CHILDHOOD LEUKEMIA:
PRESENT PROBLEMS AND FUTURE PROSPECTS

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PREFACE

Twenty years ago when Children's Cancer Association of Japan was born, the diagnosis of childhood leukemia amounted to a death sentence. Only 20% or so of children with leukemia survived more than 5 years. Since then, enormous improvements have been achieved regarding our understanding on the etiology, diagnosis, and the treatment of childhood leukemia. Now, 70% of children with leukemia survive and enter adult life.

Even though the improved survival rate of children with leukemia represents a medical success story, we now face new problems. The first problem is the fact that we still lose 20–30% of patients with childhood leukemia. To address this problem, we need to understand the etiology, epidemiology, and biology of leukemia; to identify the patients at greater risk; and to develop adequate treatments. The second problem is the treatment itself. Even though efficacious, the modern treatment for leukemia is a grueling experience for children and their families. We should develop a total care system for families and children based on a deep understanding of their needs. The third problem is the aftereffects of the treatment and of cured leukemia. Extensive radiation and chemotherapy have an entirely different spectrum of long–term effects on children than on adults. These treatments in the early stage of life, when the mind and body are developing, create many physical and psychological problems. These are the present problems of childhood leukemia.

On December 7–9, 1989, a symposium was held in Tokyo, sponsored by Children's Cancer Association of Japan, commemorating its 20th anniversary. The purpose of The Symposium on Childhood Leukemia was to promote free exchange of recent findings and ideas pertinent to the etiology, biology, epidemiology, and treatments of childhood leukemia, and also the care of affected children and their families. Invited speakers of the symposium included 13 active researchers from the Federal Republic of Germany, United Kingdom, and United States, and 20 from Japan. The participants of the symposium were pediatricians, oncologists, pediatric surgeons and physicians, nurses, social workers, and parents. Up-to-date findings and in-depth discussions were presented with respect to the present problems, as well as future prospects in our war against childhood leukemia.

Ten years ago, the International Symposium on Children's Cancer was held in Tokyo, commemorating the 10th anniversary of the Children's Cancer Association of Japan and also the International Year of the Child. The results were published as *Recent Advances in Management of Children with Cancer.* The proceedings of the Symposium on Childhood Leukemia is the sequel to the above volume. Readers of these volumes should realize the progress that we have made in 10 years, while recognizing the fact that we still have many problems today. The editors trust that the dissemination of information presented during the symposium will hasten progress in solving these problems.

A venture like this could not have succeeded without the enthusiastic participation of the invited speakers and audience, and also without additional aid from members of organizing and program committees and Mr. Mitsuo Uematsu of the Children's Cancer Association of Japan. We are also deeply indebted to the members of the executive board of the Children's Cancer Association of Japan, in particular Mr. Keiji Iwata. Finally, but not lastly, we acknowledge with appreciation that the Second International Symposium on Children's Cancer was made possible by a grant from the Japan Shipbuilding Industry Foundation, a public service corporation funded by the proceeds of motorboat racing.

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I. Biology of Childhood Leukemia

ETIOLOGICAL MECHANISMS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Childhood acute lymphoblastic leukemia (ALL) is a biologically diverse malignancy. The major subsets correspond to B or T precursor cells, although some ALL may originate in lympho–myeloid stem cells. Multiple molecular alterations contribute to the pathogenesis of ALL, including some that are subset specific. In those cases (~5%) with a Philadelphia chromosome, the genetic basis of karyotypic alteration and the resultant activated *ABL* kinase have been determined and offer new strategies for molecular diagnosis and monitoring. Although these changes at the DNA level are likely to be functionally relevant to the underlying mechanisms of leukemogenesis, the etiology of childhood ALL remains an enigma. A hypothesis is proposed that takes into account geographic and time trend variations in incidence rates, unusual properties of lymphocyte precursors, and promoting effects of immune responses in infancy.

INTRODUCTION

Acute leukemia is the major single cancer type in children, with an annual incidence in Western countries and Japan of 2–4 cases/10^5 (1). Infant and childhood deaths from malnutrition and infection are orders of magnitude more serious on a global, scale but as these problems have been controlled in developed countries, childhood cancer has achieved much more prominence, with only deaths from accidents or genetic malformations posing a similar risk to life (2).

Despite remarkable advances in treatment over the past 30 years [mostly between 1960 and 1975 (3)], a substantial morbidity and mortality is still associated with childhood leukemia. We need to discover whether or not childhood leukemia is a preventable disease, and this requires that we understand its cause(s) or etiology. The molecular mechanisms being uncovered in leukemia are central to the etiological mechanism involved, but, in the absence of any clear identification of other genetic and environmental factors involved, we are unlikely to have a satisfactory or complete

etiological explanation. In this paper, we review our current knowledge of the cellular and molecular biology of childhood acute lymphoblastic leukemia (ALL) and present a hypothesis for its etiology.

BIOLOGICAL DIVERSITY OF ALL: THE ASSOCIATION WITH NORMAL DEVELOPMENTAL BIOLOGY OF LYMPHOCYTES

Approximately one third of pediatric cancers are acute leukemias and ~80% of the latter are ALL. This leukemia is itself biologically diverse, and subgroups can be identified by cytochemical, immunological, karyotypic, and molecular criteria (Fig. 1). These subtypes may have prognostic relevance in the context of particular therapeutic regimes (4,5). Detailed immunophenotypic and molecular analysis indicates that ALL subtypes correspond to clonal derivatives of B or T *precursor* cells (6). Equivalent normal cells proliferate in substantial numbers in fetal tissues, and in normal or regenerating pediatric bone marrow or thymus (6,7).

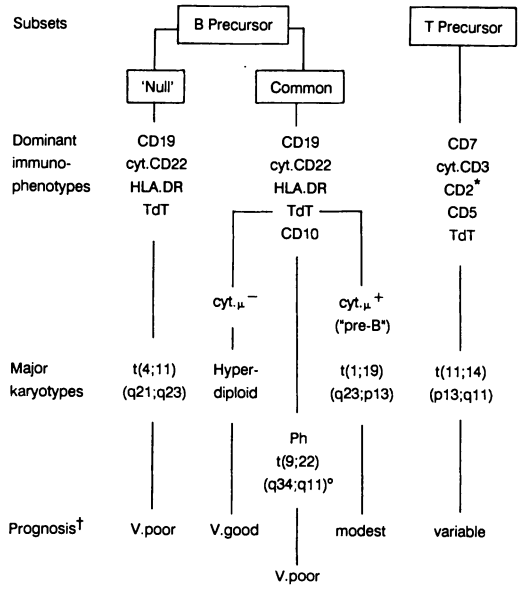

Figure 1. Biological diversity of childhood acute lymphoblastic leukemia.
cyt.= cytoplasmic; * 90% of cases; ° 5% of cases.
+ Dependent upon therapy; overall responses shown. T-ALL responses (remission duration) considerably improved with more intensive chemotherapy regimes.
• Other karyotypic changes may not be subset specific or may occur infrequently, e.g., abnormalities of 9p (99), i17q (100), hypodiploidy (101), 12p12 breaks (102), and 6q- (103).

Leukemic cells are not, however, the exact replicas of normal cells, and their composite phenotypes may show *asynchrony* of gene expression with respect to the expected normal developmental sequence. For example, the "pre-B" subset of common ALL has cytoplasmic mu chains in combination with nuclear terminal transferase (8);

this is a rare immunophenotype in normal B–cell ontogeny, as TdT activity is usually turned off at the time that the fully rearranged IgH gene is expressed (7). A similar discordance occurs with cell surface antigens (9). These observations are in accord with the view that leukemogenesis in ALL involves an uncoupling of growth from differentiation in precursor cells, rather than a stringent maturation arrest at a precise developmental stage (6).

A small proportion of childhood and adult acute leukemias exhibit "mixed lineage" (i.e., lymphoid + myeloid) phenotypes. There has been considerable debate over whether these are due to genetic *mis*-programming ("lineage infidelity") arising as a direct result of leukemogenic mutations (10,11) or, alternatively, that the leukemic process can immortalize a normal multipotential progenitor cell, which when proliferating without maturation can express a promiscuous pattern of lineage–specific genes (12). Although both possibilities may be at least in part correct, recent evidence indicates that normal multipotential stem cells proliferating continuously in response to IL3 can have a multilineage pattern of gene expression (13; Ford *et al.*, submitted for publication).

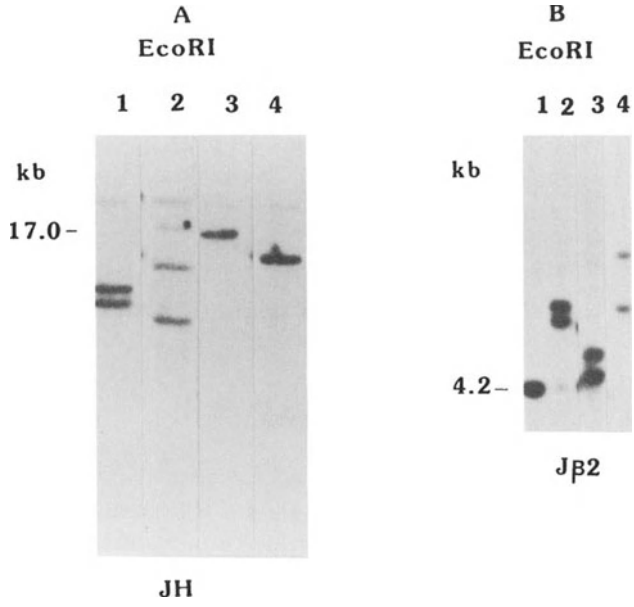

Figure 2. Cross-lineage rearrangements of IgH and TCRβ genes in ALL. DNA blots were hybridized to the JH probe in panel A and the Jβ2 probe in panel B. Sizes refer to germ line fragments of *Eco*RI digests with the appropriate probe.
Lane 1 = cALL *not* showing cross-lineage rearrangement; two rearranged IgH genes; germ line for TCRβ.
Lane 2 = cALL showing cross-lineage rearrangement; two rearranged IgH genes; two rearranged TCRβ genes.
Lane 3 = T-ALL *not* showing cross-lineage rearrangement; germ line IgH; two rearranged TCRβ genes.
Lane 4 = T-ALL showing cross-lineage rearrangement; one deleted, one rearranged IgH gene; two rearranged TCRβ genes. (Taken from ref. 17 with permission).

ALL cells have diverse patterns of immunoglobulin (H, kappa/lambda) and T–cell (gamma, delta, beta, alpha) receptor gene rearrangement, which may include incomplete (DJ but not VDJ) or aberrant rearrangements and cross-lineage rearrangement of inappropriate genes (14–17) (Fig. 2). These complex patterns of rearrangement are not surprising from the viewpoint of developmental biology. The recombinase enzyme system responsible in lymphocyte precursors for gene rearrangement can recognize the heptamer-nonamer signal sequences in both Ig and TCR V, D and J regions (18), and normal T–cell precursors have a transcriptionally active IgH gene with the enhancer region in open or accessible chromatin (19). Additionally, when T-ALL do rearrange IgH genes (in ~15% of cases), they often rearrange one allele only and then may preferentially use DQ52, the D region closest to J (20). This preference in a cross-lineage setting is probably a reflection of constraints of chromatin structure and parallels the preferential use of this same D region by very immature normal (21) and leukemic (ALL) B precursor cells (20). Obviously, normal mature T and B cells only have the appropriate Ig or TCR gene functionally rearranged. Strict lineage–specific control of V region transcription probably ensures the specificity of functional rearrangements (22), and any normal precursor cells with aberrant or cross-lineage rearrangements are probably eliminated. In ALL, there may be more of such cells, due to their expansion and retention in the precursor compartment and perhaps a failure of the normal cell death mechanism.

Ig and TCR gene rearrangement patterns in Southern blots provide an indication of the clonality of ALL cells. In most cases, one or two non-germ line restriction fragments are observed, although one allele can be deleted (Fig. 2A, track 4). In some cases, the pattern of restriction fragments suggests oligoclonality (17,23). The likely explanation of this latter observation is that, following leukemic transformation, some ALL clonogenic cells continue to rearrange their Ig or TCR genes. Other evidence confirms that ALL cells are almost always monoclonal (see below).

"TARGET" CELLS FOR ALL

Although the composite phenotypes of ALL cells may correspond to lymphocyte precursor subsets, they do not necessarily identify the precise developmental level of the original target cell population for the disease – an important distinction from both an etiological and therapeutic viewpoint (24). Studies with glucose 6–phosphate dehydrogenase X-linked polymorphisms in girls with ALL have confirmed that these are monoclonal disorders and have shown that, in most cases, the myeloid lineage is not involved (25), in contrast to CML, where both B and myeloid cells are progeny of the transformed stem cell (26). We have confirmed the former observation using DNA restriction fragment length polymorphisms and methylation patterns in the HPRT (27) and PGK (28) genes.

As shown in Fig. 3, the leukemic blasts of a patient with cALL have two HPRT alleles, but only one is actively expressed (i.e., methylated). Where investigated, the granulocytes and the remission marrow of the same patients express both HPRT alleles (Table 1). These data suggest that most ALL probably do originate in a lymphoid progenitor cell committed to the T or B lineage. Other data indicate, however, that some ALL subtypes may, however, have an origin in a more primitive multilineage

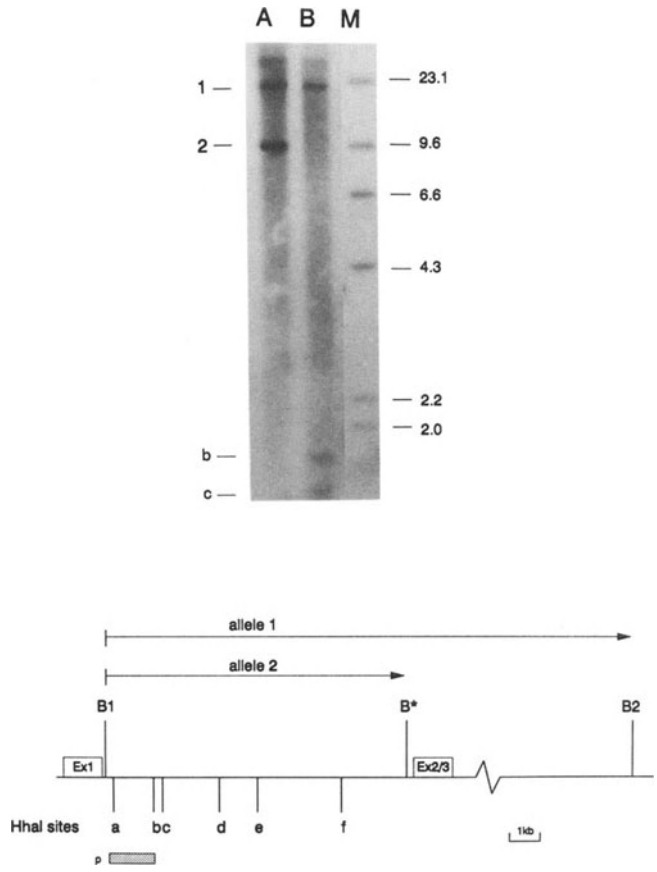

Figure 3. HPRT analysis of X chromosome inactivation patterns in cALL.
Top panel: lanes A and B represent equal amounts of DNA isolated from the leukemic blasts of a patient with cALL.
Lane A = DNA digested with *Bam*HI alone to show heterozygosity of the HPRT alleles (i.e., two bands).
Lane B = DNA digested sequentially with *Bam*HI and *Hha*I to show clonality by exclusive demethylation (activation) of HPRT allele 2 (cut at sites b + c).
Lower panel: Restriction map of the 5' end of the human HPRT gene. B1, B2 are *Bam*HI sites; the polymorphic *Bam*HI site is starred. a – f are restriction sites recognized by the methylation sensitive enzyme *Hha*I.
P = probe used; ex = exon.

progenitor or stem cell. Thus, the mixed lineage immunophenotype ALL most probably arise in a common lymphomyeloid stem cell, although this is not proven. Similarly, a proportion of young (<1 year) infant ALL with the t(4;11) translocation (29) may have a link with the myeloid lineage. Most of these cases have the *bona fide* B cell precursor "null" ALL immunophenotype (see Fig. 1), but a minority have myeloid features also (30). Most ALL with a Ph [t(9;22)] chromosome probably originate in a common lymphomyeloid progenitor. The evidence for this includes the expression of mixed lineage phenotypes (at least in adult ALL; 31), the switch of lineage in relapse

(32), and, most significantly, the demonstration of the Ph chromosome in myeloid colonies (33). Table 2 summarizes the conclusions from these studies on possible target cells for ALL.

Table 1. HPRT/PGK allelic polymorphisms as clonal markers in acute lymphoblastic leukemia (female).

Patient	Heterozygous (2 alleles)	Number of alleles expressed in cell population		
		Leukemic blasts	Blood granulocytes	Remission bone marrow
1	+	1		
2	+	1		
3	+	1		
4	+	1	2	2
5	+	1	2	2
6	+	1		
7	+	1		
8	+	1		
9	+	1		
10	+	1		
11	+	1		
Controls[a]				
1	+		2[b]	
2	+		2[b]	

[a] Normal adults.
[b] Blood mononuclear cell fraction (lymphocytes/monocytes)

Table 2. Candidate "target" cell populations for childhood acute lymphoblastic leukemia.

"Target"	Diagnosis
1. Common lymphomyeloid stem cells	Ph-positive ALL "Mixed lineage" ALL[a] Some infant null ALL with t(4;11)?
2. Lymphoid (T and B) restricted progenitors	Most common ALL (B precursor) and T ALL/T lymphoma (T precursor)

[a] ALL with myeloid plus lymphoid gene expression in individual cells.

MOLECULAR MARKERS IN ALL

The various subtypes of ALL (Fig. 1) are associated with distinctive karyotypes that are presumed to reflect molecular events of etiological importance. In most cases,

the genes and the proteins they encode have not yet been identified. The breakpoint on chromosome 11 involved in the translocation t(4;11)(p21;q23) is an interesting genetic region in which several genes reside that might be relevant to leukemogenesis, including c-ETS, Thy-1, N-CAM, CD3 (34), and an inherited fragile site (35), as well as a gene mapped by familial linkage analysis of ataxia telangiectasia (36). The same breakpoint is involved in other translocations in myeloid leukemia (37). Along with other groups, we have looked for a possible role of any of the above genes in leukemia. By Southern blot analysis with a variety of restriction enzymes, none of these genes appear to be structurally altered (LCC and LMW, unpublished observations). This does not, of course, rule out an involvement of any one of these genes.

The translocation t(1;19)(q23;p13) has a unique association with the subset of B precursor ALL that have cytoplasmic mu chain expression (38). Recent data indicate that the break at 19p13 disrupts a gene coding for an immunoglobulin enhancer binding protein E12/E47 (39). In T–cell ALL, all four TCR genes, but especially TCR delta, may be involved in translocations (40–42). In a few cases, the reciprocal gene that becomes fused to a portion of the TCR gene has been identified, cloned, and sequenced (42-44), but the function of such a candidate oncogene remains unknown. Exceptions to this are rare cases of T-ALL in which *c*-MYC becomes associated with T cell receptor loci (45,46).

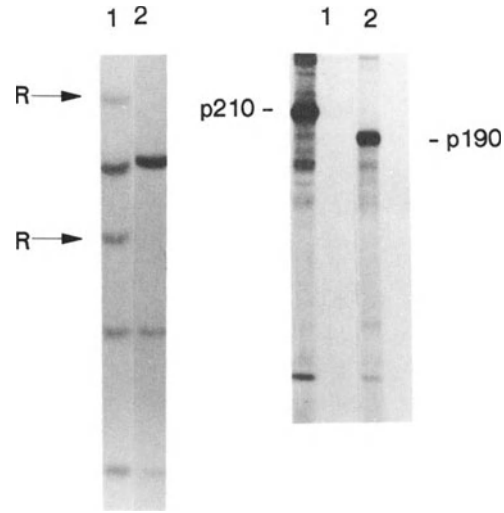

Figure 4. DNA and protein analysis of Ph-positive ALL.
Left: Example of DNA analysis with the *BCR*-3 probe. Genomic DNA was digested with *Bgl*II and, after size separation on a 0.8% agarose gel, hybridized to a radioactively labeled probe. Rearranged bands (R) are indicated for the Ph–positive and *BCR*–positive (rearranged) ALL sample in lane 1. Lane 2 contains DNA from a Ph-positive but M-*BCR negative* (unrearranged) ALL.
Right: Analysis of *ABL*-related protein in *in vitro* protein tyrosine kinase assay. *ABL*-related proteins are immunoprecipitated with anti-*ABL*-antibody and allowed to phosphorylate in the presence of [^{32}P]ATP (50). Examples of p210 (M-*BCR* positive ALL, lane 1) and p190 (M-*BCR* negative ALL, lane 2) are shown.
(Taken from ref. 54 with permission).

Approximately 5% of childhood ALL has a classical Ph chromosome: t(9;22)(q34;q11) (47). It was anticipated that the underlying molecular events in formation of the Ph chromosome in ALL would be the same as found in Ph-positive CML, i.e., the formation of a *BCR-ABL* fusion gene and production of a p210 *BCR-ABL* protein with activated protein tyrosine kinase activity (reviewed in ref. 48). Although some patients with Ph-positive ALL have an identical *BCR* gene rearrangement and activated p210 as found in CML, others do not (49) and instead produce a smaller activated *BCR-ABL* protein of p190 (Fig. 4) (50,51). At least 90% of children with Ph-positive ALL have this latter molecular phenotype. Subsequent molecular studies established that in these patients the break occurs in the first intron of the *BCR* gene, the so-called minor breakpoint region (m-*BCR*), in contrast to the major breakpoint region (M-*BCR*) that is used in CML (Fig. 5) (52,53).

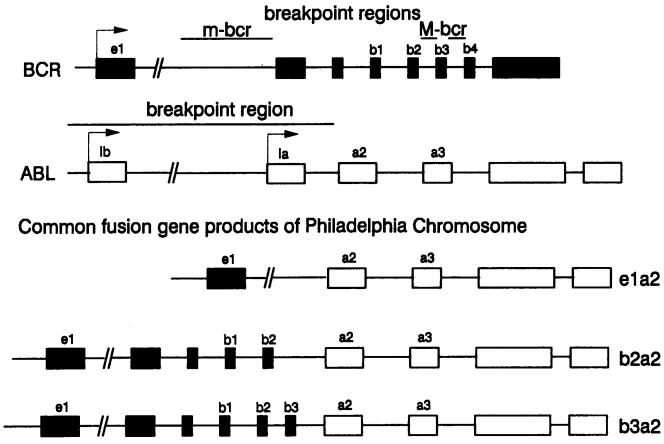

Figure 5. Gene rearrangements in Philadelphia chromosome positive leukemias. Solid boxes = *BCR* exons; open boxes = *ABL* exons; M-*BCR* = major breakpoint region - for CML and some (mostly adult) Ph-positive ALL. Two alternative junctions: b2a2 and b3a2 can be formed in frame for expression. m-*BCR* = minor breakpoint region (generating e1a2 junction) - for some ALL (including most children with Ph-positive ALL).

Although the breakpoints within the first intron may be distributed over a region of >20 kb, the consistent splicing of exon 1 of *BCR* with exon a2 of *ABL* (Fig. 4) means that the same fusion product is generated in all p190 Ph-positive ALL. This provides, therefore, a unique hybrid mRNA and protein sequence that can be used as a leukemia clone–specific marker using the PCR technique or antibodies to the *BCR-ABL* junction (54,55). Fig. 6 illustrates the detection of p190 *BCR-ABL* mRNA using the PCR method. This approach is extremely sensitive (56) and should therefore be useful in monitoring patients for residual or re-emerging disease during remission (57,58). Ig, and particularly the TCR gamma, delta gene probes, offer a similar opportunity but, unlike the situation with Ph-positive ALL (p190), where the *same* unique *BCR-ABL* junction sequence exists (at the mRNA level) in all Ph-positive ALLs, different ALL patients have distinct TCR gene rearrangements, and therefore some sequencing to produce patient specific oligonucleotide primers is necessary (59–61).

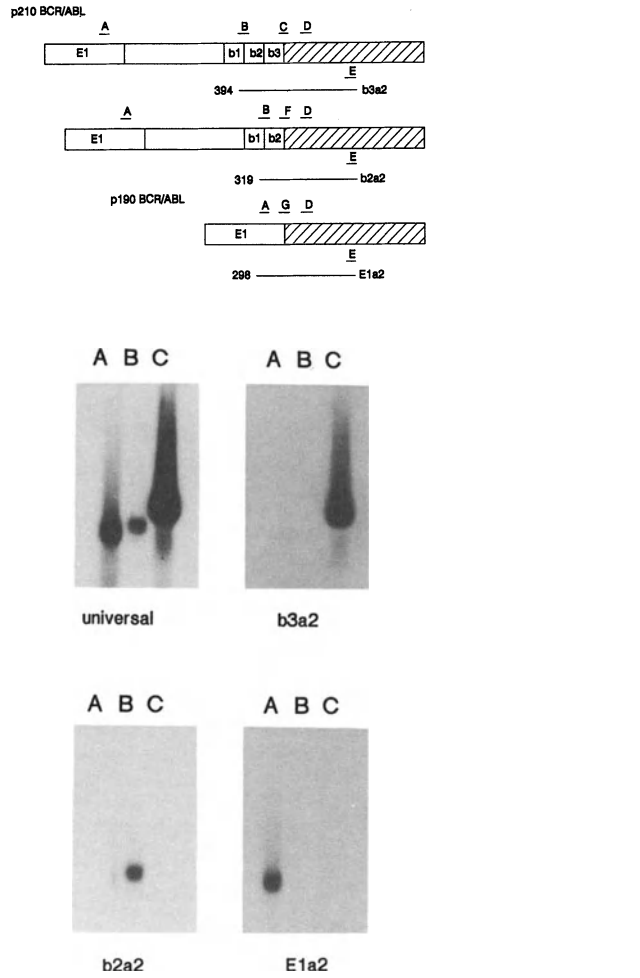

Figure 6. PCR analysis of DNA and RNA products of Ph translocations.
Example of PCR analysis of mRNA products representing a b3a2, b2a2, and E1a2 *BCR/ABL* junction hybridized to different oligomers designed to distinguish among these products. Lanes A, B, and C contain the products from e1a2, b2a2, and b3a2 junctions, respectively, and the corresponding structures of the RNAs are shown in the schematic drawing. The positions of the primers used are also shown in the drawing. Primer D represents a universal oligomer that will hybridize to most of the authentic products amplified with the primer pairs A/E or B/E. Oligomers C, F, and G represent primers recognizing the b3a2, b2a2, and e1a2 junctions, respectively, in hybridization analysis. The sequence of the oligomers is given in ref. 53.

The functional differences between p210 and p190 *BCR-ABL* are not yet clear. One possibility is that the p190 variant is more acutely transforming, especially for lymphoid progenitors, and produces *de novo* ALL; the p210 variant, which is much more common in adults, may be associated with a blast crisis developing from a clinically silent CML.

ETIOLOGICAL MECHANISMS

Despite the biological and clinical importance of these molecular investigations, they do not in themselves reveal the etiological mechanisms. First, we do not yet know the functional role of the deregulated or structurally altered protein products (e.g., the relevant substrate for *BCR-ABL* kinase) and hence the mechanism by which the genetic alterations lead to clonal growth advantage. Second, we cannot be confident that the molecular marker identified (e.g. *BCR-ABL*) initiated leukemic transformation, rather than occurring as a later, albeit essential, event. Third, the nature of the genomic rearrangements identified does not allow us to decide whether they arise spontaneously or are induced by any one of many potential environmental leukemogenic substances. From the etiological viewpoint, the most important implication of the identification of distinct subsets of ALL is that they might have different etiologies and that any epidemiological studies must take this possibility into account. The identification of T–cell subsets of lymphoid malignancy in Japan and the subsequent discovery of HTLV-I-associated ATL provides a clear precedent for the importance of defining discrete disease subsets.

Epidemiological evidence has not so far revealed any striking associations in childhood ALL. Many of the past studies can be criticized on the grounds that too few cases were involved, no specific hypotheses were tested, and no account was taken of disease heterogeneity. Nevertheless, it may be significant that inherited, familial components in ALL are very weak and no evidence exists for increased risk for ALL in immunodeficient children (64,65). Previous evidence for an inherited genetic component based upon the high concordance rate (up to 25% in infants) of ALL in identical twins (66) is most likely explained by the clonal origin *in utero* of ALL in one twin spreading via their common, shared circulation to the other twin (67). Karyotypic (68) and immunoglobulin gene data (69) strongly support this interpretation.

One useful epidemiological approach to identifying etiologic factors in disease is to study geographic variation and time trends. It is important in this respect to note that the incidence rate of childhood ALL does vary internationally and that higher rates are generally associated with higher living standards (70,71). Similarly, the incidence rate of ALL has increased during this century in the U.K. and U.S.A. (72,73). Figure 7 illustrates data from the U.K. It is striking that the increase was most marked for children between the ages of 2 and 5, and moreover that it is ALL in this same age group that is deficient in black African children (74,75). In the U.S.A., black children also had an apparent low rate of ALL in this age range up until the 1960s (76) (Fig. 8), but this situation has changed, with these children now having an age-associated incidence pattern similar to that of white, caucasian children (1,71), with an incidence rate still less than that of white children but considerably greater than that of black African children (Table 3). This observation clearly reduces the likelihood that the marked deficit of ALL in the latter population is due to genetic factors. Note also that the 2–5 year peak of childhood ALL only began to emerge in Japan during the 1960s.

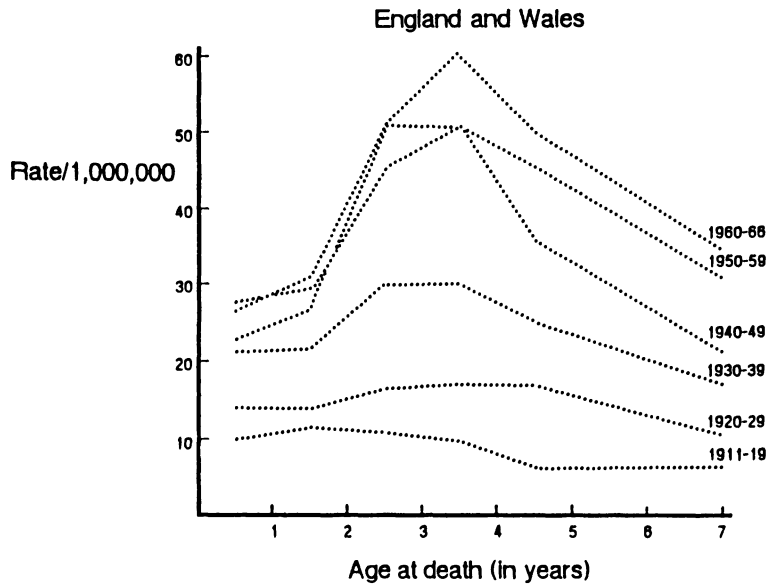

Figure 7. Leukemia mortality (0 – 7 years) in six consecutive decades (official statistics, England and Wales). (Redrawn from ref. 80)

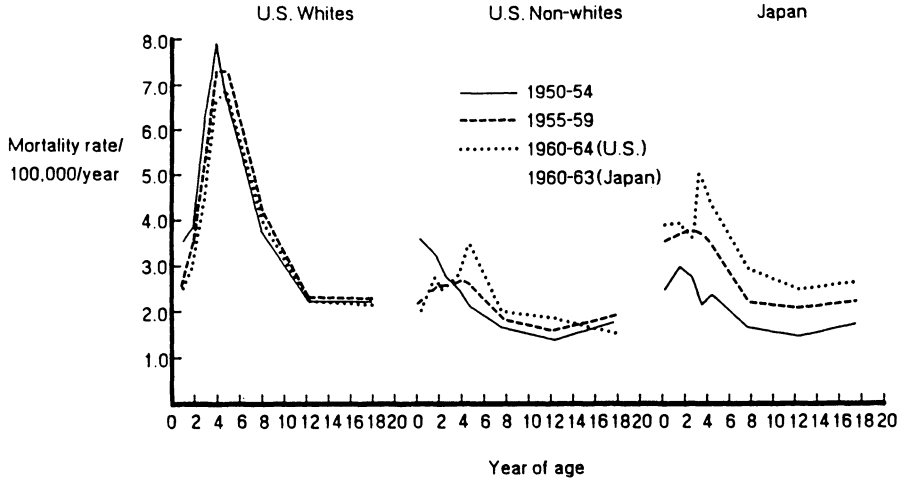

Figure 8. Childhood leukemia mortality rates by age and calendar period in the U.S.A. (1950–1964) and Japan (1950–1963). (Taken from ref. 76.)

Table 3. Incidence rates of childhood acute leukemia

		Numbers per 10^6/yr		
		ALL total	Bp	Tp
A	U.K.	27.0	22.1	4.0
	African (Bl)	5.0	2.5	2.04
			(=11.2 %)	(=51.0 %)
B			ALL	AML
	U.S.A. white (SEER)		34	6
	U.S.A. black (SEER)		16	5
			(47 %)	(83 %)
	Nigeria (Bl)		5	45
			(=15 %)	(=75 %)

A. Data taken from ref. 77 and unpublished observations of, MFG, SMP, and L. Macdougall.
B. Data taken from ref. 71.
Bp = B–cell precursor ALL; Tp = T–cell precursor ALL; Bl = black.

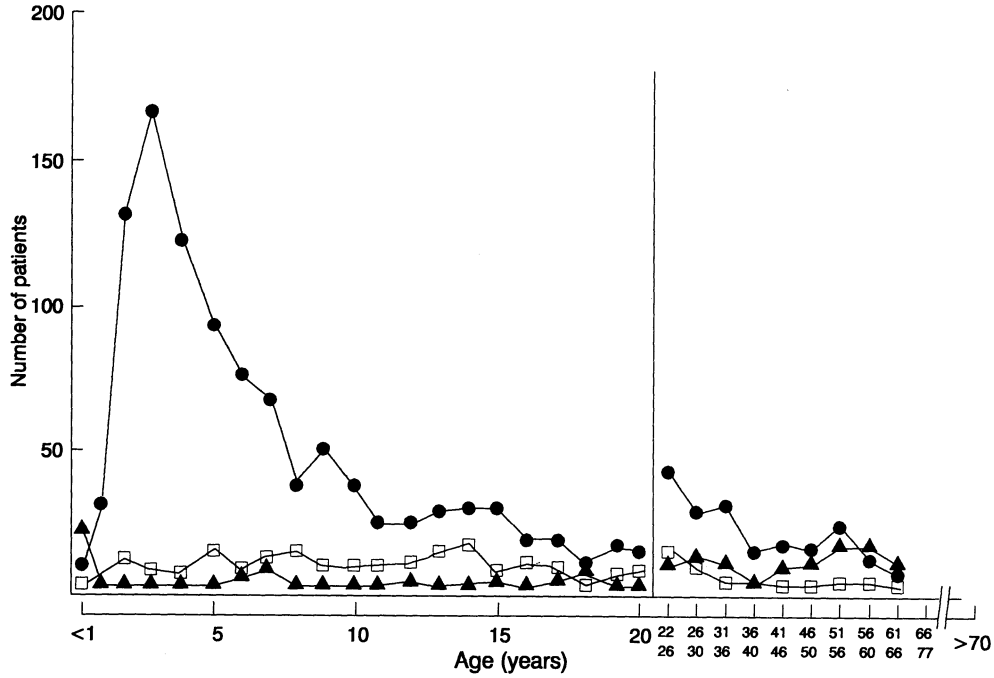

Figure 9. Age distribution of ALL subsets.
Circles = common (B precursor) ALL; triangles = null (B precursor) ALL; Squares = T (precursor) ALL. (Redrawn from ref. 77.)

When the age distributions of immunologically defined subsets of ALL are compared, it is significant that the common ALL subtype accounts entirely for the 2–5 year age peak (Fig. 9) (77), and it seems likely, therefore, that it is the incidence rate of this subset that shows geographic and time trend variations. We sought to confirm this possibility by establishing an international collaborative group study of childhood

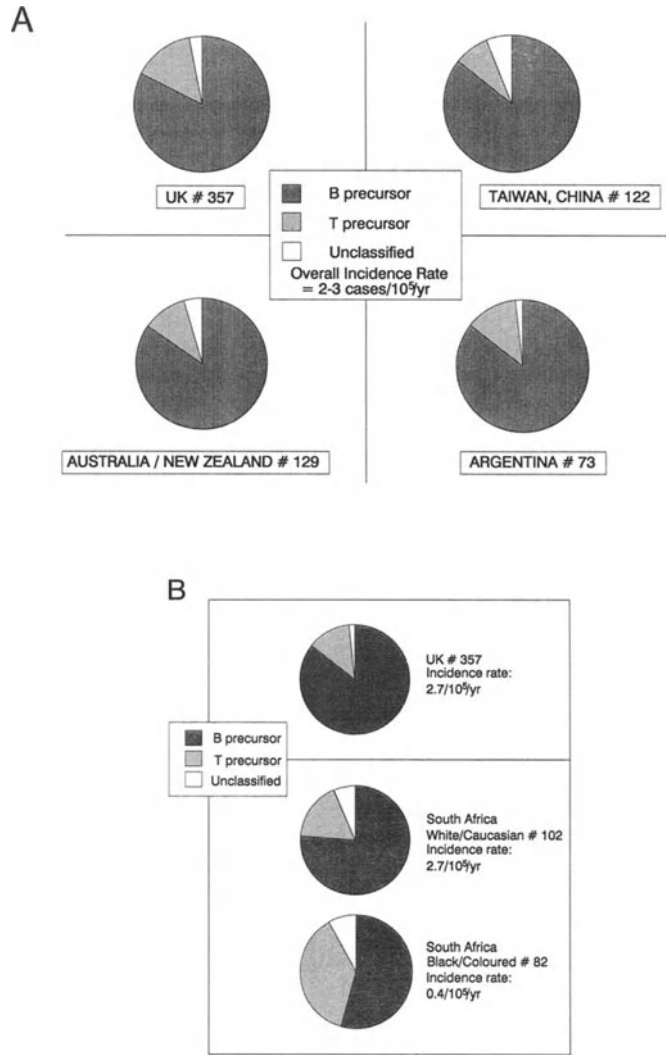

Figure 10. Geographic comparisons of ALL subsets.
Taken from ref. 77 and unpublished observations: MFG, SMP, P.M. Chen (Taiwan), K. Bradstock (Sydney, Australia), M. Beard (Christchurch, New Zealand), L. Fainboim (Buenes Aires, Argentina), L. Macdougall (Johannesburg, South Africa), and P. Jacobs (Cape Town, South Africa).
Note that for these analyses, null ALL with a B precursor phenotype were pooled with common ALL.

ALL subtypes in Europe, South America, Africa, Israel, Southeast Asia, and Australasia (77). Over 1000 patients with ALL have now been successfully phenotyped by standardized panels of monoclonal antibodies and their subset status has been defined using the strict rules and criteria now laid down by an expert system computer program (78). Some of the data are illustrated in Fig. 10. In "developed" countries, both oriental and occidental, the relative proportions and incidence rates of the major ALL subsets are the same (Fig. 10A). This is true also of Japanese ALL. In contrast, in children in

the Gaza Strip and in black children in Nigeria, Kenya, and South Africa, there is a deficiency of common ALL (77,79; MFG, SMP, and L Macdougall, unpublished data) (Fig. 10B). When these data are translated into absolute incidence figures, it becomes apparent that the acute leukemia deficit is predominantly cALL, and that T-ALL and AML are only marginally less common in black vs. white children (Table 3). Note also that pediatric AML varies much less in the incident rate internationally than does ALL (71). This *selective* deficit suggests that the historically established time trends concern primarily cALL and also helps in interpreting the nature of the deficit. It has been argued by Stewart and colleagues (80) that the earlier deficiency of ALL in the U.K. and the substantial deficiency in black African children has been due to pre-emptive death from infection, i.e., the children died from infections such as pneumonia (in the U.K.) or measles, or pneumonia or parasitic diseases (in Africa), *before* their leukemia could be diagnosed *and* because their leukemia had made them very vulnerable to infection. This is a very reasonable interpretation, which we originally favored (64), and may indeed account for some of the deficit of ALL; however, it cannot easily account for a selective effect on common ALL incidence rates. It is hard to conceive that infants with acute leukemia (<2 years, mostly with null ALL), T-ALL patients, and especially children with AML would be less vulnerable to the same infections. We therefore draw the conclusion that the predominance of common ALL as a pediatric cancer is a relatively recent development.

