by autoradiography. Figure 3 shows the one-dimensional patterns for newly synthesized proteins of the human leukemia cell lines KG-1, KG-1a, ML-3, and HL-60. Each cell line exhibits a distinct pattern, and many qualitative as well as quantitative protein differences are apparent between the lines. Several of the differences between the lines could be due to genetic polymorphisms, since with the exception of KG-1 and KG-1a the lines were derived from different individuals. Most changes, however, probably represent differences in gene expression. Changes in gene expression could occur as a result of arrest at different stages of maturation or commitment to different differentiation pathways.

In the presence of dimethylsulfoxide (DMSO), HL-60 but not KG-1a, KG-1, nor ML-3 cells undergo terminal differentiation to neutrophils. A variety of changes in the polypeptide synthesis patterns of HL-60 cells were observed by electrophoresis following treatment with DMSO (Figures 3 and 4). Using two-dimensional electrophoresis, more than a dozen qualitative or large quantitative polypeptide changes could be resolved. Most of the observed changes in polypeptide synthesis pattern occurred between days 4

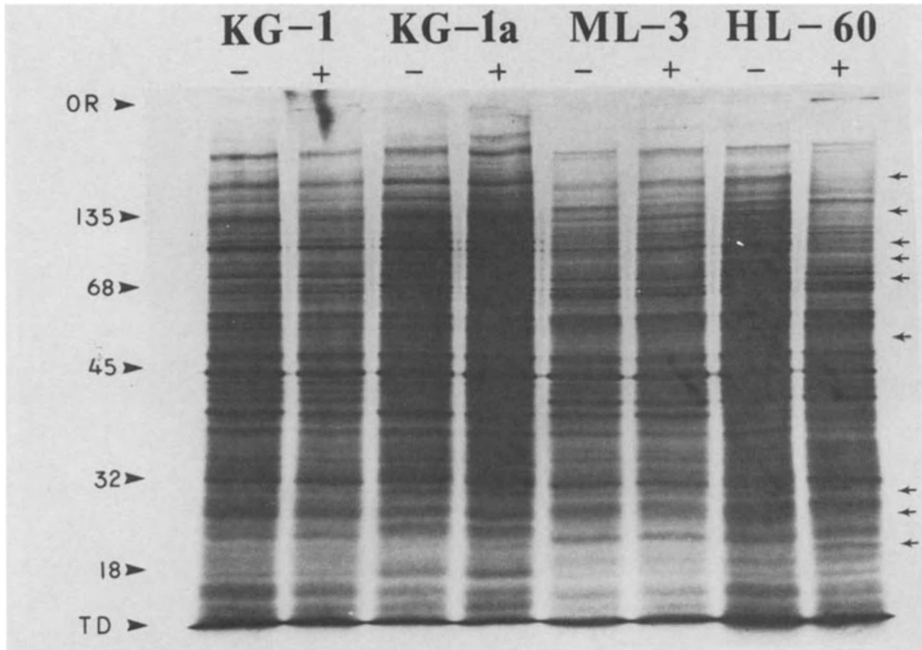


Figure 3. One-dimensional gel electrophoresis of newly synthesized proteins of various human leukemic cell lines before (−) and 7 days after (+) exposure to 1.25% dimethylsulfoxide. Proteins were labeled with ^{35}S-methionine, extracted, electrophoresed, and subjected to autoradiography. Qualitative or large quantitative differences between the two patterns are indicated by arrows.

and 6 of treatment with DMSO, and few if any changes were observed until after 3 days of treatment (data not shown). In accordance with morphological studies, DMSO treatment had little or no effect on the polypeptide synthesis patterns of KG-1, KG-1a, and ML-3 (Figure 3). Retinoic acid induced HL-60 cells to differentiate to neutrophils, and generally retinoic acid induced changes of protein synthesis of HL-60 were similar to those observed after DMSO treatment (data not shown). Treatment of KG-1 and HL-60 cells with the tumor promoter 12-0-tetradecanolylphorbol-13-acetate (TPA) has been shown by morphological and functional criteria to induce the cells to exhibit various macrophage-like characteristics [74, 75]. Numerous quantitative and approximately one dozen qualitative or large quantitative changes in polypeptide synthesis were observed between the TPA-induced and uninduced HL-60 cells (Figure 4). Our studies suggest that polypeptide synthesis patterns change with triggering of HL-60 cells to differentiate to macrophage- or granulocyte-like cells. Possibly, polypeptide synthesis patterns may be a useful means of characterizing differentiation-specific markers of blood cell progenitors.

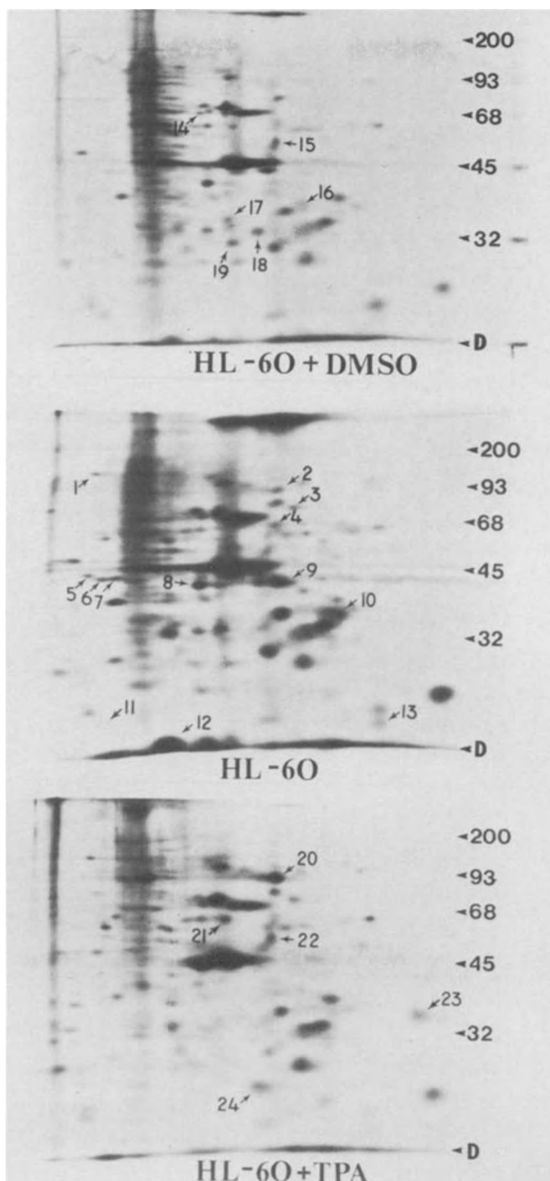


Figure 4. Two-dimensional gel electrophoresis of newly synthesized proteins of HL-60/HL-60 differentiated to granulocyte-like cells with 1.25% dimethysulfoxide (DMSO) or to macrophage-like cells with 5×10^{-8}M 12-O-tetradecanolylphorbol-13-acetate (TPA). Isoelectric focusing was from left to right (basic pH is on the left and acidic pH is on the right side of photographs) and electrophoresis in sodium dodecyclosulfate was from top to bottom. Qualitative or large quantitative differences between the 3 patterns are indicated by arrows.

3.2. Physiological Inducers of Granulocyte Differentiation of HL-60 Cells

3.2.1. *Differentiation Inducing Factor.* A member of the CSF family of molecules or a substance closely related to CSF, known as differentiation inducing factor (DIF) can trigger HL-60 cells to undergo partial differentiation to monocytes and granulocytes [76–78]. Three groups of factors are necessary for development of normal granulocytes (G) and macrophages (M): M-CSF stimulates macrophage formation; GM-CSF stimulates both macrophage and granulocyte formation; G-CSF stimulates granulocyte formation. The CSFs are required at each stage in the proliferation and differentiation sequence from committed myeloid stem cells to mature neutrophils and macrophages; therefore, in normal hematopoiesis, it is not possible to distinguish between a proliferation promoting versus a differentiation inducing action of CSF. In the majority of myeloid leukemia cell lines and fresh leukemic cells from patients, an uncoupling of proliferation and differentiation has occurred. This uncoupling in leukemic cells provides the opportunity to study the ability of DIF to induce differentiation. In several purification procedures, DIF appears to differ from both M-CSF and probably GM-CSF, but coelutes with G-CSF [76, 79–82]. Differentiation inducing factor is also known by several other names including macrophage-granulocyte inducer (MGI-2), differentiation (D) factor, granulocyte macrophage differentiation factor (GM-DF). The DIF proteins have not been purified in man, but probably have a molecular weight of 40 000 and 25 000 daltons. The cell source of DIF is not known but indirect evidence suggests that T-lymphocytes and/or macrophages may synthesize the polypeptide [76, 79, 83]. Mouse spleen cells stimulated with various mitogens produce DIF that induces murine myeloid leukemia cells (M-1) to mature into macrophage-like cells [79]. A murine myelomonocytic leukemia line (WEHI-3) can be induced to synthesize DIF which triggers differentiation of itself. Several investigators have found that conditioned medium from human blood mononuclear cells, probably T-lymphocytes, produces DIF which induces the partial differentiation of HL-60 with approximately 20% of cells differentiating toward granulocytes and 30% of cells maturing to macrophage-like cells [76, 78].

The effect of DIF on fresh human leukemic cells is unknown. Sera from many patients with active myeloid leukemia, and especially if infected, have higher than normal DIF activity when measured by the ability to induce macrophage differentiation of the murine WEHI-3 myelomonocytic leukemia line [84]. Likewise, other investigators have found that normal volunteers injected with endotoxin synthesize large quantities of DIF [83]. Infection induced endotoxemia is probably a potent stimulus for DIF production in leukemia patients. These findings are of interest because before the era of chemotherapy, spontaneous remissions occasionally occurred in

acute leukemia patients, usually following severe infections [85]. Although the studies of DIF using myeloid lines are of interest, the true importance of DIF in modulating primary myeloid leukemia is not known. Most leukemic patients receiving remission-induction chemotherapy develop bacterial and/or fungal infections and probably have a number of episodes of endotoxemia.

3.2.2. *Retinoids.* Vitamin A, its metabolites and analogs (retinoids) may participate in normal and abnormal hematopoiesis. As early as the 1920's, investigators reported that experimental animals developed marrow hypoplasia when depleted of vitamin A [86, 87]. Anemia is produced in humans who are intentionally depleted of vitamin A [88]. We found that all-*trans* retinoic acid ($3 \times 10^{-8} - 3 \times 10^{-7}$ M) stimulates at least 2-fold the clonal growth of normal human granulocyte-monocyte and early erythroid precursors [89, 90]. Retinoids appear to enhance hematopoietic proliferation by increasing the responsiveness of the stem cells to the action of CSF and erythropoietin [89, 90]. Myeloid stem cells have CSF and erythropoietin receptors on their cell membranes and retinoic acid might alter the number or affinity of the receptors. Retinoic acid could alter the membrane of blood cells by its known increase in glycosylation of membrane proteins.

In contrast to the enhancement of normal hematopoietic proliferation, retinoids inhibit carcinogenesis in a variety of *in vivo* models, suppress *in vitro* malignant transformation of certain cell lines and inhibit the growth of several tumor cell lines [91]. Retinoids induce differentiation of murine embryonal carcinoma cell lines [92]. Recent evidence suggests that retinoids are involved in differentiation of hematopoietic cells. The HL-60 cells can be triggered to differentiate to granulocytes after a 6 day exposure to 10^{-9} M all-*trans* retinoic acic [93]. Maximal HL-60 differentiation (90%) occurs at 10^{-6} M retinoic acid. Continuous exposure to the retinoid is necessary for optimal differentiation of HL-60. The 13 *cis* form of retinoic acid is equally effective as the all-*trans* form in induction of differentiation. Retinoic acid is unable to trigger differentiation of the KG-1, ML-3 or K562 cells. Retinoic acid (10^{-6} M) triggered *in vitro* differentiation of blast cells from only 2 of 21 acute myelogenous leukemic patients [48]. Each of the samples were obtained from patients with promyelocytic leukemia. The findings suggest that blast cells from patients with promyelocytic leukemia may be easily triggered to differentiate to granulocytes similar to cells from the HL-60 promyelocytic leukemia line. Presently, the weight of evidence suggests that most AML cells are not capable of substantial granulocyte maturation *in vitro* in the presence of retinoids.

Retinoids inhibit the proliferation of leukemic cells *in vitro*. The KG-1 myeloblastic leukemic cells are extremely sensitive to growth inhibition by

retinoids with 50% inhibition of clonal growth occurring at 2.4×10^{-9} M all-*trans* retinoic acid [94]. A 50% clonal growth inhibition of HL-60 is achieved by 2.5×10^{-8} M retinoic acid. Complete clonal inhibition of growth of both leukemia cell lines occurs with 1×10^{-6} M retinoic acid. Exposure of KG-1 cells to retinoic acid (10^{-7} M) for only 3 days is sufficient to inhibit all clonal growth. The all-*trans* and 13 *cis* forms of retinoic acid are equally effective in inhibiting proliferation. Retinal, retinyl acetate, and retinal (vitamin A) are less potent inhibitors. Growth of the human K562 and mouse M-1 myeloid leukemic cell lines is not affected even after exposure to 10×10^{-6} M retinoic acid.

Retinoids inhibit the *in vitro* proliferation of blast cells from acute myelogenous leukemia patients [94]. Retinoic acid inhibited the clonal growth of leukemia cells from 5 of 7 patients with acute myeloid leukemia. A concentration of $5 \times 10^{-8} - 3 \times 10^{-7}$ M retinoic acid inhibited 50% clonal growth; and 1×10^{-6} M retinoic acid inhibited 64–98% of the leukemic colonies in the sensitive patients. The 13 *cis* retinoic acid is given systemically to patients with dermatologic diseases with few side-effects and serum levels of 10^{-6} M are attainable. In a preliminary report [95], 13-*cis* retinoic acid was given to a promyelocytic leukemia patient whose leukemic cells differentiated *in vitro* in the presence of retinoids. His blood neutrophil count rose from 54 μl to 3870 μl but his bone marrow continued to contain a high percentage of malignant promyelocytes and the patient succumbed to a preexisting fungal infection. Nineteen patients with myelodysplastic syndrome received daily oral 13-cis retinoic acid in a phase I clinical trial [96]. Choeilitis and hyperkeratosis occurred in nearly all patients. Hepatotoxicity developed in patients who received more than 100 μg/M^2/day 13-cis retinoic acid. Three of 16 evaluable patients showed improvement of pancytopenia and decrease in the marrow blast count. Carefully controlled clinical trials of retinoids in the treatment of patients with preleukemia or myeloid leukemia in remission are warranted. Retinoids have no clear role in frank leukemia.

The mechanism of induction of differentiation and inhibition of leukemic growth by retinoic acid is not clear, but the following observations suggest that growth inhibition is probably more than a nonspecific toxic effect: (1) concentrations of retinoic acid that inhibit clonal growth of leukemia cells enhance clonal growth of normal human myeloid stem cells; (2) inhibition of growth of leukemia cells occurs at very low concentrations of retinoic acid; and (3) the growth of K562 and M-1 myeloid leukemia cells is not affected by high concentrations of retinoic acid (1×10^{-5} M). The recent discovery of specific cytoplasmic retinoic acid binding protein (cRABP) in various tissues has led to the hypothesis that the biological effect of retinoic acid might be mediated through this receptor. Differentiation is probably

mediated through cRABP in normal epithelial tissue and embryonal carcinoma cells. Takenaga *et al.* suggested that HL-60 cells may contain cRABP although their cold competition studies did not completely support the finding [97]. We were unable to detect cRABP in HL-60, KG-1 or leukemic blasts from patients [94]. Possibly the hematopoietic cells possess very low levels of cRABP that our assay was not sensitive enough to detect. However, other mechanisms to explain biological activities of retinoic acid should be considered. For example, retinoic acid may trigger differentiation or inhibit growth of myeloid leukemic cells by alteration of the cell membrane.

3.2.3. *Vitamin D Metabolites.* Vitamin D is a major regulator of calcium transport. The main sources of vitamin D are food and exposure to ultraviolet light. Over 20 metabolites of vitamin D have been identified; the 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the most active hormonal form of vitamin D. The effect of $1,25(OH)_2D_3$ on growth and differentiation of leukemic cells recently has been examined [98–100]. Low concentrations of $1,25(OH)_2D_3$ (10^{-9} M) slightly enhanced growth of the HL-60 cell line in liquid culture; however, at 10^{-7} M the vitamin D metabolite inhibited growth of HL-60, KG-1 and ML-3 leukemic cells. The $1,25(OH)_2D_3$ was a potent inducer of HL-60 cell differentiation to monocytes and macrophages (Table 4). As little as 10^{-10} M $1,25(OH)_2D_3$ induced maturation of 20% of HL-60 cells and 10^{-6} M of the drug triggered differentiation of nearly 85% HL-60 cells to morphological and functional monocytes and macrophages. Likewise, approximately 70% of the human ML-3 myelomonocytic leukemic cells became phagocytic and morphologically differentiated in the presence of 10^{-7} M $1,25(OH)_2D_3$. In contrast, the KG-1 cells were not triggered to differentiate by vitamin D metabolites including $1,25(OH)_2D_3$. Likewise, the $1,25(OH)_2D_3$ induced only partial differentiation of myelogenous blast cells from leukemic patients. We found that $1,25(OH)_2D_3$ triggered between 20–50% of the leukemic cells to partially or completely differentiate to monocytes and macrophages in 3 of 7 acute myelogenous leu-

Table 4. Summary of action of the vitamin metabolite, $1,25(OH)_2D_3$ on cells from leukemic cell lines

	Enhanced proliferation	Monocyte differentiation	$1,25(OH)_2D_3$ receptors
HL-60 cells	–	+	+
HL-60 blast cells*	–	–	markedly decreased
ML-3	–	partial +	+
KG-1	slight +	–	+

* A less differentiated variant of HL-60.

kemic patients. No patient with acute promyelocytic leukemia was examined. The HL-60 cells have approximately 4,000 high affinity $1,25(OH)_2D_3$ receptors in the cells [100]. Scatchard analysis showed an equilibrium dissociation constant (Kd) of 5×10^{-9} M which was close to the $1,25(OH)_2D_3$ concentration that induced 50% of HL-60 cells to reduce NBT. In contrast, a variant blast cell of HL-60 did not differentiate in the presence of $1,25(OH)_2D_3$ and had less than 10% of the number of receptors for the vitamin D metabolite as compared to HL-60 cells. The KG-1 cells had nearly the same number of $1,25(OH)_2D_3$ receptors as HL-60 but were not triggered to undergo differentiation after $1,25(OH)_2D_3$ exposure; therefore presence of D_3 receptors does not assure that the cell can be induced to differentiate in the presence of the vitamin metabolite. The $1,25(OH)_2D_3$ probably induces differentiation through initial binding to the cytoplasmic D_3 receptors but how $1,25(OH)_2D_3$ triggers differentiation after binding to its cytoplasmic receptor is not known. If analogous to steroids, the vitamin-receptor complex migrates to the nucleus, binds to a segment of DNA and alters transcriptional control of the cell.

The major side-effect of $1,25(OH)_2D_3$ is hypercalcemia. The drug can be taken orally. A trial of $1,25(OH)_2D_3$ in patients with the myelodysplastic syndrome or in AML patients in remission is appropriate.

3.2.4. *Miscellaneous Physiological Inducers of Myeloid Leukemia Differentiation.* The effect of interferon (IFN) on leukemic and normal myeloid differentiation is not as yet clear. Studies using alpha (leukocyte) and beta (fibroblast) human IFN showed that IFN alone does not induce differentiation of HL-60 or ML-3 myeloid cells, but the compounds enhance the induction of differentiation triggered by known inducers of HL-60 such as DIF, TPA and retinoic acid [101, 102]. Using alpha-2 (a_2) recombinant interferon, I found that this compound enhanced differentiation of HL-60 and ML-3 myeloblast cells in the presence of either PHA-conditioned media or conditioned media from several T-cell lines but the IFN was not able to enhance leukemic cell maturation by suboptimal concentrations of other known inducers of HL-60 such as DMSO or $1,25(OH)_2D_3$ [102]. The a_2 IFN alone or in combination with other compounds had no effect on differentiation of KG-1 early myeloblast leukemic cells. Colleagues and myself have found that γ-interferon can induce HL-60 promyelocytes to transform to macrophage-like cells and express the Ia antigen. One study on human normal bone marrow cells suggested that alpha-IFN inhibits terminal myeloid differentiation but this finding needs further confirmation [103]. The leukocyte and fibroblast IFN inhibited with similar efficacy the proliferation of both normal and chronic myeloid leukemic cells [104].

Few models exist to study the differentiation of human leukemic cells *in*

vivo. Leukemic cells can be placed in diffusion chambers in the peritoneal cavity of mice [30–32, 105–107]. Approximately 75% of HL-60 cells matured to granulocytes when placed in diffusion chambers in the murine peritoneal cavity [105]. In contrast, the KG-1 cells maintained in mice treated with cyclophosphamide, glucan or endotoxin did not change their degree of maturation despite enhanced cell proliferation [107]. An uncharacterized diffusible humoral factor stimulates differentiation of HL-60 cells, but does not affect maturation of all leukemic cells [105].

Another potential model to study differentiation, proliferation and therapy of human myelogenous leukemia cells *in vivo* is the injection of human hematopoietic cell lines into athymic (nude) or athymic-asplenic (lasat) mice [108]. The KG-1, HL-60 and K562 cells form tumors in these mice.

3.3. *Therapeutic Implications of Inducers of Granulocyte Differentiation of HL-60*

Non-physiological compounds that are capable of inducing granulocyte differentiation of HL-60 cells are usually incapable of inducing differentiation of patients' myeloid leukemic cells. Also, retinoids may induce *in vitro* the maturation of patients' acute promyelocytic leukemia cells but these compounds are not potent inducers of less mature blast cells. The 13-cis retinoic acid has improved the hematopoietic parameters in several preleukemic patients. A limited *in vitro* study of $1{,}25(OH)_2D_3$ showed that the agent induced the differentiation of less than 50% of the blast cells of less than 50% of patients. We are testing $1{,}25(OH)_2D_3$ in preleukemic patients at this time. The alpha and beta IFN by themselves do not induce the differentiation of HL-60 cells; alpha-IFN does enhance HL-60 maturation induced by DIF. The γ-IFN by itself can induce differentiation of HL-60 cells and further studies are warranted. *In vivo* studies of the effect of IFN on growth and differentiation of leukemic cells is in progress at several institutions. The influence of DIF on differentiation of myeloid leukemic cells of patients has not yet been critically examined. Purification and eventual cloning of DIF is proceeding and future testing of the ability of the compound to induce maturation of patients' leukemic blast cells will surely be attempted.

Novel approaches to use and to study biological modifiers of leukemic cell differentiation are needed. Studies in a murine leukemia model (e.g. the M-1 line) have shown that leukemic clones that are resistant to differentiation by a single compound occasionally can be induced to differentiate by combined exposure to several different compounds [109–111]. Sachs and colleagues have reported that different compounds can induce different, specific changes in gene expression of the murine leukemic clones and therefore, the induction of differentiation in resistant clones by combined expo-

sure to several compounds might be due to complementation of changes in gene expression. Sachs reports that all his murine myeloid leukemic resistant clones can be induced for at least several differentiation-associated properties *in vitro* by the appropriate combination of compounds [111]. It remains unclear if induction of only partial differentiation of leukemic cells *in vivo* will translate into a prolongation of survival. Nevertheless, studies *in vitro* using combined treatment of differentiation-resistant human myeloid leukemic cells with several compounds should be studied in an attempt to control the proliferation of the leukemic population by enforced differentiation to post-mitotic cells.

Human myeloid leukemia is a heterogeneous group of diseases with different morphologies, karyotypes, etc. Various leukemia subtypes (e.g. acute promyelocytic leukemia) may have cells amenable to induction of terminal differentiation by specific compounds. Careful studies are needed to correlate leukemic subtypes and the competence of those leukemic cells to undergo differentiation after exposure to potential inducing agents.

Another area of investigation is the development or use of more reliable and sensitive assays to study the ability of a compound to both inhibit self-renewal and induce the process of commitment to differentiation of leukemic cells. Only a minority of the leukemic cell population has the capacity for clonal self-renewal [112] and these are the cells in which we wish to induce commitment to terminal differentiation. The studies of adding various compounds to fresh AML cells in short-term liquid culture and examining for differentiation functions may not be the best assay to test agents. Most of the fresh leukemic cells in culture are replicatively inactive and terminal commitment of the few self-renewing leukemic blast cells may not be discernible either because of the rarity of these cells in the entire population or because removal of these cells from the replicative pool is not necessarily associated with either a change in morphology, or a measurable change in function. The former hypothesis assumes that the non-replicating leukemic cell population is unable to undergo differentiation. However, some evidence suggests that HL-60 cells do not have to go through the cell cycle in the presence of a compound in order to be induced by the compound [61, 113, 114].

A clonal assay of leukemic cells may be an effective means to identify potential compounds that will inhibit self-renewal and induce terminal differentiation of the blast cells. This system has been used effectively by Metcalf and colleagues to study the induction of differentiation of cells from a murine myelomonocytic leukemic cell line known as WEHI-3 [115]. A difficulty in interpreting human leukemic cell maturation in soft-gel cloning assays, however, relates to the possibility that the normally differentiated colonies arise from residual nonleukemic precursors. This problem is of

major concern because low cloning efficiencies are usually observed in soft-gel cultures. Without a cytogenetic or enzymatic marker, the origin of the mature cells in the colonies remains in question.

Leukemic cell lines provide a unique model to study differentiation of blast cells to granulocytes and macrophages and provide the opportunity to examine the genetic defects that may be responsible for the maturational arrest of leukemic cells *in vivo*. A strong note of caution is needed concerning the therapeutic role of agents that are able to induce HL-60 differentiation. The HL-60 cells, similar to Friend cells of the erythroid lineage are blocked at a more mature stage of development than cells from most myeloid leukemic patients. Leukemic promyelocytes (HL-60) are poised on the edge of undergoing terminal differentiation. The evidence suggests that compounds that trigger differentiation of HL-60 and fresh promyelocytic leukemia cells are usually not capable of triggering differentiation of leukemic cells of the great majority of patients. Acute myelogenous leukemia usually is a neoplasia of the myeloid pluripotent hematopoietic stem cell and compounds that trigger differentiation of promyelocytic leukemia cells may not be expected to induce maturation of less differentiated cells.

Careful, controlled patient trials of agents that trigger differentiation of HL-60 are possibly appropriate, especially in patients with preleukemia, AML in remission, and maybe in frank leukemia when the inducing agent is used in combinations with chemotherapy. Preleukemic patients represent a particularly interesting group for testing of possible inducing agents. Data suggest that neoplastic preleukemic cells can mature *in vitro* and while preleukemic cell differentiation is less than normal it is clearly greater than that seen in acute myelogenous leukemia cells [116]. No effective therapy exists for preleukemic patients at this time. The progression of their disease to acute myelogenous leukemia is usually slow enough to allow a trial of a potential inducer of differentiation with appropriate monitoring for changes of hematopoietic parameters including the per cent of karyotypically abnormal marrow cells. When studying frankly leukemic patients, the ability of the compound to induce differentiation of the neoplastic blasts cells should be examined *in vitro* before testing the agent *in vivo*. At this time the therapeutic role of biological inducers of granulocyte differentiation of HL-60 remains undefined.

4. INDUCTION OF MACROPHAGE-LIKE DIFFERENTIATION OF HUMAN ACUTE MYELOGENOUS CELLS

4.1. *Phorbol Diesters*

The tumor promoting phorbol diesters including 12-0-tetradecanoyl-phorbol 13-acetate (TPA) induce human AML cell lines blocked at the

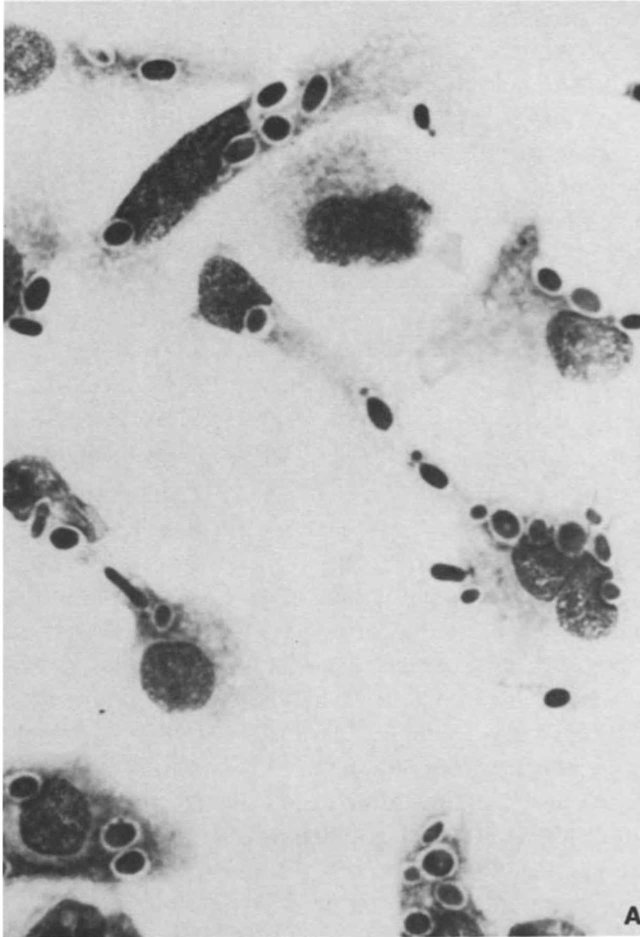
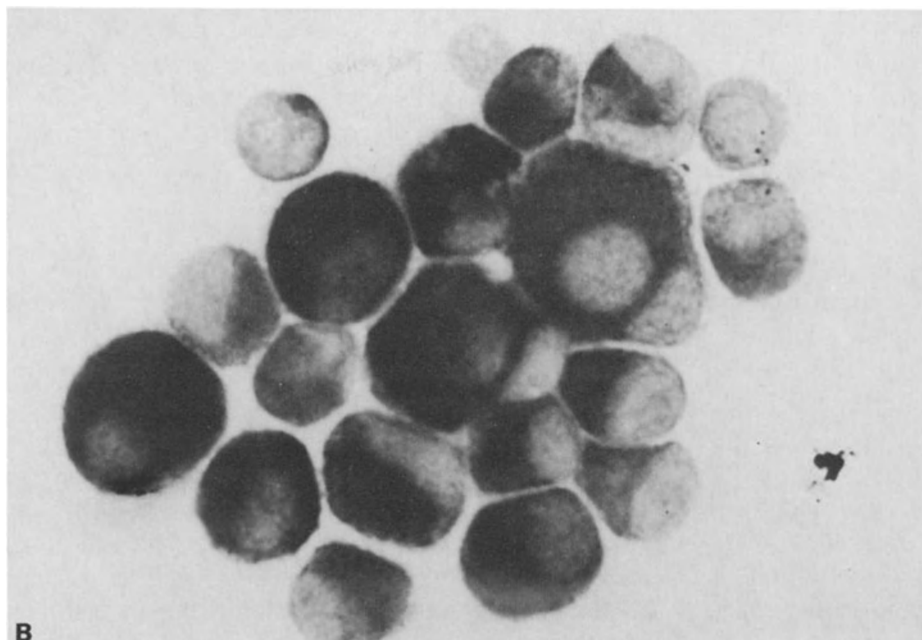


Figure 5. Macrophage-like differentiation of KG-1 cells after 3 days exposure to 5×10^{-8}M TPA:
5a.) Giesma stain of KG-1 cells that have phagocytosed yeast.

myeloblast-promyelocyte stage of maturation (KG-1, ML-3, HL-60) to differentiate to macrophage-like cells [74, 75, 117] (Figure 5). In the presence of 10^{-7} to 10^{-8} M TPA, the cells become adherent (95%), develop pseudopodia (40%), display macrophage characteristics by light microscopy (95%), synthesize nonspecific acid esterase (95%), phagocytize yeast (60%), slightly reduce nitroblue tetrazolium (NBT, 40%), display Fc immunoglobulin G receptors (20%) and kill bacteria. Lysozyme secretion and enzyme activity for beta-glucuronidase and acid phosphatase increase 2- to 20-fold concomitant with macrophage differentiation. The cells cease to divide after 10^{-8} M TPA exposure and macrophage differentiation of the blood cells does not



5b.) Staining of KG-1 cells for non-specific acid esterase activity.

require DNA synthesis [113, 114]. The myeloid leukemia cells blocked at a less differentiated myeloid blast stage of maturation (KG-1a, K562) are completely resistant to TPA-induced macrophage differentiation [75], and likewise the proliferation of the cells is not affected by phorbol diesters. The phorbol diesters probably trigger differentiation through an interaction with high affinity phorbol diester receptors on the cell membrane of the leukemic cell [118–120]. However, expression of the receptors does not assure responsiveness to the compound. The KG-1a and S-95 are less mature variants of KG-1 and HL-60 cells, respectively and do not differentiate after exposure to phorbol diesters; nevertheless, the variant cells have the same number ($\approx 5 \times 10^5$) of high affinity (Kd $\approx$ 2 nm) phorbol receptors as the parent lines [118–120]. Absence of downregulation of phorbol diester receptors by the variant cells has been suggested as the cause of lack of differentiation of these cells [118]; however, Cooper [119] and we [120] have found little down-regulation of phorbol diesters by either the differentiating or nondifferentiating leukemic lines. Studies suggest that the phorbol diester receptor may be a protein kinase C [121].

Liquid culture studies have shown that acute myelogenous leukemia cells from almost all patients can undergo macrophage-like differentiation after 2–3 days exposure to TPA (10^{-7}–10^{-8} M) if the fresh leukemic cells are blocked at the myeloblast, myelomonoblast, monoblast or promyelocyte

stage of development [122, 123]. The TPA-exposed leukemic cells become adherent (90%) and develop pseudopodia, phagocytize yeast (30%), and slightly reduce NBT (40%). Leukemic cells from patients with less differentiated myeloid leukemia do not undergo macrophage-like maturation after TPA exposure [123].

4.2. *Teleocidins*

Recently, a naturally occurring tumor promoter, known as teleocidin B, has been isolated from the mycelia of Streptomyces [124]. Teleocidins are indole alkaloids and, although chemically distinct from the phorbol diesters, exhibit the same effects *in vitro* as phorbol diesters. Specifically, teleocidins inhibit the binding of tritiated phorbol dibutyrate to specific cell surface receptors, compete with epidermal growth factor for its receptor on fibroblasts, stimulate DNA synthesis in murine fibroblasts exposed to various growth factors, induce ornithine decarboxylase activity in mouse skin, aggregate human lymphoblastoid cells and inhibit DMSO induced terminal differentiation of Friend erythroleukemia cells. Others as well as us have studied the effects of teleocidins on a number of human leukemic cell lines *in vitro* [125, 126]. The myeloid cell lines unresponsive to differentiation with phorbol diesters (KG-1a, U937, K562) are also unresponsive to teleocidins. Similarly, HL-60, KG-1 and ML-3 cells, which express macrophage functions after exposure to TPA, differentiate into macrophage-like cells after exposure to teleocidins. The concentration of teleocidin (5×10^{-8} M) and the time course (3–4 days) required for leukemic cell differentiation are similar to that seen with TPA. Teleocidin in nanomolar amounts is able to competitively inhibit the binding of phorbol diesters to their cell surface receptors on the HL-60 promyelocytic leukemic cells. Teleocidin is also able to induce macrophage differentiation of patients' leukemic cells blocked at the myeloblast and myelomonoblast stage of maturation [126]. The induction of macrophage maturation of leukemic cells of patients by teleocidin was comparable to induction of maturation induced by TPA, both in the percent of cells induced to mature (50–70%) and the concentration (5×10^{-8} M) of teleocidin required to trigger maturation. The phorbol diester and teleocidin studies provide evidence that myelogenous leukemia cells that are morphologically at the myeloblast and promyelocyte stage of maturation are not irreversibly blocked in differentiation, but can be induced to develop into macrophage-like cells. Also, the capacity of the leukemic cells to differentiate in the presence of TPA implies the genetic information for terminal macrophage differentiation is present and capable of expression in these cells.

Could phorbol diesters or teleocidins have therapeutic value in the treatment of leukemias? Several facts suggest not: 1) TPA in low concentrations

(10^{-11}–10^{-10} M) in the presence of CSF stimulates the clonal proliferation *in vitro* of human cells from patients and the KG-1 leukemic cell line [127, 128]. Also low concentrations of TPA (10^{-12}–10^{-10} M) increase the number of cells in myeloid colonies derived from chronic myelogenous leukemia patients [129]; 2) TPA inhibits normal human myeloid colony formation almost in parallel with the inhibition of leukemic clonal growth *in vitro*, suggesting little therapeutic advantage of these inducers [131, 132]. This is only a theoretical limitation because most chemotherapies that produces leukemic remissions do not usually have a differential kill of leukemic versus normal hematopoetic cells; 3) phorbol diesters and teleocidins are the most potent tumor promoters known at this time [132]. Tumor promotion usually requires frequent exposure to the promoter and usually causes tumor formation only after preexposure to a carcinogen. Nevertheless, these agents are probably risky to give to man; 4) phorbol diesters have very strong inflammatory activity; 5) the parent alcohol of phorbol diester induces lymphoblastic leukemia in SWR mice [133]; 6) both TPA and teleocidin induce differentiation of leukemia myeloblasts, promyelocytes, and myelomonoblasts but the agents do not trigger differentiation of less mature cells. The pluripotent myeloid stem cell is probably involved in the leukemic clone in many patients, and the phorbol diesters probably have little effect on maturation of these cells.

5. GENETIC ANALYSIS OF MYELOID DIFFERENTIATION

Genetic analysis using Friend cells and M-1 cells and their mutant subclones demonstrate that cell inducers do not act by a common mechanism. The studies have also shown that different stages of differentiation are under separate genetic controls. For example, the use of M-1 mutants that differ in their competence to be induced by MGI-2 has shown that there are separate controls for the induction of receptors for C3 and Fc, lysozyme synthesis, and formation of mature granulocytes and macrophages. Mutant clones with various capacities to differentiate have not yet been identified in the human HL-60 line. Several sublines of HL-60 which are more immature than the parental cells have been established [134, 135], but the sublines cannot be induced as yet by any compound to undergo differentiation.

Somatic cell hybridization provides a method to examine genetic control of gene regulation. The intraspecific hybrid of two specialized cells might express the phenotype of both parents, only one of the parents, neither parent, or activate new genes not normally expressed by one of the parents. Our laboratory has studied the expression of tissue specific features in hybrids between a human myeloid cell (HL-60) or erythroid cell (K562) and a

human Burkitt's lymphoma cell (P3HR-1) [136, 137]. Two hybrid lines, HP-1 and HP-2, and 18 hybrid clones of the myeloid HL-60 and the lymphoid P3HR-1 were established and confirmed by karyotype, isozyme, and surface antigen analyses. The hybrids extinguished the 10 myeloid (HL-60) features that were examined including myeloid morphology, histochemistry and functions that included response to colony stimulating factor and ability to differentiate to granulocytes or macrophages. In contrast, the hybrids synthesized immunoglobulin and expressed Epstein-Barr nuclear, early, and viral capsid antigens similar to the P3HR-1 lymphoid parental line. In contrast, the hybrids of P3HR-1 lymphocytes and the erythroid-myeloid cells (K562) expressed only the K562 phenotype including fetal and embryonic hemoglobin but did not express any of the lymphoid tissue specific genes. The combined findings on hybrids of human hematopoietic cells found that differentiated gene expression of only one parental type could be expressed at one time. The results suggest that phenotypic differences between human myeloid and lymphoid cells in the hematopoietic lineage involve mutually exclusive programs and may possibly be mediated by the activity of diffusible, trans-acting molecules.

6. HORMONAL MODULATION OF LEUKEMIC CELL GROWTH

The granulocyte and/or macrophage colony stimulating factor (CSF) is the name given to a family of molecules that stimulate hematopoietic precursor cells to proliferation in semisolid culture to form colonies containing granulocytes and/or macrophages. Colony-stimulating factor stimulates colony formation by the HL-60 and KG-1 cells *in vitro.* A striking characteristic of the KG-1 cells during their early passages (usually less than 75 passages) is their nearly complete dependence on CSF for colony formation in soft-gel culture [4]. In the absence of added CSF, only a rare KG-1 colony forms in soft-gel culture. There is a clear dose-response relationship between CSF concentration, and the number of colonies formed. At optimal CSF concentrations, the KG-1 cells have a cloning efficiency of 3%. The KG-1 cells also respond to CSF exposure in liquid culture with an increased ^{3}H-thymidine labelling index (L1) and an increased rate of precursor incorporation into RNA and DNA [5, 138]. We have utilized the CSF-dependent stimulation of thymidine incorporation by KG-1 to develop a sensitive microassay for human CSFs; the assay is quantitative and requires only 1 day as compared with 10–14 days for conventional colony formation assays [138]. CSF has no effect on KG-1 cell maturation as judged by morphology, functional tests and 1–2 dimensional electrophoresis of *in vivo* ^{35}S-methionine labelled proteins before and after CSF exposure. The cells have no dramatic change in

cAMP and cGMP levels after exposure to CSF [139]. A stable subline of the KG-1 line (KG-1a) spontaneously developed from the parent line and shows little response to CSF [5]. These cells are morphologically and functionally undifferentiated blast cells. The cells retain a number of constitutive markers of the parent cells. The HL-60 cells are capable of forming colonies of promyelocytes in the absence of any added factor with a plating efficiency of approximately 3%. The addition of CSF increases colony number approximately 3–4 fold in very early passages [5, 140]. The K562 cells form colonies in soft-gel culture, but do not respond to CSF. In later passages (>75) both HL-60 and KG-1 form an increased number of spontaneous colonies [139, 140]. The number of CSF stimulated colonies decreased over the same period of time. The KG-1 and HL-60 lines and their variants provide a homogeneous cell population to study the mechanism of CSF action at the cellular level and to investigate cellular CSF receptors.

The effect of other hormones on myeloid leukemia cell proliferation has not been extensively studied. The KG-1 and HL-60 clonal proliferation *in vitro* is inhibited by prostaglandins of the E series, but not of the F group [58]. The KG-1 cell growth is inhibited by dibutyryl cyclic AMP and the extracellular agonists of this nucleotide, but not by dibutyryl cyclic GMP and its extracellular stimulators. These findings parallel the response of human granulocyte-monocyte progenitor cells (CFU-C). Likewise, KG-1 clonal growth is slightly inhibited by pharmacologic concentrations of dexamethasone (10^{-7} M), while HL-60 cells are resistant to the effect of glucocorticoids [141]. Both KG-1 and HL-60 have high affinity (Kd = 3–4×10^{-9} M) glucocorticoid receptors with a mean of 10,300 and 15,600 ^{3}H-dexamethasone binding sites per cell, respectively [141]. These binding data are comparable to the glucocorticoid receptor activity seen in acute lymphocytic leukemia cells. In contrast to ALL cells, however, no correlation was observed between the number of glucocorticoid receptors and *in vitro* inhibition of myelogenous leukemic cell proliferation. Dexamethasone inhibits the expression of Fc receptors on HL-60 cells [142] and has been reported to increase the number of surface receptors for the f-met-leu-phe chemotactic peptide after exposure of the HL-60 cells to DMSO or dimethylformamide but not retinoic acid [142–144].

The HL-60 cells have receptors for both insulin [145] and estrogen [146]. Insulin is needed to grow HL-60 cells in serum free media and estradiol (10^{-9}–10^{-7} M) has been reported to stimulate growth of HL-60 cells [146]. One report suggests that HL-60 cells can produce small amounts of immunoreactive calcitonin [147].

Can leukemic cells make and respond to a self-renewal factor? The ability of adherent cells to stimulate their own growth is well described. In 1954, Earle found that CM from adherent fibroblast cells slightly stimulated the

multiplication of fibroblasts [148]. Puck, in 1955, noted that irradiated non-dividing HeLa cells produced a short-lived diffusible factor which stimulated the multiplication of single HeLa cells arranged in close juxtaposition to the irradiated feeder layer [149]. Recently, a series of low molecular weight, acid-stable, polypeptides have been isolated from neoplastic and normal adherent cells [150, 151]. The polypeptides cause non-transformed, adherent indicator cells to lose density dependent inhibition of growth in monolayers and to form colonies in soft agar. Hypothetically, cells might escape normal growth controls by endogenous production of growth factors for which they have receptors and to which they can respond. As previously suggested, these growth polypeptides might be called autocrines because the production of the polypeptides might serve as a constant stimulus for cell division by the cell [150, 151].

Little is known about the ability of myeloid hematopoietic cells to synthesize their own self renewal factors. Proliferation of normal and leukemic myeloid stem cells *in vitro* is absolutely dependent on CSF [115, 116]. A group of related CSF molecular species exists [152, 153]. Likewise, a variety of cell types can synthesize CSF: macrophages, mitogen-stimulated lymphocytes, fibroblasts and endothelial cells.

An appealing hypothesis has been that leukemic cells might respond preferentially to a minor subset of CSF or even an abnormal CSF, perhaps of leukemic origin. One investigative group has suggested that normal and leukemic human peripheral blood leukocytes can produce low molecular weight growth factors able to stimulate myeloid clonal proliferation, and the growth factors produced by the leukemic cells may differ from those produced by the normal cells [154]. To date, no other data has shown that a unique form of CSF exists in leukemic cells from patients.

Cells from patients with acute myelomonocytic and monocytic leukemia can synthesize CSF [155, 156]. This finding, however, falls short of conclusively demonstrating that the CSF production arose from cells of the neoplastic clone and not from the normal clone of nonleukemic cells. Peripheral blood cells from patients with acute myeloblastic leukemia usually do not produce CSF [156, 157].

Myeloid leukemia cell lines provide evidence that leukemic cells can produce self renewal factors. A murine myelomonocytic leukemia cell line, WEHI-3, produces a growth factor, probably CSF, that stimulates the clonal growth of itself and normal murine myeloid stem cells [157, 158]. Brennen *et al.*, found that the human promyelocytic leukemia HL-60 cells produced an approximate 13,000 dalton growth factor(s) which stimulated the clonal growth of HL-60 cells, but had no stimulatory effect on normal human or mouse bone marrow GM-CFC [122]. In contrast, the human myeloblast leukemia, KG-1, cells can secrete a growth factor(s) which stimulates self

renewal and also stimulates clonal proliferation of CSF responsive normal and leukemic cells but cannot stimulate growth of CSF unresponsive cells [159]. The KG-1 growth factor has not yet been purified but the data suggest the factor is a member of the CSF family.

Autostimulation of tumor cell growth might occur in hematopoietic malignancies. The malignant cells might inappropriately synthesize a growth factor for which the cell has receptors. Production of the factor may enhance cell division and serve as a continuous endogenous stimulus leading to sustained, inappropriate proliferation. Myeloid leukemia cells *in vivo* possibly synthesize a minor subset of the CSF molecules which provides the cells with a proliferative advantage over the normal hematopoietic clone. To date, however, no convincing data suggest that leukemic cells can synthesize a unique CSF.

7. AN IN VITRO MODEL FOR CHEMOTHERAPY OF ACUTE MYELOGENOUS LEUKEMIA

Tumor cells of therapeutic importance are those capable of sustained replication. Known as clonogenic cells, they frequently comprise 1% or less of the entire tumor mass. The *in vitro* colony formation assay allows detection and study of these clonogenic cells.

The development, characterization, and testing of different chemotherapeutic agents is frequently performed on lower mammalian cells, especially on rodent lymphoma and leukemia cells. Some drugs act on neoplastic human cells differently from their action on rodent cells. Study of the effect of various chemotherapeutic agents on human acute myelogenous leukemia (AML) clonogenic cells *in vitro* has been impeded by technical difficulties. Often, only limited proliferation occurs *in vitro* and those cells that do proliferate may be derived from the normal hematopoietic clone. The KG-1 and HL-60 leukemia cells form colonies in soft-gel culture and provide a model to study the effect of various schedules and combinations of chemotherapeutic agents [160–162].

In one study the KG-1 cells were used to test the effect of various schedules and combinations of chemotherapeutic agents [160]. For comparison, the drug sensitivity of normal human marrow myeloid clonogenic cells was tested. Cytosine arabinoside inhibited both the KG-1 and normal human colony-forming cells (CFC) approximately 25% after a 2-hour exposure of 0.1 μg/ml, 50% after a 5-hour exposure of 1.0 μg/ml, and 90% after a 24-hour exposure of 1.0 μg/ml. Daunorubicin had nearly an equal cytotoxic effect on KG-1 and normal marrow CFC after a 2- to 72-hour exposure to the drug. Daunorubicin at 0.15 ug/ml produced nearly complete inhibition

of colony-forming cells. The antileukemic agent 5-azacytidine at a concentration of 0.1 ug/ml produced approximately 60% inhibition of colony formation. Cytidine partially rescued CFC when the nucleoside was added in seven-fold excess to cultures containing 5-azacytidine. Leukemic and normal marrow clonogenic cells have nearly the same sensitivity to each chemotherapeutic agent and combination examined.

The leukemia cells are a pure cell population that permit investigation without repeated marrow aspiration and cell separation. The clonogenic assay using human myeloid leukemia cell lines, however, is not without limitations. The *in vitro* assay does not allow biotransformation of chemotherapeutic agents into active or inactive metabolites. Cells from leukemia cell lines are immortalized and therefore differ from leukemia cells *in vivo.* The KG-1 cells have a doubling time analogous to leukemic blasts *in vivo,* but the kinetics of the clonogenic cells *in vitro* may not reflect the cytokinetic heterogeneity of tumor cells *in vivo.* Even with these limitations, the clonogenic human AML cell lines may provide a useful model for detection of biologically active new anti-cancer agents, for the development of new chemotherapeutic regimes, and for the investigation of various modes of drug resistance in leukemia cells.

8. CONCLUSION

Leukemic cell lines provide a unique model to study leukemic cell proliferation and differentiation, provide a system to study differentiation of blast cells to granulocytes and macrophages and provide a homogeneous population of cells with different proliferation potentials to CSF which should help elucidate mechanisms of action of CSF. The HL-60 cells provide a unique model for granulocyte differentiation. The HL-60 cells are more mature than committed stem cells, and represent the later stages of granulocyte maturation. The cells probably do not allow studies of the process of commitment to the granulocyte versus macrophage line of differentiation. The HL-60 line has only a limited response to normal hormonal regulation such as CSF. Likewise, the cells are unable to express proteins normally seen in the secondary granules such as lactoferrin, leukocyte alkaline phosphatase or vitamin B_{12} binding protein. This means that the HL-60 cells may not be useful for the study of certain aspects of normal proliferation and differentiation of granulocyte precursors. Likewise, as mentioned earlier, leukemic promyelocytes are poised on the edge of undergoing terminal differentiation. Inducers of HL-60 cells and leukemic promyelocytes from patients are usually not inducers of the great majority of human leukemic cells which are blocked at an earlier stage of differentiation. Caution

must be exercised in extrapolating the *in vitro* data showing that a variety of compounds can trigger differentiation of HL-60. Probably most fresh leukemic cells cannot be triggered to differentiate and to leave the proliferative pool after exposure to known inducers of HL-60 differentiation.

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3. Biological Markers in Lymphoproliferative Disorders

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1. INTRODUCTION

Lymphoproliferative disorders include, in the broadest sense, all neoplasias of lymphoid cells, from immature lymphoblasts to mature-looking lymphocytes, even when they transform to immunoglobulin (Ig) secreting cells or plasma cells. In clinical practice, however, this concept refers mainly to chronic lymphoproliferative disorders and these encompass several B and T cell leukemias, some non-Hodgkin lymphomas (NHL) with bone marrow and blood involvement, such as follicular lymphoma (centroblastic/centrocytic) and cases showing lymphoplasmacytic differentiation, including Waldenstrom's macroglobulinemia. Multiple myeloma can be excluded from this group because of its distinct clinical manifestations and the easily identifiable nature of its proliferating cells. Acute lymphoblastic leukemia (ALL), with its unique clinical, biological and epidemiological features, can also be considered as a separate group. This disease is a neoplasia arising from lymphoid precursors and is best studied with other forms of acute leukemia.

Biological markers reflect differences between cell types and stages of maturation which are not readily recognized by the methods used for routine diagnosis, e.g. cytomorphology and histology. It is in the characterization of lymphoid cells (mature and immature) that membrane and enzyme markers have made the greatest contribution. It is now possible, by means of immunological methods, to recognise antigens, Ig molecules and receptors on the cell membrane that are specific for different lymphocyte subsets. In the context of this Chapter we will also refer to chromosomes as biological markers in B and T cell malignancies because the abnormalities observed in these conditions are highly specific and are often not seen in proliferative disorders of other cell lineages.

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2. IMMUNOLOGICAL MARKERS

Before the development of the hybridoma technology, the only reagents available for studying the membrane phenotype of lymphocytes were rosetting tests and heteroantibodies to human Ig [1]. Whilst the use of these 'conventional' markers has persisted, monoclonal antibodies (McAb) are now in the forefront of immunological research and clinical practice [2]. A large number of McAb are now available for the purposes of cell marker studies. Many react with well defined antigens which are specific for certain cell lineages or even particular stages of maturation, others detect antigens more widely distributed but which, when studied as part of a battery of tests, can provide diagnostic information. Ahead of these developments are studies on the molecular biology of Ig genes which can demonstrate the rearrangement of these genes as the earliest sign of B-cell differentiation [3]. Such studies have now shown conclusively that in non-T ALL, that includes common-ALL and null-ALL (absence of the common-ALL antigen), there is rearrangement of the heavy chain Ig genes before the expression of B-cell antigens and Ig molecules on the cell membrane [4]. Thus, lymphoblasts in non-T ALL are precursor cells committed to B-cell differentiation, whilst in T-cell ALL no such Ig rearrangement has been demonstrated [4]. Similar studies in other lymphoid malignancies may clarify their cell origin when this is not clear from cell marker studies. A good example is hairy cell leukemia (HCL) whose cell nature has been debated in the last 10 years. Korsmeyer *et al.* [5] have shown in elegant studies the rearrangement of Ig genes for heavy and light chains, as predicted by the expression in most cases of monoclonal Ig on the cell membrane (SmIg). Work by L. Foroni and L. Luzzatto at our Institution (to be published) has confirmed that Ig genes are always rearranged in B-lymphoproliferative disorders, including HCL, even in exceptional cases in which SmIg or cytoplasmic Ig (CyIg) are not demonstrable in the malignant B-cells.

2.1. *Conventional B and T Markers*

The demonstration of SmIg on B-lymphocytes and of receptors for sheep erythrocytes (E-rosettes) on T-cells over 10 years ago constituted a major advance with profound implications in the diagnosis and classification of lymphoid malignancies. The morphological characterization was linked for the first time to the immunological analysis and as a result the heterogeneity of lymphoproliferative disorders became apparent. Further progress was the demonstration of receptors for mouse erythrocytes on B-lymphocytes [6] which became a useful marker (M-rosettes) for the diagnosis of chronic lymphocytic leukemia of B-cell type (B-CLL) [5–8].

These rosetting methods [1] are still widely used and have retained their

high specificity for T-cells (E-rosettes) and B-cells (M-rosettes), particularly after further developments in the methodology. For example E-rosettes can be best demonstrated by using AET-treated sheep erythrocytes [9] and this has increased the sensitivity of this method for the detection of immature T-cells (T-ALL). In some chronic T-cell malignancies we have observed that the receptor for sheep erythrocytes needs to be exposed by pretreatment of the lymphocytes with neuraminidase. A similar procedure enhances the binding of M-rosettes in B-CLL [7]. A McAb against the E-receptor (T11) is now available [10] and its use can substitute the need for performing this rosetting test in many cases.

The combination of M-rosettes and SmIg is still valuable for the differential diagnosis between B-CLL and other B-cell disorders (Table 1). In a study of more than 1000 samples from lymphoproliferative disorders tested in our laboratory, 70% of which were CLL, we have observed a weak expression of SmIg and a high percentage of M-rosettes (usually > 50%) in the majority of cases (> 95%) classified as B-CLL. A reverse pattern: strong SmIg and low or negative M-rosettes are features of the other B-cell leukemias. Performing M-rosettes may be inconvenient because of the requirement of relatively fresh mouse erythrocytes (up to 2 days old). Nevertheless, this test is particularly useful in B-CLL when SmIg are undetectable (5–10% of cases) although the B-cell nature of the lymphocytes can be demonstrated by a wide range of McAb (see below). A high proportion of M-rosettes (rarely over 50%) can occasionally be shown in hairy cells [12] and in follicular lymphoma (FL) cells [13]. The binding of mouse erythrocytes to B-cells with weak SmIg has been used as an argument for the immature nature of B-CLL lymphocytes [8, 14] and to suggest their possible derivation from a small subpopulation of germinal centre cells [15].

The most common SmIg class in B-CLL is IgM, often sharing a single light chain with IgD [16]. In contrast, HCL cells have multiple heavy chain isotypes but always share a single light chain [17, 18]. The demonstration of Ig on cytoplasm of fixed cells (CyIg) is also used as a marker of B-cell differentiation. Pre-B-cells lack SmIg but have a rim of cytoplasmic μ

Table 1. B-cell markers in B-lymphoproliferative disorders

Marker	B-CLL	B-PLL	FL	LPL	HCL
M-rosettes	++	−/+	+	−/+	−/+
SmIg (Intensity)	±	++	++	++	++

B-CLL: B-chronic lymphocytic leukemia; B-PLL: B-prolymphocytic leukemia; FL: follicular lymphoma (centroblastic/centrocytic); LPL: lymphoplasmacytic lymphoma, including Waldenström's macroglobulinemia; HCL: hairy cell leukemia.

chain; plasma cells at the other extreme of the B-cell maturation pathway, have, characteristically, heavy and light chains in the cytoplasm but no SmIg. Cells at intermediate stages of B-cell maturation have variable amounts of CyIg. This is rare in B-CLL (10% of cases) and more frequent in B-prolymphocytic leukemia (B-PLL) and lymphoplasmacytic lymphoma (LPL). In general, there is a good correlation between the presence of paraprotein in the serum and the degree of CyIg staining in the neoplastic B cells, the later reflecting the late maturation stage of these cells.

2.2. *McAb to B-Cell Antigens*

A wide range of reagents is now available and this has resulted in an improved knowledge of the maturation stages of B-lymphocytes and in a better characterization of the neoplasias which arise from B-cells 'frozen' at particular maturation stages. As shown in Table 2, many McAb are specific for B cells but others (i.e. FMC4, FMC8, RFB1) are also expressed in cells

Table 2. Monoclonal antibodies against B-cell antigens [1]

McAb	Specificity	Mol wt*	Reference(s)
FMC4**	HLA-Dr; all B-cells, one-third of monocytes	28–33 complex	19
FMC7	50% PB B-lymphocytes	unknown	20, 21
FMC8 ‡	B-cell precursors; most PB B-cells; platelets, monocytes, granulocytes	24	22, 23
RFB1	Precursor cells; T-lymphocytes; negative in PB B-cells	35 ‡‡	26, 27, 28
J5	Common-ALL antigen; immature BM cells (TdT+); follicular centre cells	100	29, 30, 13
B1	B-cell precursors; all B-cells	35	31, 32
B2	Most B-cells; negative in plasma cells (as B1 and B4)	140	33
B4	Early B-cell precursors; most B-lymphocytes	40 & 80	34
Y29/55	PB and tissue B-cells	unknown	36
BA-1	B-cell precursors; B-lymphocytes	30	36, 37

1 Selected list of reagents referred to in this chapter.
* Mol wt: molecular weight in kilo daltons.
** Equivalent McAb: OKIa (Orthoclone); HLA-DR (Becton Dickinson).
‡ Similar McAb: BA-2 (24) reactive with a p24, and CALL 1 (25), cytotoxic antibody reactive with a p26.
‡‡ Dr. M.F. Greaves, personal communication.
PB: peripheral blood; BM: bone marrow.

from other lineages. Some antibodies are B-cell specific (i.e. FMC7) but react only with a subset of these cells (in this case only late maturation stages) [23]. Several other McAb (i.e. B4, B1, BA-1, J5) are expressed on B-cells from early stages of differentiation before the development of CyIg or SmIg, but in cells in which the Ig genes have been already rearranged [4]. Some of these McAb persist on B-lymphocytes through all maturation stages (i.e. B1, BA-1, FMC4) thus being generally considered as reliable pan-B reagents, but are not expressed on plasma cells. The value of a particular McAb in the study of B-lymphoproliferative disorders will depend on its B-cell specificity, the range of cells it reacts with (mature or immature), and the particular cell(s) that it helps to define. For example FMC8 [22, 23], a McAb with which we have experience and its equivalent BA-2 [3], is positive in 80% of ALL but it reacts also with 60 to 70% of acute myeloid leukemias (AML) and with 25% of T-ALL. In contrast, the McAb B4 reacts with all non-T ALL and appears to be negative in other acute leukemias including T-ALL [34].

The McAb J5 has a similar reactivity to the rabbit anti-ALL serum [1] originally described by Greaves; both reagents precipitate a similar glycoprotein (gp 100 or common-ALL antigen) [30]. This reagent has been used for many years to define a distinct form of ALL, common-ALL [1] with characteristic clinical and prognostic features. In recent years, and as a result of studies in tissue sections with J5, the common-ALL antigen has been

Table 3. Monoclonal antibodies in B-lymphoproliferative disorders*

McAb	B-CLL	B-PLL	FL	LPL	HCL
T1, Leu1**	**+**	−/+	−	−/+	−
RFB1	**+**	−/±	−	−/+	**++**
FMC7	−/+ ‡	**++**	+	**++**	**++**
RFA4	−	**+**	−	−/+	**+**
HC1 & HC2	−	−	−	−/+	**+**
anti-Tac	−	−	−	−	**+**
J5	−	−	**+**	−	−
HLA-Dr	+	++	++	−/+	++
B1	+	+	+	−/+	+
B2	+		−/+	−	
B4	+		+	−	
T10	−	−	−/+	**+**	−

* Abbreviations as in Table-1

** And other McAb (RFA-1, UCHT2, T101, etc.) that react with a gp complex of 65–69,000 dalton mol. wt.

‡ Positive in cases of B-CLL in 'prolymphocytoid transformation'.

Blank spaces = no information available; the characteristic phenotype of a disease is shown in bold type.

Table 4. Results with McAb FMC7 in B-cell leukemias (180 cases)

Disease	Number	Positive*	(%+)
B-CLL	101	21	(21%)
B-PLL	31	27	(87%)
HCL	20	20	(100%)
LPL	7	6	(86%)
NHL**	21	10	(48%)

* More than 30% positive cells.
** Non-Hodgkin lymphoma with blood and bone marrow involvement.

shown to be present in follicular centre cells [13] and, in the context of the chronic lymphoproliferative disorders, it has become a useful marker for the diagnosis of FL in blood and/or tissue specimens (Table 3).

Table 3 illustrates how a combination of McAb could help to define the characteristic membrane phenotype of the most common types of B-cell malignancies. Some of the reagents shown in Table 3 are not strictly B-cell specific and will be described later. The McAb of greater interest are those which show different reactivity in the various B-cell disorders. For example FMC7 [20, 21] which is characteristically positive in B-PLL and HCL whilst it rarely reacts with B-CLL lymphocytes. In fact, the rare positive B-CLL cases are either those which have an increased proportion of prolymphocytes [39] (one-third of such cases) or show a strong expression of SmIg [21], which is also an atypical feature in B-CLL. A summary of our findings with this antibody is shown in Table 4.

Results with FMC8, on the other hand, show a non-specific pattern (Table 5), only half of the cases of B-cell disorders being positive. We could not demonstrate any correlation between the reactivity of this McAb and any clinical or laboratory feature in B-CLL patients. As mentioned earlier, FMC8 probably reacts with the same protein (p24) [23] as BA-2, and the results reported with the latter McAb also suggest a similar non-specific pattern in the B-cell malignancies [40].

We can conclude from the findings summarised in Table 3, that two

Table 5. Results with McAb FMC8 in B-cell leukemias

Disease	No. of cases	FMC8 positive	(% of + cases)
B-CLL	90	45	(50%)
B-PLL	13	7	(54%)
HCL	16	10	(62%)
NHL*	19	9	(47%)

* Cases of non-Hodgkin lymphoma with blood and bone marrow involvement.

categories of anti-B cell McAb may be useful for the study of B-cell disorders 1) the pan-B reagents which are positive in most conditions (anti HLA-Dr, B1, BA-1) and thus help distinguishing these diseases from the T-cell malignancies and 2) McAb that have a variable pattern of reactivity (FMC7, J5) but one which is consistent in certain diseases of this group and often reflects the stage of maturation of the neoplastic B-cells. Despite their apparent disease specificity, the latter reagents are best applied as part of a battery of cell markers in order to define objectively the membrane phenotype of normal and malignant B-cells.

2.3. *McAb Reactive With B and T Cells*

There are three antigens, detected by the McAb T1 (Leul), RFB1 and T10 (OKT10), that are expressed in T and B cells, but when examined in the context of B cell disorders (SmIg) they seem to provide useful information for the purposes of disease classification (Table 3).

RFB1 is of interest because it reacts with haemopoietic precursor cells [26], including early myeloid and erythoid cells and common-ALL blasts, thymocytes and mature T-cells [28] and is unreactive with pre-B cells and mature B-lymphocytes in blood and bone marrow. Nevertheless, this McAb is positive in all cases of B-CLL and HCL tested by us and is negative or weakly expressed in cells of other B-cell malignancies [27].

T1 (Leul) reacts with an antigen present in variable density throughout the T-cell lineage (as RFB1) [28, 41–44] and in B-CLL lymphocytes. This McAb is frequently negative or is inconsistently expressed in cells from other B-lymphoproliferative disorders. B-CLL is the only disorder in which RFB1 and T1 are always positive; in contrast, T1 is always negative in HCL [40, 45, 46], whilst RFB1 is maximally expressed [27, 45]. Studies in normal tissues with RFA1, a McAb analogous to T1, have suggested that B-CLL arises from a minor B-cell subset (weak IgM and M-rosette-positive) found within the light zone of germinal centres of lymph nodes and tonsils [15]. It is not known whether this putative B-CLL precursor cell is RFB1 positive. Paradoxically for a 'T-cell marker', T1 is almost always negative in T-CLL (see below).

T10 is another McAb expressed in immature T-cells and bone marrow precursors [28, 47–49], in germinal centre cells [49] and in cells in the late stages of B cell differentiation [49, 50]. This McAb reacts with cells of lymphoplasmacytic lymphoma (LPL) and multiple myeloma, but not with cells of other chronic B-cell disorders (Table 3). In rare cases of plasma cell leukemia in which the circulating cells are small and are not easily identified as plasma cells by light microscopy, the reactivity with T10 may help to establish the correct diagnosis. Most other B-cell markers, except CyIg, are negative in these late B cells.

2.4. *McAb to T-Cell Antigens*

The study of T-cell malignancies has greatly benefited from the development of a wide range of McAb specific to T-cell antigens. These antigens can be demonstrated throughout most maturation stages (pan-T reagents) or can be found only in particular stages of differentiation, e.g. T6 in cortical thymocytes [56, 73]. The McAb which can now be applied to the analysis of normal and neoplastic T-cells are listed in Table 6.

McAb that detect antigens present from early stages of differentiation, in immature T-lymphoid cells, are useful for the differential diagnosis between T-ALL and non-T ALL. Two such reagents: 3A1 [61, 74, 75] and WT1 [62, 63] detect the same polypeptide of 40K daltons mol wt and react with all cases of immature T-ALL, including pre T-ALL (E-rosette negative) [63, 75] and are negative in non-T ALL. Our experience with 3A1 is described in Table 7. It can be seen that while T-lymphoblasts and T-prolymphocytes (T-PLL) are always positive, cells from more differentiated T-lymphoproliferative disorders are usually negative although most peripheral blood (PB) T-lymphocytes are reactive with 3A1. However, the characteris-

Table 6. Monoclonal antibodies against T-cell antigens [1]

McAb	Specificity	Mol wt*	Reference(s)
T1**/Leu1 ‡	Thymocytes and PB T-lymphocytes	67–69	41–44
T3/Leu4	Mature T-lymphocytes; T-cell receptor	20	52, 53
T4/Leu3	Helper-inducer cells	50–62	54, 55
T6/NA134	Cortical thymocytes	49	56–58
T8/Leu2	Suppressor-cytotoxic T-lymphocytes	30+32	53, 59
T11/Leu5	Receptor for sheep erythrocytes	52–55	10, 53
T17	95% PB & thymic T-lymphocytes		60
3A1/WT1/Leu9	T-cell precursors, thymocytes, PB T-cells	40	61–63
NHK1/Leu7	Cytotoxic T-cells; NK cells		64
Anti-Tac	Receptor for TCGF(IL2); activated T-cells	120	65–67
BE1 & BE2	Sézary cells (Subset of T-cells)	50 & 78	68
Leu11	NK cells; Fc receptor for IgG	50–70	69
Leu15	Cytotoxic-suppressor cells; NK cells		70
TQ1/Leu8	Subsets within the T4+ and T8+ populations		71, 72

[1] Selected list of reagents referred to in this chapter.
* Mol wt: molecular weight in kilo daltons.
** OKT series (Orthoclone).
‡ Leu series (Becton Dickinson).
PB: peripheral blood; NK: natural killer; TCGF: T-cell growth factor.