Clearly the cause of ALL is enigmatic, and a variety of environmental and genetic factors may be involved. We have, however, suggested a plausible explanation or hypothesis, which both accounts for the established data and leads to some epidemiological predictions. The hypothesis is directed specifically to the common ALL subset. It incorporates critical and unusual features of lymphocyte precursor physiology and molecular biology, and takes as correct that there is a strong link between socio-economic development and the risk of disease. This model has been published in some detail before (64,81), and the essential arguments are as follows:

1. Common ALL arises by two independent *spontaneous* mutations, the first arising *in utero* or shortly thereafter and the second arising after a latency averaging 3 years that precipitates overt disease. The first mutation produces a clinically silent preleukemic clone that is very similar to that identified recently in Eμ-*MYC* transgenic mice. The mutations involved are spontaneous in so far as they are neither inherited in the germ line nor induced by leukemogens that directly interact with and damage DNA. In relatively infrequent cases, the same mutations could, however, be inherited or induced by leukemogenic agents. The first mutation could arise much more frequently than the incidence rate of ALL, and both mutations are, as in many other cancers, an obligatory requirement for ALL.
2. Lymphocyte progenitors are relatively prone to mutation by virtue of their high proliferation rate during early development (83) combined with their utilization of intrinsic somatic mutagens – TdT and recombinases(84). Proliferation in the Bcell (but not T) progenitor compartment in the bone marrow can be upregulated by the immune response (85), and therefore the natural immune response to infections in childhood could serve as a *promoter* of the genetic events concerned. Specifically, it is predicted that common infections substantially increase the risk or probability of the second mutation.

If common infections serve as a promoter of mutation in preleukemic clones, then it would at first sight seem inconsistent if the incidence rate of cALL has indeed increased substantially with improved living standards. A plausible explanation for this paradox is that a *delay* in the normal pattern of exposure of the immune system to infection may have deleterious consequences. Polio, appendicitis, and infectious mononucleosis are all 20th century illnesses of developed countries (86,87) and provide examples of pathological responses associated with delayed infections that would otherwise be innocuous in their normal time frame.

A "biologically unnatural" delay in exposure to common infections would have become common in developed countries because of improvements in hygiene, vaccination procedures, feeding habits, reduction in sibship size and family grouping, and other associated social factors, all of which compound to immunologically insulate infants and to reduce microbiological exposure. For many children, the first frank exposure to common microorganisms may now come later in life when they first mix with other children at school or in playgroups. This delay might have the effect of leaving the immune system in a naive or immature state. During such a "sterile" period, any preleukemic clone arising by chance alone *in utero* or shortly thereafter will receive little drive to differentiate and little negative feedback from an established immune system. A subsequent infection (say, in a 3–year–old child) might then elicit a strong positive proliferative feedback signal to B–cell precursors in the bone marrow, *including* the already expanded preleukemic clone, and thereby greatly increase the risk of a second critical mutation occurring in the latter cell population. This aspect of the hypothesis should be testable in experimental animals.

The postulated role of the pattern of infection in relation to socioeconomic circumstances is also amenable to epidemiological investigation. In this regard, some predictions of the hypothesis are supported by data that have already been published, including the increased risk for first born children (88,89), a deficit of infections in the first year of life in patients with ALL, and, in two studies, a deficit of vaccinations in children with ALL (90,91). A large scale epidemiological study of childhood ALL in the U.K. is being planned to test these ideas. On the basis of epidemiological data, Gutensohn and Cole have suggested (92) an etiological hypothesis for Hodgkin's disease in young adults that is very similar to that proposed here for childhood ALL.

Finally, the model discussed here is relevant to the current concerns and controversy concerning the apparent excess cases or clustering of childhood leukemia around nuclear installations in the U.K. (93–95). That radioactivity (gamma) can greatly increase the risk of ALL (and presumably induce ALL) is very clear from the Japanese experience (96), with supporting, if more contentious, evidence for x-rays derived from studies on the increased risk associated with diagnostic pelvimetry in pregnancy (97). These recent observations around nuclear plants in the U.K. are, however, very difficult to reconcile with the very low levels of exposure to radioactive emissions and, whilst leukemogenic effects of plutonium and of other high–energy alpha particles has not been completely ruled out, other possible explanations must be considered (93,95). Several theoretical possibilities exist (93,95,98), including the hypothesis for cALL described here, which, as pointed out previously, could give rise to clusters of disease under certain demographic and social circumstances. Kinlen similarly argues that geographic isolation (of nuclear installations and associated communities), combined with high living standards, may increase the risk of childhood leukemia via mini-epidemics of infection (98). This is an important issue to resolve.

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THE EFFECTS OF HEMOPOIETIC FACTORS ON STEM CELLS *IN VITRO*

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ABSTRACT

Blast cell colonies that we have developed in our laboratory have proved to be effective in studies of the mechanisms of stem cell proliferation. The progenitors of blast cell colonies appear to be the earliest to be identified in culture and reside in the G_0 phase of the cell cycle in the steady-state marrow. Using a "mapping" technique, we have characterized the biological properties of the progenitors for the blast cell colony-forming cells and the mechanisms of growth factor interactions supporting stem cell proliferation. When the blast cell colony-forming cells remain dormant in the steady-state marrow, they do not appear to require the presence of early-acting factors such as interleukin (IL)-1, IL-3, IL-6, or granulocyte colony-stimulating factor (G-CSF) for their survival. Our evidence indicates that IL-6, G-CSF, and leukemia-inhibitory factor (LIF)/differentiation-inhibiting activity (DIA) are involved in the initiation of cell divisions of the dormant stem cells. Once stem cells exit from G_0 , their proliferation is supported by IL-3 and/or IL-4. Transforming TGFβ has been shown to preferentially inhibit colony formation from early progenitors. We have observed that the inhibitory effect of TGFβ on colony formation from dormant stem cells may be abrogated by IL-6 or G-CSF. Stem cell proliferation may be regulated by interactions of stimulatory and inhibitory factors.

INTRODUCTION

Daily cell turnover of the hemopoietic system is supported by a small number of cells termed hemopoietic stem cells. Although estimates vary depending on which assay is used, the incidence of stem cells is thought to be approximately 1 per 100,000 nucleated bone marrow cells. The majority of the early studies of hemopoietic stem cells were carried out *in vivo* using a spleen colony-forming assay (1) or hematopoiesis reconstitution assays in lethally irradiated mice or genetically anemic mice of W/W^V. However, the reconstitution assays are only semiquantitative, and there are controversies as to whether or not the colony-forming units in spleen (CFU-S) are true

stem cells. In our laboratory, we have developed a blast cell colony assay technique that is dependent on the proliferation of early hemopoietic progenitors (2). The progenitors for blast cell colonies appear to be the earliest to be identified in culture and reside in the G_0 phase of a cell cycle in the steady–state marrow. This assay technique provided a quantitative method to examine the mechanisms of differentiation and to study the effects of growth factors on the proliferation of hemopoietic stem cells. In this review, we will first define the blast cell colony assay, discuss biological properties of the progenitors for the blast cell colonies, and then describe the mechanisms of interactions of growth factors supporting the proliferation of hemopoietic stem cells *in vitro*.

BLAST CELL COLONY ASSAYS

The picture shown in Fig. 1 presents the typical morphology of a blast cell colony. Blast cell colonies may be defined as small colonies consisting of fewer than 200 to 300 blast cells with no apparent sign of differentiation. Defined as such, they do not signify specific lineages or stages of hemopoietic development. However, the blast cell colonies of interest are the blast cell colonies that develop from stem cells that are dormant in cell cycling. Murine blast cell colonies were first identified as late-appearing, small colonies consisting of undifferentiated blast cells with high replating potentials in cultures of marrow and spleen cells of normal mice (2). The incidences of the blast cell colonies in these cultures were 1 and 5 per 10^5 nucleated spleen and bone marrow cells, respectively. Because of coexisting large colonies derived from maturer progenitors, such as erythroid bursts, granulocyte/macrophage (GM) colonies, and granulocyte/erythrocyte/macrophage/megakaryocyte (GEMM) colonies, it was very difficult to identify small blast cell colonies in cultures of cells from normal mice. Injection of high-dose 5-fluorouracil (5-FU), described by Hodgson and Bradley (3), provided a simple and effective method for enriching the murine marrow and spleen cells for the blast cell colony-forming cells (4). Originally proposed as the selective method for pre-CFU-S (progenitors for the late-appearing spleen colonies, cells), this method effectively eradicated all cycling progenitors (monopotential as well as multipotential), while leaving progenitors for the murine blast cell colonies unaffected. Subsequently, our serial observation of the development of individual blast cell colonies (mapping studies) established the concept that blast cell colonies are

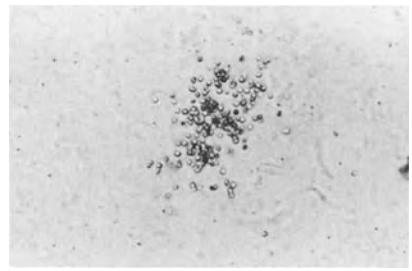

Figure 1. A representative murine blast cell colony consisting of undifferentiated cells and showing no sign of terminal differentiation.

transient colonies developing from early progenitors in G_0 and are programmed to develop into multilineage colonies or large GM colonies.

Development of a human blast cell colony also required the use of a selective technique for the growth of blast cell colonies, since the ratio of blast cell colonies to other types of colonies is approximately 1:200 in the CD34-positive bone marrow cells. We have tested the use of cycle-specific chemotherapeutic agents such as 5-FU and 4-hydroperoxycyclophosphamide (4-HC) *in vitro* as purging agents for cycling progenitors without success. Therefore, we used a biological method for the selective growth of blast cell colonies (5). This assay is based on the following hypotheses. (a) Hemopoietic stem cells are in a noncycling state and they do not require growth factors for their survival in G_0 (2). The maturer progenitors are actively cell cycling and require growth factors for their survival and continued cell proliferation. In this method, CD34-positive bone marrow cells were plated in the presence of 2% fetal calf serum (FCS), bovine serum albumin of high purity, lecithin, cholesterol, transferrin, and sodium selenite and incubated for 2 weeks. The absence of growth factors in the culture medium abolished colony formation from cycling maturer progenitors. On day 14 of culture, either medium conditioned by the human T lymphoblast cell line, C5MJ, or medium conditioned by human lymphocytes stimulated with phytohemagglutinin (PHA) was layered over the culture dishes. Blast cell colonies consisting of generally fewer than 50 cells were identified on days 21–28. The only background growth was small clusters of macrophages. Subsequently, we observed that the conditioned medium may be substituted by interleukin (IL)-3 (6). Once blast cell colonies were identified, individual blast cell colonies were replated in more permissive culture medium containing 30% FCS, erythropoietin (Ep), and either crude conditioned media or a mixture of growth factors for the expression of the full lineage potentials of the colonies. Although the majority of the human blast cell colonies thus identified revealed secondary colony formation, the relative incidence of GEMM colonies in the secondary colonies was much lower than that in the murine blast cell colonies. At the present time we do not know the reasons for this difference.

EFFECTS OF IL-3 AND IL-4 ON PROLIFERATION OF EARLY PROGENITORS

The blast cell colony assay was applied to the studies of the effects of growth factors on stem cell proliferation. Specifically, we used the "mapping technique" of individual blast cell colonies. In this method, the culture plates were scanned daily on an inverted microscope and the location of newly identified individual blast cell colonies was recorded. Subsequently, proliferation and differentiation *in situ* of each blast cell colony was examined daily. Representative mapping data of murine blast cell colonies from day-2 post-5-FU marrow cells supported by IL-3 are presented in Fig. 2A. New blast cell colonies continued to appear almost daily until the later days of incubation, and once they were identified their subsequent proliferation was characterized by constant doubling times of approximately 16–17 hours. This observation was interpreted to mean that IL-3 does not trigger the proliferation of dormant stem cells but rather passively supports the proliferation of progenitors after their exit from G_0. This point was demonstrated more clearly by the observation that delayed addition of IL-3 on day 7 of incubation to cultures of day-4 post-5-FU spleen cells did not synchronize the late-appearing blast cell colonies (7). Most recently, we observed that murine IL-4

supports a comparable number of blast cell colonies to that supported by IL-3 (8). When we replated the blast cell colonies of similar sizes grown in the presence of IL-3 or IL-4, the types of secondary colonies were similar. Interestingly, human IL-4 did not support the formation of multipotential blast cell colonies.

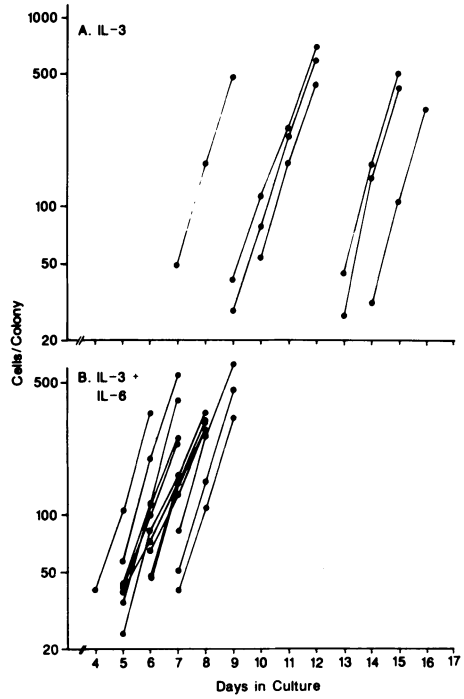

Figure 2. Time course of emergence and development of blast cell colonies from a total of 8 x 10^4 day-2 post-5-FU marrow cells in the presence of IL-3 (A) and a combination of IL-3 and IL-6 (B).

SYNERGISTIC FACTORS

Although the concept that hemopoietic stem cells are dormant in cell cycle had been proposed more than two decades ago (9), mechanisms that initiate cell proliferation of the dormant cells remained to be elucidated. In our laboratory, we identified three factors – IL-6 (10,11), granulocyte colony-stimulating factor (G-CSF) (12,13), and leukemia inhibitory factor/differentiation-inhibiting activity (LIF/DIA) (14)– that appeared to be involved with the exit of stem cells from G_0. Neither IL-6 nor G-CSF as single agents have the ability to support blast cell colony formation (11,13). However, both factors enhance IL-3- and IL-4-dependent blast cell colony formation, and part of the synergism is to shorten the G_0 period. The synergistic activity of IL-6 on IL-3-dependent murine blast cell colony formation from day-2 post-5-FU marrow cells is presented in Fig. 2B. In contrast to the culture with IL-3 alone shown in Fig. 2A, more blast cell colonies developed and they appeared earlier in the presence of IL-6 and IL-3. Their doubling times, however, were similar to those in culture with IL-3 alone, which are shown in Fig. 2A. Based on these observations, we proposed that IL-6

shortens the G_0 period of the stem cells (10,12). The synergistic ability of G-CSF on IL-3-dependent blast cell colony formation was identical to that of IL-6 in every aspect and appeared to be directed to the same target cells as those responding to IL-6 (15). As shown in Figs. 2A and B, the number of blast cell colonies from day-2 post-5-FU marrow cells supported by IL-3 alone was significantly fewer than that supported by a combination of IL-3 and IL-6. It appears that spleen cells are a source of factors, such as IL-6 and G-CSF, that stimulate the cell division of dormant stem cells. The enhancing effects of IL-6 and G-CSF were also observed with IL-3-dependent human blast cell colony formation (11,13). In the presence of synergistic factors, not only was the development of blast cell colonies earlier, but also the total number of blast cell colonies identified was almost twice that of the IL-3 control. It is possible that the stem

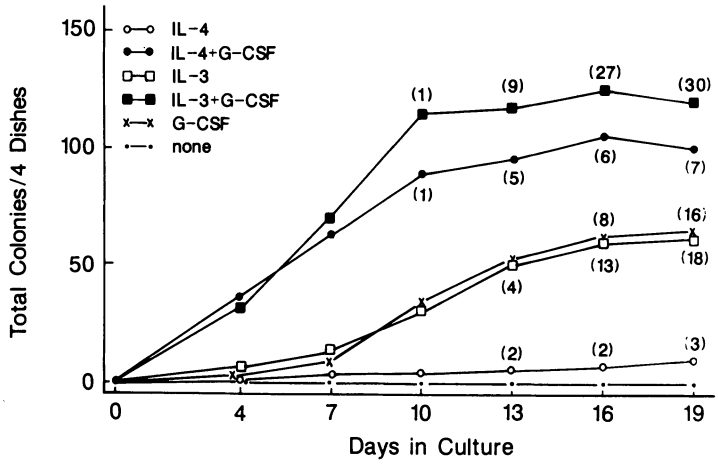

Figure 3. Synergy between IL-4 (10 ng/mg) and G-CSF (100 ng/ml) and between IL-3 (20 ng/ml) and G-CSF (100 ng/ml) on colony formation from day-2 post-5-FU murine marrow cells. The numbers in brackets represent GEMM colonies. The number of GEMM colonies in the IL-4 plus G-CSF group represents a cumulative number of GEMM colonies, because GEMM colonies tend to degenerate during observation.

cell exit from G_0 (initiation of cell proliferation) may be totally dependent on growth factors such as IL-6 and G-CSF. Earlier, investigators in three laboratories reported that IL-1 is a synergistic factor for stem cell proliferation (16,18). When we examined the effects of IL-1α and β on murine blast cell colony formation using day-4 post-5-FU spleen cells, a clear-cut synergism that was similar to that of IL-6 and G-CSF was observed (15). However, when tested in the human system, in which only 10,000 CD34-positive marrow cells were plated, there was no enhancing effect of IL-1 on human blast cell colony formation (11). This observation indicated that the synergistic effect of IL-1 demonstrated in the murine system was indirect and was mediated in part by IL-6 and G-CSF. The latter factors are probably released by accessory cells, such as fibroblasts and endothelial cells, upon stimulation by IL-1. Most recently we observed that LIF/DIA possesses synergistic activity on human blast cell colony formation (14). Although the number of blast cell colonies is significantly increased in the presence of LIF/DIA, we have not confirmed that it shortens the G_0 period. Further studies are necessary, including dose-response studies with purified material, for the characterization of the precise mechanisms of the synergistic effects of LIF/DIA.

In an earlier section of this review, we have alluded that IL-4 supports blast cell colony formation from murine marrow cells. Two synergistic factors, IL-6 and G-CSF, augment IL-4-dependent colony formation from day-2 post-5-FU marrow cells. Our report of the synergistic effects of IL-6– on IL-4-dependent colony formation has been published previously (8). In Fig. 3 the synergistic effects of G-CSF on IL-4-dependent colony formation from day-2 post-5-FU marrow cells are presented. Although the synergism between IL-4 and G-CSF on the total number of colonies is clear, the numbers of multilineage colonies supported by the combination at later periods of incubation were less that those supported by G-CSF alone. This observation is similar to the observation with IL-6 and IL-4 reported previously (8), and appears to be due to the inhibitory effects of IL-4. In order to test this hypothesis, we carried out blast cell colony transfer experiments. We plated 5 x 10^4 day-2 post-5-FU marrow cells per dish in the presence of IL-4 (10 ng/ml) and IL-6 (100 ng/ml). On day 10 of incubation, all 28 colonies detected in a dish were individually transferred to secondary cultures containing 10 ng/ml IL-3 and 2 U/ml Ep. The secondary dishes were analyzed on day 3 for the presence of erythroid colonies and on day 6 for erythroid bursts, and GM and GEMM colony formation. Twenty out of 28 primary colonies revealed granulocyte and erythroid lineage expression. These results indicate the synergistic effects of IL-6 and G-CSF on IL-4-dependent proliferation of multipotential progenitors and support the premise that the apparent lack of enhancement of multilineage colony formation later in culture is due to the inhibitory effects of IL-4.

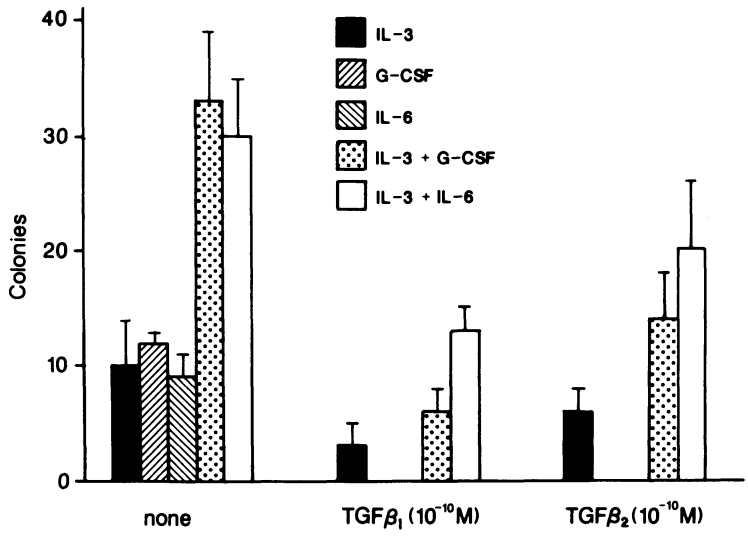

Figure 4. Neutralizing effects of IL-6 and G-CSF on the inhibitory effects of TGFβ on colony formation from primitive progenitors. 5 x 10^4 marrow cells from day-2 post-5-FU mice were cultured with factors in a dish. Data represent mean ± SD of colony numbers in triplicate cultures on day 16 of incubation. In the presence of TGFβ, neither IL-6 nor G-CSF supported colony formation. (Courtesy of Williams and Wilkins.)

Investigators in several laboratories (19–22) reported that type β transforming growth factor (TGFβ) inhibits the proliferation of early hemopoietic progenitors. We have examined the dose effects of bovine TGFβ on murine hemopoietic colony formation and observed that it shows preferential inhibition of early hemopoietic progenitors (23). The most notable suppression was colony formation from day-2 post-5-FU murine marrow cells. We then examined the interactions between IL-6, G-CSF, and TGFβ in support of colony formation from dormant progenitors using day-2 post-5-FU marrow cells. First, we carried out an experiment in which the TGFβ was added on day 0 to cultures containing various combinations of factors. We observed that the addition of IL-6 or G-CSF partially neutralized the inhibitory effect of TGFβ. The results are presented in Fig. 4.

We then exposed day-2 post-5-FU marrow cells to these factors for 2 days in serum-free suspension culture, and then washed and replated aliquots of cells for analysis of the surviving fractions of the progenitors. While TGFβ almost totally abolished the colony-forming ability of the dormant progenitors, IL-6 and G-CSF abrogated the inhibitory effects of TGFβ on colony formation. These results indicated that TGFβ and early-acting synergistic factors, such as IL-6 and G-CSF, possess opposing effects on early progenitors. It has been postulated that in the presence of TGFβ, hemopoietic stem cells are kept in a dormant state (24). The effects of TGFβ on the kinetic state of early progenitors need to be investigated further.

CELL CYCLE DORMANCY OF STEM CELLS

For over two decades it was generally held that stem cells in the steady state marrow are in the G_0 period. However, this concept was based on indirect experimental observations, such as sensitivity to ^{3}H-thymidine with high specific activity, survival of stem cells after injection of cell cycle specific chemotherapeutic agents such as 5-FU, and our mapping studies of blast cell colony development in culture. There was no direct evidence for the long residence in G_0 of the hemopoietic stem cells. Recently, we carried out observations of the development of human blast cell colonies from single cells by daily observations of mapped individual candidate progenitors (25). Our observations clearly demonstrated that human hemopoietic progenitors remain as single cells for more than 2 weeks in incubation. Once the progenitors began proliferation, the subsequent growth was characterized by steady cell doubling, similar to that reported for murine blast cell colony development. In an earlier section of this review, we presented the hypothesis that survival of blast cell colony progenitors in G_0 does not require early-acting growth factors as the rationale for the development of human blast cell colony assay. We have now demonstrated that the survival of hemopoietic progenitors in G_0 does not require early-acting hemopoietic regulators. In this series of experiments, we used neutralizing antibodies prepared against IL-1α, IL-1β, IL-3, IL-6 and G-CSF. In these experiments, cultures were initiated with individual antibodies or a combination of four antibodies, and on day 14, IL-3 or a combination of IL-3 and IL-6 was added to the cultures. Comparable numbers of blast cell colonies developed in the antibody and control groups. These results demonstrated that for the survival of early hemopoietic progenitors in G_0, IL-1α, IL-1β, IL-3, IL-6, and G-CSF are not necessary.

ACKNOWLEDGMENTS

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3

BONE MARROW STROMAL CELLS AND HEMATOPOIESIS

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ABSTRACT

Five clonal stromal cell lines were established from 6-week-old human long-term bone marrow cultures (KM-101-105). They expressed none of the cell surface antigens characteristic of hematopoietic cells and endothelial cells, and showed different radiosensitivity at a low dose rate of irradiation. These cell lines produce hematopoietic growth factors (IL-6, G-CSF, M-CSF, GM-CSF, and EPA) and adhere to the hematopoietic cells from established cell lines and primary bone marrow cultures. Transfer of Lucifer Yellow CH was observed between the primary or established stromal cells, and their neighboring cells. The dye injected into the stromal cells sometimes moved into the attached round hematopoietic cells in primary bone marrow culture, and even in coculture of KM cells, and established hematopoietic cells. These results suggest that marrow stromal cells modulate hematopoiesis through the secretion of hematopoietic diffusible factors, direct stromal cell-hematopoietic cell contact, and intercellular gap junctions.

INTRODUCTION

The bone marrow is, at least in humans, the sole site of proliferation and differentiation of hematopoietic stem cells in health, despite the fact that they are present in the circulation (1–4). Therefore, it has been indicated that the hematopoietic microenvironment, or stroma, plays a critical role in the proliferation and differentiation of the stem cells. Nevertheless, the precise role of stroma in hematopoiesis has not been clearly understood. The hematopoietic marrow stroma is composed of complex cellular elements, including reticular cells, fat cells, fibroblasts, macrophages, and vascular endothelia (5). Stromal cells and the surrounding extracellular matrix substances are usually physical supportive elements of parenchymal cells. Collagen and other macromolecules of the extracellular matrix are found in lesser amounts in normal marrow than in other organs, probably because the bone marrow is surrounded by hard bone tissue. Accordingly, it is likely that the marrow stromal cells act mainly as a functional moderator of hematopoietic stem cells rather than as their physical supportive tissue.

A long-term liquid culture system of bone marrow (LTBMC) has facilitated the

study of the proliferation and differentiation of hematopoietic stem cells and their cellular environment (6,7). In the LTBMC of the Sl/Sl^d mouse, which has a marrow microenvironmental defect, hematopoiesis is maintained in lower levels than in the normal mouse (8). Therefore, the LTBMC simulates the *in vivo* state. In this *in vitro* system (9), hematopoietic cell lineages consisting of granulocytes, monocytes, macrophages, megakaryocytes, erythrocytic, and lymphocytic cells are generated. The marrow adherent stromal cells of LTBMC support hematopoiesis *in vitro* by their effects which are presumably mediated by cell–to–cell interaction and/or regulator production. In elucidating the mechanism of hematopoiesis in *in vitro* culture, a major obstacle is the complexity of the adherent stromal cells, i.e., which cell type possesses which functions. Therefore, the establishment of clonal marrow stromal cell lines and the analysis of the hematopoietic functions would clarify the role of the hematopoietic microenvironment, since the cultured adherent stromal cells from the bone marrow are thought to represent an *in vitro* counterpart of the stromal tissue that controls hematopoiesis. This communication, therefore, focuses on the hematopoietic effects of clonal human marrow stromal cell lines that were established using the rib bone marrow of a 48-year-old hematologically normal male patient who underwent lung surgery (10).

ISOLATION OF CLONAL HUMAN MARROW STROMAL CELL LINES

The adherent cell populations in 6-week-old human LTBMC were transfected with recombinant plasmid pSV3gpt DNA containing the coding sequence of the early region of simian virus 40 (SV 40). Five clonal marrow stromal cell lines were established from the cultures (KM-101 – 105) (10). They went through more than 180 passages, but they did not form any tumor when they xenotransplanted into nude mice. All the clonal cells were fibrocytic in appearance and showed the presence of SV 40 T antigen in the nuclei.

CHARACTERISTICS OF THE HUMAN MARROW STROMAL CELL LINES

The radiosensitivity of the marrow stromal cell lines was examined (11). It is known that dose-rate independent x-ray killing is observed with marrow hematopoietic stem cells. A significantly increased radiation resistance of KM-102, 103, 104, and 105 was observed at a low dose rate (5 cGy/min), compared with high–dose rate irradiation (120–200 cGy/min.). In contrast, cell line KM-101 demonstrated no significant change in radiosensitivity relative to different dose rates. This evidence is compatible with the physiological changes observed in plateau phase bone marrow cells after low–dose rate irradiation *in vivo* and *in vitro*. This suggests that marrow stromal cells might be heterogenous in low–dose rate radiation repair. With immunocytochemical staining, each clone showed the presence of collagen type I and III, laminin, fibronectin, and HLA class I antigen, with the exception of specific cell–surface antigens expressed in hematopoietic cells and endothelial cells. They did not exhibit conversion to adipocytes, even when they were exposed to hydrocortisone or insulin.

EFFECTS OF HUMAN MARROW STROMAL CELL LINES ON HEMATOPOIESIS

The evolution of multicellular organisms has depended on the ability of cells to communicate with each other. Cells in higher animals are thought to communicate in three different ways: (a) they secrete chemicals that signal cells some distance away; (b) they display plasma-membrane-bound signaling molecules that influence other cells that make direct physical contact; and (c) they form gap junctions that directly join the cytoplasm of the interacting cells (12). By the same logic, stromal hematopoiesis is proposed to occur via the three aforementioned ways: stromal cell secretion of hematopoietic diffusible factors, direct stromal cell-hematopoietic cell contact through the plasma-membrane-bound signaling molecules, and intercellular gap junctions.

Production of Cytokines from Human Marrow Stromal Cell Lines

The human marrow stromal cell line feeders and conditioned media stimulated a lot of CFUc-derived colonies (10). When 1 aliquot of the conditioned medium was added to 9 aliquots of growth medium containing methylcellulose for early erythroid stem cells (BFUe), the number of BFUe-derived colonies increased by 20%–40%. In this culture, several mixed colonies consisting of granulocytic, erythrocytic, monocytic and megakaryocytes were formed. The addition of the conditioned medium induced the survival of BFUe in the culture without erythropoietin for a period of time (burst promoting activity, BPA). In the culture without the conditioned medium, the number of BFUe–derived colonies quickly decreased compared to the ones in the cultures with the conditioned medium. In order to purify BPA, 6 l of serum–free conditioned medium was harvested from the marrow stromal cell line, KM-102. The purification was performed by five-step sequential fractionation using ammonium sulfate precipitation, DEAE-ion exchange, lentil lectin affinity chromatographies, high–performance gel filtration chromatography, and reversed–phase high performance liquid chromatography. Purified BPA gave a single broad protein band of 17,000–19,000 MW on sodium dodecyl sulfate polyacrylamide gel electrophoresis in the presence or

Table 1. Production of cytokines by human stromal cell lines.

	hGM-CSF	hG-CSF[a]	hM-CSF	EPA	hIL-3	IL-6	Murine CSA
KM-101	+(↑)	−(+)	+[b]	+	−(−)	+(↑)	+
KM-102	+(↑)	−(+)	+[b]	+	−(−)	+(↑)	+
KM-103	+(↑)	−(ND)	ND	+	−(−)	ND	+
KM-104	+(↑)	−(ND)	ND	+	ND	ND	+
KM-105	+(↑)	+	ND	+	ND	ND	−

+ = produced; − = not produced; ↑ = the production increased; ND = not done.

[a] RIA.

[b] Northern blot hybridization of poly-A messenger RNA from cell lines showed the presence of 4.0 and 2.0 kb messages.

The sizes of M-CSF bound to membrane were 66 and 31 kD in the reduced condition. The symbol inside the parenthesis represents the change of cytokine production when the cultures were stimulated by IL-1.

absence of 2-mercaptoethanol (13). The half maximum concentration of this activity, which was detected among the survival BFUe for 8 days, was approximately 7 pg/ml, and the lowest maximal dose was about 28 pg/ml. The amino acid analysis of purified material showed that this molecule had a similar N-terminal sequence and amino acid composition to that deduced from cDNA of human GM-CSF. Up to now, it has been shown that these cell lines produce various kinds of hematopoietic factors, as listed in Table 1.

GM-CSF, M-CSF, erythroid potentiating activity (EPA), and IL-6 were constitutively produced by the human marrow stromal cell lines. The production of GM-CSF and IL-6 increased when the cultures were supplemented with IL-1. G-CSF was secreted when the cultures were stimulated by IL-1, while KM-105 constitutively produced it. The marrow stromal cell lines did not produce IL-3, even when the cultures were stimulated. Rennick *et al.* reported that the addition of either IL-1 or LPS to murine marrow stromal cell culture resulted in a tremendous increase of poly A messenger RNA of GM and G-CSF (14). These results suggest that, in an infected state, bacterial endotoxin and/or IL-1 released by macrophages stimulates the marrow stromal cells, and they increase the production of hematopoietic factors. Recent observation has suggested that heparan sulfate, a major sulfated glycosaminoglycan secreted by marrow stromal cells, adsorbs part of the hematopoietic factors and thus effectively presents them to target hematopoietic cells attached to the stromal cells (15).

In one sense, it is possible that collagen and other macromolecules of the extracellular matrix could be considered special types of local mediators, playing a role in the formation of a suitable environment for hematopoiesis. They are secreted by stromal cells and signal other local cells to alter their behavior. These molecules differ from other diffusible hematopoietic factors in that they are insoluble and therefore do not diffuse from the region where they are synthesized.

Direct Stromal Cell-Hematopoietic Cell Contact through the Plasma-Membrane-Bound Signaling Molecule(s)

A direct cellular interaction was investigated in a coculture of the human clonal marrow stromal cell line, KM-102, and the myeloid leukemia cell line, HL-60 (16). In the coculture a large number of HL-60 cells remained in the supernatant but some of them became firmly attached to KM-102 cells. The attached HL-60 cells showed little positive reaction in the nitroblue tetrazolium solution test when the culture was supplemented with 10^{-9} to 10^{-7}M 1,25-dihydroxyvitamin D3. In contrast, differentiation in the supernatant HL-60 cells was strikingly responsive to the agent in a dose-dependent manner. Furthermore, complete inhibition was observed in the incorporation of ^{3}H thymidine (^{3}TdR) into attached HL-60 cells with autoradiography, but 23.60% of the supernatant cells moderately incorporated ^{3}H TdR into their nuclei. There was little attachment between HL-60 cells and stromal cells from human thymus and lymph nodes. The modulation by marrow stromal cells was more strongly affected by this interaction than by exogenously added differentiation-inducing agents. Apparently marrow stromal cells provide a definitive milieu, possibly through membrane-bound signaling molecules, for the proliferation and differentiation of nonleukemic and leukemic immature myeloid cells.

When 10^{6} KM-101 and 7 x 10^{6} HL-60 cells were incubated together – as in the

rosette formation of T cells – and suspended in culture medium, it was frequently seen that three or more HL-60 cells adhered to each large stromal cell (Fig. 1). Similar rosettes were formed between marrow stromal cell lines and other myeloid hematopoietic cell lines. A coculture of KM-105 and the human hematopoietic cell line, CMK cells, was examined with electron microscopy. The CMK cell line expresses surface antigens specific for granulocytic, erythrocytic, and megakaryocytic lineages (17). Round CMK cells with a lot of microvilli adhered well to KM-105 cells, and frequently their cytoplasmic processes extended beneath the stromal cells (Figs. 2, 3).

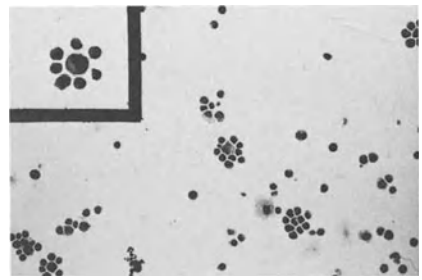

Figure 1. Rosette formation between HL-60 and KM-101 in suspension. Smaller HL-60 cells bind to large KM-101 cells.

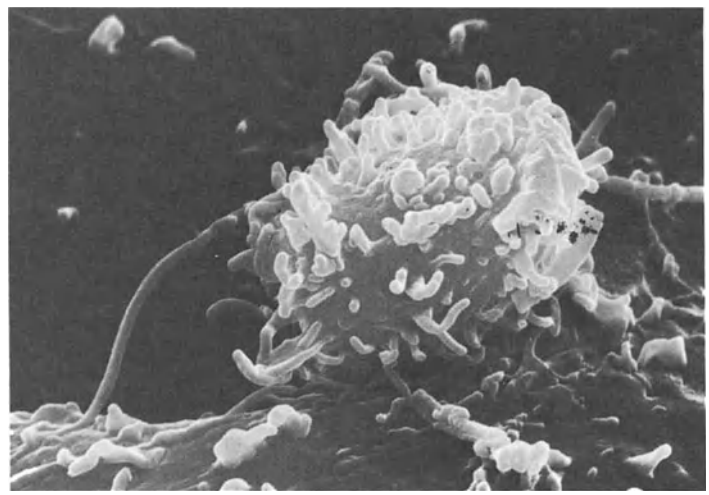

Figure 2. Scanning electron micrograph of a coculture between CMK cells and KM-105 cells.

Electron–dense spots were sometimes seen where the membranes fitted together between the cytoplasmic processes of CMK cells and stromal cells (Fig. 3). These observations prompted us to investigate the molecule or molecules that governed the heterophilic cell adhesion between hematopoietic cells and stromal cells. A lot of monoclonal antibodies were made against the stromal cell lines, KM-101 and KM-102. The Fab fragment of one of the monoclonal antibodies, C-F-9, significantly blocked the heterophilic cell adhesion between myeloid cell lines and KM-101, and between KM-101 and RPMI8402 cells, which are presumably at the prethymic stage of

development according to the examination of surface antigens (Table 2).

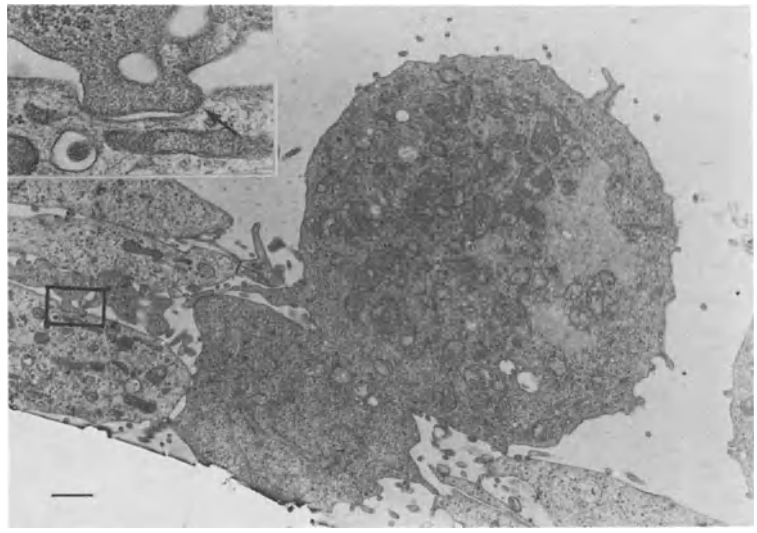

Figure 3. Transmission electron micrograph of a coculture of CMK cells and KM-105. A CMK cell is situated between KM-101 cells and extends its cytoplasmic processes beneath the KM-101 cells. The membranes of both cells fit together (inside square) and an electron-dense spot (intermediate junction) is seen (arrow).

Table 2. Effects of C-F-9 monoclonal antibody on heterophilic cell adhesion between stromal cell lines and hematopoietic cell lines.

	CMK	CMK7	KOPM	K562	HL-60	U937	RPMI 8402	JM	B-ALL	ELM-I-1	L-8057
KM-101	++	+++	+	+	++	++	+	−	−/+	−	−
+Fab(C-F-9)	↓↓↓	↓↓↓	↓↓↓	↓↓↓	↓	↓	↓	−	−/+	−	−
WI-38	−	−	−	−	−/+	+	−/+	++	−	−	−
+Fab(C-F-9)	−	−	−	−	−/+	+	−/+	++	−	−	−
WI-38 va13	−	−	−	−	−	+	−/+	++	−	−	−
+Fab(C-F-9)	−	−	−	−	−	+	−/+	++	−	−	−

CMK, CMK-7, KOPM, K562, HL-60, and U937 are human myeloid hematopoietic cell lines. RPMI8402 and JM are human T-lymphoid cell lines, and B-ALL is a human B-lymphoid cell line. ELM-I-1 and L-8057 are murine hematopoietic cell lines established from radiation-induced leukemia of C3H/He mice. Fab(C-F-9) is a culture supplemented with a Fab fragment of monoclonal antibody C-F-9.
+ = weakly adhesive to stromal cells; ++ = moderately adhesive to stromal cells; +++ = strongly adhesive to stromal cells; − = not adhesive; −/+ = partly adhesive; ↓ = weakly to moderately blocked the adhesion; ↓↓↓ = strongly blocked the adhesion.

However, this blocking was not seen in the incubation with monoclonal antibody against LFA-1. The human T-lymphoid cell line, JM, at the thymic stage of development did not adhere to KM-101 but bound well to WI-38, a human diploid fibroblast cell line. The Fab fragment did not block the heterophilic cell adhesion

between WI-38 and JM. A similar pattern of heterophilic cell adhesion in WI-38 cells was observed in that of WI-38 va 13, which was a subline of WI-38 and was infected with SV 40 virus. This evidence suggests that the antigen recognized by C-F-9 would be an adhesion molecule related to bone marrow stromal cells and immature hematopoietic cells. The investigation of the antigen recognized by C-F-9 is underway.