Table 7. Results with the McAb 3A1 in ALL and T-cell malignancies

Disease	Number	Positive
Non-T ALL*	13	–
pre T-ALL	5	5
T-ALL/LbLy	6	6
T-PLL	10	10
T-CLL	5	–
Sézary	5	1
ATLL	4	–

* Common and null-ALL.
LbLy: T-lymphoblastic lymphoma;
Sézary: Sézary syndrome.

tic unreactivity of Sezary cells with this McAb [74] may reflect that the putative normal counterpart for these cells is also 3A1 negative [76]. Because 3A1 and WT1 also react with blast cells from acute myeloid leukemia (AML) [63, 77], it is essential that the enzyme terminal transferase (TdT) [78–80] should always be investigated in cases of acute leukemia. The reactivity with these two markers is as follows: AML 3A1+, TdT−, T-ALL 3A1+, TdT+ and non-T ALL 3A1−, TdT+; thus their use in combination may be valuable in the characterisation of immature blast cells. In the mature T-cell leukemias this reagent-combination has also a distinct pattern of reactivity. For example T-PLL that occupies an intermediate position between late thymic and post thymic malignancies [75, 81] is 3A1+, TdT−, different from the findings in the other post-thymic T-cell proliferations (T-CLL, ATLL, and Sezary syndrome), which are 3A1−, TdT−.

OKT17 reacts strongly with an antigen expressed on T-cells throughout all stages of maturation [60]. Our results with this McAb (Table 8) suggest that it is an excellent pan-T reagent, although we have not yet been able to test cases of pre T-ALL. In all these cases the proportion of T-cells reactive with OKT17 was the highest compared with all the other T-cell markers examined. E-rosettes [19] and the McAb T11 [10, 53, are also good pan-T markers, the only negative exception being pre-T ALL, that represents 25% of the leukemias of T-cell precursors.

Studies with the OKT series of McAb T3, T4, T6 and T8 or with equivalent reagents are well known. A review of the findings in normal and leukemic lymphocytes, the relation to functional subsets and preferential tissue distribution, can be found in references 28, 48, 53, 56, 73 & 82. The antigens detected by these McAb appear at different stages of T-cell maturation, are coexpressed in cortical thymocytes (T4, T6 and T8) and later characterise

Table 8. Results with the McAb OK17 in ALL and T-cell malignancies

Disease	Number	Positive*
Non-T ALL**	8	0
T-ALL/LbLy ‡	5	5
T-PLL	6	6
T-CLL	4	4
Sézary	4	4
ATLL	2	2

* 80 to 99% positive cells.
** Common and null-ALL.
‡ Pre-T ALL not tested yet.

two distinct post-thymic subsets, T3+, T4+ or 'helper/inducer' and T3+, T8+ or 'cytotoxic/suppressor'. The T3 antigen, contrary to expectations, is variably expressed in the mature T cell leukemias. The T1 antigen detected by the McAb Leul, T101, etc is present throughout the T-cell lineage but it is weakly expressed in immature T-cells, is strong in peripheral blood T-lymphocytes but is negative in T-CLL lymphocytes which have a mature phenotype (T3+, T8+, TdT−) [83].

Table 9 summarises the findings with the anti-T McAb in the T cell lymphoproliferative disorders. The main distinction between the latter conditions and T-ALL and T-lymphoblastic lymphoma (T-LbLy), the two prototype proliferations of imature T-cells, is the presence in the latter (and

Table 9. Monoclonal antibodies in T-lymphoproliferative disorders

McAb	T-CLL	T-PLL	ATLL	CTL
T1/Leu1	**−**	+	+	+
T3/Leu2	**+**	+/−	−/+	+
T4/Leu3	−	++/−	**+**	**+**
T6/NA134	−	−	−	−
T8/Leu2	**++**	+/−	−	−
T11/Leu5	+	+	+	+
T17	++	**++**	++	++
3A1/WT1	−/±	**++**	−	−
HNK/Leu7	**+/−**	−	−	−
Anti-Tac	−	−/+	**+**	−
BE2	−	−	−	**+**

The membrane phenotype characteristic of a disease is shown in bold type.
T-CLL: T-chronic lymphocytic leukemia (chronic T-cell lymphocytosis);
T-PLL: T-prolymphocytic leukemia;
ATLL: Adult T-cell leukemia/lymphoma;
CTL: Cutaneous T-cell lymphoma (Sezary syndrome and mycosis fungoides)

absence in the former) of TdT activity in the nucleus of the neoplastic cells [48, 75, 78, 81, 83]. Several groups have shown that the marker phenotype of T-LbLy often corresponds to that of cortical thymocytes, with coexpression of T6, T4 and T8, whilst the blast cells of T-ALL are more immature [48, 56, 83, 84]. From Table 9 it is apparent that some reagents show specificity for particular types of mature T-cell leukemias. T4, that characterises a distinct subset of immunocompetent T-lymphocytes, is positive in all cases of ATLL, CTCL (data chiefly from Sezary syndrome) and most T-PLL. T8, on the other hand, is positive in almost all cases of T-CLL [83, 85–87] and a minority of T-PLL [81]. The cells in a small group of T-PLL cases coexpress T4 and T8 but are T6 and TdT negative, so they differ from the phenotype of cortical thymocytes [81, 83].

A new generation of McAb has now become available for the study of T-lymphocyte populations. It is apparent that the categories of 'helper' and 'cytotoxic/suppressor' cells, as known until now, are too broad and that there is significant functional and membrane phenotypic heterogeneity between the subsets defined by T4 and T8. The combination of new reagents like Leu7, Leu8, Leu11 and Leu15 (Table 6) with the old ones (T3, T4, T8) is likely to help define normal T-cell subsets with functional homogeneity [64, 69–72]. Such studies may be helpful to prove the 'monoclonality' of some T-cell proliferations, e.g. T-CLL [85] and should, if correlated with functional tests, ascertain the real value of these new McAb for the characterization of disorders of immunocompetent T-cells. A good example for the need of such reagents can be found in the observation that the cells of ATLL, a disorder predominant in the Caribbean and in Japan, have been shown to lack helper function and to act *in vitro* as potent suppressors of B-cell differentiation in a pokeweed mitogen (PWM) driven system [88–90]. This apparent paradox may be explained by the observations of Thomas *et al.* [91] who demonstrated that a minor subset of radiosensitive T4+ cells may exert potent feedback suppression upon activation by PWM. It is of interest that another McAb, anti-Tac [65, 66], which reacts with the receptor for T-cell growth factor is positive in fresh or cultured ATLL cells [92–94], as well as in the normal subset of radiosensitive suppressor cells (after activation by PWM), but negative in Sezary cells [66, 94]. Thus, it is possible that ATLL cells may derive from the minor T-cell subpopulation included within the T4+ subset.

2.5. *McAb to Cell 'Specific' Antigens*

A recent development in the field of McAb is the production of reagents against particular types of neoplastic cells. These McAb have been raised against Sézary cells (BE1 and BE2) [68], hairy cells (HC1 and HC2) [95–97] and Hodgkin's cells (Ki-1) [98–100].

Table 10. Results with the anti-Sézary cell McAb BE2

Disease	Number	Positive*
Sézary(T4+)	4	3
ATLL (T4+)	1	–
T-PLL (T4+)	2	–
T-CLL (T8+)	2	–

* More than 30% reactive cells.

BE1 and BE2 react with cells from cutaneous T-cell lymphomas (CTCL) and EBV transformed B-cell lines but do not react with any normal tissues screened [68]. BE2 also reacts with a CTCL-derived cell line (HUT78), with MOLT3 cells (T-ALL derived) and a low percentage (mean 18%) of B-CLL lymphocytes. Our limited experience with BE2, shown in Table 10, confirms the view that this reagent appears to have selectivity for Sézary cells and thus may contribute to the cell characterization of the T-cell disorders (Table 9).

The McAb HC1 and HC2 have been shown to have specific reactivity against HCL cells [95–97]. HC2 also reacts with some AML cells and with mitogen stimulated B-lymphocytes [97]. Our experience with HC2 (Table 11) confirms the apparent specificity of this McAb to HCL within the framework of the B-lymphoproliferative disorders.

Studies in our group with the immunogold method (D. Robinson *et al.*, to be published) have shown that 1% of PB lymphocytes react with HC1 and that these cells have a hairy cell-like morphology under the electron-microscope (EM). HC2 also reacts with these normal 'hairy cells' as well as with lymphoplasmacytoid B-lymphocytes. The hairy cells present in normal PB also react with FMC7, another marker for HCL (Table 4), thus leading to the suggestion that they may constitute the normal counterparts of the neoplastic B-cells of HCL. Recent studies by Posnett and Marboe [97] have

Table 11. Results with the McAb HC2

Disease	Number	Positive*
HCL	6	4
HCL-Variant**	2	–
B-PLL	5	–
B-CLL	4	–
LPL	6	–

* More than 30% reactive cells.
** Intermediate features between HCL and B-PLL (153).

shown that 2.2% of normal PB lymphocytes are HC2+ and that this proportion raises to 10–20% after the induction of B-cell differentiation with PWM.

The McAb Ki-1 was raised against the cell line L428, derived from a patient with Hodgkin's disease [68], and has been shown to react against the characteristic Reed-Sternberg cells as well as with mononuclear Hodgkin's cells [99]. No reactivity with Ki-1 was observed in other lymphoid cells or tissues with the exception of a minority of large cells around normal lymphoid follicles [100]. All histological types of Hodgkin's disease as well as a proportion of large cell lymphomas are positive with this McAb. Within the latter group Stein *et al.* [100] identified a minority of cases (c. 10%) with negative B and T cell markers and positive with Ki-1, thus showing an identical phenotype as Hodgkin's cells. Other large cell lymphomas with positive B or T markers may also show reactivity with Ki-1. B-cell lymphomas and leukemias of low grade malignancy (B-CLL, B-PLL, HCL, centroblastic/centrocytic NHL) are Ki-1 negative [100].

It is hoped that more 'cell specific' reagents are going to be described in the near future and that they may throw further light on the nature of the cells proliferating in the lymphoid malignancies.

2.6. *T-Functional Assays*

Studies on the function of T-lymphocytes have provided useful additional information to the membrane phenotype analysis with McAb. This could be ascertained by observations showing that the major immuno-regulatory T-cell subsets, whether defined by T4 and T8 or by the presence of Fc receptors for IgG (Tγ) or IgM (Tμ), are functionally heterogeneous. Although Tμ cells contain helper cells and Tγ cells do not, there is no strict correspondence between those subsets and those defined by T4 or T8. In fact it is now known that the true helper function resides in a small subset which constitutes 25-30% of the T4+ lymphocytes [101]. These helper cells could be identified by their negativity with TQ1 and Leu8 [71, 72] and by their ability to form rosettes with ox-erythrocytes coated with IgM after treatment with theophylin [101]. On the other hand, the T4+ cells responsible for the induction of suppression ('inducer' cells) are TQ1 and Leu8 positive. The relevance of functional tests is well exemplified in the studies with ATLL cells mentioned earlier. ATLL cells are T4+ but act as potent suppressors [88–90, 102, 103]. Similarly T-CLL lymphocytes which usually are Tγ+ and T8+ can be shown *in vitro* to function as: 1) killer (K) cells in antibody dependent totoxicity [104] or 2) natural killer (NK) cells that lyse target cells without the mediation of antibodies [105], or 3) suppressor cells [86, 106], or 4) NK, K and suppressors [107] or even 5) K and helper cells [108]. No correlation has been observed either between these functions

and the presence or absence of 'myelomonocytic' markers like OMK1 [105, 107]; rare cases with T8−, Tγ+ lymphocytes, which are either M1+ or M1−, have NK function but no suppressor activity [104, 109]. It is likely that a better correlation between membrane antigens and function can be found with the new generation of McAb that appear to be more specific for cells with K and NK function, such as Leu7, 11 and 15 [64, 69, 70]. For example by combining Leu7 and Leu11 it is possible to define 3 cell subsets with increasing NK function [69]: Leu7+, Leu11− (NK±), Leu7+, Leu11+(NK+), Leu7−, Leu11+(NK++). Morphologically, Leu11+ cells are large granular lymphocytes [69], like normal Tγ cells and T-CLL lymphocytes [110, 111]. The McAb Leu15 [70] may, alternatively, identify the suppressor cells within the T8+ (Leu2+) population [70]. Other functional activities that can be measured in T-cells are the response *in vitro* to polyclonal mitogens (PHA, ConA, PWM) [104, 112] and the formation of T-colonies [112]. In general both proliferative responses have been found to be decreased in the chronic (mature) T-cell leukemias [112]. The low proliferation of Tγ+, T8+ cells may correspond to their functional capability in normal individuals; the low responses in Tμ+, T4+ cells may be an abnormality associated with the leukemic state.

Other functions that can be assessed in T-cell populations are the production of factors which can be measured directly *in vitro* or indirectly through their effect in the host. One of them is osteoclastic activating factor which may be responsible for the hypercalcemia in ATLL patients [113, 114]. Another is the inhibition of erythropoiesis [115] or granulopoiesis [116] which may be responsible for the erythroaplasia [85, 115, 117] or neutropenia [83, 116] which are common in T-CLL patients. The production of γ-interferon has also been described in patients with T-cell proliferation [109, 118] but its clinical relevance is not clear at the present time.

2.7. *B-Cell Differentiation In Vitro*

The concept that cells in the lymphoproliferative disorders are 'frozen' at particular stages of maturation has led some workers to explore the possibility of inducing these cells to move further along the B-cell pathway. For this purpose, a number of polyclonal B-cell activators have been used: PWM, lipopolysaccharide (LPS), Epstein-Barr virus (EBV), Protein A- from *Staphylococcus aureus* and the tumor promoter phorbol ester (TPA) [119–124].

TPA can differentiate B-CLL and B-PLL cells *in vitro* by transforming them into blasts and plasmacytoid cells and by inducing several cellular changes: decrease in SmIg, synthesis of CyIg, loss of the receptor for M-rosettes and increased expression of HLA-Dr [120–122]. Juliusson *et al.* [119] observed increased secretion of Ig in cells from 4 out of 6 B-CLL

cases and a switch from IgM to IgG, with similar light chain, a feature which characterises norm al B-cell differentiation. Robert *et al.* [123], on the other hand, described changes in B-CLL cells suggestive of B-PLL and LPL. These cells transform into blasts, lose the receptor for M-rosettes, synthesize CyIg, express strong SmIg and react with FMC7 to which they were negative before the 5-day culture with LPS [123]. These studies also suggest that cells in clinically more aggressive disorders (e.g. B-PLL) may be immunologically more mature whilst relatively more immature cells may predominate in more benign disorders (e.g. B-CLL). Furthermore, the changes that can be observed in B-CLL in 'prolymphocytoid transformation' [39] or developing 'immunoblastic' lymphoma (Richter's syndrome) suggest that the new malignant clones are immunologically more mature, expressing strong SmIg, CyIg and FMC7.

Of interest here are 3 cases of B-CLL with a pre-B phenotype (SmIg−, Cyμ+, B1+, J5−) in which TPA induced secretion of IgM (K or L) and the appearance of SmIg IgMK [124]. The cells in these rare cases were clearly more immature than usual in B-CLL and TPA induced them to mature beyond pre-B cells.

The induction of differentiation and the patterns of antigenic expression in early B-cells has been analysed by Nadler *et al.* [125]. Cells from 4 common-ALL derived cell lines, which were negative with the McAb B1 (Table 2), were stimulated with TPA or PHA conditioned media and this resulted in the expression of B1 in all of them. B1 negative cells from common-ALL patients could also be induced to express B1 as well as Cyμ chains [125]. These studies also suggest a hitherto unrecognised heterogeneity in non-T ALL blasts which reflects the spectrum of early B-cells at different stages of maturation.

3. CHRONIC B-CELL MALIGNANCIES

The most immature blast cells of the B-cell lineage are seen in non-T ALL (null and common-ALL). The earliest feature of differentiation in these cells is the rearrangement of Ig genes [3, 4] and the expression of some characteristic B-cell antigens (Table 12). Pre-B features (Cyμ) are seen in 25% of cases of common-ALL [126] and in a minority of B-ALL (Burkitt-type). The membrane phenotype of the majority of B-ALL cases is that of a mature B-cell with expression of SmIg (usually IgM K or L) and absence of TdT. Because the cells in non-T ALL are blasts, it is important to distinguish them from T-lymphoblasts, and this can be achieved by means of the battery of reagents shown in Table 12. The McAb 3A1 (and WT1), an early T-cell marker, does not react with any case with Ig gene rearrangement [4]

Table 12. Differential diagnosis of lymphoblastic leukemias

Marker	B-cell lineage		T-cell lineage	
	Null-ALL	Common-ALL	pre-T ALL	T-ALL
TdT	+	+	+	+
3A1/WT1	−	−	+	+
HLA-Dr	+	+	−	−
B4	+	+	−	−
J5*	−	+	−/+	−
B1	−	−/+	−	−

* Common-ALL ag (gp100 K daltons).

and recognizes all the leukemias of T-cell precursors (63, 75; Table 7).

The chronic B-lymphoproliferative disorders include diseases of mature B-lymphocytes which affect, as a rule, adult patients. Although they can be classified in many ways, the key elements for a precise diagnosis are cell morphology (or histology in the case of NHL) and immunological markers. The utility of the latter has been outlined earlier (Tables 1 and 3). The delimitation of whether the primary process is leukemia or lymphoma may not always be possible or necessary. In fact, there is good evidence that a monoclonal B-cell population can be demonstrated in the PB by SmIg staining of K and L chains in one-third of cases of follicular lymphoma [127]. Involvement of the bone marrow (BM) is demonstrated in over half of these cases by trephine core biopsies.

3.1. *B-Chronic Lymphocytic Leukemia (B-CLL)*

This is the most common of the B-cell disorders, affecting adults over the age of 50 years [128]. The disease affects the blood (lymphocytosis of 10×10^9/l or over), the bone marrow (at least 40% of lymphocytes), lymph nodes and spleen. The BM histology is characteristic [129] and so are the lymph node [130] and spleen histologies [131]. A good correlation has been demonstrated between clinical staging in B-CLL and the patterns of BM infiltration [129]. The key element for diagnosis is the morphology of peripheral blood lymphocytes which show a characteristic clumped chromatin pattern. The disease can be divided in clinical stages [132, 133] that imply that progression follows an orderly development: first BM then lymph nodes and/or spleen and finally more extensive (diffuse) BM infiltration. This sequence may not always proceed in that order as there are patients with splenic forms of the disease without lymphadenopathy, and others that develop severe bone marrow failure without splenomegaly or lymphadenopathy. Furthermore, experimental evidence suggests that the cell of origin of B-CLL is found in germinal centres and not in the bone marrow [15, 49].

The membrane phenotype of B-CLL (Tables 1 and 3) is characterised by a triad which helps to distinguish this disease from other B-lymphoid leukemias (weak SmIg, M-rosettes and T1 antigen). There are two major forms of malignant transformation that can be seen in B-CLL [128]: 1) 'prolymphocytoid' change, characterised by an increase in prolymphocytes (usually over 20%), is relatively common and is associated with raising WBC, enlarging spleen and/or lymph nodes and poor response to therapy [39], and 2) 'immunoblastic' transformation which can take the form of a large cell lymphoma (Richter's syndrome) [134, 135] or a 'blast crisis' resembling acute leukemia, with blood and bone marrow involvement by large blasts with dark basophilic cytoplasm [136, 137]. Evidence of a residual population of B-CLL lymphocytes is usually demonstrable in these cases. This may facilitate the diagnosis when transformation occurs early in the disease, without a preceding chronic phase, as in one of the original cases described by Richter [134]. Although in most cases of 'prolymphocytoid' transformation the membrane markers are similar to chronic phase B-CLL and distinct from B-PLL (Tables 1 and 3), cases with an intermediate membrane phenotype between the two diseases can be seen [138]. In such cases the expression of SmIg is strong, the McAb FMC7 may be reactive with a high proportion of cells and/or a lower affinity for M-rosettes can be demonstrated [128]. These changes are often associated with resistance to conventional therapy and karyotypic changes (see below).

Cell marker studies are also helpful to identify the malignant immunoblasts, both in Richter's syndrome and in the acute leukemic transformation [128], as evolving from the original B-CLL clone [135–137]. Often the amount of SmIg is stronger in the new clone but shows the same class of heavy and light chains as in the original one; the latter may also show CyIg staining which is secreted and detected in the serum as an 'M' band or as free light chains in the urine (Bence Jones protein).

3.2. *B-prolymphocytic Leukemia (B-PLL)*

This variant of B-CLL constitutes a distinct clinicopathological entity [12, 128, 131, 138–140]. The typical cell, the prolymphocyte, has peripheral nuclear chromatin condensation and a prominent, centrally placed, nucleolus. Ultrastructural and cell volume studies may facilitate the identification of this cell [12]. In our series [128], the incidence of PLL within the lymphoproliferative disorders is 8%, with B-PLL constituting 76% of cases and T-PLL 24%. There are subtle morphological and cytochemical differences between B and T-prolymphocytes that may be useful if membrane markers are not available [see below and references 12 and 128].

The most typical membrane features of B-PLL cells are: strong expression of SmIg, low binding of mouse RBC and reactivity with the McAB

FMC7 (Tables 1 and 3). Clinically there are also differences between the two types of PLL [138]. B-PLL is characterised by WBC > 100×10^9/l and massive splenomegaly without lymphadenopathy. T-PLL has WBC > 200×10^9/l and, in addition to splenomegaly, the patients have lymphadenopathy, skin deposits and pleural effusions. The survival is worse in T-PLL, with over 50% of patients dying within the first year. B-PLL patients may respond to splenic irradiation and benefit from splenectomy [12, 141]. T-PLL requires intensive combination chemotherapy but complete responses are rare [138, 142].

3.3. *Hairy Cell Leukemia (HCL)*

In the majority of cases (99%) the cells have B-cell characteristics with a membrane phenotype close to that of B-PLL (Tables 1 and 3). The disease affects mainly middle-aged men who present with anaemia and/or thrombocytopenia and splenomegaly. Hairy cells can be recognised in well prepared blood films and by EM analysis. The bone marrow aspirate usually yields a dry tap but the trephine biopsy shows a characteristically loose cellular infiltration with marked increase in reticulin fibers [143]. Three additional features may help to make the diagnosis of HCL: i) the demonstration of tartrate resistant acid phosphatase (TRAP) activity [12], ii) the presence of ribosome-lamella complexes at EM level [12, 144] and iii) the histology of the spleen which shows predominant involvement by hairy cells in the red pulp and the formation of pseudosinuses filled with RBC [145]. Neither i) or ii) are specific for HCL but are more common in this disease than in any other lymphoproliferative disorder; iii) is more specific but cannot be used as a diagnostic test although, in fact, splenectomy is often one of the first therapeutic measures in this disease [12, 146]. Spleen histology has been found to be useful to distinguish HCL from cases of B-PLL and of LPL (immunocytoma) with 'hairy' lymphocytes in the peripheral blood. In these cases, the spleen histology shows nodular proliferation predominantly in the white pulp [131, 147, 148].

The B-cell nature of hairy cells has been debated for many years but this matter is now largely settled. In addition to the presence of monoclonal SmIg, and occasionally CyIg, and the expression of several B-specific antigens demonstrated by McAB (Table 3, references 40, 45, 46), it has now been demonstrated that the Ig genes for heavy and light chains are rearranged in the cells of this disease [5]. Furthermore, the studies with the McAB HC2 [97] suggest that the normal counterparts of hairy cells are lymphocytes in the late stages of B-differentiation. Such HC2 positive cells are found in interfollicular spaces of lymph nodes and tonsils [97] as well as in normal peripheral blood. This McAB appears to be specific for HCL within the B-cell disorders (Tables 3 and 11).

3.4. *Non-Hodgkin Lymphomas (NHL)*

The most common NHL of B-cell type is centroblastic-centrocytic lymphoma [13] also known as follicular lymphoma (FL). Bone marrow and blood involvement at presentation is frequent and this raises the question of the differential diagnosis with the chronic B-cell leukemias. In our experience the morphology of the lymphoid cells, in particular the presence of small-cleaved cells (centrocytes), facilitates the diagnosis in cases that present with lymphocytosis. PB and BM involvement in FL does not necessarily imply a worse prognosis. Late involvement, on the other hand, relates to disease progression and in these cases, large-cleaved (centroblasts) are also seen together with centrocytes and other non-cleaved cells. Analysis of membrane markers on PB and BM cells permits the differential diagnosis from B-CLL, the main difference being the strong expression of SmIg in follicular centre cells (Tables 1 and 3). In one-third of FL cases the percentage of mouse-RBC rosettes on PB cells may be high but in the majority the values are usually lower than in B-CLL.

Histology is, of course, essential to establish the correct diagnosis and immunohistology contributes significantly to define precisely the cell phenotype of the NHL lymphoma [13]. Bone marrow biopsy shows mainly paratrabecular involvement which is different from the pattern seen in the lymphocytic leukemias [129]. The presence of the common-ALL antigen in normal and neoplastic follicular centre cells [13], demonstrable with the McAB J5 [29, 30] and now also with VIL-A1 [149] and AL2 [150], is extremely useful for the diagnosis of FL within the chronic B-lymphoproliferative disorders (Table 3).

3.5. *Lymphoplasmacytic Lymphoma (LPL)*

Cases with this diagnosis are considered by Lennert [151] within the broader group of immunocytomas, that includes Waldenstrom's macroglobulinemia. The chief findings are the presence of an IgM serum paraprotein and the infiltration of the BM with cells with lymphoplasmacytic morphology. Less commonly, cases with IgA or IgG paraproteins and morphological and clinical features of LPL rather than of myelomatosis, can be seen. Some cases of LPL have splenomegaly and lymphocytosis with cells showing an irregular cytoplasmic outline suggestive of HCL [147, 148]. We have studied 7 such patients (aged 57–89) with splenomegaly and WBC between 11 to $31 \times 10^9/l$ with $\geq 50\%$ 'hairy' lymphocytes. The cells in some of them were TRAP positive, as previously described [147, 148], but the EM morphology of these lymphocytes was clearly distinct from that of HCL, B-PLL and the recently described HCL-variant [152, 153].

Cells in LPL have a strong expression of SmIg and often of CyIg as well, and have membrane antigens as other NHL (Table 3); the McAB FMC7 is

almost always positive in these cases (Table 4). The reactivity with OKT10, a McAb also positive in myeloma cells, may help in the diagnosis of LPL [50, 51].

4. CHRONIC T-CELL MALIGNANCIES

There are two major groups of T-cell leukemia/lymphoma: i) those in which the cells are blasts and have an immature (thymic or pre-thymic) phenotype, and which affect predominantly children and young adults. These include pre T-ALL, T-ALL and T-LbLy [48, 83]. A common feature of all these cases is the presence of TdT in the cell nucleus [78] and a membrane antigen demonstrated by the McAb 3A1 and WT1 [63, 75] (Tables 7 and 12); ii) those in which the cells are both morphologically and immunologically mature and have a post-thymic T-cell phenotype with negative TdT, 3A1 and WT1 (except T-PLL which is 3A1+) (Tables 7 and 9). This group is relatively rare and corresponds to the chronic T-lymphoproliferative disorder to be described here. These diseases affect almost exclusively adults and include T-PLL [81], T-CLL [85], Adult T-cell lymphoma/leukemia (ATLL) [89, 90, 114, 154, 155] and the cutaneous T-cell lymphomas, chiefly Sézary syndrome because of its consistent involvement of the peripheral blood; other peripheral T-cell lymphomas, like T-zone lymphoma [151] may be related to ATLL [83, 155].

4.1. *T-Chronic Lymphocytic Leukemia (T-CLL)*

This condition, also described as chronic T-cell lymphocytosis or Tγ-lymphocytosis, was originally described by Brouet *et al.* [157]. The cells are characterized morphologically by abundant cytoplasm, slightly eccentric nucleus and prominent azurophil granules [83, 85]. These lymphocytes, which correspond to the so-called granular lymphocytes, have numerous lysosomal granules (that contain acid hydrolases) and parallel tubular arrays in the cytoplasm which can be identified at EM level [110]. Most patients with T-CLL have splenomegaly of variable degree; skin infiltrates and lymphadenopathy are rare. The lymphocyte counts range between 5 and $25 \times 10^9/l$ and there is a moderate degree of bone marrow infiltration (40–50% lymphocytes) which is disproportionate to the frequent peripheral blood cytopenia: neutropenia is the most common finding [83, 85, 104, 105], followed by thrombocytopenia [85] and erythroid hypoplasia [83, 85, 117, 158]. The presence of pancytopenia with splenomegaly without a heavily infiltrated bone marrow often results in the decision of splenectomy, which rarely corrects the cytopenia and is regularly followed by a rise in the lymphocyte counts [85]. In 2 cases hypogammaglobulinemia

was one of the main features and this resulted from the proliferation of T-suppressor cells [106, 158]. Despite arguments about the non-neoplastic nature of this condition no cases of spontaneous regression have ever been recorded. Of interest, and perhaps relevant to the pathogenesis of this disease, is the observation, in a series of 21 cases collected from several London hospitals [85], that 7 patients had long standing rheumatoid arthritis before the diagnosis of T-CLL was made.

The most common membrane phenotype in this disease is T3+, T8+, M1− [83, 85–87, 104]. Few cases are T3+, T8+, M1+ [105, 107] and rare ones are T3−, T8−, M1+ [104] or T3+, T8−, M1− [109]. Most cases are E+, Fcγ+ (Tγ lymphocytes) and often Leu7+ [87, 159]; the expression of HLA-Dr is variable. T1 and 3A1 are usually negative or very weak [75, 83, 160], an unusual feature for mature 'post-thymic' cells. These large granular lymphocytes may function as K cells or NK cells or as suppressors with or without K or NK function. The morphology, membrane phenotype (Table 9) and cytochemistry (see below) of these cells are different from those of all other T-cell disorders. These and the unique clinical features: cytopenias, splenomegaly, and chronic course, suggest that this disease should be recognised as a distinct entity. The designation T-CLL should be used in the understanding that the neoplastic nature of the proliferation may not always be clinically apparent. An animal model for this disorder, a naturally occuring leukemia of large granular lymphocytes in aging F344 rats, has recently been described [161]. The disease appears to arise in the spleen and spreads to other organs and, infrequently, to the bone marrow. As in human T-CLL the membrane phenotype and function may be slightly heterogeneous but the clinico-pathological features are very consistent.

4.2. *T-Prolymphocytic Leukemia (T-PLL)*

Out of 100 cases of PLL studied in our laboratory over a 10 year period, 24 were found to have T-cell markers (T-PLL). All the patients were over 50 years of age and the M:F ratio was 2:1. McAb against T-cell antigens were tested in 22 of them and the results suggest some heterogeneity in membrane phenotype. The cells in all cases formed E-rosettes and were SmIg−, HLA-Dr−, TdT−, T1+, T3−/+, T6−, T10−/+, T11+, T17+ and 3A1+ (Table 9). The heterogeneity was observed with the expression of T4 and T8. The most common phenotype was T4+, T8−, seen in 14 cases; 4 cases were T4−, T8+, 3 cases coexpressed T4 and T8 and 1 case was T4−, T8−; several features of T-PLL cells suggest immaturity [81]. The prolymphocytes of 1 out of 3 cases (T4+, T8−) studied with functional assays were shown to have strong helper function (Dr. F. Miedema, personal communication). Other cases of T-PLL reported in the literature have been

found to have either 'helper' (T4+) [162, 163] or 'suppressor' (T8+) [164] markers. T-PLL is the only chronic T-cell disorder in which we have observed the coexpression of T4 and T8, a feature seen in cortical thymocytes, but, as distinct from the latter cells, T-prolymphocytes are always TdT− and T6−. An unusual case of T-cell leukemia (or CTCL) with skin infiltration and T4+, T8+ convoluted cells was reported by Staven *et al.* [165].

In half of the cases of T-PLL the cells resemble typical prolymphocytes (as seen in B-PLL). The cells in the other cases are smaller, have an irregular nucleus and a deep basophilic cytoplasm due to a high ribosomal content. The nucleolus may not be easily visible by light microscopy examination in all cases, but it is always seen by EM. Azurophilic granules are not seen in Romanovsky stained films although these cells are rich in acid hydrolases (see below) and EM analysis has identified large electron dense granules [110]. The clinical and prognostic differences between B and T-PLL have been referred to above [see also references 12, 128, 138].

4.3. *Cutaneous T-Cell Lymphomas (CTCL)*

Sézary syndrome and mycosis fungoides are closely related CTCL. Because of the characteristic involvement of the PB, Sézary syndrome can be considered a T-lymphoproliferative disorder. The main clinical features of this CTCL are erythroderma, with typical epidermal infiltration, sparing of the bone marrow in early stages of the disease and lymphocytosis of variable degree. Sézary cells may be small or large and can be recognised in peripheral blood films. However, because the space between the nuclear convolutions is narrow, the characteristic cerebriform appearance may be overlooked. Examination by EM [76, 83, 110] permits a better evaluation of the morphology of these cells. The membrane phenotype of Sézary cells is that of mature T-helper cells (OKT4+) (Table 9), a feature that has been confirmed in functional studies [166–168]. Although there is some resemblance in the morphology and membrane phenotype between Sézary and ATLL cells, a detailed analysis shows that they belong to distinct but related T-cell subsets. Both ATLL and Sézary cells are T4+, T17+ and 3A1− (Tables 7, 8 and 9), but only ATLL are anti-Tac+ [66, 67, 94] whilst Sézary cells are the only ones reactive with BE2 (Table 10). As mentioned earlier, ATLL cells lack help function and act as potent suppressors [88–90, 102, 103]; Sézary cells often act as helper cells [166–168]. Both cell-types may arise from distinct but related T-cell subsets which have been characterized in normal PB samples by immunoelectronmicroscopy studies [76].

4.4. *Adult T-Cell Leukemia/Lymphoma (ATLL)*

ATLL is a rapidly progressive T-cell disorder characterized by lymphad-

enopathy (70–80%), moderate hepatosplenomegaly (40–50%), skin lesions (45–55%), early blood and bone marrow involvement in the majority of cases and a high incidence of hypercalcemia (50–80%) [113, 114, 154]. The hypercalcemia, a rare feature in other NHL, is not associated as a rule with osteolytic lesions and may be due to the production of an 'osteoclast activating factor' by the tumor cells [113]. The disease is the most common form of lymphoproliferative disorder in Japan where it predominates almost exclusively in South Western regions [154, 169, 171]. Although initially recognized in Caribbean black residents in the UK [114], recent studies suggest that ATLL may be the most common lymphoid neoplasm in Jamaica and other islands of the Caribbean basin [171]. A series describing cases diagnosed in the USA has recently been published [155].

ATLL has unique clinical, morphological, histological and immunological features that permit its distinction from other lymphoproliferative disorders [154–156, 169–172]. Seroepidemiological studies have now shown conclusively that the clustering of birth places for ATLL cases in South Western Japan and the Caribbean basin is related to the existence of a new human retrovirus, HTLV [173], which is the likely causative agent of the disease (see chapter by Gallo *et al.*, 1). The majority of ATLL patients have a high titer of serum antibodies to the major structural core proteins of HTLV, p19 and p24 [174]; elevated titers to HTLV are also demonstrated in normal residents of the endemic areas and in patients' relatives [175]. Although a constellation of clinical and laboratory features facilitate the diagnosis of ATLL, in some patients these may not be typical. In addition, the lymph node histology is not consistent in all cases [156]. In these circumstances, the serological tests for detecting antibodies to HTLV, may have diagnostic potential [171, 174, 175].

The proliferating cells in ATLL display a remarkable pleomorphism, both in size and nuclear shape, having as a most prominent feature a highly convoluted, often polylobed, nucleus. A small proportion of ATLL cells could be, however, indistinguishable from Sézary cells [94, 172, 176]. Nevertheless, pleomorphism and nuclear multilobulation or fragmentation are more characteristic of ATLL cells [177] whereas Sézary cells display a more typical cerebriform nucleus [76, 94]. Blast cells may also be present in ATLL and when they infiltrate lymph nodes the histological sections show the appearances of a large cell, immunoblastic, NHL [156, 172]. The membrane phenotype of ATLL cells (Table 9) corresponds to those of mature (post-hymic) T-lymphocytes [83, 89, 114] which, despite being T3+, T4+, display suppressor activity and lack helper function *in vitro* [88–90]. As discussed earlier ATLL cells may derive from a minor subset of radiosensitive T4+ suppressor cells [60]. Despite this, the disease is not associated with hypogammaglobulinemia. Major differences from cells of the other T-cell

disorders, in particular Sézary syndrome, are the reactivity with anti-Tac in ATLL [65–67, 94] and with BE-2 in Sézary syndrome [68, 95; Table 10]. Anti-Tac recognizes the receptor for Interleukin-2 (TCGF) which is expressed spontaneously or after short term culture in ATLL cells and in T-lymphocytes following activation by mitogens [67]. The addition of anti-Tac in culture prevents the expression of the receptor in normal cells (due to modulation). The TCGF receptor in ATLL cells fails to modulate even in the presence of high concentrations of anti-Tac in the culture media [67]. This abnormality may play a part in the pathogenesis of the lymphoproliferation presumably triggered by HTLV.

The clinical and laboratory similarities of ATLL in the various geographical clusters extend also to the immunology, ultrastructure and karyotype of the neoplastic T-cells [94, 154, 176, 177] and are compounded by the close association of the disease, in those areas, with the same etiological agent: HTLV-I [154, 178].

5. CYTOCHEMISTRY OF ACID HYDROLASES

Although cytochemical reactions have been employed for many years, only since lymphocyte markers became available was it possible to correlate enzyme activity with immunological phenotype (for a review see ref. 179). The most useful reactions for the study of lymphoid malignancies are those detecting acid hydrolases which show differences between normal lymphocyte subsets as well as between B and T lymphoproliferative disorders [179–186]. In general, T-lymphocytes have a higher content of acid hydrolases than B-cells and the corresponding T-cell leukemias tend also to be richer in those enzymes [180]. A closer understanding of the relationship between B and T-cell subsets and enzyme reactions can now be achieved by combining McAb, by means of immunogold method, with cytochemistry [179, 181]. It is worth noting that the enzyme content varies with maturation in both B and T cells and that these changes may be different for each enzyme.

Table 13 shows the main findings with 5 acid hydrolases in the lymphoproliferative disorders described in this chapter. It can be seen that negative or weak reactions are observed with 4 of them in B-CLL and B-PLL whilst positive reactions are shown in T-CLL and T-PLL; dipeptidylaminopeptidase IV (DAP IV) is negative in the B-cell leukemias but its reactivity in the T-cell leukemias appears to be limited, in our experience, to T4+ T-PLL (see below).

5.1. *Acid Phosphatase (AP)*

Early studies in ALL have shown a significant difference between the AP

Table 13. Cytochemistry of acid hydrolases in chronic lymphoproliferative disorders

Disease	Cytochemical reactions				
	ANAE	AP	B-glucur	B-glucos	DAPIV
T-cell malignancies					
T-CLL (T8+)	−/±	++	++	++	−
T-PLL (T4+ and/or T8+)	++	+	++	++	++ ‡
CTCL(T4+)*	+	+	++	++	−
ATLL (T4+)	+	+	+	++	−
B-cell malignancies					
B-CLL	−	−	−/±	−	−
B-PLL	−	−/+	−/±	−	−
HCL	−/+	++ **	−/±	−	−
LPL	+/++	++	++	++	−

* Reaction in Sézary cells.
** Tartrate resistant;
‡ seven T4+ cases; a T8+ case was DAP IV - (Table 14).

reaction in T-ALL (strong and localized) and in non-T-ALL (negative or weak) [187, 188]. These observations correspond to findings in normal thymic cells which are always AP positive [189]. Although some E-rosette negative ALL were reported in the past as having a strong AP reaction new observations using McAb that identify early T-cells (e.g. 3A1 WT1) have demonstrated that such cases correspond in fact to pre-T-ALL. Thus positive AP reaction in lymphoblasts correlates well with T-cell differentiation.

Of the mature T-cell proliferations, T-CLL shows the strongest AP reaction (Table 13). This reactivity correlates to the number of lysosomal granules demonstrable by EM techniques in the cytoplasm of these cells. In T-CLL both the granules and the parallel tubular arrays, which are azurophilic in Romanovsky stained films, are AP positive [110, 190]. In B-CLL and B-PLL the AP content is low, but the small amount of enzyme present in B-PLL may be resistant to tartaric acid (TRAP) [12, 187]. A strong TRAP activity is characteristic of HCL cells [12, 180] and is known to correspond to a specific isoenzyme 5 demonstrable by polyacrylamide gel electrophoresis [191]. Cells in the late stages of B-cell differentiation (i.e. plasma cells) develop numerous lysosomal granules in the Golgi zone and, as a result, show a strong reactivity with AP which is not seen in mature B-cells [185]. It is possible in this context to relate the high AP content of hairy cells to their membrane phenotype which shows features of late B-cells, as discussed above.

5.2. *Alpha-Naphthyl Acetate Esterase (ANAE)*

Interest in this enzyme as a possible discriminant between B and T cells stems from studies describing a weak ANAE activity in thymic cells and a strong reaction in mature T-lymphocytes [192], and from reports of a dot-like reaction on Tμ lymphocytes, which was absent in Tγ (large granular) cells. In general, Tγ-cell proliferations (i.e. T-CLL) show negligible ANAE activity [110, 180] whilst cells in proliferative disorders with Tμ or T4 markers display a strong dot-like reaction (Table 13). The findings in T-PLL are of interest because the ANAE reaction is also positive in cells with membrane phenotype: T4+, T8+ and T4−, T8+. Studies at EM level show that this enzyme is localised in large cytoplasmic granules and in Gall bodies [190].

ANAE activity is absent in B-cell leukemias but increases during maturation, both in normal and neoplastic B-cells [185, 186]. ANAE is not consistently positive in leukemic lymphoblasts and therefore it is not as useful as the AP reaction for the characterization of pre-T and T-ALL [179, 192].

5.3. *Dipeptidylaminopeptidase IV (DAP IV)*

DAP IV is the most recently studied of the acid hydrolases and, as distinct from the other cytochemical reactions, it is absent from B-cells and monocytes and appears to be highly specific for T-cells [182–184]. Feller *et al.* [183] reported that DAP IV is confined to T4+ lymphocytes and T4+ leukemias. Our studies and those of Crockard with the immunogold method [179] suggest that, indeed, most T4+ normal lymphocytes are DAP IV+ but that reactivity can be detected in up to 40% of T8+ cells. In the lymphoid leukemias we have confirmed the absence of this enzyme in B-

Table 14. DAP IV activity in lymphoid malignancies*

Disease (marker)	No. of cases	Positive cells % (range)
Non-T ALL (TdT+)	4	0
B-ALL (SmIg+)	1	0
T-ALL LbLy	2	0–100
T-CLL (T9+)	4	2–10
T-PLL (T4+)	7	95–98
(T8+)	1	0
Sézary (T4+)	2	0
ATLL (T4+)	1	0
HCL (SmIg+)	3	2–19**
LPL (SmIg+)	2	0–10**

* Work carried out by Carolyn Andrews in our laboratory.

** Hairy cells negative; only T-lymphocytes positive.

CLL [182, 183] and observed no reaction either in HCL or LPL (Table 14). DAP IV is negative in B-lineage ALL (182; Table 14) and shows variable reactivity in T-ALL and T-LbLy [179].

In the differentiated T-cell malignancies (Table 14) DAP IV was positive in all our cases of T4+ T-PLL and this agrees with the published observations in T4+ leukemias [183, 184]; this enzyme reaction was negative in T8+ proliferations (T-CLL and T8+ TPLL; Table 14) as reported in two other cases [183, 184]. However, and confirming the findings of Wirthmuller *et al.* [184], we have been unable to demonstrate DAP IV reactivity in Sézary cells (Table 14) and we have also shown no reaction in a case of ATLL. From these studies it would appear that DAP IV is selectively expressed in a population of T4+ lymphocytes and is absent from B-cells in all stages of maturation. This reaction may thus be of value for the characterisation of T-lymphoproliferative disorders (Table 13).

5.4. *Other Acid Hydrolases*

Other enzyme reactions which have been applied to the classification of lymphoid malignancies are β-glucuronidase (β-glucur) and β-glucosaminidase (β-glucos) [179, 180]. Both appear relatively late during T-cell maturation [179, 189] and are absent or weakly expressed on B-lymphocytes, but become positive in normal and myeloma plasma cells. As shown in Table 13, both these enzymes can be broadly used as 'pan-T' markers only within the context of the chronic lymphoid leukemias [180]. Reactivity with both enzymes (stronger with β-glucos) is seen in T- and B-lineage lymphoblasts [179], thus, they do not seem to have a useful application for differential diagnosis in ALL.

6. CHROMOSOMES

The relevance of chromosomes as markers of disease is supported by evidence of a close association between specific nonrandom abnormalities and particular types of leukemias and lymphomas. Data on the acute leukemias has been analysed in several International Workshops (Third and Fourth International Workshops on Chromosomes in Leukemia). The biological significance of an abnormal karyotype has been emphasised by studies showing that specific breakpoints coincide with regions of the chromosomes where oncogenes have been localized [194]. Furthermore, a direct correlation between the breakpoints on chromosomes 14(q32), 2(p12) and 22(q11), which correspond to the loci for the heavy and light chain genes, seen in the specific translocations of Burkitt's lymphoma, t(8; 14), t(2:8) and t(8; 22), and the expression of light chains on the surface of the malig-

Table 15. Chromosome abnormalities in chronic lymphoproliferative disorders

Lineage	Disease	Abnormalities	Reference(s)
B-cell	B-CLL	trisomy 12, 14q+, t(11; 14)	198, 201, 202*
	B-PLL	14q+, t(11; 14), 6q−, t(6; 12), trisomy 12, Abn. No 1	199, 203
	HCL	trisomy 12, 14q+	204, 205, 206*
	FL	t(14; 18)	197
	WDLL	trisomy 12	197
	LPL	trisomy 12	207
T-cell	T-CLL	trisomy 22, 14q+, Abn. No 2 and No 8	108, 208, 209
	T-PLL	Abn. No 2 and No 8	209
	ATLL	trisomy 7q, 14q+, 6q−	176, 210, 211
	CTCL	6q−, i(17q), Abn. No 2	94, 176, 212, 213

* Pittman and Catovsky (to be published).
FL: follicular lymphoma; WDLL: well differentiated lymphocytic lymphoma; Abn.: abnormal.

nant B-cells, has recently been documented [194–196]. This together with evidence for the localization of the oncogene *c-myc* at the breakpoint of chromosome 8(q24) has arisen considerable interest in relation to the pathogenesis of human B-cell malignancies [194, 196].

Reproducible chromosome analysis of the lymphoid malignancies is now possible as a result of improved culture techniques [197] and the use of polyclonal B-cell mitogens for the stimulation of B-lymphocytes [119, 198–200]. This methodology has permited the demonstration of non-random chromosome abnormalities in the B and T-lymphoproliferative disorders discussed in this chapter (Table 15). It is apparent from the table that three abnormalities: 14q+, trisomy 12 and 6q− are a frequent feature of B-cell malignancies; 6q− and 14q+; have also been reported in T-cell malignancies, particularly ATLL [210, 211], and are commonly found in NHL [214]. On the other hand, trisomy 7q is an abnormality characteristic of ATLL [176, 210] and is rare in other T or B-cell disorders. Non-random abnormalities of chromosome 2 and 8 are frequent in chronic T-cell leukemias [94, 209]; chromosome 2 does not appear to be frequently involved in B-cell disorders [214].

6.1. *Abnormalities in B-CLL*

The advent of polyclonal B-cell mitogens (PWM, LPS, EBV) made possible the demonstration of chromosome abnormalities in 40–55% of B-CLL cases [198, 200–202]. Trisomy 12 is the most frequent numerical

change being observed in 25 to 30% of cases [198, 202]; we have seen trisomy 12 in 7 out of 33 patients (21%) (Pittman and Catovsky, to be published). The most common structural abnormality is a marker 14q+ which has been demonstrated with variable frequency in the published series [198, 202]. In a study using PWM as a mitogen following treatment of the cells with neuraminidase and galactose oxidase [199] we have observed a 14q+ marker in half of the B-CLL cases studied (Pittman and Catovsky, to be published). Other abnormalities such as trisomy 3, trisomy 18, abnormal No 1, 6q− have also been reported in B-CLL but with much less frequency than trisomy 12 and 14q+.

Questions relating to the clinical significance of the chromosome abnormalities in B-CLL and to the origin of the normal metaphases have been raised. Our observations (Pittman and Catovsky, to be published) and those of Han *et al.* [202] suggest that there is a strong correlation between abnormal karyotypes, advanced stages of the disease and short survival. As Han *et al.* [202], we did not observe a relationship between trisomy 12 and poor prognosis. In our cases, the presence of 14q+ (breakpoint 14q32) was associated with features of progressive B-CLL: prolymphocytoid or Richter-type transformation, refractoriness to therapy, high WBC and advanced staging and we have also documented a significant difference in survival between patients with 14q+ and those with other abnormalities. Our findings suggest that trisomy 12 may be an early change in B-CLL whilst 14q+ is possibly acquired during disease progression. Of interest too is the fact that 14q+ is not seen as an additional change in patients with trisomy 12 [202] suggesting that both abnormalities may be markers of different types of B-CLL. The accumulated data suggest that the chromosome abnormalities in B-CLL are not a consequence of previous therapy or mitogen effects. Our studies suggest also that the presence of normal metaphases in this disease usually correlates with the proportion of E-rosette forming cells present in the sample and that direct analysis of mitotic figures in combination with T and/or B markers are necessary, in cases reported as having only normal metaphases, in order to determine whether or not these cells belong to the neoplastic population. A relationship between, abnormal karyotypes and the expression of some membrane antigens could now be investigated as a result of work by Katz *et al.* [215]. These authors have mapped the p24 antigen, which has a variable expression on B-CLL lymphocytes and is demonstrated by the McAb BA−2 and FMC8, to chromosome 12. Thus, it may be feasible to examine whether the expression of this antigen is increased in cases with trisomy 12. In this respect Gahrton *et al.* [216] have recently suggested that the segment q13 and q22 of chromosome 12 carries the genes which may be important in the pathogenesis of lymphoproliferations associated with trisomy 12.

6.2. *Abnormalities in B-PLL*

Two reports have recently described chromosome abnormalities in this variant form of B-CLL [199, 203]. Sadamori *et al.*al. [203] described an unusual abnormality, t(6; 12) (q15; p13) as possibly specific for B-PLL in 5 patients. 6q− and 14q+ were also observed in 1 patient [203]. Our observations on 9 patients [199] failed to show the marker reported by Sadamori *et al.* [203]. Instead we observed a marker 14q+ (q32) in 7 cases, which in 2 resulted from t(11; 14) [199], and a deletion 6q− (q21) in 5, but with a different breakpoint from the cases of Sadamori *et al.* (q15). Although we documented trisomy 12 in three cases, this abnormality was not seen in the stem line in any of these patients, thus suggesting that B-PLL in them did not evolve from a pre-existing B-CLL with trisomy 12. In 3 of the 5 cases described with t(6; 12) [203], the origin of the complex marker was an extra chromosome 12 and an extra No 6, thus raising the possibility that B-PLL in those patients could have arisen as a result of karyotypic evolution from B-CLL with trisomy 12 [203]. In the majority of our cases [199] we have documented evidence of karyotypic evolution, which in 2 appears to be more significant in the spleen than in the PB cells.

6.3. *Abnormalities in HCL*

A report by Golomb *et al.* [204] in 26 cases using unstimulated 24–48 h PB cultures, identified one case and suspected another with trisomy 12. No other clonal abnormalities were clearly documented in the remaining cases. More recently, Khalid *et al.* [205] described a marker 14q+ resulting from a t(1; 14), with a portion of the long arm of chromosome 1 translocated to the long arm of chromosome 14, in one cell of a single case; because PHA was used as mitogen, the great majority of metaphases in that study were all normal. Polyclonal B-cell mitogens were used by Sadamori *et al.* [206] to study the karyotype of hairy cells in a PB sample from an atypical case of HCL (high WBC and TRAP negative). These authors obtained many metaphases with LPS and demonstrated multiple clones, the predominant one (44% of cells) had a small ring chromosome and another (4% of metaphases) showed a 14q+ marker. Five out of 20 patients with HCL studied with B-cell mitogens [199] in our laboratory (Pittman, Brito-Babapulle and Catovsky, to be published) had a clonal 14q+ marker as the major abnormality; the origin of the material translocated to chromosome 14 could not be established in these patients. It is obvious that more studies with several B-cell mitogens are necessary before the significance and frequency of chromosome aberrations in HCL can be ascertained.

6.4. *Abnormalities in NHL*

The most common marker in B-cell NHL is 14q+ seen in just over 50%

of cases [214, 217]. Using high resolution chromosome banding techniques, Yunis *et al.* [197] have succeeded in recognising the origin of the donor chromatin responsible for the 14q+ marker. As a result of these studies new associations between particular histological types of NHL and specific abnormalities have been established [197]. Thus FL has been shown to have, in the majority of cases, a translocation between chromosomes 18 and 14, t(14; 18) [203, 218], whilst Burkitt's lymphoma, small non-cleaved and large cell immunoblastic lymphoma have a t(8; 14) [203, 218]. The breakpoint of chromosome 14 in all these cases is at 14q32. Small-cell (well differentiated) lymphocytic lymphoma and Waldenstrom's macroglobulinemia, disorders closely related to B-CLL have, like this disease, trisomy 12 [203, 207]. Plasma cell proliferations are also associated with 14q+, often resulting from t(11; 14) [214, 217]. Other abnormalities frequent in NHL, but apparently not related to a particular histological type are 6q−, abnormalities of chromosome 1, trisomy 3 and i(17q) [197, 214, 217, 218].

6.5. *Abnormalities in T-Cell Leukemias and CTCL*

Chromosome abnormalities in T-cell disorders tend to be complex. Sometimes they appear to be non-clonal and, in some cases, several unrelated clones may be demonstrated [176, 209].

Findings in T-CLL have been scanty because of the difficulties in eliciting a mitogenic response in T8+ lymphocytes. Non-random abnormalities have, nevertheless, been documented (Table 15). There is a special interest in demonstrating clonal abnormalities in T-CLL because of the arguments questioning the neoplastic nature of this disorder [83] and the difficulties in demonstrating the monoclonal nature of T-cell proliferations by membrane markers. The disease has a protracted chronic course but even this cannot be used against the process being leukemic as shown by a patient reported by Siegal *et al.* [108] in whom trisomy 22 was demonstrated in the T-lymphocytes despite the very slow clinical evolution of the disease over an eleven year period.

Few cases of T-PLL have been reported and in each of them one or more marker chromosome, but different in each case, have been demonstrated in all abnormal metaphases obtained with PHA [209]. Both in T-CLL and T-PLL we observed frequent involvement of chromosomes 2 (e.g. 2q−) and 8 (e.g. monosomy 8) in structural and numerical rearrangements [209]. Abnormalities of chromosome 2 are rare in other human neoplasias [214] but are not uncommon in T-cell leukemias and in CTCL [208, 209]. We have recently observed structural changes of No 2 (2q+, 2p−, 2p+) in 3 cases of Sézary syndrome and one ATLL [94].

The 3 most common abnormalities in ATLL are: i) 14q+ (with breakpoint 14q32 but variable donor chromosomes), shown in 50% of Japanese

ATLL [201, 211]; we have observed a marker 14q+ resulting from t(11; 14) in a Caribbean ATLL but with the breakpoint in the region q22-24 [176]; ii) trisomy 7q in 15% of cases, both from Japan [210] and the Caribbean [176]; this abnormality has been reported, but more rarely in CTCL [94, 176], but not in other T-cell malignancies; iii) 6q−, which is known to occur in ALL and in B-cell NHL (Table 14) has been reported in 17% of ATLL cases [176, 211]. In a patient with ATLL we have shown two 6q− markers in 3 distinct clones; the breakpoint was q21 in one of them and q25 in the other [176]. A 6q− marker occurs also frequently in CTCL, in approximately 27% of cases (reviewed in 176). A marker (17q) has been described in a variety of hematological malignancies, chiefly CML in blast crisis, and has also been observed, although more rarely, in B-cell disorders (see above) and in several patients with Sézary syndrome [176, 213]. It is of interest that we have demonstrated i(17q) in one of the clones from a Caribbean ATLL in whom 30% of the neoplastic cells were indistinguishable from Sézary cells [176]. A relationship can be suggested between trisomy 7q in ATLL and the presence of the receptor for T-cell growth factor (TCGF or IL2) demonstrated by the McAb anti-Tac (see above) in ATLL cells as well as in normal T-lymphocytes infected by HTLV [178]. The gene coding for the TCGF receptor, which is essential for the immortalization of T-cells in culture, has not been mapped to any chromosome; it is possible that trisomy 7 may play an important role in the pathogenesis of ATLL, as it has been suggested for other hemopoietic malignancies with non-random abnormalities in relation to the location of oncogenes [194, 196].

7. CONCLUSIONS

Careful morphological, cytochemical and immunological analysis, has enabled a more objective diagnosis of lymphoproliferative disorders. Both, in the B and T-cell lineages, the neoplastic lymphocytes have features that resemble those of their normal counterparts. The use of McAb has greatly facilitated the analysis of membrane antigens, some of which are specific for certain cell types and others for particular stages of differentiation. The resulting picture is one of a more rational classification which has already proved fruitful by identifying disease entities with specific geographical clustering, such as ATLL, and newly defined etiological agents, such as HTLV. The study of the karyotype in the lymphoid malignancies, now possible by improved laboratory methods, has added a new marker with important prognostic implications and of value for the analysis of pathogenetic mechanisms.

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4. Surface Marker Analysis of Acute Myeloblastic Leukemia

JAMES D. GRIFFIN

1. INTRODUCTION

Acute leukemias have long been recognized as heterogeneous diseases on the basis of their morphologic appearance, clinical presentation, and response to therapy. This is particularly true of acute myeloblastic leukemias (AML) which are traditionally classified into subgroups by morphology and cytochemical staining patterns [1, 2]. For the most part, however, classification of AML by morphology has not been successful in predicting outcome of disease [2–5], and other methods have been sought to identify subgroups in this disease.

During the last decade, considerable progress has been made in the identification and characterization of surface membrane antigens that are expressed by human leukemic cells [6]. This immunological approach was initially applied to malignancies derived from lymphocytes, and has been successfully used to improve diagnostic accuracy, and to investigate leukemic cell heterogeneity in acute lymphoblastic leukemia (ALL) [7–11]. It has been demonstrated, for example, that leukemic lymphocytes express a wide variety of surface antigens that are shared by various normal lymphocyte-lineage cells. It has become possible to precisely relate a leukemic cell to a normal cell counterpart by analysis of the pattern of surface antigens which are expressed [6]. For example, the majority of cases of ALL express surface antigens characteristic of normal B lymphocyte lineage cells, while a smaller number express antigens of T cells. Rapid identification of the T cell leukemias is important clinically because the prognosis is generally worse, and therapy is different [7, 8]. Thus, surface marker analysis has become standard practice in ALL because the distinction between B-lineage and T-lineage leukemias is not generally possible using morphological analysis. Initially these surface antigens were detected with carefully prepared heteroantisera raised against purified populations of normal lymphocytes or

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ALL cells. More recently, the ability to produce monoclonal antibodies reactive with specific cell surface antigens has permitted a dramatic expansion in the field of surface marker analysis. Large panels of well characterized, highly-specific monoclonal antibodies are available that can be used to identify lineage-restricted surface antigens of B cells, T cells, and other lymphocyte populations (6). The same reagents can now be used in multiple laboratories, and the usefulness of monoclonal antibodies in the phenotyping analysis of ALL is well established.

In a similar effort to supplement the morphological classification systems of AML, a number of laboratories have investigated the utility of surface marker analysis using heteroantisera [12–22] and monoclonal antibodies [23–46]. Initial studies with heteroantisera demonstrated that myeloid lineage-specific antigens exist, and that myeloid leukemias express some of these antigens. This article summarizes current knowledge regarding the clinical utility of surface marker analysis in AML. The contributions of heteroantisera will be briefly reviewed, followed by a description of the production of monoclonal antibodies reactive with AML cells and a summary of the major monoclonal antibodies produced thus far that appear to have potential clinical diagnostic utility. Finally, the biologic implications of surface marker heterogeneity in AML, and the potential therapeutic implications will be considered.

2. DIFFERENTIATION OF NORMAL MYELOID CELLS

Any analysis of surface markers in AML must take into consideration the relationship of myeloid leukemic cells to normal myeloid cells, and a brief review of normal myelopoiesis is relevant. More detailed reviews are available [47, 48]. Mature myeloid cells are derived from a population of pluripotent hematopoietic stem cells that have the capacity for both self-renewal and differentiation (Fig. 1). The progeny of these stem cells include several committed precursor cells that give rise separately to erythroid cells, megakaryocytes, and to granulocytes and monocytes. Identification of these progenitor cells has been accomplished by *in vitro* colony assays, where it has been demonstrated that large aggregations of mature cells of each lineage are derived from single progenitor cells which proliferate in response to specific growth factors [49–52]. Differentiation of the granulocyte-monocyte precursor cell into later myeloid cells depends, at least *in vitro,* on the presence of colony-stimulating activity, a group of substances produced by monocytes, activated T cells, and certain other cells [53–55]. Although later stages of myeloid differentiation are readily identifiable by changes in morphology and cytochemistry, the detailed study of early myeloid differentiation has

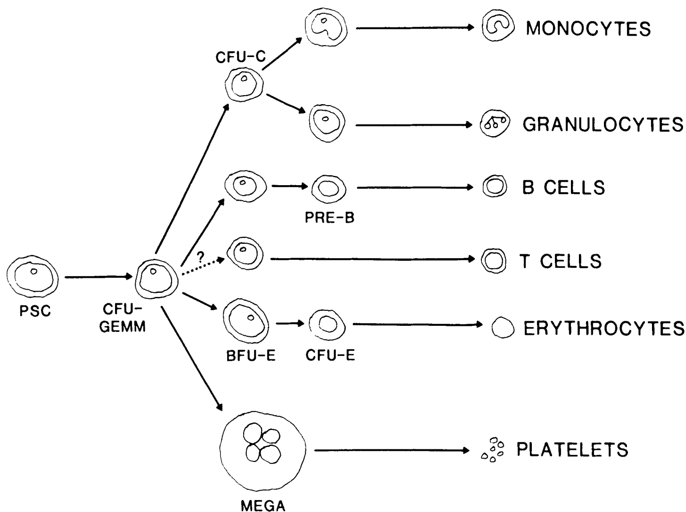

Figure 1. Model of human hematopoiesis. PSC, pluripotent stem cell; CFU-GEMM, multipotent stem cell capable of forming mixed hematopoietic colonies; CFU-C, committed myeloid progenitor cells; BFU-E, erythroid burst forming unit; CFU-E, Erythroid colony forming unit; Mega, megakaryocyte.

been difficult because of the small numbers of these precursor cells and their lack of distinctive morphological or biochemical features.

The earliest morphologically recognizable myeloid cell, the myeloblast, constitutes approximately 0.5–1% of bone marrow cells, and it is clear that the majority of myeloblasts are not capable of forming colonies, since CFU-C generally constitute only 0.05–0.1% of bone marrow cells. The stages of differentiation which follow the myeloblast are well characterized morphologically and functionally. In the granulocyte lineage, morphological progression from myeloblast to promyelocyte, myelocyte, metamyelocyte, band, and poly is accompanied by an orderly acquisition of new cell functions such as the ability to ingest foreign particles and to follow a chemotactic gradient. The frequent finding *in vitro* of colonies containing both monocytes and granulocytes suggests that at some point in differentiation, monocytes and granulocytes share a common committed progenitor cell. Monocyte precursors (monoblasts and promonocytes) are less well defined morphologically than are granulocyte precursor cells, but commitment to monocyte differentiation is presumed to occur at the level of the CFU-C or soon thereafter. It is important to note that in normal myelopoiesis, proliferation of immature cells is tightly coupled to differentiation, and that the proliferative potential of each succeeding cell stage falls rapidly from the CFU-C to the myelocyte or monocyte. Although earlier hemaotopoietic progenitor cells than the CFU-C may have some self-renewal capacity, the CFU-C does not, and each round of cell division is inevitably associated

with increased differentiation and loss of proliferative potential. This is in contrast to AML cells, where proliferation appears to proceed in the absence of any commitment to terminal differentiation. However, as noted above, the ability to morphologically distinguish differentiation stages of very immature myeloid cells is very limited and better techniques to identify early myeloid cells are required. The ability of antibodies to define differentiation antigens of immature as well as mature myeloid cells is therefore likely to be of considerable benefit in the analysis of AML cells, both in relating AML cells to normal counterpart cells (particularly to cells such as the CFU-C which can not be morphologically identified) and in the identification of differentiation levels within individual cases of AML. Considering this goal, the use of polyclonal antisera defining myeloid cell surface antigens will be reviewed as background to the use of monoclonal antibodies.

3. DETECTION OF MYELOID-LINEAGE ANTIGENS BY HETEROANTISERA

Studies with heteroantisera have demonstrated that myeloid-lineage-specific antigens exist, and that myeloid leukemias may express some of these antigens. Antisera to human myeloid cells have been raised in mice [12], rabbits [14], primates [15, 17], goats and in patients receiving immunotherapy [18, 19]. Immunization with normal myeloid cells, AML cells or with myeloid leukemia cell lines has been effective, although extensive absorption is usually necessary.

Baker *et al.* described production of heteroantiserum to human macrophages reactive with blasts from 25 to 27 patients with acute myelomonocytic leukemia, but only 1 of 20 patients with undifferentiated acute myeloblastic leukemia, and 0 of 13 patients with ALL [13]. Activity of the serum against myelomonocytic leukemia cells could be removed by absorption with monocytes, thus demonstrating that some AML cells bear differentiation antigens characteristic of normal mature myeloid cells. Roberts and Greaves described an antiserum raised in rabbits immunized with myelomonocytic and monocytic leukemia cells (anti-M serum) [20]. After absorption with erythrocytes and tonsil lymphocytes, the serum reacted with granulocytes, monocytes, and myeloid bone marrow cells as early as promyelocytes, but not erythroid progenitors or lymphocytes. Forty-five of 46 AML patients' cells were reactive, and ALL and CLL were not reactive. No leukemia-specific antigens were identified, as absorption with normal bone marrow cells removed all reactivity with leukemic cells.

Several laboratories have generated antisera that are reactive with myeloid leukemic cells but are without apparent reactivity to normal hemato-

poietic cells. Mohanakumar and colleagues have generated several simian antisera to human AML, AMML, and CML cells [16, 17]. AML antisera were prepared that had no reactivity for normal lymphocytes or lymphoid malignancies, but reacted with 25 of 32 AML patients, 16 of 16 AMML patients and 18 of 34 CML patients. Billing *et al.* have described a rabbit antisera to AML cells which reacted with leukemic cells from 10 of 46 AML patients and 2 of 23 ALL patients, without reactivity to normal peripheral cells, CML cells, or the normal bone marrow myeloid stem cells (CFU-GM) [14]. The cell line K562, established by Lozzio from a patient with CML blast crisis [21], has been used to produce a goat antiserum reactive with CML, ALL, AML, and CLL cells, but not normal cells [22].

Thus, several AML heteroantisera have been produced which lack significant reactivity with normal myeloid or lymphoid cells, suggesting the existence of leukemia-specific antigens. However, the existence of such antigens is difficult to establish using heteroantisera, which have low titers, and using methods such as complement mediated cytotoxicity testing, which may not detect reactivity with small populations of cells, or cells with low antigen density. However, reactivity of some of these antibodies with malignant cells is restricted to myeloid leukemias, and they have thus proven diagnostically useful. The problems of low titer and poor reproducibility of heteroantisera noted above, however, have limited the utility of these reagents for diagnostic studies or for research. The generation of monoclonal antibodies, however, to myeloid cell surface antigens has resulted in the rapid development of this field.

4. PRODUCTION OF MONOCLONAL ANTIBODIES TO MYELOID CELL SURFACE ANTIGENS

In 1975 Kohler and Milstein described a technique for fusion of murine B lymphocytes with murine myeloma cells resulting in the production of hybridoma cell lines secreting antibodies of predefined specificity [56]. These studies were preceded by a series of important observations demonstrating that a single B lymphocyte produced an antibody specific for a single antigenic determinant [57, 58], that the plasma cell was the endstage of B-cell differentiation [59], that myeloma cells could be grown *in vivo* [60, 61], cultured *in vitro* [62], and that stable hybrids could be obtained following fusion of two parental cell lines [63–65]. A schematic representation of the immunization, fusion, and selection of hybrid clones producing monoclonal antibodies is shown in Figure 2.

Approximately 4 weeks after the initial immunization, the immunized mice are boosted intravenously with the immunizing cell and within three

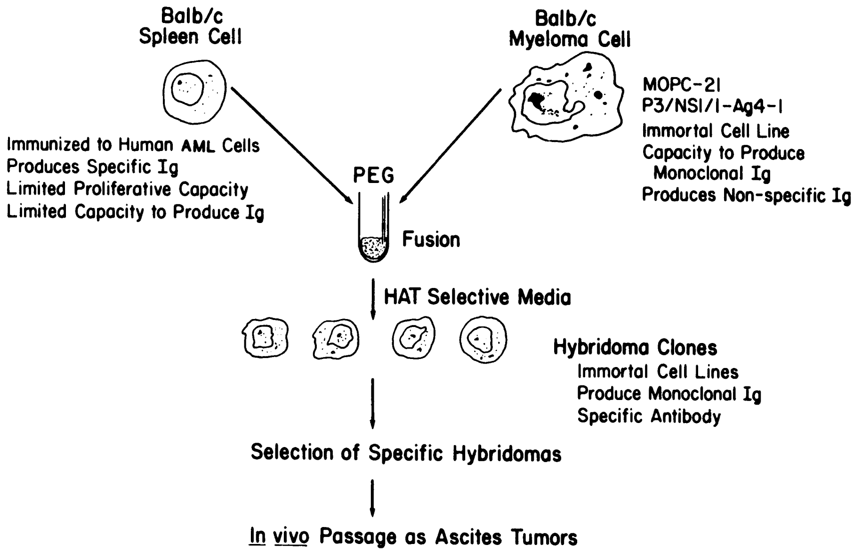

Figure 2. Production of monoclonal antibodies reactive with human AML cells.

to seven days are sacrificed (Figure 2). A single cell suspension of mouse lymphocytes is then prepared from the spleen and is combined with the murine myeloma cells. Specific myeloma cell lines have been selected because they lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) and are therefore unable to survive in media which contain aminopterin (HAT) [63]. Once these cells are combined, they are fused in the presence of polyethylene glycol (PEG) [56]. The fusion of an individual murine B lymphocyte with a HAT-sensitive murine myeloma cell is a random event. This mixture of fused cells, unfused B lymphocytes, and myeloma cells is then plated into individual microtiter wells and after 24 hours the unfused myeloma cells are killed by adding aminopterin (HAT media). The unfused mouse B lymphocytes die spontaneously in culture after 5 to 7 days. Hybrid cells are the only ones capable of proliferating under these conditions. After hybridoma cells are grown for approximately 10–14 days, the supernatants of visible clones are then tested for the production of specific mouse immunoglobulin reactive with the immunizing cells by indirect immunofluorescence, ELISA, radioimmunoassay, or microcytotoxicity. Clones producing reactive supernatants are passaged in culture and recloned to assure the presence of only a single antibody. Supernatants are then screened for reactivity with non-myeloid cells (lymphocytes, erythrocytes, ALL cells, etc.), gradually selecting antibodies with the desired spectrum of reactivity.

The majority of monoclonal antibodies which have been produced have been generated following immunization with either cryopreserved AML

cells or with AML cell lines. The promyelocytic leukemia cell line HL60 has been particularly popular [66], as has been KG-1 [67] and K562 [21]. A large number of anti-myeloid monoclonal antibodies have been produced in this fashion, and a selection of these antibodies will be considered individually in the following section.

5. MONOCLONAL ANTIBODIES REACTIVE WITH HUMAN AML CELLS

More than 40 monoclonal antibodies reactive with AML cells have been reported over the last few years. All of these antibodies also react with subsets of normal myeloid cells, and there appears to be considerable overlap among antibodies. This is a major problem at this time, and until antibodies from different laboratories can be grouped functionally or biochemically, the literature is likely to remain confusing. Very few antibodies identify structures of known function, and even the molecular weights of most antigens studied have not been determined. However, efforts to directly compare different antibodies are underway, and some preliminary results from the First International Conference on Human Leukocyte Differentiation Antigens (Paris, November 1982) will be discussed at the end of this section.

Antibodies with extensive reactivity outside the myeloid system will not be considered here, unless they have been particularly useful in the characterization of myeloid leukemias. Particular attention will be given to those antibodies that have been characterized with regards to their reactivity with normal hematopoietic colony forming cells. The reactivity of this group of antibodies with normal myeloid cells is summarized in Table 1.

5.1. *B4.3, B13.9, B2.12.* Van der Reijden *et al.* have described three monoclonal antibodies obtained as side products of an attempt to raise T-gamma cell specific antibodies [43]. These antibodies react strongly with granulocytes but not with platelets or red blood cells. B2.12 also reacts with monocytes and with a small population of lymphocytes. B13.9 is an IgG1 while the other two are IgM antibodies. Reactivity with normal bone marrow cells and colony forming cells was not recorded. However, these antibodies are included in this review because of their extensive characterization using AML cells.

5.2. *B9.8, B13.4, B34.3, B43.4.* Perussia and her colleagues have reported the characterization of a series of monoclonal antibodies reactive with both peripheral blood monocytes and granulocytes as well as with bone marrow myeloid cells of varying degrees of maturation. B43.4, B9.8, B13.4, and

Table 1. Reactivity of anti-AML monoclonal antibodies with normal peripheral blood and bone marrow myeloid cells

Antibody	Gran[a]	Mono	Bands	Myelo	Pro	Blast	CFU-C	% Positive bone marrow cells	Reference
AML-2-23	+[b]	+	+	+	+	–	–	17	38, 39, 41
B9.8	+	+	+	+	–	–	NR	NR	80
B13.4	+	+	–	–	–	–	NR	30	80
B34.3	+	+	+	–	–	–	NR	NR	30
B43.4	+	+	+	+	+	–	NR	20	30
D5	–	+	+	+	+	–	NR	52	29
D5D6	–	+	NR	NR	NR	NR	+	NR	31
5F1	–	+	–	–	–	–	–	14	32
FMC10	+	–	+	+	+	–	NR	NR	27
FMC11	+	–	+	–	–	–	NR	NR	27
FMC12	+	–	+	+	+	–	NR	NR	27
FMC13	+	–	+	+	+	–	NR	NR	27
1G10	+	–	+	+	+	+	+	48	32
80H.1	+	+	+	–	–	–	–	6	44
80H.3	+	+	+	+	+	+	–	40	44
80H.5	+	–	+	+	+	+	+	85	44
L4F3	–	+	–	+	+	+	+	25	42
MMA	+	+	NR	NR	NR	NR	+	25	54
Mo1	+	+	+	+	–	–	–	34	24, 35, 37
Mo2	–	+	–	–	–	–	–	7	24, 35, 37
Mo3	–	+	–	–	–	–	–	6	35
Mo4	–	+	–	–	–	–	–	4	35
Mo5	+	+	+	+	+	–	–	31	35
MY1	+	–	+	+	+	–	–	50	25, 69
MY4	–	+	–	–	–	–	–	6	26
MY7	+	+	+	+	+	±	±	10	26, 70
MY8	+	+	+	+	+	–	–	30	26
MY9	–	+	–	±	+	+	+	28	71
MY10	–	–	–	–	–	+	+	2	45,46
PMN6	+	–	+	+	+	–	–	18	41
PM81	+	+	+	+	+	+	–	45	40
TG-1	+	–	+	+	+	–	–	55	28

[a] Gran, granulocytes; mono, monocytes; myelo, myelocytes; pro, promyelocytes.
[b] Determined by fluorescence-activated cell sorting; NR, not reported.

B34.3 antigens arise in sequence on promyelocytes, myelocytes, metamyelocytes, and granulocytes respectively. B43.4 is also expressed on bone marrow null cells giving it a distribution similar to that of Mol. The molecular weights of these antigens have not been reported.

5.3. *D5.* Majdic *et al.* have reported the generation of an IgM monoclonal antibody raised against K562 cells [29]. The D5 antigen is expressed by peripheral blood granulocytes but not by monocytes, erythrocytes, or platelets. Fifty-two percent of bone marrow mononuclear cells are positive including granulocyte lineage cells as immature as the promyelocyte. Normal myeloblasts were only weakly positive or negative. Morphologically recognizable erythroid cells appeared to be D5 negative.

5.4. *D5D6.* Linker-Israeli and colleagues described D5D6 monoclonal antibody which was reactive with peripheral blood monocytes but not granulocytes or other circulating cells [31]. The immunizing cells were AML cells. D5D6 was described as being lytic for the blast cells of the majority of patients tested with AML while cells from chronic myelogenous leukemia or lymphocytic leukemias were negative. The percent of normal bone marrow cells which express D5D6 was not indicated but the CFU-C cells were inhibited by 90% following treatment with anti-D5D6 and complement.

5.5. *5F1.* 5F1 is a lytic IgM antibody raised against AMML cells by Andrews *et al.* 5F1 is strongly reactive with peripheral blood monocytes but not granulocytes or lymphocytes [32, 42]. In the bone marrow 14% of cells are positive and this fraction is composed primarily of erythrocytes and monocytes. A small fraction of polys are also found in the positive fraction by cell sorting. CFU-C and BFU-E are negative. Bone marrow CFU-E are positive while peripheral blood CFU-E are 5F1 negative.

5.6. *FMC10, FMC11, FMC12, FMC13.* Zola *et al.* have described a series of four monoclonal antibodies reactive with human granulocytes [27]. FMC11 is of the IgG1 isotype while the remainder are of IgM subtypes. None of these antibodies react with monocytes in the peripheral blood. FMC10, 12, 13 react with granulocyte lineage cells in the normal bone marrow as immature as some promyelocytes while FMC11 reacts with cells more mature than the myelocyte.

5.7. *1G10.* Bernstein and co-workers have also reported the development of 1G10, an IgM antibody reactive with granulocytes in the peripheral blood and very weakly with monocytes [32, 42]. 48% of bone marrow cells react with 1G10 antibody including myeloid cells as immature at the myeloblast. Bone marrow monocytes, lymphocytes, and erythroid progenitors are 1G10 negative. CFU-C in the bone marrow are 1G10 positive while peripheral blood CFU-C are 1G10 negative. 1G10 appears to identify a carbohydrate determinant (((GAL1-4)Fuc 1-3)G1cNAC).

5.8. *80H1, 80H3, 80H5.* Mannoni *et al.* have reported a series of monoclonal antibodies following immunization with normal granulocytes or with CML leukocytes [44]. These antibodies react with granulocytes from all donors tested but not with lymphocytes, platelets, or red cells. 80H.1 and 80H.3 react with monocytes in addition. In the normal bone marrow 80H.1 antigen is expressed on bands and granulocytes while 80H.3 antigen and 80H.5 antigen are expressed on cells as early as the myeloblast. 80H.5 antigen was expressed by CFU-C but not BFU-E or CFU-E as determined by complement lysis experiments. 80H.1 and 80H.3 are not cytotoxic antibodies and have not been tested for reactivity with colony forming cells.

5.9. *L4F3.* L4F3 is an IgM antibody described by Andrew *et al.* produced after immunization with AML cells [42]. In the peripheral blood, monocytes are weakly reactive while granulocytes and lymphocytes are negative. 25% of normal bone marrow cells express L4F3 antigen and this includes the majority of metamyelocytes, myelocytes, promyelocytes, myeloblasts, and monocytes. This antigen is further expressed by CFU-C in the bone marrow and peripheral blood and by BFU-E but not CFU-E. The distribution of this antigen appears to be similar to that of MY9.

5.10. *MMA.* Hanjan *et al.* have described an IgM monoclonal antibody produced against the human histiocytic cell line U937 that has reactivity with AML cells [34]. In the peripheral blood MMA is reactive with granulocytes and monocytes. Although resting T cells in the peripheral blood are negative, 80% of activated T4 cells and 10% of T8 cells expressed MMA. In the bone marrow 25% of the mononuclear cells were positive. This includes the CFU-C cells as tested by complement lysis. The T cell lines HSB-2 and Molt 4 are MMA positive.

5.11. *Mo1.* Mo1 is an antigen defined by a lytic IgM antibody [24, 35, 36] whose reactivity appears to be identical to that of 0KM1 antibody [23]. This antigen is expressed on all peripheral blood monocytes, granulocytes, and a population of non T, non B lymphoid cells operationally defined as null cells. This latter population includes the cells in the peripheral blood responsible for natural killer activity. The cell surface antigen precipitated by anti-Mo1 antibody consists of two polypeptide chains of 94 and 155 Kd. The Mo1 antigen is expressed by the majority of bone marrow myeloid cells from promyelocyte to mature granulocyte but has not been detected on CFU-C. In recent functional studies, anti-Mo1 treatment of normal monocytes and granulocytes blocks binding of the C3bi component of complement [68]. This treatment further inhibits the phagocytosis of opsonized particles.

5.12. *Mo2.* Mo2 is a monocyte specific antigen identified by a lytic IgM monoclonal antibody which binds selectively to peripheral blood monocytes, macrophages derived from cultured monocytes, and peritoneal macrophages [24, 35, 36]. Mo2 is not expressed by other circulating human cells including T, B and null lymphocytes and granulocytes, platelets, or erythrocytes. It has not been detected on any cultured human cell line. The molecular weight is 55 Kd. Treatment of human mononuclear cells with anti-Mo2 and complement has been shown to eliminate monocyte specific functions. In the bone marrow, Mo2 is expressed exclusively on cells with morphologic features of monocytes.

5.13. *Mo3.* Todd *et al.* have described anti-Mo3 which is an IgM lytic antibody identifying an antigen expressed weakly by freshly harvested peripheral blood monocytes but which becomes strongly expressed by the majority of cells which have been cultured overnight at 37 °C [35, 37]. In the peripheral blood Mo3 antigen is expressed only by monocytes.

5.14. *Mo4.* Anti-Mo4 antibody was also described by Todd *et al.* [35, 37]. The Mo4 antigen is expressed by a variable fraction of peripheral blood monocytes (40–80%) and platelets. Lymphocytes, granulocytes and erythrocytes are negative. The molecular weight of Mo4 antigen is 100 Kd. In the bone marrow, megakaryocytes and monocytes are positive.

5.15. *Mo5.* Anti-Mo5 was also described by Todd *et al.* and is also an IgG2a monoclonal antibody which defines a 94 Kd protein antigen similar in its distribution to the expression of MY8 [35, 37]. It is not found on CFU-C and is not found on natural killer cells.

5.16. *MY1.* MY1 is a granulocyte specific antigen identified by monoclonal antibody 1/12/13 described by Civin *et al.* [25]. A series of monoclonal antibodies apparently binding to the same surface antigen structure have been described by the same authors [69]. These include anti-MY24, MY5, MY7, and MY18. All five are of the IgM kappa subtype. 1/12/13 was produced by immunizing with HL60 leukemic cells. In the bone marrow, MY1 is expressed on granulocyte lineage cells as early as promyelocyte. It has not been detected on the CFU-C. Blocking experiments suggest that all five antibodies react with a sugar sequence found in lacto-N-fucopentaose III [69]. This sugar sequence has been found in glycolipids and glycoproteins. It has therefore not been established whether all of the hematopoietic cells which bind anti-MY1 antibody are expressing the same or different antigen structures.

5.17. *MY3, MY4.* Anti-MY3 and MY4 were described by Griffin *et al.*

and were produced following immunization with acute myelomonocytic leukemia cells [26]. Both MY3 and MY4 antigens are strongly expressed on all peripheral blood monocytes but very weakly on peripheral blood granulocytes. These antigens are not detected on lymphocytes, erythrocytes, or platelets. In the bone marrow the positive cells are morphologically identifiable as monocytes. The monocyte colony forming cells (CFU-C) are not positive. Although anti-MY3 and MY4 have different immunoglobulin subtypes, they appear to identify a similar antigen and are therefore considered as a group. Like Mo2 antigen, MY3 and MY4 are not found on cultured human cell lines. They are distinguished from the Mo2 antigen by the slight expression on granulocytes and by their more widespread distribution in acute myeloblastic leukemia patients.

5.18. *MY7.* Anti-MY7 is an IgG1 monoclonal antibody described by Griffin *et al.* which was produced after immunization with acute myelomonocytic leukemia cells [26]. The antigen is detected on both granulocytes and monocytes in the peripheral blood but is not found on platelets, erythrocytes or lymphocytes. In the bone marrow a variable percentage of the cells express MY7 antigen ranging from 6% to more than 30%. The higher figures are seen in situations where marrow cells are most likely to be actively proliferating, including recovery from chemotherapy. Preliminary biochemical characterization shows that the MY7 antigen has a molecular weight of approximately 160 Kd. The bone marrow cells which express MY7 antigen include myeloblasts, promyelocytes, and myelocytes in particular. By fluorescence activated cell sorting and immune rosetting techniques a fraction of CFU-C also expressed MY7 antigen [70]. This is the first antigen of the CFU-C cell which has been described to identify a subset of CFU-C. The $MY7^+$ CFU-C appear to contain those cells which are most actively proliferating, i.e. those cells traversing the cell cycle. In contrast, the MY7 negative cell fraction appears to contain very few cells which are actively synthesizing DNA. Erythroid progenitors including BFU-E and CFU-E are MY7 negative. Thus, MY7 is unlike many of the other antigens considered here in that it does not define a discrete stage of differentiation. Rather it appears that MY7 antigen may be related to cell proliferation at least of bone marrow cells.

5.19. *MY8.* Anti-MY8 monoclonal antibody, also described by Griffin *et al.*, is reactive with both granulocytes and monocytes in the peripheral blood but not with non-myeloid cells [26]. In the bone marrow the antigen is expressed on granulocyte lineage cells as immature as the promyelocyte. The CFU-C cell is MY8 negative. MY8 antigen is therefore a classical differentiation antigen of myeloid cells.

5.20. *MY9.* The MY9 monoclonal antibody was produced by Griffin *et al.* following immunization with the cells of a patient in the blast phase of chronic myeloid leukemia [71]. Anti-MY9 is an IgG2b antibody which is lytic with rabbit complement. It reacts with monocytes in the peripheral blood but is not reactive with granulocytes or non-myeloid cells in the peripheral blood. In the bone marrow, MY9 antigen is expressed on about 30% of cells. These include myeloblasts, promyelocytes, myelocytes, and monocytes. Erythroid cells, lymphoid cells, and the more mature granulocyte elements are MY9 negative. Greater than 90% of CFU-C cells are reactive with MY9 antibody as determined by complement lysis, fluorescence activated cell sorting, or immuno-rosetting techniques. Approximately 50% of BFU-E cells are MY9 positive. CFU-E are MY9 negative. Unlike MY7 antigen, MY9 is also expressed on the CFU-C cells in the peripheral blood. The mixed colony forming cell (CFU-GEMM) are MY9 positive in preliminary experiments

5.21. *MY10.* MY10 antigen is a cell surface protein with apparent molecular weight of 115 Kd, produced after immunization with KG-1a human leukemic cells [45–46]. Peripheral blood cells do not express the MY10 antigen. Two-four percent of normal bone marrow low density cells express MY10 by indirect immunofluorescence. Cell sorting and panning experiments demonstrated that CFU-C and BFU-E are MY10 positive. MY10 appears to be unique in that its expression on normal myeloid cells is limited primarily to progenitor cells.

5.22. *PMN6, PMN29, PM81, and AML-2-23.* Ball and Fanger have described the production of four monoclonal antibodies which react with AML cells [38–41]. PMN6 and PMN29 react with granulocytes in the peripheral blood but not with monocytes. AML-2-23, PM81 react with most peripheral blood monocytes and granulocytes as well as with bone marrow myeloid cells more mature than the CFU-C. PMN6 and PMN29 were expressed on 18% and 50% bone marrow mononuclear cells respectively. PMN6 was reactive with myeloid cells more mature than the myelocyte. PMN29 had a similar distribution on bone marrow cells. AML-2-23 reacted with myeloid cells more mature than the promyelocyte as well as with bone marrow monocytes. None of these antibodies is reported to react with the CFU-C or with other colony forming cells.

5.23. *TG1.* Beverly *et al.* described antimyeloid monoclonal antibody TG1 which recognizes an antigen present on peripheral blood granulocytes and eosinophils but not on lymphocytes, erythrocytes, or thymocytes [29]. In the bone marrow it is reactive with all granulocyte lineage cells as immature as promyelocytes. It is not reactive with CFU-C cells.

5.24. *UCHM1, UCHALF.* Allan, Hogg, and Beverly and colleagues have described two monoclonal antibodies which are monocyte specific. UCHM1 was generated by immunizing with peripheral blood mononuclear cells and is reactive only with monocytes in the peripheral blood. Reactivity with bone marrow cells has not been reported. Antibody UCHALF was generated against purified breast milk lactoferrin and is reactive with peripheral blood monocytes and with dendritic reticulum cells. Fixed granulocytes are also positive but unfixed cells are not. The antibody is reactive with lactoferrin and presumably identifies by the basis of surface lactoferrin. The ractivity of this antibody with bone marrow cells has not been reported.

6. EXPRESSION OF MYELOID DIFFERENTIATION ANTIGENS ON AML CELLS

6.1. *Expression of Myeloid Differentiation Antigens on Cultured Human Myeloid Cell Lines.* One of the most useful approaches for both immunization and characterization of monoclonal antibodies reactive with myeloid cells is the study of myeloid leukemia cell lines. Although human myeloid leukemia cell lines have been particularly difficult to develop, there are now a number of such cell lines available with widely different properties. The HL60 cell line was derived by Gallo and coworkers from a patient with acute promyelocytic leukemia [66]. These cells have the morphologic appearance of promyelocytic leukemia cells and have been commonly used to produce monoclonal antibodies. HL60 cells are particularly interesting because they can be induced to undergo terminal differentiation to either granulocyte-like or monocyte-like cells in response to exposure to a variety of inducing chemicals. For example, exposure of HL60 cells to DMSO [74, 75] or retinoic acid [76] results in a change in morphology and functional characteristics of these cells such that they have the appearance of granulocytes and acquire certain functional properties of mature granulocytes. Similarly, exposure of HL60 to phorbol ester [77] or cytosine arabinoside [78] causes morphological and functional differentiation of HL60 cells to monocyte-like cells. A number of investigators have noted that changes in the expression of surface antigens can be readily observed as HL60 cells are induced to differentiate [26, 79, 80]. For example, exposure of HL60 cells to phorbol ester induces expression of monocyte surface antigens Mo2 and MY4 over a period of about 4 days [26, 79]. Neither of these cell surface antigens are detectable prior to induction of differentiation. The acquisition of new surface antigens detected by monoclonal antibodies can be used to quantify the degree of differentiation of HL60 cells in a variety of functional studies. Detailed description of this research is beyond the scope of this review. The KG-1 cell line was developed by Koeffler and col-

leagues [67] and has the morphological appearance of an undifferentiated blast. Like HL60 cells, KG-1 cells can be induced to differentiate following exposure to compounds such as phorbol ester. The differentiation pathway is primarily along the monocyte lineage. The U937 cell line was developed from a patient with histiocytic lymphoma [81]. This cell is generally believed to have morphologic, cytologic, and functional characteristics most consistant with a monoblast-like cell. The K562 cell line was derived by

Table 2. Expression of myeloid cell surface antigens on cultured human leukemic cell lines

Antibody[a]	HL60 (Promyelocyte)	KG-1 (Myeloblast)	U937 (Promonocyte)	K562 (Pluripotent) (stem cell)
AML-2-23	±	–	–	–
B9.8	–	–	NR	–
B13.4	–	–	NR	–
B34.3	–	±	NR	–
B43.4	+	+	NR	–
D5	+	NR	+	+
D5D6	+	–	NR	–
5F1	–	NR	NR	–
FMC10	+	NR	–	+
FMC11	±	NR	–	+
FMC12	+	NR	–	+
FMC13	+	NR	+	+
1G10	+	NR	NR	–
80H.1	+	–	–	–
80H.3	+	–	–	–
80H.5	+	±	+	+
MMA	NR	NR	+	+
Mo1	±	+	±	–
Mo2	–	–	–	–
Mo3	–	–	+	–
Mo4	–	–	–	–
Mo5	–	–	+	–
MY1	+	–	+	+
MY4	–	–	–	–
MY7	+	+	+	±
MY8	±	+	+	–
MY9	+	+	+	+
MY10	NR	+[b]	–	NR
MM81	+	+	+	+
PMN6	+	–	–	–
PMN29	+	±	–	–
TG-1	+	+	+	+

[a] See Table 1 for references.
[b] KGla.

Lozzio and colleagues [21] from a patient with chronic myeloid leukemia in blast crisis. This cell line has been described to have features of myeloid, erythroid, and megakaryocyte lineage cells. It may be representative of a very immature hematopoietic stem cell.

The distribution of the myeloid antigens described in Table 1 on the myeloid leukemia cell lines HL60, KG-1, U937, and K562 is shown in Table 2.

6.2. *Expression of Myeloid Antigens on Cells from Acute Myeloblastic Leukemia Patients.* Prior work with heteroantisera recognizing surface antigens of myeloid cells suggested that the expression of these antigens on myeloid leukemic cells would be quite heterogeneous. Studies reported so far with myeloid monoclonal antibodies have supported that notion entirely. In considering the clinical utility of these antibodies, several questions should be borne in mind. First, can the antibody be used to reliably distinguish between AML and ALL. Second, can the antigen or pattern of antigens, expressed by each patient's cells be used to identify subgroups of AML patients. Third, what is the correlation of these immunologically defined cell groups with standard morphological and cytochemical classification systems. Fourth, can information be obtained from the immunological phenotype which is not already obtainable from standard classification systems.

The ability of a large series of anti-myeloid monoclonal antibodies to discriminate AML from ALL is shown in Table 3. Many of the antibodies have not been tested on large numbers of patients and their ultimate utility as clinical tools remains to be determined. A number of the antibodies, however, appear to have excellent ability to distinguish AML from ALL. For some antibodies, such as anti-MY7 and anti-MY9, sufficient testing has been carried out such that strong reactivity with one of these antibodies in a case where standard morphological and cytochemical diagnostic tests are uncertain can be used as reliable evidence that the leukemia is of myeloid derivation. Such antibodies will also have a valuable role in the confirmation of diagnoses established by standard criteria. If a leukemia which is morphologically felt to be most consistent with ALL reacts with a well characterized anti-myeloid antibody then reconsideration of the diagnosis should be undertaken. Other diagnostic tests such as electron microscopy and biochemical assays should then be considered before final diagnosis is made. However, although many of the antibodies reported in Table 2 appear to have excellent discriminatory ability, no diagnostic test can be taken as infallible. It is unlikely that any anti-myeloid antibody will be found which will never react with ALL cells. It should also be noted that a small number of 'biphenotypic' leukemias have been reported [82–85]. Most often these have been cases which had two populations of leukemic

Table 3. Use of monoclonal antibodies to distinguish AML from ALL

Antibody[a]	AML				ALL
	AML (unclass)	(No. Positive/No. Tested)			(No. Positive/No. Tested)
		AML	APL	AMML/AMoL	
AML-2-23	–	0/14	–	12/14	0/3
B9.8	–	0/2	0/2	–	0/12
B13.4	–	0/2	0/2	1/1	0/23
D5	–	5i9	2/2		0/8
D5D6	44/50	–	–	–	0/15
5F1	–	4/7	–	8/9	0/17
FMC10	–	2/5	–	0/1	0/4
FMC11	–	0/5	–	0/1	0/4
FMC12	–	1/5	–	0/1	0/4
FMC13	–	1/5	–	0/1	0/4
1G10	–	4/7	–	8/9	0/17
80H.1	0/14	–	–	–	0/6
80H.5	–	–	–	(50%)	0/6
MMA	9/9	–	–	–	1/25
Mo1	–	21/56	–	24/30	1/25
Mo2	–	9/57	–	14/31	0/25
Mo3	–	0/27	–	3/21	0/10
Mo4	–	7/27	–	7/21	0/10
Mo5	–	13/44	–	13/24	2/16
MY1	–	1/9	1/1	0/4	0/20
MY4	–	9/36	0/3	20/31	0/25
MY7	–	29/36	3/3	27/31	1/109
MY8	–	14/36	1/3	24/31	0/25
MY9	–	46/54	4/6	30/37	0/109
MY10	3/18	–	–	–	–
MPN6	–	0/14	–	7/14	0/3
PMN29	–	0/14	–	9/14	0/3
PM81	–	10/12	–	9/10	3/3
TG-1	–	8/17	–	39/40	0/20
UCHALF	–	1/17	–	39/40	0/10
UCHM1	–	1/17	–	38/40	0/10

[a] See Table 1 for references.

cells: often a predominant population of small lymphoid blasts which express the CALLA antigen, and a smaller population of myeloid blasts expressing myeloid antigens. It is also possible to have leukemias which express bilineage markers on the same cell [83]. Although this is uncommonly observed in many immunological phenotyping laboratories, some other investigators commonly observe this phenomenon [84]. Our own laboratory has observed a single case of CML blast crisis in which the blasts

coexpressed T cell antigens (including T3) and myeloid antigens such as Mol and MY7 [86]. Interestingly, this patient responded to treatment with high dose cytosine arabinoside and returned to a stable phase chronic myeloid leukemia. The myeloid cells in this 'remission' did not express T cell markers. Subsequently, the same patient entered a second blast phase during which the blasts expressed only myeloid markers.

The utility of anti-myeloid monoclonal antibodies as diagnostic reagents is enhanced when used in conjunction with certain markers of ALL cells. The CALLA antigen is expressed by approximately 80% of cases of childhood ALL [87–91]. This marker as defined by monoclonal antibody J5 [89] is rarely if ever detected on clear cut cases of AML. Of more than 300 cases of AML studied in our laboratory, there have been no cases identified which are CALLA positive. However, there have been two cases of CML blast crisis in which populations of $CALLA^+$ lymphoblasts were present at the same time as equal populations of $MY7^+$ myeloblasts. The 20% of cases of non-T cell ALL which lack the CALLA antigen can be particularly difficult to distinguish from AML. The anti-B4 monoclonal antibody described by Nadler *et al.* [92] appears to be an extremely valuable reagent for the identification of this subset of ALL's. The B4 antigen was detected on 35 of 35 non T cell ALL's including 10 cases which were $CALLA^-$. It is not expressed by T cell ALL and has not been detected on more than 50 AML's studied so far. Thus, the combination of anti-CALLA, B4 and T11 with antimyeloid antibodies such as anti-MY7 and anti-MY9 are likely to provide a clear cut diagnosis in more than 95% of cases of acute leukemia. A small percentage of acute leukemias will be derived from neither myeloid cells nor lymphoid cells but from erythroblasts or megakaryocytes. Recently surface markers of these lineages [93–95] have also become available and ultimately monoclonal antibodies which can discriminate erythroleukemias and megakaryoblasts from AML and ALL are also likely to find clinical use.

It is anticipated, however, that immunological phenotyping of AML cells will ultimately not only be useful diagnostically to separate AML from ALL, but also be used to define subgroups of patients with either different clinical characteristics or prognosis. The studies which have addressed this issue will be discussed in Section 6.3.

As is evident from this review there has been a large number of anti-AML monoclonal antibodies which have been partially characterized. It is clear that there is a considerable amount of overlap and that many antibodies from different laboratories may well be recognizing the same determinant. In an effort to assist investigators in comparing B cell, T cell, and myeloid monoclonal antibodies, the First International Workshop on Human Leukocyte Differentiation Antigens was convened by Jean Dausset, Cesar Mil-

stein, and Stuart F. Schlossman in Paris in November 1982. Antibodies from many different investigators were submitted to a central laboratory, where they were coded and redistributed to a series of test laboratories. Antibodies submitted for study in the myeloid protocol were then tested by the reference laboratories for reactivity with peripheral blood T cells, monocytes, granulocytes, bone marrow mononuclear cells, and then with fresh samples from AML patients, CML patients or with AML cell lines. The pattern of reactivity with normal cells and with leukemic cells was then used to identify groups of antibodies with similar reactivity. Eight such groups were identified. The first cluster contained nine antibodies including 80H.3, B13.3, FMC10, FMC11, FMC12, and FMC13. This group had minimal reactivity with monocytes but strong reactivity with granulocytes and bone marrow cells. There was minimal reactivity with AML cells, moderate reactivity with monocytic leukemic cells, and moderate reactivity with stable phase CML cells. The second cluster contained eight antibodies including TG1, 1G10, and D5. This group showed moderate reactivity with monocytes, strong reactivity with polys and strong reactivity with bone marrow mononuclear cells. There was intermediate reactivity with AML samples, very strong reactivity with monocytic leukemia samples, and moderate reactivity with chronic myeloid leukemia. The third group included only MY8. There was strong reactivity with monocytes and granulocytes and moderate reactivity with bone marrow cells. There was moderate reactivity with AML cells, strong reactivity with monocytic leukemia cells and minimal reactivity with CML cells. The fourth group included three antibodies; none of which are included in this review. The fifth group contained three antibodies, including MY7. There was strong reactivity with monocytes and granulocytes and weak reactivity with bone marrow mononuclear cells. There was strong reactivty with AML cells, AMOL cells, and CML cells. The sixth group contained ten antibodies including 5F1, Mo2 and MY4. There was strong reactivity with monocytes, minimal reactivity with granulocytes and minimal reactivity with bone marrow cells. These antibodies had low to moderate reactivity with AML cells, minimal reactivity with CML cells and strong reactivity with acute monocytic leukemia cells. The seventh group included three antibodies, including B2.12 and Mol. These antibodies displayed strong reactivity with monocytes, granulocytes, and bone marrow cells, moderate reactivity with AML cells and CML cells, and strong reactivity with monocytic leukemia cells. The final group contained one antibody only which was not part of this review. Thus, as expected, a considerable amount of functional overlap was present amongst the antibodies contributed to this workshop for analysis. Until more detailed biochemical information is available about the antigens which various anti-AML monoclonal antibodies identify, it will be impossible to determine the exact

relationship of different antibodies to each other. Biochemical analysis will be the focus of the Second International Workshop which is scheduled for September 1984.

6.3. *Use of Myeloid Monoclonal Antibodies to Identify Subgroups of AML Patients.* Although many monoclonal antibodies have been produced which react with AML cells, only a few studies have been reported with large numbers of patients. In order to determine if the expression of one antigen or a group of antigens (immunologic phenotype) can be used to define a clinically useful subgroup of AML patients, it will be necessary to study large numbers of similarly treated patients with similar prognostic features. At the present time, the FAB classification appears to be the most widely accepted classification system for AML. As noted above, this classification system has so far provided only minimal information regarding prognosis. Although it is useful to compare the surface markers on each leukemia to the FAB diagnosis, it should be born in mind that antigens expressed only by a single FAB group, or phenotypes which correlate very highly with FAB groups, are unlikely to provide any new information compared to what is learned from the morphology alone. The ideal immunological classification system would identify discrete subgroups of AML patients by differences in surface antigen expression that will correlate with response to treatment, relapse rate, or survival. This system might correlate with the FAB system partially or not at all. As will be seen from the studies discussed below, there are so far insufficient data to predict whether or not surface antigen analysis will ultimately fulfill these criteria.

Ball and Fanger have examined the expression of three monoclonal antibodies, PMN-6, PMN-29, AML-2-23, on acute myeloid leukemia cells and correlated that expression with blast cell morphology and normal myeloid cell antigen display [41]. As noted above, PMN-6 and PMN-29 are reactive with granulocytes and their precursors as immature as the promyelocyte but not with monocytes or with CFU-GM. Twenty-eight patients with AML were classified by the FAB classification and then examined for surface antigens. It was noted that none of fourteen cases expressed either PMN-6, PMN-29 or AML-2-23. In contrast, of nine patients with M4 morphology, six expressed all three antigens, two expressed two of the three antigens, and the last patient expressed none of the antigens. Five patients with M5 leukemia were studied. While all five reacted with AML-2-23, only one patient reacted with PMN-29, and no patient's cells reacted with PMN-6. It was concluded that PMN-6 and PMN-29 are likely to be reactive with AML cases of the FAB M4 subgroup but not with the less differentiated M1 and M2 classifications. Further, the pure monocytic leukemias (M5) were unlikely to express either of these granulocyte surface markers. AML-2-23

antibody reacted with both M4 and M5, but also did not react with the less differentiated leukemias. Consistant with this was the observation that none of these antibodies reacted with a significant number of leukemic cells from patients with CML in blast crisis. It is of interest that while neither PMN-6 nor PMN-29 react with morphologically identifiable normal myeloblasts, both of these antibodies are expressed on at least a fraction of the cells of acute monomyelocytic leukemia. The authors concluded that this may reflect the presence of a normal cell more differentiated than the CFU-GM which co-expresses antigens of the monocyte pathways (such as AML-2-23) and granulocyte series (antigens such as PMN-6 and PMN-29). They correctly point out, however, that drawing conclusions about normal myeloid differentiation from the behavior of leukemic cells is not necessarily accurate. It is also concluded by these authors using HL60 as a model system that monocyte lineage cells could be derived from normal promyelocytes. This was based on the observation that HL60, which expressed all three of these myeloid markers, can be driven to differentiate to monocyte-like cells which express only AML-2-23 in the presence of phorbol ester. This study demonstrates that monoclonal antibodies can be used to identify subsets of cells which in this case correlated highly with FAB morphological classification. A larger study will be required to determine if clinically significant subgroups of these patients are identified with these markers.

Van der Reijden *et al.* studied 55 patients with AML with a panel of monoclonal antibodies including anti-Ia, OKM-1, B2.12, B13.9, B4.3 as well as certain other monoclonal antibodies and heteroantisera [43]. This panel did not include any antibodies reactive only with monocytes. There was a tendency for antibodies which reacted only with granulocytes not to react with monocytic AML cells (M4, M5a, M5b). Antibodies reactive with both granulocytes and monocytes, such as B2.14, tended to react equally well with all FAB classifications, especially M5b. It was also noted that Ia antigen was never expressed in any of the acute promyelocytic leukemia patients studied, and was less likely to be expressed in M4 leukemia than M5 or M1. Using a computer program to compare the immunologic pattern to the various FAB subgroup classifications, it was found that the immunological characterization could correctly predict the FAB classification in 69% of the cases. It was concluded by the authors that the immunological phenotypes reflect differentiation levels of the AML cells and that these differentiation levels could correlate roughly with the morphological level of differentiation. These authors did not further analyze these subgroups to determine if immunological classification provided additional information.

Linch *et al.* studied blast cells from 70 cases of AML which included a preponderence of M4 and M5 subtypes [73]. Several antibodies which react strongly with normal human monocytes, UCHM1, UCHALF, and

E11, were found to react with nearly all cases of M4 and M5 leukemias. Antibody UCHALF detects surface bound lactoferrin [72] and thus indirectly detects the lactoferrin receptor. Antibody E11 reacts specifically with the C3b/C4b receptor. UCHM1 was positive in all sixteen cases of M5 leukemia, 22 of 24 cases of M4 leukemia, and only one of 17 cases of M1 and M2 leukemia. UCHALF was similarly positive in 15 of 16 cases of M5 leukemia, 23 of 24 cases of M4 leukemia, and in one of 17 cases of M1 and M2 leukemia. E11 antibody stained 11 of 13 cases of M5 and 17 of 23 cases of M4 leukemia. It was not positive in any case of M1 and M2 leukemia. In contrast, antibody TG1 [28] which is strongly expressed on granulocyte lineage cells was reactive with nearly every case of M5 leukemia and also a large number of cases of M1 and M2 leukemia. It was noted by the authors that a negative result with TG1 suggests a lack of monocytic involvement. It was concluded by the authors that certain antibodies which react with monocyte lineage cells are of great value in detecting monocytic differentiation in the myeloid leukemias. It was further suggested that the use of such a panel of monoclonal antibodies would allow classification with a reasonable degree of confidence in cases of AML where morphology is uncertain or misleading. This can be of particular importance in prognosis as in some series monocytic features are felt to convey a poorer prognosis than in other FAB subtypes.

Civin and colleagues have studied 33 patients for expression of MY1 and MY10 antigens [46]. Twenty-six of these 33 patients achieved complete remission. All 13 patients whose blast cells expressed MY1 achieved complete remission while only 12 of 19 patients with MY1$^-$ blast cells attained complete remission. MY10 was determined on 18 patients. It was detected on 3, two of whom failed to enter complete remission. In contrast, 13 of 15 MY10$^-$ patients entered complete remission. These differences for both MY1 and MY10 antigens were reported as statistically significant.

Our own laboratory has reported the results of analysis of 70 cases of AML studied with a panel of monoclonal antibodies including anti-Ia, MY4, MY7, MY8, MY9, and Mol [97]. In our initial studies using this series of antibodies [26], it was noted that certain phenotypes tended to occur quite often and that the distribution of these antigens in different AML patients was not at all random. In an effort to compare the phenotype of AML patients' cells with possible corresponding normal myeloid cells, the distribution of each of these antigens on normal myeloid cells was first carefully determined by fluorescence activated cell sorting experiments of normal bone marrow. The distribution of each antigen was determined by morphological analysis of the positive and negative cell fractions. It was then possible to determine a surface antigen phenotype of several levels of normal myeloid cells.

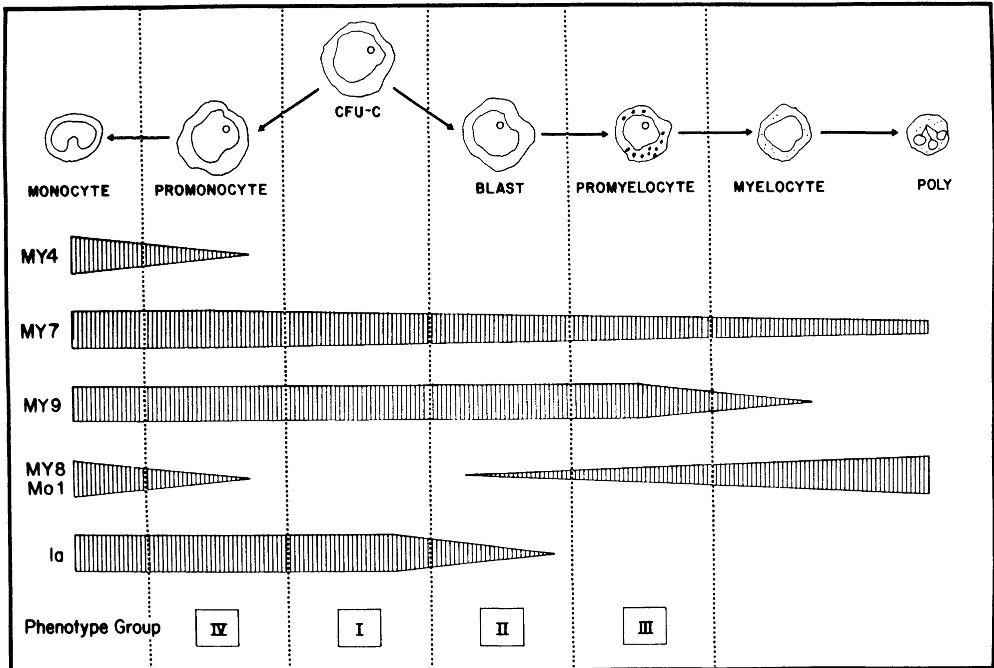

Figure 3. Expression of a series of cell surface antigens during normal myeloid differentiation. Normal bone marrow cells were separated into positive and negative cell fractions by fluorescence activated cell sorting following staining with individual monoclonal antibodies and a fluoresceinated second antibody; cells expressing each antibody were recovered in the positive cell fraction and are identified by the shaded bars. The distribution of antigens as a group (phenotype) can be used to identify four distinct phenotype groups of early myeloid cells as shown.

The distribution of MY4, MY7, MY9, MY8, Mol and Ia antigens on normal myeloid cells is illustrated schematically in Figure 3. Ia antigen is not restricted to myeloid cells and is also detected on B cells, activated T cells, and the majority of bone marrow and peripheral blood hematopoietic stem cells. Mol antigen, identified with the C3bi receptor [68], is also expressed by a subset of peripheral blood large granular lymphocytes which include the natural killer cell. Using this panel of antibodies to determine phenotypes of immature myeloid cells however, it can be seen from Figure 3 that there are several distinctive levels of differentiation identified. The most immature level corresponds approximately to the normal CFU-GM. This cell expresses Ia antigen, MY7 antigen, and MY9 antigen but lacks MY8, Mol and MY4. With differentiation along the granulocyte pathway, Mol and MY8 are gradually acquired, thus identifying a second level of differentiation. With further granulocytic maturation Ia is lost at the level of the promyelocyte while MY8 and Mol increase in antigen density. With monocyte maturation from the CFU-GM, MY4 antigen is gradually ac-

quired. Similarly, MY8 and Mol antigens are also acquired while MY7, MY9, and Ia antigens are retained. Thus, using these antigens, phenotype group I cells (CFU-GM) are readily distinguished from more mature types of cells such as group IV (promonocyte).

Since many AML cells morphologically resemble myeloblasts, promyelocytes, or promonocytes, it was of interest to determine the relationship between morphological differentiation and state of maturation determined by the cell surface antigen phenotype. Seventy patients with AML were analyzed with respect to morphology, cytochemistry, and surface antigens [97]. Sixty-two of the 70 patients expressed a phenotype that was identical to one of the four phenotypes characteristic of normal immature myeloid cells (Figure 3). The most immature cell phenotype (group I, CFU-C) included 13 AML patients. Seventy-nine percent of this group were considered by morphological criteria as having M1 or M2 subtypes of AML. Group II (myeloblasts) included 16 patients of which 73% were considered to have M1 or M2 AML, while 27% were considered to have AMML. The smallest group was group III (promyelocyte). This group included all of the patients with acute promyelocytic leukemia but also included some patients with AML or AMML that were Ia^-. Group IV (promonocyte) was the largest group and contained 28 patients. Eighty-one percent of the patients in this group were considered to have monocytic morphology (AMML, AMOL) while 19% were considered to have AML. This analysis shows that although there is a tendency for the morphology to correlate with the surface antigen phenotype, each phenotypic group contains patients having different morphological types. This suggests that the surface antigen phenotype reflects a related, but somewhat different, view of the state of leukemic cell differentiation than does morphology. Hopefully, this will provide useful supplemental information to the standard morphological classification. The relationships which we observed between surface antigen phenotype of a leukemic population and normal cell counterparts are schematically illustrated in Figure 4.

The four phenotype groups were also analyzed for differences in certain clinical and laboratory parameters. For example, the expression of myeloperoxidase and non-specific-esterase activity was studied. The promonocyte group was significantly lower in the expression of myeloperoxidase and significantly higher in the expression of non-specific-esterase when compared to the total population of leukemic patients [97]. The CFU-C group was significantly lower in the expression of non-specific esterase. The frequency of detecting Auer rods was similar in phenotype groups I-III, but significantly lower in the promonocyte group. The frequency of high presenting white count (100,000/cumm) was similar in all groups. All of these patients were treated on one of two very similar protocols at the same institution. The

ACUTE MYELOBLASTIC LEUKEMIA
RELATIONSHIP OF SURFACE ANTIGEN PHENOTYPE TO MORPHOLOGY

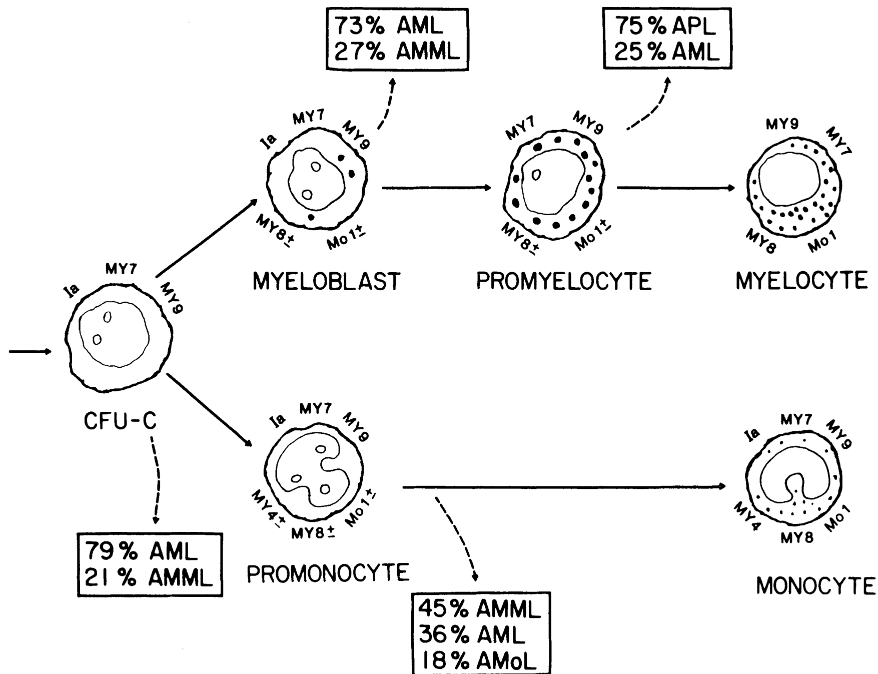

Figure 4. The relationship of surface antigen phenotype of AML cells to morphological classification. The phenotype of normal bone marrow cells is shown for comparison. The phenotypes expressed by the cells of seventy patients with AML were then compared to the phenotypes expressed by normal myeloid cells. Within each immunological phenotype group, two or more morphologies were observed. The percentages of each type of AML morphology expressed by the patients within each immunological group are shown in the boxes.

complete remission rate for group I was 79%; group II, 88%; group III, 60%; and group IV, 60%. These differences did not achieve statistical significance and a study of larger numbers of patients is currently underway to determine if these trends will hold up.

At the present time the use of surface marker analysis in AML remains primarily an investigational tool. It is likely, however, that immunological typing will supplement the standard morphological and cytochemical analysis to some extent. It is already clear that in the small percentage of patients where the distinction between AML and ALL is unclear, surface marker analysis can provide rapid and usually definitive evidence of lineage association. It is also likely that surface marker analysis can be useful in a larger percentage of cases to confirm the morphological and cytochemical diagnosis. The utility of surface markers to define clinically useful subgroups

has not been established. As emphasized from the data from our laboratory, however, the pattern of surface antigen expression on leukemic cells does appear to reflect the level of differentiation, but not necessarily in exactly the same way as does morphology. This may ultimately prove to be a distinct advantage, as the subsets of patients identified by the surface markers will therefore be distinct from those identified by morphological analysis. Studies are currently underway to determine the relationship of surface antigens and phenotype to a large number of clinical variables, including response to treatment, overall survival, and chromosome analysis. In addition to clinically useful information it will be of considerable biological interest to relate surface antigen phenotype to expression or activation of cellular oncogenes, as that information becomes available.

7. FUTURE DIRECTIONS

Monoclonal antibodies are currently being investigated in the therapy of acute lymphoblastic leukemia and B cell lymphomas [98]. Although it is not possible to conclude at this time that this therapy is useful, there is adequate experimental and preliminary clinical evidence to suggest that this approach is very promising. Antibodies can be either infused directly into a leukemic patient (serotherapy) or used to 'clean up' residual leukemic cells for autologous bone marrow transplantation. Serotherapy is likely to be most useful in B cell malignancies where anti-idiotype antibodies can be prepared [99, 100]. This has been shown to produce complete remission in certain patients with B cell tumors. However, there is no evidence that myeloid cells or myeloid leukemic cells express any surface antigens which are equivalent to the idiotype of the B cell. So far, no 'leukemia-specific' antigens have been identified on either AML or ALL cells. The CALLA antigen of ALL, however, has proven to be extremely useful therapeutically because this antigen does not appear to be expressed on any hematopoietic stem cells [98]. Therefore, although the antigen is expressed on certain normal B cell precursor cells [101], reconstitution of hematopoiesis following elimination of $CALLA^+$ cells from a bone marrow autograft does not seem to be impaired. The search for ideal antibodies in AML which would have these characteristics is currently underway. As noted above however, the frequency with which AML-reactive antibodies cross react with normal hematopoietic stem cells is high.

Another problem to be considered in the use of monoclonal antibodies for immunotherapy in AML is the frequent observation that AML blast populations in individual patients are frequently heterogeneous. This can frequently be observed morphologically, particularly in cases of M2 and M3

leukemia, where populations of relatively immature appearing blasts are mixed with promyelocyte like cells. Also, in patients with acute myelomonocytic leukemia (M4) it is common to see cells with very monocytoid features mixed with cells which appear to be undifferentiated blasts. This morphological heterogeneity is reflected in the expression of surface antigens. For example, in AMML, the expression of monocyte specific antigens MY4 and Mo2 is commonly restricted to a subset of the leukemic cells. Cell sorting experiments in our laboratory have demonstrated that the leukemic cells which express MY4 tend to be the more 'monocytoid' leukemic cells. The blast forms are likely to be MY4^{-} [102]. This heterogeneity of surface markers in AML may be of particular importance therapeutically if it extends to the so called leukemic stem cell. Although the concept of stem cells in AML is not firmly established, there is growing evidence that in most AML patients there is a small subset of cells which may act *in vivo* as progenitor cells to maintain the rest of the leukemic cell population [103–105]. These leukemic stem cells are highly proliferative and have been described to give rise to the remainder of the leukemic cells in an analogous situation to the production of granulocytes and monocytes by normal hematopoietic progenitor cells. In fact, these leukemic stem cells can be assayed by their ability to form colonies of leukemic blasts in semi-solid medium [103]. If this concept is correct, then the cell which is critical to treat either with chemotherapy or immunotherapy is the leukemic stem cell and not the bulk of leukemic cells which are apparently 'less malignant' in terms of their proliferative potential. Using the expression of MY4 antigen in AMML as a model, we have investigated the surface markers of leukemic colony forming cells. Leukemic cells from AMML were separated by fluorescence activated cell sorting into MY4 antigen positive and MY4 antigen negative cell populations. The leukemic colony forming cells were found entirely in the MY4^{-} fraction [102]. In contrast, the colony forming cells were found to express Ia antigen and MY7 antigen. These results emphasize the fact that there is considerable surface antigen heterogeneity in patients with AMML and that ideal reagents for immunotherapy must take this observation into account. More information is needed about the surface antigens of both normal and leukemic progenitor cells in order to select antibodies for therapeutic trials. None the less, it is quite possible that immunotherapy with specific anti-myeloid monoclonal antibodies may play a useful role in the therapy of acute myeloblastic leukemia.

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5. Classification of 358 Cases of Acute Myeloid Leukemia by FAB Criteria: Analysis of Clinical and Morphologic Features

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1. INTRODUCTION

In 1976, a new morphologic classification for the acute leukemias was proposed by a working committee of French, American, and British hematologists [1, 2]. Since its introduction, the FAB classification has been widely accepted internationally because of its underlying logic and relative ease of application. It has served as the basis for classification in several studies evaluating the results of chemotherapy in acute myeloid leukemia (AML) [3–8].

The present study was undertaken to assess the facility and clinical relevance of the FAB classification in a relatively large group of cases of acute myeloid leukemia, all diagnosed in one laboratory, and to compare survival statistics for the various subtypes. The study population consists of 358 untreated patients, children and adults, with a diagnosis of acute myeloid leukemia established in the hematopathology laboratory of the University of Minnesota Hospitals in the period January 1970 to December 1983. The laboratory findings and subsequent clinical course of these 358 patients were evaluated. All leukemias occurring in patients previously treated with radiotherapy or chemotherapy were excluded from this study. Cases of AML in which ultrastructural and ultracytochemical evidence of megakaryoblastic differentiation was present were also excluded.

The morphologic classification of the 358 cases was based on examination of Wright's-Giemsa stained blood and bone marrow smears, bone marrow trephine biopsy sections and smears stained with relevant cytochemical techniques. The evaluation included 300 cell differential counts on the blood smears and 1,000 cell differential counts on the bone marrow smears. Cytochemical reactions performed included myeloperoxidase [9], Sudan black B, chloroacetate esterase [10], non-specific esterase [10] and periodic acid-Schiff (PAS). All specimens were routinely processed by methods pre-

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viously described [11]. Ultrastructural and ultracytochemical studies were performed on several cases of M1 and M5A to clarify the myeloid nature of the leukemic process [12].

The criteria for diagnosis and subclassification were, with the modifications to be noted, based on the criteria published in 1976 and 1982 by the FAB committee [1, 2, 13]. The criteria for diagnosis of M1, M2 and M4 were essentially identical to the FAB criteria and included the presence of 30% or more type I and type II myeloblasts in the bone marrow. Cases in which blasts with Auer rods were identified but with fewer than 30% type I and type II myeloblasts in the marrow were excluded from the study. The distinction between M1 and M2 AML was based on the presence of more than 10% marrow leukemic cells showing maturation beyond the myeloblast stage of development in M2. The diagnosis of M3, hypergranular or microgranular acute promyelocytic leukemia, was based on the typical morphologic features of this type of leukemia [14]; the percentage of type I and type II myeloblasts exceeded 30% in only 2 of 36 cases. The diagnosis of M5A, acute monocytic leukemia, poorly differentiated, was based on the presence of 60% or more monoblasts in the bone marrow or blood [15]. M5B, acute monocytic leukemia differentiated, was characterized by the typical morphologic features of this type of leukemia [16, 17]; the percent of type I and type II myeloblasts and monoblasts was less than 30% in 64% of the cases. The percent of type I and type II myeloblasts in the bone marrow in M6 was calculated on the basis of non-erythroid cells excluding lymphocytes [18]. An additional modification for the M6 class was the inclusion of cases with greater than 30% erythroid cells with marked morphologic abnormalities [19]. The number and percentage of each of the subtypes of AML in the 358 patients are shown in Table 1.

Table 1. Acute myeloid leukemia (358 patients)

			Number		Percentage
M1			35		9.8
M2			160		44.7
M3			36		10.0
	Hypergranular	24		6.7	
	Microgranular	12		3.3	
M4			68		19.0
M5			35		9.8
	M5A	21		5.9	
	M5B	14		3.9	
M6			24		6.7

2. CLINICAL AND MORPHOLOGIC CHARACTERISTICS OF THE FAB CLASSES

2.1. *FAB-M1: Acute Myeloid Leukemia Without Maturation*

Patients. Thirty-five cases were classified as FAB-M1. The male:female ratio was 1 to 1 (Table 2). The patients ranged in age from 1 to 83 years, median (m) 46. Twenty-nine percent were 20 years of age or less; 31% were 60 or older.

The most common symptomatology was constitutional and included fever, fatigue and malaise. Two patients presented with leukemia cutis. Hepatomegaly was present in 22% of patients, splenomegaly in 26% and lymphadenopathy in 30% (Table 3). Forty-seven percent of patients had no evidence of organomegaly or lymphadenopathy.

Peripheral Blood. Table 4 summarizes the hemoglobin, leukocyte and platelet counts at presentation. Fifty-seven percent of patients had leukocytosis, 23% were leukopenic and 20% had a normal leukocyte count. The blood myeloblasts ranged from 3 to 100% (mean 65%) of the leukocytes. In 31% of cases the leukocyte count exceeded 100×10^9/L. Ninety-one percent of patients were anemic; in 65% the hemoglobin was less than 10 gm/dl. Seventy-five percent of patients were thrombocytopenic; 62% had a platelet count of less than 100×10^9/L, 47% less than 50×10^9/L.

Bone Marrow. Myeloblasts ranged from 70 to 97% with less than 10% promyelocytes and maturing neutrophils. The remaining marrow cells consisted of normoblasts and lymphocytes. Evidence of myeloid differentiation included the following: myeloperoxidase positivity, Sudan Black B positivity, Auer rods and dysmyelopoietic neutrophil maturation. Auer rods were present in 49% of cases. Abnormal maturation of marrow elements was present as follows: dysmyelopoiesis in 37% of cases, dyserythropoiesis in 29% and abnormal megakaryocytes in 11%. Two cases which lacked evidence of myeloid differentiation by light microscopy were classified on the basis of electron microscopic findings.

Trephine biopsy section material was available in 30 cases. The marrow was hypercellular in all cases; cellularity ranged from 60 to 100%. Marrow cellularity and mitotic counts are shown in Table 5.

Coagulation Studies. Coagulation studies were performed on specimens from 20 patients at diagnosis. There was evidence of DIC in four (20%); none of these had a history of hemorrhagic problems and there were no petechiae or purpura on physical examination.

Additional Laboratory Studies. Serum lysozyme studies were performed in 12 cases. The value was normal in 8 and increased in 4 (15.9, 24, 47 and 67.3 μg/ml) (normal range 2.0 to 11.9 μg/ml). Leukocyte alkaline phosphatase (LAP) was performed in 11 cases; three were normal and eight were increased.

Two patients had leukemic blasts in cerebrospinal fluid samples; neither had CNS symptoms.

Patient Survival and Induction Chemotherapy. Survival data were available for 29 patients and are summarized in Table 6. Survival ranged from <1 to 126 months, m = 3.5. Five patients are living at 5 to 76 months from diagnosis. Twenty-four patients are dead in less than one to 126 months. Forty-one percent survived one month or less; 76% less than one year.

Seventy-two percent of patients were treated with a regimen that included an anthracycline. The median survival for this group was 4 months, range (r) = <1–126. Twenty-four percent of the patients were treated with varying combinations of chemotherapy agents that did not include anthracyclines. The median survival for this group was 2 months, r = <1–120. One patient was not treated with chemotherapy and survived less than one month.

The median survivals of patients with and without Auer rods were both short, 4 months and 2.5 months, respectively.

2.2. *FAB-M2: Acute Myeloid Leukemia With Maturation*

Patients. One-hundred-sixty cases were classified as FAB:M2. The male to female ratio was 1.6:1 (Table 2). The age ranged from one month to 87 years, m = 48; 20% of the patients were 20 years of age or less, 40% were 60 or older.

The most common presenting symptoms were constitutional and hemorrhagic. Hemorrhagic manifestations included easy bruising (27 cases), epistaxis (12), gingival bleeding (9), petechiae (9), ecchymoses (6), melena (1), menorrhagia (1) and conjunctival bleeding (1). Fourteen percent of the patients manifested hepatomegaly, 14% splenomegaly and 24% lymphadenopathy (Table 3); two patients had extramedullary masses.

Peripheral Blood. Peripheral blood counts are summarized in Table 4. The majority of patients were anemic (96%), thrombocytopenic (91%) and had circulating myeloblasts (97%). Leukocytosis was more common (46%) than either a normal leukocyte count (28%) or leukopenia (27%). Eleven percent of patients had a leukocyte count $>100 \times 10^9/L$, 2.7%$>200 \times 10^9/L$ and 1.3%$>300 \times 10^9/L$.

Bone Marrow. The type I and type II myeloblasts in all cases exceeded 30% of the nucleated marrow cells (mean 60%); more than 10% of the nucleated marrow cells evidenced maturation beyond the myeloblast stage. The myeloblasts exhibited a broad spectrum of size, ranging from approximately 12 to 35 micra in diameter. The nuclei were usually round or oval with one or more prominent nucleoli and finely reticular chromatin. The cytoplasm was basophilic and had a variable number of azurophilic granules. Auer rods were present in 70% of cases. Dysmyelopoiesis, dyserythro-

poiesis and abnormal megakaryocytes were present in 87%, 67% and 33% of cases, respectively. In one case, the number of promyelocytes exceeded the myeloblasts so that the pattern resembled FAB-M3. However, the other criteria for the diagnosis of M3, to be discussed below, were not present. Increased plasma cells were occasionally present; in one case the plasma cells constituted 24% of the marrow cells.

Six cases showed increased basophils in the bone marrow ranging from 3.4 to 28%. This was a heterogeneous group in which age, symptoms, and survival varied widely. Two of these cases also had increased marrow eosinophils. Fifteen other cases also had increased marrow eosinophils, ranging from 3 to 10%. This was a diverse group of patients in which the median age and survival were similar to the entire group of M2 cases.

In trephine biopsy sections cellularity ranged from 30 to 100%; in six of the cases the marrow was hypocellular. The cellularity and mitotic counts in trephine biopsy sections are shown in Table 5.

Coagulation Studies. Coagulation studies were performed at the time of diagnosis in 115 cases. Twenty-one patients (18%) had evidence of disseminated intravascular coagulation (DIC).

Additional Laboratory Studies. Serum lysozyme determinations were performed in 46 cases. Thirty-seven percent of these were within the normal range and 63% were increased. In the group with increased values 31% were less than 30 μg/ml and 72% were less than 50 μg/ml; two were greater than 100 μg/ml [122, 165]. Data for the cases with increased serum lysozyme are summarized below.

	Blood monocyte $\times 10^9$/L	Bone marrow % monocytes	Lysozyme*
Range	0–4.6	<1–10	13–165
Median	0.3	<1	31.5

* normal = 2–11.9 μg/ml.

The degree of monocytosis in the peripheral blood or bone marrow varied widely in the 29 patients with increased serum lysozyme levels; in twelve cases neither the blood nor marrow showed increased monocytes.

LAP determinations were performed in 77 cases. The value was decreased in 17%, normal in 49% and increased in 34%.

Patient Survival and Induction Chemotherapy. The median survival for all patients was 8.5+ months (Table 6). Eighty-five percent of the patients are dead from <1 to 74 months after diagnosis. Twenty-two percent of patients died in one month or less; 64% died within one year. Sixteen percent survived two years or longer; 5% lived more than 5 years.

Treatment data were available for 125 of the patients. A wide range of chemotherapeutic regimens was administered to this group of patients. Ninety-one patients were treated with a chemotherapeutic regimen that included anthracyclines. The median survival for this group was 10.5 months, r = <1 to 132+. Twenty patients were treated with a chemotherapy regimen that did not include anthracyclines. The median survival for this group was 4 months, r = <1–33. Fourteen were not treated; median survival for these patients was <1 month, r = <1–13.

The survival correlated with presence of DIC, percent of type I and II myeloblasts in the marrow and presence of Auer rods is illustrated below.

Survival (months) for M2 AML

	Median	Range
Auer rod positive (70%)	10	<1–90+
Auer Rod Negative (30%)	6.5	<1–132+
DIC present (20 patients)	9	<1–88+
DIC absent (79 patients)	8+	<1–72+
30–60% BM blasts (47%)	10	<1–74
>60% blasts (53%)	6.3	<1–132+

None of these factors significantly influences survival although the group in which Auer rods were detected had a slightly longer median survival than the group without Auer rods and the patients with lower myeloblast counts had a slightly longer median survival than the patients with higher blast counts.

2.3. *FAB-M3: Acute Promyelocytic Leukemia*

Patients. Thirty-six cases were classified as FAB-M3; 24 (67%) were of the typical hypergranular type and 12 (33%) were microgranular or hypogranular variants [14, 20, 21]. The male to female ratio was 2:1, 1.6:1 for typical hypergranular and 4:1 for the microgranular variant (Table 2). The age ranged from 1.3 to 82 years, m = 38; only one patient was less than ten years of age. The median age for hypergranular M3 was 39, (r = 1.3–82) and 38 for microgranular M3 (r = 20 to 63).

The presenting symptoms included a wide variety of constitutional, infectious and hemorrhagic problems. The most common symptoms related to bleeding (86%) and fatigue (43%). Bleeding manifestations included easy bruisability, bleeding gums, hemoptysis, hematuria, epistaxis, petechiae, vaginal bleeding, hematemesis, melena and intracranial hemorrhage. The incidence of hepatomegaly, splenomegaly and lymphadenopathy, shown in

Table 3, was low for both types of M3. No patients with the microgranular M3 variant had hepatomegaly or lymphadenopathy.

Blood Findings. Table 4 summarizes the hematologic findings. Fifty-five percent of the patients were leukopenic at diagnosis and 38% had leukocytosis. The median leukocyte count was significantly higher in microgranular M3 (42.5×10^9/L) than in the hypergranular M3 group (1.8×10^9/L) ($p < 0.001$). In three cases of hypergranular M3 and three cases of microgranular M3 there were no or very rare blasts and promyelocytes in the blood. These were the only three cases of microgranular M3 with less than 10% blasts and promyelocytes; 8 of the 24 cases of hypergranular M3 had less than 10%. Eighty-six percent of the patients were anemic; 48% had a hemoglobin of less than 10 g/dl. Ninety percent of patients were thrombocytopenic; all of these had a platelet count of less than 100×10^9/L, 76% were less than 50×10^9/L. No statistically significant differences in hemoglobins or platelet counts were observed between hypergranular M3 and microgranular M3.