Intercellular Gap Junctional Communications

Gap junctions that couple cells ionically and metabolically are important in coordinating cells. The cell apparatus is composed of clusters of protein channels that allow ions and molecules of molecular weights less than 1500 to pass directly from the inside of one cell to that of another. Since Lucifer Yellow CH, which has a molecular weight of 457, cannot diffuse through membranes, its transfer into neighboring cells is believed to occur by gap-junctional communication (18). In murine and human bone marrow cultures, marrow stromal cells were dye coupled (Fig. 4) (19,20). Electron-microscopically gap-junctional communication has also been demonstrated in murine bone marrow stromal cells (21). This indicates that the stromal cells have direct communication between neighboring cells. From the study of the murine marrow preadipocyte cell line, H-1, it has been demonstrated that marrow preadipocytes have gap-junctional communication, and the differentiation to adipocyte causes the interruption of communication (20). We have previously observed that H-1/A cells contribute to granulopoiesis during the fibrocytic stage through the production of colony-stimulating factor, while the CSF released by H-1/A cells decreases when they are converted to adipocytes (22). It is conceivable that the stromal cells quickly synchronize their functional state via gap junctions and work in collaboration on hematopoiesis upon demand. Gap-junctional communication was frequently observed in human marrow stromal cell lines, and sometimes the dye injected into stromal cells moved into attached round hematopoietic cells in primary bone marrow culture (Fig. 4) and even in coculture of KM-102 and HL-60 cells (16). This indicates that there is a direct communication of low molecular substances between stromal cells and hematopoietic cells. But, in view of the low frequency, this communication may not be the aforementioned inhibitory mechanism.

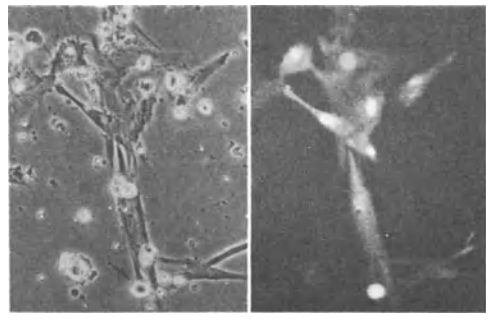

Figure 4. Dye coupling between human marrow stromal cells. Dye was injected into a stromal cell outside of the field and transferred to neighboring cells. The arrow indicates the dye transfer path from fibrocytic stromal cells to a round hematopoietic cell. A: phase contrast micrograph; B: fluorescent micrograph.

CONCLUSION

Reticular cells are a major cellular component of hematopoietic cord, form a complex meshwork, and through their fine processes are in contact with almost all hematopoietic cells. Cultured marrow stromal cells are thought to correspond with an *in vitro* counterpart of reticular cells in hematopoietic cord. Therefore, if the results in this paper represent events taking place *in vivo* in bone marrow, it would appear that reticular cells harmonize their functional state through gap junctional communication. Under steady-state hematopoiesis, reticular cells exert an inhibitory effect on the proliferation and differentiation of hematopoietic stem cells through direct cellular contact (membrane-bound signaling molecule). A small fraction of stem cells is released from the inhibitory regulation of reticular cells at a certain rate, is hit by diffusible factors secreted from marrow stromal cells, and progresses down the proliferation and differentiation pathways. Some of the divided stem cells happen to attach to the specific domain of reticular cells again and then become dormant. Upon urgent hematopoietic demand, as in infection, bacterial endotoxins and/or IL-1 released by activated macrophages stimulate the reticular cells on site. The signals are then quickly transmitted to a large number of adjacent reticular cells one after another, through gap junctional communication, to functionally coordinate and cooperatively make a contribution of urgent hematopoiesis, including increased production of hematopoietic factors.

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GENE TRANSFER INVESTIGATIONS OF ONCOGENES ACTIVATED BY CHROMOSOMAL TRANSLOCATIONS IN HUMAN LYMPHOMAS AND LEUKEMIAS: *BCL2* AND *C-MYC*

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ABSTRACT

Chromosomal translocations involving immunoglobulin gene loci activate the *BCL2* (B-cell lymphoma/leukemia-2) and *C-MYC* oncogenes in many non-Hodgkin's B-cell lymphomas. *BCL2* and *C-MYC* encode membrane-associated GTP-binding and nuclear DNA-binding proteins, respectively, whose mechanisms of action remain poorly understood. Using gene transfer approaches, we contrasted the actions of these and other oncogenes. In a human B-cell line, *BCL2* and *C-MYC* enhanced *in vitro* growth and tumorigenicity, providing direct evidence for their importance in the pathogenesis of the human B-cell lymphomas. Introduction of these two oncogenes into a human T-cell line revealed synergy between *BCL2* and *C-MYC*, suggesting that they control complementary growth pathways in lymphoid cells. In contrast, *BCL2* and *C-MYC* behaved similarly in rodent fibroblasts where they (a) rendered NIH 3T3 cells tumorigenic without inducing morphological transformation; (b) complemented with a RAS oncogene in the malignant transformation of the rat embryo fibroblasts; and (c) partially abrogated the dependence of NIH 3T3 cells on platelet derived growth factor (PDGF) for *in vitro* growth. These findings begin to define the biological actions of *BCL2* and *C-MYC*, and suggest that they control overlapping but distinct pathways involved in the regulation of normal and neoplastic cell growth.

INTRODUCTION

Chromosomal translocation is a common mechanism for activating cellular oncogenes in human lymphoid neoplasms. Often these translocations involve the antigen receptor gene loci in B and T cells, and result in the deregulated expression of the juxtaposed oncogene. This mechanism has been well documented for the *BCL2* and *C-MYC* genes in non-Hodgkin's lymphomas, where the translocations involve the transcriptionally active immunoglobulin loci in many B-cell tumors (reviewed in 1). For example, follicular lymphoma cells containing t(14;18) translocations that bring the

BCL2 oncogene into the heavy chain locus and Burkitt lymphoma cells having a t(8;14) translocation involving *C-MYC* contain high levels of *BCL2* and *C-MYC* mRNAs, respectively.

Previously we and others have examined the mechanisms that account for the deregulated expression of translocated *BCL2* and *C-MYC* genes, and have contrasted this with the normal regulation of *BCL2* and *C-MYC* expression in normal lymphocytes. These studies demonstrated that transcriptional mechanisms largely account for the high levels of *BCL2* mRNAs found in t(14;18)-containing cells (2). In lymphoid malignancies having t(8;14) translocations involving *C-MYC*, transcriptional mechanisms also play a major role in maintaining high levels of *C-MYC* mRNA, but in many cases significant regulation also occurs at the levels of transcript elongation and stability (2,3). Similar to neoplastic cells, we found that in normal human lymphocytes freshly isolated from peripheral blood or tonsils, accumulation of *BCL2* mRNA is regulated primarily at the transcriptional level, whereas both transcriptional and post-transcriptional mechanisms contribute significantly to the regulation of the expression of *C-MYC* in these cells (4,5). Unlike neoplastic lymphoid cells, which continuously maintain high levels of *BCL2* or *C-MYC* mRNAs, in normal lymphocytes expression of *BCL2* and *C-MYC* is transient and can be induced when these cells are stimulated to proliferate in culture (4,5).

These findings have revealed some of the mechanisms responsible for the deregulation of *BCL2* and *C-MYC* expression that occurs through chromosomal translocations and have provided circumstantial evidence that *BCL2* and *C-MYC* play important roles in the regulation of both normal and neoplastic lymphocyte growth. To better understand the consequences of deregulated *BCL2* and *C-MYC* expression in human lymphomas and leukemias, we have employed gene transfer methods as a means of directly exploring the functions of *BCL2* and *C-MYC*. Though only in their early stages, these studies are beginning to shed light on the cellular and molecular mechanisms of action of *BCL2* and *C-MYC* oncogenes.

RESULTS

Effects of BCL2 and C-MYC in a Human B-Cell Line

Since in humans translocations involving *BCL2* and *C-MYC* are most often found in neoplasms of B-cell origin, clearly it is of interest to investigate the function of deregulated expression of these two oncogenes in human B lymphocytes. For this purpose, we have used the approach of Lombardi *et al*, (6), and have introduced expression plasmids containing *BCL2* or *C-MYC* into an EBV (Epstein Barr virus)-immortalized human B–cell line GM607B. These B cells provide a number of convenient endpoints for assessing oncogene function, including morphological changes, serum dependence for *in vitro* growth, colony formation in semisolid medium or growth in limiting-dilution cultures, and tumorigenicity in athymic mice.

For experiments with GM607B cells, we made use of expression plasmids that contain the origin of replication from EBV. When introduced into EBV-immortalized human B cells, such as GM607B, these plasmids replicate episomally at a high copy number (7), thus facilitating high-level expression and obviating the need for subcloning

to obtain high expressors. These plasmids also contained a hygromycin resistance gene for use as a dominant selectable marker.

Expression plasmids containing *BCL2* sequences under the control of immunoglobulin heavy chain enhancer or *C-MYC* with a powerful promoter/enhancer from cytomegalovirus (CMV) were introduced into GM607B cells by electroporation (500 V/cm; 1048 μF), selected in 75 μg/ml hygromycin for 3 weeks, and the presence of intact, unrearranged episomal plasmids was then confirmed by Southern blot analysis of low molecular weight DNA isolated from these cells. The expression of the *BCL2* plasmid was confirmed by immunoblotting, showing high levels of the expected 26 kD *BCL2-α* protein. Expression of the *C-MYC* plasmid was demonstrated by RNA blotting, revealing high levels of recombinant *C-MYC* transcripts and suppression of endogenous *C-MYC* mRNA accumulation.

B cells with deregulated *BCL2* and *C-MYC* expression exhibited enhanced growth in culture, as indicated by their ability to proliferate in reduced serum and to grow in limiting-dilution cultures. Both of these oncogenes also increased the ability of GM607B cells to form tumors in athymic mice, although *C-MYC* was more potent in this regard. Unlike *BCL2*, deregulation of *C-MYC* expression also altered the appearance of GM607B cells, in culture in that these cells no longer grew as cellular aggregates ("clumps"), probably reflecting an additional change in their growth factor requirements. Based on cellular appearance and tumorigenicity results, *C-MYC* and *BCL2* appear to regulate the growth of human B cells through different mechanisms. These findings (8) provide direct evidence that *BCL2* and *C-MYC* can alter the growth characteristics of human B lymphocytes, thus strengthening arguments for their role in the pathogenesis of human lymphomas.

Synergistic Effects of BCL2 and C-MYC in a Human T-Cell Line

A role for *BCL2* and *C-MYC* in the regulation of T-cell growth is implied by the observation that expression of both of these oncogenes is transiently induced in normal human T cells upon stimulation of their proliferation in culture (4). Though chromosomal translocations involving *BCL2* have not yet been described in T-cell neoplasms, rearrangements between *C-MYC* and T-cell antigen receptor genes have been reported (see ref. 1 for review). We wished, therefore, to determine the effects of deregulated *BCL2* and *C-MYC* expression in human T cells.

For these studies, we made use of the Jurkat T-cell line, an acute lymphocytic leukemia with a mature helper T-cell phenotype. Previously, we investigated the regulation of proto-oncogene expression in these cells, and found that the phorbol ester tetradecanoyl phorbol acetate (TPA) rapidly diminished the levels of *BCL2* and *C-MYC* mRNAs and resulted subsequently in the cessation of cellular growth (9). We wondered, therefore, whether introduction into Jurkat T cells of expression plasmids that drive high levels of *BCL2* or *C-MYC* expression could abrogate the TPA-mediated reduction in cellular proliferation.

Accordingly, we prepared expression constructs for *BCL2* in a retroviral vector that contained a neomycin-resistance gene for selections in the antibiotic G418, and used a *C-MYC* plasmid that contains a CMV promotor/enhancer for driving oncogene expression and a hygromycin-resistance gene. Subcloning of *BCL2* and *C-MYC* into plasmids having G418 resistance and hygromycin-resistance genes, respectively, allowed

us to also simultaneously introduce *BCL2* and *C-MYC* plasmids into Jurkat cells using a double selection approach.

After the introduction of *BCL2* and *C-MYC* expression plasmids into Jurkat T cells by electroporation (1 kV/cm; 14 μF), selection in appropriate antibiotics at limiting dilution, and investigation of levels of expression by RNA blotting, several independent clones were discovered that expressed at high levels *BCL2*, $_{\text{C-MYC}}$, or both of these oncogenes. When these cells were stimulated with the phorbol ester TPA, we found that deregulated expression of neither *BCL2* nor *C-MYC*, nor the combination of *BCL2* and *C-MYC*, was capable of abrogating the cessation of Jurkat cell growth, despite the maintenance of high levels of recombinant *BCL2* and *C-MYC* transcripts. Thus, even the combined expression of *BCL2* and *C-MYC* appeared to be insufficient for the growth of Jurkat T cells in TPA-containing cultures. In retrospect, this observation is perhaps not surprising, since TPA also inhibits the expression of other proto-oncogenes in Jurkat T cells including N-RAS (9). On the other hand, Jurkat T cells containing *BCL2* and *C-MYC*, or the combination of these oncogenes, also did not consistently display enhanced growth in culture relative to control cells, thus suggesting that this T-cell line may not provide a sensitive indicator of *in vitro* oncogene function.

Though *in vitro* studies with Jurkat T cells were disappointing, tumorigenicity studies with these cells in athymic mice revealed synergistic actions of *BCL2* and *C-MYC*. When small numbers ($n = 10^6$) of control Jurkat T cells were injected into irradiated nude mice, for example, tumors formed slowly, requiring 12-14 weeks to appear. In contrast, injections of Jurkat T cells containing the combination of *BCL2* and *C-MYC* expression plasmids reproducibly led to tumor formation within 4-5 weeks. Because Jurkat cells that contained either *BCL2* or *C-MYC* alone had no effect on tumor latency, these findings demonstrate *in vivo* synergy between *BCL2* and *C-MYC* in these T-lymphoid cells. These observations (10), therefore, extend to T lymphocytes the reports by others showing a synergistic effect of *BCL2* and *C-MYC* in human and murine B-cells (11,12).

Comparisons of BCL2 and C-MYC with Other Oncogenes in Murine Fibroblasts

Rodent fibroblasts have been used extensively for transfection studies of oncogenes because of the ease with which genes can be stably transfected into these cells and because of the convenient endpoints they provide for assessing oncogenic function, such as cellular morphology and anchorage-independent growth (see ref. 13, for review). We therefore contrasted the effects of *BCL2* and *C-MYC* with other oncogenes in NIH 3T3 mouse fibroblasts, and in early passage rat embryo fibroblasts (REF cells). We were particularly interested in comparing *BCL2* with RAS oncogenes in these studies, because both encode membrane-associated, low molecular weight G proteins (14). Similarly, in some experiments *C-MYC* was contrasted with the E1A oncogene of adenovirus, which encodes a nuclear DNA binding protein having some sequence homology with *C-MYC* (15).

Though *BCL2* and *C-MYC* rendered NIH 3T3 cells tumorigenic in athymic mice, neither induced morphological transformation of these cells *in vitro*. In contrast, transfections with a human *ha-c-ras* oncogene did result in transformation *in vitro* and tumors *in vivo* (16). The molecular basis for this difference between G proteins encoded by *BCL2* and *ras* with regards to morphological transformation remains

unknown, since both *BCL2* and RAS were found to partially abrogate the dependence of NIH 3T3 fibroblasts on PDGF for *in vitro* growth (Reed *et al.*, in preparation).

In REF cells that (unlike immortalized NIH 3T3 cells) require two complementary oncogenes to achieve malignant transformation (13), *BCL2* and *C-MYC* cooperated with *ras*. For these experiments, we employed plasmids containing a hygromycin-resistance gene and the CMV immediate-early region promotor for driving expression of either *BCL2* or *C-MYC* (p*BCL2*/HYG and p*C-MYC*/HYG). Other plasmids were also used that contained a G418-resistance gene, together with either a human *ha-c-ras* or adenovirus *e1a* gene, whose expression was dependent on the natural promoters associated with *ha-c-ras* and *e1a* (pRAS/NEO and pE1A/NEO). By cotransfecting either p*BCL2*/HYG or p*C-MYC*/HYG together with either pRAS/NEO or pE1A/NEO, we were able to easily confirm the stable introduction of both plasmids by sequential antibiotic selections using G418 and hygromycin.

REF cells containing the combination of p*BCL2*/HYG and pRAS/NEO invariably displayed classical transformed morphology when adjacent suppressive normal cells were removed from cultures by antibiotic selection. These morphologically transformed cells were capable of high-efficiency colony formation in semisolid medium and rapidly produced tumors in syngeneic rats, thus providing functional confirmation of their malignant phenotype. Cotransfections with *BCL2* and *C-MYC* plasmids, in contrast, failed to transform these cells, whereas the combination of *C-MYC* and ha-c-ras was fully transforming (17).

These findings suggest that *BCL2* and *ras*, despite the biochemical similarities of their encoded proteins, can under some circumstances regulate distinct complementary pathways involved in malignant transformation. Furthermore, the failure of *C-MYC* and *BCL2* to act synergistically in REF cells, when contrasted with findings in lymphoid cells(10–12), raises the intriguing possibility that the phenomenon of oncogene complementation may be subject to tissue specificity.

Though in most cases two complementary oncogenes are required to produce morphological transformation of early passage rodent fibroblasts, transfection of a single oncogene can immortalize these cells. In our experiments, when a single oncogene-containing plasmid was transfected, we observed that e1a was by far the most potent at inducing immortalization. In contrast, *C-MYC* had moderate activity, and *BCL2* and ras had weak activity where immortalization is concerned. Taken together with the finding that the combination of *BCL2* and ras transforms REF cells, these data indicate that oncogenes cannot necessarily be differentiated into complementary groups based on their ability to immortalize early-passage fibroblasts, contrary to earlier reports (13).

DISCUSSION

Using gene transfer approaches, we have begun to explore the biological actions of *BCL2* and *C-MYC*, as well as other oncogenes that are found activated by chromosomal translocations in human lymphomas and leukemias. These studies have provided direct experimental evidence that deregulated expression of *BCL2* and *C-MYC* can alter the growth of human B cells (8), the cells wherein translocations involving *BCL2* and *C-MYC* are most commonly found in humans (1). Gene transfer into various types of cells has also permitted comparisons of the activities of *BCL2* and

C-MYC with other oncogenes, yielding information about the capacity of these genes to regulate cellular transformation, immortalization, growth–factor dependence, and tumor formation (8,10,16,17). These findings, thus, are helping to structure our thinking about the functions of *BCL2* and *C-MYC*, and are pointing the directions for future investigations.

It should be recognized, however, that the endpoints measured in cells transfected with *BCL2* and *C-MYC*, such as morphological transformation and tumorigenicity, are distal events that merely reflect the net effects of the complex networks of biochemical pathways perturbed through the introduction of these oncogenes. Accordingly, issues such as cell lineage and the stage of differentiation, host cell karyotypic abnormalities, and the role of viruses must be considered when interpreting and comparing such data. For example, the human B–cell line used in our gene transfer investigations of *BCL2* and *C-MYC* was immortalized by EBV and, in addition, contained an abnormal chromosome 19. EBV may be particularly relevant for *C-MYC*, since many Burkitt lymphomas with *C-MYC* translocations are infected with EBV (1). Potentially, genes expressed by EBV or as a result of EBV infection could complement with *C-MYC*, accounting for the greater tumorigenicity of *C-MYC*-transfected B cells relative to *BCL2* (8). Similarly, the chromosomal aberration found in the GM607B cells may provide an explanation for the *BCL2*-mediated tumorigenicity that has not been observed by other investigators using other EBV-immortalized cell lines (12,18).

The use of morphological transformation and tumorigenicity as endpoints for assessing oncogene function may provide both insight and confusion where the phenomenon of oncogene complementation is concerned. We and other investigators, for instance, have obtained evidence for synergism of *BCL2* and *C-MYC* in lymphoid cells (10–12) a result anticipated by reports of low-grade follicular lymphomas with *BCL2* rearrangements undergoing clinical and histological progression after acquiring a second translocation involving *C-MYC* (19). Our studies in fibroblasts, in contrast, failed to demonstrate complementation by *BCL2* and *C-MYC*. Instead, *BCL2* and *C-MYC* behaved similarly in NIH 3T3 and REF cells in most regards (16,17). Of particular note was the observed complementation of both *BCL2* and *C-MYC* with a *ras* oncogene (17) that provided strong evidence for *BCL2* and *C-MYC* controlling similar, rather than complementary pathways involved in the transformation of these cells.

Several potential explanations exist for these alternative findings with regards to *BCL2* oncogene complementation in lymphoid and fibroblast cells. First is the possibility that, depending on the experimental system, the phenomenon of oncogene complementation can reflect either quantitative or qualitative synergism of two oncogenes. Several examples are available from the literature wherein expression of a single cellular oncogene at extremely high levels is sufficient for inducing transformation, mediating immortalization, abrogating growth factor requirements or enhancing tumorigenicity. Perhaps the most striking example of this quantitative aspect to malignant transformation by oncogene transfection concerns the *ras* oncogene in the REF cell complementation assay. In these early–passage rodent fibroblasts, an activated *ras* gene can be sufficient for malignant transformation if it is expressed at extremely high levels through the use of strong promoters and enhancers (20). In such circumstances, a second complementary oncogene like *C-MYC* is not necessary.

With regards to our findings, therefore, we cannot at this time determine whether the observed synergism of *BCL2* and *C-MYC* in lymphoid cells reflects a qualitative difference between the growth pathways regulated by *BCL2* and *C-MYC*, or alternatively whether *BCL2* and *C-MYC* regulate similar pathways with the simultaneous expression of both of these oncogenes being equivalent to overexpressing, for example, *C-MYC* alone. To differentiate between these possibilities, we are currently attempting to maximize *C-MYC* expression in Jurkat cells through the use of alternative expression constructs and through gene amplification. Similarly, our findings in REF cells showing complementation by *BCL2* and ras remain without explanation, but suggest the intriguing possibility that expression of a ras oncogene at extremely high nonphysiological levels in these cells may permit ras proteins to substitute for other G proteins that are similar to *BCL2* and that control transformation pathways complementary with ras.

A final possible explanation for our data, however, must be entertained. It is possible that the phenomenon of oncogene complementation is subject to tissue specificity, with the same oncogene playing different roles in lymphocytes and fibroblasts. In this regard, ABL oncogenes have been shown to complement with *C-MYC* in a rat fibroblast cell line (21) but not in murine lymphoid cells (22).

To resolve these questions, clearly a more detailed understanding of the molecular events regulated by *BCL2* and *C-MYC* is needed. To this end, we are currently using the cells described here as a result of our gene transfer approaches to examine the effects of deregulated expression of *BCL2* and *C-MYC* on a variety of specific biochemical events, including kinase activity and gene transcription. These forthcoming investigations should continue to provide insights into the molecular and cellular mechanisms responsible for the pathogenesis of human lymphomas and leukemias.

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5

MOLECULAR STUDIES OF CHROMOSOMAL TRANSLOCATIONS IN CHILDHOOD LEUKEMIAS

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ABSTRACT

Leukemia is a genetic disease in the sense that at its most fundamental level it involves primary abnormalities of DNA, the genetic material. In acute leukemias, somatically acquired DNA changes frequently take the form of structural alterations of the chromosomes, most notably translocations. Some of these abnormalities have been shown to result in the oncogenic conversion of genes normally involved in the control of cellular growth and differentiation. Two novel translocated genes have recently been described in pediatric acute leukemias. One of these, *lyl*-1, was activated by juxtaposition with the β T-cell receptor gene. The other, *prl*, was activated by formation of a fusion gene that codes for a predicted fusion protein. The proteins encoded by both the *lyl*-1 and *prl* genes have features that indicate they normally function as transcription factors. The data suggest that genetic aberration of transcription factors plays a significant role in the pathogenesis of some acute leukemias.

INTRODUCTION

Chromosomal translocations have been shown to be important pathogenetic events in childhood leukemias. The best studied examples are translocations involving the c-*myc* and c-*abl* genes in acute lymphoblastic leukemias (1). Molecular studies of these recurrent genetic abnormalities in childhood leukemias have helped establish an important paradigm: The role of cellular genes involved in the control of growth and differentiation may be usurped by certain chromosomal events resulting in their oncogenic conversion. The mechanisms by which gross chromosomal changes may result in dominant oncogenic conversions are of two general types. The first, typified by *myc* gene translocations, involves transcriptional deregulation by juxtaposition with an antigen receptor gene, i.e., the immunoglobulin or T-cell receptor loci (2,3). The second, typified by *bcr-abl*, involves truncation and activation of a proto-oncogenic protein through formation of a fusion gene and chimeric protein, the latter possessing unique biochemical properties not associated with its normal counterpart (4,5).

Although studies of translocations involving *myc* and *abl* have been important

for our understanding of somatic genetic events leading to malignancy, the fact remains that abnormalities of these genes are observed in only a small fraction of childhood leukemias. Given the complexity of the growth and differentiation processes, it is likely that a number of different cellular genes may be altered to contribute to leukemogenesis. The presumption is that some of these previously undescribed genes will be located at translocation breakpoints and will play important roles in the pathogenesis of at least a subset of childhood leukemias.

We have recently isolated two novel translocated genes from acute childhood leukemias. One of these genes was activated by juxtaposition with the beta T-cell receptor locus and the other by formation of a fusion gene and chimeric protein. The proteins encoded by both of these leukemia-associated genes have structural features indicating that they normally function as transcription factors, thus establishing an important link between aberrations of such factors and certain malignancies.

THE *lyl*-1 GENE AND PREDICTED PROTEIN

In published studies (6), we have described the isolation of t(7;19) (q35;p13) breakpoint DNA from a T–cell lymphoblastic leukemia cell line carrying this cytogenetic abnormality. Molecular analyses of the t(7;19) breakpoint DNA showed that a fragment of chromosome 19p13 was juxtaposed adjacent to the beta T–cell receptor locus at 7q35 (Fig. 1). The breakpoint on chromosome 7 occurred at joining segment Jβ1.1, suggesting that the translocation occurred during T–cell receptor gene rearrangement as an error in this process. The chromosome 19p13 DNA adjacent to the t(7;19) breakpoint was shown to contain a transcription unit, since a 1.5 kb RNA was observed by Northern blot analysis using a fragment of chromosome 19 DNA adjacent to the breakpoint as a probe. The name *lyl*-1 (for lymphoblastic leukemia) was proposed for this previously undescribed transcription unit.

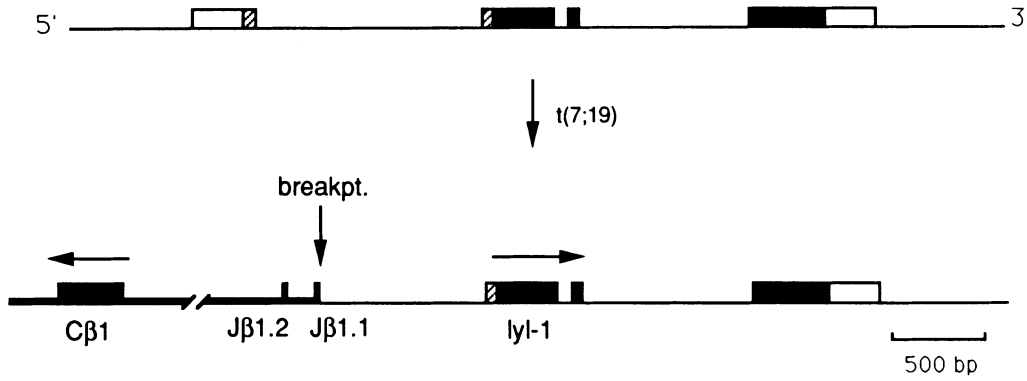

Figure 1. Juxtaposition of the *lyl*-1 and beta T-cell receptor genes following t(7;19). The physical structure of the normal *lyl*-1 gene is shown on the top line. The head-to-head configuration of the beta TCR gene and the truncated *lyl*-1 genes is shown below. Boxes denote exons, solid areas are protein coding sequences, open boxes are untranslated sequences, and cross-hatched areas denote potential coding sequences downstream of alternative start CTG codon in exon 1.

The *lyl*-1 transcription unit was further characterized by molecular cloning of its cDNA (7). Nucleotide sequence analysis showed that the cDNA contained a single open reading frame encoding potential proteins of 29 and 33 kD, depending upon which one of two alternative translation start codons was used. The most notable feature of the predicted Lyl-1 proteins was a 54 amino acid region that showed striking though limited sequence similarity to a region conserved in all of the Myc proteins (Fig. 2). Comparison of Lyl-1 to Myc showed 30% identity to a region that lies directly adjacent to the so-called leucine zipper in each Myc protein.

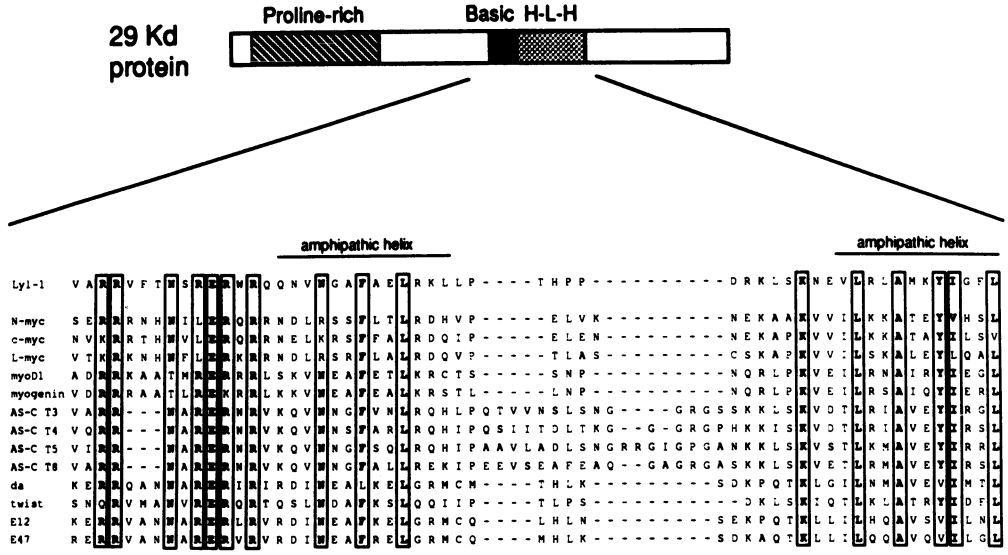

Figure 2. Amino acid similarities between Lyl-1 and a variety of other proteins. Top line shows schematic of Lyl-1 protein as predicted by cDNA sequence. Proline-rich, basic, and helix-loop-helix regions are denoted. Lower panel shows amino acids shared by Lyl-1 and various proteins. Identical amino acids (bold) shared by most or all proteins are enclosed within boxes.

This region of Myc has been shown by several recent studies to be conserved in a number of proteins involved in growth control and differentiation, including MyoD and myogenin, which are involved in myoblast conversion, several *Drosophila* proteins, including the *achaete-scute* complex involved in neural differentiation, *twist* involved with the determination of embryologic germ layers, *daughterless* involved in neural and sex determination, and two immunoglobulin enhancer binding proteins, E12 and E47 (see ref. 7 and 8 for detailed discussion).

This sequence motif is notable for the conservation of several hydrophilic residues and two short clusters of hydrophobic residues, and has the features of an amphipathic helix-loop-helix domain (8). In addition, this motif appears to define a new DNA-binding and dimerization domain, as shown by recent studies on the immunoglobulin enhancer binding proteins E12 and E47 (8). This region of MyoD has also been shown by deletion mutagenesis studies to be necessary and sufficient for myoblast induction and mediates its sequence-specific DNA-binding properties (9). For c-Myc, presence of this domain is required for *in vitro* transformation (10). By analogy we suggest that the equivalent region of Lyl-1 mediates some of its biological effects

and that Lyl-1 will prove to be a nuclear protein with sequence-specific DNA-binding properties. Some of the helix-loop-helix proteins have recently been shown to form heterodimers with each other, resulting in complexes with increased avidity for specific DNA recognition sequences (11). This has led to the notion that these proteins are components of an enhancer binding regulatory system whose specificity is determined at least in part by the combinatorial interaction of helix-loop-helix proteins. In light of this, it will be of particular interest to determine what proteins Lyl-1 interacts with and which genes it may regulate.

The requirements for oncogenic conversion of the *lyl*-1 gene are not well defined, but our studies show that the structural effects of t(7;19) on *lyl*-1 are remarkably similar to some *myc* gene translocations. The *lyl*-1 gene was structurally interrupted by t(7;19), since the breakpoint occurred within the first intron of the gene, resulting in truncation of the gene and head-to-head juxtaposition with the T–cell receptor beta chain gene. The translocated *lyl*-1 gene remains transcriptionally active, giving rise to shortened transcripts that appear to initiate from cryptic promoter sequences in the first intron downstream of the t(7;19) crossover site (7).

Recent studies confirm that there is also a deregulation of *lyl*-1 expression, since only the translocated gene is transcriptionally active in t(7;19)-carrying cells. In addition, like *myc* (12), there are two potential translation products of the *lyl*-1 gene, one of which initiates at a translation start codon present in the first exon. This form of the Lyl-1 protein cannot be synthesized by the t(7;19)-truncated mRNAs, whereas another shorter form of the protein continues to be made. It is not clear what role if any this perturbation in the ratio of the two potential Lyl-1 (and Myc) translation products plays in the pathogenesis of leukemias carrying translocations of the respective genes.

MOLECULAR BIOLOGY OF THE t(1;19) CHROMOSOMAL TRANSLOCATION

The observations that both the Lyl-1 and Myc proteins were helix-loop-helix proteins suggested that they might normally function as transcription factors. This prompted studies to determine whether the genes encoding other helix-loop-helix proteins, particularly those implicated in lymphoid-specific gene expression, might also be involved in genetic abnormalities in various leukemias. To further investigate this possibility, the chromosomal localization of the gene encoding immunoglobulin enhancer binding factors E12 and E47 was mapped using both *in situ* chromosomal hybridization and Southern blot hybridization of human-rodent hybrid cell lines (13). Both analyses showed that the gene (*E2A*) encoding these factors mapped to chromosome band 19p13, a region associated with cytogenetic abnormalities in acute leukemias. The most frequently observed cytogenetic abnormality involving 19p13.3 is the t(1;19) (q23;p13) chromosomal translocation reported to be present in up to 35% of acute lymphoblastic leukemias (ALLs) with a pre-B cell phenotype (14,15).

To assess the potential involvement of the *E2A* gene by t(1;19), DNA isolated from cell lines and leukemias carrying this translocation was examined using Southern blot techniques. Rearrangements of the *E2A* gene were observed in DNA from 8 of 8 leukemias and 2 of 2 cell lines (13). Thus, rearrangements were detected in all cases carrying a cytogenetically confirmed balanced or unbalanced t(1;19). More recent studies mapping several t(1;19) translocation breakpoints showed that they cluster on chromosome 19 within a single intron of the *E2A* gene. Localization of the breakpoints

indicates specific truncation of the 3' end of the *E2A* gene, resulting in deletion of the helix loop-helix DNA binding domain by all t(1;19) translocations analyzed.

Cell lines carrying a t(1;19) were also observed to contain a novel *E2A* transcript that was significantly larger than the 2.9 and 4.4 kb E2A mRNAs expressed in several other hematolymphoid cell lines (13). As predicted from the breakpoint mapping data, these abnormal transcripts lacked the sequences coding for the helix-loop-helix DNA binding motif, since they did not hybridize with a probe containing only this portion of the E2A cDNA. RNAse protection studies of mRNA from several t(1;19) carrying cell lines showed specific truncation of the E2A mRNA, consistent with clustering of breakpoints in a single E2A intron (16). The size of the abnormal E2A mRNAs in conjunction with the breakpoint mapping data suggested that a fusion mRNA that crossed the breakpoint may be synthesized as a result of t(1;19) in acute leukemias.

Cytogenetic studies have shown that the t(1;19) is most frequently unbalanced, since the derivative chromosome 1 is lost by the leukemia cells in most cases (17). This observation suggested that the translocation of material from chromosome 1 to chromosome 19 is the genetically important rearrangement. We have shown previously that in leukemias and cell lines with an unbalanced t(1;19) carrying only the 19p+ chromosome, only one rearranged E2A DNA fragment could be detected, whereas two E2A rearrangements were observed in cells with a balanced t(1;19). The single rearranged fragments corresponding to the der19 chromosome in unbalanced t(1;19) variants contained sequences for the 5' end of *E2A* but lacked 3' sequences of *E2A* containing the helix-loop-helix DNA-binding motif. These observations confirmed that t(1;19) breakpoints fall within the *E2A* gene but also indicated that the role of the E2A proteins in the pathogenesis of t(1;19) was associated with alteration or more likely loss of their DNA binding properties.

The effects of t(1;19) on the E2A transcription unit were further studied by cloning E2A-homologous cDNAs from a t(1;19)-carrying cell line (16). Structural analysis of several cDNAs showed that they consisted of the 5' half of E2A cDNA fused to 1.5 kb of unknown DNA (Fig. 3). The 3' half of these cDNAs was shown to be derived from chromosome 1, thus confirming that the cloned cDNAs crossed the t(1;19) breakpoint. Sequence analyses showed that they coded for a predicted protein of 85 kD consisting of the amino-terminal two thirds of *E2A* fused in frame to a previously undescribed polypeptide. The fusion point in *E2A* was amino-terminal of the helix-loop-helix motif, thus confirming previous studies suggesting loss of this motif following t(1;19).

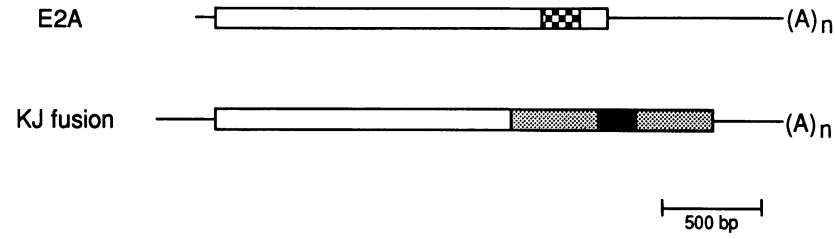

Figure 3. Schematic diagram of fusion cDNA isolated from t(1;19)-carrying cell line. The physical structures of the E2A and 1;19 fusion cDNAs as deduced from their nucleotide sequences are shown. Open areas = E2A coding sequence; checkered box = helix-loop-helix motif; stippled areas = non-E2A sequences from chromosome 1; filled box = homeobox motif; solid lines = 5' and 3' untranslated sequences.

Analysis of the chromosome 1-derived protein sequence showed it to have significant similarity to a number of proteins, all of which were homeoproteins (Fig. 4). Alignment of the fusion protein with several homeoproteins showed that the region of similarity corresponded to their homeodomains demonstrating that the chromosome 1-derived portion of the fusion cDNA coded for a protein with a homeodomain (18). Comparative analyses showed that the fusion protein was no more than 25% identical to any of the 80 previously described homeoproteins. In addition, it shared with the yeast MATa2 a three amino acid insertion within the homeodomain, which is a unique feature of only these two sequences, indicating significant divergence from all other members of the homeoprotein family. The observed similarity to homeoproteins did not extend beyond their homeodomains. These data indicate that the chromosome 1q23-encoded portion of the fusion protein, which we have named Prl (for Pre-B–cell leukemia), should be considered a new member of the homeoprotein family.

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                                                  TURN
                                          HELIX       HELIX
Prl       DARRKRRNFNKQATEILNEYFYSHLSNPIPSEEAKEELAKKCGITVSQVSNWFGNKRIRYKKNI   human
             :      .   :   :                        .    :  :: : : . :
Oct2      RR-K--TSIETNVRFA-EKS-   -A-QK-TS-EILLI-EQLHMEKEV-RV--C-R-QKE-RIN   human
Pit1      RK-KR-TTISIA-KDA-ERH-   GEHSK--SQEIMRM-EELNLEKEV-RV--C-R-Q-E-RVK   rat
c1        XRK-G-QTYTRYQ-LE-EKE-   HY-RYLTRRRRI-I-HALCL-ER-IKI--Q-R-MKW--EN   human
Hu1       -GK-A-TAYTRYQ-LE-EKE-   HF-RYLTRRRRI-I-HALCLSER-IKI--Q-R-MKW--EN   human
huhox2.5  XS-K--CPYT-YQ-LE-EKE-   -F-MYLTRDRRH-V-RLLNLSER--KI--Q-R-MKM--MN   human
huhox2.7  XSK-A-TAYTSAQLVE-EKE-   HF-RYFVRPRRV-M-NLLNLSER-IKI--Q-R-MK---DQ   human
huhox2.4  XR--G-QTYSRYQ-LE-EKE-   -F-PYLTRKRRI-VSHAL-L-ER--KI--Q-R-MKW--EN   human
k8        E---L-TAYTNTQLLE-EKE-   HF-KYLCRPRRV-I-ALLDL-ER--KV--Q-R-MKH-RQT   human
en        -EK-P-TA-SSEQLAR-KRE-   NE-RYLT-RRRQQ-SSEL-LNEA-IKI--Q---AKI--ST   fly
ant       ERK-G-QTYTRYQ-LE-EKE-   HF-RYLTRRRRI-I-HALCLTER-IKI--Q-R-MKW--EN   fly
MATa2     TKPYRGHR-T-ENVR--ESW-AKNIE--YLDTKGL-N-M-NTSLSRI-IK--VS-R-RKE-TIT   yeast

Consensus -----R--Y---Q---L---F   ----Y-----R---A--L-L---Q-KIWFQNRR-K-K---
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Figure 4. Similarity of predicted fusion protein with various other homeodomains. Amino acid identities are indicated by dashes. A three amino-acid gap has been introduced in most sequences to maximize the similarities with Prl.

The potential contributions of the E2A/Prl fusion protein to leukemia pathogenesis is suggested by the structural and functional properties of E2A and homeoproteins in general. The *E2A* gene encodes two helix-loop-helix proteins, E12 and E47, originally implicated in kappa gene expression (8). However, it is now clear that these proteins are expressed in many different tissues and are implicated in the expression of nonlymphoid genes, such as insulin and creatine kinase. Based on their ability to form heterodimers with tissue–specific helix-loop-helix proteins such as MyoD, they are thought to be ubiquitous components of an enhancer-binding regulatory system.

Prl also has features suggesting that it might normally play a role in the regulation of gene expression. Homeodomains have a predicted helix-turn-helix secondary structure that is similar to that of bacterial DNA-binding proteins (18). There is good evidence showing that homeotic proteins regulate the expression of developmentally important genes. In addition, the mammalian transcription factors Pit1, Oct1, and Oct2 contain homeodomains necessary for their specific binding to DNA recognition motifs (19–21). Based on its homeodomain homology, it is tempting to speculate that Prl may play an important role in the development of the lymphoid lineage. Alteration of Prl by chromosomal translocation likely results in maturation

arrest at the pre-B–cell stage, contributing to the development of an acute leukemia.

The molecular pathogenesis of t(1;19) appears to involve an activation of Prl by at least two potential mechanisms. One may be the transfer of the E2A effector domain to Prl, resulting in a chimeric transcription factor consisting of the Prl DNA-binding domain fused to the E2A effector domain. The E2A/Prl chimeric protein would therefore retain its ability to bind to DNA sites normally recognized by Prl, but would presumably result in aberrant activation of these same genes. This mechanism implies an important function for the amino terminal two thirds of E2A in transcription activation for which there is some preliminary data.

A second mechanism likely involves a transcriptional deregulation of *prl* by t(1;19). Preliminary data suggest that Prl expression is normally very restricted, since it was not observed in various lymphoid cell lines, including those with a pre-B–cell phenotype. The *E2A* gene, however, appears to be constitutively expressed in a wide variety of tissues. Following t(1;19), fusion of the 5' end of *E2A* to the 3' end of *prl* brings the *prl* transcription unit under the control of the *E2A* promoter. Thus the chimeric gene is expressed at levels typical of *E2A*, whereas the nontranslocated *prl* gene appears to be transcriptionally silent.

These studies emphasize the importance of transcription factors in the pathogenesis of human acute leukemias. It now seems apparent that some chromosomal translocations represent important yet fortuitous mutations of transcription factors. Study of these abnormalities should provide useful insights into the mechanisms of transcription control in mammalian cells and the consequences of their aberration in certain neoplasms.

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MOLECULAR ANALYSIS OF CHROMOSOMAL ABNORMALITIES IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Three recurrent chromosomal deletion syndromes have been identified in ALL– 6q-, 9p-, and 12p– each of which is apparent in leukemic cells from about 10% of patients with this disease. We examined the cells from patients with ALL both at diagnosis and remission by using probes with restriction fragment length polymorphisms (RFLPs), which were assigned to 6q and 12p. Our data suggested that the critical deleted region of 6q was between q16 and q21, and that mitotic recombination of 6q might not be involved in childhood ALL. Further studies are needed to clarify the role of 6q and 12p abnormalities. Moreover, we examined a series of cases with t(1:19) and have preliminary results confirming previous reports.

INTRODUCTION

A number of nonrandom chromosomal abnormalities have been specifically associated with acute lymphoblastic leukemia (ALL), including t(8;14) (q24;q32) in B-cell ALL (1), 14q11 and 7q35 translocations in T-cell ALL (2,3), t(1;19) (q23;p13) in pre-B-cell ALL (4-6), and abnormalities of the short arm of chromosome 12 in early pre-B cell ALL (7). Phenotypic-specific abnormalities provide a unique opportunity to define new regulatory gene products. Recent molecular analysis of the retinoblastoma (RB) gene has focused attention on tumor suppressor genes or anti-oncogenes (8). Similar mechanisms have been implicated in the pathogenesis of other tumors, including Wilms' tumor (9), embryonal rhabdomyosarcoma (10), hepatoblastoma (10), small cell lung carcinoma (11), renal (12) and colorectal (13) carcinoma, meningioma (14) and acoustic neuroma (15). Although these mechanisms have not been well documented in the hematological malignant diseases, the high frequency of specific chromosomal loss or deletion (16,17) suggests that these genetic mechanisms may also be important in the pathogenesis of ALL.

Three recurrent chromosomal deletion syndromes have been identified in ALL–

6q-, 9p-, and 12p- - each of which is apparent in leukemic cells from about 10% of patients with this disease. Molecular studies with currently available probes that detect restriction fragment length polymorphisms (RFLPs) localized to the involved chromosomal regions are needed to define the true frequency of allelic loss in cases without cytogenetically detectable abnormalities. Homozygous deletion of interferon α and β genes, which have been assigned to the affected region (9p21-p22), have been reported in leukemic cell lines with or without 9p- abnormalities (18). We examined bone marrow cells from patients with ALL both at diagnosis and remission by using RFLP probes assigned to 6q and 12p, and also studied the rearrangement of leukemic cells with t(1;19).

CHROMOSOMAL ABNORMALITIES IN CHILDHOOD ALL

The frequency of chromosomal abnormalities detected in childhood ALL at St. Jude Children's Research Hospital is shown in Table 1. The subgroup with more than 50 chromosomes in their leukemic cells comprises about 25% and has a favorable prognosis. Each chromosomal deletion syndrome– 6q-, 12p-, and 9p- – is found in about 10% of patients. The frequency of each of the nonrandom translocations is low: 11q23 translocations are found in about 6%, and t(1;19) and t(9;22) in about 5%. Other translocations, including 14q11 and 7q35 translocations in T-cell ALL, are very rare.

Table 1. Frequency of chromosomal abnormalities in childhood ALL.*

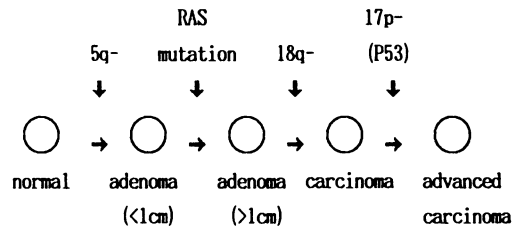
Chromosome abnormalities	Lineage	Frequency (%)
>50	early pre-B	25
6q–	T and B	10
12p–	early pre-B	10
9p–	T and B	10
11q23 translocations	early pre-B	
t(4;11) t(11;19)	mixed	6
t(1;19)	pre-B	5
t(9;22)	B or mixed	5
14q11 translocations	T	3
7q35 translocations	T	2
t(8;14) t(2;8) t(8;22)	B	2
Others		22

*St. Jude Children's Research Hospital, 1989.

CLONAL EVOLUTION HYPOTHESIS IN ALL

Vogelstein *et al.* (19) have devised a clonal evolution model of colon cancer supported by molecular studies, describing a sequence in which 5q- developed in normal or proliferative cells, then RAS mutations in small adenomas, 18q- in large adenomas, and finally 17p-, which involved the p53 gene (Fig. 1). Multistep oncogenesis

was required for the development of colon cancer, and recessive tumor suppressor gene may play a role in the causation of both childhood and adult-type neoplasms. Based on cytogenetic and molecular analyses, we hypothesize that multistep leukemogenesis, including inactivation of recessive suppressor gene located in the 6q-, 12p-, and 9p- chromosomal regions, may be involved in childhood ALL (Fig. 1).



[Clonal Evolution Hypothesis of Childhood ALL]

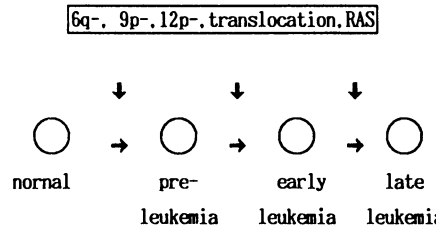


Figure 1. Clonal evolution model of colon cancer (19) and clonal evolution hypothesis of childhood ALL.

6q- ABNORMALITY IN ALL

The 6q- abnormality has previously been reported in 5–20% of ALL patients (20-22) and in 10% at St. Jude (17). This abnormality has been reported to be associated with the T-cell phenotype(20). However, our study suggested that this abnormality was mainly found in blasts with the early pre-B–cell phenotype, and was not restricted to the T-cell phenotype. CALLA$^-$ early pre-B and mixed-lineage expression were not observed in any cases with the 6q- abnormality. Interestingly, all three cases with 12p-, in addition to 6q-, had the early pre-B phenotype, and all four cases with 9p-, in addition to 6q-, had the T-cell phenotype.

Barletta *et al.* (23) have reported that *c-myb* was retained in six leukemic cell lines with 6q- using *in situ* hybridization, and that C-MYB mRNA was overexpressed in some of these cell lines. In contrast, no association of *c-myb* with 6q- has been reported in 2 T-cell cell lines (24).

RFLP STUDY OF 6q

Figure 2 shows a map of 6q. The breakpoints for the 42 cases with 6q- can be

subgrouped into four groups: q13 to q21 in 17 cases, q15 to q21 in seven cases, q21 to q23 in nine cases, q15 to q23 in seven cases, and the others in two cases (17). Notably, deletions in all 42 cases involved the 6q21 band, suggesting that this might be the locus of a recessive tumor suppressor gene. We found three probes assigned near 6q21 band (25). Superoxide dismutase 2 (SOD2) is assigned to 6q21 and has an RFLP with *Taq*I restriction endonuclease. The *c-myb* proto-oncogene is assigned to 6q23 to q24 and has RFLP with *Eco*RI, and an anonymous gene D6S37 is assigned to 6q22 to q23, and is known to be a VNTR marker with *Eco*RI and *Taq*I.

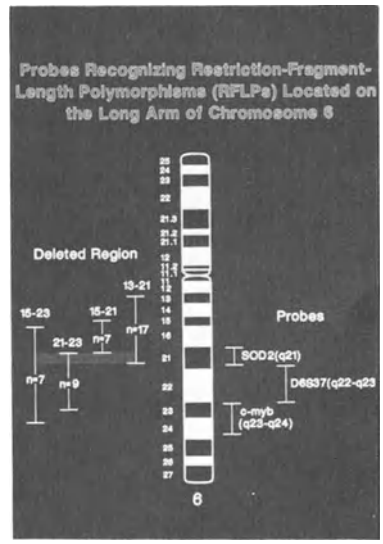


Figure 2. The distribution of the deleted region and probes recognizing RFLPs on the long arm of chromosome 6.

Table 2. Molecular analysis of the deleted region in 6q- cases. Loss of heterozygosity in informative cases.

Probe	Deleted region		
	q13-q21	q15-q21	q15-q23
SOD2(q21)	0/5	0/3	2/2
D6S37(q22-q23)	0/5	0/3	2/3
C-MYB(q23-q24)	0/1	0/3	NI

Analysis of 14 cases with 6q- using three probes is shown in Table 2. Two cases with deletion q15 to q23 had loss of heterozygosity. No loss of heterozygosity was observed in any cases with other deletions, suggesting that all three probes are located distal to the critical locus. Analysis of 40 ALL cases lacking 6q- is shown in Table 3. Among 26 informative cases, one case showed loss of heterozygosity of SOD2 (Fig. 3). This case lacked heterozygosity of D6S37, suggesting loss was caused by microscopic

deletion, rather than mitotic recombination. Loss of heterozygosity of the other two probes was not found in any informative cases. In conclusion, *c-myb* was retained in all ALL cases with or without 6q-, and SOD2 was retained in 10 ALL cases with deletion 6q15-q21. Combined with cytogenetic findings, the crucial deleted region was between 6q16 and q21. Our data suggest that mitotic recombination of 6q is not involved in childhood ALL, which is different from Wilms's tumor and retinoblastoma.

Table 3. Allelic loss of loci on the long arm of chromosome 6 in ALL cases lacking 6q−.

Probe	Informative cases	Allelic loss
SOD2(6q21)	26	1*
D6S37(6q22-q23)	28	0
C-MYB(6q23-q24)	16	0

* *D6S37* was retained in this case, suggesting that loss was caused by microscopic deletion, rather than mitotic recombination.

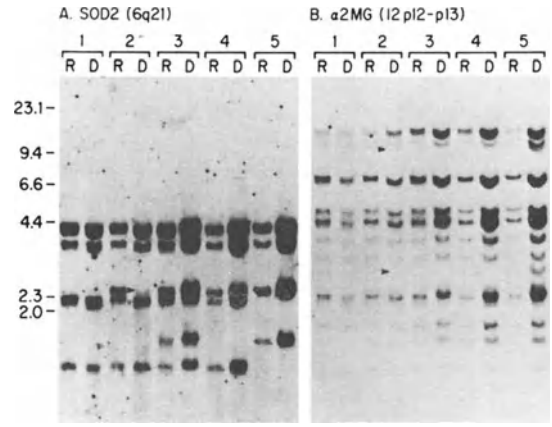


Figure 3. Southern blotting hybridized with SOD2 (A) and Southern blotting hybridized with A2MG (B).
R = at remission; D = at diagnosis.

12p ABNORMALITIES IN ALL

12p abnormalities have been reported in 5–15% of childhood ALL (21,22) and at about 10% in the St. Jude Total XI study. We identified two chromosomal patterns: one was simple deletion, and the other was a derivative chromosome resulting in partial monosomy of 12p (Fig. 4). Raimondi *et al.* (7) have reported 21 cases with

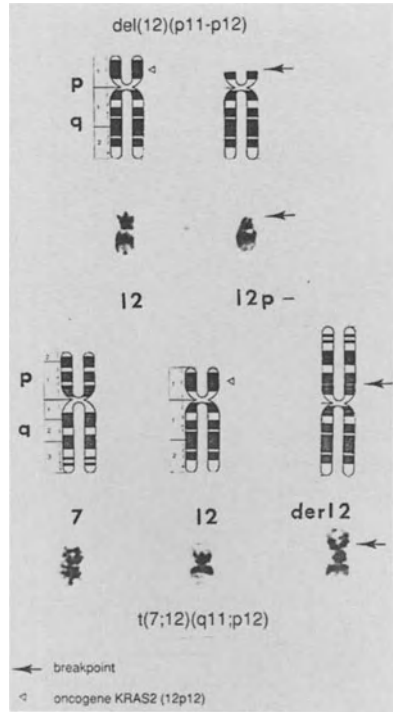


Figure 4. Partial karyotype and schema of simple deletion, del (12) [top], and unbalanced translocation der (12) (7;12) resulting in 12p monosomy [bottom].

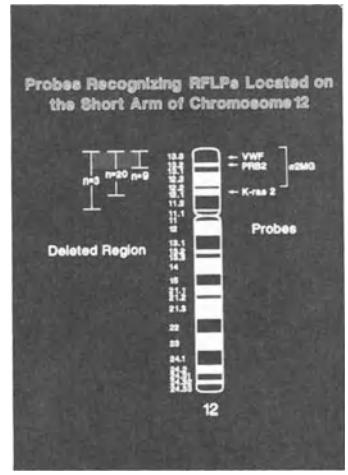


Figure 5. The distribution of the deleted region, and probes recognizing RFLPs on the short arm of chromosome 12.

CALLA-positive B-lineage ALL. Recently our preliminary studies have suggested that these abnormalities are mainly restricted to the CALLA–positive early pre-B

phenotype. Figure 5 shows a map of 12p. The breakpoints were divided into three subgroups. The common deleted region of 12p was the p13 band. We used three probes that detected *Taq*I RFLP. K RAS-2 is assigned to p12, A2MG is assigned to p12 to ter, and VWF is assigned to p13.

RFLP STUDY OF 12p

Analysis of seven cases with 12p- using three probes is shown in Table 4. Loss of heterozygosity was not observed in any informative cases. One case has der(12), resulting in monosomy for 12p13 band. Heterozygosity for each of the three probes was retained. Thus, the breakpoint was considered to be distal to these three probes. The result of 39 cases lacking 12p- is shown in Table 5. Among 26 informative cases, one case had loss of heterozygosity of A2MG (Fig. 3). Two cases out of 31 informative cases had loss of heterozygosity of VWF. Our data suggests that mitotic recombination of 12p is rarely involved in childhood ALL.

Table 4. Allelic loss of loci on the long arm of chromosome 12.

	Informative cases	Allelic loss
K ras-2	2	0
A2MG	3	0
VWF	2	0

One case; −6, −12, +der(12) t(6;21)(p21;p13).
Three probes were retained. The breakpoint was considered to be distal to these three probes (p13.3.).

Table 5. Allelic loss of loci on the long arm of chromosome 12 in ALL cases lacking 12p−.

Probes	Informative cases	Allelic loss
K-RAS 2	6	0
A2MG	26	1[a]
VWF	31	2[b]

[a]; #12$_s$ are normal. This case has 6q−.
[b]; #12$_s$ are normal. One case has 14q− and t(20;22).
The other case has t(15;22).

9p ABNORMALITIES IN ALL

9p abnormalities have been reported in 7–12% of childhood ALL patients (26-29) and in about 10% of those in the St. Jude Total XI study. Chilcote *et al.* have reported that these abnormalities are associated with a T-cell phenotype or lymphomatous features (26). However, this association has not been confirmed (28,29).

Diaz *et al.* (18) have reported that among 21 leukemic cell lines, hemizygous

deletion was found in six cell lines and homozygous deletion was found in three cell lines using interferon α and β probes. Mitotic recombination of 9p may be involved in childhood ALL.

t(1;19)(q23;p13)

t(1;19) has been reported in 4–6% of childhood ALL (4-6) and in 30% of pre-B ALL in children (30). Our study suggested that immunophenotype was restricted to the pre-B phenotype. The chromosomal pattern can be subgrouped: (a) balanced t(1;19) in about 29% of cases and (b) unbalanced der(19)t(1;19) in about 71% (Fig. 6). Cases with t(1;19) have poor prognostic features (5,30). Previous molecular studies showed that the insulin receptor and c-SKI were not involved in t(1;19)-ALL. Recently enhancers of Ig heavy chain and light chain were characterized. Leonardo *et al.* (31) have reported that among Ig *k* light chain enhancers, *k*B and *k*E2 are important to Ig light chain gene transcription. Murre *et al.* (32) have isolated two cDNAs, E12 and E47, that encode proteins that bind to the *k*E2. These were found to be derived from one gene, which was called E2A. Mellentin *et al.* (33) have reported that E2A is

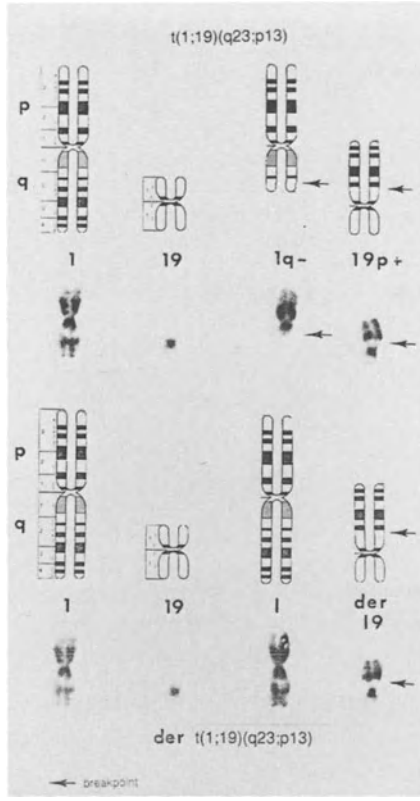


Figure 6. Partial karyotype and schema of balanced translocation t(1;19) [top], and unbalanced translocation der(19)t(1;19) [bottom].
← indicates the breakpoint.

located in the 19p13.2 to p13.3 region using hybrid cells and *in situ* hybridization. They have also reported that E2A is rearranged in ALL with t(1;19) by DNA blot analyses and that a larger sized transcript was found. We examined a series of cases and have preliminary results confirming these findings. Our findings are compatible with those of ALL with t(9;22), which produced a unique bcr-abl fusion transcript and protein.

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IMMUNOLOGICAL PROPERTIES OF HEMATOPOIETIC MALIGNANCIES IN CHILDHOOD

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ABSTRACT

Childhood non-Hodgkin's lymphomas were immunohistochemically analyzed utilizing monoclonal antibodies. Eighty-two cases were studied and 98% cases were histologically subtyped into lymphoblastic (LB,39%), Burkitt's lymphoma (BL,31%), and large cell (LA,28%) types. BLs were all positive for pan B markers (immunoglobulins and CD 19/20/22), as well as the activated B–cell antigens, CD10 and B5. However, BLs were phenotypically heterogenous with the combination of L30 and L29, which detect resting and activated B cells, respectively. With L30 and L29, BLs were subgrouped into three phenotypes ($L30^+L29^-$, $L30^+L29^+$, and $L30^-L29^+$). Study on the distribution of these antigens on normal B cells, as well as on *in vitro* activation, revealed that the heterogenous antigen expression of BLs corresponds to the dynamic phenotypic change during early activation of mature B cells. Therefore, it is likely that BLs are tumors of early activated B cells. In LAs, 14 anaplastic large cell lymphomas (Ki-1 lymphoma) were included. Phenotypically, Ki-1(CD30), epithelial membrane antigen (EMA), HLA-DR, and the IL–2 receptor were frequently identified. The tumor cells expressed no specific T– cell (CD3/5/8) or B–cell (CD19/20) markers, but coexpression of CD4 and alpha-1-antitrypsin was frequently seen. The results indicate that Ki-1 lymphoma is a heterogenous group of tumors including those of non-lymphoid cell origin.

INTRODUCTION

Monoclonal antibodies have been widely applied in pediatric oncology. Immunophenotyping, for example, has now become an invaluable examination, not only for proper diagnosis, but also for the determination of suitable therapy. Immunophenotyping, however, is not limited to the diagnostic field. What is important is that we can identify a certain subpopulation of hematopoietic cells and understand the precise histogenesis of tumor cells. The present study describes the immunophenotypic analysis on non-Hodgkin's lymphomas in childhood. By utilizing a variety of monoclonal

antibodies, their histogenesis is precisely demonstrated.

NON-HODGKIN'S LYMPHOMA IN CHILDHOOD

Eighty-two cases of non-Hodgkin's lymphomas (NHL) were studied (Table 1). Histological classification was done according to the working formulation (1) and it was found that the major types were lymphoblastic (LB), Burkitt's (BL), and large (LA) cell types. The incidence for each type was 39%, 31%, and 28%, respectively. Childhood NHLs were male predominant with the ratio of 2:1. Immunophenotyping was performed principally on acetone-fixed frozen sections by the indirect immunoperoxidase method, but in some cases typing was done on paraffin sections. LBs were mostly T cells with frequent mediastinal involvement. However, there were some B–cell LBs, which tended to arise from extranodal sites. BLs also tended to arise from extranodal sites, including intestine and retroperitoneum. LA is a heterogenous group with respect to the cell marker phenotype. Of particular, mediastinal mass is frequently observed, most of which were classified as Ki-1 lymphoma.

Table 1. Histology, immunophenotype and clinical characterization of non-Hodgkin's lymphomas in childhood.

Histology	No. (%)	Phenotype	M:F	Mean age	Site Examined					
					Med	LN	Ton	GIT	RP	Others
Lymphoblastic	32 (39%)	T:28	19:9	8.5y	12	15	-	1	-	-
		B:4	1:3	6.0	-	1	-	-	-	3 (B)
Burkitt's	25 (31%)	B:25	19:6	8.3	-	12	2	6	4	1 (O)
Large	23 (28%)	B:7	3:4	7.8	1	2	1	2	-	1 (T)
		Ki-1:14	8:6	9.4	6	6	-	-	-	2 (S,B)
		U:2	2:0	8.6	-	2	-	-	-	-
Small cleaved	2 (2%)	T:2	2:0	12.0	-	-	-	1	-	1 (S)
	82 (100%)	T:30	54:28	8.5	19	38	3	10	4	8
		B:36								
		Ki–1:14								
		U:2								

U = unclassified; Med = mediastinum; LN = lymph node; Ton = tonsil;
GIT = gastrointestinal tract; RP = retroperitoneum.
Others: B = bone; O = oral mucosa; T = testis; S = skin.

PHENOTYPIC HETEROGENEITY OF BURKITT'S LYMPHOMA

All BLs showed uniform histology that was technically indistinguishable from each other. For immunophenotyping, the antibodies listed in Table 2 were used. The summary of the immunophenotype of BLs is shown in Table 3. All cases were terminal deoxynucleotidyl transferase negative but were positive for immunoglobulin as well as

Table 2. Antibodies used for immunophenotying of Burkitt's lymphoma.

Immunoglobulin	Pan B
CD19 (Leu12)	Pan B
CD20 (Leu16)	Pan B
CD22 (B3)	Pan B
HLA-DR	Pan B
L30*	Resting B
CD23	Receptor for IgE
CD10 (CALLA)*	Activated B, Immature B
L29*	Activated B
B5	Activated B
CD25 (IL2R)	Activated B
TdT	Immature lymphocyte

Table 3. Immunophenotype of Burkitt's lymphomas.

Case		sIg	CD 19/20/22	B5	CD 10	L30	L29	CD 23	CD 25
1	4y,M	-/K	+	+	+	+	−	−	−
2	10y,M	M/K	+	+	+	+	−	−	−
3	10y,M	M/K	+	+	+	+	−	−	−
4	10y,M	M/K	+	+	+	+	−	−	−
5	10y,M	M/K	+	+	+	+	−	−	−
6	11y,M	M/K	+	+	+	+	−	−	−
7	13y,M	M/K	+	+	+	+	−	−	−
8	4y,M	M/L	+	+	+	+	+	−	−
9	6y,M	M/-	+	+	+	+	+	−	−
10	7y,M	M/K	+	+	+	+	+	−	−
11	9y,M	M/K	+	+	+	+	+	−	−
12	10y,M	M/−	+	+	+	+	+	−	−
13	12y,M	M/−	+	+	+	+	+	−	−
14	10y,M	M/K	+	+	+	−	+	−	−
15	12y,M	M/K	+	+	+	−	+	−	−

sIg = surface immunoglobulin; M = IgM; K = kappa; L = lambda.

other pan B–cell markers (CD19, CD20, and CD22). In addition, they were all positive for activation antigens such as CD10 and B5. CD10 was previously considered to be an antigen expressed on primitive lymphocytes (2), but recent studies have clearly shown that the molecule is expressed on activated B cells (3,4). Consistent with previous reports (3), BLs seemed to express the homogeneous phenotype when these antibodies were used. However, interesting results were obtained when L30 and L29 were applied (5). Most cases (cases 1–13) expressed the resting B–cell antigen, L30. In this $L30^+$ group, six cases showed L29 positivity. The remaining $L30^-$ cases (case 14 and 15) were $L29^+$. Thus, with L30 and L29 BLs could be subclassified into three groups : $L30^+L29^-$, $L30^+L29^+$, and $L30^-L29^+$. This result was striking because

previous study showed that cell lines of BL were $L30^-L29^+$ (5,6). From a pure histological point of view, BLs are thought to be derived from the germinal center B cells (7). In order to know more precisely the histogenesis of BLs, the distribution of L30, L29, and CD10 on normal mature B cells was carefully studied. On frozen section of peripheral lymphoid organs, L30 distributed on mantle zone B cells of lymphoid follicles. L29, on the other hand, was expressed on germinal center B cells (4,5). By two color flowcytometrical analysis, however, a different result was obtained. As expected, L30 and L29 single positive cells were identified. In addition, a L30/L29 double positive cell population was also identified. The results suggest that phenotypic change during B cell activation occurs as follows: $L30^+L29^- \rightarrow L30^+L29^+ \rightarrow L30^-L29^+$. Next, we examined the distribution of CD10 on normal B cells. By immunoperoxidase staining on frozen section, CD10 distributed in germinal center B cells. Consistently, by two color immunofluorescence study, the majority of $CD10^+$ B cells were $L30^-$ and $L29^+$. However, a small number of B cells with the $CD10^+L30^+$ phenotype were also identified, suggesting that CD10 is an activation antigen expressed in the early phase of activation. Indeed, by *in vitro* activation study, CD10 appeared shortly after stimulation, reached its peak at 5 hours, and disappeared quickly (4). Therefore, it is now evident that CD10 is an inducible molecule on mature B cells by activation and has the characteristics of an early activation antigen. The results established that the heterogenous expression of resting and activation related molecules on BLs reflects the dynamic phenotypic change during the early activation pathway of mature B cells of lymphoid organs.

Table 4. Phenotypic and genotypic analysis of Ki-1/EMA "lymphoma."

Activation-related	CD30 (Ki-1)	13/14*
	EMA	14/14
	HLA-DR	9/9
	IL2R	7/8
	T9	8/8
T cell	CD1/3/5/8	0/8
	CD4	5/8
B cell	CD19/20, Ig	0.8
	SL26	0/14
Myeloid cell	CD14 (My7)	0/8
	CD33 (My9)	1/8
Monocyte/histocyte	CD13 (My4)	0/8
	Alpha-1 AT	5/11
	Lysozyme	0/11
	S100	0/11
Leukocyte common	CD45	11/11
DNA analysis	Ig R(+)	0/5
	TCR-β R(+)	2/5
	TCR-gamma R(+)	4/5
	EB virus genome	0/3

*No. of positive/no. tested.

ANAPLASTIC LARGE CELL LYMPHOMA, Ki-1 LYMPHOMA

Recently, Stein *et al.* and Kadin *et al.* reported the existence of a hematopoietic malignancy that is distinguishable from other types of tumors by the expression of a cell surface molecule called Ki-1 antigen (8,9). The tumor is called anaplastic large cell lymphoma, or briefly Ki-1 lymphoma. The Ki-1 lymphoma shows unique histological characteristics, as well as immunophenotypic markers (8-15). Immunophenotypic as well as genotypic analysis has shown that Ki-1 lymphoma is a heterogenous group of tumors consisting of a variety of cell lineages. In this study, 14 cases of childhood Ki-1 lymphomas were analyzed. Phenotypically, all cases were positive for Ki-1 or epithelial membrane antigen (EMA) (Table 4, Figs. 1C and 1E). Ki-1 (CD30) and EMA are both now considered to be activation-related molecules on various hematopoietic cell lineages including T cell, B cell, and macrophage. In addition, HLA-DR, IL2R, and OKT9 were frequently positive. Their phenotypic characteristics indicate that the Ki-1 lymphoma corresponds to the activated hematopoietic cell. As illustrated in Figs. 1A and 1B, tumor cells in most cases were large with highly atypical nuclei. However, in some cases, tumor cells had round to oval nuclei. The infiltration pattern in the lymph node is usually characteristic. Medullary and marginal sinus involvement is a typical pattern, as illustrated in Fig. 1D by hematoxylin-eosin and as more dramatically demonstrated by immunoperoxidase staining, as shown in Fig. 1E. By electron microscopy, the details of tumor cell structure are more precisely demonstrated. The tumor cell nuclei were irregularly deformed and cytoplasmic organella were abundant. These features indicate that tumor cells are derived from well–developed cells. In some cases, particularly, marked interdigitation of cell membrane was noted, as shown in Fig. 1F by immunoelectron microscopy. Cell lineage specific markers, such as CD3/5/8 for T cells and CD19/20 for B cells, were completely negative in all cases, but CD4 and alpha-1-antitrypsin were frequently positive (5 cases out of 8) (Table 4). In addition, coexpression of CD4 and alpha-1-antitrypsin was observed in four cases. The rearrangement pattern for immunoglobulin as well as T–cell receptor DNA was examined in five cases (done by T. Miyashita and S. Mizutani at the Department of Virology, National Children's Medical Research Center), and it was found that all cases showed the germ line pattern for immunoglobulin heavy chain but two cases and four cases respectively, had rearrangements for T–cell receptor beta and gamma chains. In these five cases examined for genotype, two cases were positive for CD4. The resultant DNA analysis may favor the suggestion that tumors showing T–cell receptor DNA rearrangement are derived from T cells. However, no case showed any definitive T–cell marker, CD3. In the literature, the presence of the T–cell type Ki-1 lymphoma with CD3 positivity and T–cell receptor gene rearrangement has been reported, but usually phenotype and genotype do not correlate well (11,15). For example, CD4 is a general marker for helper or inducer T cells, but recent reports clearly indicate that histiocytes, including monocyte/macrophage, can express this molecule (16). Thus, coexpression of the histiocyte–related protein, CD4 and alpha-1-antitrypsin, may favor that some Ki-1 lymphomas arise from nonlymphoid hematopoietic cells. Very recently, Andreesen *et al.* reported that macrophages can express Ki-1, EMA, and IL2R in addition to HLA-DR and OKT9, the phenotype identical to that of K-l lymphoma (17). Also, in our research, one case (case 2) was shown to produce granulocyte-colony stimulating factor, as is characteristic of macrophage (K. Nishihira, Kanagawa Children's Medical Center, personal communication). These results strongly suggest that some "Ki-1

lymphoma" may represent the malignant tumor of nonlymphoid cell lineage. At this moment, therefore, Ki-1 lymphoma seems to include a wide range of hematopoietic tumors deriving from lymphoid cells and nonlymphoid cells. Further analysis, including the study of the presence of functional RNA transcript for lymphocyte antigen receptor protein, is clearly necessary to subclassify Ki-1 lymphoma.

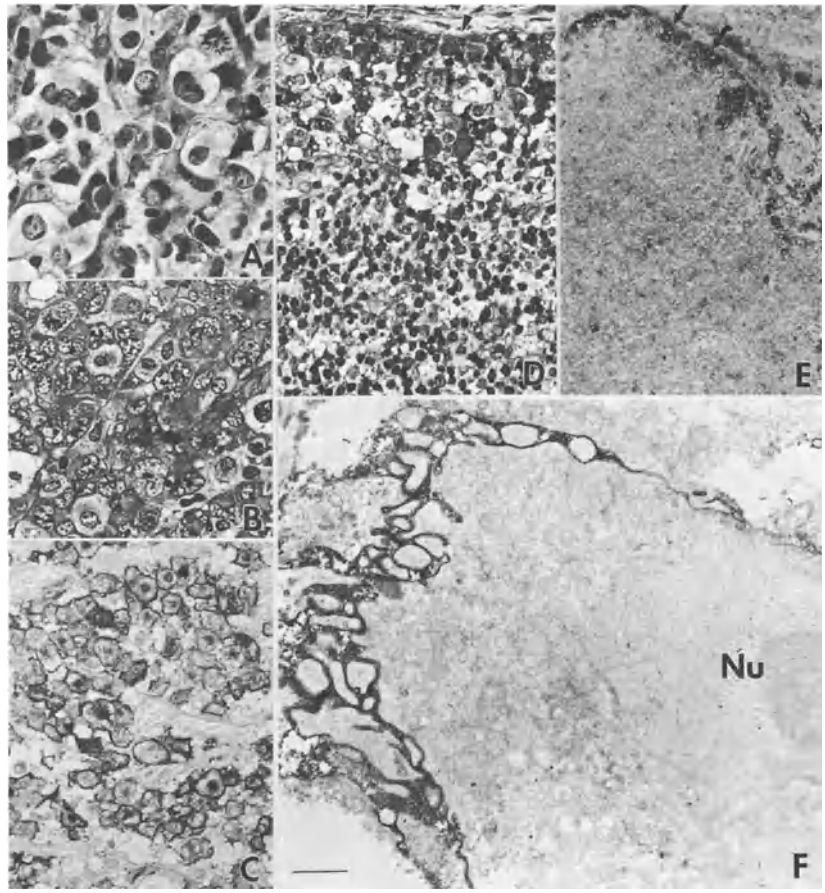

Figure 1. Histological and immunohistochemical characteristics of Ki-1 lymphoma. A: Testis of case 2 (H & E stain, xl70); B: Lymph node of case 4 (H & E stain, xl70); C: EMA immunoperoxidase staining of case 4 (x200). Note the cell surface and cytoplasmic positive staining; D: Infiltration of tumor cells into marginal sinus (arrow head) is shown (H & E stain, case 4, xl50); E: Ki-1 immunoperoxidase staining of tumor cells (arrow) in marginal sinus (case 4, xl00); F: Immunoelectron microscopy of case 4 (xl2500). Note the marked interdigitation of cell membrane in the left part. Nu = nucleus of the tumor cell; Bar = 1 μm.

Finally, the clinical features of 14 cases are summarized in Table 5. Most cases included children whose ages ranged from 8 to 14 years. Lymph node was the most frequent site involved, but it must be emphasized that mediastinal mass was also observed in many cases (six cases). Other characteristic features were also noted.

Table 5. Clinical manifestation of Ki-1/EMA+ "large cell lymphoma."

Case	Age,sex	Extent of disease	Other characteristics	Prognosis
1	2y,F	Med, skin (forehead)	Lymphadenitis	Alive (9m)
2	3y,M	LN (abd), testis	Granulocytosis (PB)	Dead (1y 2m)
3	5y,M	LN (neck)	Necrotizing lymphadenitis	Alive (1y 6m)
4	8y,M	LN (neck, abd)		Alive (1y 7m)
5	8y,F	LN (axillar)		Alive (9y)
6	9y,M	Skin (leg)		Alive (10y)
7	10y,F	Bone, LN (abd)	Granulocytosis (PB)	Dead (8m)
8	11y,M	Med	Granulocytosis (PB)	Alive (10m)
9	11y,M	Med		Alive (7m)
10	11y,F	Med		Dead (8m)
11	12y,F	Med, LN (abd)		Dead (1m)
12	14y,M	Med, LN (systemic)		Alive (4m)
13	14y,M	LN (lng), bone		Alive (4m)
14	14y,F	LN (axillar)		Alive (2m)

Med = mediastinum; LN = lymph node; abd = abdominal; ing = inguinal; PB = peripheral blood.

Lymph node swelling, which was histologically benign and regressed with no anti-tumor therapy, was observed in two cases. Furthermore, peripheral blood granulocytosis with no evidence of an infectious agent was observed in three cases, either at the time of the initial diagnosis or during the course of the disease, indicating that the tumor may appear as a functional tumor producing some specific cytokines. Lastly, the most important point–the natural history–is summarized, although the observation period has not yet been long enough. Four cases died in a short period of time, despite the intensive radio- and chemotherapy. However, two cases are alive after 9 years and are now in an off– therapy condition. These two cases, previously diagnosed as malignant histiocytosis and large cell lymphoma (cell lineage not specified), were newly diagnosed as Ki-1 lymphoma on immunostaining on paraffin sections with Ki-1 and EMA. Indeed, Kaneko *et al.* described one case of Ki-1 lymphoma that regressed without any therapy (18). Therefore, Ki-1 lymphoma can be divided into two forms, one with a good prognosis and one with a poor prognosis, although no definitive phenotypical characteristics have been identified that can distinguish between these two forms. It is particularly important to collect clinical information, including various laboratory data, in order to know the biological characteristics of Ki-1 lymphoma and to establish the proper therapeutic regimen.

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POLYMERASE CHAIN REACTION STUDIES ON CHRONIC MYELOGENOUS LEUKEMIAS AND DETECTION OF RESIDUAL Ph1 CLONE AFTER BONE MARROW TRANSPLANTATION

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ABSTRACT

Thirty–four cases of chronic myelogenous leukemia (CML) were studied for the expression of breakpoint cluster region (bcr)/abl fused transcripts using the polymerase chain reaction (PCR). In all samples the bcr-abl chimeric mRNA was detected. Seven cases showed bcr exon 2/abl exon 2 (b2-a) chimeric mRNA, 15 cases showed bcr exon 3/abl exon 2 (b3-a) chimeric mRNA, and 12 cases showed both of these mRNAs. None of the 34 samples with expression of b3-a and/or b2-a showed either the expression of BCR-abl chimeric transcripts or b1-a chimeric mRNA, which is due to the alternative splicing of chimeric mRNA, as is the case for b2-a/b3-a mRNA. Taking advantage of the high sensitivity of this method, 24 CML patients, after bone marrow transplantation (BMT), were analyzed for the presence of Ph1 clones. Thirteen out of 24 patients showed residual Ph1 clones detected by PCR assay. The incidence of positive Ph1 clones detected by PCR is also discussed in relation to conditioning regimens for BMT.