Bone Marrow. The range and median values of bone marrow differential counts for type I myeloblasts, type II myeloblasts, and promyelocytes is shown below.

	Type I Myeloblasts (%)	Type II Myeloblasts (%)	Type I and Type II Myeloblasts (%)	Promyelocytes (%)
Range	0.2–32	0.2–20	0.6–42	32.3–92.0
Median	1.2	7.1	7.7	75.2

Ninety-four percent of cases had less than 30% type I plus type II myeloblasts; 69% had less than 10%. Mean blast counts were similar in hypergranular M3, 9% and microgranular M3, 15%.

The leukemic cells in hypergranular M3 were large, 18 to 25 micra in diameter. Some of the nuclei were round to oval, but most showed irregularity of contour ranging from slight indentation to bilobulation. One or more nucleoli were present. The nuclear chromatin was usually finely reticular. The cytoplasm was densely packed with large, deeply staining azurophilic granules which in some instances partially obscured the nucleus. The granules often appeared to be concentrated in the nuclear indentation or about the bridge joining nuclear lobes. The marrow from most cases of hypergranular M3 contained cells with smaller granules as well as a small number of agranular myeloblasts.

In microgranular M3 the cytoplasm contained large numbers of granules which were very fine, but which manifested perinuclear accentuation in some cases. In some instances, the granulation was so delicate that its effect

was that of a patchy alteration of the tinctorial properties of the cytoplasm rather than obvious granulation.

An interesting feature of microgranular M3 was the frequent presence of small cells with hyperbasophilic cytoplasm [14]. These cells, which predominated in two cases, measured approximately 12 to 15 micra in diameter and had a thin rim of basophilic cytoplasm which was agranular or contained very fine granulation. The nuclei were frequently lobulated and hyperchromatic with small or inapparent nucleoli. Similar cells were observed as a minor component in most of the cases of hypergranular M3.

Auer rods were detected in 35 of the 36 (97%) cases of M3 in this study. A frequent finding was cells with more than ten Auer rods which often occurred as bundles or stacks (Faggot cells). Cells with multiple Auer rods were present in 97% of cases; in one of these they were found only in a relapse marrow.

Trephine biopsy sections were hypercellular in all cases; cellularity ranged from 70 to 100%. Marrow cellularity and mitotic counts are shown in Table 5. The range of cellularity was similar for both hypergranular M3 and microgranular M3. The mitotic count and mitotic index appear to be higher in microgranular M3 than hypergranular M3.

Coagulation Studies. Thirty-one of the 36 patients had coagulation studies at diagnosis; 84% had evidence of DIC, 80% of microgranular M3 and 86% of hypergranular M3. All patients had DIC with initiation of chemotherapy.

Additional Laboratory Studies. Serum lysozyme was measured in 5 patients with hypergranular M3 and in 2 with microgranular M3. One case of hypergranular M3 had an elevated value, 54 µg/ml.

Patient Survival and Induction Chemotherapy. Survival data were available for 33 patients, 23 hypergranular M3 and 10 microgranular M3, and is summarized in Table 6. Survival ranged from <1–100 months (m = 16) for all M3 cases. Median survival for microgranular M3 was 11.5 months (r = <1–32) and 21.5 for hypergranular M3, r = <1–100. Three patients with hypergranular M3 are living at 13, 19 and 20 months, and one patient with microgranular M3 is alive 13 months following diagnosis. Forty percent of patients with microgranular M3 and 19% with hypergranular M3 were dead within 1 month of diagnosis. Fifty percent of cases of microgranular M3 died within one year of diagnosis; only one patient (10%) lived more than two years. Thirty-five percent of patients with hypergranular M3 died within one year; 45% of patients survived more than two years. Eighty percent of patients were treated with an anthracycline and other chemotherapeutic agents. The median survival for this group was 21 months (r = <1–100), 24.5 months for the hypergranular M3 (r = <1–100) and 13.5 for microgranular M3, r = <1–32. Three patients (10%) were treated with a chemotherapy regimen that did not include anthracyclines. The survivals

for this group were <1, <1 and 2.5 months. Three patients were not treated; all of these presented with intracranial hemorrhage and died within 48 hours of admission to the hospital. All three of these patients had M3 microgranular.

2.4. *FAB-M4: Acute Myelomonocytic Leukemia*

Patients. The study included 68 cases of M4. The male to female ratio was 1.4:1 (Table 2). The age range was 7 months to 87 years (m = 50); 12% of patients were 10 years of age or younger and 16% were over 70.

The most common symptoms were fatigue (32%), bleeding disorders (28%), fever (20%), pharyngitis (12%) and gingival hyperplasia (10%). Hemorrhagic problems included easy bruisability (10 cases), gingival bleeding (4 cases), ecchymoses (3 cases), epistaxis (1 case) and hematemesis (1 case). No patient was asymptomatic at presentation. Initial physical findings included hepatomegaly 30%, splenomegaly 34%, lymphadenopathy 46%, and soft tissue leukemic infiltrates 5% (Table 3).

Peripheral Blood. Table 4 summarizes the leukocyte count, hemoglobin and platelet counts. Eighty-seven percent of patients had leukocytosis and 11% were leukopenic. Seventy-seven percent of the patients had peripheral blood absolute monocyte counts of 5×10^9/L or greater. Monocytes were not seen in one case. There were no myeloblasts in the peripheral blood of 2 patients. Ninety-two percent of patients were anemic; 53% had a hemoglobin of less than 10 g/dl. Eighty percent of patients were thrombocytopenic; 76% had a platelet count of less than 100×10^9/L and 46% less than 50×10^9/L.

Bone Marrow. All patients had greater than 30% monoblasts and type I and type II myeloblasts in the bone marrow. The diagnosis of M4 was based on the presence of 20% or more monocytes and promonocytes in the marrow or blood, more than 5×10^9/L monocytes and promonocytes in the blood or non-specific esterase (NSE) positivity in 20% or more of the bone marrow cells. There was always a component of 20% or more granulocytic elements in the marrow.

Promonocytes were usually large, up to 35 μm, with abundant light gray or lightly basophilic cytoplasm. In most cases, fine, evenly distributed, azurophilic granulation was present. Round cytoplasmic vacuoles were present in occasional cells but were not a prominent feature in any case. The nuclear chromatin varied from finely stippled in the less mature cells to dense, reticular or 'lace-like' in more mature monocytes. The nuclear outline varied from oval to reniform to deep indentation. In many cases the nuclei were characterized by delicate folding or creases. Auer rods were present in 64% of cases; dysmyelopoiesis was present in 84%, dyserythropoiesis in 42% and abnormal megakaryocytes in 31%.

The percent of cells reacting with myeloperoxidase ranged from 18 to 95, m = 60. Two cases showed less than 20% positive cells; both were Auer rod positive and had evidence of neutrophil maturation. The percent of NSE positive cells ranged from 0 to 65, m = 25. In 4 cases the percentage of NSE positive cells plus the percentage of myeloperoxidase positive cells totaled greater than 100% indicating that some cells were positive with both reactions.

Trephine biopsy sections were hypercellular in all but one case. The cellularity in the hypercellular marrows ranged from 70% to 100%. Data on cellularity and mitotic counts are shown in Table 5.

Coagulation Studies. Coagulation studies were performed in 60 of the 67 cases; evidence of DIC was present in 23%. Twelve of the 17 patients with hemorrhagic manifestations had thrombocytopenia but no evidence of DIC.

Additional Laboratory Studies. Serum lysozyme values were increased in 95% of cases studied and normal in 5%. Values ranged from 9.2 to 340 μg/ml, m = 77 (normal 2–11.9 μg/ml). In 5% of cases the value exceeded 300.

LAP scores were available in 41 cases; 20% were decreased, 51% normal and 29% increased.

Cerebrospinal fluid was positive for leukemic cells in 8% of the cases.

Patient Survival and Induction Chemotherapy. The median survival for all patients was 8+ months. Eighty-two percent have died in less than one to 63 months. Nineteen percent died in one month or less and 66% within one year. Twenty-one percent survived 2 years or longer. Eighteen percent of the patients are living from 3 to 92 months from diagnosis. Treatment data were available for 53 of the patients. Forty-five patients were treated with chemotherapy including an anthracycline drug; the median survival for this group was 11 months, r = <1–92+. Six patients were treated with chemotherapy that did not include an anthracycline; median survival for this group was 5 months, r = <1–6. Two patients were not treated; both survived one month or less.

The median survival for the 64% of cases with Auer rods was 12 months r = <1–92+, and 4 months for those without Auer rods, r = <1–29. The survival difference is not statistically significant ($0.05 < p < 0.10$). No statistically significant difference in survival was noted between the patients with and without evidence of DIC at diagnosis.

M4 With Increased Bone Marrow Eosinophils. There were 21 cases (32%) in the M4 group that had increased bone marrow eosinophils. Of these, 10 (15% of M4 cases) were known to have a deletion or inversion of the long arm of chromosome number 16 [22, 23]. The bone marrow eosinophil counts in this group ranged from 9–42%. Auer rods were found in all 10 of

these cases. Eleven cases (17% of M4 cases) in which cytogenetic studies were not performed had 3 to 38% bone marrow eosinophils.

The median survival of patients with M4 with known 16q abnormalities, M4 with increased bone marrow eosinophils without cytogenetic studies, M4 without increased marrow eosinophils and the entire group of M4 is contrasted in the table below. Median survivals are longer in the 16q group and in M4 with increased marrow eosinophils.

Survival (months)

	Median	Range
M4 with a 16q abnormality and increased marrow eosinophils	24	<1–92+
M4 with increased eosinophils (without chromosome studies)	12.5	<1–78+
M4 without increased marrow eosinophils	7	<1–90+
M4 total	8+	<1–92+

2.5. *FAB-M5A: Acute Monocytic Leukemia Poorly Differentiated (Monoblastic)*

Patients. Twenty one cases were classified as FAB:M5A. The male to female ratio was 0.7:1 (Table 2). The age range was 1.3 to 87 years, m = 16; 74% were less than 25 years of age and 16% were 75 or older.

The most common symptoms at presentation were weakness (24%) and bleeding disorders (18%). One patient presented with each of the following problems: paraplegia due to an epidural leukemic mass at the L2 level in a 5 year old male, leukemic infiltration of an eyelid in a 16 year old female, proptosis due to an orbital mass in a 15 year old male, a testicular mass in a 3 year old male. A 25 year old male had aplastic anemia 12 years previously, presumably secondary to chloramphenicol therapy, which had remitted following treatment with prednisone and androgens.

Initial physical findings included hepatomegaly 50%, splenomegaly 44%, and lymphadenopathy 33% (Table 3). Six patients (29%) were found to have extramedullary masses. These patients will be discussed later in this section.

Peripheral Blood. Peripheral blood counts are summarized in Table 4. Thirty-three percent of the patients were leukopenic and 39% had leukocytosis; the leukocyte count exceeded 100×10^9/L in 11%. Monoblasts were present in the blood in all cases. Mature monocytes were present in 36%. Anemia was present in 78% of patients; in 66% the hemoglobin was less than 10 g/dl. Thrombocytopenia was present in 67%; the platelet count was less than 100×10^9/L in 50% of patients and less than 50×10^9/L in 22%.

Bone Marrow. In all cases monoblasts exceeded 65% of the bone marrow cells. Eighty-two percent of patients had more than 80% and 41% of patients more than 90% monoblasts. The monoblasts were characteristically large, up to 40 micra in diameter, with variably basophilic cytoplasm; pseudopod formation was prominent in some cases [15]. Fine azurophilic granulation was frequently abundant. Cytoplasmic vacuoles were generally rare. Most monoblasts had round or oval nuclei. The delicate nuclear folding, characteristic of the promonocytes in M4 and M5B was very uncommon. Occasional cells had slight nuclear folds. The nuclear chromatin was generally finely reticular. Most cells had one prominent nucleolus which was often accentuated by a rim of chromatin condensation; occasional cells contained multiple nucleoli.

In one case most of the cells showed large, irregularly shaped or round vacuoles which tended to coalesce near the periphery of the cytoplasm. These vacuoles were intensely PAS positive. In another case the cytoplasm showed peripheral and radiating basophilia to the extent that these cells resembled large reactive lymphocytes.

Mature monocytes were present in 24% of cases. These cases showed only a small number of mature monocytes with no evidence of transition from monoblasts to promonocytes to monocytes in contrast to the marrows in M5B in which there was a predominance of promonocytes and a clear transition from less mature to more mature monocytes. Auer rods were not found in any of the cases. Maturing neutrophils were seen in 91% of cases but always numbered 9% or less of the marrow cells. Dysmyelopoiesis and dyserythropoiesis were not noted in any case.

The range of NSE positive leukemic cells varied from <1 to 95%, $m = 71\%$. Two cases showed less than 50% NSE positive cells (<1 and 42%); the remaining cases had from 50 to 95% positive cells. Occasional cases showed rare myeloperoxidase positive leukemic blasts. The case with rare NSE positive cells was also negative with myeloperoxidase, chloroacetate esterase and sudan black B. Electron microscopy confirmed the monoblastic nature of the leukemic cells by the presence of membrane bound granules, abundant fine filaments and ribosome-lamella complexes [15].

The cellularity and mitotic counts in trephine biopsy sections are summarized in Table 5. All cases were hypercellular, 70–100%.

Coagulation Studies. Coagulation studies were performed in 17 cases at diagnosis. Evidence of DIC was present in 9 cases (53%).

Additional Laboratory Studies. Serum lysozyme studies were performed in 9 cases; five were within the normal range and four were increased, 14, 26, 70 and 91 μg/ml.

LAP studies were done in five cases. Three were normal and two were increased. Monoblasts were present in the CSF of 17% of the cases.

Patient Survival and Induction Chemotherapy. Survival data was available for 18 patients. The median survival was 8 months, range <1 to 36+. Three patients are living at 16, 19 and 36 months after diagnosis. Fifteen died in from less than one to 18.5 months. Fifteen patients were treated with a chemotherapy regimen that included an anthracycline. The median survival for this group was 11 months, r = <1–36+. Two patients were treated with a regimen that did not include anthracyclines. These patients both survived less than one month. One patient was not treated and survived less than one month. There was no difference in survival between patients with and without DIC.

Patients Presenting with Extramedullary Masses. Clinical and hematologic data on the 6 patients with extramedullary disease, all 16 years of age or less, are summarized in the table below.

#	Age & sex	Organomegaly* H S L	Mass	WBC ($\times 10^9$/L)	Absolute blood monoblast count ($\times 10^9$/L)	Marrow % monoblasts	CNS cytology	DIC	Survival (months)
1	5M	+ − −	Paraspinal	20.8	11.6	67	+	+	<1
2	1F	+ + +	Skin	6.8	1.4	88	+	+	3
3	16F	− − +	Eyelid	5.2	NA	89	+	NA	8
4	7F	+ + +	Orbit	2.2	0.5	74	−	+	NA
5	3M	+ + −	Testis Orbit	117	91.3	88	−	+	<1
6	1F	+ + −	Renal	3.5	0.03	82	−	−	16+

* H = Hepatomegaly, S = Splenomegaly, L = Lymphadenopathy, NA = Not Available.

A one year old female patient not included in the study because of failure to meet the criteria for leukemia presented with an orbital mass that had the cytologic and cytochemical features of neoplastic monoblasts. Only rare monoblasts were present in the bone marrow smears and none were detected in the blood. Cytogenetic analysis of the marrow specimen showed a t(9; 11) chromosome rearrangement. Serum lysozyme was 102 μg/ml and monoblasts were present in the CSF. No evidence of substantial marrow involvement occurred for 21 months. The leukemic cells in the marrow at that time had the typical features of a monoblastic leukemia.

2.6. *FAB-M5B: Acute Monocytic Leukemia with Differentiation*

Patients. Fourteen cases were classified as FAB-M5B. The male to female ratio was 1.8:1 (Table 2). The patients ranged in age from 1 month to 82

years, m = 49. The most common presenting symptoms were diffuse erythematous skin rash and hemorrhagic problems. Physical findings included hepatomegaly in 54% of patients, splenomegaly in 46% and lymphadenopathy in 54% (Table 3). Extramedullary masses were present in 4 cases (29%). Two of these were cutaneous, one maxillary and one patient presented with both maxillary and gingival infiltration.

Peripheral Blood. Table 4 summarizes the leukocyte and platelet counts and hemoglobin values. Fourteen percent of patients were leukopenic and 57% had leukocytosis. The leukocyte count exceeded $100 \times 10^9/l$ in five cases and $300 \times 10^9/l$ in two. No blasts were identified in one case. In all cases monocytes were identified in blood smears. In 57% of cases the monocyte count was greater than $5 \times 10^9/l$. Absolute myeloblast and monocyte counts are shown below.

	Absolute blast count $\times 10^9/L$	Absolute monocyte count $\times 10^9/L$
Range	0–85	0.2–216
Median	2.4	8

Ninety-two percent of patients were anemic. The hemoglobin was < 10 gm/dl in 77% of cases. Seventy-five percent of patients were thrombocytopenic; 67% had a platelet count of less than $100 \times 10^9/L$, 33% less than $50 \times 10^9/L$.

	Myeloblasts & monoblasts (%)	Promyelocytes (%)	Promonocytes (%)	Monocytes (%)
Range	8–46	0.4–6	15–46	6–40
Median	26	2	29	25

Bone Marrow. Differential counts of bone marrow cells are summarized below.

Sixty-four percent of the cases had less than 30% myeloblasts and monoblasts. Monocytes plus promonocytes ranged from 32 to 73%, m = 54%; in 8 cases these cells were $> 50\%$. The morphology of the promonocytes and monocytes showed the same spectrum as described in M4. Monocyte maturation was frequently more pronounced in the blood than the bone marrow. Auer rods were present in 29% of cases. Maturing neutrophils were less than 15% in all cases. Dysmyelopoiesis was present in 64% of cases, dyserythropoiesis in 43% and abnormal megakaryocytes in 21%.

NSE stains showed from 12 to 93% positive cells, m = 67%. In 67% of cases >50% of nucleated marrow cells were positive. In two cases with 12 and 14% NSE positive cells, Wright's stained marrow smears revealed 60% and 85% promonocytes and monocytes; the blood smears contained 9×10^9/l and 153.9×10^9/l monocytes respectively. Myeloperoxidase activity was present in 4 to 88% of the bone marrow cells; in 44% of the cases, >30% of the cells were positive.

Trephine biopsy sections were hypercellular in all cases. The cellularity ranged from 80 to 100%. Data on cellularity and mitotic counts are summarized in Table 5.

Coagulation Studies. Coagulation studies were performed at diagnosis in 9 cases; four had evidence of DIC.

Additional Laboratory Studies. Serum lysozyme values ranged from 7 to 1080 µg/ml, m = 148. The value was increased in 80% of cases; values were greater than 100 µg/ml in 57% and greater than 200 µg/ml in 14%. LAP scores were available in 5 cases, three were increased and two within normal limits. The CSF from one of 12 patients examined contained leukemic cells.

Patient Survival and Induction Chemotherapy. Survival data were available in 11 cases and are summarized in Table 6. Survival ranged from less than one to 37 months with a median of 5. Three patients (27%) survived 1 month or less, 9 (82%) died within one year and 1 (9%) lived for 37 months. Treatment data were available in 10 cases. Four (40%) patients were treated with a chemotherapy regimen that included an anthracycline drug. Survivals for these four patients were <1, 1, 11 and 21 months. Four (40%) patients were treated with a chemotherapy regimen that did not include anthracyclines. Survivals for these patients were <1, 3, 5 and 37 months. Two (20%) patients were not treated; they survived <1 and 5 months.

2.7. *FAB-M6: Erythroleukemia*

Patients. Twenty-four cases were classified as FAB-M6. The male to female ratio was 1.4:1 (Table 2). The age ranged from 3 to 79 years, m = 54; 54% of patients were fifty years of age or older. The most common presenting symptoms were constitutional or hemorrhagic (33%). Physical findings included hepatomegaly in 25% of patients, splenomegaly in 13% and lymphadenopathy in 17% (Table 3).

Peripheral Blood. Table 4 summarizes the hematologic parameters. Fifty-five percent of the patients were leukopenic and 20% had leukocytosis. In two cases no myeloblasts were found in the blood. The highest absolute blast counts occurred in the three patients with the highest leukocyte counts. Two of these also showed numerous nucleated red cells, 45 and 73 per 100

leukocytes. All patients were anemic; 85% had a hemoglobin of less than 10 gm/dl. All patients were thrombocytopenic; 84% had a platelet count of less than 100×10^9/L, 47% less than 20×10^9/L.

Nucleated red cells ranged from rare to 160 per 100 leukocytes, m = 28. They were rare in one case, less than ten per 100 leukocytes in 29% of cases, between 10 and 50 per 100 leukocytes in 37% and greater than 50 in 29%. In 24% of cases normoblastemia was associated with increased numbers of polychromatophilic red cells. Polychromatophilic red cells were rare or absent in 29% of cases. The red cell morphology varied from normal to extremes of anisopoikilocytosis with macrocytes, oval macrocytes and hypochromic microcytes. Two cases showed marked red cell fragmentation. In neither case was there laboratory evidence of a coagulopathy. Atypical platelets were present in several cases.

Bone Marrow Morphology. Bone marrow smear differential counts for myeloblasts and nucleated red cells are shown below.

	Percentage of Type I and Type II blasts (Excluding erythroid cells and lymphocytes)	Percentage of nucleated red cells
Range	30–95	30–95
Median	50	54

The degree of maturation in the erythroid line was variable and ranged from a marked predominance of early erythroblasts to abundant polychromatophilic erythroblasts. Dyserythropoiesis was present in every case to varying degrees and was characterized by gigantism, multinucleation, nuclear lobulation, pyknosis and megaloblastoid changes. Cytoplasmic vacuoles were often prominent in the basophilic erythroblasts and varied from diffusely scattered small single vacuoles to several confluent vacuoles; these vacuoles were positive with the PAS stain. Diffuse PAS staining varied in intensity and usually was confined to the polychromatophilic erythroblasts or anucleate red cells.

Auer rods were found in myeloblasts in 58% of cases. Abnormalities of granulocyte development were present in 67% of the cases. Abnormalities in the megakaryocyte series were present in 71% of cases and consisted primarily of small megakaryocytes with one or two nuclei and variable but usually small amounts of platelet producing cytoplasm.

Two cases were classified as erythremic myelosis. In these cases there was no evidence of abnormalities of granulocytic or megakaryocytic maturation and no increase in myeloblasts and promyelocytes. Both of these had greater than 90% erythroblasts in the bone marrow.

All trephine biopsies were hypercellular. The sections reflected the cellular

composition of the smears. The proerythroblasts and basophilic erythroblasts had large round nuclei with open chromatin and prominent nucleoli; the cytoplasm was deeply basophilic and symmetrical. More mature erythroblasts appeared smaller with variable degrees of nuclear pyknosis. Data on cellularity and mitotic counts are shown in Table 5.

Coagulation Studies. Laboratory coagulation data were available for 13 patients; one showed evidence of DIC.

Additional Laboratory Data. Serum lysozyme values were available in 4 cases; one was slightly elevated and 3 were within normal limits.

LAP scores were available in eleven cases; 5 were elevated, 6 were within the normal range.

Induction Chemotherapy and Patient Survival. Survival data is summarized in Table 6. Survival ranged from <1 to 125 months, m = 11. Forty-eight percent of patients died within one year and 61% within 2 years; 39% survived two years or longer. Treatment data were available on 20 of the 24 patients. Twelve patients received an anthracycline with varying combinations of antimetabolites and prednisone. The median survival for this group was 22 months. Six patients were treated with varying combinations of chemotherapy that did not include an anthracycline. The median survival for this group was 11 months. Two patients received no treatment; one patient with >90% erythroblasts in the marrow died in six months and one patient was lost to follow up.

Ten patients without organomegaly or lymphadenopathy had a median survival of 23 months compared to 8 months for those with organomegaly or lymphadenopathy. The median survival was longer for patients with less than 70% nucleated red cells in the marrow, 22+ months, compared to the median survival for patients in whom the marrow nucleated red cells exceeded 70%, 8 months. The median survival was longer, 22 months, in cases in which Auer rods were detected than in cases without Auer rods, 7 months; the difference was not statistically significant.

3. COMPARISON OF FAB CLASSES OF AML

The distribution of the various FAB classes of AML is shown in Table 1. M2 (44.7%) and M4 (19%) were the most common and together comprised approximately 64% of all cases. All other classes were much less common, with microgranular M3 (3.3%) and M5B (3.9%) being the least frequently encountered.

Although many of the presenting clinical and hematologic features were relatively uniform for the various types of AML, there were certain characteristics that distinguished several of the classes.

3.1. *Age and Sex Distribution* (Table 2)

The median age at diagnosis was similar for M1, M2, M4 and M5B, 46 to 50 years. The youngest median patient age, 16 years, was in M5A; there were no patients between 35 and 75 in this group. M3 was also associated with a relatively young median age, 38 years; however, only one patient was less than 10. The oldest median age, 54 years, occurred in M6. All classes showed a male predilection except M1, in which the sex distribution was approximately equal and M5A, in which there was a slight female predominance. There was a notable male predominance in M3 micrinfo granular, 4:1.

Table 2. Age and sex distribution in acute myeloid leukemia

	Age in years		
	Median	Range	Sex ratio (M:F)
M1	46	1-83	1:1
M2	48	0.1-87	1.6:1
M3	38	1.3-82	2:1
Hypergranular	39	1.3-82	1.6:1
Micrinfo granular	38	20-63	4:1
M4	50	0.5-87	1.4:1
M5A	16	1.3-87	0.7:1
M5B	49	0.1-82	1.8:1
M6	54	3-79	1.4:1

3.2. *Clinical and Laboratory Findings*

Presenting symptoms were similar in all classes and generally related to the abnormal blood counts; hemorrhagic manifestations were most common in M3. Organomegaly was most common in M5A and M5B and least common in M3 (Table 3). Fifty-four percent of the patients with M5B had lymphadenopathy and 54% hepatomegaly; no patients with microgranular M3 had either lymphadenopathy or hepatomegaly. Extramedullary tissue masses and CSF involvement were most frequent in leukemias with a predominantly monocytic component (M4, M5A, M5B) and M1; 29% of cases of M5A and M5B presented with extramedullary tissue masses. Similarly, CNS involvement was most frequently observed in M5A (17%) and M5B, M4 and M1, 8% each. Extramedullary masses and CNS involvement were not observed in M3 and M6.

There was considerable variation in the presenting leukocyte counts (Table 4). The median presenting leukocyte counts were increased in M1, microgranular M3, M4 and M5B and decreased in hypergranular M3 and M6. The highest median counts were in M4, 45.5×10^9/L and microgranular

Table 3. Percent of cases with organomegaly in acute myeloid leukemia

	Splenomegaly	Hepatomegaly	Lymphadenopathy
M1	26	22	30
M2	14	14	24
M3 Hypergranular	11	16	16
M3 Microgranular	20	0	0
M4	34	30	46
M5A	44	50	33
M5B	46	54	54
M6	13	25	17

M3 42.5×10^9/L. In contrast the median leukocyte count for patients with hypergranular M3 was 1.8×10^9/L. The tendency of hypergranular M3 for leukopenia and microgranular M3 for leukocytosis has been previously noted [14]. M6 was also associated with a relatively low median leukocyte count, 3.4×10^9/L. Anemia and thrombocytopenia were frequent in all groups. Median hemoglobin levels were highest in M4 and hypergranular M3, both 9.8 g/dl, and lowest in M6, 7.5 g/dl. Platelet counts were highest in M5A and M5B, 99×10^9/L and 80.5×10^9/L, respectively and were lowest in hypergranular M3, 20.5×10^9/L and M2, 22×10^9/L.

Table 4. Blood counts at diagnosis of acute myeloid leukemia

	Leukocyte count ($\times 10^9$/L)		Hemoglobin (g/dl)		Platelet count ($\times 10^9$/L)	
	Median	Range	Median	Range	Median	Range
M1	16.5	0.82-442	8.6	4.8-13.9	66	13-325
M2	8.3	0.4-390	9.1	2.4-15.4	50	2-352
M3	2.5	0.5-144	9.6	6.6-13.2	21	5-170
Hypergranular	1.8	0.5-31	9.8	6.7-13.2	20.5	5-170
Microgranular	42.5	2.6-144	8.9	6.6-12.3	25	8-94
M4	45.5	0.9-270	9.8	4.3-13.	51.5	3-408
With 16q abnormality	35	2.8-105	9.8	5.9-13.5	50.5	18-127
M5a	7.3	1-365	9	4.8-14.5	99	2-282
M5b	21	1.7-438	9	6.7-15.4	80.5	11-250
M6	3.4	1-44	7.5	3.9-10.9	22	8-139

Comparison of classes for presence of laboratory evidence of DIC at diagnosis is shown below.

Percentage of cases with laboratory evidence of DIC at diagnosis

M1	M2	M3	M4	M5A	M5B	M6
20%	18%	84%	23%	53%	44%	8%

Evidence of DIC varied from 8% of cases of M6 to 84% of cases of M3. M5A and M5B also had a relatively high incidence of DIC. Severity of DIC was generally greatest in M3 and M5.

Serum lysozyme levels were increased in the highest percentage of cases in two of the three classes with a predominantly monocytic component, M4 (95%) and M5B (80%). Only 44% of the cases of M5A studied for lysozyme had increased values. In 63% of cases of M2 studied for serum lysozyme the value was increased. In 41% of the M2 cases with increased lysozyme, monocytes were not increased in either the blood or marrow, in the other 59% there was a slight to moderate increase in monocytes which never exceeded $4.6 \times 10^9/L$ in the blood or 10% of marrow cells. In the M2 cases the lysozyme elevation was slight or moderate in most instances; higher levels tended to occur in M4 and M5B.

3.3. *Morphologic Findings*

The percent of cases with Auer rods for the various classes is shown below.

Percentage of cases with Auer rods

M1	M2	M3	M4	M5A	M5B	M6
49%	70%	97%	64%	0	29%	58%

Auer rods were most frequent in M3, 97% of cases, and not found in M5A.

There was no difference in bone marrow cellularity between groups; all presented with hypercellular bone marrows except occasional cases of M2. Similarly the mitotic counts and indices showed only minimal differences except within M3 where hypergranular M3 was associated with a mean mitotic index of 2.3, the lowest for all classes of AML, and microgranular M3, a mitotic index of 8.5, the highest of all classes (Table 5).

3.4. *Survival*

Median survival varied from 3.5 months for M1 to 21.5 months for hypergranular M3 (Table 6). The subgroup within the M4 class associated

Table 5. Bone marrow cellularity and mitotic activity in acute myeloid leukemia

	Percent cellularity		Mitoses/10 HPF		Mitotic index	
	Mean	Range	Mean	Range	Mean	Range
M1	87	60-100	7.2	1-20	6.4	0.9-16
M2	81	30-100	5.4	0-18	4.4	0-16.2
M3	88	70-100	4.7	0-16	4.4	0-16
Hypergranular	87	70-100	2.5	0-7	2.3	0-7
Microgranular	90	70-100	8.8	3-16	8.5	2.7-16
M4	90	50-100	6.7	0-18	4.9	0-16.2
M5a	92	70-100	6.0	0.5-20	5.6	0.5-20
M5b	89	80-100	6.2	1-12	5.3	0.8-9.6
M6	89	70-100	7.2	1.5-16	6.2	1.4-12.8

Table 6. Survival in acute myeloid leukemia

	Months survival from diagnosis	
	Median	Range
M1	3.5	<1 to 126
M2	8.5+	<1 to 132
M3	16	<1 to 100
Hypergranular	21.5	<1 to 100
Microgranular APL	11.5	<1 to 32
M4	8+	<1 to 92
With Chromosome 16 abnormality	24	<1 to 92
M5a	8	<1 to 36+
M5b	5	<1 to 37
M6	11	<1 to 125
All cases	8	<1 to 132

with a chromosome 16 abnormality had a median survival of 24 months. The short median survivals for M1 and M5B were the result of frequent early death. There was no significant difference in median survival in any of the FAB classes between patients who presented with and without DIC.

Survival related to presence or absence of Auer rods and treatment with anthracycline drugs is illustrated below.

Median survival related to Auer Rods and treatments with Anthracycline drugs (months)

	M1	M2	M3	M4	M5A	M5B	M6
Auer rods present	4	10	*	12	‡	5	22
Auer rods not present	2.5	6.5		4		3	7
Treated with regimens including anthracyclines	4	10.5	21	11	11	6	22
Treated with regimens not including anthracyclines	2	4	<1	5	<1, <1	4	11
Not treated	<1 (1 pt)	<1	<1	<1,1 (1 pt)	<1	<1,5 (1 pt)	6

* Only 1 case without Auer rods.
‡ No cases with Auer rods.
pt = patient.

In all FAB classes in which a comparison could be made cases with Auer rods had a longer survival than those without; the difference was not statistically significant in any class. In all FAB classes except the small M5B group, patients treated with an anthracycline drug had a longer median survival than those treated with regimens that did not include anthracyclines.

4. DISCUSSION

Since its introduction in 1976, the French-American-British (FAB) Leukemia Cooperative Study Group morphologic classification of the acute leukemias has gained increasing acceptance by oncologists, hematopathologists, and hematologists [1, 2]. Prior to this classification, there was considerable variability in the terminology applied to various forms of acute leukemia, particularly of the myeloid type, and difficulty was encountered when attempts were made to compare treatment results from different institutions. Revised criteria for the distinction between the myelodysplastic syndromes and the various forms of acute myeloid leukemia (AML) published in 1982 by the FAB committee addressed some of the problems inherent in the original paper and improved the utility of the classification [13].

One of the most serious problems with the original classification, which was readily recognized by experienced observers, was the criteria of greater than fifty percent marrow blasts and promyelocytes to establish a diagnosis of AML. This figure was unrealistically high and considerable confusion occurred over the distinction between M2 AML and the myelodysplastic syndromes. In the 1982 publication by the FAB committee [13], the percent

of marrow Type I and Type II blasts necessary for a diagnosis of acute myeloid leukemia was revised to thirty percent. Although the revision improved the applicability of the FAB classification, there are still several problems which were apparent in the present study.

The first major problem was the definition of the type II myeloblast. The type I myeloblast is relatively easily defined; it is a blast without granules. The criteria for a type II myeloblast are ambiguous. This cell has the nuclear and the cytoplasmic features of a myeloblast but in addition has a 'few' azurophilic granules, in contrast to the promyelocyte which has 'numerous' azurophilic granules. Even to the experienced observer, the distinction between few and numerous may be very troublesome in an individual case. In this study, we attempted to limit the definition of a type II myeloblast to a blast with 10 or fewer azurophilic granules. However trivial this distinction may appear to be, it is important because the classification of a case as refractory anemia with an excess of blasts transforming to acute leukemia (RAEB-T) or FAB-M2 is predicated on the percentage of type I and II blasts and treatment may be unwisely instituted or withheld because of morphologic imprecision.

The second problem was the distinction between a myelodysplastic syndrome, primarily RAEB-T, and M2 based on the total blast percentage. This problem was most evident in a group of 13 individuals under the age of 40 studied in this laboratory who had 5.2 to 26% type I and II myeloblasts with Auer rods in the bone marrow; 5 of these patients were less than 10 years of age. In this laboratory, the process in these patients was interpreted as M2 on the basis of the presence of Auer rods. However, by FAB criteria these cases would be viewed as RAEB-T and they were excluded from this study.

As originally defined, RAEB was a disorder usually occurring in individuals over the age of fifty [24]. The hematologic pattern was usually characterized by varying combinations of cytopenias, dyserythropoiesis and dysmyelopoiesis, and a somewhat prolonged clinical course. The percentage of myeloblasts and promyelocytes in the marrow was increased up to thirty percent. However, the number of myeloblasts and promyelocytes necessary to establish a diagnosis of AML was fifty percent [1, 2]. In the 1982 revision the criteria for RAEB were modified [13]. The maximum percentage of blasts, defined as type I and type II, was reduced to 20. The number of type I and type II myeloblasts necessary for a diagnosis of FAB-M2 was reduced to 30%. RAEB-T was introduced to bridge the area between RAEB and M2, M4 and M6. Although RAEB-T implies that the patient had RAEB with its typical clinical and hematologic characteristics at one time, the term RAEB-T was applied to patients of all ages without any reference to clinical findings or hematologic status except the percentage of marrow and blood type I

and type II blasts and possible presence of Auer rods. As a result, there are now studies of myelodysplastic syndromes being reported in which children with increased blasts in the marrow are being classified as RAEB or RAEB-T [25] although the term RAEB was originally intended to be used for a relatively specific entity occurring in older individuals [24]. Part of this problem relates to the interpretation of cases in which Auer rods are found in blasts. In one of the original articles detailing the features of the FAB classification, it was stated that the Auer rod is 'unequivocal evidence of AML' [2]. Subsequently, in the 1982 publication, one of the criteria for RAEB-T was the presence of Auer rods in blasts [13]. However, recent articles published by individual members of the FAB committee continue to emphasize the diagnostic significance of the Auer rod for AML [4, 8].

From the observations in this study, it is apparent that the minimum of thirty percent type I and type II myeloblasts is not always satisfied in cases in which all observers would agree on the diagnosis of AML. This is best illustrated by the data from the patients with FAB-M3. The bone marrows from 90% of the cases of M3 contained less than 30% type I and type II myeloblasts; in approximately 70% of the cases, the percentage of type I and type II myeloblasts in the marrow was less than 10%. If the FAB criteria were rigidly or even loosely adhered to, 90% of the cases of M3 would be classified as RAEB-T.

The cases of M5B are somewhat analogous to the cases of M3 in that M5B is morphologically distinctive and readily recognized. Similar to the cases of M3 in the present study, there frequently were less than thirty percent type I and type II myeloblasts in the bone marrow. The marrow and blood from these patients usually contained a very high percentage of promonocytes. The relationship of this cell to the blast population has been imprecise. In an article on the FAB classification authored by one of the signatories to the classification, the term promonocyte is applied to blast forms with twisted or folded nuclei [4]. However, in the 1982 publication detailing the revision of the myelodysplastic syndromes, the promonocyte is clearly excluded from the blast category [13]. Examination of the photomicrograph illustrating M5B in the original FAB publication will show only a very small percentage of cells which could be even loosely interpreted as type I or type II blasts [1]. If this photomicrograph is representative, the case illustrated clearly would not meet the minimum blast percentage of 30 necessary for a diagnosis of AML, much less 50% as was specified in the original article [1]. The same statement would apply to the illustration of M4 [1]. From our observations in this study, it would appear that the promonocyte, as it occurs in M5B should be considered analogous to the type II blast.

M6 presents several problems both in regard to the myelodysplastic syn-

dromes and to other forms of AML, most notably M2 and M4. The minimum requirement of 30% type I and II myeloblasts in M6 may be particularly troublesome in cases of obvious erythroleukemia in which the percentage of erythroblasts exceeds the suggested 50% minimum. This problem has been partially resolved by a recent decision of the FAB committee to calculate the blast percentage on the basis of the marrow cells which are non-erythroid precursors [18]. As a result, if 50% of the marrow cells are erythroid precursors the percent of blasts would be doubled, i.e., 15 blasts in a count of 100 would be 30%. This modification for M6 has partly ameliorated the problems inherent in the original criteria for this subtype.

The other major difficulty with the criteria for M6 is the unrealistically high requirement of 50% erythroid precursors in the marrow for a diagnosis. There are cases of AML in which the marrow contains between 30 and 50% red cell precursors which are markedly abnormal in appearance and which are positive with the PAS reaction. The designation of these cases as M2 or M4 is inappropriate. The presence of 30% or more markedly abnormal erythroid cells in a marrow specimen is clearly evidence of involvement of the red cell series. The adequacy of 30% erythroid precursors for a diagnosis of M6 has been recognized by one of the authors of the FAB classification in a recent publication [26].

Although extremely uncommon, there are cases of AML which present with essentially pure red cell involvement. Formerly these processes were designated erythremic myeloisis or Di Guglielmo's disease. These disorders are often marked by a rapidly progressive course. Because of the very high number of erythroid precursors, often in excess of 80%, the number of myeloblasts may be very low. Even utilizing the revised method for determining the blast percentage in M6, the minimum of 30% type I and II myeloblasts may not be present. As a result these cases would be classified as a myelodysplastic syndrome. The M6 category should have a provision for this, admittedly rare, type of case.

In a substantial number of cases M5A has features which may lead to problems in recognition. Several patients in this study presented with extramedullary masses. Although the cases had 60% or more monoblasts in the marrow, earlier bone marrow specimens from two of the patients with extramedullary involvement contained less than 20% leukemic cells. Another patient, a one year old child not included in this study, initially presented with an orbital lesion which cytologically and cytochemically had the features of monoblasts. At the time of initial presentation only occasional monoblasts were noted in the bone marrow although metaphases with a t(9; 11) chromosome abnormality were present. Monoblasts were also noted in the cerebrospinal fluid at this time. Frank marrow involvement in this patient did not occur for 21 months following initial presentation.

It is apparent from the patients with M5A in this series and other patients that have been studied in this laboratory, that this form of leukemia has a high incidence of extramedullary involvement [27]. In some patients, the extramedullary lesion may be the presenting manifestation and the criteria for a diagnosis of leukemia may not be present. As a result, some of these cases may be misclassified and inappropriately treated. It is important to recognize that these cases represent incipient leukemia and therapeutic decisions should be predicated on an eventual leukemic evolution.

Two types of AML observed in this laboratory but not included in this study were acute megakaryoblastic (7 cases) [28, 29] and acute basophilic leukemia (1 case) [30]. There is no provision for inclusion of these cases in the present FAB classification system. With the increasing use of ultrastructural cytochemistry and monoclonal antibodies for the study of morphologically undifferentiated leukemias undoubtedly more of these cases will be recognized.

The FAB classification of AML has been deservedly widely accepted internationally as a working morphologic classification; its utility has been demonstrated in several studies. However, as exemplified in this study, there are still several problem areas. These relate primarily to impreciseness in definition of some of the classes and the specific cell types (i.e. type II myeloblast-promyelocyte, promonocyte) and ambiguities regarding the significance of the Auer rod. This lack of precision still allows for considerable variation in the application of this classification.

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6. Chemosensitivity of *In Vitro* Colony Forming Units as a Predictor of Response to Antileukemic Drugs

JOHN J. HUTTON

1. INTRODUCTION

Leukemia is a clonal disease secondary to malignant transformation of one or more normal hematopoietic stem cells. These leukemic stem cells are capable of proliferation and self renewal. They give rise to one or more dominant clones of cells that eventually fill the bone marrow and suppress normal hematopoiesis in the patient. At this time clinical disease becomes apparent with signs and symptoms of marrow failure. The population of blasts in acute leukemia contains cells capable of colony formation in culture [1-6]. These cells are called leukemic colony forming units, L-CFU. If the colonies are harvested from culture and replated, some also contain cells capable of forming colonies so that proliferation of stem cells capable of self renewal can be demonstrated. There is substantial evidence that L-CFU which replicate extensively *in vitro* and form large colonies are identical or closely related to the leukemic stem cell responsible for maintenance of the leukemic clone in the patient [7, 8].

Properties of the leukemic stem cell can be deduced by manipulating a population containing them *in vitro* and then measuring the effects by a colony forming assay. As a specific relevant example, aliquots of mononuclear cells from the bone marrow or peripheral blood of a leukemic patient can be incubated with cytotoxic drugs and the effects on the leukemic stem cell can be assessed by colony assay. Theoretically, this type of chemosensitivity testing can be used to select optimal chemotherapy for an individual patient. Drugs cytotoxic to the leukemic stem cells would be administered and those to which the leukemic stem cell was resistant would be avoided. The patient could be spared the toxicity of therapy with ineffective agents. Despite the attractiveness and conceptual simplicity of *in vitro* chemosensitivity testing of leukemic cells, it has been extremely difficult to standardize methodology and to prove its relevance to the clinical management of

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patients. All *in vitro* chemosensitivity assays based on colony formation by tumor stem cells share several practical limitations when applied to patients [9–11]: (a) cells from the majority of tumors do not grow well, (b) few satisfactory therapeutic options are available, and (c) results of the assays are not available for several weeks after the tumor specimen is obtained. The underlying theory and specific problems of *in vitro* chemosensitivity testing by assay of leukemic colony forming units are the subjects of this review.

2. COLONY ASSAYS

2.1. *General Features*

Essential requirements for growth of normal and leukemic colony forming hematopoietic precursors *in vitro* are: (a) a semisolid (agar) or viscous (methylcellulose) culture medium so that dispersion of loose aggregates of cells is prevented and (b) the presence of special macromolecular growth factors produced by other cells. Growth of malignant colony forming cells from acute lymphoblastic leukemia, but not acute myelogenous leukemia, also requires low oxygen tension [5].

Several kinds of colony forming cells are present in normal bone marrow and peripheral blood. These include early progenitors of granulopoiesis (CFU-GM), erythropoiesis (BFU-E and CFU-E), megakaryopoiesis (CFU-M), and lymphopoiesis, as well as the multi-potent stem cell (CFU-GEMM). Mature B and T lymphocytes can also form colonies under certain conditions. Mature granulocytes, erythrocytes, and platelets cannot proliferate. Under most circumstances investigators want to study a specific type of colony forming unit so that methods of selectively growing one class of progenitors are essential. Specificity can be conveyed by: (a) choice of source of cells, (b) pretreatment of cells before plating, and (c) culture conditions when growing the progenitors. When studying properties of leukemic colony forming units, the major problem is to grow these and not to grow normal hematopoietic progenitors. Peripheral blood is an excellent source of leukemic cells except in patients with small numbers of circulating blasts. Peripheral blood contains very few normal progenitor cells (CFU-GEMM, CFU-GM, CFU-E, BFU-E) that will grow *in vitro*. Unfortunately, it is a rich source of T-lymphocytes which proliferate and form colonies under conditions of culture favorable to growth of myeloid blasts. Colonies of T-cells and leukemic blasts cannot be reliably distinguished by simple techniques so that it is necessary to separate T-lymphocytes from blasts in peripheral blood before colony forming cells are cultured. Removal of peripheral T-cells also improves the cloning efficiency of L-CFU from most patients [12]. Bone marrow, on the other hand, does not contain large numbers of mature T-cells, but it does contain the whole array of normal hematopoietic progen-

itors that can proliferate. Specificity of growth *in vitro* is conveyed by use of appropriate stimulators, although specificity for leukemic blasts is never absolute. In particular, most cultures that support growth of L-CFU also support growth of the normal myeloid colony forming units from marrow, such as CFU-GM and sometimes CFU-GEMM or CFU-E. Because L-CFU cannot be grown with absolute specificity, it is essential that the nature of colonies grown from each patient be determined.

Several methods are commonly employed to establish the nature of colonies grown *in vitro*. Individual colonies can be examined microscopically following staining for characteristic proteins such as hemoglobin, myeloperoxidase, and esterase [13, 14]. This permits assessment of the stage of differentiation of cells within the colony, as well as whether the cells are morphologically uniform and belong to the same lineage. Proliferating lymphocytes can be detected by E-rosetting or by immunofluorescence staining for immunoglobulins or T-cell markers. Cytogenetic studies can be performed on cells from colonies, as well as from the patient. Demonstration that the same cytogenetic abnormality is present in both leukemic blasts from the patient and in cells within colonies grown *in vitro* is definitive proof that CFU are derived from leukemic blasts and are in fact L-CFU [2, 15]. Otherwise, the investigator is left with weaker evidence based on morphological correlations, presence or absence of markers of differentiation, and quantitative relationships between number of blasts plated and number of colonies grown.

2.2. *Colony Assay of Blasts in Acute Myelogenous Leukemia*

Most assays of myeloid L-CFU are modifications of the procedure reported by Buick, Till, and McCulloch in 1977 [2]. A general outline of one current version of this method is shown in Figure 1 [8]. Heparinized peripheral blood is removed from the patient and the mononuclear cells are obtained by separation on a Ficoll-Hypaque density gradient. The mononuclear cells are then incubated with sheep red blood cells. T-lymphocytes form rosettes which are then separated from myeloid blasts by centrifugation through a density gradient. The T-lymphocyte depleted mononuclear cells are plated in methylcellulose (0.8% v/v final concentration) containing 20% fetal calf serum and 5% PHA-LCM (phytohemagglutinin-leukocyte conditioned medium). The PHA-LCM consists of the supernatants of cultures of normal human leukocytes incubated for 7 days at a concentration of 10^6 cells/ml in medium containing 10% fetal calf serum and 1% phytohemagglutinin. Cultures are generally grown in tissue culture dishes, 1 ml volume per dish, at a concentration of 2×10^5 cells per ml. Colonies of 20 or more cells per aggregate form after 5-7 days growth at 37 °C in a humid atmosphere of 5% CO_2-air. The initial plating efficiency, PE-1, is a mini-

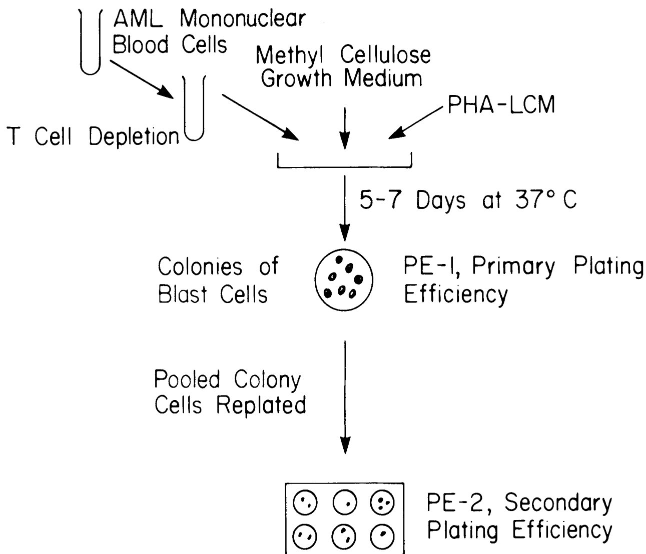

Figure 1. Assay of the primary and secondary plating efficiencies of human Myeloid L-CFU. Modified and taken with permission from: McCulloch EA: Stem cells in normal and leukemic hematopoiesis. Blood 62:1–13, 1983.

mum estimate of the relative frequency of progenitor cells, L-CFU. For patients with acute myelogenous leukemia, PE-1 is highly variable and ranges from 0 to 8000 L-CFU per 2×10^5 cells plated. Colonies grown from L-CFU consist of cells which, morphologically, are blasts. Under these conditions of culture the T-cell depleted mononuclear fraction from *normal* peripheral blood grows from 0 to 200 colonies per 2×10^5 cells. Colonies from normal CFU consist largely of maturing granulocytes. There is considerable variation among investigators in the length of time plates are incubated before colonies are counted and in the minimum number of cells required to constitute a colony. In some scoring systems, aggregates of 10 to 40 cells are scored as clusters, and those of greater than 40 cells are scored as colonies, after 7 to 14 days of incubation.

Cells forming colonies *in vitro* must be able to proliferate, else multicellular colonies would not be formed. It requires at least 6 divisions for a single cell to form a colony of 40 cells. In terms of proliferative potential, however, cells are divided into two fundamentally different classes, stem cells and progenitor cells. Stem cells are capable of both self renewal and maintenance of a differentiating cell compartment, while progenitor cells are incapable or have a very limited capacity for self renewal. Stem cells are capable of a reasonably large, but not infinite, number of mitotic divisions.

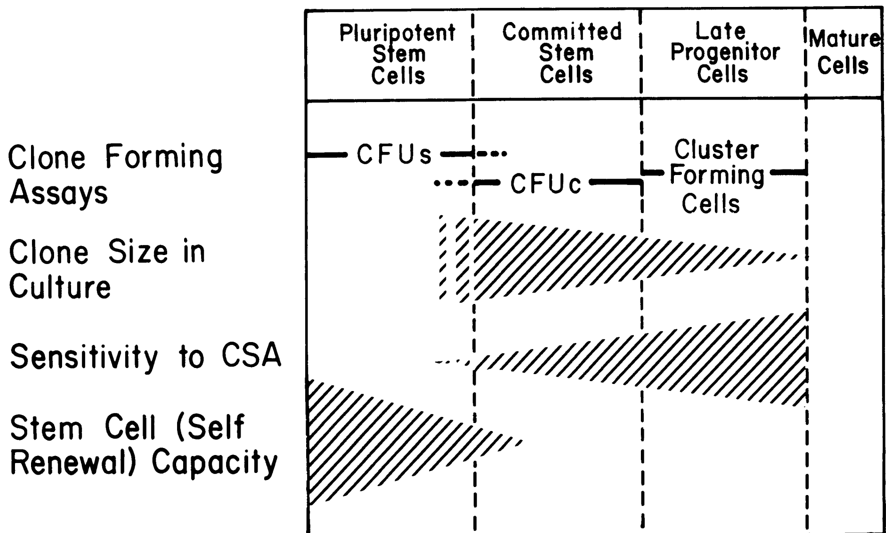

Figure 2. Biological properties of various types of colony forming units. 'CFUs' is the multipotent stem cell defined by transplantation experiments in mice. CFU-GEMM may be the *in vitro* equivalent of this cell in humans. 'CFUc' is the granulocyte-macrophage progenitor that is currently termed CFU-GM. Modified from: Francis GE: Response to therapy in acute myeloid leukemia. Medical Hypotheses 5:999–1006, 1979.

Self renewal and differentiation are mutually exclusive in myelopoiesis, that is, cells capable of self renewal are not committed progenitors of specific lineages (Figure 2). Conversely, once a cell is committed to a specific lineage, it progressively loses its capacity for self renewal. The process of commitment of stem cells to differentiation along a specific lineage may be stochastic and irreversible [8, 16]. L-CFUs in each patient arise by malignant transformation of a single normal hematopoietic progenitor and then expand clonally. The properties of L-CFU differ from patient to patient, but in each case appear to be reasonably similar to a normal CFU at some point in differentiation. Blast progenitors retain some of the stem cell properties of their normal ancestors, but their descendants do not follow a normal program of differentiation leading to fully functional mature blood cells [7, 8, 16]. Proliferative capacity (colony size) decreases and sensitivity to colony stimulating activities increase as cells differentiate [17]. In terms of *in vitro* L-CFU assays, there is evidence that colonies containing relatively large numbers of cells are usually derived from stem cells capable of self renewal, whereas clusters are usually derived from committed transitional cells capable of a limited number of divisions, but not capable of self renewal (Figure 2) [8, 16]. If this theory is correct, then L-CFU which form colonies are fundamentally different from L-CFU which form clusters. Colony forming L-CFU could self renew and clonally expand in a patient to give recurrence of disease, whereas a few residual cluster forming L-CFU

could not. This contrasts with the view that clusters merely reflect poor growth *in vitro* for any of a number of reasons and are not fundamentally different from colonies [18]. Mackillop *et al.* [19] have developed a mathematical model of human tumor growth which may permit both a test of whether clusters are fundamentally different from colonies and also a way to establish the 'cut off' point between stem cell colonies and other types of colonies.

Figure 1 illustrates one method of measuring the capacity for self renewal. Cells are pooled from many colonies, mechanically dissociated to form a single cell suspension, and replated. Secondary colonies are indistinguishable from primary colonies with regard to size, morphology, and biochemical or immunological markers [20, 21]. If the number of individual colonies contributing to the pool of cells is large, the value of the secondary plating efficiency (PE-2) is a measure of the capacity of the cells in the colonies to self renew. The problem with this type of pooled replating experiment is that an average value of PE-2 is found, rather than the fraction of colonies which contain stem cells. Plucking and testing individual colonies is more informative, but extremely tedious. Cells also grow poorly when plated at very low density. The extent of renewal capacity of human leukemic cells is limited to 2 or 3 serial transfers and is affected by chemotherapy [21, 22]. Measurement of the effects of drugs on both PE-1 and PE-2 may be relevant, when predicting the effects of chemotherapy by *in vitro* assays.

One common modification of the myeloid L-CFU assay shown in Figure 1 is the replacement of PHA-LCM with another type of conditioned medium. There are several disadvantages to PHA-LCM. First of all the phytohemagglutinin in the medium stimulates T-cells to form colonies *in vitro* along with colonies formed by L-CFU. The mixture of two types of colonies complicates interpretation of experiments in which removal of T-cells is incomplete. This is particularly likely in specimens of peripheral blood that contain a low percentage of blasts. Secondly, PHA-LCM is made by incubation of normal human peripheral leukocytes with PHA and varies considerably from batch to batch in its ability to support the growth of colony forming units. Each batch must be individually titrated to estimate the optimal concentration for use in experiments. Both too little and too much give poor growth of cells [20]. The identity of the macromolecule(s) in PHA-LCM required for growth of L-CFU is/are not known, but they are presumed to be identical to normal colony stimulating factors [23].

Historically, the earliest source of colony stimulating factors was peripheral blood leukocyte feeder layers [1]. Living feeder layers, however, have several important disadvantages when compared to PHA-LCM and other conditioned media: (a) they must be used within one week of preparation, (b) preparations from different donors vary in efficacy so that feeder layers

from several donors must be prepared each time to insure that at least one gives maximal stimulation, and (c) feeder layers do not produce standard amounts of colony stimulating factors so that each set has to be standardized, usually by reference to response of a standard batch of frozen progenitor cells. Two commonly used conditioned media, besides PHA-LCM, that can be standardized and stored are human placental conditioned medium [24] and giant cell tumor (GCT) cell line conditioned medium [25]. These do not contain phytohemagglutinin and do not stimulate proliferation of mature T-cells. Leukocyte feeder layers, PHA-LCM, placental conditioned medium, and GCT conditioned medium differ considerably in their ability to support the growth of leukemic cells and appear to contain different growth factors [26–28]. Their ability to support growth of normal granulocyte-macrophage progenitors does not correlate closely with their ability to support growth of L-CFU, and in fact L-CFU from different patients respond differently to the various sources of colony stimulating activity. The different conditioned media may support different subpopulations of leukemic cells. For these reasons the choice of conditioned media for use in experiments such as chemosensitivity testing must be made with care and with the knowledge that results obtained with one medium may not be duplicated when another medium is tested. No single medium is absolutely the best under all circumstances.

One particular modification of the conventional myeloid L-CFU assay shown in Figure 1 is of relevance to chemosensitivity testing. While it is not widely used, a system of feeding cells on a daily basis has been developed by Park *et al.* [29] and permits experiments that cannot be done with other techniques. The culture system consists of double agar layers in a plastic Petri dish with 6 small holes in its bottom. The lower layer of 0.5% agar in conditioned medium prevents loss of cells through the holes. The upper layer of 0.3% agar in conditioned medium contains the cells. Cultures are incubated at 37° in a humid atmosphere flushed continuously with 7% CO_2. Cultures are fed daily by overlay of 0.5 ml of medium containing any desired additives such as drugs. The medium percolates through the agar layers and drains out through holes made in the bottom of the Petri dish. Although the system is technically more difficult to work with and more prone to contamination than conventional systems, it appears to offer several advantages: (a) the plating efficiency of L-CFU seems improved, (b) the number of cells per colony is increased, (c) the cell cycle can be manipulated by the feeding schedule [30], and (d) complex regimens of *in vitro* chemotherapy can be 'administered'.

2.3. *Colony Assay of Blasts in Acute Lymphoblastic Leukemia*

Clonal growth of leukemic lymphoblasts has only recently been accom-

plished. Smith *et al.* [5] grew lymphoid colonies from 26 of 45 bone marrow samples from children with acute lymphoblastic leukemia in relapse. The colonies grew in agar overlaying a leukocyte feeder layer. No mitogens, antibiotics, or thiols were added. The observation critical to success was that L-CFU from acute lymphoblastic leukemia require a hypoxic atmosphere containing 7% oxygen rather than the 20% oxygen present in air. Colonies grew slowly over a period of 18-21 days. The plating efficiency was 11 to 209 colonies per 10^6 cells. Most (perhaps 80%) of the colonies appear to have been derived from leukemic progenitors based upon cytochemical staining, the presence of specific cell surface markers, and the ability to grow without mitogens. Cytogenetics and capacity for self renewal (PE-2) were not studied. This same assay can also be used to quantitate malignant colony forming cells in the bone marrow of patients with non-Hodgkin's lymphoma [31]. However, normal CFU-GM from bone marrow can also form colonies in this and in similar assays. Careful monitoring of type of colony being grown is always necessary.

A completely different, more complex, but more efficient technique for clonal growth of lymphoblastic progenitors has been developed by Izaguirre *et al.* [6]. The method is based on their procedure for growing normal and malignant B-lymphoid progenitors [32], coupled with incubation at low oxygen tension [5]. T-cell depleted mononuclear fractions are isolated from marrow or blood by E-rosetting and density gradient centrifugation. Conditioned media are prepared by culturing purified normal T lymphocytes with PHA. Living feeder cells are also necessary and are comprised of normal T lymphocytes irradiated with 2000 rads so they cannot proliferate. Assays consist of mixtures of T-lymphocyte depleted leukemic cells, irradiated feeder cells, conditioned medium, methylcellulose, and growth medium. After 5-7 days of culture in 5-7% O_2, colonies containing more than 20 cells are counted and characterized. In the original study, colonies were grown from marrow or blood of 16 of 18 patients. Cells in colonies had the same markers (E^-, sIg^-, $cALL^+$ and $cIgM^+$ or $cIgM^-$) as the cells from the patient. The primary plating efficiency (PE-1) ranged from 0 to 276 colonies per 10^4 cells plated and the secondary plating efficiency (PE-2) ranged from 7 to 441 per 10^4 cells. Because of the presence of living feeder cells that might affect or be affected by drugs, the complex assays required to quantitate lymphoblastic L-CFU are difficult to recommend for use in chemosensitivity testing.

3. QUANTITATION OF SENSITIVITY TO ANTILEUKEMIC DRUGS

Leukemic stem cells are believed to be responsible for the initial expansion of the leukemic clone which eventually replaces normal hematopoietic

cells in the patient. Similarly, regrowth of leukemic stem cells not killed by chemotherapy leads to recurrence of disease. This simple model predicts that effective therapy of leukemia depends upon eradication of the leukemic stem cells. These comprise a very small proportion of the leukemic cells within the patient. *In vitro* tests aimed at predicting the sensitivity of a leukemia to chemotherapeutic agents must, one would assume, measure the effects of the agents on the leukemic stem cell rather than the leukemic population in general. The L-CFU which replicates extensively and forms a large colony *in vitro* appears to be identical or closely related to the leukemic stem cell [8, 19]. Clonogenic assays of L-CFU, then, represent a way of distinguishing effects of drugs on the small population of stem cells from effects on the large population of non-stem cells. They form the basis of the human tumor stem cell assays proposed as *in vitro* methods by which anticancer drugs can be selected for activity against tumor cells from a patient [9–11].

3.1. *In Vitro Clonogenic Assays of Drug Sensitivities*

It is easy to measure the effect of a drug on the plating efficiency of L-CFU. However, it is difficult to choose clinically relevant conditions of drug exposure and to interpret the results. A typical example of *in vitro* chemosensitivity testing is shown in Figure 3 [33]. The drug, cytosine arabinoside, forms a part of most regimens used to treat acute myelogenous leukemia (AML). L-CFU were cultured as shown in Figure 1. Various concentrations of cytosine arabinoside were added to the methylcellulose growth medium. A dose dependent decrease in colony formation was observed and in most instances, the dose-response curve could be approximated by a negative exponential. The sensitivity of the L-CFU to cytosine arabinoside can be characterized by the dose required to reduce survival to 10% (Ara D_{10}). For 42 patients tested in one laboratory [33], sensitivity of L-CFU to cytosine arabinoside varied from very sensitive (Ara $D_{10} < 0.1$ µM) to resistant (Ara $D_{10} > 10$ µM). The concentration of cytosine arabinoside regularly achieved in human plasma during remission induction of AML ranges from 1 to 10 µM [34]. L-CFU classified as sensitive by having the Ara $D_{10} < 0.1$ µM should be killed *in vivo* by standard cytosine arabinoside containing induction regimens. Obviously, the dose response curve is an exponential function with some fraction of cells surviving at any given dose of drug so that total cell kill would not be expected. Marked patient to patient variation is observed when sensitivities of L-CFU to drugs are examined by *in vitro* colony forming techniques. However, repeated measurements of D_{10} on the same specimen of cells tested in the same laboratory are reproducible, even when cells have been cryopreserved.

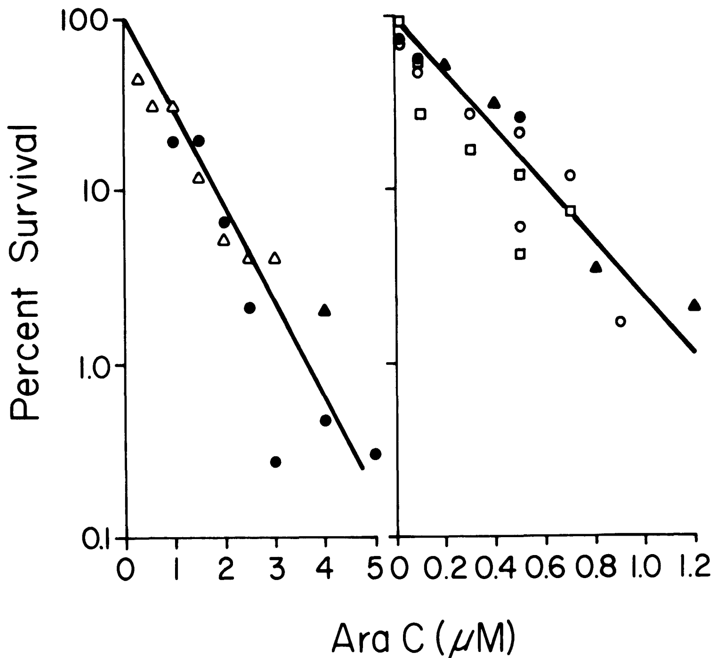

Figure 3. Survival of L-CFU exposed continuously to cytosine arabinoside while growing in methylcellulose as shown in Figure 1. Two patients with AML were studied repeatedly. Closed symbols are fresh and open symbols cryopreserved cells throughout. Panel (a): ● In relapse 8 months after induction, had received many drugs including cytosine arabinoside and adriamycin; △ Restudied at 9 months, still in relapse, no response to further treatment. Panel (b): ○ At diagnosis, before treatment; □ at 6 months, after many drugs, no remission; ▲ at 10 months, continued relapse, more drugs. Taken with permission from McCulloch EA, Buick RN, Curtis JE, Messner HA, Senn JS: The heritable nature of clonal characteristics in acute myeloblastic leukemia. Blood 58:105–109, 1981.

Although studies on very few patients have been reported, it appears that serial measurements on patients who are treated with cytotoxic drugs, but remain in relapse (Figure 3), or who achieve remission and then relapse, do not usually show major changes in drug sensitivities of the L-CFU. In this sense, sensitivities to chemotherapeutic drugs are heritable traits of leukemic clones little affected by chemotherapy [33]. An interesting type of exception to this statement has been documented by Preisler [35] who showed that chemotherapy *in vivo* may affect the cycling of L-CFU and dramatically affect the subsequent drug sensitivities of the L-CFU to cycle active drugs *in vitro.* It is not known whether the drug sensitivities of L-CFU generally change during chemotherapy, as would be expected if heritable drug resistance developed. This point needs definitive clarification by longitudinal study of a much larger number of patients than have been studied thus far. The results of these studies could affect our approach to

maintenance and consolidation chemotherapy, depending upon whether residual leukemic cells were found to be genetically resistant to drugs used to induce a remission.

The data shown in Figure 3 were obtained by adding cytosine arabinoside to the growth medium so that L-CFU were continuously exposed to the drug and/or its metabolites throughout the entire culture period. When administered to patients, cytosine arabinoside is rapidly deaminated to uracil arabinoside and has a short biphasic half life in plasma [34]. There is no information about the fate of cytosine arabinoside or any other drug added to culture medium in colony forming assays. One can guess that the pharmacokinetics of exposure *in vitro* do not mimic pharmacokinetics *in vivo*. Whether these differences are important in assessing whether L-CFU are sensitive or resistant to drugs and in extrapolating *in vitro* results to the *in vivo* situation is not known with any certainty.

Because of a lack of information about the relevance of pharmacokinetics, a number of methods of exposure of cells to drugs have been utilized empirically. Pulse exposure to drugs is probably the most commonly used technique. Purified mononuclear cells containing the leukemic blasts are incubated for 10 to 60 minutes with different concentrations of drug. Cells are then washed and plated for colony formation in growth medium free of drug. As in the case of continuous exposure to cytosine arabinoside, a dose dependent decrease in colony formation is observed. In most cases the dose response curve can be approximated by a simple negative exponential and the D_{10} can be calculated. When the D_{10} values for a 10 minute exposure to Adriamycin were determined on L-CFU from 52 patients with AML, they ranged from very sensitive (Adria $D_{10} < 0.1$ µg/ml) to resistant (> 5 µg/ml) [33].