INTRODUCTION

The Philadelphia (Ph1) chromosome results from a reciprocal translocation between chromosomes 9 and 22, and occurs in more than 90% of patients with chronic myelogenous leukemia (CML). The oncogene *c-abl* is translocated from chromosome 9 to 22, where it is linked to the clustered region of the breakpoint cluster region (*bcr*) gene (1). The breakpoint on chromosome 9 may vary by more than 100 kilobases (kb) from case to case (2,3). But, in spite of this wide variation in the chromosomal breakpoints at the DNA level, the fused *bcr-abl* gene is consistently transcribed into two types of chimeric mRNA: one consisting of exon 2 of the bcr region linked to exon

2 of the *c-abl* gene (b2-a), and the other consisting of exon 3 of the bcr region linked to exon 2 of the *c-abl* gene (b3-a). These transcripts give rise to 8.5 kb bcr/abl mRNA, which codes for a protein of 210 kD (P210) (4,5). The Ph1 chromosome is also observed in 25% of adult and 10% of childhood ALL. In 50% of adult Ph1 ALL and in almost all childhood Ph1 ALL, the breakpoint on chromosome 22 is located 50 kb 5' upstream from the breakpoint in CML (BCR). Translocation of c-abl to this position results in transcription of a smaller BCR/abl mRNA of 7 kb, encoding a hybrid protein of 190 kD (p190) (6–8).

We have conducted a study of bcr/abl chimeric transcripts by using the PCR method (9). This technique enabled us to detect bcr/abl transcripts with high sensitivity and specificity. Using this highly specific assay, we studied the expression of chimeric mRNA of exons 1, 2, 3 or 4 of the *bcr* gene linked to the *c-abl* gene. We also addressed the correlation of the localization of the breakpoint with the expression of the chimeric mRNA. Residual Ph1 clones in CML patients after bone marrow transplantation (BMT) were also studied by the polymerase chain reaction technique.

MATERIALS AND METHODS

Thirty four samples from 34 patients with the diagnosis of CML were studied. Cell lines without Ph1 chromosome or bcr rearrangement were included for negative controls. Five patients were in blastic crisis at initial presentation, eight cases progressed to blastic crisis during this study. For the study of residual Ph1 clones in BMT patients 24 cases were included; seven patients received cyclophosphamide (Cy) + total body irradiation (TBI), nine cases received cytosine arabinoside (Ara C) + Cy + TBI, and five cases received busulfan (Bu) + Cy. Twenty–one patients received BMT at chronic phase, two at accelerated phase, and one at blastic phase. Total RNA was isolated by the guanidium/CsCl method. DNA was extracted according to the method described previously.

Table 1. The oligonucleotides used for primers and probes.

3' primers	
a; abl ex II (antisense)	5'-GCTGAAAGTCAGATGCTACA-3'
5' primers	
b1;bcr ex 1 (sense)	5'-ATCTCCTCTGACTATGAGCG-3'
b2;bcr ex 2 (sense)	5'-ATTCCGCTGACCATCAATAA-3'
b3;bcr ex 3 (sense)	5'-CACTCAGCCACTGGATTTAA-3'
b4;bcr ex 4 (sense)	5'-AAAGCAAAGACGCGCGTCCA-3'
B; BCR ex I (sense)	5'-GTTGTCGTGTCCGAGGCCAC-3'
Probes	
b2-a:b2-a junction (antisense)	5'-GCTGAAGGGCTTCTTCCTTATTGATA-3'
b3-a;b3-a junction (antisense)	5'-GCTGAAGGGCTTTTGAACTCTGCTTA-3'
B-2; B-a junction (antisense)	5'-GCTGAAGGGCTTCTGCGTCTCCAT-3'
Pr a;abl exon II (sense)	5'-AAGCCCTTCAGCGGCC-3'

3' primer and 5' primers are primers used for amplification of DNA.
b2-a, b3-a, and B-a are probes that identify the junction sequence of bcr exon 2 and abl exon II, bcr exon 3 and abl exon II, and BCR exon I and abl exon II, respectively.
Pr-a is a probe that identifies abl exon II.

The oligonucleotides used for the primers and probes were synthesized by an Applied Biosystem 381A DNA synthesizer based upon the published sequence data and are shown in Table 1. The sizes of amplified cDNA fragment predicted by the sequence data of the *bcr* and *c-abl* genes are also shown in Fig. 1.

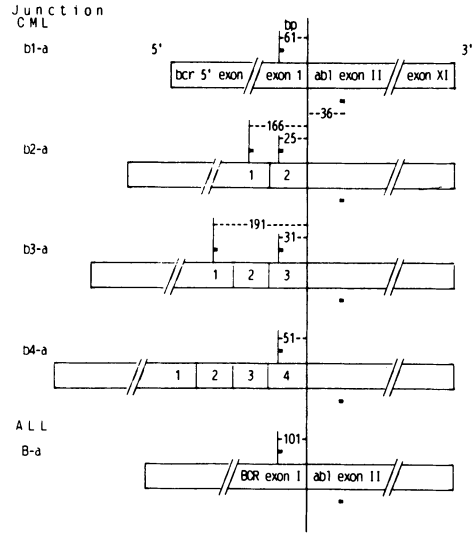


Figure 1. CML and ALL mRNA structure.
b1-a, b2-a, b3-a, b4-a, and B-a denote chimeric mRNA consisting of bcr exon 1/abl, bcr exon 2/abl, bcr exon 3/abl, bcr exon 4/abl, and BCR exon I/abl, respectively. The sizes of the amplified cDNA fragment predicted by the sequence data are shown.

Five to 10 μg total RNA was dried under vacuum. The pellet was dissolved in 9 μl of annealing buffer (250 mM KCl; 10 mM Tris-HCl, pH 8.3; 1 mM EDTA) and 100 pmol of abl minus strand primer was added. The sample was heated for 3 minutes at 80°C and subsequently incubated for 60 minutes at 31°C to allow annealing of primer. After the addition of 15 μl of cDNA buffer (24 mM Tris-HCl, pH8.3; 16 mM $MgCl_2$, 8 mM DTT, 0.4 mM per dNTP mix), 5 units of reverse transcriptase (Rous–associated virus 2, RAV-2, Takara, Kyoto, Japan), and 0.5–1.0 unit/μl RNasin (Promega, Seikagaku-kougyou, Japan), the samples were incubated at 42°C for 60 minutes. Five μl of cDNA was amplified by adding 2.5 μl of X10Taq polymerase buffer (500 mM KCl; 100 mM Tris-HCl, pH 8.3; 150 mM MgCl2; 0.1% gelatin), 2 μl of 2.5 mM each dNTP, 100 pmol 5' primer, DDW to 25 μl, and mineral oil to prevent evaporation. The sample was denatured for 6 minutes at 94°C, and 2.5 units of Taq polymerase (Ampli Taq TM, Cetus) were added. PCR (denaturation at 94°C for 1 minute, annealing at 51°C for 1 minute and extension at 72°C for 2 minutes) was then performed for 30 cycles using a Perkin Elmer Cetus DNA Thermal Cycler. Eight microliters of each reaction mixture was electrophoresed on composite gels containing 1% Seakem agarose plus 3% Nusieve agarose (FMC) in TBE (tris/borate/EDTA)

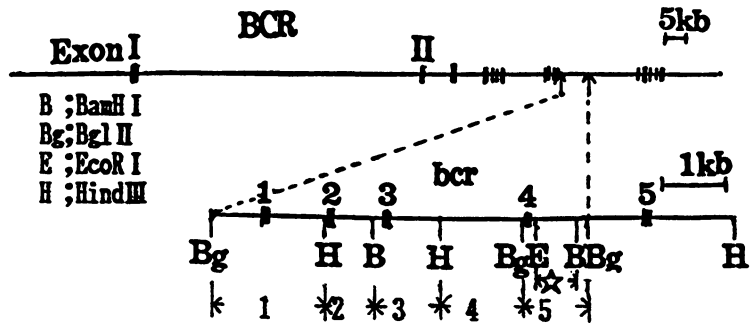
buffer. Gels were transferred onto nylon filters. The membrane was prehybridized and hybridized with either a 5' –end ^{32}P-labeled b2-a junction probe or a b3-a junction probe at 65°C in hybridization solution (5 μg/ml SSSDNA, 5XSSPE, 0.6% SDS). Hybridization was performed overnight at 65°C and washing was done at 60°C with 5XSSPE and 0.1% SDS. The blots were autoradiographed using Kodak XR film.

For Southern hybridization analysis 4–10 μg of DNA was digested with at least two different restriction enzymes (*Bgl*II, *Bam*HI, *Hind*III, or *Eco*RI), electrophoresed through 0.7% agarose, and transferred to a nitrocellulose or nylon filter. Hybridization probes were radiolabeled (α^{32}P-dCTP) by the random primer method. The 3' bcr probe, a 1.2 kb *Hind*III/*Bgl*II fragment (Oncogene Science), was used for all samples. A 5' probe, a 2.0 kb *Bgl*II/*Hind*III fragment [kindly provided by Dr. C. Bartram (10)], was used for samples with deletion of the 3' bcr fragment. A "large bcr probe" (kindly provided by Dr. J. Groffen) was also exploited for the analysis of bcr rearrangements. Prehybridization and hybridization were essentially the same as reported previously. The bcr was divided into five zones based on the published restriction map (Fig. 2), and the breakpoint of each patient was localized into one of the zones.

RESULTS

Correlation of the Breakpoint in the bcr Region and the Transcription of Chimeric mRNA.

We investigated whether or not a specific subtype of the bcr/abl mRNA junction was found in samples with a specific chromosomal breakpoint in the bcr region (Table 2, Fig. 2). With combinations of the restriction enzymes *Eco*RI, *Bgl*II, *Bam*HI, or *Hind*III, we were able to identify the chromosomal breakpoints in the bcr region in 30 Ph1 positive CML patients but not in two samples with deleted bcr regions. One CML sample had breakpoints within zone 2, 16 samples within zone 3, 10 samples within zone 4, and 3 samples within zone 5. The results were further correlated with the subtypes of bcr/abl mRNA junctions detected by the PCR assay. b2-a junction mRNA was detected in the sample with the breakpoint occurring within zone 2. Among 16 samples with the breakpoint occurring within zone 3, we found six samples with the b2-a junction, five samples with the b3-a junction, and five samples with both the b2-a and b3-a junctions. Among the 10 samples with the breakpoint within zone 4, six showed the b3-a junction and four both the b2-a and b3-a junctions. None of these 10 cases expressed b2-a chimeric mRNA alone. All three samples with the breakpoint occurring within zone 5, including one that demonstrated the breakpoint at the 3'region to bcr exon 4, had the b3-a junction and no chimeric mRNA consisting of b4-a was identified (data not shown). Thus all the samples studied could be categorized into three groups based upon the type of chimeric mRNA expressed: (a) those expressing b2-a mRNA, (b) those with the simultaneous expression of b2-a and b3-a mRNA, (c) those expressing b3-a mRNA (Fig. 2).



zone	2	3	4	5
b2-a	1	6	0	0
b3-a	0	5	6	1*
b2-a/b3-a	0	5	4	2
b1-a	0	0	0	0
b4-a				0

* No b4-a junction identified in spite of the break point localized in ☆

* No b4-a junction identified in spite of the breakpoint localized at the asterisk.

Figure 2. Zones identified by the combination of the restriction enzymes *Bam*HI, *Bgl*II, *Eco*RI, and *Hind*III probed by the bcr probe.
Zones of the breakpoint of 30 cases are also shown in relation to the results of chimeric RNA expression.

Analysis of Simultaneous Expression of More than Two mRNA Junctions

In order to determine if b1-a is simultaneously expressed with other mRNA junctions, as is the case for b2-a and b3-a, b1-a chimeric mRNA expression was examined in 34 CML patients (Table 2). None of the samples examined showed b1-a chimeric 97bp mRNA. The positive signals obtained at 202 bp and/or at 227 bp show the amplification of b1b2-a and/or b1b2b3-a chimeric mRNA, respectively (data not shown). These results indicate that, even in the samples with the simultaneous expression of b2-a and b3-a, there is no machinery for the simultaneous expression of spliced short b1-a mRNA.

Simultaneous expression of b2-a and b3-a mRNA was observed in 12 out of 34 CML samples (35%), including those with deleted bcr and those in which the bcr region could not be studied.

Table 2. Clinical and laboratory data of the patients and cell lines studied.

pt. no.	age	sex	Status at sampling	clinical characteristics	follow up	zone in bcr	b2-a	b3-a	b1-a	b4-a	B-a
1	12	F	bp(My)	B	1y10m	3	++	+	-		-
2	14	F	cp	C	3m	3	++	+	-		-
3	9	F	cp	C	1y 5m	3	++	+	-		-
4	10	M	cp	D	8m	3	+	++	-		-
5	14	M	cp	D	3y 9m	3	+	++	-		-
6	36	F	cp	D	6m	4	+	++	-		-
7	31	M	cp	D	3y 2m	4	+	++	-		-
8	44	F	cp	C	8y 0m	4	+	++	-		-
9	52	F	cp	C	1y11m	4	+	++	-		-
10	41	M	cp	C	8m	5	+	++	-		-
11	23	M	bp(?)	A	10m	5	+	++	-	-	-
12	9	F	bp(Mgk)	A	2y 4m	nd	+	++	-	-	-
13	81	F	cp	C	7m	2	+	-	-		-
14	12	M	cp	D	2y10m	3	+	-	-		-
15	8	M	bp(My)	B	4y 3m	3	+	-	-		-
16	49	M	bp(Ly)	B	1y 7m	3	+	-	-		-
17	22	M	cp	B	5y 5m	3	+	-	-		-
18	23	M	cp	C	1y 1m	3	+	-	-		-
19	12	F	cp	C	1y 1m	3	+	-	-		-
20	4	M	cp	B	2y 0m	3	-	+	-		-
21	5	M	cp	D	1y 7m	3	-	+	-		-
22	13	M	cp	C	4y 0m	3	-	+	-		-
23	8	M	cp	C	1y 4m	3	-	+	-		-
24	22	M	cp	D	1y 0m	3	-	+	-		-
25	14	M	cp	B	11m	4	-	+	-		-
26	8	F	bp(Ly)	A	7m	4	-	+	-		-
27	28	F	cp	D	8m	4	-	+	-		-
28	3	M	cp	D	10m	4	-	+	-		-
29	46	F	bp(Ly)	B	4y 4m	4	-	+	-		-
30	54	F	bp(Ly)	B	3y 3m	4	-	+	-		-
31	21	M	ap	E	1y 6m	5	-	+	-	-	-
32	8	F	bp(mix)	A	1y 0m	G	-	+	-		-
33	54	F	cp	A	9m	G	-	+	-		-
34	?	F	cp	D	?	nd	-	+	-		-

cp = chronic phase; bp = blastic phase; ap = accelerated phase; (My) = myeloblastic crisis; (MgK) = megakaryoblastic crisis;(Ly) = lymphoid crisis; (Mix) = mixed lineage crisis; (?) = no information available about the lineage at blastic crisis;
A = blastic crisis at onset; B = blastic crisis during the study; C = chronic phase maintained; D = bone marrow transplantation in 1st chronic phase; E = accelerated phase; Zone = breakpoint region within bcr determined by Southern blot hybridization; G = germline with no rearrangement by Southern hybridization.

Molecular Studies of the Cases with a Masked Philadelphia Chromosome

Among 34 clinical samples, three clinical samples showed no Ph1 chromosome microscopically. In order to elucidate whether these cases also express bcr-abl chimeric mRNA, PCR analysis was employed. The karyotype of the three cases with a masked Ph1 chromosome was 46XY t(22;22)(q13;q11) (case 19), 46XX t(9;12)(234;q24) (case 27), and 46XX t(7;9;22)(p13;q34;q11) (case 33), respectively. As shown in Fig. 3, all the cases showed either a b2-a (case 19) or a b3-a junction (cases 27,33).

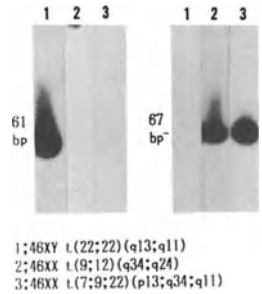


Figure 3. PCR analysis of the cases with a masked Ph1 chromosome.
Lanes 1, 2, and 3 are cases 19, 27, and 33, respectively in Table 2.

Table 3. Relation of Ph1 clones detected by PCR in the presence or absence of graft-versus-host reaction (GVHD) and the conditioning regimens for BMT.

MRD and GVHD

GVHD	n	Positive bcr/abl
+	16	6/16
−	6	5/6

MRD and type of GVHD

GVHD	Positive bcr/abl
Acute	4/8
Chronic	5/11

MRD and conditioning regimens

Regimens	bcr/abl status by PCR		
	n	+	−
CY+TBI	7	6/7	1/7
AraC+CY+TBI	9	4/9	5/9
Busulfan+CY	5	1/5	4/5

MRD = minimum residual disease; Cy = clycloclophosphamide; AraC = cytosine arabinoside; TBI = total body irradiation; n = Number studied.

Among 24 samples studied, 13 patients showed positive Ph1 clones detected by PCR assay. Eleven out of 21 patients who received BMT at the chronic phase were identified to be positive for the Ph1 clone. Two out of 2 who received BMT at the accelerated phase and 1 out of 1 who received BMT at the blastic phase were positive for the Ph1 clone.

When analyzed with reference to the presence or absence of GVHD, those who had GVHD showed less frequent Ph1 clones detected by PCR. Conditioning regimens consisting of busulfan and cyclophosphamide were associated with less frequent Ph1 clones detected by PCR (Table 3)

DISCUSSION

In this study we demonstrate that the PCR assay offers a highly specific method for the detection of chimeric mRNA in Ph1-positive CML. Using this specific assay, we investigated the correlation of the breakpoint in the bcr region on chromosome 22 and the expression of chimeric bcr/abl mRNA subtypes. Among 34 clinical samples obtained from CML patients, 1 showed the breakpoint to be within zone 2, 16 within zone 3, 10 within zone 4, and 3 within zone 5. In the case with the breakpoint within zone 2, the subject is expected to have either b1-a or b2-a chimeric mRNA. Our result, however, showed that this case expresses b2-a, which indicates that the breakpoint of bcr lies in intron 2, between 3' to exon 2 and the *Bam*HI site 5' to exon 3. Five out of 16 cases with a breakpoint in zone 3 also produced b2-a mRNA, and no b3-a mRNA was detected. This is compatible with the results obtained when the breakpoints lie in intron 2, between the *Bam*HI site 5' to exon 3 and exon 3 of bcr. As the remaining 10 cases produced b3-a mRNA with or without b2-a mRNA, their breakpoints must lie in intron 3, between exon 3 and the *Hind* III site 3' to exon 3.

All the cases with breakpoints within zone 4 expressed b3-a mRNA with or without b2-a mRNA, which is in accordance with the results expected from the locus of the breakpoint. The three cases with the breakpoint in zone 5 were shown to express b3-a with or without b2-a RNA, and no b4-a mRNA was expressed, which suggests that the breakpoint in these cases must lie in intron 3, between the *Bgl*II site 5' to exon 4 and exon 4 itself. Alternatively, although the breakpoints lie in intron 4, bcr exon 4 is spliced out, resulting in the expression of b3-a chimeric mRNA, which is indeed the case for one of the three samples that demonstrated bcr rearrangement with *Bam*HI but not with *Eco*RI digestion (pt. 11 in Table 2). Together, these data indicate that the heterogeneity of the breakpoint in bcr is not associated with that of the expression of bcr/abl chimeric mRNA, which can be either b3-a or b2-a chimeric mRNA or both.

The simultaneous expression of b2-a and b3-a mRNA has been reported and has been explained as the result of alternative splicing by Shtivelman *et al.* (5). We have also found 12 such cases out of 34 clinical samples. In order to determine if similar transcribing and splicing into chimeric b1-a mRNA is taking place, PCR analysis was done using b1 and abl primers. None of the 34 cases showed this truncated form of mRNA (97 bp). The 202 bp (b1b2-a) and/or 227 bp (b1b2b3-a) species identified in these samples indicate that our result is not due to the failure of the reaction. As

the results show, alternative splicing, as has been observed in b2-a/b3-a, is not the case for b1-a. Further study is needed to determine the precise mechanism for the specific alternative splicing of b2-a/b3-a.

We found three samples clinically diagnosed as CML with a masked Ph1 chromosome to express b2-a (case 19) and b3-a (case 27 and 33). None of these three cases demonstrated the Ph1 chromosome, but they did show t(22;22)(q13;q11) (case 19), t(9;12)(q34;q24) (case 27), and t(7;9;22)(p13;q34;q11) (case 33). These results indicate that they are associated with the translocation and juxtaposition of the *bcr* and *c-abl* genes at the molecular genetic level without an apparent Ph1 chromosome.

Twenty–four CML patients were included in the study for detection of Ph1 clones by the PCR method, and of these 13 patients were identified to be positive for the Ph1 clone, in spite of no clinical or karyotypical relapse. When analyzed in relation to the presence of GVHD, the cases with GVHD showed less frequent positivity for the PCR assay. Although the difference is not statistically significant, our results may suggest the clinical importance of GVHD for the eradication of malignant clones.

The positivity for the PCR–detected Ph1 clones was also analyzed with regard to the conditioning regimens for BMT. This study suggested that the regimen consisting of busulfan+cyclophosphamide is superior with regard to the incidence of residual Ph1 clones. Although it is an issue to be answered in a future study whether or not those patients with the PCR–detected Ph1 clone develop clinical relapse, PCR can be one of the tools used for the study of the superiority of the conditioning regimens with regard to tumor cell eradication.

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LINEAGE–SPECIFIC CLASSIFICATION OF CHILDHOOD LEUKEMIA AND ITS CLINICAL IMPLICATIONS

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ABSTRACT

Cells from 203 childhood leukemia patients were analyzed using a broad panel of markers, such as immunological marker studies and Southern blot and Northern blot analyses to define the lineage–specific classification of childhood leukemia. Phenotypically, they were divided into B lineage (62.6%), T lineage (9.8%), nonlymphoid (14.3%), and uncertain lineage (13.3%). Two of the B-lineage ALL and two of the T-lineage ALL studied did not show immunoglobulin (Ig) and T-cell receptor (TCR) gene rearrangements, respectively. Therefore those four cases were excluded from the final classification. The uncertain lineage leukemia, which includes undifferentiated leukemia and mixed lineage leukemia, could be further subclassified at the DNA and RNA levels. On the basis of these findings, we discuss the definition for B-lineage and T-lineage cells, the incidence of dual genotypes or spillover, heterogeneity of undifferentiated leukemia, and a new classification for mixed–lineage leukemia. In addition, NK–lineage leukemia associated with Epstein-Barr viral infection is also presented.

PHENOTYPE AND GENOTYPE

Of the 203 patients studied by conventional morphological, histochemical, and immunological marker studies, 127 were diagnosed as B-lineage acute lymphoblastic leukemia (ALL), 19 as T lineage, 29 as nonlymphoid, and 27 as uncertain lineage, consisting of 13 of undifferentiated type and 14 of mixed lineage (Fig. 1).

Ig gene and TCR gene rearrangements seem to be an essential property for establishing B-lineage and T-lineage cells, respectively (1-3). Among 88 B-lineage ALL studied, two cases showed a germline configuration of the Ig gene. Similarly, two cases from T-lineage ALL in Fig. 1 revealed a germline configuration of the TCR gene. Therefore, those four cases did not fall into our current lineage–specific classification.

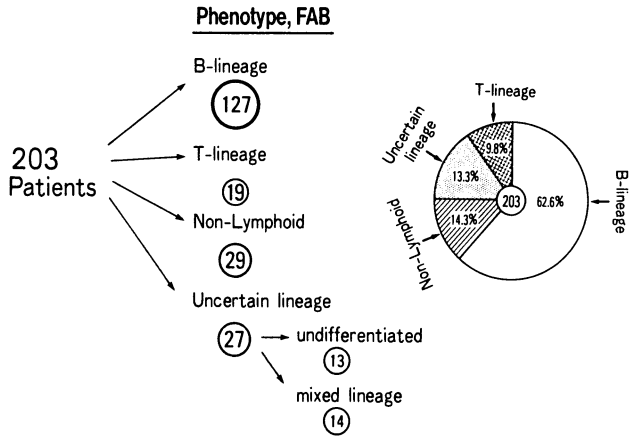

Figure 1. Classification of 203 cases of childhood leukemia.

INCIDENCE OF DUAL REARRANGEMENTS

TCR gene rearrangements were frequently observed in B-lineage ALL (Fig. 2). Among TCR genes, TCR-delta rearranged in 74%, TCR-gamma in 41%, and TCR-β in 29%. On the other hand, about 16% of T-lineage ALL were shown to have IgH rearrangement (Table 1). One of the three rearranged cases also revealed Igk rearrangement, as reported previously (4).

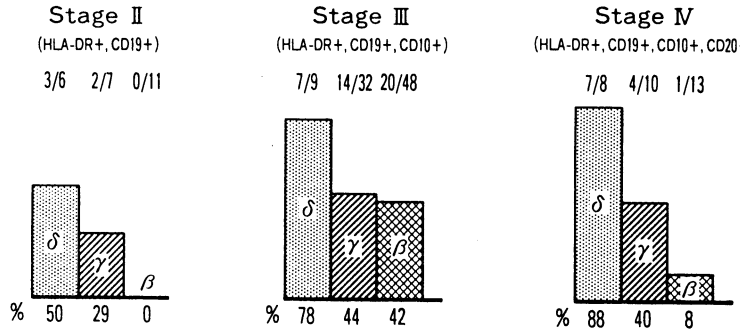

Figure 2. TCR gene rearrangement in B-lineage ALL.

IgH and TCR-β rearrangements were observed in about 13% and 19% of ANLL, respectively. No TCR-delta rearragement was detected in the present study (data not shown).

To explain the presence of these spillover or bigenotypic leukemias, the following two reasons seem to be the more likely (a) Ig and TCR gene rearrangements occur before lineage commitments, and (b) gene rearrangements continue after leukemic transformation (active recombinases) (5).

Table 1. Diversity of Ig and TCR gene rearrangements.

	B-lineage ALL	T-lineage ALL
IgH	Biallelic	Single allelic D-J_H (close doublet)
TCR-β	Single allelic germline or deletion of $C_{\beta 1}$	Biallelic Rearrangement of $C_{\beta 1}$
TCR-*gamma*	Single allelic $J_{gamma\ 1}$ ($C_{gamma\ 1}$) V_{gamma}, variable	Biallelic $J_{gamma\ 2}$ ($C_{gamma\ 2}$) CD3+, $V_{gamma\ 2,3,4,5}$ CD3−, $V_{gamma\ 8,9,10,11}$
TCR-δ	V-D_δ or D-D_δ (not $J_{\delta 1}$)	$J_{\delta 1}$

Table 2. Ig and TCR gene rearrangements in T-lineage ALL/NHL.

	J_H	TCR-δ	TCR-γ	TCR-β
Stage I, 6 ($CD3^-$, $CD4^-$, $CD8^-$)	0/6	4/6	2/6	2/6
Stage II, 9 ($CD3^-$, CD4 and/or $CD8^+$)	2/9	9/9	9/9	9/9
Stage III, 4 ($CD3^+$)	1/4	4/4	4/4	4/4

Although Ig and TCR gene rearrangements are necessary to establish B- and T-lineage cells, they are not sufficient to determine an individual lineage by themselves. However, when one carefully looks at the pattern of such dual rearrangements, it may be possible to distinguish nonproductive rearrangements from productive rearrangements, as summarized in Table 2.

HIERARCHY OF TCR GENE REARRANGEMENTS

Two cases from stage 1 T-lineage ALL showed only TCR-delta rearrangements (Table 1), while TCR-delta, gamma and β genes concomitantly rearranged in rather mature T-lineage ALL (stage II and III). Together with frequently rearranged TCR-delta in B-lineage ALL, the TCR-delta rearrangement seems to be the earliest event in T–cell differentiation, preceding rearrangements of the other TCR gene (6), as well as in the case of Ig genes (IgH → Igk → Ig*lambda*).

AUL (ACUTE UNDIFFERENTIATED LEUKEMIA)

Eleven cases failed to express T-lineage–associated, B-lineage–associated, and myeloid-associated antigens, as shown in Table 3. Six cases from these AUL showed

rearrangements of both the IgH and TCR-delta genes, frequently accompanied with Igk, TCR-α, gamma and β rearrangements, and the remaining five cases did not display any rearrangements (Table 4). Therefore, those phenotypically classified AUL can be divided into two subgroups at the DNA level (7).

Table 3. Summary of phenotypic analysis of AUL.

Patient No.	CD2 T11	CD3 T3	CD5 Leu-1	CD7 Leu-9	CD10 J5	CD19 B4	CD20 B1	sIg	CD13 My7	CD14 Mo2	CD33 My9	CD41w PLT1	HLA-DR I_2	Negative Control
1	2	2	2	ND	0	0	ND	1	2	ND	ND	ND	0	0
2	13	ND	11	ND	0	3	4	11	4	ND	ND	ND	2	3
3	0	3	0	ND	9	18	0	3	0	ND	ND	ND	60	1
4	0	ND	0	ND	0	0	2	0	0	0	ND	ND	78	0
5	1	3	2	ND	0	0	7	1	0	ND	0	ND	64	0
6	1	0	ND	0	0	0	0	1	0	0	0	ND	80	0
7	6	2	5	5	2	1	1	0	2	ND	ND	5	4	2
8	5	ND	4	ND	0	0	0	0	15	ND	ND	ND	27	2
9	1	3	ND	6	14	0	0	0	4	0	ND	7	38	1
10	2	2	1	ND	0	3	6	0	3	0	ND	ND	74	2
11	0	2	0	0	0	0	0	0	2	0	68	2	26	1

Abbreviation: ND, not done.

Numbers are specific percentages of positive cells. The background staining was not subtracted.

ND = not done; Numbers are specific percentages of positive cells. The background staining was not subtracted.

Table 4. Summary of Southern and Northern blot analyses of AUL.

	Rearrangement													
	Ig		TCR				Total Number of	Transcription						
Patient No.	H	L (κ)	δ	α	γ	β	Rearranged Alleles	MPO	TdT	C_μ	TCR-γ	TCR-β	CD3-γ
1	R/D	G	R/D	R/G	R/R	R/D	9*/12†	–	+	–	+	+(1.3, 1.0)	+
2	R/R	G	R/D	R/G	R/R	R/G	8/12	ND	ND	ND	ND	ND	ND
3	R/R	G	R/D	R/G	G	R/G	6/12	–	+	+	–	+(1.2)	–
4	R/R	G	R/R	G	G	R/D	6/12	–	ND	+	ND	–	ND
5	R/R	ND	R/D	R/G	G	G	5/10	–	+	–	–	–	–
6	R/D	R/D	R/R	G	G	G	6/12	ND	ND	ND	ND	ND	ND
7	G	G	G	G	G	G	0/12	–	ND	–	ND	–	ND
8	G	G	G	G	G	G	0/12	ND	ND	ND	ND	ND	ND
9	G	ND	G	G	G	G	0/10	–	ND	–	–	–	–
10	G	G	G	G	G	G	0/12	–	–	+	–	–	+
11	G	ND	G	G	G	G	0/10	–	–	–	–	+(1.0)	+

Abbreviations: G, germline; R, rearranged; D, deleted; +, expression was detected; –, expression was undetected; ND, not done.

Size is in kilobases.

*Rearranged alleles.

†Examined alleles.

G = germline; R = rearranged; D = deleted; + = expression was detected; – = expression was undetected; ND = not done.
Size is in kilobases. [a] = rearranged alleles. [b] Examined alleles.

MLL (MIXED LINEAGE LEUKEMIA)

About 6% of leukemia patients were diagnosed as having so-called mixed lineage leukemia (MLL). Since numerous terms are used to refer to this type of leukemia (5) and the definition for it remains ambiguous, we dissected 17 cases of MLL, including an additional three cases referred to us. According to our criteria, these 17 cases were further divided into four subtypes, as shown in Table 5.

Table 5. Mixed lineage leukemia.

Type I:	Cells differentiating along a specific lineage, but coexpressing other lineage-associated markers
	ex. B-lineage cells coexpressing myeloid markers (CD13, CD14, or CD33)
Type II:	Biphenotypic, but not well differentiated
	ex. CD7(+) stem cell leukemia
	Low percentage of MPO positivity
Type III:	Specific chromosome translocation
	ex. Ph^1
	11q23
Type IV:	Biclonal

Nine cases were classified as Type I, that is, they contain leukemic cells differentiating along a specific lineage but coexpressing other lineage-associated markers. Although the incidence of this type of MLL was the highest, patients of Type I responded well to conventional treatment, and we do not see any clinical significance among them. Two cases with specific chromosome translocations were classified as Type III and those had Ph1 chromosome and translocations involving 11q23. A single case without any such kind of chromosome translocation was diagnosed to have biclonal leukemia at the level of phenotype and genotype (Type IV). Finally, five cases belonging to Type II displayed as biphenotypic, but were not well differentiated. As shown in Table 4, CD7+ (double negative) stem cell leukemia and leukemia with a low percentage of MPO positivity are included in this group (8,9). Interestingly, three of the five cases presented with a mediastinal mass. As reported previously, those are leukemias of immature hematopoietic cells, and recognizing them seems to be very important in terms of prognosis, because they respond poorly to conventional treatments.

NK LEUKEMIA ASSOCIATED WITH EB VIRUS

Lymphoproliferative disease of granular lymphocytes (LDGL) is a heterogeneous disorder and the pathogenesis is likely to be complex. The clonal nature of LDGL has been established in CD3+LDGL in terms of TCR gene rearrangement, but not in all cases including CD3-LDGL.

Table 6. LDGL and EB viral genome.

		EBV genome	
		+	−
CD3+ LDGL	CD4−8+	0	8
	CD4−8−	0	1
CD3− LDGL		10(*4)	2

*patients with CAEBV

Table 7. Heterogeneity of LDGL.

1. Polyclonal (reactive) → benign course / aggressive course
2. Monoclonal-stable (chronic NK leukemia) → benign course ? / aggressive course
3. Monoclonal, aggressive course (acute NK leukemia)

More recently, we reported that EB viral DNA was detected in some cases of CD3-LDGL and were able to show the clonal nature of those LDGL on the basis of the pattern of Southern blot analysis using an EB virus terminal probe (10). As shown in Table 6, so far 21 cases of LDGL have been studied, and EB viral DNA has been detected in 10 cases. All of the EB virus positive cases were CD3-LDGL and four of them have been diagnosed as chronic active EB viral infection (CAEBV). Among four patients with CAEBV, two showed the monoclonal nature, and the remaining two revealed the polyclonal nature, indicating reactive proliferation of the increased granular lymphocytes. From a clinical point of view, monoclonality of EB viral DNA does not seem to correlate with the outcome in these CAEBV patients. The remaining six patients without any underlying disorders also consisted of four of a monoclonal and two of a polyclonal nature. Based on these findings, the heterogeneous nature of LDGL can be summarized as shown in Table 7.

Table 8. Lineage-specific classification of leukemia.

1. B lineage:	slg (or clg) positive
	or
	Ig gene rearrangement + (CD19, CD20, CD10, HLA-DR, etc)
2. T lineage:	CD3/TCR positive
	or
	TCR gene rearrangement + (CD7, CD5, CD2, CD1, CD4, CD8, etc)
3. Non lymphoid:	
	Myeloid-lineage: MPO (≤25%)
	or
	MPO (<25%), NSE + (CD13, CD33, CD14, etc.)
	Megakaryoblastic
	Erythroblastic
4. NK lineage (CD16, NKH-1, Leu7, CD2, etc.)	
5. Uncertain lineage:	
	Undifferentiated
	Mixed lineage

It seems to be essential to further dissect the heterogeneity of leukemias using a broad panel of markers in order to design more sophisticated and effective treatments. Our findings, together with those of others, raise the possibility of

establishing a lineage–specific and clinical-oriented classification of leukemias, as shown in Table 8.

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II. Epidemiology of Childhood Leukemia

10

THE EPIDEMIOLOGY OF LEUKEMIA: RESULTS FROM THE MANCHESTER CHILDREN'S TUMOUR REGISTRY

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ABSTRACT

The Manchester Children's Tumour Registry gathers clinical and epidemiological information on all childhood cancers from a defined population base. Between 1954 and 1988 there were 1107 leukemias (879 ALL, 203 ANLL, 21 CML, and 4 other unspecified). The rates per 10^6 child years for ALL, ANLL, CML, and all leukemias were 26.2, 6.1, 0.6, and 33.1, respectively. Linear regression analyses of the annual incidence showed an upward trend in ALL, but this was not statistically significant. Comparison of ALL rates during 5–7 year periods (1954–1960, 1961–1967, 1968–1974, 1975–1981, 1982–1988) showed no significant differences among children aged <1, 5–9 and 10–14, but a significant increase in children aged 1–4. Rates were 41.8, 45.2, 53.1, 54.2, and 55.5 during each successive period, respectively (chi squared trend = 4.8, $p < 0.05$). In order to explore these time trends for ALL in more detail, 5-year running means of the annual incidence by age group were plotted. While in children aged <1, 5–9 and 10–14 there was little apparent variation, the incidence pattern for the 1-4 year age group appeared to be cyclical, i.e., there were successive increases and decreases in incidence. This observation is compatible with the idea that in childhood common ALL the pattern of infections in early life may be etiologically important and a fluctuation in incidence may reflect fluctuations in the prevalence of infectious agents. The rates for ANLL were stable throughout the study period overall and within each age group.

INTRODUCTION AND METHODS

The Manchester Children's Tumour Registry (MCTR) was founded in September 1953. The registry is population based and includes all cases of malignant, and certain benign, neoplasms in children aged less than 15 years at diagnosis, and who are resident within the North Western Regional Health Authority area of England (formerly Manchester Regional Hospital Board area). The region has a child population (ages 0–14) of approximately 1 million. The MCTR is located within the

Christie Hospital & Holt Radium Institute, which is a specialized cancer hospital and provides radiotherapy and oncology services for the North West Region.

Notifications are received directly from clinicians, and frequent contact is maintained with pathologists, pediatricians, surgeons, radiotherapists, hematologists, and certain other hospital personnel, in order to achieve rapid and accurate ascertainment of eligible cases. A national cancer registration scheme has been in operation since 1962, and the MCTR also routinely receives copies of the cancer registration documents relating to children. Copies of death certificates for children, on which a neoplasm is recorded as a cause of death, are also obtained routinely.

Notifications are checked for eligibility, and a copy of the hospital notes, or a detailed summary of these, containing information on presenting symptoms, diagnostic tests, treatment and progress, is obtained for each eligible case. Histopathological slides of solid tumors are circulated to a panel of expert pathologists, who each independently report back to the Registry. In cases where opinions differ, a consensus diagnosis is reached at meetings of the pathologists' panel. Histopathological material is retained by the Registry, so that diagnoses may be reviewed and revised in line with current ideas on classification. For leukemias, diagnosis is based on bone marrow examinations. Annual follow-up is carried out by writing to the clinician in charge. Since the outset, ascertainment has been consistently high. For a more detailed description of the Registry, see Birch (1).

In the present study, descriptive data on leukemias included in the MCTR from January 1, 1954 to December 31, 1988, have been reviewed. The incidence of leukemias by cell type, age, and sex has been estimated, using the annual midyear estimates of the child population as the denominator, and temporal trends in incidence have been examined. Data on immunophenotype in acute lymphoblastic leukemia (ALL) are available from 1979 onwards, and the incidence of ALL by immunophenotype has also been estimated. Finally, the case records were searched for factors of etiological interest.

INCIDENCE BY AGE AND SEX

The incidence of the main types of malignant disease in children, estimated from MCTR data, is shown in Table 1. Leukemias represent approximately one third of all cases. Central nervous system tumors comprise a further quarter of the cases. Lymphomas, neuroblastoma, Wilms' tumor, and bone and soft tissue sarcomas represent other major groups. Other tumors, for example, retinoblastoma, hepatoblastoma, and certain germ cell tumors, including sacrococcygeal yolk sac tumor and orchioblastoma, are rarely seen beyond the age of 5, but are nevertheless relatively infrequent, compared with other typical childhood malignancies. Carcinomas, which form the overwhelming majority of cancers in adults, are rarely seen in children.

Leukemias were classified as acute lymphoblastic leukemia, acute nonlymphoblastic leukemia (ANLL), chronic myeloid leukemia (CML), and other and unspecified leukemias.

A total of 1107 cases of leukemia were included in the MCTR during the study period (621 males, 486 females). Of these, 879 (513 males, 366 females) were ALL, 203 (92 males, 111 females) were ANLL, 21 (15 males, 6 females) were CML, and 4 (1 male, 3 females) were other and unspecified leukemias.

The sex-specific incidence per 10^6 child-years is shown in Table 2A. For ALL the rate in males (29.9 per 10^6) was significantly greater than the rate in females (22.4 per 10^6); $p \leq 0.001$, giving a male to female ratio of 1.33. Chronic myeloid leukemia was also more common in males than in females, although the rates in both sexes are very low. In contrast, there was no significant difference in the rates for ANLL in males (5.4 per 10^6) compared with that in females (6.8 per 10^6).

Table 1. Manchester Children's Tumor Registry. Malignant disease in children aged 0–14, 1954–1988.

	M	F	M + F
	Incidence per 10^6		
Leukemias	36.2	29.8	33.1
Lymphomas	15.0	8.0	11.6
CNS tumors	26.6	22.7	24.7
Heuroblastoma	7.3	6.3	6.8
Wilms' tumor	6.1	5.8	5.9
Retinoblastoma	3.3	2.9	3.1
Hepatoblastoma	0.8	0.4	0.6
Soft tissue sarcomas	6.1	4.6	5.4
Bone tumors	4.4	5.7	5.1
Germ cell tumors	2.5	3.3	2.9
Carcinomas	2.2	2.5	2.4
Other and unspecified	1.5	1.2	1.3
Total	112.0	93.2	102.9

Table 2A. Sex specific incidence of leukemia. Manchester Children's Tumor Registry.

	M	F	M + F
	Incidence per 10^6		
ALL	29.9	22.4	26.2
ANLL	5.4	6.8	6.1
CML	0.9	0.4	0.6
Other and unspecified	<0.1	0.2	0.2
All leukemia	36.2	29.8	33.1

Table 2B. Age-specific incidence of leumemia. Manchester Children's Tumor Registry.

	0	1-4	5-9	10-14	0-14
	Incidence per 10^6				
ALL	13.8	49.4	22.7	13.4	26.3
ANLL	5.8	6.5	5.6	6.2	6.1
CML	0	0.5	0.4	1.0	0.6
Other and unspecified	0.4	0	0.3	0	0.1
All leukemia	20.0	56.4	29.0	20.6	33.1

Table 2B shows the age-specific incidence among the various cell types. The most striking feature is the very marked peak in the incidence of ALL in children aged 1–4, with a relatively low incidence in infants aged under 1 and in children aged 10–14, and an intermediate rate among children aged 5–9. In ANLL the pattern is quite different, with similar rates in all age groups.

TEMPORAL TRENDS IN INCIDENCE

In 1981 we published data from the MCTR relating to the leukemia incidence over the period 1954–1977 (2). The main finding at that time was of a significant increase in the incidence of ALL, while the incidence of ANLL had not changed. The observed increase in ALL was most marked among boys and in children aged 1–4. In view of these previous findings, we carried out an analysis of temporal trends over the period currently under study to gain an overall impression of whether rates were tending to increase, decrease, or remain stable. Linear regression analyses of the annual incidence of ALL were carried out with respect to all cases, males, females, and the age groups 0, 1–4, 5–9 and 10–14. Figure 1 shows the results for all cases of ALL.

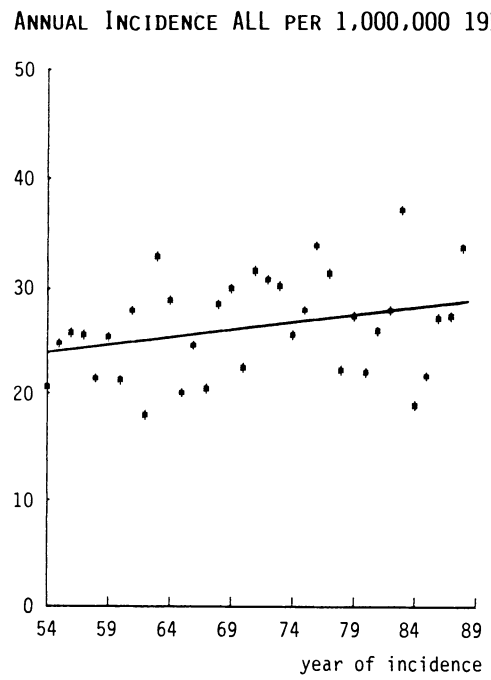

Figure 1. Regression analysis of annual incidence. ALL, Manchester Children's Tumor Registry, 1954–1988.

There was considerable fluctuation in the annual incidence, but an upward trend was observed overall. However, this failed to reach statistical significance ($p < 0.1$). When the annual incidence in males and females was examined separately, no significant increase in incidence was observed (p in both groups > 0.1).

In our previous analyses of the 1954–1977 data, the most pronounced increase

in incidence was seen among children aged 1–4. Linear regression of the annual incidence of ALL in this age group with respect to the current data demonstrated an increase in incidence that was of borderline statistical significance ($p<0.1$), again demonstrating that the earlier observed increase in rates has not been sustained (Fig. 2).

In order to explore these trends in more detail, the incidence during five consecutive 7-year periods was compared with respect to all cases, males and females, and the various age groups. The expected numbers of cases during each time period were estimated, assuming a uniform incidence throughout the 35-year study period. These expected numbers were compared with the observed number of incident cases. To test for changes in incidence, chi squared values for heterogeneity and for trend were calculated. Table 3 shows the results for all cases of ALL.

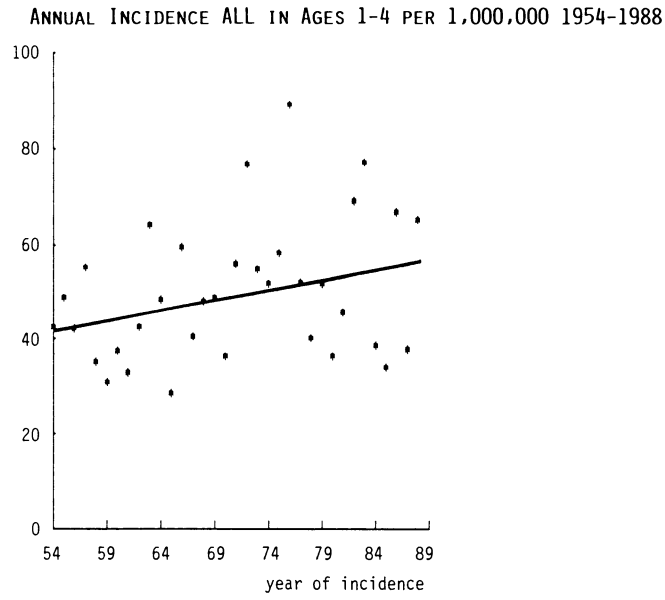

Figure 2. Regression analysis of annual incidence of ALL in the age group 1-4 years. Manchester Children's Tumor Registry, 1954–1988.

Table 3. Incidence of ALL by 7-year time period. Manchester Children's Tumor Registry.

Time period	1954–1960	1961–1967	1968–1974	1975–1981	1982–1988
Incidence per 10^6	23.5	24.6	28.4	27.3	27.6
No. cases observed	161	179	213	173	153
No. cases expected	179.8	191.0	196.6	166.2	145.4
Observed:expected	0.90	0.94	1.08	1.04	1.05

Chi square = 4.7; trend = 3.1.

The incidence during the first two 7-year periods was lower than in the latter three 7-year periods. However, tests for heterogeneity and trend were not significant. Similarly, no significant differences were found when the data for males and females were examined separately. When the age groups were analyzed in a similar fashion, no significant differences over the five 7-year periods were observed among children aged <1, 5–9, and 10–14. However, among children aged 1–4, there was an increase in incidence with each consecutive 7-year time period, and the trend was statistically significant ($p < 0.05$). These results are shown in Table 4. The incidence in this age group was 33% higher during 1982–1988, compared with the incidence during 1954–1960.

Table 4. Incidence of ALL age 1–4 by 7-year time period. Manchester Children's Tumor Registry.

Time period	1954–1960	1961–1967	1968–1974	1975–1981	1982–1988
Incidence per 10^6	41.8	45.2	53.1	54.2	55.5
No. cases observed	82	96	110	79	81
No. cases expected	96.9	104.8	102.3	71.9	72.1
Observed:expected	0.85	0.92	1.08	1.10	1.12

Chi square hetero = 5.4; trend = 4.8; $p < 0.05$.

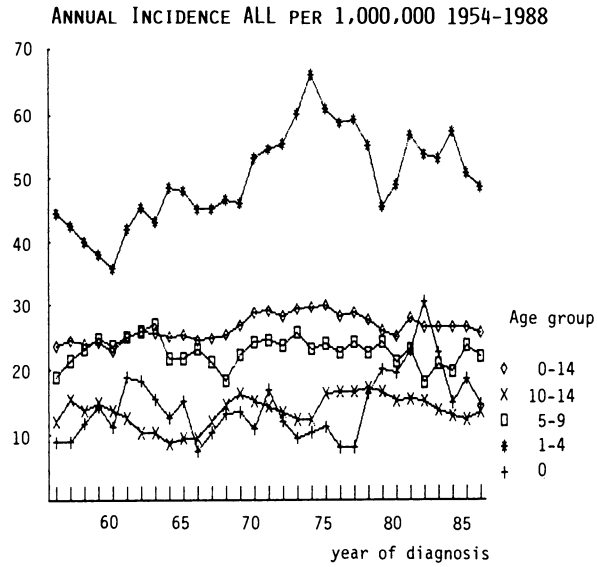

Figure 3. 5-year running mean of incidence of ALL by age group. Manchester Children's Tumor Registry, 1954–1988.

The pattern of incidence in ALL overall, and in subgroups defined by age and sex, was examined by plotting 5-year running means. The mean incidence during each 5 years was calculated by dividing the number of incident cases by the total child-years

over each respective 5 years. The effect of a running mean is to smooth out the annual fluctuations such that any underlying pattern of incidence becomes more obvious. Figure 3 shows plots for the annual incidence of ALL overall and by age group. The figure clearly shows that there has been little variation in incidence with time when all age groups are considered together, and for the age groups 5–9 and 10–14. Although some fluctuation is seen in the incidence of ALL in children aged <1, these results are based on very small numbers (approximately 1 case per year on average).

The most interesting results are found in the 1–4 year age group, where rather than a linear trend in incidence with time, the incidence pattern appears to be cyclical, with a decline in incidence during the 1950s, followed by a rise in incidence, reaching a peak during the mid-1970s, with a subsequent decline in incidence during the late 1970s, followed by a second increase, but with rates currently falling. Similar patterns were observed when males and females were analyzed separately.

Rates for ANLL were stable throughout the study period, overall and within each age and sex group.

INCIDENCE BY IMMUNOPHENOTYPE

Incident data by immunophenotype are available from 1979 onwards. During the period 1979–1988 there were a total of 218 cases of ALL. One hundred and sixty of these (73%) were of the "common" cell type; 26 (12%) were T cell; 14 (7%) were null cell; and only 5 (2%) were classified as B cell. Thirteen (6%) were classified as non-T, non-B, or were unclassified. These were mainly in the early part of the period.

Table 5A shows the sex-specific incidence of ALL by immunophenotype. A higher incidence in males than in females was seen in both common ALL and T-cell ALL, whereas there were no marked sex differences in the incidence of the B-cell, null-cell, and unspecified groups. Table 5B shows the age-specific incidence of ALL by immunophenotype. This table clearly shows that the peak in incidence in the 1–4 age group is largely accounted for by common ALL. T-cell leukemia and B-cell leukemia were not observed in children aged less than 1 year in the present series, but were otherwise fairly evenly distributed among the remaining age groups. In contrast, the majority of cases of null-cell ALL occurred in infants.

Table 5. Incidence of ALL per 10^6 by sex and immunophenotype (A) and age and immunophenotype (B). Manchester Children's Tumor Registry, 1979–1988.

	M	F	M+F	0	1-4	5-9	10-14	0-14
T-cell	3.6	2.8	3.2	0	3.4	3.1	3.7	3.2
B-cell	0.7	0.5	0.6	0	1.0	0.4	0.7	0.6
Common	21.1	18.2	19.7	5.6	41.6	18.2	8.4	19.7
Null	1.7	1.8	1.7	13.0	2.0	0.8	0.3	1.7
Unspecified	1.7	1.5	1.6	0	4.4	0	1.4	1.6

FAMILIAL ASSOCIATIONS OF CHILDHOOD LEUKEMIA WITH OTHER PEDIATRIC AND ADULT CANCERS

The most interesting finding to emerge came from the examination of case records of each child related to the family history of cancer. There were six children with ALL, who between them had seven siblings with cancer diagnosed below the age of 15. The details of these cases are shown in Table 6. Assuming on average two siblings per case child, this represents approximately a threefold increase above the expectation. Three of the siblings had brain tumors, compared with an expected number of 0.6. Furthermore, two other children with ALL had parents with malignant brain tumors, and in other families brain tumors were seen in second–degree relatives. One child had two siblings with soft tissue sarcomas.

A further striking feature, shown in Table 6, is the distribution of age at diagnosis in the index children with ALL. Each of these children was relatively old at diagnosis, compared with the median age at diagnosis of children with ALL in the present series.

Table 6. Acute lymphoblastic leukemia cases with siblings having childhood cancer. Manchester Children's Tumor Registry.

Index patient		Sibling with cancer		
Sex	Age	Diagnosis	Sex	Age
Male	8 years	Hodgkin's disease	Male	6 years
Male	11 years	Astrocytoma	Female	12 years
Male	12 years	Astrocytoma	Male	2 years
Female	12 years	Ependymoma	Male	4 years
Female	13 years	Leukemia + Down's syndrome	Male	2 years
Male	14 years	1. Malignant hemangiopericytoma	Female	Congenital
		2. Rhabdomyosarcoma	Male	4 years

DISCUSSION

Leukemias numerically form the most important group of childhood malignancies in most countries where population-based data are available (3). Acute lymphoblastic leukemia appears to be the most common cell type seen in children worldwide. The rates observed in the present study are similar to those found in many populations in western Europe and the U.S. white population. However, the recent International Study of Childhood Cancer Incidence, which was sponsored by the International Agency for Research on Cancer (IARC), found a ninefold difference between the highest rates, seen in Costa Rica, and the lowest rates, seen in Nigeria (4). In the U. S. black populations, the incidence of ALL was about half that in whites. This difference in incidence in these two populations of the United States could be mainly accounted for by the less marked peak in the 2–4 year age range in the black population. The childhood peak in ALL appears to be absent in African children.

The high rates of ALL in young children characteristically seen in developed countries is accounted for by the "common" subtype of ALL (cALL). The low incidence

of cALL in populations with poor socioeconomic conditions is consistent with Greaves' ideas about the cause of this type of childhood leukaemia (5). Greaves speculates that the development of this particular subtype of leukemia depends on spontaneous mutation in lymphoid precursor cells occurring prenatally, with a second mutational event that precipitates leukemia development and that depends upon the pattern and timing of common infections during infancy and early childhood.

International comparisons of childhood leukemia incidence can provide clues to etiology. Similarly, studies of temporal trends within a given population can also form the basis of etiological hypotheses. However, consistent complete or unbiased ascertainment of cases over a very long time period is necessary for the study of trends in incidence with time, and few cancer registries have data of this quality.

A recent report examined trends in childhood cancer incidence in Connecticut from 1935 to 1979 based on data from the Connecticut Tumor Registry (6). The most striking finding from this study was a three-fold increase in the incidence of ALL in boys aged under 5. Smaller increases in rates were also seen in boys aged 5–9 and in girls aged <10. These observations were consistent with the earlier report based on MCTR data (2) and on national data from the UK (7), which demonstrated increases in the incidence of childhood ALL.

The current observations on the MCTR data, which showed that this increase has not been sustained, are particularly interesting in the light of a recent paper from the Netherlands, which demonstrated a stable incidence of ALL between 1973 and 1978, an increase in rates between 1979 and 1984, with a subsequent decrease to the previous level during the 2 most recent years under study (8).

Close scrutiny of the data presented in Fig. 3 suggests that this fluctuation in incidence may be cyclical over a long time period, specifically in the 1–4 year age group, with periods of high incidence and low incidence. The restriction of this phenomenon to this particular age group would suggest that it is the "common" subtype that is showing these variations in incidence. This would give some support to Greaves' ideas on the involvement of common infections in the etiology of cALL. The fluctuations in incidence may be a reflection of fluctuations in the prevalence of infectious agents.

We have incidence data by immunophenotype for the most recent 10 years of data only in the present study, and many more years of data collection are required before time trends in the incidence by immunophenotype can be examined in sufficient detail.

In the study of cancers in close relatives of children with leukemia, of particular interest was the apparent association with central nervous system tumors, both in siblings and other close relatives. This may be an independent association in its own right, but another possibility is that this represents a manifestation of the Li-Fraumeni syndrome. This cancer family syndrome is characterized by bone and soft tissue sarcomas in children and young adults, and early onset breast cancer in their mothers and other close relatives. Certain other cancers occur to excess in these families, including leukemia and brain tumors (9). In this context the family in which two siblings of a child with ALL had soft tissue sarcomas may also represent an example of the Li-Fraumeni syndrome.

In the present series, the familial cancers were found in association with ALL, but not with ANLL. Furthermore, the age distribution among the case children who had close relatives with cancer was unusual, and it may be that familial cancer risk is

associated with a specific and relatively uncommon subtype of ALL.

In the future, it will be important to determine the immunophenotypes and karyotypes of leukemias involved in familial clusters of cancers. Although the present data are largely anecdotal, it would seem an important and potentially fruitful area for further study.

In conclusion, population-based registries are not only useful in the planning of clinical services, but can also provide valuable insights into etiology. However, many years of data must be accumulated before the full value of a registry can be realized.

ACKNOWLEDGEMENTS

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11

CONTRIBUTION OF JAPAN CHILDREN'S CANCER REGISTRY TO MEDICAL RESEARCH AND CARE OF CHILDHOOD LEUKEMIA AND RELATED CONDITIONS

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ABSTRACT

The Japan Children's Cancer Registry was established in 1969 as an annual nationwide survey to clarify the status not only of mortality, but also morbidity in childhood malignancies and among long-term survivors. We found that childhood leukemia, which is the most frequent malignancy, accounted for 43% of the total cases in this hospital-based registry. Leukemia showed male dominance and an age peak at 2–3 years, whilst most solid tumors showed no sex predominance and an age peak within 12 months after birth. High concordance in monozygotic twins and a high incidence of specific chromosomal translocation were observed in leukemia. These findings suggest that the etiological mechanism of leukemia differs from some blastomas in which loss of heterozygosity of the genes has been reported. Heterogeneity of leukemia was also suggested by comparisons of characteristic features, among types classified both by FAB and immunological markers, in relation to age distribution, sex ratio, type of chromosomal aberration, frequency of complication of Down syndrome, and family history. The registry will continue to contribute to the study of the etiology and biological nature of leukemia, as well as to the improved care of children with leukemia in general.

INTRODUCTION

The Japan Children's Cancer Registry (1) is a program to provide an annual nationwide survey as a means of clarifying the status and analyzing the risk and causative factors in childhood malignancy, including leukemia. As in other developed countries, childhood malignancy is not a common disease but remains a major cause of death in children, and leukemia accounts for nearly half of the mortality. The registry was established in January 1969 by the Children's Cancer Association of Japan,

and it is controlled by the Committee of Japan Children's Cancer Registry, which is composed of representative members of the cancer committees of both the Japan Pediatric Society and the Japanese Society of Pediatric Surgeons, as well as other specialists and epidemiologists.

A registry of patients with malignancies who survived over 5 years was also started in 1980. Information from the retinoblastoma registry of ophthalmologists has also been linked since 1971. Unfortunately, brain and bone tumors are registered by different systems maintained separately by neurosurgeons and orthopedic surgeons.

Recent progress in the treatment of childhood malignancies has increased the number of curable cases by more than half, and life expectancy has been appreciably prolonged. The registry provides information on morbidity, mortality, and long-term survivors. Moreover, our hospital-based registry is based on pathohistological classification and contains various medical and social information.

In this report we wish to outline the registration system used and show what kind of information can be provided from the registry for research and improved treatment of leukemia. We present gross statistics and the characteristic features of registered cases of leukemia in comparison with solid tumor and a possible heterogeneity for leukemia. Information on long-term survivors and social problems is not presented here.

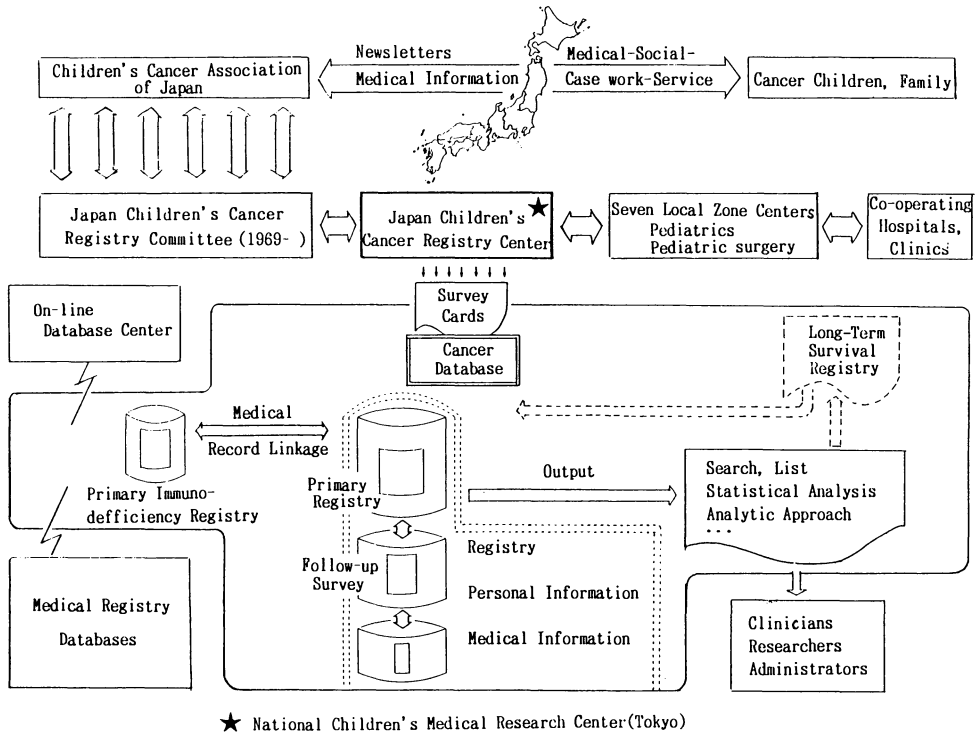


Figure 1. System of Children's Cancer Registry.

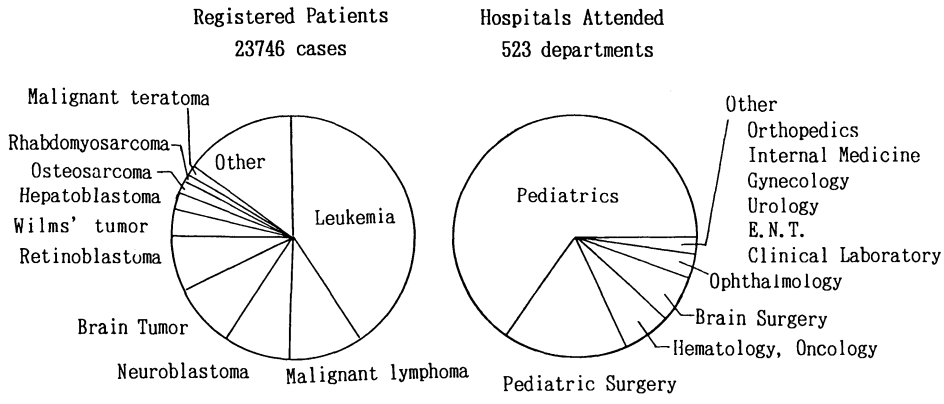
REGISTRATION SYSTEM

All 47 prefectures of Japan are divided into seven zones from north to south. Each zone has its own Regional Registry Office to handle the registration within its zone. The Central Office (Registration Center of the Japan Children's Cancer Registry) has been located in the Department of Child Ecology, National Children's Medical Research Center, Tokyo, since 1985. Registered data on file since the beginning of the registry have been accumulated in the database in the Central Office (Fig. 1). Some 23,746 cases of malignancy have been registered to date, with the cooperation of 523 departments of various institutions all over Japan (Fig. 2). The reporting rate of the registry was estimated to be about 50–60% in relation to the estimated overall incidence of childhood leukemia in Japan (2).

Major items of the survey are (a) personal information and diagnosis; (b) family history, ray exposure, pregnancy course, and delivery; (c) past medical history; and (d) major complications and detailed physical examinations (1). A case is reported to the registry when a child under the age of 15 with malignant neoplasia makes the first visit to the hospital.

GROSS STATISTICS OF CHILDHOOD MALIGNANCIES

Figure 2 shows the distribution of the types of malignancy in the registered cases. Leukemia is the most frequent (43%), followed by malignant lymphoma, neuroblastoma, brain tumor, retinoblastoma, Wilms' tumor, and others.



Estimated Registration Rate = 50 ~ 60% (Hanawa, 1974)

Figure 2. Registered cases in Japan Children's Cancer Registry.

DIFFERENCE IN SPECIFIC FEATURES OF REGISTERED CASES WITH LEUKEMIA AND SOLID TUMOR

Age Distribution and Sex Ratio in Leukemia and Solid Tumor

The age distribution and sex ratio of leukemia and solid tumor in our registry are shown in Fig. 3. Leukemia more frequently affects males, with a peak at 2–3 years. Neuroblastoma, retinoblastoma, and other blastoma-type cancers have a peak within the first 12 months, and there is no prominent sex predominance. Teratoma shows female predominance and the age distribution differs in terms of the tissue type. Chromosome syndromes, particularly Down syndrome, are closely associated with leukemia as compared with solid tumors.

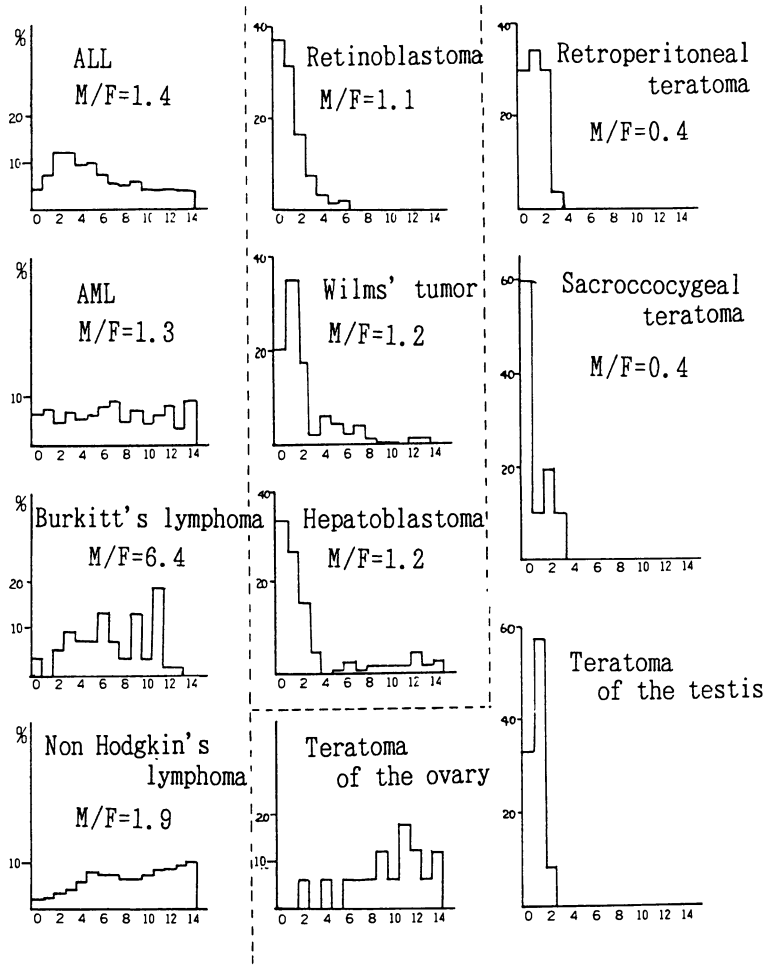


Figure 3. Age distribution and sex ratio of leukemia and solid tumor cases.

Leukemia and Solid Tumor in Twins

Thirty-three sets of twins were reported to the registry between 1985 and 1987 (Table 1). The twinning rate is nearly the same as that in the general population. Concordant pairs account for only 3 out of 6 pairs of monozygotic twins with leukemia and 1 out of 3 pairs of monozygotic twins with neuroblastoma. This high concordance in monozygotic twins with leukemia suggests genetic factors and also a good possibility of twin-to-twin transfusion for leukemia.

Table 1. Twins reported in Japan Children's Cancer Registry during 1985-1987.

Entity	Monozygotic twins		Dizygotic twins		Zygosity uncertain		Total pairs
	Concordant	Discordant	Concordant	Discordant	Concordant	Discordant	
Leukemia	3	3		5		2	13
Lymphoma		2		2			4
Neuroblastoma	1	2				1	4
Retinoblastoma				1	1		2
Ovarian Teratoma		1		1			2
Other (Brain tumor, etc.)		1		0	1	6	8
Total (pairs)	4	9	0	9	2	9	33

Twin rate: 0.85% (33 pairs of twin / 3893 cases) in children's cancer
0.70% in Japan

Parental Exposures in Leukemia and Solid Tumor Cases

The rates of parental exposure to irradiation, chemicals, and drugs taken by the mother before and during her pregnancies and also of maternal smoking and drinking habits during pregnancies (Fig. 4) are statistically significant in some blastomas (3) in which loss of heterozygosity of the genes has been reported (4). On the other hand, there is no significant correlation with these factors in leukemia and malignant lymphoma (3), in which a high incidence of specific chromosomal translocations has been reported (5).

These results of the difference between leukemia and other cancers suggest that the etiological mechanism of leukemia and malignant lymphoma differs from that in a group of blastomas such as retinoblastoma, neuroblastoma, or Wilms' tumor, etc. (Table 2).

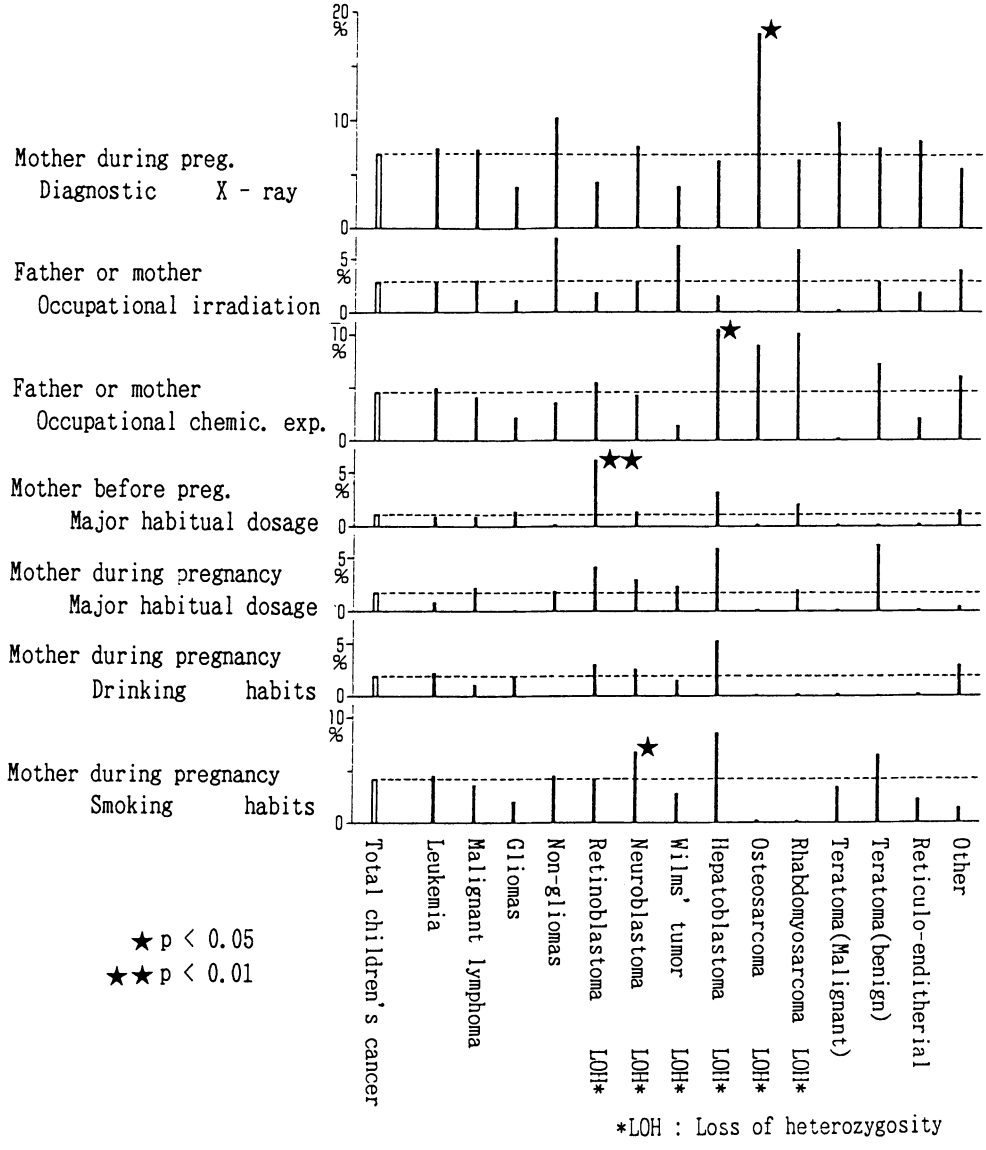


Figure 4. Parental exposure to mutagenic factors before and during pregnancies.

Table 2. Major difference between leukemia and other children's cancer.

	Tumor cells	Age at onset	Sex ratio	Possible mutagenic effects
Leukemia	translocations	depend	M > F	−
Malignant lymphoma	translocations	on types		
Neuroblastoma				
Retinoblastoma	loss of	0 - 1	M ≒ F	+
Wilms' tumor	heterozygosity	year		
Hepatoblastoma				
Teratomas			M < F	±

HETEROGENEITY OBSERVED IN REGISTERED CASES OF LEUKEMIA

In order to investigate the heterogeneity of leukemia, we compared some characteristics among types classified both by FAB and immunological markers. Some 1424 leukemic cases with this information registered between 1985 and 1987 were used.

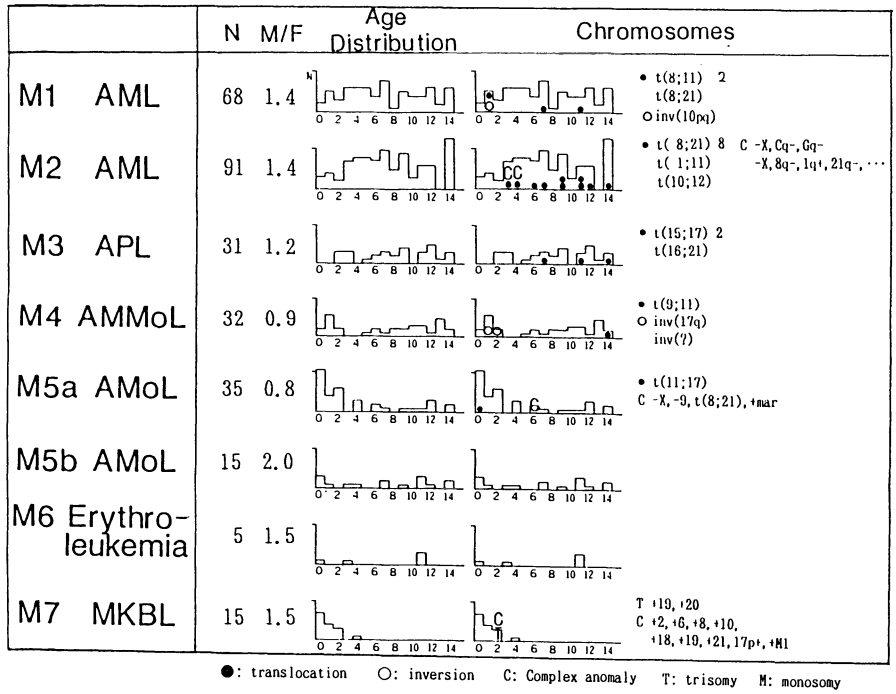


Figure 5. Age distribution, sex ratio, and chromosomal aberrations in FAB types of ANLL.

Specific Features in Type of ANLL

Figure 5 shows the age distribution of ANLL based on the FAB classification. M1 and M2 are most frequent in ANLL. The age at onset is widely distributed from 0 to 14 in any type of M1, M2, and M3, and sex ratios in these types indicate male dominance. M4 and M5a types show a peak at a very young age (0–3 years) and are female dominant, and the M7 type is concentrated in the first 2 years.

The chromosome aberrations, particularly special types of translocation, are shown in each type of M1, M2, M3, M4, M5a, and M7 (Fig. 5). Down syndrome is highly concentrated in M7 and in the preschool age of M1. One case with Down syndrome in M3 and two in M5a have also been registered. One case of M2 type with Rubinstein syndrome has been reported, and several other malformations are registered in cases of M2, M3, M4, and M5a types. Family history is negative, except for one female M5a type with dwarfism whose elder sister is also AMoL with dwarfism. There is no consanguineous marriage in their parents.

These results suggest that all eight types of ANLL are heterogenous. It is of interest that M1 is particularly associated with Down syndrome with 21 trisomy and M2 is very closely associated with translocation between chromosomes 8 and 21 in this registry.

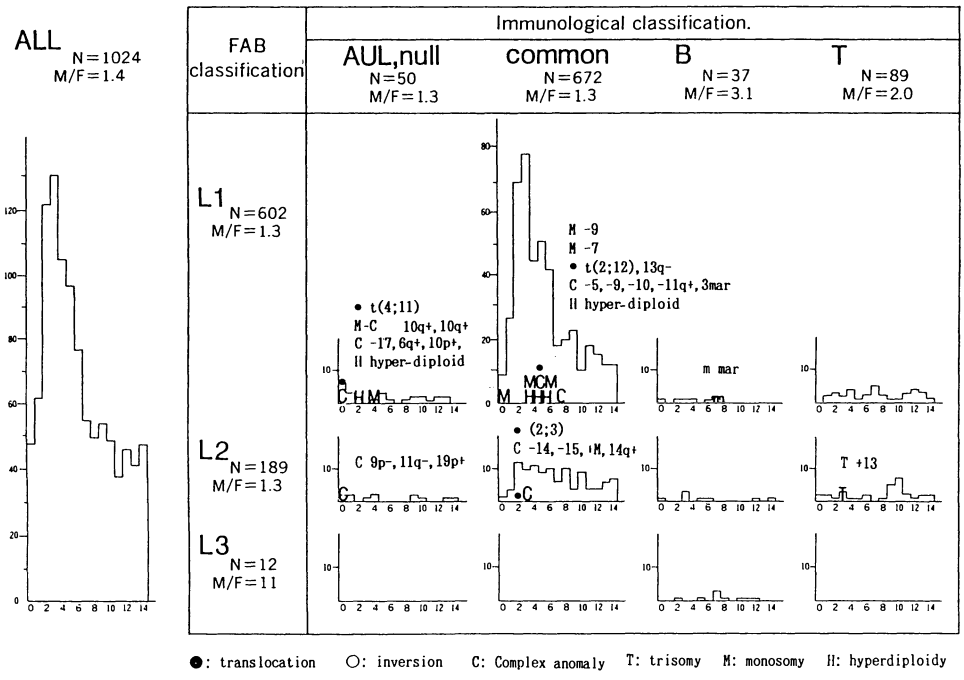


Figure 6. Age distribution of ALL and chromosomal aberrations.

Specific Features of FAB Type and Immunological Characteristics of ALL

Figure 6 presents the age distribution of ALL by immunological and FAB classifications. Null and L1 type show a peak at 0–1 year. In the common type, the age distribution is clearly different between L1 and L2; L1 peaks at age 2 and 3 but L2 has no peak, although there has been some difficulty in distinguishing L1 and L2. Common L1 is the majority type in ALL, and the age distribution of whole ALL reflects that of common L1 type. B–cell and T–cell types are considered to have a relatively late onset and show marked male predominance. Chromosomal aberrations are not frequent in ALL (Fig. 6). Some hyperdiploidy or monosomy types are reported, but the translocation type is rare. These features are distinctly different from those of ANLL. Down syndrome was reported in six cases (three common L1, two common L2, and one in T L1). There are no age clusters. Twenty-eight cases with ALL have major malformations. Six cases had mental retardation or CNS malformations, but there are no specific patterns of malformations.

Table 3. Twin leukemia cases (registers 1985-1987).