While D_{10} is relatively reproducible, survival curves may not be linear and it may not be possible to calculate D_{10} precisely. Low concentrations of a cytotoxic drug sometimes stimulate, rather than kill L-CFU. Because the estimation of D_{10} is based upon survival of L-CFU at several different drug concentrations, the measurement also poses serious practical problems. In a typical experiment, mononuclear cells are plated at a concentration of 2×10^5 cells/ml which is equivalent to 2×10^5 cells per culture dish. Depending upon plating efficiency, the number of plated cells is adjusted to yield 50 to 300 colonies. This adjustment is permitted only if previously assayed, cryopreserved cells are used because the plating efficiency varies from patient to patient and cannot be predicted *a priori*. Cells are usually plated in quadruplicate at each drug concentration. If D_{10} is based on measurements at 5 concentrations of drug, then 20 plates containing a total of 4×10^6 mononuclear cells would be required. If several drugs are to be tested or if the experiment needs to be repeated, then the number of cells

required rapidly escalates. Perhaps a fourth of leukemic patients do not have a large number of circulating blasts or marrow may not be aspirable. Reduction in the number of cells required for chemosensitivity testing is desirable. One approach is to test the sensitivity of L-CFU at one or two critical concentrations of drug. The concentrations are usually related either to levels of drug achieved in patients' plasma during chemotherapy or to concentrations of drug that reproducibly kill 25% and 75% of CFU-GM from normal cryopreserved bone marrow.

Preisler [36] for example tested the sensitivity of myeloid L-CFU to 0.3 and 3 μg/ml of cytosine arabinoside. Cells were exposed to drug for one hour, washed thoroughly, and then plated. After 7 days, the plates were fixed with 3% glutaraldehyde and the number of cluster-colonies (> 4 cells/group) was counted. Of note is the small number of cells that could constitute a cluster-colony. If the concept of a fundamental difference between the growth potential of CFU giving rise to clusters versus colonies is correct (Figure 2), then the chemosensitivity of a variety of types of CFU would be measured in this assay. In particular, the predominant CFU giving rise to clusters would not be a leukemic stem cell capable of self renewal. A leukemic patient receiving standard doses of cytosine arabinoside by continuous infusion (100 mg/M^2/day) might sustain a plasma level of 0.1 μg/ml. The 0.3 μg/ml of cytosine arabinoside in the *in vitro* L-CFU assay would be similar to this plasma level, whereas the 3 μg/ml *in vitro* would be at least 10-fold higher than the usual concentration achieved clinically. In 7 of 23 specimens tested at 0.3 μg/ml and in 4 of 27 tested at 3 μg/ml, the number of L-CFU cluster-colonies actually increased after exposure to drug. In 13 of the 22 trials in which leukemic cells were exposed to more than a single concentration of cytosine arabinoside, the proportion of L-CFU killed by the high and low concentrations of the drug were indistinguishable, while for 9 the high drug concentration killed substantially more L-CFU than the low concentration.

Given these kinds of mixed responses, it is difficult to express drug sensitivity in a simple way and to establish a threshold that clearly separates drug-sensitive from drug-resistant cells. The goal of most *in vitro* chemosensitivity tests is to predict the *in vivo* response of the leukemia to chemotherapy. Under these conditions, one approach to establishing *in vitro* thresholds separating drug-sensitive from drug-resistant cells is to correlate the outcome of therapy with the *in vitro* killing of L-CFU. In Preisler's study the proportion of L-CFU killed by 0.3 μg/ml of cytosine arabinoside ranged from 0 to 60% for drug-sensitive patients with ⩾ 30% killing for 5 of 7 specimens. The range of killing for drug-resistant patients was from 0 to 51% with ⩽ 13% killing for 7 of 8 specimens. The mean ±SE for percent L-CFU killed for drug-sensitive and drug-resistant patients were 30%±9%

and 9%±6%, respectively ($p = 0.05$). As a result of this type of analysis, one would test whether L-CFU from a leukemic patient were killed at 0.3 µg/ml cytosine arabinoside. Drug sensitivity *in vitro* would be defined by the percentage killed, using previously established clinical correlates, but there would obviously be a 'gray zone' in which cells were neither clearly sensitive nor clearly resistant. In the example described, the gray zone would extend from approximately 15% to 21% killing. There are no published studies of the reproducibility of these kinds of drug sensitivity assays among different laboratories testing the same batch of leukemic cells. At the present time one cannot assume that correlates established by one laboratory are generally applicable in other laboratories.

A potential objection to the chemosensitivity assays discussed thus far is their dependence on either a limited pulse exposure of L-CFU to drugs, followed by washing before plating, or a continuous exposure to drug incorporated in the growth medium. Theoretically, pulse exposure might be best used to test drugs which are not cycle active, are rapidly inactivated *in vivo*, or are administered to patients in a limited number of widely spaced pulses. Continuous exposure *in vitro* might be most appropriate to test drugs which are cell cycle dependent, have a long half life *in vivo*, or are generally administered by continuous infusion. If the goal is to mimic *in vitro* the pharmacokinetics observed *in vivo*, then the laboratory problem becomes unmangeably complex. Chemotherapy given to patients with acute leukemia is invariably a mixture of drugs and schedules except for specialized Phase I and II trials of single agents. Host factors modify drug metabolism so that each person is pharmacologically unique [18]. The culture system described by Park *et al.* [29] permits limited flexibility both in drug exposure times and in the mixture of drugs to which cells are exposed. New medium is fed to cells daily and the old medium is drained from the culture plates. This change in medium readily allows addition and washout of drugs at prescribed times. The cell cycle of the L-CFU can be manipulated by changing the composition of the medium [30]. Leukemic cells can be kept out of cycle by withholding PHA-LCM and put back in cycle by adding PHA-LCM to the daily feeding. The interaction between cell kinetics and chemotherapy can be studied. While flexible, the system is more technically demanding than conventional assays. Whether it mimics the *in vivo* situation sufficiently well to provide important new insights into the design of optimal chemotherapy for leukemia, is not yet known.

3.2. *Differential Sensitivities of Normal and Leukemic Colony Forming Units*

When administering cytotoxic chemotherapy to patients with leukemia, one determinant of outcome might be the relative sensitivity of the normal

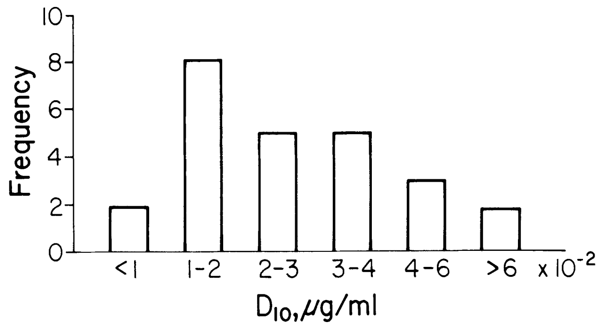

Figure 4. Frequency distribution of D_{10} values of cytosine arabinoside on CFU-GM from non-leukemic marrow. Buffy coat cells were obtained from 22 patients without malignant disease. Cells were continuously exposed to cytosine arabinoside during culture. The concentration of drug required to reduce survival to 10% (D_{10}) was calculated for each individual specimen. Taken with permission from Hiho Y, Till JE, McCulloch EA: Effect of arabinosyl cytosine on granulopoietic colony formation by marrow cells from leukemic and non-leukemic patients. Exp Hemat 4:63–69, 1976.

and leukemic cells to drug, rather than the absolute sensitivity of the leukemic population alone. In addition, there is systematic variation from experiment to experiment in the plating efficiency and relative survival of cells. For these reasons, a number of investigators have tested the effects of drugs on both normal and leukemic CFU in the same experiment and a sensitivity index has been calculated. While the survival fractions of L-CFU and CFU-GM can fluctuate, they tend to fluctuate together from experiment to experiment. The ratio of these two fractions tends to remain relatively constant from experiment to experiment [37]. Interestingly, the sensitivities of non-leukemic CFU-GM to cytotoxic drugs can vary at least 10 fold from individual to individual [38] (Figure 4). Whether this affects the kinetics of repopulation of marrow with normal cells after antileukemic therapy is not known. The variation does not appear to have a technical basis because survival curves are reproducible when the same marrow specimen is tested repeatedly. It also does not seem to be related generally to the proliferative state of the cells at the time drug is added to cultures [38], although pronounced changes in sensitivity to drugs can be caused by changes in cell cycling. This is particularly true in specimens from leukemic patients receiving chemotherapy [35]. Because of variation among individuals in sensitivity of CFU-GM to drugs, marrow mononuclear cells from several normal donors are generally pooled and cryopreserved. These can be repetitively tested and serve as a normal standard in experiments carried out over a long period of time.

The simplest way to express the relative sensitivities of normal and leukemic colony forming units is:

Table 1. Comparison of 4 methods of determining the relative sensitivity of leukemic cells to drugs[a]

Patient	Complete remission	Sensitivity index[b]			
		Single drugs[c]		Mixture of drugs[d]	
		Ara-C	Anthracycline	Method A	Method B
1	Yes	1.0	3.0	2.9	3.5
2	Yes	2.8	3.4	1.7	2.5
3	Yes	0.6	0.9	5.9	6.7
4	No	0.7	0.6	0.8	0.8
5	No	0.8	1.0	0.8	1.2
6	No	1.2	1.3	1.3	0.9

[a] Data abstracted and simplified from Park CH, Amare M, Morrison FS, Maloney TR, Goodwin JW: Chemotherapy sensitivity assessment of leukemic colony-forming cells with *in vitro* simultaneous exposure to multiple drugs. Cancer Treat Rep 66:1257–1261, 1982.

[b] Sensitivity index = % survival of normal myeloid CFU/% survival of leukemic CFU.

[c] The sensitivity index of single drugs was calculated from survival of clonogenic cells following a 1 hour exposure to one drug concentration.

[d] The sensitivity index of multiple drugs (a four drug mixture) was determined by two different methods. The four drug solutions contained anthracycline, cytosine arabinoside, vincristine, and prednisone, each in a concentation that singly reduced the average survival of normal myeloid CFU to 75%. Cells were exposed to drug for 1 hour before being washed and plated. In Method A the sensitivity index was calculated from survival at one concentration of the drug mixture. In Method B, survival was also measured at 5,10 and 20 times this drug concentration. The sensitivity index was calculated from the ratio of areas under the survival curves.

$$\text{Sensitivity index (SI)} = \frac{\text{\% survival of CFU-GM}}{\text{\% survival of L-CFU}}$$

SI values > 1 indicate that L-CFU are more sensitive than normal CFU-GM, and SI values < 1 indicate that L-CFU are more resistant than normal CFU-GM. The neutral point indicating equal killing of the two types of CFU is 1.0. Examples of SI calculated in this manner are shown in Table 1. Certain statistical problems associated with this method of expressing relative sensitivities have been resolved by Park and coworkers [39]. In their method the sensitivity index is calculated as a log odds ratio,

$$\text{SI} = \ln \left\{ \frac{\dfrac{\text{\% survival of CFU-GM}}{\text{100-\% survival of CFU-GM}}}{\dfrac{\text{\% survival of L-CFU}}{\text{100-\% survival of L-CFU}}} \right.$$

where leukemic cells are more (> 0) or less (< 0) sensitive than normal cells with a neutral point of 0. With this method of calculation, the relative magnitudes of SI fall symmetrically around the neutral point and the sensitivity indices obtained at two different concentrations of a drug can be averaged. A disadvantage is the derived nature of the value, which investigators may have difficulty relating directly to the results of an experiment.

3.3. *Effects of Drugs on Capacity for Self-Renewal*

The defining property of stem cells is their capacity for self-renewal because this property provides them with the ability to originate self-maintaining clones. The capacity for self-renewal (secondary plating efficiency, PE-2) can be assayed by replating cells from primary colonies and quantitating new colony formation (Figure 1). Secondary colonies are similar to primary colonies in size, morphology, cellular markers, and culture requirements for growth [20]. There is marked variation from patient to patient in the PE-2 of L-CFU, but PE-2 is a stable characteristic of each leukemic clone [33]. In a series of 44 previously untreated patients with acute myelogenous leukemia, Buick *et al.* [21] found a highly significant correlation between a low capacity for self-renewal (PE-2) and successful remission induction. They proposed that drugs which reduce the capacity for self-renewal might play useful roles in the treatment of leukemia. Moreover, agents that inhibit self-renewal might be those that induce hematopoietic differentiation rather than kill cells directly [22]. Theoretically, measurement of the effect of a drug on PE-1 would assess its cytotoxicity to all L-CFU, whereas the effect on PE-2 would assess its ability to inhibit self-renewal, perhaps by triggering terminal differentiation of L-CFU. These concepts are complicated by the fact that primary growth is necessary before self-renewal can be measured. While the hypotheses have not been proved, they provide a provocative framework for evaluation of cancer chemotherapeutic agents.

An example of the effects of two commonly used anti-leukemic drugs is shown in Table 2. Both Adriamycin and cytosine arabinoside killed L-CFU and reduced the primary plating efficiency, PE-1, of cells from the patient. When the cells from surviving colonies were harvested and replated to assay capacity for self-renewal, PE-2, the two drugs differed. Adriamycin did not appear to affect PE-2 so that primary colonies at all levels of survival to the drug had an equal probability of self-renewal. Cells from 6 of 6 patients tested with Adriamycin confirmed this result [21]. Cytosine arabinoside did appear to affect PE-2 and to decrease self-renewal. Of 5 patients tested [21], cells from 3 had lower and 2 had unchanged PE-2 in survivors of cytosine arabinoside treatment. In another series of experiments cells from 4 of 4

Table 2. PE-1 and PE-2 of clonogenic cells treated with cytotoxic drugs in culture

Drug	Drug concentration (μg/ml)	Primary plating efficiencies PE-1 (colonies/10^5 cells)	Secondary plating efficiencies PE-2 (colonies/10^4 cells)
Adriamycin	0	193 ± 8	38 ± 3
(10 min	0.5	100 ± 5	38 ± 3
pre-exposure)	2.5	17 ± 5	32 ± 8
	0	202 ± 18	38 ± 4
Cytosine arabinoside	0.01	78 ± 9	7 ± 2
(continuous exposure)	0.1	34 ± 6	14 ± 1

Taken with simplification from Buick RN, Chang LJ-A, Messner HA, Curtis JE, McCulloch EA: Self renewal capacity of leukemic blast progenitor cells. Cancer Res 41:4849–4852, 1981.

patients treated in culture with m-AMSA had increased PE-2 in the surviving fraction [21]. Buick *et al.* [21, 22] speculate that the apparent selectivity of cytosine arabinoside in inhibiting self-renewal might explain its extraordinary effectiveness in remission induction. Within this theoretical framework, Adriamycin and m-AMSA would have their major favorable effects purely because of cytoreduction. The enhancement of PE-2 by m-AMSA would not be a desirable characteristic of an anti-leukemic drug. There have been reports that interferon inhibits self-renewal [40] and that low doses of 12-0-tetradecanoyl phorbol acetate increase self-renewal [41] of human myeloid L-CFU. Too few patients have been studied to arrive at firm conclusions about relationships between effects of drugs on PE-1 or PE-2 *in vitro* and their mechanism of action in producing clinical remission of acute leukemia. This area requires a great deal of additional research.

3.4. *What Is a 'Drug Resistant' L-CFU?*

Many dose-survival curves of the type shown in Figure 3 are not linear. Maximal inhibition of growth may occur at a low dose of drug and an increase in the dose may no longer increase cell kill in a predictable fashion. This is the type of result that would be predicted, if a large drug resistant subpopulation were present. It is important, however, to eliminate common artifactual causes of non-linear survival curves such as a non-linear relationship between the number of cells plated and the number of colonies obtained, the scoring of small clusters of cells as colonies, and the failure to disperse cells thoroughly so that clumps of cells are plated on day 0. Clusters seen after exposure to drug may represent damaged cells with very limited replicative potential or cells triggered to terminal differentiation. The term 'drug resistant' must be precisely defined in a given context. A subpopulation of L-CFUs could appear resistant to cytosine arabinoside, if it did not

enter the S-phase of the cell cycle during or within a few hours after exposure to the drug. Cytosine arabinoside only kills cells when it is converted to its triphosphate and is present during DNA synthesis. It does not remain within the cell indefinitely, but is rapidly degraded. Resistance to the antimetabolite would be a transient kinetic phenomenon and would not be heritable. This type of *in vitro* drug resistance of cells out of cycle is real, but would not predict *in vivo* drug resistance to cytosine arabinoside administered over several days. Non-linear dose-survival curves secondary to kinetic phenomena are most likely to be seen when cells are 'pulse' exposed to drug for a short time and are probably minimized in systems which permit continuous or repeated exposure to drug.

Of greater potential therapeutic import than kinetic resistance to drugs is heritable resistance caused by spontaneous genetic mutation [42]. This is the type of drug resistance seen in bacteria resistant to antibiotics and is also the type studied by mammalian somatic cell geneticists. An example is lymphoblasts resistant to 6-thioguanine (6-TG) because of genetically deficient hypoxanthine-guanine phosphoribosyl transferase (HGPRT). HGPRT is an X-linked enzyme which must be present to convert 6-TG to its metabolically active ribonucleotide. Other examples of genetic mutations causing drug resistance include loss of transport proteins necessary for drugs to enter cells, loss of kinases needed to activate drugs, and gene amplification with overproduction of enzymes inhibited by the drugs. These mutants typically are resistant to 5–10 fold higher concentrations of drug than kill their normal counterparts. Absolute drug resistance is occasionally seen. Examples of genetic drug resistance are shown in Table 3 [43]. Stably resistant clones are observed at a frequency of about 10^{-7} after a single drug exposure. Resis-

Table 3. Relative resistance of mutant mammalian cells to a variety of cytotoxic agents.

Cytotoxic agent	Relative resistance	
	Mel^R	CH^RC5
Melphalan	4	15
Chlorambucil	5	2
Nitrogen mustard	5	3
cis-DDP	2	–
Puromycin	1	167
Adriamycin	1	90
Colchicine	–	167

The Mel^R and CH^RC5 mutants of CHO cells were selected for resistance to either Melphalan or colchicine, respectively. The resistance of the wild type parent was 1.0. Data were taken with modification from Elliot EM, Ling V: Selection and characterization of Chinese hamster ovary cell mutants resistant to Melphalan. Cancer Res 41:393–400, 1981.

tance is heritable. The progeny of a drug resistant cell are also drug resistant. Cells selected for resistance to one drug may also be resistant to other drugs. For example, in Table 3 Mel^R cells selected for resistance to Melphalan are also resistant to Chlorambucil, nitrogen mustard, and cis-platinum. CH^RC5 cells selected for resistance to colchicine are also highly resistant to such apparently unrelated drugs as Adriamycin and Puromycin. The cause of multiple drug resistance is not always known, but in the case of CH^RC5 cells it is attributed to reduced drug accumulation due to a plasma membrane alteration [44]. Knowledge of patterns of multiple drug resistance would be helpful in the design of clinical trials. Limited studies of this problem have been carried out on L-CFU, *e.g.* Table 1, but much more information is needed.

Somatic mutations constantly occur in the human genome and one would expect approximately one in every million haploid cells to be genetically resistant to any given drug. The situation is complicated by the fact that humans are diploid except for the single active X-chromosome and have two copies of most genes. The average frequency of cells resistant to drugs because of mutations in homologous genes on each of a pair of chromosomes ranges from 1 in 10^{10} to 1 in 10^{12} cells. Nevertheless, patients with cancer can harbor many drug resistant mutants because one gram of tumor contains 10^9 cells and most patients harbor much more than one gram of malignant cells. Perhaps 0.1% of these would be the critically important tumor stem cells capable of repopulating the host. Because the leukemic cells in a patient are clonal and are all derived from a single transformed cell, one would actually expect major fluctuations from patient to patient in the fraction of cells that are genetically resistant [19, 42]. If these hypotheses are correct, it follows that drug resistant mutants: (a) will be present in essentially all large populations of cancer cells, (b) will vary in relative frequency from patient to patient, (c) will be rare, (d) will pass the drug resistance to their progeny, and (e) could be responsible for drug resistant disease in some patients, particularly at the time of relapse after chemotherapy.

The question of whether there is a relationship between genetic drug resistance and survival of L-CFU in chemosensitivity assays can now be examined. The critical experiment for distinguishing genetic from non-genetic drug resistance is to test whether survivors of drug exposure give rise to progeny that are also more resistant than the original population, *i.e.*, is the resistance heritable. There are inadequate data to answer this question with regard to L-CFU. It would require that L-CFU which grow unexpectedly well in the presence of drug be reassayed for drug sensitivity as they are retested for their secondary plating efficiencies, PE-2 (Figure 1). It should be pointed out that survivors of drug exposure in an experiment that gives a negative exponential as a survival curve (Figure 3) would not be

expected to be drug resistant. The kinetics of killing of a normal population generates such an exponential, so that a plot of the logarithm of cell survival against dose of drug is a straight line. Drug resistance would be expected only when a plateau is seen, where additional drug does not kill the expected proportion of cells. If the drug resistance were caused by genetic mutants, then the plateau should be seen after most L-CFU had been killed, *i.e.* at well below 1% survival because mutants do not occur frequently in most populations of cancer cells. Plateaus are rather commonly seen, typically after a small percentage of cells have been killed. In most cases these plateaus are probably technical artifacts. The frequency of their occurrence and the large percentage of resistors certainly rules out new genetic mutation as the cause of resistance. There are many drugs to which most human cancer cells are intrinsically resistant. It is not the presence of rare drug resistant mutants that causes this type of resistance, but the biochemical characteristics of the normal population.

If selection of drug resistant mutants were responsible for failure of chemotherapy, then one would expect that the chemosensitivity of L-CFU assayed at diagnosis and relapse would differ. Unfortunately, there have not been a sufficient number of systematic longitudinal studies of patients to prove or disprove this hypothesis. McCulloch *et al.* [33] measured drug sensitivity (Adriamycin and cytosine arabinoside) repeatedly during the course of therapy of 7 patients with acute myelogenous leukemia. Examples of their data are shown in Figure 3. In 5 of their 7 patients drug sensitivities appeared to be stable, whereas in 2 of the 7 drug resistance appeared to be developing. There was no close correlation with outcome of therapy. The general question of the nature of 'drug resistant' L-CFU, as defined by survival curves, remains unanswered.

4. CORRELATIONS BETWEEN IN VITRO CHEMOSENSITIVITIES OF L-CFU AND CLINICAL RESPONSE OF THE PATIENT

Any laboratory test which could reliably predict drug sensitivity or resistance in individual patients could have extremely important clinical applications. Optimal chemotherapy, including the possibility of no chemotherapy, could be chosen prospectively for each person and investigational drugs could be screened for activity without the need for extensive Phase II clinical trials. Validation of *in vitro* chemosensitivity assays as predictors of *in vivo* responses requires well designed clinical trials. At the present time the assays appear to identify patients who are likely to do well or to do poorly, but there is no prospective trial proving that: (a) the assay is better than other prognostic indicators, (b) it measures true drug resistance, or (c) it

permits better selection of drug combinations than is possible empirically. Several relevant issues will be discussed: (a) what is clinical drug resistance, (b) is drug resistance a frequent cause of treatment failure, (c) what problems occur when L-CFU assays are carried out in clinical trials, (d) what is the relationship between L-CFU growth and outcome of therapy, and (e) do dose-survival curves of L-CFU predict clinical outcome.

4.1. *Clinical Drug Resistance*

As pointed out in Section 3, there are no absolute criteria for *in vitro* sensitivity or resistance of L-CFU to drugs. Definitions must be arrived at empirically and are usually based on retrospective comparisons of *in vitro* dose-survival curves with clinical outcome. As Preisler has pointed out in several papers [18, 35, 36, 45, 46], it is extremely important to define 'clinical outcome' precisely. If it is proposed that *in vitro* dose-survival curves can identify patients who fail therapy because of drug resistant leukemic cells, then clinical criteria for identification of these patients must be specified. During induction of remission, leukemic patients may die of bleeding, infection, or organ failure quite independently of whether they have drug resistant disease. When correlating *in vitro* and *in vivo* drug resistance, it is an error to consider death or remission failure as synonymous with drug resistant disease. Practical criteria for distinguishing drug resistant disease from other kinds of treatment failure are listed in Table 4. It is important that these or similar criteria be employed in clinical evaluation of *in vitro* chemosensitivity assays.

4.2. *Drug Resistance and Treatment Failure*

If *in vitro* dose-survival curves simply served to distinguish drug resistant from drug sensitive L-CFU, then what practical impact would the assays have on the clinical management of leukemia? One approach to this question is shown in Table 5. Preisler *et al.* [47] analyzed the outcome of remission induction in 200 patients with acute myelogenous leukemia, using

Table 4. Criteria for classification of the cause of failure of remission induction in acute leukemia, as defined by Preisler (35).

Drug resistant disease	The patient survives for >7 days after the end of a course of remission induction therapy, but the marrow is still cellular with >5% leukemic cells or the leukemia recurs within 4 weeks of therapy that produced severe marrow hypocellularity.
"Other" failures	The patient expires <7 days after the end of a course of chemotherapy regardless of the characteristics of the marrow or the patient expires with a severely hypocellular marrow, regardless of the time of death.

Table 5. Overall outcome of therapy for patients with acute nonlymphocytic leukemia
Adapted from: Preisler HD and 12 others: Prediction of response of patients with acute nonlymphocytic leukemia to remission induction therapy: Use of clinical measurements. (Brit J Haemat, 1985).

Therapy	Number of patients	Outcome		
		Complete remission	Drug resistant	'Other' failure
'7+3'[a]	60	28 (47%)	14 (23%)	18 (30%)
'10+3'[b]	140	87 (62%)	13 (9%)	40 (29%)

[a] Seven day infusion of cytosine arabinoside at 100 mg/m^2/d + 3 days anthracycline antibiotic.
[b] Ten day infusion of cytosine arabinoside at 100 mg/m^2/d + 3 days anthracycline antibiotic.

two different treatment regimens. For the two regimens, 23% and 9% of the patients were classified as drug resistant, respectively, using criteria shown in Table 4. In each regimen, other causes of failure were more important than drug resistance. Improvements in supportive care might have a more immediate impact on outcome of induction chemotherapy than prospective identification of drug resistance, but drug resistance does appear to be important in 10–20% of patients. The alternate forms of antileukemic therapy that could be offered these patients are limited, but they would be reasonable candidates for trials of new forms of therapy. Interpretation of the data shown in Table 5 is more complex than discussed thus far. For example, host factors can profoundly affect metabolism of drugs used in chemotherapy [18]. Disease classified as 'drug resistant' may actually consist of drug sensitive leukemic cells in a host that rapidly metabolizes cytosine arabinoside so that adequate plasma levels are not achieved. The 'disease' in the patient is drug resistant, but the malignant cells are not.

If drug resistant genetic mutants were present in the patient, they should be rare and constitute a very small proportion of all leukemic cells. For practical reasons related to numbers of colonies per plate, dose-survival curves are set up to measure between 1% and 100% survival. Differences among patients at this level of L-CFU survival are probably secondary to multiple genetic and epigenetic factors, rather than to the presence or absence of a single kind of genetic mutant. If rare drug resistant mutants were present, they would not be detected in the assay as presently performed. Their detection depends upon demonstration of a change in slope of the dose-survival curve and this would not be expected to occur until survival was well below 1%. Because of their rarity, drug resistant mutants would be associated with relapse of disease rather than clinical induction

failure. They might be detected by comparison of L-CFU dose-survival curves at relapse with those seen before therapy. As noted previously, so few patients have had long term repeated studies of *in vitro* drug sensitivities that this hypothesis can neither be confirmed nor refuted.

Drug resistance *in vitro* and treatment failure *in vivo* are both complex phenomena. Neither is usually caused by one simple thing. Each can be empirically defined and useful correlations between the two may be found. Correlations do not establish causal relationships and cannot be precisely interpreted.

4.3. *L-CFU Assays and Clinical Trials*

Clinical trials of L-CFU assays as predictors of response to therapy are complex. Specimens of marrow or peripheral blood must be systematically collected, L-CFU must be assayed, and dose-survival curves must be constructed. Patients must receive the planned chemotherapy and clinical data must be collected. Most clinical studies have not included a large number of patients. Examples of problems encountered during one interinstitutional trial are listed in Table 6 [39]. This was an experienced group of investigators and represents the best results that might be expected in an interinstitutional setting at the present time. Of 108 specimens of leukemic cells which arrived in the laboratory, only 53 (49%) grew and had sufficient cells for some chemosensitivity testing. This is similar to the proportion (58%) of human solid tumors that grow with a plating efficiency sufficient for assessment of drug activity [10, 11]. Both complete chemosensitivity testing *in vitro* and administration of planned chemotherapy *in vivo* were accomplished in 28 (26%) of the patients. The high percentage of patients with data that cannot be analyzed complicates statistical evaluation of this type

Table 6. Problems with *in vitro* chemosensitivity studies on leukemic clonogenic cells, the experience of one established laboratory (39)

Total number of specimens	108	(100%)
Specimens grew >10 colonies per plate *in vitro*	73	(68%)
Specimens grew and had sufficient cells for some chemosensitivity testing	53	(49%)
Specimens grew, but there was microbial contamination of cultures, technical problems with the assay, inadequate clinical data, or planned therapy was not given	25	(23%)
Specimens grew, chemosensitivity testing was complete, planned chemotherapy was given, *in vitro/in vivo* correlation could be made	28	(26%)

Plates were fed daily and were counted after 2 to 3 weeks. To be scored, a colony was required to contain 50 or more cells.

of trial. In addition, poor growth of cells with present techniques would prevent adequate chemosensitivity testing of most patients, even if the assay were validated as a predictor of therapeutic response.

4.4. *L-CFU Growth In Vitro and Outcome of Therapy*

Evidence discussed in Section 2.2 supports the theory that myeloid L-CFU which produce different sizes of colonies are biologically different (Figure 2). Under these circumstances interpretation of 'no growth' of a clinical specimen becomes complex. Does 'no growth' occur for technical reasons related to quality of the laboratory assay or is growth pattern an intrinsic, meaningful biological characteristic of L-CFU cultured under specific conditions? Biological significance has been suspected both for theoretical reasons outlined in Figure 2 and because of correlations between growth pattern and the results of antileukemic therapy [3, 46, 48, 49]. For example, Preisler *et al.* [46] cultured bone marrow cells from 166 patients with acute myelogenous leukemia. Cells were grown in soft agar. Giant cell tumor conditioned medium served as the source of colony stimulating activity. Plates were counted after 7 days and clones were grouped according to whether they contained 4–10 cells, 11–20 cells, 21–40 cells, or greater than 40 cells. The number and size of clones differed among patients, but was not related to the following: FAB type of leukemia, age of the patient, cytogenetic or cell cycle characteristics of the leukemic bone marrow cells, and, finally, whether the patient was studied at the time of initial diagnosis or at relapse. Higher cloning efficiencies were correlated with a lower chance of attaining a complete remission, although differences were not quite significant statistically. For example, the complete remission rate for patients whose leukemic cells failed to grow *in vitro* was 68%, while it was 44% for those growing >100 clones/10^5 plated ($p = 0.078$). There was no general relationship between plating efficiency *in vitro* (PE-1 in Figure 1) and remission duration except that patients whose cells failed to grow at all (PE-1 $= 0$) had a median duration of complete remission of 93 weeks versus 47 weeks, if they grew at least one cluster-colony per 10^5 cells plated ($p = 0.07$). The remarkable finding was the highly significant relationship between the maximum number of cells per clone and the median duration of complete remission (Figure 5). The median durations of remission were progressively shorter with median values of 34, 27, and 27 weeks for patients whose marrow cells produced at least one clone consisting of $\geqslant 10$ cells, $\geqslant 20$ cells, or $\geqslant 40$ cells respectively. When compared to the median remission duration of patients whose cells failed to grow (93 weeks), these differences in remission duration were significant with p values of 0.04, 0.02, and 0.05, respectively. The number of cells per clone is a reflection of the proliferative potential of the clonogenic cells and, in Preisler's study, was a prognostic

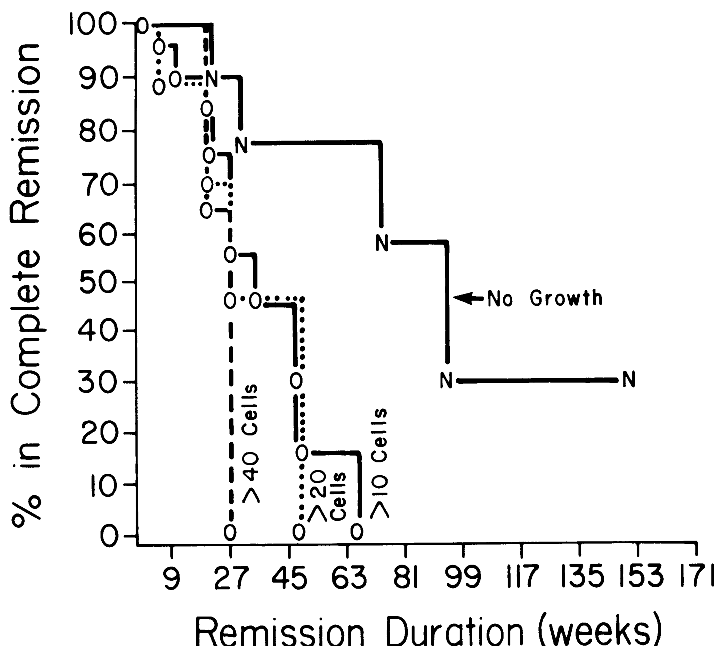

Figure 5. Life table comparison of remission duration for patients with acute myelogenous leukemia whose leukemic cells produced at least one clone consisting of ⩾10 cells, ⩾20 cells or ⩾40 cells with patients whose marrow cells did not clone *in vitro.* The remission durations for patients whose cells failed to clone *in vitro* are statistically significantly different from patients whose cells produced at least one clone consisting of ⩾10 cells, ⩾20 cells, ⩾40 cells with p values of 0.04, 0.02, and 0.05, respectively. Taken with permission from: Preisler HD, Azarnia N, Marinello MJ: Growth of leukemic cells *in vitro*: Relationship to patient and leukemic cell characteristics and to outcome of therapy. (Cancer Res 44:1712–1717, 1984).

indicator unrelated to commonly measured leukemic cell characteristics (FAB type, labelling index, cytogenetics) or to other prognostic indicators such as age and past history of chemotherapy. These observations are in agreement with those of Francis [49] who found that patients whose L-CFU formed large colonies and were least sensitive to colony stimulating activity were most refractory to chemotherapy.

Whether cells grow and the size of colonies do not necessarily reflect the technical care with which experiments are done or the quality of reagents, but may be intrinsic biological properties of the L-CFU. In view of these results, the problems shown in Table 6 should be reconsidered. The fact that colonies could not be grown from a significant number of patients may reflect characteristics of the L-CFU and not defects in a particular experiment. If the L-CFU have limited replicative potential, then modification of the conditions of growth are not likely to improve the results, *i.e.* it is not reasonable to expect to find conditions under which all patients will grow

colonies containing large numbers of cells. This implies that it is unreasonable to expect to perform *in vitro* chemosensitivity tests on all leukemic patients, given present knowledge of growth factors and conditions. It is also possible that colony-forming L-CFU are present in specimens showing 'no growth', but are rare and are not detected because of the limited number of cells that can be plated. Development of methods of isolating cell subpopulations might permit their assay.

Data shown in Figure 5 emphasize once again the importance of defining the number of cells necessary to form a colony before reporting and analyzing dose-survival curves of myeloid L-CFU. Small and large colonies on a growth plate may not be descendants of the same type of L-CFU (Figure 2). The chemosensitivities and other properties of the L-CFU may differ and may have a different impact on prognosis. A variety of sizes of colonies may be present on the same assay plate and size may be distributed continuously so that discrete subpopulations cannot be identified. Establishing size criteria becomes arbitrary, but failure to do so can create serious artifacts in dose-survival curves if cytotoxic drugs damage cells but permit a limited number of divisions. The data could also be misinterpreted if a drug stimulated terminal differentiation. Small colonies might be composed of differentiated cells, rather than blasts that could not replicate because of damage to their DNA. A distinction between stimulation of differentiation and inhibition of replication can be made by morphological evaluation of cells within clusters and colonies on plates with and without drug.

Mackillop *et al.* [19] have outlined a method of analyzing colony sizes to establish a cut-off point between clones derived from stem cells and those derived from transitional cells. Transitional cells have limited replicative potential, whereas a stem cell has the ability to break the constraints of the maximum number of divisions. Stem cells give rise to more new stem cells by self renewal and if cultures are incubated sufficiently long to permit many cell divisions, one would expect to see large colonies that clearly fall outside the size distribution characteristic of other clones. A cut off point can then be established between colonies from transitional and stem cells. In practice this approach has been hindered by the inability to grow L-CFU for long periods of time. Colonies usually begin to die after 7–14 days in culture. Park's [29] technique of refeeding cultures sustains longer periods of growth and might permit this type of analysis. Of final note is the fact that these types of analyses have only been carried out on myeloid L-CFU and myeloid leukemias. Lymphoid precursors may behave quite differently. Terminal differentiation and limited replicative potential of transitional cells may not be characteristic of normal and malignant lymphoid precursors.

4.5. *Chemosensitivities of L-CFU In Vitro and Outcome of Therapy*

There have been numerous reports of *in vitro* chemosensitivities of myeloid L-CFU [12, 21, 33, 35–41, 50–59] and a few reports of chemosensitivities of lymphoid L-CFU [31, 60, 61). In some publications there is good evidence that the colony forming units are derived from malignant cells, and are in fact L-CFU, but in others it is not clear whether normal or malignant CFU are being grown. Similarly, there is frequent failure to specify the size of the clusters or colonies of cells being counted in the presence and absence of drug. Changes in the size of colonies can be as important as changes in number. Clinical data may be inadequate because drug resistance is not distinguished from other causes of treatment failure. These deficiencies make it difficult to assess correlations. Under the best of circumstances chemosensitivity of L-CFU can be measured and clinical correlations made only on the subgroup of patients whose L-CFU grow well *in vitro.* As indicated in Section 4.4, patients with this type of L-CFU do not behave as if they were randomly selected from all leukemic patients, but represent a subpopulation of patients with a poor prognosis. The better the L-CFU grow, the larger the colonies, and the more likely the patient's disease is to be drug resistant with early relapse after therapy. This correlation of colony size with drug resistance seems to hold for commonly used induction regimens employing cytosine arabinoside and an anthracycline, sometimes in combination with other agents [46, 49]. The patient's disease is really resistant to multiple drugs. 'Drug resistance' is not likely to reflect the presence

Table 7. Comparison of the sensitivity of leukemic clonogenic cells to drugs *in vitro* with the clinical response of the patient to induction chemotherapy with the same drugs.

a) The leukemia was considered sensitive (S) to drugs *in vitro* if the clonogenic cells were killed more readily by either or both of the two drugs (cytosine arabinoside and an anthracycline) than normal marrow clonogenic cells. Otherwise, the leukemia was considered resistant (R). Clinically, the leukemia was considered sensitive if a complete remission was achieved, otherwise the disease was considered resistant regardless of the cause of treatment failure

Method of drug exposure	Number of correlations	In vitro/clinical sensitivity				Overall concordance
		S/S	S/R	R/S	R/R	
SIC[a]	21	8	3	3	7	15/21 (71%)
SIP[b]	22	7	5	2	8	15/22 (68%)

[a] SIC = Sensitivity index assessed by comparison of killing of normal myeloid versus leukemic clonogenic cells, when cells were continuously exposed to drug throughout the entire period in culture.

[b] SIP = Sensitivity index assessed by comparison of killing of normal myeloid versus leukemic clonogenic cells, when cells were pulse exposed to drug for 1 hour in liquid culture before being washed and plated in agar without drug.

b) The effects of modifying criteria for *in vitro* sensitivity or resistance are shown.

Method of drug exposure	*In vitro*/clinical sensitivity			
	Gray zone excluded [a]	Mixed results excluded [b]	Ara-C only [c]	Anthracycline only [c]
SIC	12/13 (92%)	12/15 (80%)	15/21 (71%)	15/20 (75%)
SIP	13/16 (81%)	9/14 (64%)	12/21 (56%)	14/20 (70%)

[a] Establishing more rigid criteria of *in vitro* sensitivity (gray zone excluded) improves correlations, but excludes 8 of 23 patients whose sensitivities fall in the gray zone. Mathematically, the gray zone was defined as a sensitivity index between -1.0 and $+1.0$ when calculated as a log odds ratio (Section 3.2).

[b] Includes only those cases in which the sensitivity index for 2 drugs were both positive or both negative.

[c] Sensitivity or resistance was determined by response to one drug only, either cytosine arabinoside or an anthracycline.

Data were abstracted with permission from: Park CH, Wiernik PH, Morrison FS, Amare M, Van Sloten K, Maloney TR: Clinical correlation of leukemic clonogenic cell chemosensitivity assessed by *in vitro* continuous exposure to drugs. Cancer Res 43:2346–2349, 1983.

of genetic drug resistant mutants. Clinical correlations of *in vitro* chemosensitivities with outcome of therapy should always be evaluated with these comments in mind. This is particularly true when asking how much the outcome of therapy is improved by having performed *in vitro* chemosensitivity tests in addition to other measurements. Merely observing patterns of growth of L-CFU gives the information shown in Figure 5 with regard to induction therapy with cytosine arabinoside and an anthracycline. Because this is the best available standard therapy, the question to ask is whether chemosensitivity assays can help select more effective non-standard chemotherapy. This important question has not yet been addressed in a prospective fashion.

An example of a recent, well performed, study [39] of *in vitro* chemosensitivity assays and their clinical correlates is given in Table 7. The purpose of the study was to compare prolonged, as opposed to short pulse, *in vitro* exposure of L-CFU to drugs as predictors of clinical response. Clinical response was defined by whether the patient achieved a complete remission, so that drug resistance was not distinguished from other causes of treatment failure. Correlations with median duration of complete remission were not sought. The 21 patients in the study all had newly diagnosed acute myelogenous leukemia and all were treated with intensive combination chemotherapy that included cytosine arabinoside and an anthracycline. When L-CFU were exposed continuously to drugs throughout the entire culture period, 8 of 11 patients whose cells were sensitive to drugs *in vitro* achieved

a complete remission. Of 10 whose cells were resistant *in vitro*, 3 achieved a complete remission and 7 did not. The overall concordance between the *in vitro* and clinical results was 15/21 or 71%. When L-CFU were pulse exposed for 1 hour to drugs and were then plated in media without drugs, the concordance was 13/22 or 68%. Overall, there was no significant difference between pulse and continuous exposure as predictors of outcome. In each method both false positive and false negative results were seen. The concordance could be improved (Table 7b) by increasing the stringency of criteria for *in vitro* sensitivity and resistance. However, this excluded approximately 30% of the patients whose sensitivity indices fell in the 'gray zone'. Whether the concordance would improve if more stringent clinical criteria for drug resistant disease were employed (Table 4) is not known. This would eliminate from analysis those patients who died of causes other than drug resistance. It is interesting that defining *in vitro* chemosensitivity by the sensitivity index of cytosine arabinoside alone, anthracycline alone, or both together all gave similar concordance with clinical outcome. If the *in vitro* assay were a true measure of chemosensitivity of L-CFU and if drug resistance were an important cause of treatment failure, then one would have expected concordance in the sensitivity indices to two drugs to have been more predictive of the outcome of combination chemotherapy than the sensitivity index to one drug alone. Major modifications in the conditions of drug exposure, *i.e.* pulse versus continuous exposure, and major changes in drug chosen for test, *i.e.* sensitivity to cytosine arabinoside or to anthracycline or to both, did not greatly alter the correlations seen. It seems unlikely that continued tinkering with the details of *in vitro* chemosensitivity assays, as presently performed, will greatly modify the strength of *in vitro* - *in vivo* correlations. What remains to be seen is whether the assays can predict in a prospective fashion whether new drugs and new combinations will be effective in individual patients, and do this better than physicians who select drugs on the basis of more readily available clinical and laboratory criteria.

Additional very convincing evidence for the relative weakness of *in vitro* chemosensitivity assays as a predictor of outcome of antileukemic therapy comes from the work of McCulloch *et al.* [54]. They determined the sensitivities of L-CFU to cytosine arabinoside and Adriamycin and their capacity for self-renewal (PE-2 of Figure 1). These properties together with clinical risk factors were tested as attributes contributing to the variation in remission induction and survival of 56 patients with acute myelogenous leukemia (Table 8). As univariate parameters, self-renewal, age, and the percentage of marrow blasts contributed significantly to both remission induction and survival. Sensitivity to cytosine arabinoside was predictive of remission induction, although the strength of the statistical association as

Table 8. Univariate contribution of laboratory and clinical attributes to remission and survival outcomes. Modified with permission from: McCulloch EA, Curtis JE, Messner HA, Senn JS, Germanson TP: The contribution of blast cell properties to outcome variation in acute myeloblastic leukemia (AML). Blood 59:601–608, 1982

Attribute	n	Remission *		Survival †	
		X^2	p	X^2	p
PE ‡	36	7.4	0.007	22.8	0.000002
D_{10}Ara ‡‡	34	5.2	0.02	0.1	0.71
D_{10}Adria ‡‡	29	0.2	0.65	0.4	0.50
Age	37	6.9	0.009	8.7	0.003
Marrow blasts	37	9.8	0.002	7.9	0.005

* Logistic regression.

† Cox regression.

‡ Capacity for self renewal as measured by replating cells from pooled colonies.

‡‡ Sensitivity to cytosine arabinoside was measured by continuous drug exposure; sensitivity to Adriamycin by 1 hour pulse exposure. Dose response curves were simple negative exponentials from which the amount of drug required to reduce survival to 10% (D_{10}) was calculated.

measured by Chi-square was less than for the other parameters, *i.e.* self-renewal, age, and marrow blasts. Sensitivity to Adriamycin did not contribute to remission induction, and neither sensitivity to cytosine arabinoside nor to Adriamycin contributed to survival. In a multivariate analysis, self-renewal, age and percentage of blasts in the marrow contributed to outcome variation, whereas drug sensitivities were not significant. While the data shown in Tables 6 and 7 were obtained with different types of *in vitro* assay systems, there is no evidence that one method is superior to the other, and, in particular, both confirm the basic weakness of *in vitro* chemosensitivity assays as predictors of patients' responses to antileukemic therapy. Perhaps the capacity for self-renewal rather than drug sensitivity is the predominant determinant of remission induction and survival in acute myelogenous leukemia [22], given present regimens of chemotherapy that are heavily dependent on cytosine arabinoside and anthracycline. This need not be true for all drugs and all combinations. Of more significance is the fact that the capacity for self-renewal, PE-2, can be measured and the effects of drugs on PE-2 can be assessed (Table 2). Unfortunately, measurement of PE-2 is extremely laborious, but it should be possible to mount limited clinical trials testing whether individual variation in response of PE-2 to drugs is correlated with outcome of therapy and whether new drugs affecting PE-2 have the expected beneficial or deleterious effects, when administered to patients with leukemia.

5. SUMMARY

Both myeloid and lymphoid leukemic colony forming units can be grown in culture. It is not known whether some or all classes of L-CFU faithfully mimic *in vitro* the biological properties of leukemic stem cells in the patient. Drugs can have at least 3 effects on L-CFU and each can be measured: direct killing, triggering of differentiation, and changing of potential for self-renewal. Most investigators have studied cytotoxicity, although the other two effects may be at least as relevant to therapy of acute leukemia. For acute myelogenous leukemia, the growth properties of L-CFU are more closely correlated with outcome of therapy than their *in vitro* sensitivities to drugs. These relationships are not known for lymphoid leukemias. There has not been a prospective clinical trial that establishes the value of *in vitro* chemosensitivity assays in the selection of appropriate therapy for leukemia. The fundamental biological reasons for success or failure of treatment of acute leukemia are not understood, so it is difficult to know the questions to ask of an *in vitro* assay designed to improve chemotherapy of the disease. Future research should focus on the basic biology of normal and leukemic stem cells and on the causes of success or failure of therapy of leukemia. Additional retrospective correlations of clinical outcome with *in vitro* chemosensitivities, as presently measured, are not likely to contribute significant new information.

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7. Post-Remission Therapy for Acute Myelogenous Leukemia

WILLIAM R. VOGLER

1. INTRODUCTION

Over the past 25 years considerable progress has been made in the treatment of acute myelogenous leukemia (AML). Multiple factors have contributed to this success. These include the introduction of new drugs, the use of combination chemotherapy, a better understanding of the biology of the disease and bone marrow regeneration, improved supportive care including platelet and granulocyte transfusions and more effective antibiotics. As a result, complete remission rates of 80% have been obtained in some subsets of patients.

Despite these encouraging results, the fact remains that the majority of patients relapse and eventually succumb to their illness. Thus, the emphasis on treatment has shifted to developing better methods of maintaining remissions. This chapter will review the past and current studies addressing this issue, attempt to determine what factors are predictive for long term remissions and discuss the strategies designed to achieve this goal.

2. ROLE OF INDUCTION THERAPY ON REMISSION DURATION

The criteria for therapeutic response established by the Cancer and Leukemia Group B have generally been accepted by the medical community [1]. A complete remission (CR) is defined as less than 5% blasts and less than 10% blasts and promyelocytes in a normally cellular marrow, normal blood counts and absence of findings of leukemia on physical examination. Over the past two decades, the percentage of patients achieving CR has increased dramatically. Earlier studies using single agents such as 6-mercaptopurine and methotrexate produced remissions in less than 10% of the patients [2–4]. Combination chemotherapy programs using 6-mercaptopu-

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rine, methotrexate, prednisone and vincristine increased remission rates to 20% to 30% [5–7]. Prior to 1966, the median survival from diagnosis in adult patients admitted to the National Cancer Institute was 5 to 6 months. However, those achieving a complete remission survived a median of 10.5 months whereas non-responders survived 3.5 months [8]. Thus, it has been clear for sometime that remission prolongs survival.

The introduction of cytosine arabinoside resulted in remission rates similar to those achieved with the above combination chemotherapy [1, 9–13]. Even better rates were reported using the anthracycline, daunorubicin, with some schedules giving remissions in 43 to 56% of patients [6, 14, 15]. The combination of cytosine arabinoside and daunorubicin or adriamycin now appears to be the most effective combination giving remission rates of 50 to 67% among various studies reported [16–21]. Except in isolated series the addition of other drugs such as thioguanine, prednisone and vincristine has not significantly increased the remission rate [22–28].

Current thinking is that more intensive therapy early after diagnosis offers the best chance of obtaining a prolonged remission. To address the issue of early intensive therapy, examination of the evidence supporting this concept is in order. The first question to address is whether the drugs or doses of drugs used to obtain remission are important in prolonging remissions. With the supportive care now available, patients can survive prolonged periods of marrow aplasia, although it is obviously desirable to make this time as short as possible. The Southwestern Oncology Group demonstrated that cytosine arabinoside was more effective as a single agent in inducing remissions when infused over 120 hours than when infused over 48 hours and that the CR rate also correlated with the total dose of drug given [29]. However, the duration of CR between the two groups was not significantly different even though the same programs were continued as maintenance therapy. The Cancer and Leukemia Group B compared a 5 day course of therapy consisting of either a continuous infusion of cytosine arabinoside at 100 mg/M^2/day or bolus intravenous injections at 100 mg/M^2 every 12 hours and daunorubicin 45 mg/M^2 for 2 days with a 7 day course in which the cytosine arabinoside was given as above for 7 days and the daunorubicin for 3 days [18]. The 7 day program gave significantly better remission rates, but the duration of remission was influenced more by the maintenance treatment than by the induction arm. Thus, the duration of induction therapy can increase remission rates. However, once a remission is obtained, is there any evidence that the intensity of induction prolongs the duration of remission? The Southeastern Cancer Study Group compared 5 day courses of cytosine arabinoside and thioguanine given every 12 hours at a dose of 100 mg/M^2 each plus daunorubicin, 10 mg/M^2 daily for 5 days (TAD) to a 5 day continuous infusion of cytosine arabinoside at 100 mg/M^2 daily plus

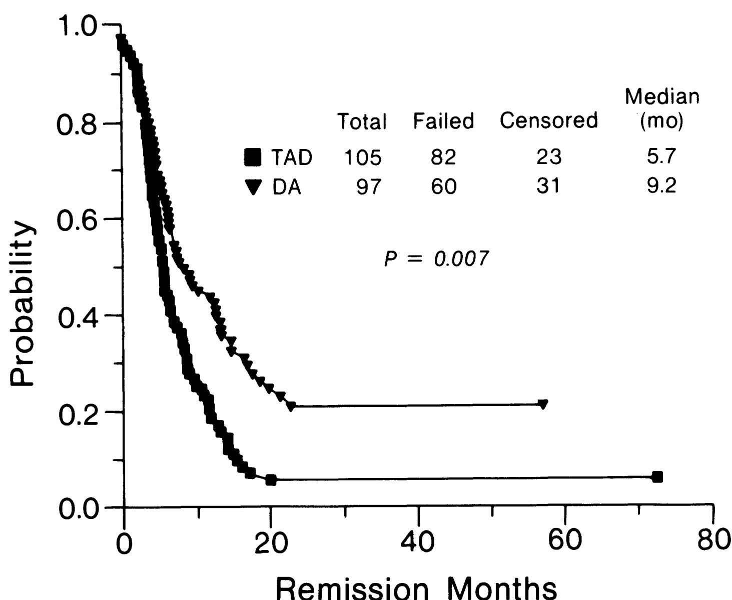

Figure 1. The duration of complete remission in AML patients induced with TAD or DA (see text). Southeastern Cancer Study Group Protocol 354R.

daunorubicin, 45 mg/M^2 daily $\times$ 2 (DA). All CR's were consolidated with 3 courses of a reduced TAD (cytosine arabinoside and thioguanine given every 24 hours) and then randomized to monthly chemotherapy consisting of bis-chlor-ethyl-nitrosourea (BCNU) and cytosine arabinoside, BCG or no treatment. The results were published by Omura *et al.* [21]. Although the remission rates were the same, 50% with TAD and 52% with DA, those patients receiving DA for induction had a significantly longer remission (Figure 1) regardless of the maintenance arm. The number of long term survivors was almost twice as high on the DA arm. This suggests that the increased dose of daunorubicin or the method of administration of cytosine arabinoside during induction increases the remission duration regardless of subsequent treatment. The Cancer and Leukemia Group B study mentioned above [18] observed longer remissions in the group given continuous infusion of cytosine arabinoside for induction followed by a maintenance program in which the drug was given by intermittent subcutaneous injection. In their best arm they had a median duration of remission of 25 months and survival of 35 months. They did not separate the 5 and 7 day induction programs regarding remission duration.

Yates *et al.* [19] reported the experience of Cancer and Leukemia Group B comparing 30 versus 45 mg/M^2 of daunorubicin daily $\times$ 3 in patients receiving continuous 7 day infusion of cytosine arabinoside at doses of

100 mg/M^2 and found no difference in remission rates overall and no difference in duration of remission and survival.

The median remission duration from a series of publications with combination programs in which cytosine arabinoside was given every 12 hours for 5 to 7 days was 11.2 months [18, 21–23, 26–28, 30, 31] and when given by continuous infusion for the same time period usually at half the dose was 14.5 months [11, 18, 21, 25, 32, 33]. Attempting to correlate the dose of anthracycline given during induction with the duration of remission in these series yielded a correlation coefficient of .381. From these above observations, one can conclude that the induction regimen doses have an effect on remission duration. The magnitude of this effect is clouded by the variety of consolidation and maintenance programs used in addition to variations in induction schedules. Whether more intensive induction therapy such as a 10 day infusion of cytosine arabinoside as done by Preisler *et al.* [17] or a 7 day infusion of cytosine arabinoside at a dose of 200 mg/M^2/day as reported by Boiron *et al.* [34], both in small groups of patients, will be more effective is unknown. The Southeastern Cancer Study Group is testing the 10 day schedule currently.

Several studies have added prednisone, vincristine and thioguanine to the induction programs [24–26, 28, 32]. No direct comparisons have been made, but the duration of remission in those induced with a 5 drug combination ranged from 9.1 to 24 months with a median of 15 months. These results are similar to programs using only cytosine arabinoside and an anthracycline in combination. Thus, there is no evidence that remission duration is prolonged by adding additional drugs.

3. ROLE OF CONSOLIDATION THERAPY ON REMISSION DURATION

Remission durations following induction therapy were noted to be short when no further treatment was given [6]. Most early studies followed induction therapy with a maintenance program consisting of either reduced doses, frequency or duration of treatment. Most remissions were relatively short ranging from 3 to 9 months [35–37]. It became evident that more needed to be done and the concept of treating with more intensive therapy during remissions developed. Some studies have called this consolidation and others have simply included this as a maintenance program. Regardless of the terminology the concept is the same. As shown in Table 1 in 5 reports which included a consolidation program followed by a maintenance program, the median duration of remission varied from 9 to 15 months and in 2 reports [31, 33], in which patients were given an intensive maintenance program, remission durations were similar, 12 and 17 months. These remis-

Table 1.

Induction	Consolidation	Maintenance	Number/ Pts	% CR	Remission/ Duration	Survival	Ref.
1. TAD	TAD	Cx, AdT, A,V or TAD	108	55	11.2	17.5	23
2. TAD	TAD	Cx, CCNU	20	85	11.2	16.3	21
3. TADVP	TADVP	TAVP or TADP	139	60	9.0	14.9	26
4. TAD	TAD	TAD or TAD+I	68	82	13.0	21.0	27
5. TADVP	TADVP	TA	65	57	15.0	–	25
6. TA, DP, DA*	–	TA	45	58	17.0	26.5	33
7. TAAd	–	TAAd**	91	54	12.0	–	31

* Various induction programs used in 26 patients.
** Total of 6 cycles including induction given every 3-4 weeks.
Abbreviations. A = cytosine arabinoside, D = daunorubicin, Ad = adriamycin, P = prednisone, T = thioguanine, Cx = cyclophosphamide, CCNU = 1-(2-chlorethyl)-3-cyclohexyl-1-nitrosourea, V = vincristine, I = immunotherapy (c. parvum).

sions appear to be longer than those earlier studies mentioned above.

Although one may argue about the semantics of consolidation the term is useful in experimental design for addressing specific issues. For instance, is consolidation with different drugs than those used for induction better than continuing with the same agents? The Southeastern Cancer Study Group addressed that issue in an early study in which patients were randomly assigned to continue induction treatment at a reduced dose or receive three different drugs [38, 39]. Both groups were given 6 courses of consolidation before being randomized to maintenance. Although there was no difference in the percentage remaining in remission after completion of the consolidation program 57% versus 61%, the median duration of remission was suggestively longer in the group receiving different drug (10.1 versus 8.5 months). In a more recent study [40] all patients were given a 7 day infusion of cytosine arabinoside at 100 mg/M^2 and daunorubicin 45 mg/M^2 daily for the first 3 days. Those achieving a complete remission were randomized to receive either a 5 day infusion of 5-azacytidine at 150 mg/M^2/day, a combination of 5-azacytidine at the same dose plus betadeoxythioguanosine at 300 mg/M^2 daily, or a combination of cytosine arabinoside (100 mg/M^2 IV every 12 hours for 5 days) and 6-thioguanine orally every 12 hours for 5 days), and daunorubicin (10 mg/M^2 daily for 5 days). Three courses of consolidation were given. Those remaining in remission were then randomized to receive either a continuous infusion of cytosine arabinoside (100 mg/M^2/day for 5 days) plus daunorubicin (45 mg/M^2 daily for 2 days), every 13 weeks for 4 courses, or monthly BCG innoculations following a twice weekly immunization program, or the combination of chemotherapy

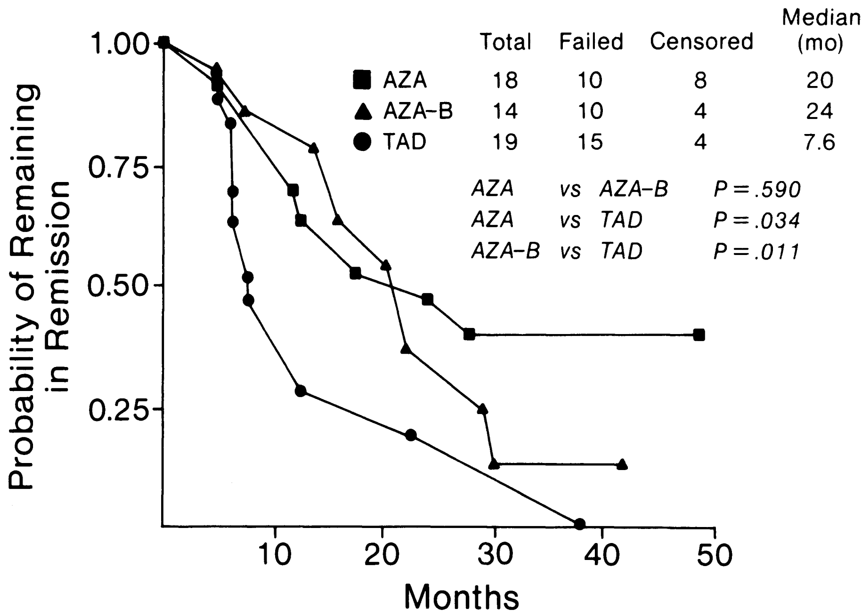

Figure 2. Duration of remission by consolidation arms. All received the same induction and maintenance treatment. (■—■) azacytidine, (▲—▲) betadeoxythioguanosine and azacytidine, (●—●) thioguanine, cytosine arabinoside and daunorubicin.

and immunotherapy. Although there were no differences in those remaining in remission through 3 consolidation courses, those receiving azacytidine or azacytidine and betadeoxythioguanosine followed by maintenance chemotherapy had longer remission durations than those receiving the same drugs during consolidation. This is illustrated in Figure 2. These observations suggest that switching drugs during consolidation either delays the development of drug resistance or else kills cells resistant to the induction drugs *de novo* and is supportive of the Goldie-Coldman hypothesis [41, 42]. This is being tested further in the current Southeastern Cancer Study Group protocol in which patients are randomized following induction therapy to consolidation with the same drugs or alternate drugs.

4. ROLE OF MAINTENANCE CHEMOTHERAPY IN PROLONGING REMISSIONS

Few randomized trials have been done assessing the role of maintenance therapy. Embury *et al.* [43] randomized 26 patients following induction and four consolidation courses to no treatment or continuing monthly consolidation courses. The median duration of remission of the treatment arm was

10.3 months which was significantly better than the 6.7 months in the no treatment arm. However, Omura *et al.* [21] randomized 97 patients who were in remission following 3 consolidation courses to no treatment, immunotherapy (BCG) or chemotherapy (monthly BCNU and weekly cytosine arabinoside) and found no differences in remission duration, medians of 6, 7 and 8 months, respectively. However, the chemotherapy program was not intensive by recent standards. In a concomitant study of 31 patients studied at 2 Southeastern Cancer Study Group institutions in which patients were given the same induction and consolidation program as reported in Omura's study, a more intensive chemotherapy arm (monthly BCNU and 5 day courses of cytosine arabinoside) plus immunotherapy (BCG with or without allogeneic leukemic cells) gave a median remission duration of 10.1 months [44]. These data support the view that maintenance therapy prolongs remission duration.

The optimal schedule for maintaining remissions which is compatible with a reasonable quality of life for the patient is unknown. Yates *et al.* [19] randomized patients to a cyclic maintenance program given either every 4 weeks or every 8 weeks and found no difference in remission duration (median 12 months). There are several reports of prolonged remissions but few randomized trials comparing various maintenance programs. Peterson *et al.* [33] reported a median remission duration of 17 months in 26 patients maintained on 6-thioguanine given at a dose of 2 mg/kg on each of 4 successive days followed on the fifth day by intramuscular cytosine arabinoside, 1.5 mg/kg. These courses were administered every week until relapse. Armitage *et al.* [28] reported a median remission duration of 15 months in 37 patients treated with monthly 5 day courses of 6-thioguanine and cytosine arabinoside given every 12 hours. Rai *et al.* [18] reported a median duration of remission of 22 months in patients given monthly cyclical courses of chemotherapy. All had received a 7 day continuous infusion of cytosine arabinoside plus daunorubicin for induction and 5 day courses of subcutaneous cytosine arabinoside given every 12 hours alternating with thioguanine, cyclophosphamide, CCNU, or daunorubicin. When comparing the intravenous route to subcutaneous route of cytosine arabinoside administration during maintenance regardless of the induction program significantly longer remissions (18 versus 8 months) and more hematologic toxicity was observed by the subcutaneous route. This study which involved 125 patients in remission clearly indicates that the more hematologic toxicity noted during maintenance the longer the remission duration.

Recently, more intensive maintenance therapy has been explored. Weinstein *et al.* [25] gave 4 sequential maintenance programs four times to a series of 58 younger patients with acute myeloblastic leukemia for 14 to 15 months and projected a median remission duration of 23 months. Oblon

et al. [45] reported a pilot study in which patients less than 60 years of age received 4 intensive courses of chemotherapy consisting of 10 day infusions of cytosine arabinoside at 200 mg/M^2 plus 3 days of daunorubicin at 40 mg/M^2 for induction (days 1–3), followed by a 7 day infusion of cytosine arabinoside at 200 mg/M^2 plus daunorubicin at 40 mg/M^2 (days 1–3), followed by a 7 day infusion of 5-azacytidine at 100 mg/M^2, plus daunorubicin at 40 mg/M^2 daily for 3 days and 6-thioguanine 100 mg/M^2 every 12 hours for 14 days and finally another seven day infusion of cytosine arabinoside and 3 days of daunorubicin. Even though not all 9 remissions received all 4 courses of treatment, prolonged remissions were observed, the median being 19 months. Obviously, more information is needed to confirm these observations, but the approach of intensive treatment early in the disease is promising. However, this is not without risk of death during the periods of marrow aplasia. The Southeastern Cancer Study Group is investigating this approach currently.

5. THE ROLE OF IMMUNOTHERAPY IN MAINTENANCE

Over the past decade numerous investigations have been conducted to define the role of immunotherapy in the treatment of acute myelogenous leukemia. These studies were initiated as a result of the report of Mathé *et al.* [46] in which vaccinations with BCG or allogeneic leukemia cells significantly prolonged remissions in acute lymphoblastic leukemia. Early randomized studies in acute myelogenous leukemia, in which BCG was combined with chemotherapy, did show a modest but not always statistically significant effect in prolonging remissions [39, 47–50]. The results of randomized trials have been summarized recently and one can conclude that the effect is only marginal [51]. Negative results in randomized studies have been reported [52, 53]. Comparing immunotherapy alone to no maintenance therapy gave varying results. Omura *et al.* [21] found no difference in remission duration between no treatment and immunotherapy. Zuhrie *et al.* [54] reported an increase in remission duration to 8.3 from 4.6 months in 41 patients randomized to receive BCG plus allogeneic leukemic cells or no therapy. In a randomized study [55] Lister *et al.* failed to show any advantage of adding allogeneic leukemic cells to BCG treatment.

The Southeastern Cancer Study Group has recently updated the results of a randomized study mentioned above [40] in which patients were given either intermittent courses of intensive chemotherapy (5 day infusions of cytosine arabinoside, 100 mg/M^2 plus daunorubicin, 45 mg/M^2 on two consecutive days) every 13 weeks for 4 courses, or BCG monthly after a 4 or 8 week course of twice weekly immunizations, or the combination of chemo-

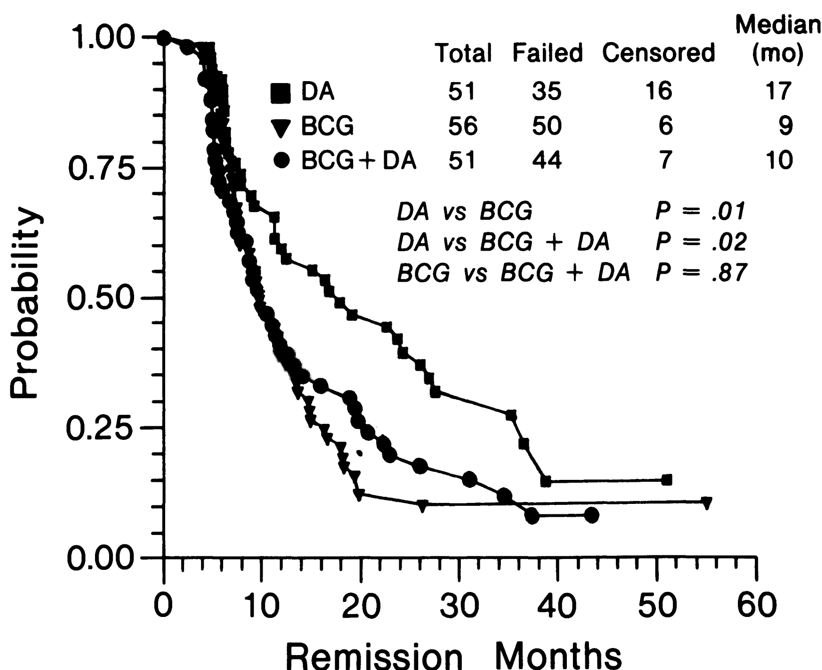

Figure 3. The effect of 3 maintenance arms on remission duration (see text)..

therapy and BCG [56]. All maintenance treatment arms were stopped after 1 year. Figure 3 shows the remission duration curves for the 158 patients randomized to the various maintenance arms. The chemotherapy only arm was significantly better than the other 2 arms. Although it might appear that BCG had an adverse effect, the remission duration of 9 months is similar to 8 months observed in a previous study [21] and that reported by Zuhrie *et al.* [54]. The advantage of this protocol appears to be the fact that remission duration was so much longer for the chemotherapy arm.