	Monozygotic twin	Dizygotic twin	Zygosity uncertain
Concordant twin	ALL, common, L1 (2Y1M) ALL, common, L1 (2Y1M)		
	ALL, common, L2 (5Y1M) ALL, common, L2 (5Y0M)	-	-
	ALL, T , L2 (28D) ALL, T , L2 (2M)		
Discordant twin	ALL, common, L2 ALL, unclassified, L1 ALL, unclassified, L1	ALL, common ALL, common ALL, common, L2 AML, M1 Acut mixed leukemia	ALL, common, L1 ALL, common, L2
Total (pairs)	6	5	2

The family history of ALL is of special interest. In a type of common L1, the registry reveals a concordant twin pair and one leukemia in a cousin of a family tree. In a type of common L2, a concordant twin pair, a family history of ALL common sib, and ALL L1 cousin are reported. In T–cell L1 type, one ALL sib and one ALL cousin in a family tree, and in T–cell L2 type, a concordant twin pair are reported. These family histories in twins, sibs, and cousins suggest genetic backgrounds in the etiology of ALL. Moreover, three sets of twins show concordance of the same immunological and FAB type, supporting the existence of heterogeneity between the common and T–cell types and between common L1 and common L2 (Table 3).

Specific Features of Chronic Leukemia

In juvenile CML, the onset age is within 1 year, and there are no cases with chromosomal aberrations (Fig. 7). In adult CML, the frequency is highest at the age 10–11 years. Translocation between chromosomes 9 and 22 is reported in two thirds of the cases, and they are distributed from 1 to 12 years. One case with CMMoL aged 0 year is registered.

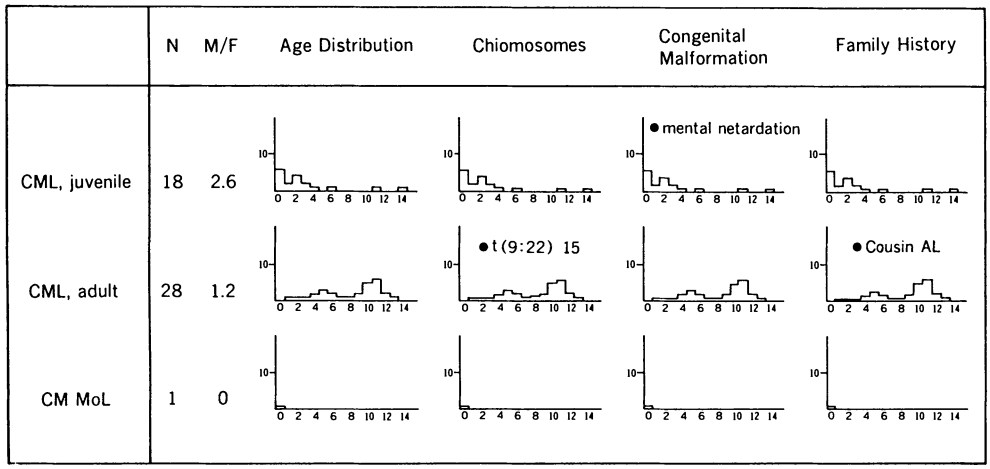


Figure 7. Observed features of chronic leukemia.

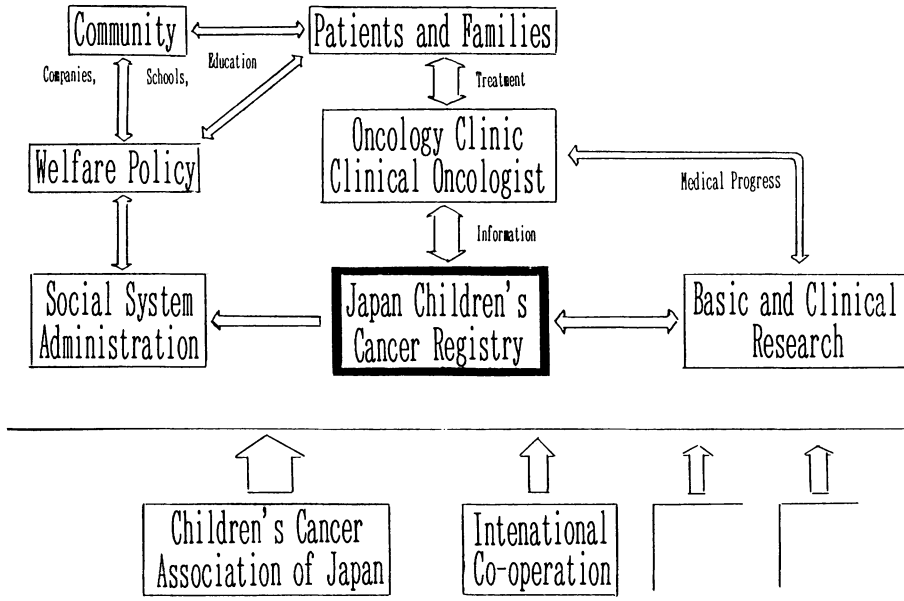


Figure 8. Function of Japan Children's Cancer Registry.

These differences in not only age distribution and sex ratio, but also in the type of chromosomal aberration and complications, including Down syndrome, suggest the etiological heterogeneity of leukemia. It is hoped that these results will be investigated in well-planned experimental, clinical, and epidemiological studies.

CONCLUSION

The Japan Children's Cancer Registry will continue to be an information center, establishing a data bank and reference center for medical and social problems of children's cancer. The flow of information relating to the registry can be summarized as shown in Fig. 8.

ACKNOWLEDGEMENTS

The author appreciates the great support provided by the Children's Cancer Association of Japan and pediatricians and pediatric surgeons with an interest in oncology and hematology, and for their cooperation and generous support for the Japan Children's Cancer Registry. Thanks are also due to Drs. I. Matsui and M. Tanimura, Department of Child Ecology, Medical Research Center, National Children's Hospital, for their assistance with registration and analysis of the database.

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12

LONG-TERM SURVIVORS OF LEUKEMIA AND LATE EFFECTS: A REPORT FROM THE REGISTRY OF LONG-TERM SURVIVORS OF CHILDREN'S CANCER OF JAPAN

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ABSTRACT

About one third of childhood leukemia patients have some abnormalities, but the majority of abnormalities are seen in relapse cases. As far as individual patients are concerned, some patients, even nonrelapsed ones, have apparent abnormalities. However, as a population, significant abnormalities are not apparent. A large proportion of the patients in this study were treated before current treatment procedures came into widespread use, and this treatment was, therefore, relatively mild. Since it is anticipated that treatment will become more vigorous, and, the proportion of long-term survivors will rapidly increase, these results will be subject to change in the future.

INTRODUCTION

The Registry of Long-Term Survivors of Children's Cancer was started in 1980 as a part of the Registry of Children's Cancer, which was established in 1969 under the sponsorship of the Children's Cancer Association of Japan.

The purpose of this registration is to elucidate various physical, psychological, and social problems of long-term survivors of children's cancer under the particular circumstances of Japan and to improve their quality of life. This registry is also expected to act as a starting point for further detailed studies in this direction.

Here described is the part of this registration relating to leukemia. Recently, plans to revise the registry of survivors of childhood cancer and to directly link it to the main registry have been made. The revision is expected to increase the number of cases, but will cause a decline in its comprehensiveness. Therefore, this will be the final report from this registry concerning leukemia.

MATERIALS AND METHODS

Registration

All patients with children's cancer who have survived for 5 or more years, regardless of disease status, are requested to register with the Registry of Long-Term Survivors. The principal medical professionals who participate in these registries are pediatric oncologists, pediatric surgeons, and ophthalmologists, who take part through the Retinoblastoma Registry. Unfortunately, there is little participation on the part of neurosurgeons, orthopedists, and doctors in other areas who treat cancers within their specialties. Therefore, only a few patients with brain tumors, bone tumors, soft tissue sarcomas in extremities, and tumors in the urogenital region other than Wilms' tumor have been registered. In this study, all registered cases with leukemia were included.

Registration Form

The registration form includes a questionnaire concerning information about the type of cancer, method of treatment, course of disease, disease status, abnormality in organ systems (including occurrence of second primary malignancies), and problems in daily life. Information about body weight and height, and status of menstruation are also requested for indices of growth. However, for the above-mentioned purpose and simplification, information about detailed biological data are intentionally excluded from the questionnaire.

Table 1. Items in questionnaire.

Answered by	
Physicians	Patients or families
Head and neck	Developmental delay
Chest	Delayed kindergarten or school admission
Abdomen	Absence from kindergarten or school
Spine	Decreased physical acuity
Extremities	Phychological status
External genitalia	
Skin	
Sphincter function	
Chronic diarrhea	
Other somatic status	
Psychological and psychiatric status	
Second primary malignancies	

The questionnaires are divided into two parts (Table 1). The first part consists of questions that are answered by the physician, and the second part consists of questions that are answered by the patients or their families. The reason for this structuring of the registration form is that patients are intentionally left unaware of the diagnoses of their own diseases in cancer practice in Japan.

RESULTS AND DISCUSSION

On the Registry of Long-Term Survivors, 1564 patients have been registered, of which 343 are leukemia patients. Leukemia is second to retinoblastoma (691 cases) in frequency. The mean duration of treatment is 5.1 years, and the mean interval between diagnosis and registration is 7 years.

Two hundred and forty-seven (72%) of these patients remain in their first remission. Eighty-one patients experienced relapses from 1 to 8 times. Three patients experienced eight relapses.

Table 2. Leukemia in registry of long-term survivors with children's cancer.

Leukemia	343
Acute lymphocytic leukemia	302 (88.0%)
Acute nonlymphocytic leukemia	32 (9.3%)
Others	9 (2.6%)
Nonrelapsed cases	247 (72.0%)
Relapsed cases	81 (23.6%)
Not stated	15 (4.4%)
Mean interval between diagnosis and registration	7.0 ± 2.5 years
Mean duration of therapy	5.1 ± 1.9 years
Retinoblastoma	691
Other	530
All cancers	1564

Among 343 leukemia patients, about 90% have acute lymphocytic leukemia (ALL) and 10% have acute nonlymphocytic leukemia (ANLL) (Table 2). This relatively large number of long-term survivors of acute nonlymphocytic leukemia is rather surprising. Although a recent paper showed that the type of distribution of acute leukemias in Japan is not different from that of Western countries (1), it has long been believed in the past that the ALL to ANLL ratio was smaller in Japan than in Western countries. This difference resulted from wide use of the wrong classification system in the past in Japan. Since many of the long-term survivors were diagnosed during the era when this classification system was used, the possibility is not excluded that the types of leukemias of these long-term survivors might have been wrongly classified.

The age distribution of long-term survivors of leukemia shows that the percentage of patients aged 2–6 at diagnosis is significantly higher than that of total cases of leukemia registered in the Registry of Children's Cancer (Fig. 1). This finding directly reflects the better prognosis for this age group (2).

The male to female ratio of long-term survivors of leukemia is lower than that for total cases of leukemia (Fig. 1). This finding is also thought to reflect the better prognosis for female patients.

In 113 patients, questions on the registration form identified at least one

abnormality. Relapsed patients had a mean number of 1.04 abnormalities. This was significantly higher than the number of abnormalities in nonrelapsed patients (0.37). The percentage of patients who had at least one abnormality was also higher in relapsed patients than in nonrelapsed patients (54.3% vs. 26.7%) (Table 3).

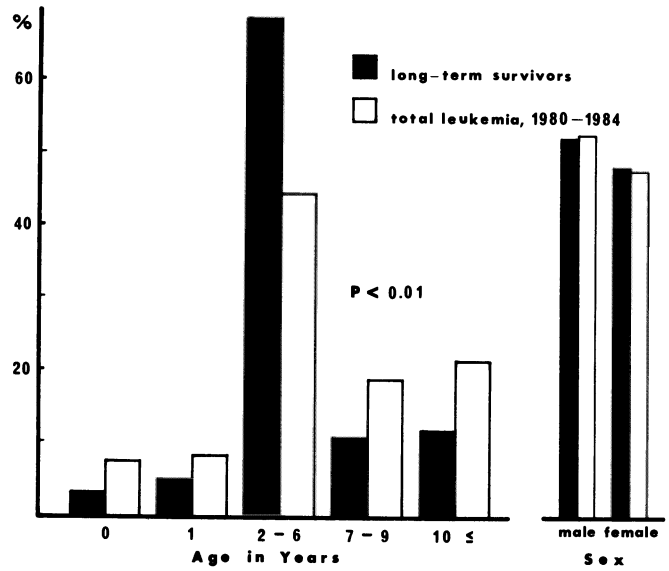

Figure 1. The distributions of sex and age in long-term survivors of leukemia. For comparison, the distributions of sex and age at diagnosis of total cases of leukemia registered to the Registry of Children's Cancer of Japan between 1980 and 1984 are also shown (white bar).

Table 3. Distribution of the numbers of "yes" answers for abnormalities in questionnaire.

		Score									% of Score not 0	
		0	1	2	3	4	5		Total	Mean		
Type	ALL	201	67	19	9	4	2		302	0.52	33.4	
	ANLL	22	5	3	1	1	0	NS	32	0.56	31.3	NS
Age	<7 yr	175	53	18	10	4	2		262	0.55	33.2	
	≥7 yr	52	8	5	0	1	0	NS	76	0.42	31.6	NS
Relapse	Yes	37	21	12	7	2	2		81	1.04	54.3	
	No	181	49	11	3	3	0	<0.01	247	0.37	26.7	<0.01
<y yr, relapsed		30	15	9	7	2	2		65	1.11	53.8	
≥7 yr, no relapse		37	12	1	0	1	0	<0.01	51	0.35	27.5	<0.01

Score = number of "yes" answers; NS = not significant.

When the incidence of abnormalities was compared among different age groups, delay of admission to school or kindergarten was seen more frequently among patients older than 7 years than among younger patients (Fig. 2).

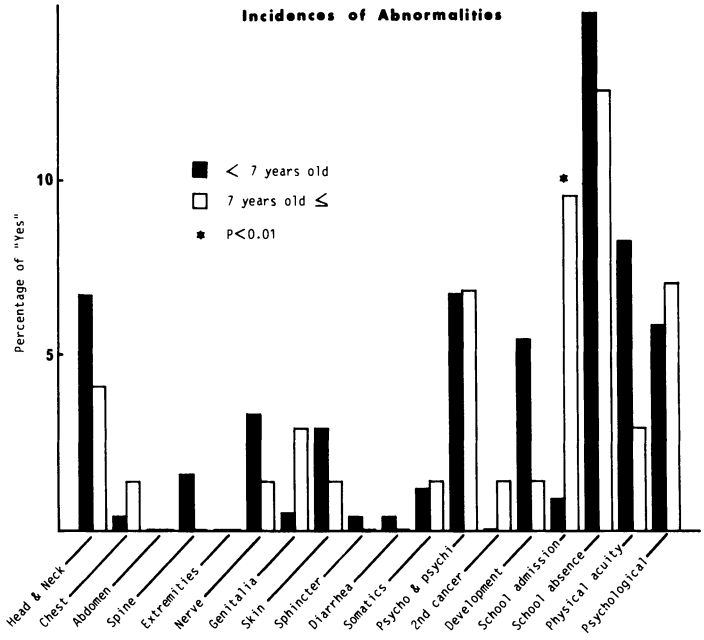

Figure 2. Incidence of abnormalities. Comparison of difference in incidence by age.

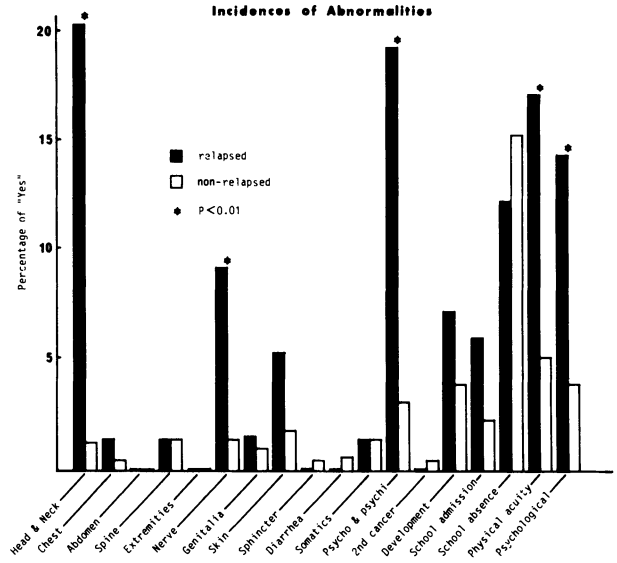

Figure 3. Incidence of abnormalities. Comparison between relapsed patients and nonrelapsed patients.

When the incidence of abnormalities was compared between relapsed patients and nonrelapsed patients, abnormalities in the head and neck region, neurological abnormalities and psychological and psychiatric abnormalities were seen more frequently in relapsed patients (Fig. 3). Decreased physical acuity and more psychological problems were also seen in this group.

Table 4. Major categories of abnormalities observed in childhood leukemia patients.

Head and neck	3	17	20
Hair	1	16	17
Other	2	1	3
Nervous system	3	7	10
Leucoencephalopathy	1	2	3
Epilespy	2	2	4
Other (transverse myelopathy, hemiplegia, etc)	0	3	3
Psychological and psychiatric status	7	14	21
Anxiety	1	5	6
Dependency	0	5	5
Other	6	4	10
Decreased physical acuity	13	9	22
Decreased exercise tolerance	10	6	16
Other	3	3	3
Psychological status	11	8	19
Anxiety	3	3	6
Other (selfishness, restless, impulsiveness, less patience, etc.)	8	5	13

Abnormalities seen in the head and neck region were mainly related to the quantity and quality of hair (Table 4). Other abnormalities included cataract, glaucoma, and blindness. Those of the nervous system included leukoencephalopathy, epilepsy, transverse myelopathy, and hemiplegia. With regard to psychological and psychiatric status, anxiety, dependency, decreased concentration, nervousness and decreased learning ability were major findings. In addition, one patient with a second primary malignancy was registered. This second malignancy was identified as thyroid cancer.

The mean age of menarche was 12.4 years (Fig. 4), which corresponds to the mean age of menarche of Japanese girls in the general population. Several patients had experienced menarche before the age of 10. It would be interesting to know whether these patients had received cranial irradiation, since it has been reported that sexual maturation is accelerated after such treatment for the prevention of central nervous system leukemia (3). This finding has also been confirmed by our own unpublished data.

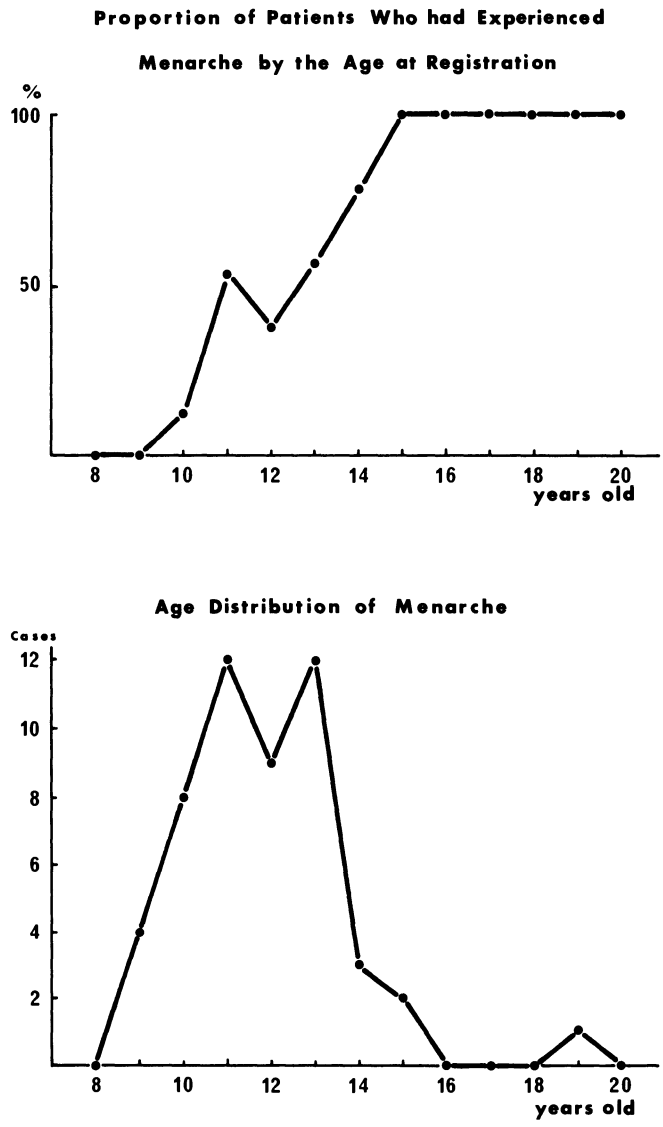

Figure 4. Distribution of age of menarche. Upper half: proportion of patients who had experienced menarche by the age at registration. Lower half: age distribution of menarche.

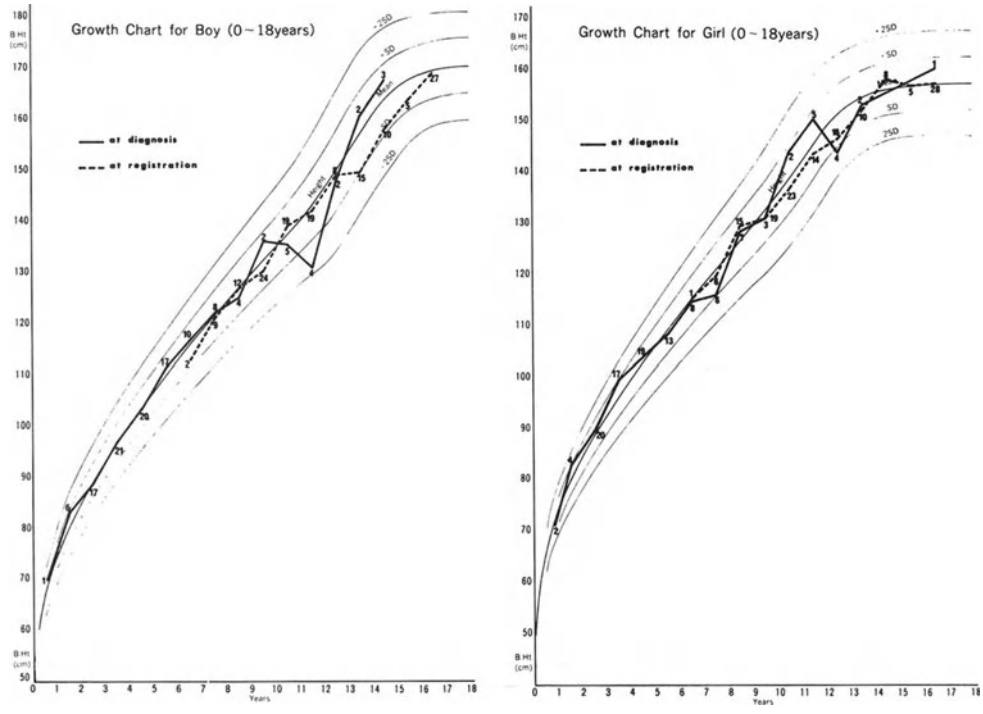

Figure 5. Distribution of mean body height of each age group.
Solid lines and dotted lines indicate the mean height at diagnosis and at registration, respectively. Numbers indicate the numbers of patients.

The mean body height of each age group at the time of diagnosis and also at registration was distributed within one standard deviation of the mean height of the Japanese general population (Fig. 5). There was no difference between the distributions of mean height at diagnosis and at registration.

ACKNOWLEDGEMENTS

I would like to express sincere gratitude to all the participants of the Registry of Long-Term Survivors of Children's Cancer of Japan.

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III. Treatment of Childhood Leukemia

13

THERAPY OF CHILDHOOD LEUKEMIA: UNDERSTANDING SUCCESS AND FAILURE

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ABSTRACT

The treatment of childhood acute lymphoblastic leukemia has revolutionized our concepts of what is possible in the management of all disseminated malignancy. From the cautious use of single–agent chemotherapy in the 1950s, therapy has evolved to include the aggressive use of multiple agents. The results have been a progressive increase in remission frequency and remission duration. However, the most important outcome has been the increased cure rate, which approximates 60% in many large studies. Progress has been slower in recent years, which has fostered the careful study of reasons for failure and the exploration of novel diagnostic, prognostic, and therapeutic techniques. We have been far less successful in acute myeloid leukemia, with cure rates of about 20%. We offer two examples of approaches to overcome the failures. We are studying pharmacodynamic variability because some failures may be due to ineffective dose-time exposure of leukemia cells; if so, more targeted dosage adjustment might improve results. Second, we are studying the application of growth factors in mitigating the negative effect of chemotherapy on normal marrow, as well as in influencing the growth of leukemia cells, particularly myeloid cells. We anticipate continued improvement in treatment outcome by empirical protocol modification, albeit at a slow rate, and potentially major improvements with help from the basic science laboratory.

INTRODUCTION

Gratifying progress has been made in improving the cure rate of acute lymphoblastic leukemia (ALL), however, at least one third of children will not be cured. Also, less success has been achieved in the treatment of acute myeloid leukemia (AML). In this presentation, we offer an analysis of success and failure by reviewing the experience of investigators at St. Jude with a variety of treatment, biological and pharmacological studies. Finally, we present an outline of future studies.

REVIEW OF TOTAL THERAPY STUDIES

From 1962 to 1988, 1707 consecutive children and adolescents with newly diagnosed ALL were treated at SJCRH on Total Therapy Studies I–XI. The only patients excluded were those with the mature B-cell ALL subtype, as determined by surface immunoglobulin evaluation. The major question addressed by this review was: Is there evidence that cure rates in childhood ALL have progressed during the 1980s?

With the exception of the investigations conducted in the 1960s, all patients received combination chemotherapy and effective CNS prophylaxis. For purposes of analysis, we recognize four major treatment eras: *Studies I–IV (n = 90)*, representing initial exploratory trials conducted from 1962 to 1966, in which patients received steroids, vincristine, 6-mercaptopurine, methotrexate and cyclophosphamide. CNS prophylaxis was not given to all subjects. *Studies V–IX (n = 833)*, representing attempts from 1967 to 1979 to increase the treatment intensity and to improve methods of CNS prophylaxis. Anthracyclines, l-asparaginase, and cytarabine (ara-C) were added to previous therapeutic regimens, and radiation doses were boosted to control CNS leukemia. *Study X (n = 428)*, representing two clinical trials conducted from 1979 to 1983, in which intensive risk-directed therapy (VM-26 + ara-C) was tested and CNS irradiation was either omitted or reduced in dose. *Study XI (n = 358)*, representing the evaluation of intensive induction therapy and novel regimens of continuation therapy for patients with worse- or better-risk ALL, classified according to the features identified retrospectively in Study X.

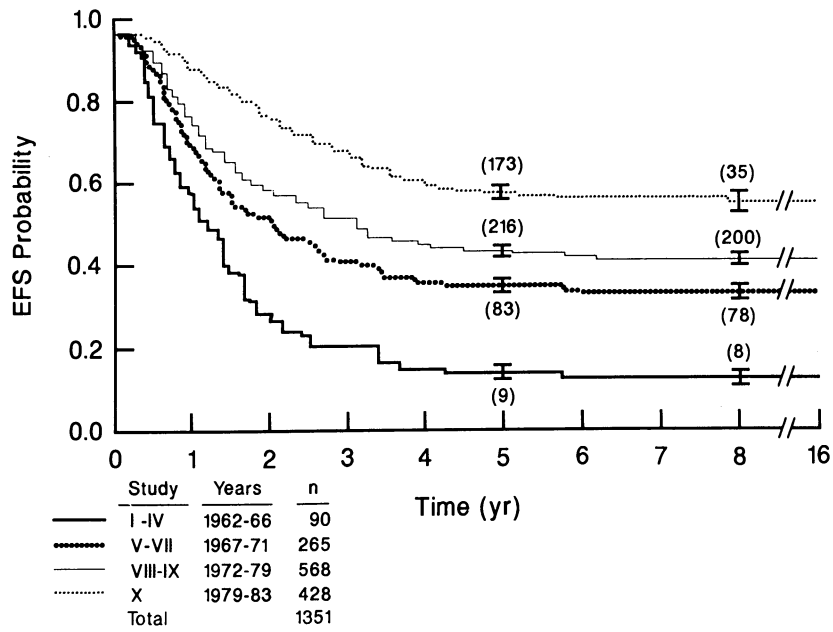

Figure 1. Event-free survival in St. Jude Total Therapy Studies for acute lymphoblastic leukemia.

Figure 1 compares event-free survival (EFS) by treatment era for Studies I–X, demonstrating a progressive improvement in outcome. Although the median follow-up time of 3 years is too short to plot, the 4-year EFS estimate in Study XI exceeds those at identical times in previous total therapy studies and compares favorably with results reported from other large centers. Thus, the likelihood of curing a child of ALL has approximately doubled since the 1970s. We attribute this improvement to the development of effective chemotherapy (VM-26 + ara-C) for high-risk patients (first demonstrated in Study X and strengthened significantly with the addition of other drug pairs in Study XI), more intensive induction therapy (Study XI), and the use of high-dose MTX to extend hematological remissions for all patients.

Of the 361 patients enrolled in Study XI from February 1984 to September 1988, only three patients were not evaluable (two, incorrect diagnosis; one, refusal of therapy); 340/358 (96%) attained complete remission (CR). One hundred eleven patients (31%) were classified as better risk and 248 (69%) as worse risk. The higher proportion of worse-risk patients can be explained partly by the use of cytogenetics in the risk model; that is, with exclusion of chromosomal translocations from the risk definition, the proportions of better- and high-risk patients would be approximately equal. All patients with ALL were eligible for the study, except those classified as having mature B-cell ALL by surface immunoglobulin evaluation. Examples of high-risk subgroups included black patients (12%), infants <1 year of age (3%), Philadelphia chromosome-positive patients (4%), and those with leukocyte counts >100 x 10^9/l (18%). The numbers of patients randomized to each treatment arm were 39 better-risk patients to standard continuation therapy (B1), 69 better-risk patients to rotational combination chemotherapy with four pairs of drugs (B2), 148 worse-risk patients to the same rotational chemotherapy (W2), and 84 worse-risk patients to every 6–week rotational combination chemotherapy using the same drug pairs as in treatment 2 (W3). As expected, the overall event-free survival for better-risk patients has so far surpassed that in worse-risk children; however, as of this early evaluation, comparison of event-free survival estimates for each treatment group has not disclosed any significant differences (1).

Chemotherapy in total therapy trials have not been associated with undue toxicity. The early death rate among all patients is 2.6%; the death rate due to nonleukemic causes is 2.9% for those in remission. There are 1351 patients in Studies I–X who are evaluable for late events, having survived for 5 or more years after diagnosis and 3 or more years after elective cessation of therapy. Twenty-two patients have developed secondary solid tumors after ALL, and 19 of these survive. Fourteen patients have had leukemias other than ALL (secondary AML) at the time of hematological relapse. Importantly, only three children have been lost to follow-up.

PROGNOSTIC FACTORS POTENTIALLY AMENABLE TO INTERVENTION

The study of prognostic factors may help standardize the comparison of studies, but most factors are beyond our control and offer no biological or pharmacological opportunities for intervention, e.g., age, leukocyte count. Two examples are given below of research efforts that may allow us to intervene and to overcome some treatment failures.

The first example deals with the possibility that pharmacodynamic variability may

lead to undertreatment. The epipodophyllotoxin VM-26 has become established as a major component of chemotherapy for childhood leukemia. Investigators demonstrated the magnitude of pharmacokinetic variability when VM-26 is administered in fixed doses and, more importantly, defined a concentration-effect relationship for both the toxicity and the efficacy of this agent (3). Twenty-eight patients with relapsed leukemia or solid tumors received 53 courses of VM-26 as 72-hour continuous infusions in doses of 300–750 mg/m^2. The variability in VM-26 plasma concentrations was more than fivefold in patients given the same dose adjusted for body surface area. It is clear that the therapeutic index is narrow and that pharmacokinetic variability plays a significant role in determining treatment outcomes. These data complement research previously reported for methotrexate (4) and provide an impetus to define the pharmacodynamic relationships for other agents, such as vincristine, etoposide, and ara-C. Using pharmacokinetic strategies, based on Bayesian estimation algorithms, to achieve target concentrations of VM-26 + ara-C and high-dose methotrexate pulses, this group of researchers has provided the framework for individualization of therapy in new studies.

The possibility that one may be able to control leukemia by interdiction of the effect of a growth factor is an exciting prospect. One such factor is CSF-1. As a first step toward determining the possible role of CSF-1R (the c-*fms* encoded receptor for macrophage colony-stimulating factor) in the pathogenesis of human leukemias, investigators analyzed the expression of this protein on leukemic blast cells from patients with leukemia, using flow cytometry and newly developed monoclonal antibodies to the receptor's extracellular ligand-binding domain (5). Twenty-seven (41%) of 67 patients with AML had detectable CSF-1R on their leukemic cells, in contrast to none of 19 children with acute lymphoblastic leukemia. Receptors on AML cells were biochemically indistinguishable from those on peripheral blood monocytes and were normally downmodulated after incubation with either human recombinant CSF-1 or phorbol esters. Monocytes and their progenitors were the only cells in normal blood and bone marrow that expressed CSF-1R, but a substantial proportion of AML cases showing cytomorphologic and immunophenotypic features of granulocytic differentiation were found to bear such receptors.

In related work, these investigators demonstrated that 4 of 12 monoclonal antibodies to different epitopes in the extracellular domain of CSF-1R specifically inhibit CSF-1 binding to receptor-bearing cells (6). One of these antibodies, MAb2-4A5, interfered with the ligand-independent proliferation of NIH-3T3 cells transformed by an oncogenic, mutant c-*fms* gene. The uniqueness of these new reagents was demonstrated by screening an entire myeloid panel of previously characterized MAbs submitted to the IV International Conference on Leukocyte Differentiation Antigens for the ability to detect normal CSF-1R molecules on mouse NIH-3T3 cell lines engineered to express high levels of human CSF-1R on their surface. The lack of reactivity in this test indicated that our recently developed antibodies define a new myeloid cluster group (7). Monoclonal antibodies that recognize the human CSF-1R and inhibit CSF-1 binding may not only prove useful for immunophenotypic diagnosis of AML, but may also have therapeutic potential by altering the growth responses of leukemic myeloblasts. MAb2-4A5, for example, is being tested for its ability to inhibit both CSF-1-induced and growth factor-independent proliferation of leukemic myeloblasts.

SUMMARY AND PLANS FOR THE FUTURE.

The acute myeloid and lymphoid leukemias of childhood present very different research problems. Currently, about 65% of children with acute lymphoblastic leukemia (ALL) can be expected to achieve prolonged event-free survival and probable cure if treated with intensive multiagent chemotherapy. Thus, the major challenge is to improve treatment without introducing undue or permanent adverse effects. Our goals in studies of ALL are to develop and test innovative strategies that will eradicate leukemic progenitor cells while protecting vital host functions, such as hematopoiesis. The major questions to be addressed include: (a) Does chemotherapy targeted to achieve plasma concentrations (systemic exposure) above the median level produced by conventional dosing improve event-free survival? (b) Will molecularly cloned hematopoietic growth factors, such as G-CSF, shorten the duration and severity of drug-induced myelosuppression, permitting intensification of chemotherapy beyond the current limits imposed by the delayed recovery of circulating granulocytes? Identification of cell biological, chromosomal, and molecular genetic features that characterize leukemic cell populations in children is a further aim being pursued, with special emphasis on defining the clonal origin of leukemic cell populations present at relapse.

In contrast to ALL, 5-year event-free survival in acute myeloid leukemia (AML) rarely exceeds 30% among patients treated uniformly in large chemotherapy trials (8-10), justifying extraordinary measures to improve results. Inability to account for the varied responses of identically treated patients (9) has severely hampered the development of risk-specific therapies; thus, all cases must be viewed as potentially high risk. We therefore propose to complete our ongoing clinical trial testing the hypothesis that intensive chemotherapy, with adjustment of doses to achieve an "optimal" systemic exposure, will result in improved event-free survival. Simultaneously, in order to identify clinically useful biochemical indicators of drug sensitivity, we will attempt to correlate intracellular cytosine arabinoside (ara-C) metabolism and the effect of etoposide (VP-16) on topoisomerase II with treatment outcome. This study will be followed by a trial capitalizing on advances in autologous bone marrow transplantation and in the understanding of certain biological response modifiers. If interleukin-2 (IL-2) infused into recipients of marrow grafts stimulates either the generation of lymphocytes directly toxic to leukemic cells or the production of cytokines that inhibit the growth of leukemic myeloid progenitors, then it should be possible to reduce the relapse hazard following marrow transplantation.

The paucity of information on the molecular events leading to malignant transformation and disordered differentiation of myeloid progenitors has clearly been a major obstacle to improved therapy. Recent characterization of numerous hematopoietic growth factors has made it possible to begin studies assessing whether the unique biologic properties of leukemic myeloblasts can be used as the basis for designing effective therapy and whether the growth factor sensitivity of these blasts predicts response to chemotherapy.

ACKNOWLEDGMENTS

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RESULTS AND SIGNIFICANCE OF SIX RANDOMIZED TRIALS IN FOUR CONSECUTIVE ALL-BFM STUDIES

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ABSTRACT

Beyond all biological properties and clinical features of individual patients with acute leukemias, therapy by itself and its quality has emerged as the most important predicting factor for success or failure. The acute leukemias still need adequate management, no matter how the geno- or phenotype of the leukemic clone is characterized. Enforced by the antileukemic efficacy of improved therapy, some risk factors of the past have lost importance, whereas others are still meaningful. Dependent on quality of chemotherapy, the proportion of survivors in acute lymphoblastic leukemia (ALL) varied between 30% and 40% with the standard therapy in the early 1970s, and between 50% and 70% with intensified therapy of the 1980s. All advanced therapy concepts are fraught with danger. They have to be balanced by knowledge, specific competence, commitment and equipment. Thus, incompetence and insufficient infrastructures in individual institutions are adverse prognostic factors. The majority of treatment failures still occur unpredictably and are rather unexpected. Only early relapses are more easily suspected as a consequence of an initial inappropriate therapy response. In addition, the prognostic significance of two chromosomal translocations (4/11, 9/22) is recognized. In ALL, allogenic bone marrow transplantation is only an option in front–line therapy for a small minority of patients, whereas after (early) relapse this method offers the only potential rescue chance. In the acute nonlymphoblastic leukemias, only a selected few patients need this method, according to risk factors that have been characterized over the last years. Only therapy distinguishes readily between success and failure. Risk factor therapy can be assessed only in adequate randomized trials. In four stratified BFM trials performed between 1976 and 1986, answers to six randomized therapy questions have been summarized as follows: In high–risk patients in studies with BFM 76 and 79 (36%, risk index >2), either early or late intensification (protocol II) improved event-free interval (EFI) dramatically if compared with the preceding study, BFM 70 (0.64 vs. 0.38, $p = 0.0001$). Non-risk patients (62%, risk index <2), three vincristine/prednisone reinductions (BFM 79 study) had no major influence on prognosis (EFI 0.76 vs. 0.71, $p = 0.44$).

Methotrexate, as applied in the BFM 81 study, could not adequately replace standard CNS preventive therapy (brain irradiation 18 Gy) if applied to patients (57%) with a risk factor of 0–1.2 (EFI 0.68 vs. 0.78, $p = 0.08$). In patients in the BFM 83 study with a risk factor of <0.8 (27%), no protocol III impaired EFI significantly (EFI 0.62 vs. 0.84, $p = 0.01$). 12 Gy brain irradiation proved to be as effective as 18 Gy irradiation in patients (25%) with a risk factor of 0.8–1.2 (BFM 83 study, EFI 0.74 vs. 0.70). Patients in BFM 81 and 83 studies (85%, n=746), randomized for 24 months total duration of therapy (vs. 18 months) had an EFI of 0.83 (vs. 0.77, $p = 0.04$). For the vast majority of patients in these trials, only tumor burden, age, and sex are important prognostic factors beyond the quality of therapy and initial response.

INTRODUCTION

In addition to all the biological properties and clinical features of individual patients with acute leukemias, the quality of therapy itself has evolved as the most relevant predictive factor for cure or failure. Leukemia is still a life-threatening condition and needs adequate management, irrespective of the phenotypic or genotypic characterization of the leukemic clone.

During the past three decades, our knowledge of optimizing therapy for acute lymphoblastic leukemia (ALL) has increased in a multistep fashion. Most improvements have been introduced empirically; some had a significant impact on outcome; others were hardly recognized. In order to avoid specific risks and side effects, changes in structure, composition, dosage, and timing of therapeutic regimens have been imperative and have been the consequence of therapy results. Some of these changes may have had obvious disadvantages for outcome or have remained prognostically unrecognized because of the countereffects of other modifications. Only randomized trials, if properly performed, supported with an adequate number of patients and critically analyzed and correctly interpreted, give unequivocal information. On the other hand, randomized trials are difficult to assess and may be ethically delicate. Only highly motivated and disciplined cooperative groups may cope with these difficulties. From an ethical point of view, some major questions cannot be asked, which certainly is a regrettable limitation of the method. In general no major therapy changes in randomized trials are possible if a significant difference in results is anticipated, no matter how relevant the question may be. Thus, major (positive) modifications of therapy design have usually been introduced uncontrolled by randomization. But these elements of progress also opened new doors and initiated questions to be answered in subsequent (randomized) trials.