Since 1971 the Southeastern Cancer Study Group has conducted 4 protocols exploring 9 maintenance programs. These included no therapy (1) chemotherapy (3 different programs), immunotherapy only (2 similar programs), and immunotherapy plus chemotherapy (3 different programs). This has involved 340 patients in documented complete remissions available for maintenance programs. The medians for duration of remission and survival are given in Table 2. As can be seen, methotrexate or a combination of BCNU and cytosine arabinoside offered no advantage over no maintenance treatment. Immunotherapy alone, reproducibly in sequential programs had only a marginal, statistically insignificant, effect on remission duration but appeared to prolong survival. Combinations of chemotherapy and immunotherapy added little. The most significant advance occurred when repeated courses of intensive therapy were administered during maintenance.

Table 2. Southeastern Cancer Study Group protocols for remission maintenance 1971-1983

Type of maintenance therapy	Protocol number	Ref.	Number patients	Median (mo) remission duration	Median (mo) survival
1. None	354	(21)	32	6.0	23
2. Chemotherapy					
Methotrexate	341	(39)	30	7.4	17
BCNU, CA	354	(21)	35	7.0	16
Daun, CA	318	(58)	51	17.0	29
3. Immunotherapy					
BCG	354	(21)	30	8.4	22
	318	(56)	56	9.0	21
4. Chemotherapy + Immunotherapy					
Methotrexate-BCG	341	(39)	24	9.4	22
BCNU, CA, BCG	A05	(44)	31	10.1	18
Daun, CA, BCG	318	(56)	51	10.0	21

BCNU = (1,3-Bis(2 chlorethyl)-1-nitrosourea), CA = Cytosine arabinoside, Daun = Daunorubicin, BCG = Bacillus Calmette Guerrin.

Other immunotherapy approaches have included use of methanol extracted residues of BCG [57], polyriboinosinic: polyribocytidylic acid [58], BCG cell wall skeletons [59], corynebacterium parvum [27, 60] and viral oncolysate [61]. None proved very effective. The use of neuraminadase modified myeloblasts as immunogens has been reported to be superior to the combination of these immunogens with MER but this study requires confirmation [62].

The failure to consistently identify an antigen or antigens unique to the leukemic myeloblast has delayed the development of more specific immunotherapy as has been developed in the lymphoblastic leukemias [53, 64]. However, these promising approaches are under investigation by many laboratories.

6. PROGNOSTIC VARIABLES PREDICTING FOR PROLONGED REMISSION DURATION

Attempts to correlate the clinical status of the patients with treatment outcome, remission duration and survival have been made by numerous investigators. With the application of statistical methods for assessing the impact of individual variables on remission, regression models have been

established which are predictive of remission induction and duration [65-68]. Using these techniques Keating *et al.* determined factors related to remission duration in 202 patients treated at M.D. Anderson Hospital [69]. They evaluated 36 pretreatment characteristics as well as numbers of courses of chemotherapy required to obtain remission and the time to halving the leukemic cell population. Significant favorable factors included lactic dehydrogenase (LDH) of less than 400 mU/ml, fibrinogen less than 250 mg/dl, 1 course to remission, AML diagnosis, no leukemic cells after 1 day of treatment and age less than 65. A multivariate analysis of factors relating to remission duration was reported by Passe *et al.* [70]. The only variables which proved to be significant for remission duration were intermediate age range (25 to 54), 2 courses of induction therapy and a low platelet count. In contrast to Keating's observations, the LDH level was not significant. Furthermore, Keating found 1 course of induction therapy to be favorable. The Southeastern Cancer Study Group analyzed 25 pretreatment variables and found higher initial hemoglobin concentration, higher platelet count, absence of respiratory disease and bone pain and the diagnosis of myelomonocytic leukemia (FAB M4) to be favorable characteristics for prolonging remissions [56]. Keating *et al.* [71] analyzed 457 patients treated between 1965 and 1976 using the same statistical methods as described above. The pretreatment bone marrow characteristics associated with longer remission were a diagnosis of progranulocytic leukemia, more than 4% eosinophils, and a high percentage of blasts and promyelocytes. A low serum LDH was a favorable characteristic. A WBC below 50 000/μl favored a 5 year remission duration. Treatment was a major variable affecting remission duration. Those patients receiving cytosine arabinoside or a combination of cytosine arabinoside and an anthracycline had a greater chance of being in remission at 5 years. A rapid fall in circulating blasts and an SGOT elevation during consolidation favored prolonged remission. These findings are based on 207 patients who obtained a remission, but only 36 remained in remission at 5 years.

Thus, these statistical methods are of value, but one must keep in mind that improved supportive care, new drugs and other advances may affect these variables. In addition, sufficiently large numbers of patients need to be studied. In a report by Brandman *et al.* [72] of 94 patients treated at a single institution, none of the clinical pretreatment variables were predictive of prolonged survival.

The duration of maintenance therapy is an unsettled issue. Keating *et al.* [71] reported that in 36 survivors of more than 5 years, 22 were treated for 16 to 18 months, 18 for 18 to 30 months, and 2 longer than 30 months. Only 3 of 39 patients at risk relapsed after 3 years. Rai *et al.* [18] reported that 70% of patients remained in remission when maintenance was discon-

tinued in the third year, 75% when discontinued in the fourth year and 78% when discontinued in the fifth year. The trend has been for more intensive treatment earlier in the disease. Weinstein *et al.* [25] and Vogler *et al.* [56] treated for approximately 15 to 16 months which included induction treatment.

The approach of very intensive therapy early in the disease followed by no maintenance therapy is being tested by the Southeastern Cancer Study Group. In this respect the results of a small series reported by Vaughan *et al.* [73] in which unmaintained remissions lasting a median of 10 months with 47% in remission at 1 year following two courses of chemotherapy given at an interval to maximize leukemic cell kill, are encouraging but need confirmation.

7. CENTRAL NERVOUS SYSTEM LEUKEMIA

Central nervous system (CNS) involvement in acute myelogenous leukemia is relatively infrequent. Wolk *et al.* [74] reported a frequency of blasts in the spinal fluid in 6.5% of 199 AML patients, similar to the 7% observed by Pavlovsky *et al.* [75] in 101 patients. Few studies have reported CNS involvement at diagnosis. Wiernik *et al.* [76] reported positive spinal fluid in 4 of 66 patients. Meyer *et al.* [77], using a cytocentrifuge, noted blasts in the spinal fluid in 7 of 39 newly diagnosed adult patients. All had myelomonocytic leukemia, elevated serum lysozyme levels and in 6 the WBC was greater than 50 000/μl. Thus, the incidence of CNS involvement at diagnosis is probably less than 10%.

The development of CNS leukemia during treatment is probably age related. Wiernik *et al.* [76] reported 7 of 62 adults (11%) developed CNS leukemia and Weinstein *et al.* [25] noted CNS as first sign of relapse in 7 of 35 (20%) patients less than 17 years of age. Dahl *et al.* [78] found CNS leukemia in 14% of children at diagnosis. In 44 children with negative CNS findings at diagnosis, 16 received prophylactic cranial-spinal irradiation, 8 received cranial-spinal plus liver and spleen irradiation and 20 received no prophylactic therapy. Four (20%) having no therapy relapsed in the CNS and 2 additional patients had a simultaneous marrow and CNS relapse. In contrast, none of the irradiated group had CNS relapse. However, the median duration of remissions were not significantly different.

The frequency of CNS relapse in adult AML patients in remission for more than a year was 20% in a small series [79]. Insufficient data are available to determine the relative risk of developing CNS leukemia in patients treated with current maintenance programs. Thus, the impact on remission duration is probably small.

8. BONE MARROW TRANSPLANTATION

This subject will be reviewed in detail in other chapters. The success of marrow transplantation in some patients with refractory leukemia [80, 81] prompted attempts at allogeneic transplantation in remission with increasing success [82–84]. Disease-free survival in excess of 18 months post transplant has been reported in 59 to 64% of patients [85, 86, 87]. Currently, marrow transplantation is limited to younger patients and those with HLA-compatible donors, although HLA mismatched transplants are underway at several centers [88–90]. Comparisons of marrow transplantation to chemotherapy maintenance programs are underway. Although early results favor marrow transplantation in 2 series [85, 91], one group found no statistical difference in survival at 3 years [92]. The long term effect of marrow transplantation and chemotherapy are unknown and the data are insufficient to judge one treatment to be superior as yet.

The development of autologous transplantation is an approach which appears promising and remissions of some sort are obtained in the majority of patients [93, 94]. Two issues are under investigation. One is more effective antileukemic treatment in the patient, such as multiple drug combinations [95, 96]. Another is *in vitro* treatment of stored marrow to eliminate any residual leukemia [97]. It is too early to fully assess the impact of this approach on remission duration and survival.

9. SUMMARY AND CONCLUSIONS

This review has attempted to assess the factors which contribute to long term remissions in adult AML. Obviously, if a constant percentage of patients who obtain remission become long term survivors, increasing the remission rate would increase that number. It would appear that the percentage of survivors is increasing as a result of improvement in therapy. Multiple factors appear to be responsible. The introduction of cytosine arabinoside and anthracyclines are of major importance. More intensive induction therapy, which can now be tolerated because of improved methods of supporting patients through periods of aplasia, appears to be important. Further treatment following remission induction is necessary. Some evidence suggests that switching to other non-cross resistant drugs may increase remission duration. The concept of intensive treatment to induce significant myelosuppression during maintenance is currently being investigated and appears promising. At issue, is the timing and duration of such treatment. The impact of immunotherapy as done in the past has been

marginal and awaits further development of new approaches such as tumor specific monoclonal antibodies or other biological modifiers.

The development of mathematical models for predicting response and survival has improved the experimental design of clinical studies. By identifying favorable and unfavorable characteristics, the reasons for treatment failure can be identified in many instances. However, it must be kept in mind that these predictive variables are derived from past experiences and are subject to change with new developments.

Central nervous system involvement in AML occurs in a minority of patients and would seem to have only a minor impact on remission duration. Prophylactic therapy is not indicated in the adult population.

The impact of bone marrow transplantation on remission duration is increasing. Although disease-free survival of patients transplanted in remission appears good, this is offset by the complications of graft-versus-host disease and interstitial pneumonitis. Furthermore, it is currently limited to a minority of patients with AML. Thus, chemotherapeutic approaches will remain the major treatment modality for the majority of patients.

The outlook for patients with AML is continually improving and as an understanding of the disease increases and new treatments develop, one can look forward to prolonged remissions in the majority of patients.

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8. The Role of Marrow Transplantation in the Treatment of Leukemia

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1. INTRODUCTION

The ability to kill leukemic cells is directly related to the dose of irradiation or 'radiomimetic' chemotherapeutic agents administered [1–5]. Unfortunately, the dose of systemic chemotherapy or radiotherapy that can be given with safety is limited by toxicity to normal tissues, and for many agents the most sensitive targets are the hematopoietic cells of the marrow.

It has been known since the mid-1950s that murine bone marrow cells transfused intravenously into an otherwise lethally irradiated host will reliably 'home' to the marrow space and there proliferate and restore normal hematopoiesis [6–8]. This remarkable behavior of marrow stem cells made it possible to consider treatment of patients suffering from otherwise incurable leukemia with doses of chemotherapy and/or radiotherapy that would normally cause lethal myelosuppression and then to rescue the patient with marrow transplantation. Except for a few transplants between identical twins, the earliest attempts to apply marrow transplantation to the treatment of acute leukemia were unsuccessful, due largely to lack of knowledge about histocompatibility and inadequate supportive care [9–11]. More recently, however, advances in both of these areas have made it possible to apply marrow transplantation widely and with considerable success to the treatment of acute leukemia.

In this chapter we will review briefly the technique of marrow transplantation, the clinical results that have been obtained with this technique, and some of the major complications encountered. A major focus of this review will be to compare the results of marrow transplantation to that of more conventional chemotherapy for individual patient groups at specific times in the course of their diseases. Such comparisons are of considerable importance in advising patients about appropriate treatment options.

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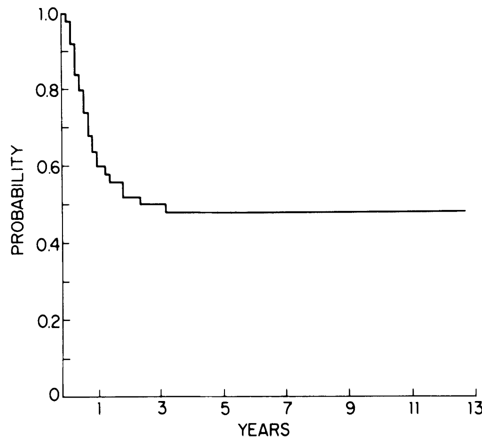

Figure 1. Kaplan-Meier product limit estimates for the probability of being in remission for 729 patients with acute leukemia treated with allogeneic marrow transplantation in Seattle between December 1971 and January 1983. The last relapse occurred at day 1373 after transplant. Seventy-two patients have been followed in continuous remission from 1374 to 4632 days after transplant without a single relapse.

When comparing marrow transplantation to more conventional chemotherapeutic approaches, several important points must be kept in mind. First, such comparisons are largely retrospective since few prospective controlled studies have been performed. Retrospective studies are inherently flawed and are, at best, only approximations of what might be expected. Second, even in retrospect, for many patient groups there are insufficient data for comparative purposes, partly because previous studies have not defined patients according to factors which have since been shown to influence prognosis and partly because these studies have not reported the results of sufficient numbers of patients followed for adequate periods of time. If the goal of therapy is cure, then studies selected for comparison should have a follow-up after completion of therapy which is long enough to determine that a true plateau in disease-free survival has been achieved. Figure 1 is a Kaplan-Meier product limit estimate of the probability of being in remission for all 729 patients treated in Seattle with allogeneic marrow transplantation for acute leukemia between December 1971 and January 1983. As shown, the last relapse occurred on day 1373 after transplantation, and thereafter no other relapse was seen among the 72 patients alive and disease-free from 3½ to 12 years after transplant. Thus, for marrow transplantation, disease-free survival beyond 3½ years can be considered equivalent to cure [12]. For conventional chemotherapy it is less clear how long off therapy patients must be followed, but at least 3½ years off therapy would seem to be a minimum requirement.

Third, comparisons between two forms of therapy are not necessarily

enduring. Certainly one hopes that the future will see major improvements in both chemotherapy and marrow transplantation so that comparisons between the two will require constant reevaluation. Finally, the decision to choose one form of therapy over another is necessarily influenced by many important issues which are as much philosophical as medical. For example, in certain situations marrow transplantation performed early in the course of the disease may, because of transplant-related mortality, decrease the probability of survival at 3 or 6 months but at the same time improve the chances of long-term survival and ultimate cure. These sorts of decisions demand a careful and in-depth dialogue between physician and patient. Such discussions require that the patient be given the opportunity to obtain as much information as possible in order to assist in making an informed decision.

2. DONOR SELECTION

An initial problem in considering marrow transplantation is the identification of an appropriate donor. Because there are no concerns of graft rejection or graft-versus-host disease, identical twins make attractive marrow donors. Therefore, although identical twins are relatively uncommon with an incidence of one in 290 live births [13], there is considerable experience with identical twin (syngeneic) transplants. This experience is important to consider because twin transplants provide the clearest evaluation of the antitumor efficacy of the preparative regimen. Unlike allogeneic marrow transplantation in which the allogeneic marrow may exert a graft-versus-leukemia effect or autologous marrow transplantation in which tumor contamination of marrow may contribute to the relapse rate, there is little evidence that any aspect of the transplant regimen other than the preparative regimen itself contributes to the antitumor effect in syngeneic transplants. Also, syngeneic transplantation allows for the clearest distinction of the toxicities associated with graft-versus-host disease and its treatment from the other toxicities seen posttransplant.

The overwhelming majority of marrow transplants have used sibling donors who are genotypically HLA-identical with the patient as determined by serologic typing of HLA-A and -B and by mutual nonreactivity in mixed leukocyte culture. Because animal studies have shown that matching between donor and recipient at the major histocompatibility complex reduces graft-versus-host disease and increases survival, most investigators have been reluctant to use other than genotypically matched donor-recipient pairs. More recently, however, a number of transplants have been performed in which related donor-recipient pairs are genotypically identical for

one HLA haplotype but only partially matched on the other haplotype [14, 15]. The impact of different degrees of histoincompatibility on the outcome of marrow transplantation for acute leukemia has not yet been determined, but initial results suggest that a single antigen mismatch may not alter the outcome of transplantation dramatically [14, 15]. Totally unrelated donors phenotypically identical with the patient at HLA-A, -B and -D have been used in a few cases with encouraging results [16].

It has been demonstrated previously in a controlled clinical trial that human marrow can be successfully aspirated, cryopreserved and reinfused, thus making autologous marrow transplantation feasible [17]. An obvious potential limitation of autologous marrow transplantation in the treatment of acute leukemia is the reinfusion of stored leukemic cells along with the remission marrow. The problems and potential promise of autologous transplantation in the treatment of leukemia are beyond the scope of this chapter.

3. PREPARATIVE REGIMENS

The preparative regimen used prior to allogeneic marrow transplantation for leukemia must be immunosuppressive enough to prevent the patient from rejecting the graft and ideally should provide a potent antileukemic effect. The first allogeneic transplants for leukemia performed in Seattle used total body irradiation (TBI) delivered at 5–8 rad/minute to a total dose of 1000 rad as the preparative regimen [18]. Of six patients with relapsed acute lymphoblastic leukemia treated, five relapsed with their leukemia and one is alive without leukemia more than 12 years later. In an effort to deliver a more potent antileukemic effect, high-dose cyclophosphamide (CY), 60 mg/kg on each of 2 days, was added to the TBI. The CY-TBI regimen has become the 'standard' Seattle preparative regimen. A number of variations on the basic CY-TBI regimen have been developed. In situations where the overwhelming reason for transplant failure has been disease recurrence, attempts have been made to increase the antitumor effect of the regimen. In other situations where the major reason for failure is transplant-related toxicities, attempts have been made to decrease the toxicity of the regimen. These variations will be discussed in the individual disease sections.

Preparative regimens using only chemotherapy without TBI are capable of providing adequate immunosuppression to allow engraftment, but it remains unproven whether such regimens provide an adequate antileukemic effect. Initial studies performed over a decade ago using preparative regimens composed only of CY failed to yield any long-term survivors [19, 20].

Another chemotherapy-only regimen combining bischloroethyl nitrosourea (BCNU), cytosine arabinoside, high-dose CY, and 6-thioguanine (BACT) was used to prepare a small number of patients with relapsed leukemia for allogeneic transplantation [20]. Of this group, one patient remains alive more than 10 years from transplant and another died more than 5 years posttransplant of infection without evidence of leukemia (unpublished observations). More recently, Santos *et al.* have used a combination of busulfan (4 mg/kg/day for 4 days) and CY (50 mg/kg/day for 4 days) [21]. Although the follow-up is short, there are a number of patients who are currently alive and disease-free more than 2 years after treatment.

4. TECHNIQUES OF MARROW TRANSPLANTATION

The general technique of marrow transplantation is quite simple [22]. Under general or spinal anesthesia and using standard marrow aspiration needles, multiple aspirates are performed from the anterior and posterior iliac crests and, occasionally, the sternum of the donor. The marrow is placed in heparinized tissue culture media and then, using metal screens, filtered to remove bony spicules and fat globules and to create a monocellular suspension. Usually within 2–24 hours after the completion of TBI, the marrow is infused intravenously, following which the marrow stem cells 'home' to the marrow space, proliferate, and eventually restore normal hematopoiesis and immune function. It is probably important to give as many marrow cells as possible. In controlled animal models it has been demonstrated that higher doses of marrow cells provide more reliable and more rapid engraftment [23]. Although in humans graft rejection is uncommon after preparative regimens employing TBI, a low number of transplanted marrow cells has been associated with graft rejection after CY conditioning and marrow transplantation for aplastic anemia [24].

Following the transplant the patient usually receives some form of immunosuppression such as methotrexate or cyclosporine, in an attempt to prevent or at least modify graft-versus-host disease. The pathogenesis and treatment of graft-versus-disease will be discussed later in this chapter.

5. CLINICAL RESULTS IN ACUTE NONLYMPHOBLASTIC LEUKEMIA (ANL)

5.1. *Refractory ANL*

In Seattle 16 patients with refractory ANL have been treated with CY, 60 mg/kg for 2 days, 1000 rad TBI, and a marrow transplant from a genetically identical twin. One patient died of interstitial pneumonia and ten

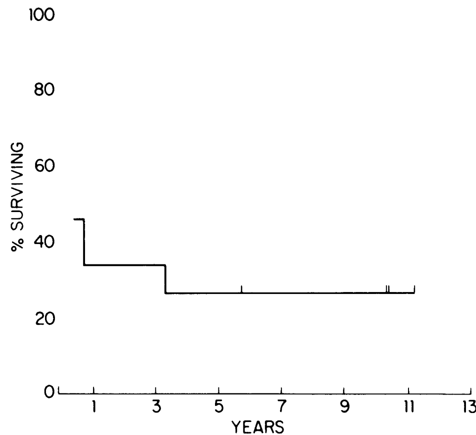

Figure 2. Survival of 16 patients following syngeneic marrow transplantation for refractory ANL. The patient who died more than 3 years after transplant had metastatic colon cancer but no evidence of leukemia at the time of death.

died of either persistent or relapsed leukemia, but five (31%) remain alive in complete remission without further therapy from 3 to 11 years after treatment (Figure 2) [25–27]. The results of allogeneic marrow transplantation for refractory ANL are not as good, owing largely to many more deaths due to graft-versus-host disease and its complications. Between 1970 and 1975, 54 patients with refractory ANL were transplanted from HLA-identical siblings, and six are living and well 8–11 years after grafting with no further antileukemic therapy (Figure 3) [28]. Given that these were patients with chemotherapy-resistant leukemia, it is impressive that even this small percentage could be cured. Not unexpectedly, leukemic recurrence was a major cause of treatment failure in this group. Based on actuarial analysis, it can be estimated that 65% of these patients would have relapsed had they not died of nonleukemic causes, a figure similar to the relapse rate after syngeneic transplantation. Efforts to reduce this high relapse rate have included the addition of more chemotherapy to the transplant regimen (dimethyl busulfan, BCNU, or daunorubicin) or the use of fractionated irradiation in order to increase safely the total dose of irradiation delivered. Unfortunately, neither of these approaches has convincingly decreased the high relapse rate or resulted in a higher proportion of disease-free survivors [29, 30].

5.2. *First Remission of ANL*

Following the initial demonstration that 10–15% of end-stage patients with ANL could be cured with marrow transplantation, a group of 19 patients was transplanted while in first remission [31]. Ten of these patients are living in remission, all more than 5 years after transplantation (Figure 4). Similar results have now been reported by a number of investigators [32–

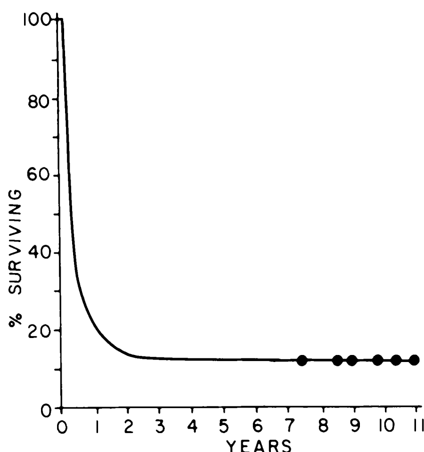

Figure 3. Survival of 54 patients following allogeneic marrow transplantation for refractory ANL. Six patients remain alive in continuous complete remission more than 7 years after transplant.

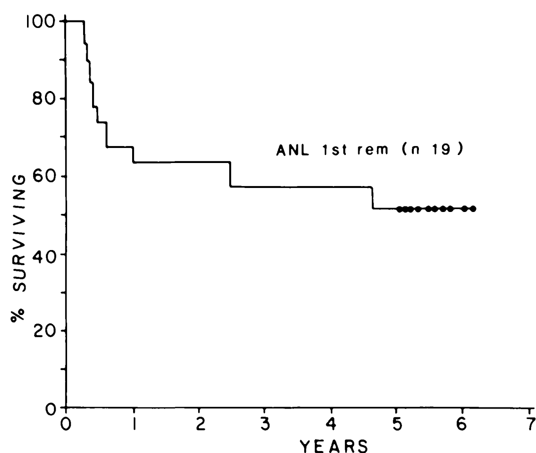

Figure 4. Survival of 19 patients transplanted for ANL in first remission. The dots indicate patients living in unmaintained remission.

36. Among the first 19 patients transplanted in first remission, the major cause of failure was interstitial pneumonia (five cases) while only two patients relapsed. It thus appears in this group that the complications of the transplant regimen, rather than recurrent leukemia, are the major problem.

Based on the concept that fractionation of TBI should provide for a better therapeutic ratio between leukemic cell kill and normal tissue tolerance, a prospective study was carried out in which 53 patients were randomized to receive either the standard CY + 1000 rad single-dose TBI or CY + 200 rad on each of 6 days [37]. Fractionated irradiation appeared to have a

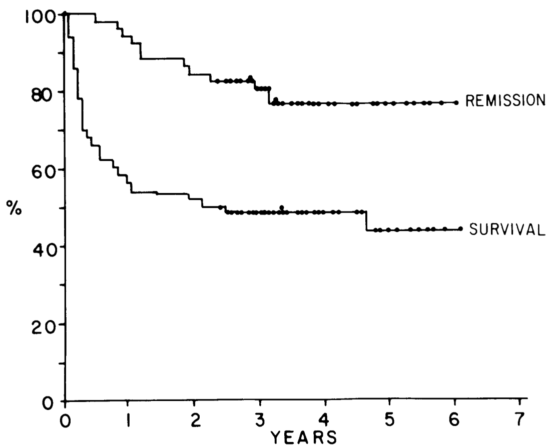

Figure 5. Kaplan-Meier product limit estimates for the percent in remission and for percent surviving for 75 patients transplanted for ANL in first remission. Patients dying of nonleukemic causes have been censored at the time of death in constructing the remission curve. The dots indicate patients living in unmaintained remission.

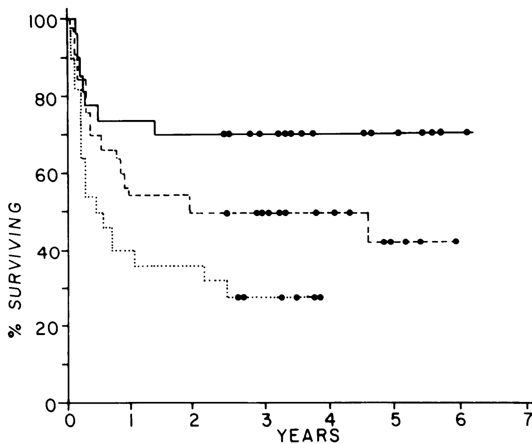

Figure 6. Kaplan-Meier product limit estimates for survival of 75 patients with ANL transplanted in first remission divided according to age. Solid line (●—●) represents age <20 (n = 23), dashed line (●- - -●) age 20–29 (n = 30), and dotted line (●.....●) age 30–50 (n = 22). The large dots indicate patients living in unmaintained remission.

slight advantage over single-dose irradiation, but no single factor (e.g. diminished incidence of interstitial pneumonia or leukemic relapse) could account for this difference. Other fractionated schedules are now being explored by other investigators [38].

Among the first 75 patients with ANL transplanted while in first remission, approximately 50% are alive in remission more than 2 years after transplantation (Figure 5) [39, 40]. The risk of leukemic relapse in this large

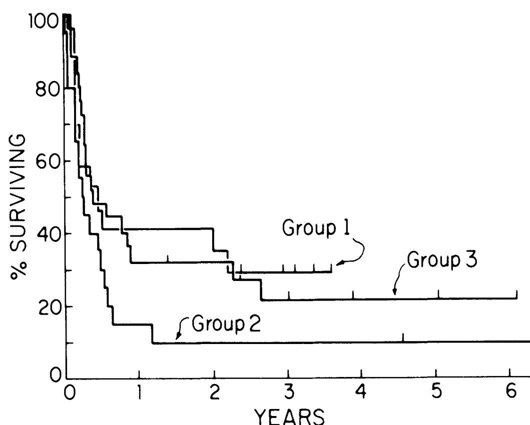

Figure 7. Kaplan-Meier product limit estimates of the probability of survival of patients with ANL transplanted without reinduction (group 1), having failed reinduction (group 2), and in second remission (group 3).

group of patients is approximately 20%. Survival is strongly influenced by age (Figure 6) [39, 40]. The influence of age on survival appears to be due largely to an increased incidence and severity of graft-versus-host disease in older patients.

5.3. *After First Relapse*

There are, of course, clinical situations other than end-stage refractory ANL and first-remission ANL in which marrow transplantation might be considered. We have recently reviewed our experience in 62 patients with ANL transplanted after first relapse [41]. In 17 patients (group 1) no attempt at reinduction of remission was made prior to transplantation. In 20 patients (group 2) attempts at inducing a second complete remission were unsuccessful, and in 25 patients (group 3) a second remission was achieved. Five of 17 patients (29%) in group 1, 2 of 20 (10%) in group 2, and 5 of 25 (20%) in group 3 are surviving disease-free 2–6 years after grafting (Figure 7). These results suggest that transplantation directly after first relapse is superior to waiting until patients have end-stage refractory disease and that there is no clear advantage to reinducing patients into second remission.

5.4. *The Role of Transplantation in ANL*

Patients with ANL who fail induction therapy or who relapse after achieving an initial complete response have an extremely poor prognosis with conventional or experimental chemotherapy [42, 43]. Therefore, because marrow transplantation can cure between 15 and 30% of such patients, it is certainly reasonable to consider this approach for any such patient less than 50 years of age with an identical-twin or matched-sibling

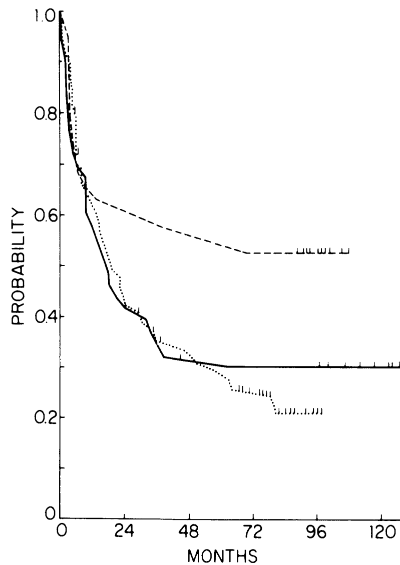

Figure 8. Kaplan-Meier product limit estimates of the probability of disease-free survival for 19 patients transplanted in first remission (- - - -), 43 patients treated on the Southwest Oncology Group (SWOG) study 7315/16 (——), and 117 patients treated on SWOG 7416/17 (. . . .). Transplanted patients were all less than age 48 and were transplanted, on the average, 16 weeks after diagnosis. The curves displayed here for the chemotherapy groups represent the length of complete remission from 16 weeks after the start of chemotherapy for patients less than age 48. By log-rank test, transplantation is superior to SWOG 7416/17 ($p = 0.03$, two-sided) but not to SWOG 7315/16 ($p = 0.12$).

donor. There is no apparent advantage to getting the patient into second remission prior to transplant. However, it may take a considerable period of time to identify an appropriate donor, to arrange finances, and to locate a bed in a transplant center. Because relapsed ANL may be a very rapidly progressive disease, some patients with ANL in first relapse will continue to require chemotherapy prior to transplantation in an attempt to keep them in reasonable clinical condition while a transplant is being arranged.

Whether to transplant a patient in first remission is a more difficult question. The results of combination chemotherapy in the treatment of ANL have been the subject of several recent reviews [42–44]. If one accepts that the goal of treatment of ANL in patients less than age 50 is cure of the disease, then in determining the effectiveness of chemotherapy it is necessary to select studies which have long enough follow-up to test whether an actual plateau in disease-free survival has been achieved. Recent reports from several cooperative group trials or single large institutions indicate that approximately 20% of all patients achieving complete remission have remained disease-free for 5 years or longer [45–52]. Figure 8 illustrates the comparison of disease-free survival of the first 19 Seattle patients trans-

planted in first remission with that of patients treated on two recent Southwest Oncology Group protocols, SWOG 7315 and SWOG 7416. Because patients in the transplant group were all under the age of 50 and the median time from diagnosis to transplant was 16 weeks, patients over age 50 and any relapsing during the first 16 weeks after therapy were removed from the SWOG data in an effort to make the groups reasonably comparable.

Although this comparison suggests an advantage for transplantation, there are several reasons to view such comparisons with caution. There may have been referral bias in such a retrospective analysis. It is possible that only healthier patients were referred for transplantation or, conversely, physicians may have referred for transplantation only those patients whom they felt would do poorly with conventional chemotherapy. Also, it is unknown if factors which predict for long remission duration were equal among the two groups of patients. Further, in such a retrospective analysis the initial induction chemotherapy differed between the two groups, and how this might affect ultimate survival is unknown.

The best way to compare marrow transplantation to conventional chemotherapy would be through a prospective study. We have been conducting such a study since 1978. Patients were entered on study and treated with a five-drug induction regimen including daunomycin, cytosine arabinoside, 6-thioguanine, vincristine, and prednisone. Patients achieving complete remission were offered allogeneic marrow transplantation if they had a matched sibling donor; if not, patients were continued for 2 years on chemotherapy including consolidation and late intensification therapy. One hundred eleven patients were entered on study and 90 (81 %) achieved complete remission. Eleven patients with HLA-matched donors refused transplantation, and two others relapsed before transplantation could be carried out. Forty-five patients without matched donors were treated with conventional chemotherapy while 32 patients were transplanted (Figure 9). Thirty-five patients in the chemotherapy arm have failed therapy, and all but two died with recurrent leukemia. The causes of failure in the transplant arm include relapsed leukemia in four cases, interstitial pneumonia in six cases, and graft-versus-host disease and infection in five. The results of this study support the retrospective analysis previously shown and suggest that the initial results obtained with marrow transplantation are not influenced by referral bias or patient selection.

More recently, several very intensive chemotherapy programs have been reported which seem to result in an improved median duration of complete remission [53–55]. However, the follow-up times on these studies have been too short to allow one to determine whether relapses have been delayed or if the more intensive therapy will truly result in an increased cure rate. As stated earlier, it is expected that no comparison of marrow transplantation

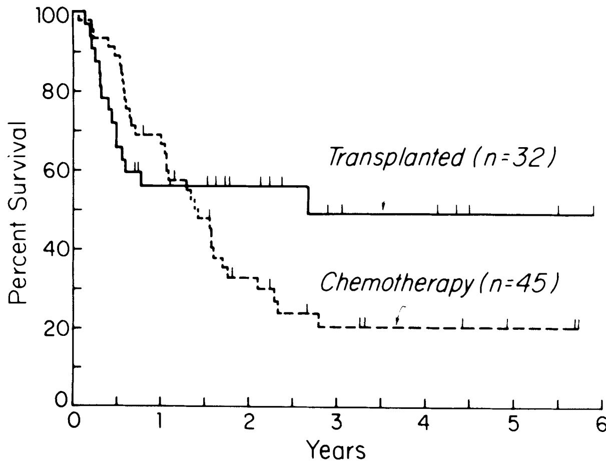

Figure 9. Kaplan-Meier product limit estimates of survival for patients with ANL in first remission treated with marrow transplantation (N = 32) or continued chemotherapy (n = 45) in Seattle.

to chemotherapy will be enduring but that advances in both treatment modalities will require constant reassessment.

Given these data, for whom can transplantation in first remission be recommended? If 20–25% of patients under the age of 30 can be cured with chemotherapy and if 20–25% of those who relapse can be cured with marrow transplantation in first relapse or second remission, the aggregate cure rate without transplantation in first remission would appear to be 40–45%, which is less than the 60% obtained with marrow transplantation in first remission. For patients aged 30–50, it is less clear that the long-term survival rate with transplantation in first remission will be significantly higher than the aggregate obtained with conventional chemotherapy followed by transplantation in first relapse or second remission.

The existence of reliable prognostic factors to determine which patients will do very well with chemotherapy would help greatly in the decision about whom to transplant. Recently attempts have been made to identify such factors. For example, Keating has reported that 5-year continuous complete remission is significantly associated with a low lactic dehydrogenase at diagnosis, a high differentiation ratio (blasts + promyelocytes)/(myelocytes + metamyelocytes + neutrophils), the diagnosis of acute promyelocytic leukemia, a low serum calcium, and a low white count at diagnosis [56, 57]. Unfortunately, not every study has found similar results. For example, Passe *et al.* in a study of 79 adults with ANL found the number of courses of induction therapy required to achieve complete remission, patient age, and a low platelet count at diagnosis were the only factors which seemed to predict for longer remission duration [58]. Other factors thought

perhaps to predict for longer remission duration include eosinophilia in the initial marrow [57], the development of 'hepatitis' during therapy [59, 60], the presence of circulating immune complexes at diagnosis and/or during remission [61], limited 'self-renewal' capacity of malignant ANL clones *in vitro* [62], and the absence of abnormal metaphases in the marrow at diagnosis [reviewed in 63]. Conversely, patients with smoldering leukemias, leukemias which follow long preleukemic phases, and leukemias secondary to prior cytotoxic chemotherapy have generally been found to respond poorly to conventional chemotherapy. If groups of prognostic factors could be developed and shown to identify reliably those patients who will do either very well or very poorly with conventional chemotherapy, the choice of whom to transplant and when could be simplified.

Another approach which might have application in the future would rely on the ability to recognize regrowth of leukemic cells prior to clinical reappearance of disease. If such techniques could be developed, it might be possible to reserve transplantation until impending relapse. Techniques which have been investigated in small series include the observation that there are a decreased number of cells in S phase in remission marrows about to relapse [64], that there are more cells in late G_1 prior to relapse [65], and that there may be an increase in the number of cells reactive with a murine antibody directed against human ANL cells just prior to relapse [66].

6. CLINICAL RESULTS IN ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

6.1. *Refractory ALL*

Among 18 patients with refractory ALL treated in Seattle with marrow transplantation from identical twins, three (17%) are alive without disease more than 5 years after transplantation [25–27]. One patient died of hepatitis and the other 14 died with persistent or recurrent leukemia. Thus, as for ANL, a small but significant proportion of end-stage ALL patients can be cured with this approach.

Among the first 46 patients with refractory ALL transplanted after CY and 1000 rad TBI with HLA-identical sibling marrow, seven are alive 7–11 years after grafting (Figure 10) [28]. For both syngeneic and allogeneic transplantation for refractory ALL, the major problem has been leukemic relapse. The use of fractionated TBI regimens was explored in an effort to deliver an increased dose of TBI without causing prohibitive toxicity. Although these studies demonstrated that fractionated TBI could be given at doses as high as 1575 rad delivered at 225 rad/day for 7 days without undue nonmarrow toxicity, no improvement in the antileukemic effect of the preparative regimen was evident [67].

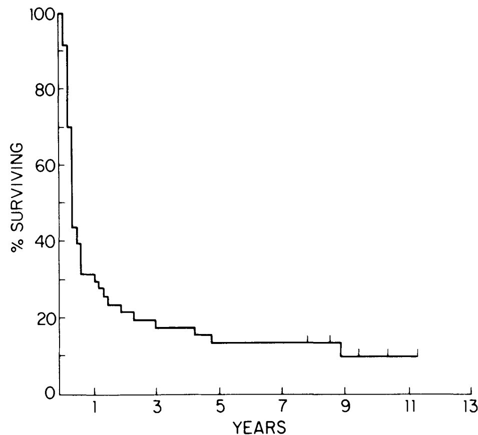

Figure 10. Survival of 46 patients with refractory ALL treated with allogeneic marrow transplantation. Ticks indicate patients living in unmaintained remission.

6.2. *Remission ALL*

Because of the excellent outlook for some patients with ALL in first remission, the initial studies of remission marrow transplantation for ALL were carried out during second or subsequent remission. The median survival for the first 22 patients transplanted in remission was 12 months with an actuarial survival of 46% at 2 years and 32% at 3 years [68]. Now, with all patients followed for more than 5 years, the actual 'cure' rate is 27% (Figure 11). A major problem after transplantation was a continued high relapse rate estimated at 50%. The use of fractionated irradiation to a total dose of 1400 rad has not diminished this rate [69].

6.3. *The Role of Transplantation in ALL*

Although the results of marrow transplantation for refractory ALL are not very good, the 10–15% long-term survival rate is better than can be achieved with chemotherapy. Therefore, if facilities and a donor are available, it is reasonable to offer marrow transplantation to any patient under the age of 50 with refractory ALL who is in reasonable clinical condition.

In 1981 we published the results of a prospective study comparing the outcome of marrow transplantation in 24 children with ALL in second or subsequent remission to that of conventional chemotherapy in 21 children in second remission [70]. All patients on this study have been followed from 3 to 6 years, and all 21 of the children treated with conventional chemotherapy have relapsed a second time. In contrast, eight of the 24 patients treated with marrow transplantation remain in complete remission more than 3 years since transplantation. Most of the patients in both groups had initially relapsed on therapy. There is some evidence that children with

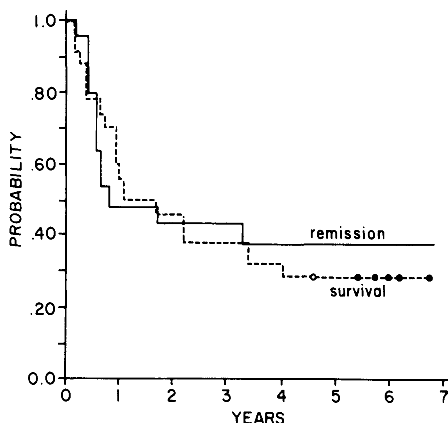

Figure 11. Kaplan-Meier product limit estimates for the probability of being in remission or for surviving for 22 patients transplanted for ALL in second or subsequent remission. The solid circles indicate patients living in unmaintained remission, and the open circle indicates a patient who was killed in a automobile accident while in remission.

ALL who relapse off therapy may do better than those who relapse while on maintenance, but whether such patients can be cured without transplantation is as yet unproven [71–73]. Thus, for children in second complete remission, especially those who relapse on therapy, marrow transplantation is probably the treatment of choice. The prognosis for adults with ALL in second remission is even less favorable than for children, and therefore marrow transplantation should be considered in all adults with ALL after first relapse [74, 75].

There are virtually no published results of marrow transplantation for patients with ALL in first remission. It is probably possible, however, to identify groups of patients with ALL in first remission with such a poor outlook that it is reasonable to consider transplantation in this setting. Factors influencing remission duration for which there is general agreement include the extent of disease at diagnosis with a white count of greater than 100,000/cu mm as a particularly adverse feature, age with patients less than age 2 or older than 10 having a poorer prognosis, and morphology, with cases classified as L2 doing worse than L1 [76–82]. Other factors in childhood ALL which are likely to be associated with a poorer prognosis include Negro race, slowness to remit, high hemoglobin, low immunoglobulin, and specific cytogenetic findings (e.g. t[4:11] and t[8:14]) [63, 81–84]. More recently, the immunological phenotype of childhood ALL has been shown to be associated with disease remission duration as well. B-cell ALL patients clearly do worse than others [85]. Also T-cell ALL patients have a poorer prognosis, but this may be due to higher initial white cell counts seen in this group [85, 86]. Among children with non-B, non-T ALL, those who are

common ALL antigen-negative do worse than those who are common ALL antigen-positive [87]. There are many problems yet to be worked out concerning prognostic factors for childhood ALL including a better understanding of which factors are independently important and how different chemotherapeutic programs may alter the impact of these variables. Nonetheless, it is probably possible to identify patients with childhood ALL whose chances of long-term disease-free survival with currently available chemotherapy is less than 20%, a figure which is less than that achievable with marrow transplantation for ALL in *second* remission. Since transplantation in *first* remission is likely to yield even better results, one might consider marrow transplantation for this group of patients. However, it is unknown if those same prognostic factors which predict for a short remission with chemotherapy will also turn out to predict for a poor outcome with transplantation.

Considerably less is known about the long-term outcome of chemotherapy for adult ALL. Large single-institution or group studies with 5-year follow-up have generally reported fewer than 20% of adults alive and disease-free at 5 years [74, 75, 88]. More recently there have been several studies with more encouraging results. Clarkson *et al.* have reported 44% predicted 5-year disease-free survival in adult ALL with the Sloan-Kettering L-10 protocol [89]. Blacklock has also reported a similar experience in a small group of young adult ALL patients [90]. Whether or not these results represent real advances in the treatment of adult ALL or statistical anomalies remains to be proven. As in childhood ALL, it may be possible to select adult ALL patients with poor prognosis. One such factor may be absence of the common ALL antigen on leukemic cells [91]. Whether or not it will be advantageous to transplant all or a subset of adult ALL patients in first remission rather than reserving transplantation for therapy after first relapse remains undetermined.

7. CLINICAL RESULTS IN CHRONIC GRANULOCYTIC LEUKEMIA (CGL)

7.1. *CGL Blast Crisis*

Ten patients with CGL have been transplanted during blast crisis with identical-twin marrow after preparation with CY, TBI and, in most cases, dimethyl busulfan. Eight patients died shortly after the transplant, four of transplant-related causes and four with rapidly recurrent disease. Two patients remain alive and disease-free, one more than 8 years after grafting [92]. Similar results have been obtained with allogeneic transplantation for CGL in blast crisis; among 11 patients transplanted, two are alive and disease-free beyond 2 years, five died of recurrent disease, and four died of

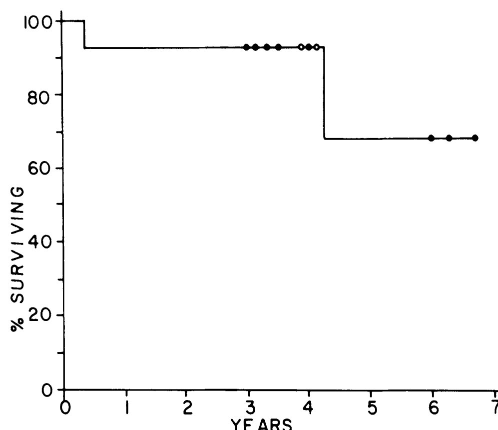

Figure 12. Kaplan-Meier product limit estimates for survival following syngeneic marrow transplantation in 12 patients with CGL in chronic phase. The solid circles indicate patients living in unmaintained remission, and the open circles represent two patients alive but with cytogenetic evidence of recurrence.

transplant-related causes [93]. These findings demonstrate that, as in end-stage ANL, a small but definite minority of patients with CGL in blast crisis can achieve prolonged complete unmaintained remission after marrow transplantation. However, because of the advanced nature of disease and the poor clinical condition of the patient, the incidence of both disease recurrence and transplant-related mortality is high.

7.2. *CGL in Chronic Phase*

We have reported the results of syngeneic marrow transplantation in 12 patients with CGL in chronic phase (Figure 12) [92, 94]. All had cytogenetically documented complete responses after transplant. One patient died of pneumonitis while in remission. Three had cytogenetic relapses 22–30 months after grafting. One of these three has entered blast crisis and died. Eight patients remain in complete remission 3–7 years after treatment.

Given these encouraging results in twins, we have extended this technique to patients with nontwin HLA-identical donors. Among the first ten patients with CGL in chronic phase given allogeneic transplants, six are alive and well in complete clinical, cytogenetic, and hematologic remission 18–42 months after transplantation [95]. The reason for failure in the four patients was interstitial pneumonia in three and severe graft-versus-host disease in one. Similar results have been published by others [96]. McGlave *et al.* reported nine patients transplanted during the accelerated phase of CGL [97]. Although all patients had been followed for less than 1 year, six of the nine were alive in remission at the time of this report; two had died of graft-versus-host disease and one had relapsed with CGL.

7.3. *The Role of Transplantation in CGL*

Since no patient with CGL can be cured by currently available chemotherapy, marrow transplantation should be considered for any such patient less than age 50 for whom an appropriate donor is available. The major question is the timing of this intervention.

The best results appear to come from transplantation during the chronic phase of the disease. Whether transplantation early in chronic phase is better than later in chronic phase is unknown, but there are reasons to suspect this may be the case. Prolonged exposure to low-dose radiomimetic alkylating agents may select for resistant neoplastic clones. Among the first 12 syngeneic transplants performed for chronic-phase CGL, there have been three cytogenetic relapses, two of which occurred in the two patients who had been followed longest in chronic phase prior to transplant. Also, prolonged exposure to busulfan may result in lung damage; whether such damage predisposes to interstitial pneumonia is not determined. Finally, by delaying transplantation there is always a chance that the patient will suddenly enter blast crisis and thus have an obviously worse prognosis for cure. However, some patients undergoing transplantation for chronic-phase CGL will die shortly after the procedure of transplant-related causes who otherwise might have lived reasonably normal lives for some months or years without transplantation. Thus, the benefits of early transplantation must be weighed against the risk of early death. Recently Tura *et al.* have published prognostic factors which may be of use in predicting the duration of chronic phase for patients presenting with CGL [98]. Another possibly useful approach would be sequential cytogenetic analyses since it has been demonstrated that new chromosomal abnormalities often predate and predict the onset of blast crisis [99]. In the end, it is likely that waiting to perform the transplant decreases the likelihood of success, but proceding early in the course of the disease is accompanied by a risk of early mortality. The ultimate decision of when to proceed should depend on the patient's attitude toward his or her disease and therapy.

8. LEUKEMIC RECURRENCE AFTER TRANSPLANTATION

Leukemic recurrence is a major reason for failure after transplantation. In the vast majority of cases, leukemic recurrence is due to failure of the transplant regimen to eradicate the malignant clone. However, by utilizing blood genetic markers and cytogenetic techniques, recurrence of morphologically identical leukemia in cells of donor rather than recipient origin has been documented [100, 101]. Among 54 Seattle patients who relapsed after marrow grafts from a donor of the opposite sex, three relapses in donor-type

cells were seen [102]. Thus, approximately 5% of recurrences may be of this type. The mechanism is, of course, unknown but may relate to the transfection of DNA between cells.

More recently, the development of an immunoblastic lymphosarcoma in cells of donor origin has been seen posttransplant [103]. This tumor was morphologically distinct from the original leukemia and was associated with Epstein-Barr viral genomes within its DNA.

9. GRAFT-VERSUS-HOST DISEASE (GVHD)

Based on animal studies, GVHD is thought to be the result of the responses of alloreactive T cells, transferred with the graft or developing from it, against target cells of the genetically different host. The immunological basis fo GVHD in humans has recently been reviewed [104]. Clinically, GVHD can be separated into two distinct syndromes, acute GVHD and chronic GVHD.

9.1. *Acute GVHD*

Acute GVHD, by definition, develops within the first 3 months after marrow transplantation and presents with characteristic lesions in the skin, liver, and gastrointestinal tract [18]. A staging system for acute GVHD has been developed in which grade I refers to an isolated skin rash, grades II and III are defined to include a rash plus increasingly severe involvement of the liver and the gastrointestinal tract, and grade IV is life-threatening.

Studies in a variety of animal models have demonstrated that immunosuppressive therapy given immediately posttransplant can diminish or prevent GVHD [reviewed in 105]. In dogs, methotrexate begun the day after transplantation and continued for 100 days was found to be the most useful approach, and therefore this regimen has been used as part of the transplant regimen for the majority of patients transplanted in Seattle. Parenthetically, it might be mentioned that methotrexate has never been compared to no therapy in a controlled trial in humans. Despite methotrexate prophylaxis, approximately 30–40% of patients will develop acute GVHD and 30–40% of those will die of GVHD and/or associated infections [106].

A number of different approaches for GVHD prevention have been tried. Ramsay *et al.* have added antithymocyte globulin and steroids to posttransplant methotrexate and were able to diminish the incidence of GVHD from 48% to 21% [107]. Unfortunately, there was no difference in the overall survival curves between the two groups. Perhaps the increased immunosuppression posttransplant also increased the incidence or severity of infectious complications.

Cyclosporine is a recently developed potent immunosuppressive agent which has been shown to be quite effective in prolonging allograft survival in a number of animal models. Pilot studies describing the use of cyclosporine as posttransplant GVHD prophylaxis were encouraging. We have been carrying out a prospective randomized study of cyclosporine versus methotrexate as GVHD prophylaxis in patients transplanted for ANL in first remission [110]. Preliminary results of the first 62 patients randomized disclose no difference in overall survival between the groups although the incidence of GVHD may be slightly less in the cyclosporine arm. Also associated with cyclosporine in this study was faster engraftment, less mucositis, and a shorter initial hospital stay. On the other hand, cyclosporine can be quite nephrotoxic and demands careful monitoring.

A somewhat different approach to the prevention of GVHD involves the elimination of donor T cells from the marrow inoculum prior to transplantation. Such an approach has been shown to be effective in murine transplant models [111]. Methods of removing T cells from human marrow have involved treating the marrow with either anti-T cell monoclonal antibodies alone, antibody plus complement, or soybean agglutinin and sheep red blood cells [112–114]. These studies are so preliminary and involve so few patients that the only conclusions so far are that patients have engrafted but GVHD still develops in some cases.

A final approach to the prevention of acute GVHD was based on the observation that there is a significant reduction in the incidence and severity of GVHD after allogeneic marrow transplantation in mice maintained germ-free from birth or decontaminated shortly after birth [115]. A recent study of 130 patients engrafted for aplastic anemia demonstrated a reduction in grades II-IV acute GVHD from 39% to 23% if patients were transplanted after decontamination and placement into laminar air flow rooms [116].

Once patients have developed grades II-IV acute GVHD, approximately one-half will go on to die of either GVHD or an associated infection. The clinician is faced with a difficult dilemma in treating patients with established acute GVHD; the only effective treatments are immunosuppressive agents and, at the same time, major threats to the patient are severe overwhelming infections. The immunosuppressive agents that have been used in this setting include antithymocyte globulin, prednisone, cyclosporine, CY, and monoclonal antibodies directed against T cells. No one approach has yet been shown to be convincingly better than others [117–119].

9.2. *Chronic GVHD*

Chronic GVHD develops more than 3 months after transplantation and is a syndrome with a spectrum of collagen vascular-like manifesta-

tions [120, 121]. Patients virtually always have some skin changes including malar erythema or sclerodermatous skin changes and in addition may have oral ulcers, alopecia, sicca syndrome, polyserositis, and photosensitivity. Esophageal involvement with sclerodermatous-like changes has been seen. Liver involvement is characterized by bile duct degeneration and profound cholestasis. Laboratory values usually include elevated liver function tests, autoantibodies, eosinophilia, and impaired immune response.

Approximately 30% of patients surviving 150 days after transplantation will develop chronic GVHD. Both increasing patient age and prior acute GVHD are associated with an increased risk of chronic GVHD [120–122]. Patients with limited chronic GVHD (involving only skin or skin and liver) usually do quite well with no therapy. Patients with extensive chronic GVHD who are untreated have a very poor prognosis with fewer than 25% alive at 1 year [120, 121]. Preliminary results have suggested that steroids combined with azathioprine may be helpful in the management of chronic GVHD [120]. In addition, supportive care (artificial tear replacement, sun-blocking agents, and prophylactic oral antibiotics) may be very helpful.

9.3. *Graft-Versus-Leukemia*

Although GVHD is generally thought to be an undesirable outcome of allogeneic marrow transplantation, there is evidence that the likelihood of being in remission 2 years after transplantation is higher among patients with GVHD than among patients without GVHD or recipients of identical-twin marrow grafts [123]. This finding implies that one target of the graft-versus-host reaction is the host leukemic cell. Prior to 1978 the advantage of a lesser probability of recurrent leukemia in patients with GVHD was offset by a greater probability of other causes of death. More recently, we have observed an apparent decrease in the fatality rate of acute GVHD without any apparent decrease in the antileukemic benefit resulting in improved survival [124]. In addition, the development of chronic GVHD is associated with a lower relapse rate and improved survival [125]. Currently we are investigating whether it might be possible to manipulate GVHD to exploit its antileukemic effects.

10. INFECTIOUS COMPLICATIONS, INCLUDING INTERSTITIAL PNEUMONIA

Infectious complications following marrow transplantation have been the subject of several recent extensive reviews [126, 127]. During the early granulocytopenic period lasting 20–30 days after transplant, the major complications are fever with bacterial or fungal infections. After recovery of granulocytes until about day 100, viral and protozoan infections become prom-

inent. After day 100, the incidence of infection decreases, but late infections with varicella-zoster virus and bacterial infections, especially in patients with chronic GVHD, may be seen.

During the early granulocytopenic phase most patients develop fever, and almost 50% will have at least one documented bacteremia. Organisms isolated are similar to those from other granulocytopenic patients; about one-half are aerobic gram-negative bacteria, predominantly *Escherichia coli*, Klebsiella and pseudomonas, while most of the remainder are gram-positive organisms. We routinely treat febrile, granulocytopenic patients with an empiric antibiotic regimen consisting of an antipseudomonas penicillin, an aminoglycoside, and, not infrequently, vancomycin. Once empiric antibiotic treatment has been initiated, we continue treatment until the patient recovers 500 circulating neutrophils/mm^3.

Many efforts have been directed at attempting to prevent infectious complications in transplant recipients. In a prospective randomized trial, laminar air flow isolation together with skin cleansing, topical and oral nonabsorbable antibioitics, and sterile food for the first 50 days after transplant were successful in preventing serious bacterial infection [128]. Also, the use of prophylactic granulocyte transfusions has been shown to decrease bacterial and fungal infections after marrow grafting [129]. It should be pointed out that in the marrow transplant setting, a fully matched granulocyte donor (the marrow donor) is often available, and this may in part account for the success of granulocyte transfusions after transplantation.

Fungal infections are also, unfortunately, common during the early post-transplant period and may be seen later as well. As many as 30% of patients who die of transplant-related causes will have evidence of fungal infection at autopsy. Candida has been the most common organism followed by aspergillus. Parenteral amphotericin remains the treatment of choice for all invasive fungal infections.

From the time of granulocyte recovery until day 100, the major infectious (or possibly infectious) complication is interstitial pneumonia [130, 131]. Interstitial pneumonia should be considered as a syndrome rather than a specific disease. Patients usually present with fever, a dry nonproductive cough, tachypnea, and hypoxia. Radiologic findings usually include a diffuse, rapidly progressive infiltrate although segmental and nodular infiltrates have been seen. The highest incidence of interstitial pneumonia is between days 30 and 70. Generally, the etiology of pneumonia can only be reliably determined by open-lung biopsy. Many cases are idiopathic while other etiologies include cytomegalovirus, other viruses (usually herpes simplex), or *Pneumocystis carinii*. The relative incidence of interstitial pneumonia of each type is shown in Table 1 for 525 recipients of allogeneic grafts and 100 recipients of syngeneic grafts [131, 132]. The incidence of idiopathic pneu-

Table 1. Incidence of Interstitial Pneumonia after Marrow Transplantation for Hematological Malignancy

	Syngeneic	Allogenic
Total penumonia	0.17 [a]	0.48 [b]
Idiopathic	0.11	0.13
Pneumocystis carinii	0.01	0.07 [b]
Cytomegalovirus	0.00	0.19 [b]
Other virus	0.01	0.03
Clinical	0.04	0.09

[a] Entries indicate proportion of patients with each pneumonia type.
[b] Differences between syngeneic and allogeneic transplants are statistically significant.

monia is virtually identical in allogeneic and syngeneic transplant recipients, suggesting that idiopathic interstitial pneumonia is due to a direct toxic effect on the lung since all other infectious complications are more common in allogeneic patients. Another point of interest is that even though syngeneic transplant recipients convert their cytomegalovirus titers as frequently as allogeneic recipients do, not a single case of cytomegalovirus pneumonia was seen in the first 100 syngeneic recipients, suggesting that GVHD and/or its treatment must have a profound effect on the development of cytomegalovirus pneumonia. Both idiopathic and cytomegalovirus pneumonias are less common after transplantation for aplastic anemia when no TBI is used, suggesting that TBI may play a role in the development of both forms of disease.

There is no known effective treatment for idiopathic or cytomegalovirus pneumonia. The mortality rate is almost 50% despite the use of steroids, adenine arabinoside, acyclovir, or interferon. More recently, attempts to prevent cytomegalovirus pneumonia with the use of cytomegalovirus immune globulin have been published with encouraging results [133, 134]. *Pneumocystis carinii,* formerly a significant problem, has been all but eliminated with the use of trimethoprim-sulfamethoxazole prophylaxis.

The most common late infection is varicella-zoster virus (VZV) which develops in nearly 50% of all marrow transplant recipients with a median time of onset of 5 months. We routinely treat patients developing VZV infections during the first 4 months after transplant with adenine arabinoside. For patients later in their course, we reserve therapy until signs of dissemination develop. Other late infections are uncommon unless the patient is suffering from chronic GVHD, in which case late bacterial infections of the upper respiratory tract and lungs are seen, usually due to gram-positive organisms such as streptococcus pneumonia and *Staphylococcus*

aureus [135]. For this reason we have more recently been placing patients with chronic GVHD on trimethoprim-sulfamethoxazole prophylaxis in the hope of preventing bacterial infections.

11. OTHER ORGAN TOXICITIES

11.1. *Cardiac*

An acute pancarditis has been seen shortly after the administration of very high-dose CY (greater than 180 mg/kg) [136, 137]. The clinical presentations included orthostatic hypotension, tachycardia, fluid retention, and dyspnea on exertion, with the subsequent development of decreased voltage on electrocardiogram, pericardial effusion, and heart failure. If patients survive the acute cardiac decompensation, the disease seems to be self-limited. The exact mechanism of this syndrome is uncertain, but electron microscopic examination of autopsy specimens suggests significant endothelial damage [137].

11.2. *Hepatic*

Veno-occlusive disease of the liver (VOD) was first observed to occur among Jamaican children after ingestion of pyrrolizidine alkaloids. A similar clinicopathologic entity has been observed among marrow transplant recipients [138, 139]. The clinical features of this syndrome include the development of ascites, tender hepatomegaly, and jaundice within 1–4 weeks of transplantation. VOD may be so mild as to be 'subclinical', may develop and regress spontaneously, or may progress to fatal liver failure. The overall incidence of VOD in our experience is approximately 10%, and perhaps one-third of these cases may be fatal. VOD is seen more frequently among patients who begin the transplant procedure with abnormal liver function and is also more frequent after conditioning regimens that include extra chemotherapy in addition to the standard CY+TBI [138].

11.3. *Gonadal Function*

The preparative regimen used prior to transplantation for aplastic anemia (CY 200 mg/kg) does not prevent the return of normal gonadal function in younger women and in most men [140]. Preparative regimens used for leukemia which include CY plus TBI lead, in the majority of women, to permanent primary ovarian failure with amenorrhea and elevated luteinizing and follicle stimulating hormone levels [140]. After CY and TBI, men usually have normal testosterone and luteinizing hormone levels, follicle stimulating hormone levels are usually elevated, and semen analyses usually

reveal no sperm. Rarely, however, men have fathered normal children after CY and TBI.

12. SUMMARY

The role of marrow transplantation in the treatment of adult leukemia is gradually becoming clearer. For certain patients, transplantation is the only form of therapy which can provide long-term disease-free survival or cure. This includes patients with ANL or ALL who fail initial induction therapy or who relapse after achieving an initial remission and patients with CGL in accelerated phase or blast crisis. Since such patients have no other prospect for cure and since transplantation can cure 15–30% of them, marrow transplantation should be considered if facilities and an appropriately matched donor are available and if the patient is less than age 50 and in reasonable clinical condition.

If transplantation is carried out earlier in the course of leukemia, the outcome is better. From 50% to 60% of patients with ANL in first remission less than age 30 can be cured with marrow transplantation and are therefore appropriate transplant candidates. For patients over age 30, the cure rate with transplantation is lower and therefore it is less clear if such patients should be transplanted in first remission or after initial relapse. The results of marrow transplantation for chronic-phase CGL are at least as good as for first-remission ANL and thus, especially for younger patients, marrow transplantation during the chronic phase of CGL is the treatment of choice for this disease.

The role of marrow transplantation in the treatment of adult leukemia in the future will depend on how successful we are in overcoming the major limitations of transplantation. At present, the majority of patients lack an appropriately matched donor. The use of partially matched donors or phenotypically identical but unrelated donors could dramatically increase the number of patients for whom transplantation is a reasonable option. Transplantation is also currently limited by the complications of the procedure, most notably GVHD and infectious complications, especially cytomegalovirus infections. It is likely that newer approaches to GVHD will be fruitful, particularly the use of cyclosporine and the removal of T cells from the marrow inoculum. Newer approaches to viral infections, including the use of newer antiviral agents, immunoglobulin to cytomegalovirus, and blood and marrow donors who are free of cytomegalovirus infections are promising, but a great deal more must be learned about the biology of viral infections in the transplant recipient. The major limitation of marrow transplantation as treatment of adult leukemia continues to be leukemic recurrence.

The only approach to this problem which has convincingly reduced the leukemic relapse rate has been to transplant patients earlier in the course of their disease. There is hope that the high recurrence rate after transplantation for end-stage leukemia can be reduced. We and others are currently studying new preparative regimens composed of fractionated irradiation and chemotherapeutic agents other than cyclophosphamide. Methods of manipulating GVHD to take advantage of its antileukemic effect are under study. The use of posttransplant therapy with interferon, monoclonal antibodies, or further chemotherapy is also under investigation.

While this research is taking place, newer, more intensive chemotherapy programs for adult leukemias are also being developed which will also, hopefully, result in more long-term disease-free survivors. The question of whom to transplant and when will, hopefully, require continued reevaluation.

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9. Hyperleukocytosis in Adult Leukemia

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1. INTRODUCTION

Patients with leukemia and hyperleukocytosis are at risk of developing leukostasis [1]. Patients with acute myeloid leukemia (AML) will tend to develop symptoms of leukostasis at a white blood count (WBC) of 100 000/µl. Patients with chronic myelocytic leukemia (CML) will tend to develop symptoms of leukostasis at a higher WBC of over 200 000/µl. Patients with acute lymphocytic leukemia (ALL) and hyperleukocytosis have a poorer prognosis than patients with a lower WBC but leukostasis is not a common finding. Patients with chronic lymphocytic leukemia (CLL) very rarely develop symptoms from hyperleukocytosis unless the WBC is extremely elevated e.g. 400 000/µl. Leukostasis was reported by us in a single patient with hairy cell leukemia and a WBC of 1 million/µl [2].

Leukostasis refers to a pathological finding of slightly dilated, thin walled vessels filled with leukemic cells. The lung and the brain are the organs most commonly involved [3, 4]. The commonest symptoms are headache, visual impairment and shortness of breath. Patients with AML and hyperleukocytosis tend to be of a certain subtype, namely acute myelomonocytic leukemia (AMML) or acute monocytic leukemia (AMOL) [5, 6]. Patients with acute lymphocytic leukemia and hyperleukocytosis also are predominantly of a certain cell type, namely T-cell [7]. This chapter will review the presentation, prognostic factors and therapy of patients with leukemia and hyperleukocytosis.

2. ACUTE MYELOCYTIC LEUKEMIA

Thirty-six newly diagnosed patients (Pts) with AML and an initial WBC of over 100 000/µl were admitted to Mount Sinai Hospital (MSH) from

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Table 1. Findings at diagnosis

Patient	Age/Sex	WBC/ul	FAB[a]	Lung[b]	CNS[c]
1	75/F	115,000	M-4	0	0
2	50/F	224,000	M-2	0	+
3	41/F	135,000	M-2	0	0
4	51/F	277,000	ND	+	0
5	51/F	253,000	ND	+	+
6	55/F	225,000	M-4	+	0
7	75/F	145,000	ND	+	0
8	50/F	254,000	M-2	0	0
9	47/M	178,000	M-2	0	0
10	76/F	215,000	M-2	+	+
11	71/F	129,000	M-4	+	0
12	17/M	278,000	M-2	0	0
13	65/M	122,000	M-4	0	0
14	29/F	137,000	M-4	0	+
15	56/F	180,000	M-4	0	+
16	53/F	190,000	M-5	+	+
17	26/M	117,000	M-2	0	0
18	17/M	438,000	M-4	0	+
19	61/M	150,000	M-5	0	0
20	51/M	215,000	M-4	0	0
21	58/M	120,000	M-5	0	0
22	45/M	160,000	M-4	0	0
23	62/M	143,000	M-5	0	0
24	15/F	102,000	M-2	0	0
25	28/F	200,000	M-4	0	+
26	25/M	263,000	M-4	0	0
27	12/M	306,000	M-2	0	0
28	16/F	134,000	M-4	0	0
29	66/M	201,000	M-2	+	0
30	74/M	224,000	M-5	0	0
31	53/F	116,000	M-5	0	0
32	48/M	211,000	M-5	+	+
33	45/M	490,000	M-4	+	0
34	22/M	420,000	M-4	+	0
35	50/M	100,000	M-4	+	+
36	57/M	112,000	M-4	+	0

[a] French American British Classification.
[b] a+means an abnormal chest X-ray compatible with leukostasis.
[c] CNS = a+sign means leukemic cells seen on cytocentrifuge specimen of cerebrospinal fluid.

1977 through 1982. Table 1 shows the findings at diagnosis. Hepatosplenomegaly was found in over 50% of these patients. Gingival enlargement was seen in about 1/3 of our patients. Thirty-three of our patients were

classified according to the French American British (FAB) classification [8]. Sixteen patients (48%) were classified as M-4, seven (21%) were M-5 and ten (30%) were classified as M-2. Thus, 70% of patients with hyperleukocytosis were classified as acute myelomonocytic or acute monocytic leukemia. These patients had a median serum lysozyme of 67 µg/ml. This association has been reported by our group and others [5]. Overall 21 patients (58%) achieved remission (19 CR, 3 PR) and 15 (42%) had no response.

2.1. *Central Nervous System Leukemia*

A lumbar puncture is indicated in patients with hyperleukocytosis at diagnosis because of the increased incidence of CNS leukemia in this group of patients. We performed a lumbar puncture on 39 consecutive newly diagnosed patients with AML of all subtypes at the time of diagnosis. Seven of the 39 patients had blast cells in their cerebrospinal fluid. All seven had acute myelomonocytic leukemia [9]. In our recent study of patients with hyperleukocytosis, one third of patients had CNS involvement at diagnosis. Of these, 7 of 9 (78%) were M-4 or M-5 subtype. All patients received chemotherapy with a seven day continuous infusion of cytosine arabinoside (Ara-C) and three days of daunorubicin (DNR). Eight patients underwent therapeutic leukapheresis prior to chemotherapy (see Table 2). Five (45%) achieved CR while none of 3 patients without leukapheresis responded.

2.2. *Lung Involvement*

Thirty-three percent of patients with hyperleukocytosis had lung involvement at diagnosis. Pulmonary leukostasis was defined as patients with dyspnea, tachypnea, pulmonary rales, non-hypercapneic respiratory failure and diffuse interstitial infiltrate. Lung involvement was associated with an extremely poor prognosis. These patients had the highest mortality of all the patients in our series. Patients with lung involvement tended to have a higher WBC (median 222 000/µl vs. 183 000/µl). Hypoxemia was common

Table 2. Effect of therapeutic leukapheresis on leukostasis in patients with hyperleukocytosis

	Lung			CNS		
	# of Pts.	# of Pts. who died in induction	% died	# of Pts.	# of Pts. who died in induction	% died
Overall	12	9	75	11	6	55
With leukapheresis	7	5	71	8	3	38
Without leukapheresis	5	4	80	3	3	100

in this group of patients. Whole blood viscosity was measured in eight patients with hyperleukocytosis. It was elevated in six of eight patients. All patients with pulmonary involvement had an elevated whole blood viscosity. This group of patients had the poorest response to therapy; only 3 of 12 patients (25%) achieved a complete remission. Patients who present with hyperleukocytosis and lung infiltrates must be considered as medical emergencies and treated as soon as possible. Treatment should include leukopheresis plus chemotherapy.

2.3. *Therapy*

Patients with AML and hyperleukocytosis are critically ill either with evidence of leukostasis or are at great risk of developing leukostasis. They must be treated immediately. Three modalities of treatment have been used namely, cranial radiation, chemotherapy, e.g., hydroxyurea, or DNR plus Ara-C, and most recently therapeutic leukapheresis. Cranial radiation though effective takes several days to administer. It, of course, has no effect on pulmonary leukostasis, which had the highest mortality in our series. Hydroxyurea has been reported to be effective therapy [10]. In our experience it is no more effective than good induction chemotherapy e.g., Ara-C and daunorubicin. If a patient presents with hyperuricemia, then chemotherapy of any kind cannot be given because of the imminent danger of renal failure secondary to massive tumor cell lysis. Therapeutic leukapheresis is a method of safely and rapidly lowering the white cell count until definitive chemotherapy can be given.

We recently reported our experience with therapeutic leukapheresis in 22 patients with newly diagnosed AML and white cell counts greater than 100 000/µl [11]. In this study all patients received one, two or three leukaphereses prior to induction chemotherapy with Ara-C and DNR. Fifteen of 22 (68%) achieved a complete remission. We found that a greater than 30% decrease in initial WBC was an important predictor of induction response. Fifteen of 17 patients (88%) with a greater than 30% reduction of initial WBC entered a complete remission, while none of 5 patients with less than a 30% reduction responded ($p = .001$). The remission rate of 68% is among the best reported for this group of patients. Dutcher and colleagues reported on 34 patients with WBC over 100 000/µl treated with an anthracycline plus Ara-C [12]. The remission induction rate was 47%. Remission duration was 3 months, and median survival for CR patients was 11 months.

The Cancer and Leukemia Group B (CALGB) entered 207 newly diagnosed, evaluable patients with AML and initial WBC greater than 100 000/µl on three randomized studies during the years 1974 to 1979. Remission induction chemotherapy was with a seven day continous infusion of Ara-C and DNR in the majority of patients or with Ara-C and

adriamycin in the remainder. Two hundred patients had data on response to induction therapy. Of these, 81 (41%) were complete responders and 28 (14%) were partial responders. In the Mount Sinai Hospital series, the complete remission rate was 68%. A median remission duration of 10.8 months was seen in the CALGB group of patients compared to 11.6 months seen in the Mount Sinai Hospital series. The median survival for responders in the CALGB series was 19.2 months compared to 20.1 months in the Mount Sinai series.

3. CHRONIC MYELOCYTIC LEUKEMIA

Patients with chronic myelocytic leukemia presenting with hyperleukocytosis may exhibit increased morbidity and mortality as a result of hyperviscosity, leukostasis and the formation of intravascular leukocyte aggregates [13]. Compromise of central nervous system [14–16], pulmonary [17–21], and cardiovascular systems may occur. McKee [13] reported 17 cases of intracerebral leukostasis in the chronic phase of CML; and 19 additional cases in blastic phase CML. Papilledema and fluctuating mental status paralleling the level of leukocytes is well reported [22]. Frost [20] reports death from respiratory distress secondary to elevated WBC while Karp [17] reports reversal of leukostatic respiratory failure with rapid decrease in WBC. Morbidity occurs in both the chronic and accelerated phases of CML with the life expectancy lessened at higher white blood cell counts. It has been speculated that leukostasis is attributable to elevation of the whole blood viscosity (WBV), due to increased concentration of circulating leukocytes, and other cells [23, 16]. We have studied the clinical effects of initial elevation of WBC and undertaken a systematic study of the clinical effects of initial WBC and viscosity, and correlated changes in viscosity with clinical progression.

From July 1980 through April 1983, whole blood viscosity (WBV) was measured in patients with elevated white cell counts. During this period, 19 patients with chronic myelogenous leukemia (CML) were seen by the Leukemia/Lymphoma Unit at The Mount Sinai Hospital. All patients had the characteristic blood picture of CML. In eleven patients with WBC greater than 50 000/µl and 1 patient with platelets greater than 1 000 000/µl WBV was measured at 37°, two using a Wells-Brookfield Cone Plate viscometer and 10 with a coaxial cylinder viscometer. Serial measurements were made during therapy. In 7 patients with WBC less than 50 000/µl, no viscosity measurements were made. Therapy was individualized in order to assure the most rapid decrease in leukocrit. All 12 patients underwent therapeutic leukapheresis. Chemotherapy differed among the group. Leukapheresis was

Table 3. Chronic myelocytic leukemia – Findings at presentation

Patient	Age/sex	Initial blood counts* WBC	Hgb	PLT	Symptoms of leukostasis**	Signs of leukostasis***	WBV pre therapy	WBV after therapy
Chronic phase CML								
A	23/M	684,000	9.0	170,000	HA, BV	OMS, RI, RH	24.2	10.1
B	34/F	21,000	7.3	3,190,000	HA, BV, SOB	RH, RI	5.7	2.8
C‡‡	27/M	620,000	8.0	350,000	HA,	PAP, PLB	15.0	3.9
D	32/F	307,000	11.6	37,000	BV, Bone pain	RET, RH	5.0	4.0
E	66/M	88,000	7.8	2,310,000	HA, BV, Bone pain	Pul. Inf.	4.6	3.3
F	70/M	51,300	14.6	1,910,000	SOB	Pul. Inf.	7.8	4.8
G	17/M	582,000	5.0	261,000	HA, Bone pain	RH	18.7	14.0
Blastic phase CML								
H‡‡	55/M	575,000	9.7	690,000	HA	RH	13.0	4.6
I	43/F	421,000	8.6	155,000	SOB, Bone pain	PLB, Pul. Inf.	23.0	6.3
J	64/M	265,000	11.6	37,000	SOB	Pul. Inf.	8.3	4.8
K	34/M	200,000	12.3	300,000	Prolonged erection	PLB, Priapism	11.0	5.2
L	18/F	372,000	7.9	99,000	BV	RET, PLB	10.8	6.0

* WBC = White Blood Count/ul, Hgb = Hemoglobin gm/dl, PLT = Platelets/ul.

** HA = Headache, BV = Blurred Vision, SOB = Shortness of Breath.

*** OMS = Organic Mental Syndrome, RH = Retinal Hemorrhage, RI = Retinal Infiltrates, PAP = Papilledema, PLB = Phlebitis, RET = Retinopathy, Pul. Inf. = Pulmonary Infiltrates.

‡ WBV = Whole Blood Viscosity measured in centipoise.

‡‡ Patients C and H measured by a Wells-Brookfield cone plate viscometer at a shear rate of 115 sec^1 at 25 °C (All other patients were measured by a coaxial cylinder viscometer at a shear rate of 52 sec^1 at 37 °C).

performed on an intermittent flow cell separator (Haemonetics Model 30 Blood Processor). Anticoagulant was ACD Formula B.

Of 12 patients where viscosity measurements were made, 7 were in the chronic phase. The mean WBC for the chronic phase was 450 000/µl and blastic phase 272 000/µl. The most common presenting symptoms were headache (50%), blurred vision (42%), bone pain (33%) and shortness of breath (33%). The most common physical findings were retinal hemorrhage (42%), phlebitis and thrombosis (33%), and pulmonary infiltrates (33%). The mean hematocrit was 28% and leukocrit was 20%. Ten patients had an elevated whole blood viscosity, 5 patients had a whole blood viscosity more than six times normal at a shear rate of 52^{-1}. (Table #3). All patients were treated with therapeutic leukapheresis. Decrease in leukocrit correlated with decreased blood viscosity. Clinical improvement was temporally related to decrease in WBV.

In the remaining group of 7 patients all were in the chronic phase of CML. The median WBC was 73 000/µl. The presenting symptoms were abdominal discomfort (28%), and malaise (14%), with the remainder being asymptomatic. Leukostasis was not seen in this group. Patients in both the chronic and accelerated phases of CML may exhibit hyperleukocytosis. We have documented the presence of hyperviscosity associated with progressive increase in leukocyte number. This may be reflected in multiple organ system dysfunction.

3.1. *Central Nervous System*

Direct central nervous system involvement with infiltration of meninges and brain parenchyma in CML are uncommon but well-described. Hyperleukocytosis in CML producing hyperviscosity with associated leukostasis and leukostatic thrombi seems far more common but has only recently been reported [14, 16]. The most common symptom of leukostasis was headache, found in 50% of our patients. Although diverse causes in CML such as anemia, bleeding and infection may cause headache, this symptom in a setting of hyperleukocytosis and rapid increase in white blood count must alert one to the need for rapid cytoreduction. Papilledema [22], exudative retinal detachment [24], and direct infiltration of the eye and meninges may occur [24, 25]. In our patients, disorientation and headache rapidly cleared with lowering of the white blood cell count. Blurring of vision and exudative retinopathy similarly cleared with appropriate therapy of elevated white blood cell counts.

3.2. *Lung Involvement*

Thirty-three percent of our hyperleukocytotic group presented with shortness of breath. Several of our patients exhibited X-ray evidence of pulmon-

ary infiltrates with no infectious etiology found. Pulmonary infiltrates cleared on lowering of white blood cell count. Autopsy studies in this setting have demonstrated leukostatic thombi in arterioles and capillaries of the pulmonary vasculature [13]. Similar case reports of pulmonary compromise and respiratory failure in the setting of hyperleukocytosis with clinical recovery corresponding to decreased white cell counts have been reported [19, 20, 21]. Rapid cytoreduction in one of our cases resulted in pulmonary improvement. This was correlated with x-ray evidence of improvement. Hyperleukocytosis in CML has been reported to result in vascular compromise of the GI tract [26], and the portal system [27]. Vascular compromise in the genito-urinary system has similarly been reported [28]. One of our cases exhibited priapism clearing with lowering the WBC. We have seen peripheral vascular compromise i.e., phlebitis.

An early study by Stephens [23] on patients with chronic myelogenous leukemia and high leukocyte counts demonstrated a marked increase in blood viscosity at room temperature by using the Hess viscometer. The probable relationship of increased leukocrit to the symptomatology of the disease has been extensively discussed, and most investigators [29, 30] considered a large increase in whole blood viscosity to be a rare event in leukemia. Our studies demonstrated that a significant elevation of blood viscosity was present in CML patients studied when their leukocyte counts were over 200 000/μl. Extreme elevations of blood viscosity were present in three patients with cytocrits ranging from 60 to 70%; their blood viscosity was 250% that of an equal erythrocrit or 450% that of normal blood. Besides an elevated whole blood viscosity, other factors which may also contribute to the pathogenesis of leukostasis are the high intracellular viscosity, the low cell deformability, the large cell volume and the large nucleus of leukemia cells [23], the vasoactive factors released from leukemic cells, and the low hemodynamic pressure of the stagnant vascular system in the organs involved [29]. The clinical features seen in CML are generally attributable to blood flow disturbances in the microcirculation.

3.3. *Therapy*

Rapid cytoreduction in our patients resulted in decreased leukocrit, decreased viscosity and resolution of clinical leukostasis. We developed therapeutic leukapheresis in the therapy of hyperleukocytotic acute myelocytic leukemia [11]. Despite theoretical possibilities, no lasting effects have been demonstrated with long-term leukapheresis in CML [31]. Rapid temporary symptomatic relief with improvement of hematological values has been reported [32]. We use intensive short-term leukapheresis in conjunction with rapidly acting cytotoxic agents, i.e., cytosine arabinoside 100 mg/m^2 intravenously soluset every 12 hours for 10 doses and 6 thiogunine

100 mg/m^2 orally every 12 hours in most cases to effect rapid cytoreduction. As we treated all patients with both chemotherapy and therapeutic leukapheresis, it is difficult to ascribe a decreased WBC to one form of therapy. It is our impression, however, that leukaphereses stabilized the WBC for the initial 4-6 days of combined therapy. Thereafter, the rapid decrease in WBC was due to the combination chemotherapy. Two to 3 weeks after the completion of chemotherapy the WBC usually was in the 10-15 000/µl range and the splenomegaly was usually either no longer present or reduced by 70-80%. Rapid clinical improvement ensued paralleled by decreased leukocrit and viscosity. Clinical leukostasis occurs in both the chronic and accelerated phase of CML.

In AML symptoms of leukostasis seem to appear at counts of greater than 100 000/µl. In our experience in CML a direct numerical correlate was not established in CML. Counts of greater than 200 000/µl seem to be related to clinical leukostasis.

Rapid increase in white blood count and/or cell type may be important. We suggest that patients with CML and WBC over 200 000/µl be observed for symptoms of hyperviscosity especially headache, visual disturbances, bone pain or phlebitis. Rapid cell reduction will result in decreased morbidity as we have shown in acute myelogenous leukemia.

4. ACUTE LYMPHOCYTIC LEUKEMIA

4.1. *Introduction*

The tumor burden, as reflected by the initial white blood cell count, is the most important determinant of prognosis in adult acute lymphocytic leukemia (ALL) [32]. This is most typified in patients presenting with white blood cell counts greater than 100 000/µl. Patients with T-cell acute lymphocytic leukemia (diagnosed by sheep RBC rosetting or more recently by the use of monoclonal antibodies) present with a mediastinal mass, high initial white blood cell counts and central nervous system (CNS) involvement. Both remission induction and median survival rates are shorter in T-ALL than in common C-ALL (CALLA) or null ALL. Thus, the proper diagnosis of adult ALL requires cytochemical stains (PAS, SBB), terminal deoxynucleotidyl transferase and lymphocyte surface markers.

Patients presenting with leukocyte counts > 100 000/µl are at risk from the complications of hyperleukocytosis with leukostasis. This represents a potential medical emergency because of the life-threatening complications of hyperleukocytosis. Lymphoblasts by increasing the viscosity of the microcirculation can affect the blood circulation in the lung, heart, brain and

Table 4. Initial WBC count and prognosis in adult acute lymphocytic leukemia

Initial white blood cells	# Pts.	Complete response (%)	Age	Median duration remission (MO)	% relapse	Median survival time (MO)	Complete response at 3y%	Four year survi-val %	Five year survi-val %	Authors
<10000	410		All			10				Zippin *et al.* [33]
>100000	122					3.6				
All	876					7.6			1	
<5000			Adult					27		Till and Hardisty [35]
5–10000								52		
10–20000								11		
20–50000								9		
>50000								1		
All	83									
<25000			Adult	27						Gee *et al.* [36]
>25000				15						
All	23	78		25						
<25000	25	72	Adult	17.5		50				Shaw and Raab [37]
>25000				4.8		33				
All				10.2						

< 10000	22		Adult				61		Lister *et al.* [38]
> 10000	13						26		
> 100000	7	57		<6		9.5			
All	51	71		18.5		21			
< 100000	75	N.S.D.*	Adult	19.3		26.1			Omura *et al.* [39]
> 100000	24	N.S.D.							
All	99	80		19.3		26.1			
> 100000	141	71	All	6		9			Harousseau *et al.* [40]
All	55	69	Adult	9		13	N.S.D. WBC		Leimert *et al.* [41]
<2000	22	86	> 11Yr		47			20	Baccarani *et al.* [42]
2–4900	66	86			54			28	
5–9900	39	85			72			11	
10–19900	39	77			63				
20–34900	31	74			61				
35–49900	20	70			86				
50–99900	27	67			89				
> 100000	46	76			86			11	
All	293	79		16				20	
< 35000	197	82			60			31	
> 35000	93	72			87			8	

* N.S.D. = no significant difference.

other organs. They may also compete for oxygen in the microcirculation causing damage to vessel walls.

Increased whole blood viscosity related to the high white cell count has also been suggested as a cause for the hyperleukocytosis syndrome. Blast cells are also thought to be more rigid than mature cells causing the blast cells to lodge in small blood vessels producing a leukocyte thrombus with ischemia and hemorrhage.

Effective cytoreductive chemotherapeutic treatment in acute lymphocytic leukemia may lead to a tumor lysis syndrome through rapid destruction of massive numbers of leukemic cells in 2-3 days causing metabolic changes such as hyperkalemia, hyperphosphatemia and hyperuricemia and the danger of producing obstructive uropathy. Thus, it is imperative to decrease the tumor mass rapidly and reverse the hyperleukocytosis syndrome without aggravating metabolic abnormalities.

Fritz *et al.* in 1959 were the first to emphasize the association of fatal intracranial hemorrhage in patients with acute leukemia and white blood cell counts over 300 000/μl [3]. Fifteen patients presented with leukocyte counts > 300 000/μl and 9 of them died rapidly of intracranial hemorrhage. Of these, there were 6 patients with acute lymphocytic leukemia, and 3 with acute myelocytic leukemia. In contrast, nine out of 85 patients with white cell counts less than 300 000/μl developed intracranial hemorrhage. Of this group 6 patients had acute lymphocytic leukemia and 3 acute myelocytic leukemia. At autopsy patients who died of intracranial hemorrhage with white cell counts over 300 000/μl had, within the hemorrhagic areas, nodules of leukemic cells. Those who died with a white blood cell count of < 300 000/μl had either subdural or subarachnoid hemorrhage as the cause of death. They also emphasized the lack of life-threatening thrombocytopenia in these patients.

4.2. *Review of the Literature*

The reported series of adult acute lymphocytic leukemia show the remission induction rate not to be influenced by the initial white cell count. The remission duration, however, is significantly shortened in patients presenting with white blood cell counts > 50 000 to > 100 000/μl. (Table 4). Zippin evaluated 876 patients of all ages and found a median survival time of 7.6 months [33]. For patients with a white cell count < 10 000/μl median survival was 10 months and for an initial white blood cell count > 100 000/μl median survival was 3.6 months. This difference was found consistently for all age groups. Jacquillet looked at 4 year survivors for all age groups and found that the complete response rate was 50–60% for patients with greater or less than 50 000/μl [34]. Till and Hardisty also looked at 4 year survivors for all age groups and found that 28 out of 83 patients survived 4 years [35].

If the initial white blood cell count was > 50 000/µl, 1% of patients survived 4 years compared with 52% survival at 4 years if the count was between 5 000/µl and 10 000/µl. Gee reviewed the Memorial Sloan-Kettering experience in both children and adults on their L2 protocol [36]. Six out of 98 patients did not respond and they had a median white blood cell count of 85 000/µl. If the white blood cell count was < 25 000/µl in the adults their median duration of response was 27 months compared with 15 months if the white blood cell count was > 25 000/µl. Of interest, 6 out of the 9 adult patients who relapsed with central nervous system involvement had an initial white blood cell count > 25 000/µl.

Shaw and Raab treated 25 adult acute lymphocytic leukemia patients with adriamycin, vincristine and prednisone and obtained a remission response of 72% [37]. If the white blood cell count was < 25 000/µl the remission duration was 17 1/2 months and survival 50 months compared with a remission duration of 4.8 months and a survival of 33 months if the initial white blood cell count was > 25 000/µl. Lister *et al.* studied 51 adult patients who were treated with vincristine, doxorubicin, prednisolone and 1-Asparaginase + radiotherapy and intrathecal methotrexate, and obtained a 71% complete remission rate and a median remission duration of 18 1/2 months [38]. Seven patients had an initial white blood cell count > 100 000/µl but none had any central nervous system complications. Of this group 3 died less than a month after diagnosis, and 4 patients went into complete remission which lasted to 25, 6, 3 and 5 months respectively. If the initial white blood cell count was > 10 000/µl, 26% of patients were in complete remission at 3 years compared with 61% if initial white blood cell count was < 10 000/µl.

Omura *et al.* studied 99 adult patients of whom 79 went into complete remission [39]. Their median time for relapse was 19.3 months and median survival was 26.1 months. They found an initial white blood cell count of > 100 000/µl in 24 out of the 99 patients. In contrast to others they could find no relationship between initial white blood cell counts and the complete remission or survival time.

Harousseau *et al.* reviewed 141 patients with acute lymphocytic leukemia of all ages who had an initial white blood cell count > 100 000/µl [40]. Seventy-one percent of the patients entered complete remission with a median duration of response of 6 months. The median survival time was 9 months for all patients and 11 months for those who went into complete remission. There did not appear to be a relationship between the white blood cell count and the response or survival once the white blood cell count rose over 100 000. They found that 91% of these patients had splenomegaly, 72% had hepatomegaly, 65% had lymphadenopathy, and 46% had a mediastinal mass. Ninety percent of these patients had a platelet

count < 100 000/μl but only 19% had a platelet count of < 20 000/μl. These patients' relapses occurred in the marrow (41 patients), meninges (21 patients), testicles (8 patients), and generalized (10 patients). Leimert *et al.* reviewed 55 adults with acute lymphocytic leukemia [41]. Sixty-nine percent went into complete remission with a median duration of remission of 9 months and median survival time of 13 months. There did not appear to be any relationship between the initial white blood cell count and the complete response, median duration of response, or median survival time.

Baccarani *et al.* reviewed 293 patients over the age of 11 with acute lymphocytic leukemia [42]. A complete response was obtained in 79.2% of patients. At 5 years, 20% of patients were alive. As noted by others their complete remission rate for patients with > 100 000/μl white blood cells was no different than patients with < 100 000/μl. However, of the 46 patients with > 100 000/μl, 41 eventually either died or relapsed giving an 11% survival at 5 years. In contrast, there were 69 out of 244 or 28% of patients surviving who had an initial white blood cell count of < 100 000/μl.

Few of the studies reviewed utilized lymphocyte marker studies. Baccarani *et al.* found that the percent relapse rate was least for E-SIg- (61%) and greatest for SIg+ (67%) and E+ (88%) [42].

Overall, these reports indicate that although complete remission rates remain reasonably good for adult acute lymphocytic leukemia patients, median duration of remission and survival are unsatisfactory. The current literature provides unequivocal evidence that current treatment for patients with elevated white blood cell counts > 100 000/μl remains unsatisfactory and other treatment modalities must be developed.

4.3 *Therapy*

Numerous therapeutic approaches for the hyperleukocytosis syndrome have been proposed based upon small numbers of patients. For prevention of intracerebral bleeding, emergency cranial irradiation utilizing 600 rads in a single dose has been recommended by Wiernik [43]. Optimum time and dose relationships for radiotherapy remain to be developed. It is apparent that single treatment radiotherapy regimens do not preclude later cranial radiation. However, radiotherapy has no effect on organ systems outside the radiation field.

Chemotherapeutic agents such as hydroxyurea have been used by Hoagland and Perry, and Grund *et al.* for a rapid cytolytic effect [44, 45]. Unfortunately, this agent takes up to four days before a 50% reduction in WBC is seen. In addition one runs the risk of aggravating metabolic disorders.

The advent of continuous and discontinuous flow centrifugation systems have permitted us to rapidly remove specific blood components from

patients who present with elevated leukemic cells. We, and others, have reported the efficiencies of these techniques in acute myelocytic and lymphocytic leukemia, chronic myelocytic and lymphocytic leukemia, and essential thrombocythemia [11, 46–49]. Between 1977 and 1982 we at the Mount Sinai Medical Center have therapeutically leukapheresed approximately 100 patients. At all times, patients tolerated the procedure well and had marked amelioration of their symptoms. Where apheresis techniques are not available, exchange transfusion has also been used effectively.

Lane reported a 5 year old patient with acute lymphocytic leukemia who presented with a leukocyte count of 285 000/μl and priapism [50]. Despite the child's small size he was able to be successfully leukapheresed reducing his peripheral leukocyte count to 42 000/μl over a 3 hour procedure. Removal of 1×10^{12} leukocytes was accomplished, which was three times the number estimated to be circulating at the initiation of the procedure. Kamen *et al.*, reported a 15 year old patient presenting with hyperleukocytosis, anemia and metabolic abnormalities who underwent an exchange transfusion over a 3 hour period producing a reduction of the initial white blood cell count from 457 000/μl to 200 000/μl [51].

Since 1976 it has been our policy to therapeutically leukapherese patients with acute leukemia presenting with > 100 000 cells/μl.

4.4. *Mount Sinai Hospital Experience*

We have treated 9 patients (> 15 years old) (6 male, 3 female) with a mean age of 39.8 years (range 16–62 years) (Table 5). All patients with initial WBC > 100 000/μl were leukapheresed prior to induction chemotherapy. Specific indications for treatment included hyperleukocytosis alone in one patient, leukemic meningitis and hyperuricemia in two patients, bleeding manifestations including diffuse petechiae in three and central nervous system bleeding in one patient, azotemia and hyperuricemia in one patient, and pregnancy in one patient.

The mean initial hemoglobin was 9.8 (range 6–13.9), platelet count was 65 000/μl (range 15–108 000/μl), and white blood count was 270 000/μl (range 109–586 000/μl). All patients had 3 or 4+ hypercellular marrows with > 75% blasts.

We have utilized a Haemonetics model 30 discontinuous blood cell separater for all our procedures. This unit is compact, easily movable and can adapt to either a one or two arm procedure. In all cases, patients were leukapheresed on an emergency basis, usually within the first 12 hours of admission. Patients were leukapheresed on a daily basis until they were stabilized. All procedures were extremely well tolerated by patients.

An average of 3.3 procedures (range 2-7) were performed over 3.6 days (range 2–9 days). The mean total of lymphoblasts removed from each

Table 5. Therapeutic leukapheresis of adult acute lymphocytic leukemia patients at the Mount Sinai Hospital

Patient	#1	#2	#3	#4	#5	#6	#7	#8	#9
Sex	M	F	M	M	F	M	M	F	M
Age	16	19	18	62	56	48	61	35	43
WBC	586000	294000	492000	109000	448000	158000	116000	113000	177000
Platelet	79000	95000	80000	108000	100000	28000	22000	15000	59000
Liver (CM)	1	3	3	0	9	0	1	3	4
Spleen (CM)	5	5	2	0	0	0	1	3	3
Bleeding	None	None	None	None	Petechiae	Petechiae	None	Retinal	Epistaxis
CNS	CSF+	None	CSF+	None	HA, LP−	None	None	CSF−	Headache
Uric acid	13.4	6.4	11.6	9.3	7.8	6.1	5.7	4.8	7.9
Renal insuff.	Bun 25	None	None	Bun 55	None	None	None	None	None
Chest X-ray	Med wide	Med wide	WNL	WNL	WNL		Hillar ade	WNL	WNL
Lymph markers		T cell	Null cell	T cell	Null cell		T cell	Null cell	Non T, PH+
TDT	+	+	+	+/−	+		−	+	+
Induction	CR	CR	CR	CR	CR		NR	NR	CR
Duration remission (MO)	91+	16	4	28	7+				1
Duration survival (MO)	91+	17	8	30	8+	5+	1	2	3
# Procedures	3	7	3	2	4	2	3	3	3
Yield WBC $\times 10^{11}$	14.5	11.76	17.4	2.54	15.57	1.36	6.79	5.07	8.52
Final WBC	100000	76000	291000	94000	138000	37400	71800	21500	128000
Initial circulating WBC $\times 10^{11}$	2.49	1.272	1.755	0.5614	23	8.37	6.38	4.18	
Final circulating WBC $\times 10^{11}$	0.427	0.3287	1.0913	0.4841	7.58	1.98	3.95	0.796	

patient was 9.28×10^{11} cells/µl (range $1.36\text{-}15.57 \times 10^{11}$ cells). The mean final white blood cell count after therapeutic leukapheresis was 106 000/µl (range 21.5-291 000 cells/µl), giving a mean reduction of white blood cells of 61%.

All patients were subsequently treated with chemotherapeutic combinations including vincristine, prednisone, daunomycin, 1-asparaginase, intrathecal methotrexate and cranial irradiation. Six patients went into complete remission and three patients failed induction chemotherapy. Of the patients who have reached complete remission, two patients are alive and remain in remission, 91+ and 7+ months after initiating therapeutic leukapheresis. Four deaths occurred at 3, 8, 17 and 30 months. In comparison with survival times obtained from reported series our patients have done extremely well.

From our experience with therapeutic leukapheresis in hyperleukocytic, acute lymphocytic leukemia of the adult, we recommend proceeding on an emergency basis when patients present with white blood cell counts > 100 000/µl. After a diagnosis is made patients are treated for any metabolic derangements and are then immediately started on therapeutic leukapheresis. Our ability to rapidly stabilize all patients treated in this manner, and our high complete response rates lead us to propose therapeutic leukapheresis as an initial treatment for all patients presenting with hyperleukocytosis in acute lymphocytic leukemia. Although, our study is limited in numbers, three of our patients have had significant durations of complete remission. Chemotherapy should be initiated as soon as the patient is metabolically stable.

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10. New Developments in the Etiology, Diagnosis, Treatment and Prevention of Infectious Complications in Patients with Leukemia

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1. INTRODUCTION

The last several years have witnessed significant improvements in the remission rate and survival of adults with leukemia. Infection, however, remains a major problem for these patients, particularly when they are granulocytopenic. During the last decade, considerable strides in supportive care have helped to reduce infection-related morbidity and mortality. The principles employed are unique to the compromised host and require a departure from those considered standard in general infectious disease. An appreciation of the approach to the diagnosis, management and prevention of infection in the patient with acute leukemia is essential for the clinical oncologist and infectious disease specialist. In this chapter we will focus on the emerging body of data which has defined the boundaries and principles of management. In so doing, we will highlight the new developments in the patterns of infection and the agents available to treat them. Our goal is to place current data and controversies in focus and perspective.

2. CHANGING PATTERNS OF INFECTIONS

2.1. *Bacteria*

While bacteria remain the most frequent cause of primary infections in patients with leukemia [1], the last decade has witnessed major changes in the organisms responsible for these infections as well as in their response to treatment. A multiplicity of factors contributed to these changes, including the selective pressure from empiric and prophylactic antimicrobial regimens, the colonization of the patient with new pathogens, the interruption of physical defense barriers by invasive diagnostic and therapeutic tech-

niques [indwelling catheters, implantable pumps, Ommaya reservoirs and mechanical ventilators (see section 5.1.)], and the stress of prolonged granulocytopenia. Once rendered granulocytopenic and on antibiotics, the compromized host is at constant risk for second or superinfections. Successful therapy demands cognizance of both the primary infectious pathogen(s) as well as the modifications or additions to therapy which are necessary to treat secondary or superinfections.

During the 1950's and early 1960's *Staphylococcus aureus* was the most frequent bacterial isolate in patients with leukemia. Availability of β-lactamase resistant antistaphylococcal penicillins provided effective therapy for *S. aureus* and during the late 1960's and 1970's, gram-negative bacillary organisms became the predominant bacterial pathogens (especially *E. coli, Klebsiella* sp. and P. *aeruginosa)* [2, 3]. Infection with these organisms was frequently accompanied by endotoxic shock and a high fatality but during the early 1970's the practice of promptly initiating empiric broad-spectrum antibiotics (particularly cephalosporins, aminoglycosides and carboxypenicillins) when the granulocytopenic patient became febrile, dramatically reduced the early morbidity and mortality associated with gram-negative sepsis [4]. During the last five years, infections due to *P. aeruginosa* have inexplicably decreased in many institutions, a phenomenom which clearly effects both the selection and probable success of initial empiric antibiotics. In some centers, gram-positive bacteria have again become the most common isolate in leukemia patients [5, 6]. *S. epidermidis,* once considered a contaminant, has emerged as a significant pathogen [7], especially in patients with indwelling venous catheters [8, 9]. A particular concern with *S. epidermidis* (and in some centers *S. aureus*) is its resistance to antistaphylococcal penicillins and cephalosporins [7, 10, 11]. Moreover, even when *in vitro* sensitivity to these antibiotics is demonstrated, clinical failures have been described [7, 12]. Thus, vancomycin has re-entered the therapeutic armamentarium [13]. While infections with *S. epidermidis* were previously considered minor, recent studies show that this organism can cause fatal pneumonia, endocarditis and meningitis, as well as uncomplicated bacteremias [14-16].

Alpha streptococci can also cause sepsis in patients with leukemia. While infrequent, some of these isolates are penicillin resistant [17]. Diptheroids, frequently skin contaminants, can also be multiply drug resistant (CDC group JK) and can result in serious infections, particularly in patients with prolonged granulocytopenia [18-22]. These organisms are also sensitive to vancomycin.

As new pathogens have emerged, so too have changes in antibiotic therapy (see section 4). Some of these changes have been to improve antibiotic spectrum or activity, others to overcome emerging antibiotic resistance. It is

of interest that antibiotic resistance has been highly variable and probably occurs more frequently with orally administered prophylactic antibiotics than with systemic therapy. For example, in spite of our constant use of cephalothin, gentamicin, and carbenicillin during the last dozen years, multiply resistant organisms have been isolated in less than 1% of cases [23]. It remains to be seen whether the present trend to use third generation cephalosporins and extended spectrum penicillins (which have less β-lactam stability) will result in greater resistance.

While anaerobic organisms are themselves an infrequent cause of infection in leukemic patients [1], the role they play in mixed infections and in maintaining host resistance to colonization by aerobic pathogens (i.e., colonization resistance) [24] has become increasingly appreciated. A number of currently employed antibiotics can eradicate the GI anaerobic flora, upsetting the patient's colonization resistance (see Section 7.2.2.).

2.2. *Fungi*

With improved control of bacterial pathogens, fungi have emerged as major pathogens in leukemic patients, especially when they experience prolonged granulocytopenia and protracted courses of antibiotics. The predominant fungal pathogens are *Candida sp, Aspergillus sp, Torulopsis glabrata, Cryptococcus neoformans* and the Phycomycetes [25]. There is considerable institutional variation in the predominant fungal pathogens, although in many centers, *Aspergillus* has increased in frequency in recent years. Unfortunately, the ability to diagnose even invasive fungal disease is difficult [26–29]. A number of investigators have attempted to use surveillance cultures [30, 31], and while these might be helpful for some organisms (e.g., nasal swabs positive for *A. flavus*) [32], they are not helpful for others (e.g., *Candida sp.*) [33].

As with bacteria, colonization of the host with fungi is an important prerequisite to infection. Once colonized, the risk for invasive disease and site(s) of infection depend upon the inherent virulence of the organism, the body burden, and the degree of host compromise. For example, while greater than 85% of patients at the NCI are colonized along their alimentary tract with *C. albicans,* only a minority develop infection [34]. In contrast, while isolation of *C. tropicalis* from body surveillance cultures is less frequent, 14 of 25 patients colonized with this organism developed a systemic infection, suggesting that *C. tropicalis* may be more invasive [35].

Improvement in blood culture technology may improve the isolation of certain fungi. For example, lysis-centrifugation (e.g., Dupont isolator system) appears more sensitive in detecting candidemia than conventional broth culture systems [36]. This is important since less than 20% of patients with disseminated candidiasis have positive fungal blood cultures by stan-

dard bottle inoculation. Similarly, the 'target' lesions seen in the liver with ultrasound or the microabscesses with abdominal CT scan provide improved methods to both diagnose and follow patients with disseminated candidiasis [37, 38]. It is still likely, however, that patients with fungal disease are being undertreated rather than overtreated. For example, in a recent study, 15% of patients with presumed transient, uncomplicated, catheter-associated fungemia had evidence of focal embolic lesions appearing 3 weeks to 8 months after catheter removal despite initially appearing asymptomatic [39]. This suggests that even these patients, once thought to be at low risk of invasive disease after catheter removal, require some systemic antifungal therapy.

Amphotericin resistant fungal organisms have been infrequent. However, Dick et al. observed that 55 of 747 isolates from 101 patients with leukemia were resistant to both amphotericin B and nystatin. These 55 isolates were from 6 patients, each of whom had received prior therapy with both these drugs [40]. While these cases remain unusual, they are of concern since amphotericin B remains the antifungal drug of proven efficacy for the immunocompromised host. There is, however, a spectrum of sensitivity for *Candida sp.* For example, some isolates of *C. parapsilosis* appear tolerant to amphotericin B [41] and all isolates of *Petryllidium boydii* are resistant to amphotericin B (although sensitive to miconazole [42]). It should be remembered that while persistent fungemia in patients receiving amphotericin B may represent drug resistance, it more likely is due to a persistent focus of infection (e.g., endocarditis, phlebitis) [43].

In some centers, *Aspergillus* has emerged as the most important fungal pathogen. This may be due to building construction resulting in the airborne spread of *Aspergillus* spores [44]. The rhinocerebral syndrome and pulmonary infection associated with *Aspergillus* are particularly troublesome since they are difficult to treat [45–47], especially in patients with prolonged granulocytopenia (see Section 5.8.).

Although less common, the phycomycetes (Mucor and Rhizopus) can also cause pulmonary disease and/or invade the sinuses causing a rhinocerebral syndrome [48]. *Torulopsis glabrata,* like *Candida sp,* can result in disseminated infection [49] and has recently been observed in patients receiving oral ketoconazole as a prophylactic agent [50] (to which *T. glabrata* is imperfectly sensitive). *Trichosporon sp,* an arthrospore forming yeast, can cause both local skin lesions as well as invasive disease in leukemia patients [51]. Unlike patients with lymphoma, cryptococcal meningitis does not appear to be increased in the leukemia patient. As well, while endemic fungal diseases such as histoplasmosis, coccidiodomycosis, and blastomycosis do not appear to be increased in frequency in leukemic patients, there appears to be an increase in their severity and invasiveness when infection occurs [52, 53].

2.3. *Mycobacteria and Nocardia*

Surprisingly, mycobacterial infection is uncommon in leukemia patients [54, 55] unlike the current prevalence of *M. avium-intracellulare* infection in patients with AIDS (acquired immune deficiency syndrome) [56]. Atypical mycobacterial infections may be increased in patients with hairy cell leukemia as compared with more common forms of acute leukemia [57]. Of note, when mycobacterial infection occurs, only half are due to *M. tuberculosis,* the remainder being atypical (particularly *M. fortuitum* and *M. kansasii*) [58].

Nocardia (both *asteroides* and *brasiliensis*) are uncommon pathogens, but are not adequately treated by most of the antibiotics used for the empiric management of the patient with leukemia. This organism should be considered in the differential diagnosis of the leukemia patient with a cavitary pulmonic infiltrate, particularly if there are skin lesions or evidence of CNS disease. Sulfadiazine is the drug of choice [59-62].

2.4. *Viruses*

The herpes viruses [*H. simplex* (HSV), *Varicella-zoster* virus (VZV), *Cytomegalovirus* (CMV)] are important as pathogens themselves [63], but may also suppress host defenses and lead to secondary bacterial or fungal infections. For example, Hamilton and colleagues demonstrated that rodents infected with CMV had a higher mortality when challenged with *P. aeruginosa* or *Candida sp.* than non-CMV infected controls [64]. The clinical corollary of this is patients undergoing cardiac transplantation, in whom CMV infections are associated with bacterial superinfection [65]. CMV is of particular importance in the leukemia patient undergoing allogeneic bone marrow transplantation. Blood products (particularly white blood cell transfusions) have been implicated with the seroconversion or infection of recipients [66]. The major target organ for CMV in the immunosuppressed leukemic patient is the lung and mortality with CMV pneumonitis ranges between 40-100% [67-69]. None of the developments in antiviral therapy (adenosine arabinoside, acycloguanosine, or interferons), alone or in combination, have demonstrated efficacy with this infection once established [70-72]. However, two recent studies, one at UCLA [73] and the other at the Fred Hutchinson Cancer Center [74] suggest a role for CMV immune plasma and CMV immune globulin for prophylaxis against pneumonitis putatively caused by this virus.

H. simplex is an important pathogen for several reasons. Firstly, it may be a primary or secondary cause of oral mucositis or esophagitis [75, 76]. Recognition and diagnosis is important since patients with HSV will respond to therapy with either adenosine arabinoside (10 mg/kg/day $\times$ 7 days) [77] or acycloguanosine (750 mg/m^2/day $\times$ 7 days) [78, 79]. Secondly,

H. simplex infections have been successfully prevented in leukemia patients undergoing bone marrow transplantation when they are treated prophylactically with systemic acyclovir [80].

3. NEW DEVELOPMENTS IN EVALUATION AND DIAGNOSIS

A continuing dilemma in the management of the neutropenic leukemic patient is the difficulty in differentiating life threatening infectious causes of fever from benign etiologies. The practical importance of this resides in the fact that 40–60% of leukemics who present with fever and granulocytopenia will not have a defined infectious etiology for their fevers [1, 3, 81]. Clearly, improved diagnostic methodologies would help in differentiating which of these patients with unexplained fever (FUO) actually required antibiotic therapy. A number of specific (e.g., serodiagnosis, improved culture technique) as well as nonspecific approaches (e.g., nuclear imaging) have been investigated.

3.1. *Nuclear Imaging*

For more than ten years gallium citrate has been known to accumulate in inflammatory lesions and has been used (with variable success) to localize sites of occult infection in non-neutropenic patients with FUO [82]. In the neutropenic cancer patient, however, gallium has several important limitations. Gallium localizes to sites of inflammation because of its avid binding to lactoferrin from granulocytes. Thus, gallium could not be expected to localize in granulocytopenic patients although this might be achieved if leukocytes were transfused together with the gallium. In general, two to three days are necessary for definitive gallium localization and nonspecific colonic accumulation and pulmonary uptake makes scanning less efficient [83]. Uptake of gallium by tumor cells (particularly lymphoid cells) is an additional problem [84].

As an alternative to gallium, indium-111 has been evaluated in neutropenic leukemic patients. In particular, indium has been used to label autologous or allogeneic leukocytes *in vitro* [85]. When transfused into animal models or surgical patients with known abdominal abscesses, these labelled leukocytes localize at the site of infection [86]. Indium oxine has been used most commonly because of its high labelling efficiency and minimal toxicity to granulocytes [87]. ABO compatible HLA unmatched leukocytes have been utilized in most studies with rapid migration of cells to sites of infection allowing scanning to be done within hours after transfusion [88–90]. Although Dutcher *et al.* reported poor trafficking of leukocytes in alloimmunized patients [91], McCullough *et al.* found anti-HLA antibodies to be

Table 1. Studies of Indium-III-labeled Donor Leukocyte Scans in Febrile Leukemics

Study **Year** [ref]	Number Patients Studied	Underlying Disease	WBC at Time of Scan	Number WBCs Transfused	Time Between Transfusion and Scan	Results and Comments
Anstall et al. **1982** [88]	8	AML 1 CML in blast crisis 1 Unspecified neoplasm 6	<300/mm^3	5.2×10^8— 1.1×10^9	24 hours	5/8 negative scans with negative abCT or abUS, negative physical and subsequent recovery without sequlae. 3/8 positive scans, 1 clinically evident at time of scan, 1 occult abdominal abscess verified by CT scan, 1 occult perirectal abscess subsequently verified by physicial exam.
Dutcher et al. **1981** [89]	14	ANLL 10 ALL 1 CML in blast crisis 1 Lymph. 1 Breast ca. 1	<500/mm^3	0.63×10^8— 1.5×10^8	30 minutes, 4 hours, + 24 hours	13/14 positive scans 30 minutes post transfusion 14/14 positive scans 24 hours post transfusion. All patients with known sites of infection prior to scanning. All scans positive at known sites at 24 hours.
Alavi et al. **1980** [90]	7	AML 7	<500/mm^3	$\sim 10^8$	1 hour + 18 hours	4/7 positive scans at presumed sites of infection. 3/7 negative scans. All patients with "presumed" sites of infection. Of the 4 positive, one was initially negative with filtration collected WBCs. Of the 3 negative scans all sites were felt to be transient and/or resolved by the time of scan.

insignificant in granulocyte migration and has supported the use of HLA unmatched ABO compatible donors [92]. Table 1 summarizes results of the recent studies which have examined the value of Indium-111 leukocyte scans in neutropenic patients. While potentially encouraging, the number of patients studied to date remains small and appropriate control patients are lacking. Even when the scan is positive, however, the microbiologic identification of the putative infection still requires additional investigation.

3.2. *Serodiagnosis*

The role of ELISA (enzyme-linked immunosorbent assay), RIA (radioimmunoassay), CIE (counter immunoelectrophoresis), and LPA (latex particle agglutination) in rapidly diagnosing infections is an area of active investigation. While these techniques have been successfully employed for a number of common viral and bacterial (e.g., *N. meningiditis, H. influenza, S. pneumonia*) infections, they have been disappointing for the rapid diagnosis of the bacterial and fungal infections which occur in cancer patients [93, 94]. Similarly, the ability to measure circulating endotoxin using the limulus assay, has been unsuccessful [95]. The obvious utility of serodiagnosis for early diagnosis of fungal infections has been met with particular enthusiasm. Initial studies focused on measurement of agglutinating and precipitating antibodies against *Candida sp.* [96, 97], but this approach had too many false negatives, presumably because of the low antigenicity of *Candida* [98].

Use of CIE to detect *Candida* precipitins has also been disappointing in differentiating patients simply colonized with *Candida* from ones with invasive infection [99]. A recent objective has been to measure circulating antigens (e.g., mannan) [100–104] or arabinatol/creatinine clearance ratios [105, 106]. As shown in Table 2, several studies appeared promising initially, but with the addition of more patients or controls now seem disappointing. We have studied patients with extensive hepatic involvement with *Candida* in whom circulating mannan antigen could not be detected, suggesting that the ease of detection may depend more on the antigen load in body fluids (blood, ascites, urine, CSF) than on the actual tissue burden,

Table 2. Serodiagnosis of Invasive Candidiasis by Antigen Detection.

Study **Year** [ref]	Underlying Diseases	Antigen-Detected	Method	Positive Results	Comments
Miller et al. **1974** [100]	Unspecified	Mannose – derived from mannan	GLC[1]	0/16 normal controls 0/11 mucosal candidiasis 0/19 bacteremia 6/6 candidemia 2/4 invasive candidiasis	Test not sensitive. Too few patients with "invasive" disease tested.
Weiner et al. **1976** [101]	Neoplasia, plus other, (no leukemics)	Mannan	PHA-I[2]	1/90 normal controls 2/144 hospitalized patients without evidence of candida 0/10 with other mycotic diseases 0/20 with candidia colonization 0/4 with catheter-associated candidemia 2/5 with invasive gastrointestinal candidiasis 4/14 with invasive candidiasis positive	Test not sensitive or specific. Few patients with invasive disease studied.
Segal et al. **1979** [102]	Leukemia Lymphoma Solid tumor	Mannan	ELISA[3]	13/20 without invasive candidiasis <10% inhibition by ELISA 7/20 without invasive candidiasis 10-20% inhibition by ELISA 3/7 with invasive candidasis >20% inhibition by ELISA 4/7 with invasive candidiasis >50% inhibition by ELISA	Few patients all studied retrospectively. Need for more patients and standard cutoffs for "positive" test.
Kerkering et al. **1979** [107]	Leukemia, Carcinoma, other diseases	Cell-wall polysaccharide	CIE[4]	0/20 normal controls 0/15 mucosal candidiasis 13/48 "suspected" invasive candidiasis 8/13 antigen positive proven at biopsy or postmortem to be true positives	Not all patients with "suspected" disease were examined by biopsy or autopsy. Of 35 "negative" patients none were treated with systemic antifungal therapy. None were subsequently proven to have invasive disease.
Weiner et al. **1979** [103]	Not specified	Mannan	RIA[5]	0/22 normal controls 0/7 colonized with candida 0/11 with mucosal candidiasis 0/2 with invasive aspergillosis 1/1 with catheter-associated candidemia 1/3 invasive gastrointestinal candidiasis 5/11 with invasive candidiasis	Less than 50% sensitive for invasive disease.

[1]GLC = gas-liquid chromatography, [2]PHA-I = passive hemagglutination inhibition, [3]ELISA = enzyme-linked immunoassay, [4]CIE = counterimmunoelectrophoresis, [5]RIA = radioimmunoassay.

Table 2. Serodiagnosis of Invasive Candidiasis by Antigen Detection.

Study **Year** [ref]	Underlying Diseases	Antigen-Detected	Method	Positive Results	Comments
Kiehn et al. **1979** [105]	Neoplastic diseases	Arabitinol	GLC	54/65 controls 0 μg/ml arabinitol 11/65 controls <0.2 μg/ml arabinitol 3/28 colonized with candida 1-2.2 μg/ml arabinitol 25/28 colonized with candida <1 μg/ml 15/20 with invasive candidiasis >1.0 μg/ml arabinitol	Overlap of serum arabinitol levels in patients with colonization and invasive disease. Renal failure nonspecifically elevates levels in some patients without invasive disease.
Meunier-Carpentier **1981** [108]	Neoplastic disease	"Candida-antigen"	PHA-I	0/49 controls 4/22 "partial" inhibition on PHA-I assay when colonized with candida 19/22 considered positive by PHA-I with invasive candidiasis (3 with partial inhibition)	Test shows overlap in patients colonized with candida and those with invasive disease (i.e. tests showing partial inhibition).
Lew et al. **1982** [104]	Leukemia, carcinoma, other	Mannan	ELISA	0/90 hospitals controls (i.e. <10 ng/ml antigen) 8/15 with invasive disease (2 with <10 ng/ml antigen)	Test shows overlap in controls and patients with known invasive candidiasis. Only 53% sensitive.
Gold et al. **1983** [106]	Leukemia and other neoplasms	Serum Arabinitol/ Creatinine	GLC	3/88 controls with arabinitol/ creatinine ratio >1.5 16/25 with invasive candidiasis had arabinitol/creatinine ratio >1.5	Neither specific or sensitive.

Table 3. Serodiagnosis of Invasive Aspergillosis by Antibody Detection

Study **Year** [ref]	Underlying Disease(s)	Method(s) of Antibody Detection	Positive Results	Comments
Young et al. **1971** [109]	13-Leukemia 1-Hodgkin's 1-"Leukopenia"	ID[1] CF[2] IEP[3] IFA[4]	1/1 non-invasive broncho-pulmonary aspergillosis 0/15 invasive aspergillosis by all methods	Early retrospective study of serum samples from patients with known invasive disease. Only positive occurred in patient without invasive disease. Tests neither sensitive nor specific.
Schaefer et al. **1976** [110]	10-Leukemia	ID[1]	7/10 invasive aspergillosis	Six additional patients found to be positive; however, autopsy or biopsy proof of aspergillus lacking. Test 70% sensitive, ?% specific.
Marier et al. **1979** [111]	8-Leukemia 11-other neoplasms and non-malignant diseases	RIA[5]	7/58 controls with other mycotic or bacterial disease 15/19 invasive aspergillosis	Despite greater sensitivity than concurrently run CIE and ID, RIA still only 79% sensitive.
Holmberg et al. **1980** [112]	10-Leukemia, aplastic anemia, and other immunodeficiency	CIE[6] ELISA[7]	0/12 by CIE, 1/12 by ELISA febrile granulocyropenic controls 7/10 by CIE, 8/10 by ELISA invasive aspergillosis	ELISA more sensitive, however less specific. Small number of patients studied.
Gold et al. **1980** [113]	55-Neoplasia	PHA[8] ID[1]	0/122 by ID, 4/122 >1:160 by PHA hospitalized cancer patients. 12/14, 1/14 with titer 1:1280 by PHA healthy microbiology lab workers. 16/55 by ID, 15/55 by PHA invasive aspergillosis	Tests neither sensitive nor specific for invasive aspergillosis.
Mishra et al. **1983** [114]	8-Not specified	ELISA[7]	1/50 healthy donors 3/69 other pulmonary diseases 35/35 ABPA or aspergilloma 1/4 systemic candidiasis 6/8 invasive aspergillosis	Test not specific for invasive aspergillosis.

[1]immunodiffusion, [2]complement fixation, [3]immunoelectrophoresis, [4]indirect fluorescent antibody, [5]radioimmunoassay, [6]counter-immunoelectrophoresis, [7]enzyme linked immunoadsorbent, [8]passive hemmagglutination.

Table 4. Diagnosis of Invasive Aspergillosis by Antigen Detection

Study **Year** [ref]	Underlying Disease(s)	Method of Antigen Detection	Positive Results	Comments
Shaffer et al. **1979** [115]	3-Leukemia	RIA[1] of serum	0/20 controls with leukemia and no evidence of fungal disease or patients with other mycosis 2/2 (?3/3) invasive aspergillosis	Few patients studied, one "positive" patient not proven to have disease by biopsy or autopsy.
Weiner **1980** [116]	2-Leukemia 1-Pancreatitis and sepsis 1-Influenza and sepsis 3-Not specified	RIA[1] of serum	0/27 normal donors 0/50 controls with non-invasive aspergillosis, sepsis, other mycosis 4/7 invasive aspergillosis	Only 57% sensitive for invasive disease.
Andrews and Weiner **1982** [117]	40 Neoplasia, and misc. pulmonary diseases	RIA[1] of broncho-alveolar lavage fluid	3/36 controls with other pulmonary disease 2/2 aspergilloma 2/2 invasive aspergillosis	Follow up of controls unknown. Only 2 patients with invasive disease studied. Test not specific for invasive aspergillosis.
Weiner et al. **1982** [118]	14 Leukemia	RIA of serum	0/24 normal controls 0/8 without aspergillosis 4/6 invasive aspergillosis	Few patients studied, all leukemia patients. Test only 66% sensitive.

[1]radioimmunoassay.

and therefore the invasiveness of the disease. It deserves mention that while kits to detect mannan are now commercially advertized and available, the number of false negatives does not make them reliable and they should be used with caution, if at all. A similar caveat applies to the detection of *Aspergillus* antigens. As shown in Tables 3 and 4, the limited sensitivity of antigen detection does not presently permit this approach to serve as a reliable tool for patient management. Thus, the current use of fungal serodiagnosis should be considered experimental.

3.3. *Surveillance Cultures*

The rationale for microbial surveillance of the leukemic patient is well established [30]. First, it has been known for more than a decade that more than 80% of the organisms ultimately responsible for infection can be found in the patient's endogenous flora. Second, nearly half of these organisms are acquired by the patient during hospitalization [119]. Fainstain *et al.*, observed that the 'normal' oral and stool flora of leukemic patients was replaced within days after admission to the hospital by gram-negative rods and fungi [120]. This microbial shift is also effected by altered epithelial and mucosal binding receptors [121] and by changes in the aerobic/anaerobic balance secondary to chemotherapy and antibiotics [24]. The practical relevance is that knowledge of colonization with certain organisms (e.g., *P. aeruginosa, C. tropicalis*) may predict whether a patient will develop a subsequent infection when rendered granulocytopenic [122, 35]. Indeed, serial surveillance can be helpful in monitoring the changing host flora and in

tracking the emergence of resistant organisms. However, it is also labor intensive and expensive, and the question must be asked of how such cultures influence initial and subsequent antibiotic therapy. A recent study assessed the utility of routine nose, throat, stool and urine cultures [31]. While the organisms shown to be responsible for infection were found in 60% of these surveillance cultures, they were not found to be helpful in guiding antibiotic therapy. In practice, no one body site could be monitored and multiple isolates were usually obtained making prospective assignment of the 'true pathogen' guesswork. However, restricted surveillance may be of benefit at some centers. For example, certain hospitals have experienced an increased incidence of *Aspergillus* infections. Nasal swabs had been used to identify patients who become colonized with *Aspergillus* (particularly *flavus*) and may help to identify patients at heightened risk for infection [32]. Similarly, monitoring stool cultures for the acquisition of *P. aeruginosa* has been found to be useful by some investigators, particularly in patients with prolonged neutropenia [122]. Serial surveillance of patients undergoing protected isolation has been found to be useful [123]. Thus, while indiscriminate surveillance is expensive and not particularly useful, well considered and limited surveillance may be useful in certain situations at certain centers.

4. DEVELOPMENTS IN ANTIMICROBIAL THERAPY

The last several years have witnessed an explosion of new antimicrobial agents. These include the third generation cephalosporins, extended-spectrum penicillins, the imidazole antifungal agents and interferons (see Tables 5-8) [124, 125]. Each offers the potential for improving therapy but at the same time provides a confusing (if not awesome) array of agents to assimilate and evaluate. It is imperative, therefore, to critically assess the merits and disadvantages of each of these agents before they are incorporated into the therapeutic armamentarium.

4.1. *Empiric Antibiotic Therapy*

The inability to distinguish the febrile neutropenic patient with a life-threatening infection from one with a non-lethal cause of fever justifies the initiation of empiric antibiotics once the patient becomes granulocytopenic (generally defined as less than 500 polymorphonuclear leukocytes per ml) and febrile. This approach has clearly led to a significant reduction in early infection related morbidity and mortality [3, 4]. As such, the primary objective of initial empiric therapy is to protect the patient from immediate death. The antibiotic regimen, therefore, must contain activity against the

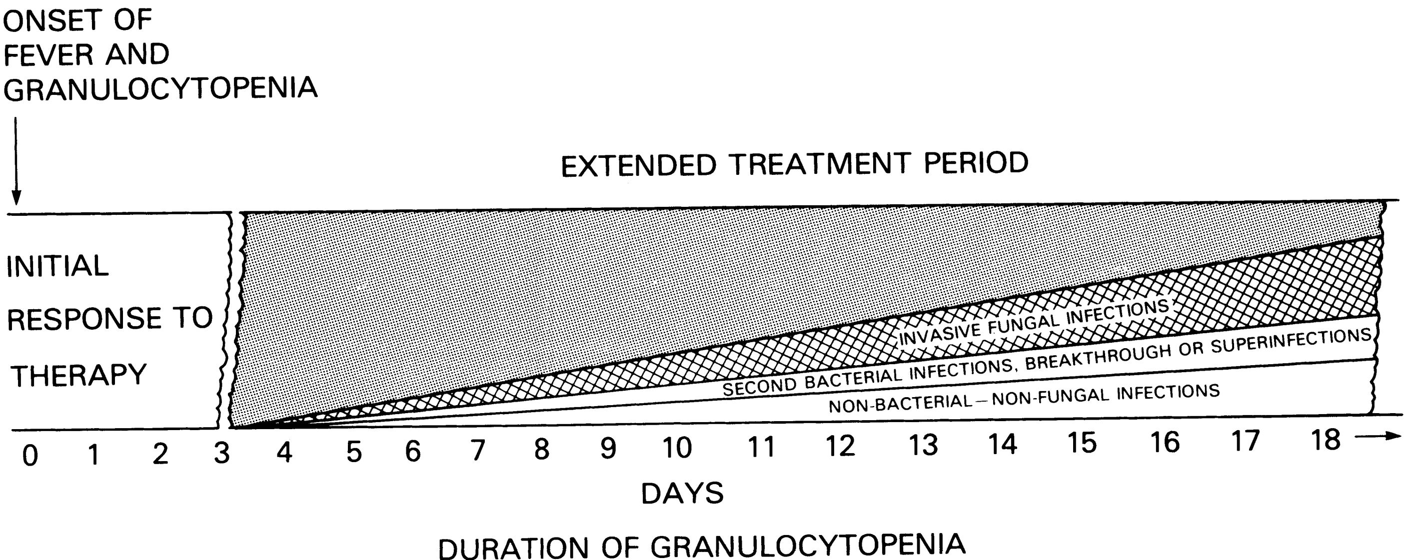

Figure 1. The efficacy of antibiotic regimens in cancer patients should be evaluated separately for initial empiric therapy (i.e., the first 72 hours after the initiation of antibiotics) and during extended therapy (i.e., while the patient remains persistently granulocytopenic and at continued risk for new infections).

major pathogens, particularly gram-negative bacilli and gram-positive cocci. Although they comprise a large component of the endogenous microflora, anaerobic bacteria are an infrequent cause of primary infection in granulocytopenic patients [1]. Fungi (particularly *Candida sp.*) can be the primary cause of an infection, but are more likely to be a problem for patients with protracted granulocytopenia who are already receiving antibiotics [25].

A large number of antibiotics have been evaluated for the primary empiric management of the febrile granulocytopenic patient. It is important to underscore that the effectiveness of an empiric antibiotic regimen must be evaluated for both the initial treatment of the febrile-granulocytopenic patient (i.e., within the first 72 hours after the initiation of antibiotics) and during extended therapy (i.e., while the patient remains persistently granulocytopenic). As illustrated in Figure 1, these are quite different periods. The success of initial therapy is focused on the efficacy of the antibiotic against bacterial pathogens. In contrast, extended therapy must take into account the fact that patients with persistent granulocytopenia are at continued risk for second infectious complications. The risk for second infections increases as the duration of neutropenia becomes more prolonged and includes other bacteria as well as non bacterial pathogens, particularly fungi.

For initial empiric therapy, the range of potential gram-positive *(S. aureus,* streptococci) and gram-negative (*E. coli, K. pneumoniae, P. aeruginosa*) organisms has traditionally necessitated two-or-three drug combinations, usually with a cephalosporin, an aminoglycoside and/or an antipseudomonal penicillin [3, 126, 127]. In addition to their spectrum of activity, other desired properties in the selection of antibiotics include the achievement of bactericidal levels and synergy. Results from a large number of trials have failed to reveal convincing differences between most of the two-or-three drug regimens used for initial empiric management. It is noteworthy that several investigators have shown that successful therapy for gram-negative bacteremia is optimal (i.e., > 80% success) if the isolate is sensitive to and treated with two antibiotics, compared to when the isolate is sensitive to only one antibiotic, in which case the success rate is approximately 59% [4, 128]. While combination therapy may be preferable, especially when antibiotics are synergistic, the benefit of this therapy must be balanced against the additive risk for organ toxicity which occurs with multiple drug treatment.

A question of current interest is whether combination therapy (and synergy) is important in light of the development of the third generation cephalosporins and extended spectrum penicillins. Several of these new antibiotics have exceedingly broad-spectrum activity, including *P. aeruginosa* as well as the Enterobacteriaceae, *Serratia, Citrobacter,* indole positive *Proteus,* and anaerobes (including *B. fragilis*). However, the third generation cepha-

losporins are not as active against *Staphylococci* and *Streptococci* as are the first generation cephalosporins, and none are effective against enterococci. Nonetheless, these agents achieve serum levels which can be more than a hundred fold higher than the MICs and MBCs of gram-negative bacteria, raising the possibility that they might be effective as single agents. The advantages of the third generation cephalosporins are their minimal toxicity and long serum half-lives. While these new drugs are expensive, their cost must be evaluated within the context of their efficacy and clinical application.

The new cephalosporins and penicillins which have been most studied to date are cefotaxime, moxalactam, cefoperazone, ceftazidime, piperacillin and azlocillin (see Tables 5 and 6). It is important to compare these agents to proven antibiotics (e.g., first generation cephalosporins, aminoglycosides, carboxypenicillins) as well as to each other in their efficacy, toxicity and cost effectiveness. Table 9 summarizes some recently completed or ongoing trials studying these new antibiotics. Wade *et al.* evaluated ticarcillin and amikacin versus piperacillin and amikacin in patients with clinically or microbiologically documented infections, and found them to be comparable [129]. In

Table 5. Extended Spectrum Penicillins Used in Patients with Leukemia

Antibiotic	Trade Name	Spectrum	Dosage	Side Effects	Cost
Carbenicillin	Pyopen Geopen	Non-penicillinase producing gram-positive cocci, gram-negative diplococci and *H. influenza*; enterococci; some *E. coli*, *Salmonella* and *Shigella sp*; *P. aeruginosa*. Anaerobic bacteria including some *Bacteroides sp*, some *Clostridium sp*, *Peptostreptococcus sp*, Peptococcus sp, *Fusobacterium sp* *Klebsiella sp* and some *Serratia sp* are resistant.	500 mg/kg/day in 6 divided doses	Allergic reactions Hypokalemia Platelet dysfunction	$ 5.49/5 gm
Ticarcillin	Ticar	Same as carbenicillin	300 mg/kg/day in 6 divided doses	Same as carbenicillin	$ 7.09/3 gm
Mezlocillin	Mezlin	Same as carbenicillin **but also** *Klebsiella sp*	300 mg/kg/day in 6 divided doses	Allergic reactions	$ 9.45/ 3 gm
Azlocillin	Azlin	Same as carbenicillin **but with** increased activity against *P. aeruginosa*	300 mg/kg/day in 6 divided doses	Allergic reactions	$11.82/3 gm
Piperacillin	Pipracil	Same as carbenicillin **plus** *Klebsiella sp* and increased activity against *P. aeruginosa*	300 mg/kg/day in 6 divided doses	Allergic reactions	$10.82/3 gm

Table 6. Cephalosporins Used in Patients with Leukemia.

Antibiotic	Trade Name	Spectrum	Dosage	Side Effects	Cost	Comments
		FIRST GENERATION				
Cephalothin	Keflin	Gram-positive cocci, including staphylococci, streptococci (except enterococci), some aerobic gram-negative rods *(Klebsiella, E. coli).*	200 mg/kg/day in 6 divided doses	Allergic reactions	$ 3.31/gm	Best gram-positive coverage
Cefazolin	Ancef Kefzol	Same as Cephalothin	100 mg/kg/day in 4 divided doses	Allergic reactions	$ 2.82/gm	Longer half-life than cephalothin
		SECOND GENERATION				
Cefamandole	Mandol	Same as cephalothin **plus** *Enterobacter sp*, indole-positive *Proteus sp.*, and *H. influenza*	200 mg/kg/day in 6 divided doses	Allergic reactions	$ 5.95/gm	
Cefoxitin	Mefoxitin	Same as cephalothin **plus** indole-positive *Proteus sp.* and *Bacteroides sp.* (including *fragilis*)	200 mg/kg/day in 6 divided doses	Allergic reactions	$ 6.06/gm	Excellent anaerobic coverage.
		THIRD GENERATION				
Cefotaxime	Claforan	Same as cephalothin **plus** *Enterobacter sp.*, indole-positive *Proteus, H. influenza, Bacteriodes sp., Citrobacter sp., Serratia sp.* and some *P. aeruginosa.*	200 mg/kg/day in 6 divided doses (max. 12 g/day)	Allergic reactions	$10.93/gm	All third generation cephalosparins are less active against gram-positive cocci than first-generation drugs. None are effective against enterococci or *Listeria*. They have variable anaerobic coverage and are different in their efficacy against *P. aeruginosa.*
Moxalactam	Moxam	Same as cefotaxime **but also** covers *B. fragilis.*	200 mg/kg/day in 3 divided doses (max. 12 g/day)	Allergic reactions. Antabuse reactions. Hypoprothrombinemia.	$12.20/gm	Same comment as Cefotaxime.
Cefoperazone	Cefobid	Same as moxalactam **but with better** *P. aeruginosa* activity.	200 mg/kg/day in 3 divided doses (max. 12 g/day)	Same as moxalactam	$ 9.50/gm	Same comment as Cefotaxime.
Ceftazidime	Investigational	Same as cefoperazone but with less activity against anaerobes. Most active against P. aeruginosa.	90 mg/kg/day in 3 divided doses (max. 6 g/day)	Allergic reactions		Same comment as Cefotaxime.

a second study at the same institution, deJongh *et al.* compared the previous 'best regimen' (i.e., ticarcillin and amikacin) to moxalactam and amikacin [130]. Interestingly, although the patient population was similar in both trials, the results with ticarcillin and amikacin was 80% in this second study compared to 56% in the first trial. The authors relate this to more careful pharmacologic monitoring and dose adjustment of amikacin, underscoring the importance of serially monitoring aminoglycoside drug levels.

Double β-lactam combinations are being evaluated at two centers (Table 9), both comparing moxalactam and piperacillin to moxalactam and amikacin [131, 132]. In the UCLA study, preliminary overall results are comparable for these two regimens, although the subset of patients who had microbiologically proven infections appeared to fare better with moxalactam and piperacillin (92%) compared to moxalactam and amikacin (69%); however, only a small number of patients have been studied to date [131]. DeJongh and his colleagues find both the double β-lactam combination and the β-lactam and aminoglycoside combination to be comparable in efficacy, but report a lower incidence of ototoxicity when an aminoglycoside was avoided [132].

Although combination therapy has served as the standard, at least three studies have addressed the possibility of using a single drug for the initial management of the febrile granulocytopenic patient. Two of these trials have employed moxalactam, one comparing moxalactam to nafcillin and tobramycin and the second to ticarcillin and tobramycin. Both trials have shown a 60-69% response to initial empiric therapy with moxalactam, although only small numbers have been studied so far. However, the study by Pickard *et al.* [133] had a surprisingly low rate of success with ticarcillin and tobramycin in comparison to other centers, while the nafcillin and tobramycin regimen utilized by Alanis *et al.* [134] would likely be problematic in larger trials since it provides poor coverage for *Pseudomonas*, relies totally on an aminoglycoside to cover enterobacteriaciae, and lacks adequate coverage for *Klebsiella sp.* Hence, while single drug therapy with moxalactam was similar to combination therapy in these two studies, this should not be interpreted as evidence for success, since neither of the comparative regimens were optimal. In assessing the results of a comparative trial, it is also imperative that the standard arm be of established efficacy.

In an ongoing study at the NCI [135], we are comparing monotherapy with an investigational third generation cephalosporin, ceftazidime, to our standard three-drug combination of cephalothin (Keflin), gentamicin, and carbenicillin (KGC). We specifically chose ceftazidime because its achievable serum level exceeds the MICs of predominant pathogens by 10–100 fold. During a fifteen month period we have randomized 204 patients (ranging in age from 1–75 years) who presented with fever and granulocytopenia to

initial empiric therapy with either ceftazidime or KGC. The initial (pre-antibiotic) evaluation revealed a defined infectious etiology for the fever in 30% of the patients while the fever was unexplained (i.e., FUO) in the remaining 70%. We have evaluated the initial response to therapy during the interval of the study: For patients with unexplained fever, the overall success (i.e., patients who recovered and came off antibiotics) was 96% for patients randomized to KGC versus 98% for patients randomized to ceftazidime. To date, a relatively small number of patients with documented infections have been studied. Nonetheless, the overall response of patients with clinically or microbiologically documented infections is comparable in patients randomized to KGC (88%) versus ceftazidime (84%). Of 21 granulocytopenic patients with bacteremia, 20 cleared their bacteremia and showed an initial objective response when treated with ceftazidime. Although our findings are preliminary, these data suggest that ceftazidime may be successful for the initial therapy of granulocytopenic patients with fever or documented infections. We are continuing this trial in order to establish more definitive conclusions.

It would seem, therefore, that the initial empiric management of the febrile granulocytopenic patient can be accomplished successfully with a variety of two or three drug antibiotic combinations. Moreover, with new antibiotic developments, even a single drug may be adequate for initial empiric therapy. The role of monotherapy requires further clinical confirmation and especially determination of whether resistant organisms will emerge to negate the apparent benefits of these new drugs.

Conversely, while present data suggest that there is no apparent advantage to three versus two (and perhaps even one) antibiotic(s) in the initial empiric therapy (i.e., within the first 72 hours), it is important to underscore that these findings might not apply to situations where protracted courses of antibiotics are necessary. In such settings, three-drug combinations may turn out to have an advantage, by providing both systemic prophylaxis as well as therapeutic benefit. Hence, the value of antibiotic regimens must be evaluated separately for initial versus continued therapy.

Another important aspect of initial antimicrobial therapy is the spectrum of microbial isolates and their antibiotic sensitivities at any given institution. For example, if the predominant isolates at a given center are *Klebsiella* or *Serratia,* the choice of initial therapy might differ from that at centers where *E. coli* and *S. aureus* are the predominant pathogens. It is also important to recognize that the increased use of indwelling catheters and the emergence of *S. epidermidis* as an important pathogen, may eventually necessitate the inclusion of antibiotics like vancomycin in the inital drug regimen [see Section 2.1].

Table 7. Antifungal Agents

Drug	Spectrum	Dose and Pharmacokinetics	Side Effects	Comments
Amphotericin-B (Fungizone)	Vitrually all yeasts and filamentous fungi (*P. boydii* is an exception)	**Intravenous** 0.5-1.0 mg/kg/day Peak level 0.5-2.0 μg/ml T½ approximately 24 hrs. Less than 5% excreted into urine and only 20% biliary excretion.	Fever, chills, rigors Hypokalemia and nephrotoxicity. Anemia and thrombocytopenia with prolonged use. Pulmonary toxicity with granulocyte transfusions.	Only drug of proven efficacy in immunocompromised hosts for treatment of invasive disease. Total dose varies from 1 gm for single organ-site infection to 2 gms for disseminated disease. Low dose therapy (0.1 mg/day) may be effective for mucositis or esophagitis.
5-Fluorocytosine (Flucytosine, Ancobon)	*Cryptococcus, Candida, Torulopois,* Chromomycosis	**Oral** 50-150 mg/kg/day Peak level is 30 μg/ml T½ is 3-4 hrs but excretion is impaired with renal failure.	Myelosuppression Hepatic and GI toxicity	Resistance emerges when used alone. However, combination therapy (amphotericin + 5-fluorocytosine) is only proven for cryptococcal meningitis.
Clotrimazole (Lotrimin)	*Candida sp*, dermatophytes	Topical therapy only: Suppositories or trouches Rapidly inactivated by hepatic enzymes if administered systemically.	None with topical therapy (with systemic administration can cause nausea, vomiting, diarrhea, GI bleeding, hepatic dysfunction, visual aberrations, depression).	Can be used for oral thrush and may be effective for esophagitis caused by candida.
Miconazole (Monistat)	*Candida sp, Aspergillus sp, Zygomycetes, Torulopsis, Cryptococcus, Petriellidium, Blastomyces, Coccidioides, Histoplasma, Paracoccidioides, Sporothrix.*	**Intravenous** 1500-3600 mg/day Peak level 2-8 μg/ml; falls rapidly to 0.1-0.2 in triphasic pattern. Crosses blood-brain barrier poorly.	Hyponatremia, Anemia, Thrombocytosis, Nausea, Vomiting, Hyperlipidemia, Pruritis, Cardiac Arrythmias and rarely Allergy, CNS toxicity.	No proven efficacy in invasive fungal infections in immunocompromised hosts. Relapses frequent after therapy.
Ketoconazole (Nizoral)	Similar to miconazole	**Oral** 200-400 mg/day Peak level 2-13 μg/ml T½ 2-8 hrs Extensively metabolized; crosses blood-brain barrier poorly.	Nausea, Vomiting, rarely Hepatic enzyme elevation, Dizziness, Drowsiness, Lethargy, Gynecomastia, Adrenal insufficiency	While effective in chronic mucocutaneous candidiasis, no proven efficacy in invasive fungal infections in cancer patients.

Table 8. Antiviral Agents

Agent	Trade Name	Formulations	Dose	Pharmacokinetics	Indications	Side Effects
Adenosine arabinoside	Vidarabine	Intravenous (poorly soluble) Topical	10-15 mg/kg/day as 12 hr. infusion	Peak = 4-8 μg/ml T½ 3-5 hr with 42-53% renal excretion 50% of plasma level in CSF Requires dose modification for renal failure	*H. simplex* encephalitis, mucositis and esophagitis. Dermatomal zoster in immunosuppressed patients Primary varicella (No efficacy against CMV)	Anorexia, nausea, vomiting, diarrhea Myelosuppression (rare) Neurotoxicity (tremors, seizures) Excessive fluid requirements Possible teratogenicity.
Acycloguanosine	Acyclovir	Intravenous Topical Oral (investigational)	10-15 mg/kg/day in 3 divided doses	Peak = 30-50 μM T½ 2.2-5.0 hrs Urinary excretion is 30-76%	Same as adenosine arabinoside (No efficacy against CMV)	Neurotoxicity (at high doses) Thymidine kinase resistant mutants have been described.
Interferons		INF-2 (leukocyte) IFN-β (fibroblast) IFN-γ (immune) (Topical and systemic formulations)	1×10^4 – 5×10^5 U/kg/day	T½ 4-6 hrs	Dermatomal zoster in immunosuppressed patient Primary varicella (No efficacy against CMV, even when used in combination with other agents)	Local pain, fever, alopecia, fatigue anorexia, bone marrow suppression *Note:* These side effects are also observed with the recombinant DNA preparations and hence are not due to impurities.

4.2. *The Patient with Continued Fever and Granulocytopenia*

The necessary modifications or durations of therapy are especially perplexing when the patient's initial evaluation has failed to reveal a defined infectious etiology for their fever. This is not a trivial problem, since 40–

70% of patients who become febrile while granulocytopenic have such unexplained fevers (FUO's).

During the past eight years, we have tried to define rational guidelines for management by comparing the common (but frequently arbitrarily applied) treatment options utilized in these patients. The major questions are how long to continue empiric antibiotics and whether, if antibiotics are continued, this places the patient at increased risk for secondary or superinfection as well as for antibiotic-related organ toxicity.

It seems clear that these patients with unexplained fever can be divided into low and high risk groups. Low risk patients have resolution of granulocytopenia within a week after becoming febrile and neutropenic [23, 81]. Virtually all of these patients recover without sequelae if antibiotics are continued until the granulocyte count has risen to above $500/mm^3$.

The dilemma of how long to continue therapy is particularly problematic for patients who remain granulocytopenic for more than a week. In a series of clinical trials, we have attempted to evaluate the risks and benefits of continuing versus stopping therapy in such patients. We chose seven days as our decision point, since a week constitutes sufficient time to evaluate the pre-antibiotic cultures and to assess the patient's response to empiric antibiotics while still being short enough to not significantly increase the risk for antibiotic-induced toxicity and superinfection.

In our studies, patients with unexplained fever who had been treated with the same broad-spectrum antibiotic regimen were randomized on day seven of therapy to either discontinue antibiotics or to continue therapy until the resolution of granulocytopenia [23, 34]. Patients were stratified according to whether they had become afebrile after starting antibiotics or whether they remained persistently febrile in spite of the antibiotics. Of the 17 patients who had become afebrile on broad-spectrum antibiotics and who were randomized to discontinue antibiotics, 7 (41%) became febrile or infected again within a median of three days after antibiotics had been discontinued. In contrast, none of the afebrile patients randomized to continue antibiotics while remaining granulocytopenic developed second infectious complications, suggesting that continued antibiotic therapy was important in patients who had defervesced on antibiotics but who remained granulocytopenic. Similarly, when we randomized patients with unexplained fever who were still febrile and granulocytopenic in spite of seven days of empiric antibiotics to discontinue antibiotics, 9 of 16 patients (56%) developed either shock or evidence of an infection within a median of three days after stopping antibiotics. In contrast, persistently febrile granulocytopenic patients with unexplained fever who were randomized to continue antibiotics had a lower incidence of acute bacterial infections (1 out of 16); however, 5 of these 16 patients developed evidence of invasive fungal disease during their episode

of fever and neutropenia. These data suggest that while continuing antibiotics in patients with protracted fever and granulocytopenia may have decreased the risk for an early bacterial relapse, fungal infections were important, either as a primary or secondary infection. Indeed, the continued fever in these patients at the time of randomization may have been due to undetected fungal disease. This is suggested by the virtual elimination of invasive fungal disease when patients were randomized to continue broad-spectrum antibiotics with the addition of empiric antifungal therapy with amphotericin B. It is also of interest that nearly half of the patients begun on amphotericin B defervesced within three days in spite of sustained granulocytopenia (median 24 days). In sum, these data suggest that patients with protracted granulocytopenia benefit from continued antibiotics, either to eradicate a previously undetected infection or to provide systemic antimicrobial prophylaxis while the patient remains persistently granulocytopenic. However, in view of the risk of invasive fungal disease, the addition of empiric antifungal therapy in patients continuing to receive antibiotics is important for both treatment and prophylaxis.

In ongoing studies, we are attempting to further define the appropriate duration of antibiotic therapy in persistently granulocytopenic patients as well as evaluate alternatives to amphotericin B for antifungal prophylaxis. For example, in patients with unexplained fever who defervesce after beginning empiric antibiotics but who remain granulocytopenic, we are studying whether a standard course of antibiotics (i.e., 14 days) is adequate (as if the patient had an occult infectious etiology for the unexplained fever), even if the patient remains granulocytopenic. Conversely, is it better to continue the antibiotics until the ultimate resolution of the granulocytopenia (as if the antibiotics were providing systemic prophylaxis)? During the past three years, we have evaluated 84 patients who were still granulocytopenic but who had become afebrile after seven days of therapy. Patients were continued on antibiotics for 14 days if they remained afebrile and persistently granulocytopenic, and were randomized to either discontinue antibiotics or to continue therapy. Of these 84 patients who were afebrile and granulocytopenic on day 7, 47 (56%) resolved their granulocytopenia before day 14. Antibiotics were continued until granulocyte return and all recovered without subsequent sequelae. However 12 (14%) of these 84 FUO patients who had become afebrile after starting antibiotics and who remained granulocytopenic, became febrile again prior to day 14. Of these 12 patients with new fever, three had evidence of localized infection (progressive necrotizing gingivitis or perirectal tenderness) which responded to the introduction of a specific anti-anaerobic antibiotic (e.g., clindamycin). The remaining 9 of 12 patients with new fevers received amphotericin B empirically. Of interest is that pre-amphotericin blood cultures eventually grew *Candida sp* in two

Table 9. New Antibiotics and Combinations for Empiric Management

Study **Year** [ref.]	Regimens Studied	Patients with Documented Infections	% Response
Wade et al.	Ticarcillin + Amikacin	34	56%
1981	vs		
[129]	Piperacillin + Amikacin	38	58%
DeJongh et al.	Ticarcillin + Amikacin	56	80%
1982	vs		
[130]	Moxalactam + Amikacin	57	83%
Barnes et al.	Moxalactam + Amikacin	44	82%
1982	vs		
[131]	Moxalactam + Piperacillin	42	71%
Alanis et al.	Moxalactam	26	69%
1982	vs		
[134]	Nafcillin + Tobramycin	22	59%
Pickard et al.	Moxalactam	25	60%
1982	vs		
[133]	Ticarcillin + Tobramycin	25	36%
NCI	Ceftazidime	31	84%
1983	vs		
[135]	Keflin + Gentamicin + Carbenicillin	25	88%

patients; all the patients responded to the addition of empiric antifungal therapy. This data further suggests that persistent fever or the development of a new fever in the granulocytopenic patient may indicate a fungal infection and that prompt initiation of antifungal therapy is appropriate. The number of patients randomized on day 14 to either continue or discontinue antibiotics is still small (25 patients) and this study is still ongoing.

The exact point at which empiric antifungal therapy should be started in the patient with persistent fever and granulocytopenia and which antifungal agent should be used still deserves additional study. At present, amphotericin B is the only drug of proven efficacy and all alternatives must be compared to it. While our starting time for empiric amphotericin B (i.e., day seven of antibiotics) was initially chosen somewhat arbitrarily, this has turned out to be a reasonable initiation point. By waiting until day seven of persistent fever and granulocytopenia, we avoid overtreating patients who defervesce before day seven. Importantly, we have not encountered cases where this delay was deleterious. However, these results depend on the particular patients under consideration and the predominant fungal isolate at a given institution. For example, at the NCI, *Candida sp* have been the predominant fungal pathogen. In contrast, while *Aspergillus* may respond to early institution of amphotericin B, this fungi is more difficult to treat or control than *Candida sp.* Hence, centers in which *Aspergillus* is the predom-

inant pathogen may find less success with empiric amphotericin B, especially in patients who remain neutropenic for extended periods [136]. It is unclear if even earlier initiation of amphotericin might be helpful in such patients.

Similar considerations pertain to the type of antifungal therapy utilized. For example, we are presently comparing amphotericin B to oral ketoconazole for empiric antifungal therapy in patients with prolonged fever and granulocytopenia. However, institutions where *Aspergillus* is a major problem may be less likely to benefit from ketoconazole, in view of its minimal activity against this organism. Hence, while empiric antifungal therapy appears beneficial, the particular agent, starting point and schedule must be considered along with the particular spectrum of fungal infections at a given institution.

It is worth pointing out that the decision of whether to stop or start antibiotics or antifungal agents can be viewed from a different vantage point [137]. For example, while stopping antibiotics in the persistently granulocytopenic patient will require re-starting therapy in approximately 60% of patients, the remaining 40% of patients will not require re-institution of therapy and may thus be spared the need for prolonged hospitalization and potential drug related complications. The main issue is whether the discontinuation of antibiotics places the patient at increased risk for infection-related morbidity or mortality. To a considerable extent, this depends upon the clinical acumen of the primary physician in rapidly intervening if the patient deteriorates after stopping antibiotics. From our perspective, the reliable endpoints which indicate the need for re-institution of antibiotics are vague and ill-defined, particularly in the patient who is already febrile. Indeed, we observed in our randomized trial and have encountered in practice, that patients may rapidly deteriorate after the discontinuation of antibiotics. Hence, unless the patient is under surveillance by skilled observers and is accessible for prompt intervention, prudence would suggest that it is preferable to continue antibiotic therapy in the persistently febrile granulocytopenic patient. While this will surely lead to over-treatment of some patients, it assures that the highest risk patients will not be compromised.

The final duration of antibiotic therapy is still difficult to determine. For the patient who has defervesced on therapy, continuation until either resolution or granulocytopenia of for a 14 day course of antibiotics (which ever comes first) is advised until additional clinical data is available. In contrast, for the persistently granulocytopenic patient, we would continue antibiotics and antifungal therapy until the resolution of the granulocytopenia.

5. NEW OR EVOLVING SYNDROMES IN MANAGEMENT OF INFECTIONS IN THE ADULT LEUKEMIC

5.1. *Foreign Bodies*

Indwelling right atrial silastic (Hickman/Broviac) catheters, initially designed for home parenteral nutrition for patients with chronic bowel disease [138], are being increasingly used to provide venous access for leukemia patients [139-142]. The advantages of these catheters for the safe and confortable administration of chemotherapy, blood products and other intravenous medications are enormous. Double and triple lumen catheters also enable repeated blood sampling and even provide a means of intensive monitoring [143]. Conversely, these foreign bodies provide a nidus for local infection and a portal for systemic invasion. While initial reports suggested that the incidence of catheter related bactermia was low [138], present observations suggest that there is a significant risk for both infectious and non-infectious complications with these catheters [144, 145]. The reasons for these variable rates of complication (3-60%) may relate to differences in techniques of catheter insertion, care and maintenance. Differences in patient populations, their therapies, degree of catheter use and definition of catheter related infection and complications also influence the appreciation of complications. These complications must, nonetheless, be balanced against the considerable advantages which the catheters offer. A question of practical importance is whether these catheters need to be removed if positive blood cultures are obtained. While classic principle of infectious disease stress the importance of removing a potentially infected foreign body, this is not a simple matter for the neutropenic (and frequently thrombocytopenic) cancer patient. Accordingly, while 15 of 25 (60%) of patients with silastic catheters and acute nonlymphocytic leukemia who were undergoing treatment at the Hospital of the University of Pennsylvania developed bacteremia, the catheter tip was positive for the same organism in only one case. The majority of bactermias were treated successfully with antibiotics alone and without removal of the catheter [139].

At the NCI, we evaluated our practice in patients with catheter associated bacteremia (defined as two or more positive blood cultures obtained from two or more separate sites or at two separate times). Positive cultures were usually obtained from both the catheter as well as a peripheral vein as part of the evaluation of a new fever. From 1979-1981, 51 catheters were placed in 43 patients, 39% of which were associated with an episode of bacteremia. Of these, 60% occurred while the patient was granulocytopenic. Gram-positive organisms (especially *S. epidermidis*) predominated, particularly in non-granulocytopenic patients. To assess the impact of indwelling catheters on the incidence of bacteremia, we compared the frequency of bacteremias in patients with and without catheters, both during the study period as well as

during the two year period prior to our routine insertion of silastic catheters. In granulocytopenic patients who had been febrile, the incidence was four-fold higher than in patients without catheters; in non-granulocytopenic patients with indwelling catheters, the incidence was forty-fold higher than for comparable patients without catheters [145]. Thus, there is no doubt that a catheter places the patient at heightened risk for developing a bacteremia, regardless of whether patients become granulocytopenic. The consequences of these bacteremias and their ease of management are, therefore, of considerable importance. Overall, we have observed that more than 80% of these bacteremias can be treated without the need for catheter removal. Similar results have recently been reported by Abrahm *et al.* in a prospective study of patients with silastic catheters [146]. However, the need for caution still exists, since long-term experience with these catheters is still relatively nascent. During the last year, with now over 100 catheter placements at the NCI, we have observed a patient with endocarditis due to *Candida sp,* and another who developed osteomyelitis due to *S. aureus* bacteremia. While the majority of patients respond to parenteral antibiotics (generally with vancomycin) we have recently observed two patients with penicillin sensitive *Bacillus sp* bacteremias, whose infections could not be cleared until their catheters were removed. Similarly, patients with significant exit site or subcutaneous tunnel inflammation require catheter removal to erradicate their infection. This probably also applies to patients with fungemia.

Subcutaneous implantable catheters are also being introduced into the clinical arena for patients who require continuous infusion therapy. To date, experience with these devices is still too early to assess their utility or complications [147].

In addition to intravenous devices, intraventricular (e.g., Ommaya) reservoirs may be used in the treatment of meningeal leukemia. Early experience with these devices suggested a high incidence of infection. During the last decade we have implanted Ommaya reservoirs in 32 pediatric leukemia patients. These reservoirs were left in place for an average of 36 weeks and had been punctured an average of 33 times. The major dilemma is distinguishing patients who develop a true infection from patients with 'ventricular catheter colonization'. Patients who have repetitive isolation of *S. epidermidis* or *P. acnes* in conjunction with a headache and ventricular pleocytosis, are likely to have infection rather than colonization. As in patients with indwelling intravenous catheters, we have been able to treat patients successfully with oral, intravenous, or intraventricular antibiotics, unless there is evidence of cellulitis around the reservoir site [148]. A trial of antibiotics and careful patient follow-up, therefore, appears worthwhile.

5.2. *Antibiotic Modifications (Broad vs. Specific Therapy)*

A common question is whether the initial empiric antibiotic regimen should the modified once a microbiological source of infection is defined. In particular, should the antibiotic spectrum be narrowed to cover just the isolate or should the original broad spectrum regimen be continued (assuming the organism is sensitive and the patient has responded). The benefits of narrowing the antibiotic spectrum include a lowered toxicity, decreased risk for superinfection and microbial resistance, and lower cost. In spite of the success of this approach in non-granulocytopenic patients, we observed a significant liability with narrow spectrum therapy in patients with persistent bacteremia. We retrospectively evaluated 78 neutropenic patients with gram-positive bacteremia (predominantly *S. aureus*) who were treated with either specific therapy (e.g., oxacillin or nafcillin) or broad-spectrum antibiotics [149]. The type of therapy was unimportant for patients who remained neutropenic for a week or less. However, patients who remained neutropenic for more than a week had a 47% incidence of a second bacterial infection. These were due to gram-negative organisms suggesting that broad-spectrum antibiotics may not only control the primary (gram-positive) infection, but may also prevent the emergence of second gram-negative infections in patients who remained neutropenic. Surprisingly, there was no difference in fungal superinfections in patients who received broad rather than narrow spectrum antibiotics. To confirm these findings, we are conducting a prospective study which randomizes patients with microbiologically defined infections to either a specific antibiotic or to continued broad spectrum therapy.

5.3. *Management of Oral Complications*

Oral complications are common in patients with acute leukemia as a consequence of chemotherapy induced mucositis and impaired local and systemic host defenses. Mucositis, gingivitis and other dental related problems may occur in up to 85% of leukemics during the course of their disease [150]. Several studies have addressed the changing oropharyngeal microflora in cancer patients and the risk this poses for both local and generalized infections [120, 151, 152]. Recently measures have been sought to lower the risk for developing oral gingivitis and mucositis [153]. Peterson *et al.* evaluated 38 febrile patients undergoing treatment for acute nonlymphocytic leukemia, and found a 32% incidence of local oral infections, more than half of which were thought to be the cause of the patient's fever. The peridonteum was the most common site of infection (Figure 2), cultures of which revealed mixed flora [154]. Pre-existing periodontitis is common, and exacerbation is likely when the patient becomes immunosuppressed. A program of careful oral hygiene may relieve this complication [153, 155].

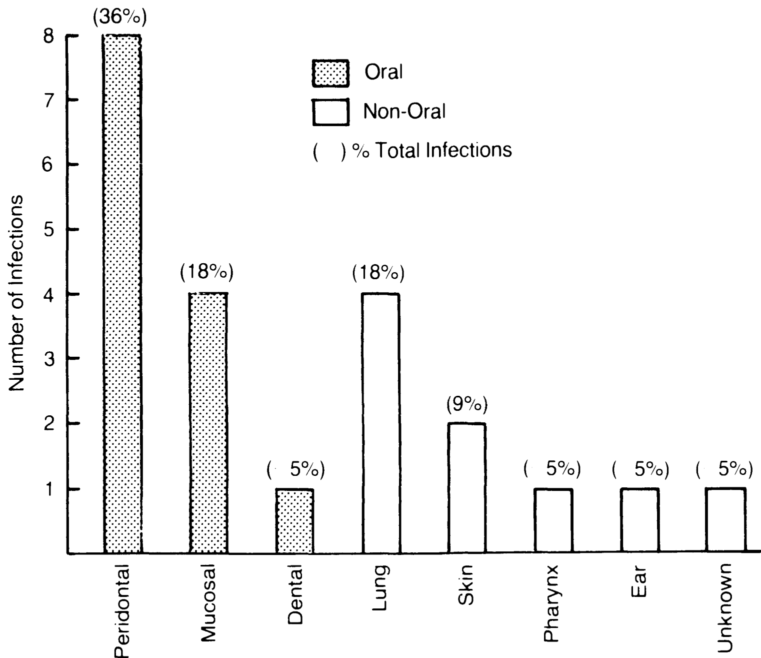

Figure 2. Frequency of infection by site in patients with acute nonlymphocytic leukemia. From Peterson *et al.*: Oral Surg 51, 1981 [154]. Reprinted with authors' permission.

Mucositis *per se* is more difficult to attribute to an infectious etiology, since a number of chemotherapeutic agents can cause erythema, sloughing and frank ulceration of the buccal and labial surfaces [156]. Superimposed infections are not infrequent, particularly with *Candida sp* [157]. *Herpes simplex* virus may also cause severe oral mucositis in the patient with leukemia, sometimes together with other infecting organisms [75]. Why the oral cavity is so suceptible to such complications is not well understood. Disease and treatment induced disturbances of local host defenses (e.g., salivary content) and the altered epithelial binding sites for microorganisms which contribute to this are under active investigation [121, 158]. Unfortunately, oral hygiene *per se* does not appear to significantly reduce the incidence of mucositis [153].

Treatment of the patient with mucositis depends largely on the proven or presumed cause. Most patients are already receiving broad spectrum antibiotics when the mucositis develops. While the antipseudomonal penicillins and some of the third generation cephalosporins provide anaerobic coverage (see Tables 5 and 6), we have observed that some patients will develop progressive gingivitis in spite of these antibiotics. The addition of a specific antianaerobic agent (e.g., clindamycin, metronidazole) has proven beneficial

in such cases. For patients with progressive oral thrush, we have found clotrimazole lozenges or trouches more effective than oral nystatin. Oral ketoconazole has been suboptimal in our experience, even at doses of 800 mg/day. Patients who fail to improve with topical therapy will generally respond to intravenous amphotericin B (0.1–0.5 mg/kg/day × 5 days). If the mucositis is caused or exacerbated by *H. simplex* virus, intravenous adenosine arabinoside (10 mg/kd/day × 5 days) or acycloguanosine (750 mg/m^2/day × 5 days) may be helpful [77, 78]. If the herpetic lesions are limited to the outer lips, topical therapy with acycloguanosine (Zovirax) may suffice [159].

5.4. *Esophagitis*

While esophagitis may be due to chemotherapy or gastric reflux, the syndrome of fever with dysphagia or odynophagia in neutropenic patients on antibiotics is most likely infectious in origin. Although *Candida sp* is the most likely infecting organism [160, 161], *H. simplex* (either alone or together with *Candida*) [76, 162] or bacteria also deserve careful consideration [161]. A common dilemma is whether endoscopy and biopsy should be performed to establish the diagnosis. While barium swallow and/or simple fiberoptic esophagoscopy can demonstrate cobblestoning or the putative 'white curtain' associated with *Candida*, both are non specific and are associated with both false positives and false negatives. The only definitive way to establish the diagnosis is with biopsy, culture, and histology. Ruxer *et al.* evaluated 11 patients with acute nonlymphocytic leukemia for 12 episodes of esophagitis with endoscopy [161]. Of seven which visually appeared to be consistent with the diagnosis of *Candida* esophagitis, biopsy revealed a nonfungal etiology for the inflammation in three. Two were bacterial in origin and one was herpetic. However, it is not always possible or safe to biopsy the patient with esophagitis, particularly if the patient is also profoundly thrombocytopenic. Not only is there a risk for bleeding, but also of bacteremia. An alternative to biopsy is to try a short course of empiric therapy. Patients with esophageal candidiasis will generally respond to either clotrimazole or to intravenous amphotericin B (0.1–0.5 mg/kg/day × 5 days) [163]. If the patient is not symptomatically improved within 48 hours after starting such empiric therapy, esophageal biopsy would be important to establish the diagnosis. Rarely, patients with esophagitis may present as if they had mediastinal adenopathy or a mass due to an inflammatory response of the surrounding tissue to *H. simplex* esophagitis [164].

5.5. *Management of Pulmonary Infiltrates*

Management of the leukemia patient with a new pulmonary infiltrate is complicated, since 17-41% of infiltrates may have a non-infectious etiology

including hemorrhage (consequent to thrombocytopenia), leukemic infiltration or toxicity from antileukemic chemotherapy [165–171]. Bacteria, fungi, viruses and protozoa are all potential pathogens. Evaluation is further complicated by the patient's altered inflammatory response. For example, sputum is rarely obtainable for analysis in the neutropenic patient, necessitating a more invasive approach for microbiologic diagnosis. The choice of procedures is controversial and based on both their yield and potential complications. An interesting study was recently reported by Burt *et al.* [172] in which patients served as their own controls. Each of 17 patients with a new diffuse pulmonary infiltrate was scheduled for an open lung biopsy and while in the operating room, also underwent transthoracic needle aspirate and biopsy as well as transbronchial brush and biopsy. A diagnosis was established from 30% of aspirates, 59% of transbronchial biopsies and 94% of the open lung biopsies. While there is likely to be considerable variation with the expertise in performing diagnostic procedures, the open lung biopsy would seem to be the most reliable and probably the safest procedure in the leukemic patient.

A continuing question is whether it is necessary to actually establish the microbiological diagnosis before initiating therapy. To an extent, this depends on the type of infiltrate and the clinical status of the patient, particularly whether the patient was granulocytopenic and/or receiving antibiotics when the infiltrate appeared. Most would agree that the high likelihood of a gram-negative bacterial pneumonia in the neutropenic patient who has a localized pulmonary infiltrate should prompt the initiation of broad spectrum antibiotics [27, 173, 174]. Most patients in this category will improve. However, if after a 48–72 hour trial of empiric antibiotics the patient has not stabilized, a definitive diagnosis should be sought, even if this necessitates open lung biopsy. The problem is more difficult when a granulocytopenic patient develops a new pulmonary infiltrate while already on broadspectrum antibiotics. For example, is the infiltrate an early indicator of granulocyte recovery with leukocyte infiltration into previous sites of infection, or is this a second bacterial or a fungal superinfection. In a retrospective analysis, we observed that patients who developed infiltrates in conjunction with bone marrow recovery did well without additional therapeutic modifications. However, when a new infiltrate appeared and progressed in patients who remained granulocytopenic, a fungal etiology was most likely [175]. Early antifungal therapy in these patients optimized their chance for recovery. Ideally, such patients should undergo an open lung biopsy to guide therapeutic modifications as well as the necessary length of therapy. However, if biopsy is not feasible because of the patient's clinical status, amphotericin B should be added empirically and continued for a full course of therapy (1.5–2 grams total dose) [176–177].

A similar consideration pertains to patients who present with diffuse infiltrates. Prior to the advent of trimethoprim-sulfamethoxazole (T-S), most clinicians agreed that biopsy proven evidence of *Pneumocystis carinii* was essential to justify the administration of potentially toxic therapy with pentamidine isothionate. Presently, the debate frequently rages as to whether it is preferable to proceed directly to some invasive diagnostic procedure in the patient with a diffuse infiltrate (since antigen assays and other non-invasive procedures are not consistantly reliable) versus simply beginning antibiotics empirically and gauging the patient's response to this therapy. While this may be successful for the patient who turns out to have *P. carinii* pneumonia [178], a number of other etiologies for a diffuse infiltrate must be considered, especially if the patient is neutropenic or has recently received other antibiotics. Accordingly, the necessary 'empiric therapy' may need to include other antibiotics in addition to T-S and in some cases even antifungal agents. The balance between the potential side effects of polypharmacy must be weighed against the risks for an invasive diagnostic procedure. This is further complicated by the slow response time (e.g., approximately 3–4 days for *P. carinii* pneumonia) making rapid changes in the therapeutic approach difficult. In light of this debate, we began a prospective study in March 1982 which addresses the merits of empiric therapy versus early definitive diagnosis. Patients presenting with a diffuse infiltrate are stratified according to their granulocyte count and prior antibiotic therapy and are randomized to either an empiric trial of therapy or to an early diagnostic study. We have chosen the open lung biopsy as our procedure of choice in view of its comparative success and low morbidity [172]. In the non-granulocytopenic patient not on antibiotics, intravenous erythromycin is given empirically in addition to T-S in those randomized to early empiric therapy. This addition was made due to the increasing recognition of *Legionella sp.* as an etiologic agent for pneumonitis in the immunocompromised host [179], although at the NCI it has not yet been a major pathogen. While this study is still in progress, it will hopefully resolve this continuing debate.

5.6. *Unique Gastrointestinal Pathogens*

Clostridia species are ubiquitous and number $10^9 - 10^{10}$ organisms/gram of feces. Surprisingly, clostridia are rarely the sole cause of infection in immunocompromised patients although serious complications have been associated with them [180, 181]. While uncommon, spontaneous clostridial sepsis (i.e., not associated with bowel surgery or trauma) has been described in the immunocompromised host receiving cytotoxic chemotherapy and/or radiation [181–183], primarily with histotoxic clostridia, such as *C. perfringens* and *C. septicum.* The classic syndrome is one of hypotension, mas-

sive hemolysis, DIC and death within 24 hours. The clostridial exotoxins (*a* toxin, lecithinase) are important in the severity of these infections. The 'classic signs' of clostridial sepsis (e.g., alertness despite hypotension, pulse/temperature deficit, subcutaneous crepitance) are the exception rather than the rule. Thus, awareness of clostridium as a potential pathogen is critical in guiding outcome. For example, each of seven leukemia patients with *C. septicum* sepsis who received inadequate antibiotics died, while five of seven patients who received adequate therapy survived [183]. Penicillin, clindamycin and chloramphenicol are the drugs of choice and some of the second and third generation cephalosporins also have proven activity. *C. tertium* and *C. inoccum* have more variable and often resistant sensitivity patterns. Although rarely isolated from the blood, these latter organisms are more commonly found in mixed infections (e.g., abdominal abscesses) and are not considered histotoxic [184].

Clostridium difficle has become recognized as the leading cause of antibiotic associated diarrhea and colitis. *C. difficle* has been found in 2.9–4.4% of stool samples from 137 healthy or hospitalized adults without diarrhea. The diarrhea caused by *C. difficle* appears to be due to the elaboration of a toxin by the organisms. The cytotoxic effect of this toxin can be demonstrated on fibroblast monolayers and serves as a diagnostic test [185]. Cancer patients are exposed to multiple courses of antibiotics which can lead to *C. difficle* overgrowth, a phenomenon which can also result from several anticancer drugs [186]. *C. difficle* can be nosocomially transmitted and patients with this syndrome should be placed on enteric precautions [187]. The true prevalence of *C. difficle* associated colitis in cancer patients is still unclear and whether both the toxin and isolation of the organism is necessary for diagnosis remains unresolved. We have encountered symptomatic patients who have *C. difficle* in their stools yet without detectable toxin. Treatment of *C. difficle* colitis requires oral vancomycin (1–2 grams/day × 10–14 days). If the patient responds quickly and is not receiving any other antibiotics, an abbreviated course of vancomycin (i.e., 7 days) can be considered. Notably a 10–20% relapse rate has been reported, although most patients respond to a second treatment course [185].

Strongyloides stercoralis, a helminth, can result in diffuse pulmonary infiltrates and severe gastrointestinal symptoms in immunosuppressed patients, especially those receiving corticosteroids. Symptoms may initially mimic an intestinal obstruction or ileus, progressing to malabsorption, electrolyte imbalance and polymicrobial sepsis as the worms convert from the rhabditiform to the filariform stage and traverse the intestinal wall carrying microorganisms with them. Although this nematode is most common in subtropical climates, ease of travel necessitates consideration of *S. stercoralis* infestation in patients who have lived in endemic regions. A heightened

index of suspicion is important and patients thought to be at risk should have their stools examined prior to beginning chemotherapy. Patients with infestation should be treated with thiabendazole (25 mg/kg bid × 2 days) to eradicate the worms and prevent the hyperinfection syndrome [188, 189].

5.7. *Perirectal Cellulitis and Abscess*

Perirectal cellulitis used to be a frequent infectious complication in leukemia patients undergoing chemotherapy [190]. Today, probably because of the aggressive initiation of empiric antibiotics when the granulocytopenic patient becomes febrile, this complication is much less common. When perirectal infection does occur, signs may range from superficial erythema to ulcerations, fissures or frank ischiorectal fossa abscess formation. Gram-negative rods are the predominant pathogens, although mixed infections (particularly with anaerobes) are probable. *Herpes simplex* can be a primary or secondary pathogen and may respond to topical or systemic therapy with acycloguanosine. When perirectal inflammation progresses to painful, red, swollen and indurated lesions, in particular extending to involve the genitalia, surgical drainage should be considered. Barnes *et al.* recently reported an increased survival in a group of 16 leukemia patients with significant perirectal infection when routine drainage was employed (10 patients) or the lesions spontaneously drained on their own (5 patients) [191]. We have observed clinical improvement in leukemia patients with early signs of perirectal inflammation who are already on or just beginning broad-spectrum antibiotics for fever and granulocytopenia with empiric additional antianaerobic coverage (e.g., clindamycin or metronidazole). Thus, early aggressive antimicrobial therapy may spare many patients a surgical drainage procedure.

5.8. *Rhinocerebral Syndrome*

Fungal sinusitis may begin as a small crusted lesion on the anterior, inferior turbinate or adjacent cartilagenous septum, but can progress rapidly to involve the paranasal sinuses with consequent facial swelling. Unchecked, the infection will cause bony erosion and destruction of the nose, paranasal sinuses and orbit(s), resulting in the 'rhinocerebral syndrome' with involvement of the brain by direct extension or vascular thrombosis [192]. *Aspergillus sp.* leads the list of etiologic agents of the rhinocerebral syndrome in leukemics [45, 192] with the phycomycetes [193] (more commonly seen in diabetics), and *Candida sp.* being less common pathogens. In some centers, *Aspergillus* infections have increased significantly in recent years. Violler *et al.* reports that *Aspergillus* has risen to 4% in patients with acute nonlymphocytic leukemia and nearly 20% in adults with acute lymphocytic leukemia during a five year period at the University of Maryland Cancer Cen-

ter [194]. A similar pattern (but lower incidence) has been observed in children with acute lymphocytic leukemia [195, 196], perhaps related to the corticosteroids administered to these patients. Patients suffering a relapse, especially if they require broad-spectrum antibiotics for management of fever and neutropenia, are at highest risk for this infectious complication. Treatment of patients with advanced *Aspergillus* sinusitis or the rhinocerebral syndrome has been disappointing. Of particular concern is that pneumonitis may occur prior to, together with or following the onset of *Aspergillus* sinusitis. Diagnosis necessitates biopsy confirmation and treatment requires debridement as well as intravenous amphotericin B [192]. Since early therapy, particularly in the profoundly neutropenic patient, is optimal, methods for early diagnosis have been sought. Serodiagnosis of invasive aspergillosis has not yet proven to be clinically useful (see section 3.2.) and although nasal culture surveillance (for *Aspergillus flavus*) has been used to detect patients at heightened risk for *Aspergillus* pneumonitis [32], the same center found the test to be neither sensitive (58% true positive) nor specific (42% false positive) when used to predict patients at risk for fungal sinusitis [194]. Therefore a high index of suspicion, a thorough nasal exam and early empiric antifungal therapy for the patient with continued fever and granulocytopenia who is already on broad-spectrum antibiotics, is needed to reduce the incidence and severity of the rhinocerebral syndrome.

6. RECENT DEVELOPMENTS IN ADJUVANT THERAPY

6.1. *Granulocyte Transfusions*

The role of granulocyte transfusions in the treatment or prophylaxis of infection has re-entered the arena of controversy. While several clinical trials during the mid 1970's suggested that granulocyte transfusions were beneficial for patients with gram-negative bacteremia if their duration of granulocytopenia exceeded a week [197–200], these studies had enrolled only small numbers of patients and utilized varying definitions of bacteremia and infection. Several important changes have taken place during the last several years which prompt the need for critical re-appraisal of granulocyte transfusion technology. First, the spectrum of bacterial infection has shifted to a higher proportion of gram-positive organisms (see section 2.1.) and the antibiotics available to treat bacterial infections have improved, so that infection-related mortality has decreased (see section 4). Thus, to demonstrate a significant survival advantage with granulocyte transfusions requires a very large number of patient entries. A recent prospectively controlled trial randomized patients with granulocytopenia and documented infection to receive continuous flow centrifugation collected leukocytes or to serve as

controls [201]. All patients had been started on comparable broadspectrum antibiotics. Patients with fever of unknown origin, viral or fungal infections were excluded from this study. Ninety-five patients (65 with acute leukemia) were evaluated, 48 receiving granulocyte transfusions and 47 serving as controls. Thirty-two of the 48 (67%) transfused patients and 36 of the 47 (77%) controls had documented gram-negative bacteremias, with *E. coli, Klebsiella sp,* and *P. aeruginosa* being the major isolates. The median duration of granulocytopenia was comparable in transfused (12 days) and control (11 days) patients. No difference in the successful treatment of infection or in survival was observed, regardless of the duration of the patients' granulocytopenia or the specific infecting organism. The reasons for these negative findings include the current limitation of leukocyte collection technology as well as improvements in antibiotic therapy and general supportive care. For example, even highly efficient cell separations are only likely to collect 2–3×10^{10} cells, which because of the short circulatory half-life of granulocytes, accounts for only approximately 5% of the turnover which might be expected from a non-granulocytopenic patient when suffering from bacteremia, pneumonitis or a significant cellulitis [202]. In addition, the survival of transfused granulocytes can be adversely altered by alloimmunization, although this can be overcome to some extent by HLA typing [203]. Although the effects of filtration leukopheresis on white cell function have been known for some time, the sequelae of routine radiation of WBC collections to prevent graft versus host disease on granulocyte function have only recently been appreciated [204]. These factors all serve to diminish the potential advantage that granulocyte transfusions might provide. It is important, therefore, to balance the questionable efficacy of granulocyte transfusions against whatever liabilities they carry for both the donor and recipient. For the recipient, at least three major complications have been described. First, the risk of alloimmunization, not only to leukocytes, but to other blood products was noted by Schiffer *et al.* when leukemia patients receiving prophylactic leukocyte transfusions developed refractoriness to platelet transfusions [205]. Secondly, pulmonary toxicity has been associated with leukocyte transfusions. In addition to leukoagglutinin reactions, patients receiving prophylactic granulocytes had a higher incidence of unexplained pneumonitis compared to controls [206]. A pulmonary complication has also been associated with patients receiving amphotericin B. Sixty-four percent of patients who were receiving both leukocyte transfusions and amphotericin B developed a syndrome of acute respiratory deterioration, hallmarked by hypoxia and new pulmonary infiltrates. This was especially pronounced for patients who were already receiving leukocyte transfusions when amphotericin B was begun (10/11 patients developing the syndrome) compared to (4/11) who had been receiving amphotericin when leukocyte

transfusions were initiated [207]. While not all centers have observed this syndrome [208], this may be due to differences in patient populations. In the NCI study, leukocyte transfusions were restricted to patients with documented gram-negative sepsis (and putative endotoxemia). In other centers, leukocyte transfusions were given to patients with FUOs who may not have been endotoxemic. An interaction of endotoxin with the leukocyte or damage of the cell wall [209] may be necessary for this complication to be manifest when amphotericin is administered. A third complication of granulocyte transfusions is the introduction of infecting pathogens. Contamination of leukocytes with *Toxoplasma gondii* was demonstrated more than a decade ago [210]. More recently, granulocyte transfusions have been implicated in the transmission of cytomegalovirus (CMV) to seronegative recipients [66]. This has been demonstrated in patients undergoing allogeneic bone marrow transplantation who received prophylactic granulocyte transfusion and is of significant concern in view of the morbidity and mortality associated with CMV pneumonitis following bone marrow transplantation. Thus, it seems clear that if granulocyte transfusions are used at all, they should be limited to patients who are failing appropriate antimicrobial therapy. Patients receiving amphotericin B should ideally have their leukocyte transfusion spaced approximately 12 hours from the amphotericin infusion. In addition, the costs of these transfusions to both the donor and recipient deserve consideration.

6.2. *Immunoglobulins*

A stimulus for passive immunization with antibacterial antibody comes from three findings. First, the observation that patients with defective antibody production (e.g., chronic lymphocytic leukemia and multiple myeloma) have an increased frequency of pyogenic infection, even when they are not neutropenic [211]. Second, patients with acute leukemia have lower levels of antibody to the core glycolipid of enterobacteriaceae than noncancer patients. Moreover, the antibody level falls in patients receiving cytotoxic therapy and patients who develop gram-negative bacteremias have lower levels of antibody than patients who do not develop infection [212]. And lastly, the protective role of antibody to the core glycolipid has been demonstrated by both laboratory studies and a recent clinical trial [213, 214]. While gram-negative bacteria derive their antigenic diversity from various oligosaccharide side chains ('O' antigens), they share in common a core glycolipid with 2-keto-3-deoxy-octonate (KDO) and lipid A (i.e., endotoxin). A rough mutant of *E. coli* O-111 exposes this core glycolipid and has been used by Braude, Ziegler and co-workers to prepare a vaccine and antisera (known as the J5 antisera). This J5 antisera has been shown to be protective in rabbits challenged with endotoxin [213]. A recent

multicenter trial randomized patients with suspected or proven gram-negative sepsis to receive passive immunization with either the J5 antisera or a pre-vaccine antisera [214]. The mortality due to sepsis was significantly reduced in patients who received post-vaccine antisera (24%) compared to the pre-immune sera (38%). More striking was the reduction in mortality in patients who presented in profound shock when they were randomized to receive J5 antisera (46%) compared to the pre-vaccine controls (76%). While these results are best considered preliminary, they are encouraging and suggest that passive immunization may become an important adjunct to the management of serious infections in immunocompromised patients. The development of commercially available immunoglobulin preparations as well as the ability to develop both hyperimmune gammaglobulin preparations and monoclonal antibodies is likely to rapidly advance this new therapeutic option.

6.3. *Immune Adjuvants*

Methods to accelerate bone marrow recovery (or to prevent it from the cytotoxic effects of chemotherapy) have been largely unrewarding. Currently available immunoregulatory reagents (e.g., lithium chloride), while successful in bone marrow culture [215] and in non-granulocytopenic psychiatric patients [216], have been largely unsuccessful in shortening the period of neutropenia in patients with leukemia [217]. Nonetheless, the possibility of selectively sparing or stimulating granulocyte precursors remains an important research goal. The recent purification of granulopoietin from the urine of patients with aplastic anemia may provide new therapeutic opportunities [218]. Obviously, the possible implications of such stimulation on the malignant clones in patients with leukemia, particularly of the myelocytic type, must be considered [219].

7. CURRENT CONCEPTS IN INFECTION PREVENTION IN ACUTE LEUKEMIA

A number of approaches to infection prevention have been explored during the last several years. We will restrict our attention to studies designed to either prevent the acquisition of new pathogens by the immunocompromised host and/or to suppress the host's colonizing endogenous microflora. In addition, we will review some of the studies aimed at improving the altered host defenses of the leukemic patient.

7.1. *Isolation*

7.1.1. *Reverse Isolation.* Housing the granulocytopenic leukemia patient in a single room with gown, mask and glove precaution is still frequently

utilized in a number of medical centers. By itself, however, this reverse isolation serves only as a reminder for handwashing [220] and affords little other benefit to the patient. Used alone, reverse isolation has little impact on the host's endogenous microbial flora (from which more than 80% of the infecting organisms arise) and does not prevent the acquisition of potential pathogens from food, air or water. A controlled study by Nauseef and Maki [221] showed no benefit of reverse isolation in preventing infection when careful handwashing was performed in all patient contacts. The addition of HEPA filters (see section 7.1.2.) adds little alone, but may help in reducing the incidence of *Aspergillus* in high risk institutions. Positive-pressure rooms with HEPA filters (i.e., laminar air flow rooms) are poor places to place contagious patients (e.g., patients with VZV or tuberculosis).

7.1.2. *Total Protected Environment.* A more sophisticated means of patient isolation is the 'total protective environment' (TPE) which is designed to comprehensively reduce the patient's endogenous microbial flora while simultaneously preventing the acquisition of new potential pathogens. To accomplish this, patients are isolated in a laminar air flow room (LAFR) which uses HEPA filters to remove particles larger than 0.3 microns. The room surfaces are disinfected and all items entering the LAFR are steam or gas sterilized. Foods are sterile or semisterile and all who enter the LAFR wear sterile garments. Patients are decontaminated with oral non-absorbable antibiotics as well as skin antiseptics, and antibiotic sprays and ointments are applied to all body orifices. During the past ten years, a number of trials have shown that leukemia patients rendered profoundly neutropenic have a significant reduction in their incidence of infections when they are treated in a TPE [123, 222–227]. However, infections still occur in patients treated in a TPE and virtually all require systemic antibiotics during their period of isolation and granulocytopenia [223]. Moreover, the simple prevention of infection has not been associated with a higher rate of complete remission in leukemia patients if they are refractory to standard dosages of chemotherapy nor has treatment in an LAFR convincingly prolonged the duration of complete remission [228].

In spite of their success in reducing infections, the TPE suffers from a number of major problems [229]. It is very costly and cumbersome to maintain and service. Patient compliance, particularly with the oral non-absorbable antibiotics, is poor. Further, antibiotic resistant organisms have emerged and present regimens afford no benefit against latent organisms (especially CMV). This is a particular concern in the leukemia patient undergoing bone marrow transplantation, since CMV pneumonitis constitutes one of the major reasons for death in the post engraftment period. While experimental data suggests that a gnotobiotic state can reduce the

incidence and severity of graft-versus-host-disease [230], and while one clinical trial has suggested that this might improve the survival for patients with aplastic anemia who are undergoing bone marrow transplantation [231], no such effect has been shown for patients with acute leukemia. Thus, the TPE is of limited utility in patients with acute leukemia.

7.2. *Antibiotic Prophylaxis*

7.2.1. *GI Decontamination.* A number of oral non-absorbable antibiotics (e.g., gentamicin, vancomycin, polymixin, nystatin, framycetin, colistin) have been used to reduce the endogenous microbial burden and ultimately, to prevent infection. While there is some rational basis for this approach, studies performed to date have not shown that oral non-absorbable antibiotics have a consistent benefit in preventing infection when used alone [123, 222–227, 232–237]. In addition to variations in study design, patient compliance has been both difficult to obtain with these antibiotics [238] (because of their unpalatability and their side effects) and poorly monitored. Since these antibiotics suppress rather then eliminate the endogenous microflora, rapid re-population of the GI tract and subsequent infection (particularly with *P. aeruginosa*) has been described when antibiotics are discontinued prematurely, further limiting this form of antibiotic prophylaxis [225]. In addition to their limited efficacy, non-absorbable antibiotics have been associated with the emergence of aminoglycoside resistant organisms as well as a prolongation of neutropenia [123, 223]. These liabilities make GI decontamination with non-absorbable antibiotics alone undesirable for the routine prevention of infection in patients with acute leukemia.

7.2.2. *Selective Decontamination.* The importance of the host's anaerobic flora in exerting a resistance to colonization by exogenous aerobic organisms was demonstrated by van der Waaij in the late 1960's [24, 239]. In specific-pathogen free rodents or animals treated with antibiotics, it has been observed that the residual intestinal flora determines whether a rodent will become colonized with orally administered gram-negative bacteria. An expression to quantify this phenomenon, Colonization Resistance (CR), has been derived and defined as the log of the oral bacterial dose which results in persistent (> 2 weeks) colonization of 50% of challenged animals. For example, the CR for an antibiotic treated rodent challenged with *E. coli* is 10^2 organisms compared to 10^9 organisms for a non-antibiotic treated animal. The potential clinical importance of 'colonization resistance' was suggested in studies showing that colonization of normal human volunteers with *P. aeruginosa* was transient, unless the volunteer was receiving an oral antibiotic (e.g., ampicillin) which affected the anaerobic flora. On the other

hand, other antibiotics (e.g., trimethoprim-sufamethoxazole, polymixin B, nalidixic acid, amphotericin B) are able to eradicate aerobes while preserving GI anaerobes, thus maintaining the animal's 'colonization resistance'. Thus, the administration of these antibiotics has the potential to decrease the endogenous microbial reservoir as well as to reduce the acquisition of new organisms without the need for isolation.

Several clinical trials have applied the concept of colonization resistance (or selective antimicrobial modulation) to leukemia patients undergoing induction chemotherapy [240–244]. The antibiotics utilized in these trials include both orally absorbed drugs (e.g., trimethoprim-sulfamethoxazole, nalidixic acid) and non-absorbable antibiotics (e.g., neomycin, polymixin, amphotericin B). Sleijfer observed that within a week after starting the regimen, the percentage of stool cultures containing *E. coli, Klebsiella sp,* other enterobacteriaceae, and *P. aeruginosa* fell significantly in the CR group compared to placebo-treated control patients. This was also accompanied by a decreased incidence of serious infections while the patients were granulocytopenic [242]. While these results are encouraging, relatively few patients have been studied to date. Moreover, infections are not entirely eliminated and successful utilization of this approach requires close microbiological surveillance to adjust the antibiotic regimen when drug resistant or new organisms appear. This is both labor intensive and expensive and the usefulness of this approach in large numbers of patients is yet to be demonstrated.

7.2.3. *Monoprophylaxis.* A number of investigators have attempted to achieve colonization resistance with one or two antibiotics rather than the

Table 10. Antibiotic Prophylactic Regimens with Trimethoprim-Sulfamethoxazole as the Major Component

Study **Year** [ref]	Number of Patients	Age	Underlying Malignancy	Antibiotic Regimen	Other Prevention Measures	Study Design	Results and Comments
Enno et al. **1978** [247]	30	45 (15-69)	Acute leukemia	FRACON: Framycetin, 2 g/day Colistin sulfate-6 M units/day Nystatin-2-4 M units/day vs. FRACON + T-S-320 mg/day	Single room with reverse isolation.	Prospective, randomized. Not blinded. No untreated control group.	Advantages: Incidence of fever or infection in 8/14 T-S + FRACON vs. 15/16 FRACON alone. Note: Why incidence of infection so high with FRACON alone?
Gurwith et al. **1979** [246]	41	49 (17-83)	Acute leukemia Solid tumors	T-S-320 mg/day vs. "Gut Sterilization" Neomycin-2 g/day Polymixin-200 mg/day Nystatin-4 M units/day	None for T-S patients.	Initially a three-way randomization: T-S vs. "gut sterilization" vs. no treatment. However, gut sterilization dropped because of patient intolerance.	Advantages: Overall decrease in infection in T-S vs. control (19 vs. 39%). Significant decrease in bacteremias in T-S (0/59 episodes) vs. control (9/52 episodes). Note: Some patients began T-S along with systemic antibiotics.

Table 10. Antibiotic Prophylactic Regimens with Trimethoprim-Sulfamethoxazole as the Major Component

Study **Year** [ref]	Number of Patients	Age	Underlying Malignancy	Antibiotic Regimen	Other Prevention Measures	Study Design	Results and Comments
Weiser et al. **1981** [248]	29	40 (20-66)	Acute leukemia (maintenance therapy)	T-S-320 mg/day	None	Prospective, randomized. Not blinded or placebo controlled.	Advantages: None. Disadvantages: Incidence of fever or infection same for T-S and control (40%). No difference in time to infection in T-S vs. control patients.
Dekker et al. **1981** [249]	52	49	Acute leukemia	T-S-480 mg/day Amphotericin-800 mg/day Nystatin-3 M units/day	None	Prospective, randomized. Not blinded or placebo controlled.	Advantages: Incidence of fever 30% for T-S vs. 46% for control. Therefore, decreased use of systemic antibiotics. Incidence of infection 16/26 for T-S vs. 31/26 for controls. Significant lowering of GI gram-negative bacilli in T-S patients. Disadvantages: Increase in T-S resistant organisms — lead to infection in some patients.
EORTC **1981** [250]	248	Adults and children	Acute leukemia Solid tumors	T-S-320 mg/day (Not controlled for oral nonabsorbable antibiotics).	Some patients received oral non-absorbable antibiotics. Some patients in protected isolation.	Prospective, randomized. Double blind placebo controlled for T-S. Multicenter trial.	Advantages: Significant decrease in bacteremia observed only for adults with lung cancer — not for patients with leukemia. Disadvantages: No overall difference in fever/infection as bacteremias between T-S vs. placebo.
Kauffman et al. **1983** [251]	55	44	Leukemia Lymphoma Solid tumors	T-S-160 mg/day Nystatin-1 M units/day	None	Prospective, randomized. Placebo controlled.	Advantages: Significant decrease in bacteremias and pneumonia in T-S (3/27 episodes) vs. control (16/26 episodes). Disadvantages: No difference in mean febrile days or fever of unknown origin. Increase colonization with T-S resistant organisms vs. control.
Gualtieri et al. **1983** [252]	47	51 (15-85)	Acute leukemia Lymphoma Myeloma	T-S-320 mg/day	None	Prospective, randomized. Double blind placebo controlled.	Advantages: Significant decrease in bacteremias in T-S (3/24 episodes) vs. control (9/23 episodes). Disadvantages: No difference in mean febrile days. Increased colonization with T-S resistant bacteria and fungi vs. control.
NCI **1983** [253]	150	17 (1-43)	Leukemia Lymphoma Solid tumors	T-S-10 mg/kg/day (max 640 mg/day for an adult). Erythromycin 30 mg/kg/day (adult dose 2 g/day).	None	Prospective, Double blind. Placebo controlled. Randomized trial.	Advantage: Incidence of fever or infection (18% for T-S - E vs. 32% for placebos) but only for patients with total compliance. Disadvantages: Benefit small and significant only for patients with leukemia. Increased GI intolerance in patients receiving T-S - E. Prolonged granulocytopenia in patients receiving T-S - E.

more comprehensive schedule described above. The major antibiotic to be used has been trimethoprim-sulfamethoxazole (T-S). In a controlled trial to prevent *Pneumocystis carinii* pneumonia in children with acute lymphoblastic leukemia, Hughes observed that patients receiving T-S also had a significant reduction in bacterial infections when compared to placebo treated patients [245]. Using T-S in adults undergoing induction therapy for acute non-lymphocytic leukemia, Gurwith [246] observed that patients randomized to T-S had a significant reduction in infections and bacteremias compared to a control group. Since then, a large number of studies have evaluated T-S as a prophylactic antibiotic for cancer patients undergoing chemotherapy. Some of these studies have utilized T-S to achieve a 'modified' colonization resistance, others to take advantage of the possible systemic prophylaxis which might be achieved with this well absorbed antibiotic. Overall the results attained with T-S have been conflicting [246–253] (Table 10). Part of the discrepancy may be due to variations in the underlying diseases of the patients studied, their duration of granulocytopenia, drug dosage and compliance as well as to variations in the administration of other antibiotics, special diets or isolation. While a modest reduction in infection has been observed with T-S prophylaxis, its limited efficacy must be balanced against the possibility for prolonged granulocytopenia and the emergence of T-S resistant organisms, some of which is plasmid mediated [249, 254]. As an alternative to T-S, Wade and co-workers [244] used nalidixic acid to achieve selective decontamination. However, nalidixic acid alone was even less successful than T-S and was also associated with the emergence of resistant organisms. Current studies are investigating derivatives of nalidixic acid (e.g., norfloxocin) but are also likely to be of limited benefit and are best reserved for patients with profound (less than 100 PMNs/mm^3) and prolonged (greater than 7 days) neutropenia.

7.2.4. *Specific Antimicrobial Prophylaxis.* Antimicrobials have been used, with variable success, for the prevention of protozoan, fungal and viral infections in immunocompromised patients. There seems little question that trimethoprim-sulfamethoxazole (T-S) can significantly reduce the incidence of *P. carinii* pneumonia when administered prophylactically [245]. Since T-S can prolong neutropenia, its use seems best restricted to high risk patients, primarily in centers where the incidence of *P. carinii* is significant.

Antifungal prophylaxis has been disappointing. In spite of its frequent use, oral nystatin is largely unsuccessful in preventing local or invasive fungal infections. While several of the newer imidazoles (clotrimazole, miconazole, ketoconazole) will decrease the number of colonizing fungi (primarily *Candida* sp), no reduction in invasive fungal infections has been observed to

date [50, 255–257].

Effective antiviral agents have only recently joined the clinical armamentarium. Adenosine arabinoside and acycloguanosine (Acylovir) have demonstrable activity against *H. simplex* and *Varicella-zoster* virus [77–79, 258, 259]. A reduction in oral *H. simplex* infection has been recently observed in patients undergoing bone marrow transplantation after prophylaxis with intravenous acyclovir [80], although resistance to this agent has also been noted [260].

Unfortunately, none of the available antiviral agents or interferons are effective in the treatment or prevention of CMV, a major cause of death in patients undergoing bone marrow transplantation [70–72]. Recent studies, however, suggest that CMV immune plasma or globulin may hold some promise for prophylaxis [73, 74].

7.2.5. *Recommendations Regarding Antibiotic Prophylaxis.* The following general conclusions can be drawn.

1. Total alimentary tract microbial suppression may be efficacious, but poor compliance with resultant inadequate suppression, rebound overgrowth when antibiotics are discontinued prematurely, and the risk of aminoglycoside resistant organism acquisition and infection makes this technique applicable only in the setting of the total protected environment.
2. Selective antimicrobial modulation to suppress pathogenic aerobes while preserving colonization resistance is theoretically promising and early studies have shown clinical benefit, making this an area which deserves additional study.
3. Systemic antimicrobial prophylaxis can be effective, but this depends upon the kinds of drugs which are being utilized. It is not clear what the relative contributions of systemic versus local suppression are at this time. Although trimethoprim-sulfamethoxazole has been used most commonly, its efficacy is not dependable in all clinical settings and there are potential side effects which may well outweigh its benefit in specific institutions. Nevertheless, the concept deserves further consideration.
4. While studies have demonstrated that total protective isolation is effective in patients with protracted granulocytopenia, its cost and cumbersomeness limit its utility to selected patients, perhaps to those undergoing bone marrow transplantation or other similar degrees of intense, prolonged marrow aplasia.
5. Finally, no antibiotic prophylaxis should be utilized without careful attention to such common sense practices as handwashing, diet, good housekeeping, etc.

7.3. *Immunoprophylaxis*

As a means of prophylaxis against bacterial infections, active immunization has been used to stimulate host antibody production against *P. aeruginosa* and *S. pneumoniae* (particularly in splenectomized patients) [261–263]. Despite an initial antibody response to vaccination in many patients, concurrent administration of chemotherapy is associated with a subsequent decrease in titers, making active immunization less useful in patients undergoing repeated cycles of chemotherapy [261–265]. While live viral vaccines are avoided in immunosuppressed patients, a live varicella vaccine is presently being evaluated in children with leukemia [266]. Although few adults would probably benefit from such a vaccine (i.e., those without a history of chickenpox and/or with low antibody titers to VZV), additional study and follow-up is needed before this vaccine can be routinely recommended in either children with leukemia or this select group of adults.

Passive immunization with zoster immune globulin (ZIG) prepared from plasma with high titers to the varicella zoster virus has been shown to reduce the incidence of severe infections (pneumonia, encephalitis) from 25% to 7% and of mortality from 5–7% to 0.5% in patients contracting primary varicella while immunosuppressed [267]. Although less common in the adult leukemic, exposure to varicella zoster virus, either in the form of chickenpox or shingles, should prompt an immediate investigation of childhood disease history. Passive immunization with ZIG or zoster immune plasma within 72 hours of exposure should be considered if the patient is found to be at risk.

Passive immunization against bacteria has been discussed earlier (see section 6.2.). Several trials designed to study the utility of routine administration of J5 antibody to patients suffering from granulocytopenia secondary to immunosuppressive chemotherapy, as a means of prophylaxis against gram-negative septicemia and shock, are currently underway.

7.4. *Granulocyte Transfusions*

Leukocyte transfusions have also been used to prevent infection in patients with leukemia. A recent cooperative trial showed that there was no overall reduction of infection in patients who were randomized to receive prophylactic leukocyte transfusions. While the transfusion group had a lower incidence of bacterial infections, they had a higher incidence of pulmonary infiltrates [206]. Thus, in light of the complications and expense of leukocyte transfusions, they are not recommended for the prophylaxis of patients undergoing induction chemotherapy.

7.5. *Summary*

There have been both advancements and disappointments in the last

decade of research for the prevention of infections in the immunosuppressed leukemia patient. In particular, most prophylactic antibiotic regimens have shown only minimal or limited protective value. While it is tempting to utilize some means to protect the patient against a potential life threatening infectious episode, the liabilities (e.g., myelosuppression, antibiotic resistance, and cost) must be kept in mind. It may be that future studies will show that rather than any single modality, a combination of prophylactic methods, such as immunization and granulocyte transfusion with or without antibiotics may prove to be a more consistent and reliable means of infection prevention.

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