During the decade from 1976 to 1986, the BFM study group asked six questions in four consecutive randomized trials. Written consent of parents and/or patients was demanded, because optional decisions for the other therapy arm also had to be accepted. Thus, not all patients could be included in the randomized trials. It was the intention of the study design to create randomized therapy arms with similar outcomes.

PATIENTS AND TRIAL QUESTIONS

Between October 1976 and September 1986, a total of 1793 patients under the age of 18 years were recruited in four consecutive BFM trials. In trial ALL-BFM 76, there were 158 patients enrolled; in trial ALL-BFM 79, 325 patients; in trial ALL-BFM 81, 633 patients; and in trial ALL-BFM 83, 677 patients. The trials included all infants, patients with B-type ALL, hybrid leukemias, and all patients with lymphomalike syndromes, providing they met the requirement of more than 25% relative bone marrow involvement with leukemic cells. A total of 1864 patients were enrolled for randomization in the four trials: 380 patients in trials ALL-BFM 76 and ALL-BFM 79, and 1464 patients in trials ALL-BFM 81 and ALL-BFM 83. In the latter two trials, the study design required two randomizations, thus the number of patients randomized was greater than the total number recruited.

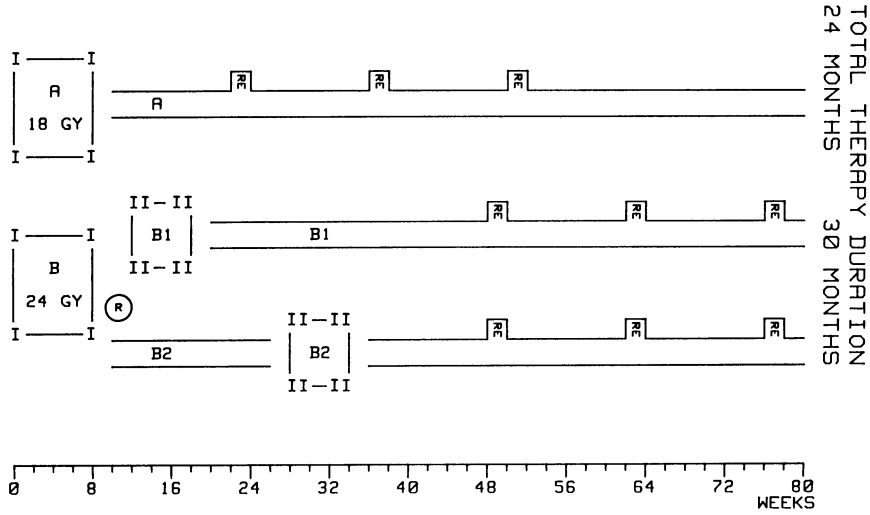

Figure 1. Design for therapy trial ALL-BFM 76. Group A patients: risk index ≤ 2; group B patients: risk index ≥ 3. Risk index (sum of points): WBC > 25,000/mm^3 : 3 points; CNS disease at diagnosis: 2 points; thymic mass: 1 point; focal acid phosphatase positivity: 1 point; negative PAS reaction: 1 point; age at diagnosis 2 or ≥ 10 years: 1 point; significant extranodal tumor: 1 point. RE = reinduction pulses with prednisone/vincristine; I = protocol I; II = protocol II. *Randomization (R) arm B 1 vs. arm B 2.*

The six questions to be answered by randomization in these four trials are as follows:

1. Trials ALL-BFM 76 and ALL-BFM 79 (Figs. 1, 2): Patients with a *risk index* of ≥ 3 (35% of all recruited patients, predominantly defined by a white blood count of ≥ 25.000/mm) were randomized to receive intensive reinduction therapy by *protocol II early* or *late* after the achievement of remission (1,2).
   Question: Is the outcome different with respect to the event-free interval (EFI)?
2. Trial ALL-BFM 79 (Fig. 2): Patients with a *risk index* of ≤ 2 (65% of patients

with standard risk features) *received* or did *not receive* three pulses of *prednisone/vincristine* during the first year of remission (2).
Question: Do both arms differ with respect to EFI?

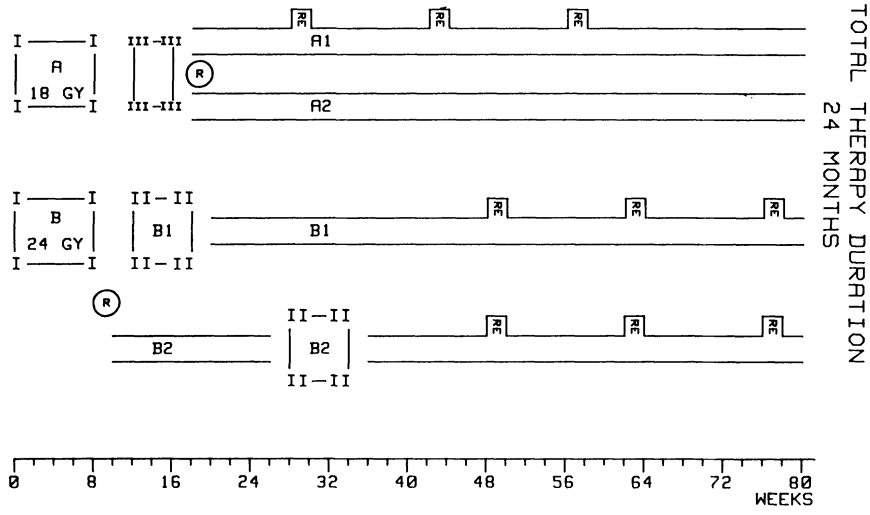

Figure 2. Design for therapy trial ALL-BFM 79. III = protocol III. Other information as in Fig. 1. *Randomizations arm A 1 vs. arm A 2 and arm B 1 vs. arm B 2.*

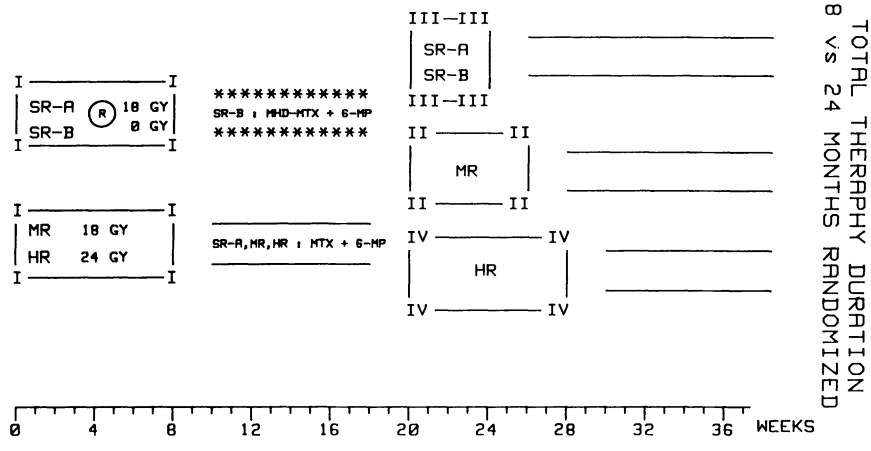

Figure 3. Design for therapy trial ALL-BFM 81. Risk factor (RF) = 0.2 log (blasts + 1) + 0.06 x liver + 0.04 x spleen (organ size in cm below costal margin). Standard risk (SR) = RF < 1.2; medium risk (MR) = RF 1.2 to < 1.7; high risk (HR) = RF ≥ 1.7. MTX = Methotrexate; 6-MP = 6-mercaptopurine; MHD-MTX = intermediate dose MTX. IV, protocol IV. Other information as in Figs. 1 and 2. *Randomization arm SR-A vs. arm SR-B.*

3. Trial ALL-BFM 81 (Fig. 3): Patients with a *risk factor* <1.2 (standard risk, 60% of all recruited patients) received either *cranial irradiation* (during protocol I, 18 Gy) or intermediate–dose *methotrexate* (after protocol I, 4 x 0.5 g/m^2 body surface) (3,4).
Question: Do both arms differ with respect to EFI, especially in events concerning the central nervous systems (CNS)?

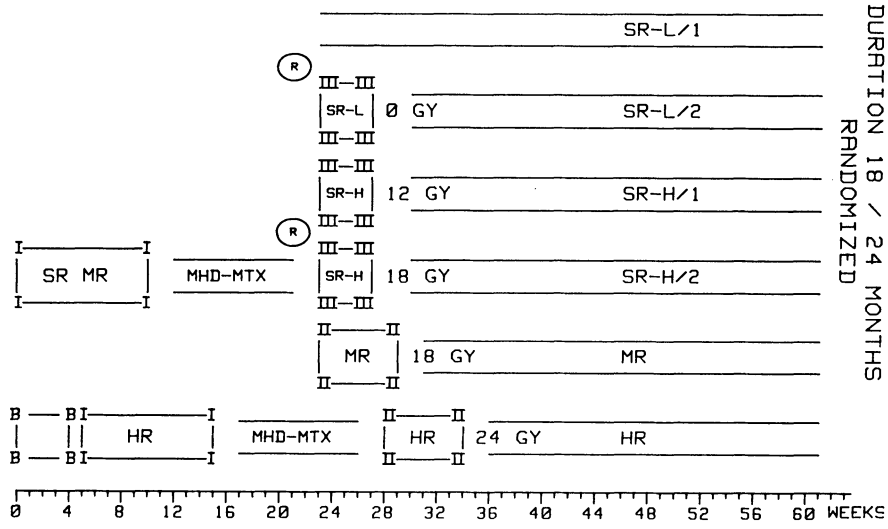

Figure 4. Design for therapy trial ALL-BFM 83. Detail information as in Fig. 3. SR-L = standard risk low; SR-H = standard risk high; B = therapy element as administered in B-type ALL/NHL. *Randomization SR-L/1 vs. SR-L/2 SR-H/1 vs. SR-H/2 and 18 vs. 24 months duration of therapy.*

4. Trial ALL-BFM 83 (Fig. 4): Patients with a *risk factor* <0.8 (standard risk low, 25% of all recruited patients) *received* or did *not receive* intensive reinduction therapy with *protocol III* after remission was obtained (3,5).
Question: Do both arms differ with respect to EFI?
5. Trial ALL-BFM 83 (Fig. 4): Patients with a *risk factor* of 0.8 to ≤ 1.2 (standard risk high, 30% of all recruited patients) received either *12Gy* or *18 Gy cranial radiation* following protocol III (2,3,5).
Question: Is the low dose ("minimal effective dose") equivalent to the standard dose with respect to EFI, especially in events concerning the CNS?
6. Trials ALL-BFM 81 and ALL-BFM 83 (Figs. 3 and 4): Patients in complete continuous remission at 18 months were or were not exposed for another 6 months of *continuation therapy* with oral methotrexate and 6-mercaptopurine (total duration of therapy *18* vs. *24 months*) (6).
Question: Is EFI different in both arms?

Thus all questions are related to the intensity or quality of therapy, four to the intensity of chemo- or radiotherapy (questions 2,4,5,6), one to the quality of chemotherapy (question 1), and one to the interaction of both (question 3). For

analysis, standard methods, including the Kaplan-Meier plot and the log-rank test, have been applied.

ANSWERS TO THE TRIAL QUESTIONS

In trial ALL-BFM 76, the uncontrolled introduction of a new therapy element – protocol II – for patients with an increased risk for therapy failure generated a dramatic improvement in EFI (1, 6, 7), if compared with the results of the historical Berlin pilot study (8) performed between 1970 and 1976 (Fig. 5, p = 0.001). In the Berlin pilot study, all patients were only exposed to protocol I and did not receive protocol II. An improvement in EFI has been interpreted to be caused exclusively by the introduction of this new therapy element (7). In order to enroll a substantial number of patients to answer trial question number one, the randomization of early or late exposure to protocol II was not only used in trial ALL-BFM 76, but was continued in the subsequent trial ALL-BFM 79 (1,2). Since both trials differed only in one minor modification (duration of maintenance therapy, Figs. 1 and 2), the answer is rather unambiguous (Fig. 6). Kaplan-Meier plots for both arms are superimposable for up to 3 years of follow-up and subsequently differ only slightly. Thus, exposure to protocol II late in remission could have been nonsignificantly preventative (p = 0.24) for late events. This information was utilized in trial ALL-BFM 81 with late, rather than early, application of protocol II, since, additionally, late application of protocol II resulted in less toxicity (6).

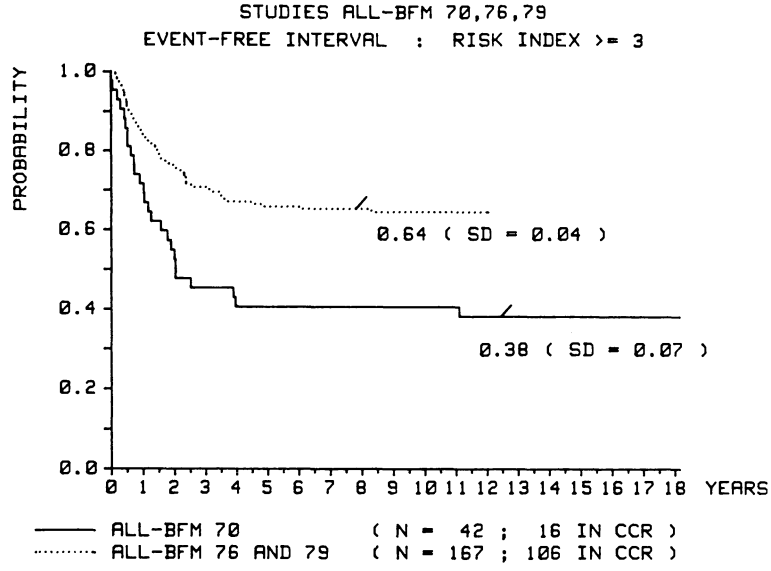

Figure 5. EFI as related to exposure to protocol II; p = 0.001.

Traditionally, reinduction pulses with prednisone/vincristine are used during maintenance therapy. The purpose of the second trial question was to quantify these pulses in the context of the BFM study design, which for the first time used systematically intensive front-line therapy. The morbidity of these pulses has been

significant at that time. Due to repeated exposures to corticosteroids, the pulses were often followed by pneumocystis pneumonia, thus creating a target for a randomized trial question (Fig. 2). Enforced by the efficacy of front-line induction therapy, patients with standard–risk features (risk index ≤2) probably did not benefit from these three pulses, as demonstrated in Fig. 7 (p = 0.44). This result prompted us to delete this treatment element in subsequent trials (2).

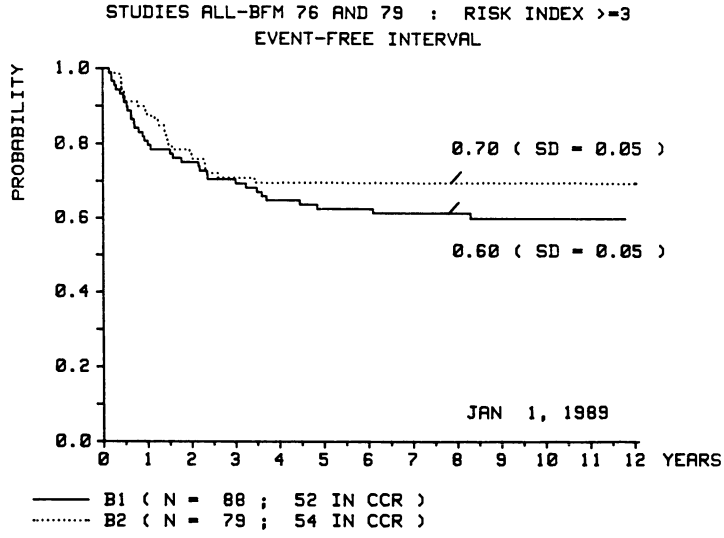

Figure 6. EFI as related to early (B 1) or late (B 2) application of protocol II; p = 0.24 (randomization).

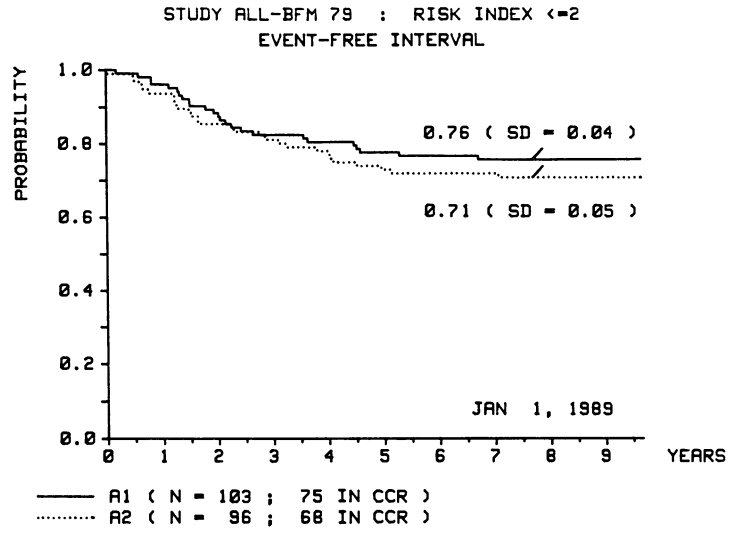

Figure 7. EFI as related to prednisone/vincristine pulses (A1) or no pulses (A2); p = 0.44 (randomization).

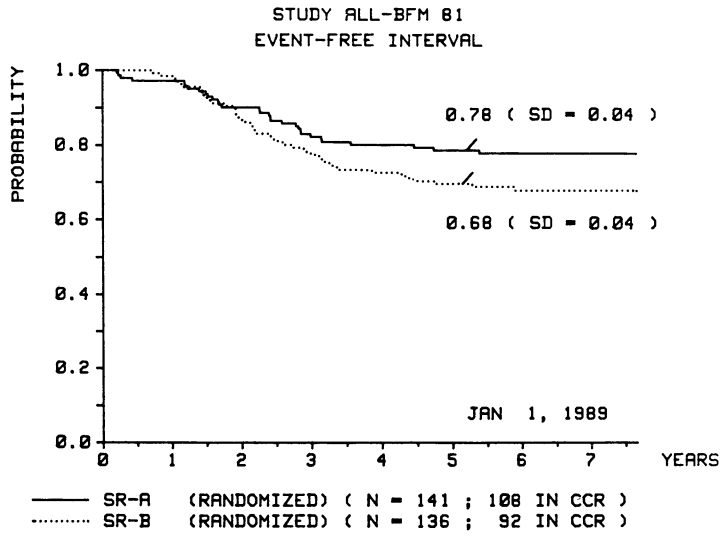

Figure 8. EFI as related to cranial irradiation (SR-A) or intermediate dose methotrexate (SR-B) for all events; p = 0.08 (randomization).

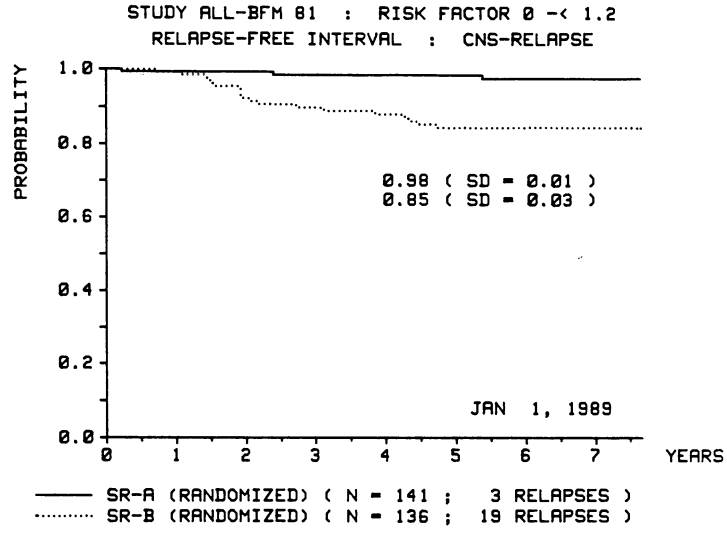

Figure 9. EFI as related to cranial irradiation (SR-A) or intermediate dose methotrexate (SR-B) only for all CNS event; p = 0.0003 (randomization).

The question of the best mode of CNS prevention is still unsettled (6,9–11). Most investigators consider it to be opportune to substitute cranial irradiation with a pharmacologically suitable drug if an almost equal efficacy can be predicted (9,12). In spite of cranial irradiation, the risk for CNS disease increases with the increase of

tumor burden at diagnosis. Thus in trial ALL-BFM 81 (4), only patients with a lower tumor load (RF <1.2) were qualified for this randomization (Fig. 3). In this randomized trial, intermediate–dose methotrexate was not an adequate substitute for cranial irradiation. This result is not highly significant (Fig. 8, p = 0.08) if all patients of this subset are considered and all events evaluated. CNS events, however, were significantly more frequent in arm SR-B (standard–risk B, no cranial irradiation) if compared with arm SR-A (standard-risk A, cranial irradiation), as demonstrated in Fig. 9 (3 vs. 19 events, p = 0.0003). Furthermore, only patients with a risk factor of 0.8 to <1.2 contributed to this negative result, whereas patients with a risk factor of <0.8 were equally protected by cranial irradiation and methotrexate with respect to CNS events(1 vs. 3 events, p = 0.30). This information urged physicians not to omit cranial irradiation in the subsequent trial of ALL-BFM 83 for the endangered subset of patients but to avoid it for patients with a risk factor of <0.8. Alternatively, the argument is well accepted that intermediate dose methotrexate is inappropriate as a substitute for cranial irradiation, but higher doses would have been protective.

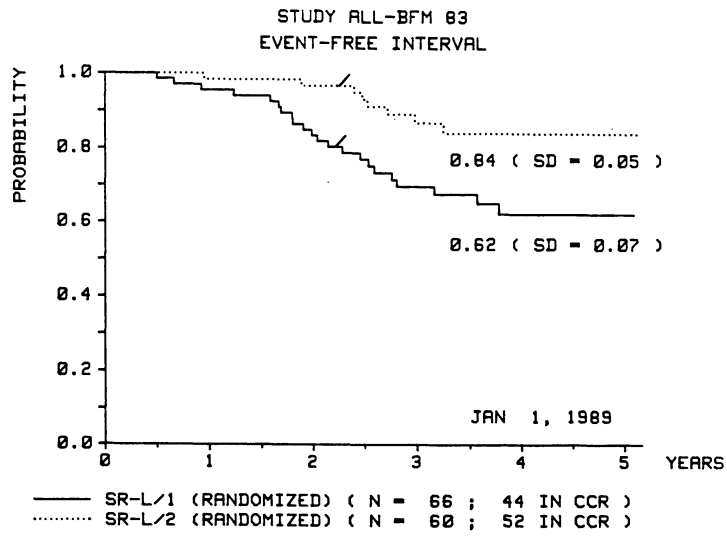

Figure 10. EFI as related to no protocol III (SR-L/1) or exposure to protocol III (SR-L/2); p = .007 (randomization).

Patients with a low tumor load at diagnosis (in the setting of the BFM trials, approximately 25–30%) have been considered to be critically overtreated and should not be exposed to intensive reinduction therapy (5). In these patients the risk/benefit ratio is very delicate, since overtreatment in a group of patients with a 75% chance of cure must be balanced against treatment-induced toxicity, morbidity, and even mortality. Thus, the question in trial ALL-BFM 83 for patients with a risk factaor <0.8 was focused on the benefit of protocol III (Fig. 4). It was anticipated that this element of therapy was unnecessary Unfortunately, the group of patients who did not receive protocol III as an intensive reinduction were critically disadvantaged compared with the complementary group (Fig. 10, p = 0.007). This negative result was only recognized

after the trial had been closed in September 1986, since both therapy arms obviously did not differ during the first 2 years of follow-up. The hypothesis that more therapy in this patients' group will not necessarily generate a better result was strikingly disproved. At the same time, most of the events in this subset occurred after the discontinuation of maintenance therapy, which may still offer a major chance of second-line rescue to these patients. If one evaluates the BFM experience of all trials in which protocol III has been used (risk index ≤2, risk factor 0 to <1.2, patients of the Berlin pilot study included), the superiority of intensive reinduction compared to no reinduction is evident (Fig. 11, p = 0.01), irrespective of other therapeutic modifications in these trials over the years.

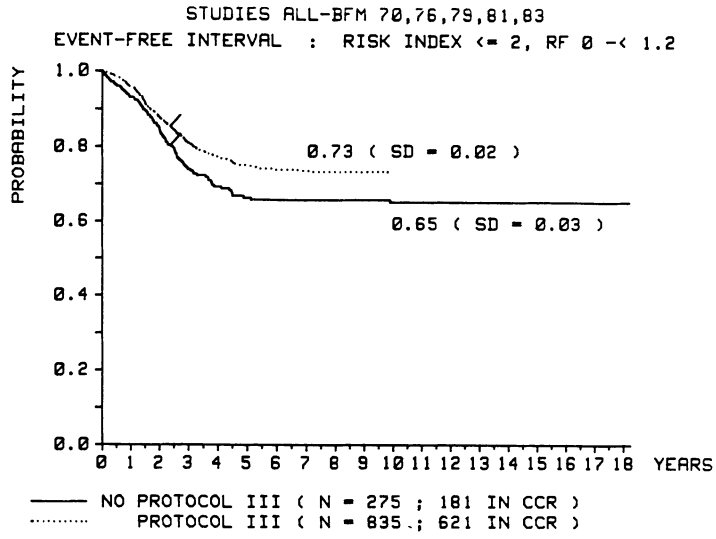

Figure 11. EFI as related to no protocol III or exposure to protocol III in four BFM trials and the Berlin pilot trial; p = 0.01.

In question five of trial ALL-BFM 83 (5), the dose of cranial irradiation was randomized as outlined in Fig. 4. Additionally, in this trial intermediate-dose methotrexate (4 times 0.5 g/m^2 body surface between protocol I and III) was introduced uncontrolled by randomization as a consequence of new information at that time (9,10). Thus, the trial result must be interpreted cautiously, if one has this introduction in mind, which by itself may have a higher potential of extracompartment disease prevention. In the setting of this trial, low–dose cranial irradiation with 12 Gy was as effective as the standard dose (18 Gy, Fig. 12, p = 0.7). In the future a "minimal effective dose" of 12 Gy may offer the possibility of successfully protecting even patients with a higher risk for CNS disease. This dose should certainly be less effective in inducing brain damage or second CNS tumors.

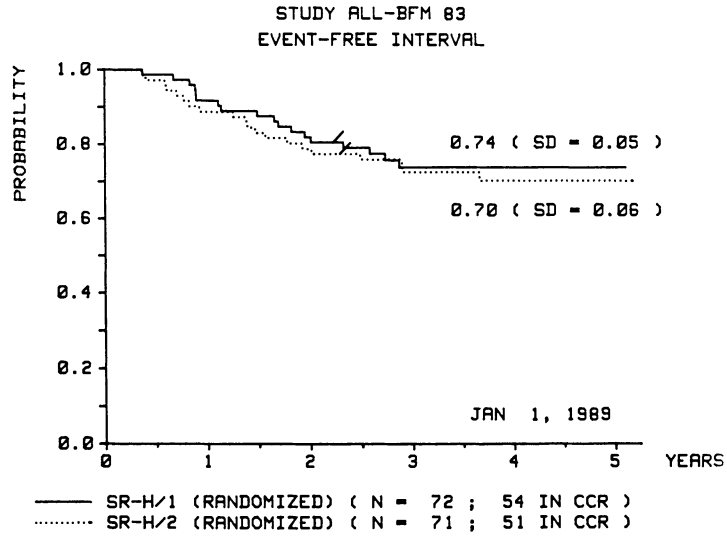

Figure 12. EFI as related to 12 Gy cranial irradiation (SR-H/1) or 18 Gy cranial irradiation (SR-H/2); p = 0.7 (randomization).

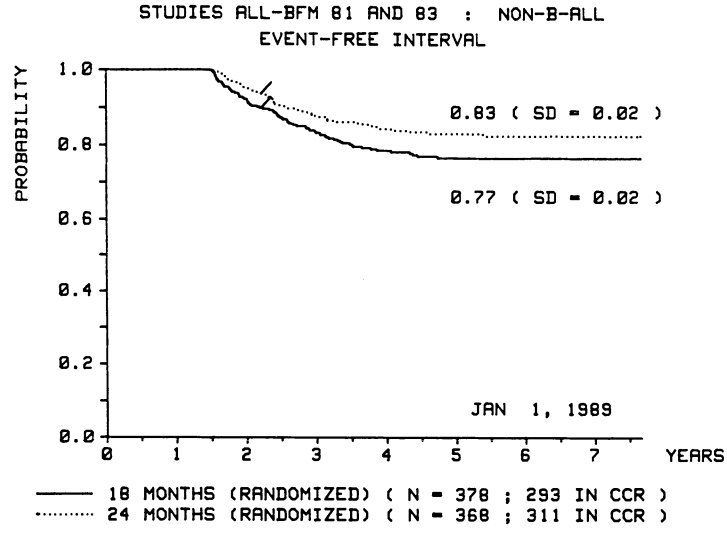

Figure 13. EFI as related to total duration of therapy of 18 months or 24 months; p = 0.04 (randomization).

In addition to the three randomizations in trials ALL-BFM 81 and ALL-BFM 83 on front-line therapy, another question for patients recruited in both trials has been asked, provided the patients were in continuous remission after an 18–month total

duration of therapy. The antileukemic efficacy of maintenance therapy with respect to the rate of long-term survivors is virtually unknown, since after the impact of intensive front-line therapy no randomized trial has been carried out throughout the duration of treatment. Therapy duration in many trials fluctuated between 2 and 5 years (1,2,9,13); never before has therapy been limited to 18 months (Figs. 3 and 4). Seven hundred and forty-six patients in both trials have been randomized, as outlined in Fig. 13. After a median duration of these trials of 6 years, with all patients no longer on maintenance therapy for at least 6 months, the difference in both branches is not impressive, but nevertheless is recognizable ($p = 0.04$). For all further subsets of patients that may be characterized (both trials analyzed separately, different strategy groups, sex, immunophenotypes), this difference disappears more or less, with p values between 0.05 and 0.5. The incidence of events in the 24 month arm is only three quarters of that of the 18 month arm, thus continuation of maintenance therapy for 6 months more in the 18 month arm would have avoided approximately 25 relapses. Again, these patients may have a substantial chance to be treated successfully with any effective second-line therapy. This result logically raises the question, of whether a therapy duration beyond 24 months may be even more effective.

CONCLUSIONS

The answers to the six questions raised in the four ALL-BFM trials are unequivocally in favor of the simple statement that, in general, more intensive treatment or longer therapy exposure was superior with respect to EFI (6,7). The hope that less therapy, if more appropriately applied, is equally or even more effective is in vain, at least in our experience. None of the therapy protocols presently in use can claim to be optimal with respect to efficacy and safety, certainly with regard to those being used in the BFM trials. If one analyzed all randomized therapy arms with favorable results separately, one might conclude ALL to be a curative disorder at a level of almost 80%, however, the actual rate in the BFM trials is only 70%. In spite of the difficulties of randomized trials, their results give – if critically evaluated and interpreted – invaluable information that is superior to that obtained by other methods.

As anticipated in 3 of 6 trial questions, the difference in therapy intensity or quality was not expressed as a difference in EFI. Thus, therapy arms with less hazards are more favorable (late protocol II, no reinduction pulses, low-dose cranial irradiation). The unexpected differences in three trial results are of ethical concern, yet include important information. None of the trial answers were available at the time the trial was closed for further entries. Cranial irradiation proved to be more effective than intermediate–dose methotrexate. Nevertheless, this potent drug – if administered at a different dosage and/or at different times – is to be preferred, considering the late effect of ionizing radiation on the child's brain. Most significant, however, has been the information that in patients with a minimal tumor burden, the omission of intensive reinduction therapy provoked an inferior outcome. This was not to be anticipated at the time, but is compatible with the hypotheses that (a) tumor load is not the only predictor for prognosis and (b) minimal residual disease may be overcome by more therapeutic intensity. The latter argument fits also with trial question six: longer therapy exposure may produce a negative blast cell turnover, which more frequently is followed by the nonreappearance of the neoplastic clone, no matter which mechanism may

additionally have been active. To repeat the phrasing of the introduction: Leukemia in childhood is a life-threatening condition and needs adequate management.

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PHARMACOKINETICS OF COMMONLY USED ANTI-LEUKEMIC AGENTS IN CHILDREN

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ABSTRACT

To treat children with acute lymphoblastic leukemia, the pediatric oncologist must have a basic understanding and appreciation of the clinical pharmacology of vincristine, methotrexate, 6-mercaptopurine, cytarabine (cytosine arabinoside), and corticosteroids. The treating physician should be cognizant of the inter- and intrapatient variabilities in the pharmacokinetics and clinical effects of each of the drugs. The pharmacokinetics of other cancer chemotherapy agents used in the treatment of childhood leukemia should also be known (doxorubicin, asparaginase, teniposide, and cyclophosphamide), but these agents are less commonly used or administered for a short period of the total treatment. Recent laboratory studies and clinical observations have suggested ways in which the agents could be administered more optimally. For the antimetabolites, these include limited bioavailability and the absence of a diurnal variation in oral methotrexate and 6-mercaptopurine, new antifolates with better pharmacological characteristics for oral administration, intravenous therapy with 6-mercaptopurine, and subcutaneous therapy with methotrexate. For corticosteroid therapy, dexamethasone appears to have an advantage over prednisone and prednisolone in treating leukemic cells in the central nervous system. Intrathecal chemotherapy for the prevention of meningeal leukemia is more effective if the dosage is based on the volume of the central nervous system instead of the body surface area. Exposure of systemic tissue to cytotoxic drug concentrations after intrathecal chemotherapy is substantial with methotrexate and negligible with cytarabine.

INTRODUCTION

To design optimally effective leukemia treatment, the pediatric oncologist must have a thorough understanding and appreciation of the clinical pharmacology of those antineoplastic agents selected for use. In childhood acute lymphoblastic leukemia, these agents always include vincristine, methotrexate, 6-mercaptopurine, and a corticosteroid (usually prednisone, prednisolone, or dexamethasone). The treating physician should have a basic knowledge of the absorption, distribution, metabolism, excretion and mechanism of action of each of these drugs (1). Cognizance of the interpatient variability of the agents, particularly with oral administration, is especially important. Anthracyclines, asparaginase, alkylating agents, and epipodophyllotoxins are variably used and are not included in this review.

ORAL 6-MERCAPTOPURINE (6MP) AND METHOTREXATE (MTX)

The route of drug administration is an important factor that may influence the pharmacokinetics of a drug, as well as its toxicity and efficacy. One of the best examples of the influence of the route of administration in pediatric oncology is found in the use of orally administered maintenance chemotherapy in the treatment of ALL. Although orally administered 6-mercaptopurine (6MP) and methotrexate (MTX) have been standard maintenance modalities for approximately 25 years, until recently little was known regarding their clinical pharmacology. In this presentation, some recent findings regarding the pharmacokinetics of these agents is reviewed. These observations highlight the potential problems associated with orally administered chemotherapy.

Several investigators have confirmed that oral administration of standard doses of MTX does not result in predictable or uniform serum MTX concentrations. One study demonstrated a fourfold difference in the peak MTX concentrations achieved and a fivefold range in the time to the achievement of peak concentrations following oral MTX administration, as well as a wide range in the fraction of orally administered drug absorbed (from 23% to 95%) (2). The significant variability in absorption of orally administered MTX has also been demonstrated by the measurement of MTX polyglutamate accumulation in the erythrocytes of patients with ALL undergoing maintenance chemotherapy (3).

Recent studies have also demonstrated that the bioavailability of oral 6MP is limited and highly variable. For example, it has been estimated that following uniform oral dosing of 75 mg/m^2 of 6MP, the mean bioavailability is only 15% (Fig. 1), only onethird of patients achieved therapeutic plasma levels (1 μM or greater), and, even in these individuals, 1 μM levels were maintained only briefly (4). Also, MTX concurrently administered with 6MP increases the area under the plasma 6MP by about onethird (5). These observations raise significant questions regarding whether maintenance therapy with 6MP and MTX is being optimally delivered.

Several additional factors purportedly influence the bioavailability of oral MTX and 6MP including diurnal variation (6) and concomitant administration of food (7) and allopurinol (8). There does not however, appear, to be a significant variation in the pharmacokinetics of the antimetabolites as a function of the time of the day at which

the drug is administered (Balis and Poplack, unpublished data).

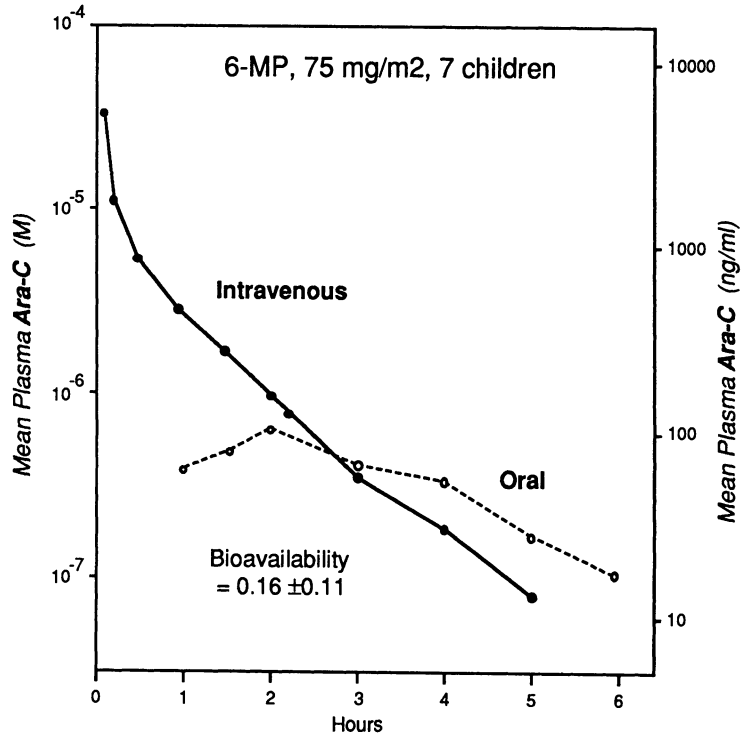

Figure 1. Plasma concentrations after intravenous (bolus) and oral administration of cytarabine (Ara-C). Modified from Zimm *et al.* (4).

CORTICOSTEROIDS (PREDNISONE, PREDNISOLONE, DEXAMETHASONE)

The absorption of orally administered prednisone, prednisolone, and dexamethasone is nearly complete. Prednisolone is the predominant form in plasma after an oral dose of prednisone. Prednisone is rapidly converted to prednisolone, but requires normal liver function. A number of oral formulations of prednisolone are now commercially available. These have the advantage of usefulness in patients with hepatic dysfunction. In children, the elimination half-lives are approximately 2.5 hours for prednisolone (9) and 4 hours for dexamethasone (10), reflecting differences in the rate of catabolism resulting from the different modifications to cortisol metabolites. Studies in a nonhuman primate model have revealed that dexamethasone achieves higher cerebrospinal fluid (CSF) to plasma concentration ratios than prednisolone, which appears to be due to differences in the CSF drug elimination rates and in plasma protein binding (11). This finding may explain the lower rate of meningeal leukemia with dexamethasone than with prednisone observed in the one trial that compared these two agents for the treatment of childhood ALL (12).

VINCA ALKALOIDS

For vincristine, there are no significant differences in the disposition of the drug according to age, with clearance values of 84 ml/min/m^2 reported for both children and adults. Dosage modifications of the vinca alkaloids are generally recommended in infants and in patients with delayed bilirubin excretion, as evidenced by an elevated direct bilirubin. However, because definitive pharmacokinetic data are lacking, these guidelines are empirically derived. Infants do appear to manifest increased toxicity with standard doses of vincristine based on body surface area. Infants and younger children do have a relatively larger ratio of body surface area to weight. Therefore, many pediatric protocols recommend that the vincristine dosage be based on weight (0.03–0.05 mg/kg) rather than on body surface area.

The standard dose for vincristine in children is 1.0–1.5mg/m^2 administered every 1–3 weeks. Because early studies suggested that absolute doses greater than 2 mg led to increased toxicity, primarily neurotoxicity, the general practice has been to limit the total single dose to 2 mg. Neurotoxicity correlates with the area under the plasma concentration curve, and substantial interindividual variation occurs in the plasma pharmacokinetics of vincristine. Thus, application of the upper limit has been questioned (13,14). Escalation beyond the 2 mg maximum, particularly at 3-week intervals, may be well tolerated in the majority of patients, including children.

INTRATHECAL CHEMOTHERAPY: METHOTREXATE AND CYTARABINE

Every child with ALL receives intrathecal (IT) chemotherapy with MTX with or without cytarabine (cytosine arabinoside, ara-C) to prevent or treat CNS leukemia. The developmental pharmacology and pharmacokinetics of these agents in the CSF and plasma after IT administration have been characterized (15). MTX is actively transported out of the CNS by energy-dependent processes (16). Inhibitors of anion transport by the choroid plexus, CSF bulk flow, and CSF production increase the MTX concentration in the CNS (16). The volume of distribution appears to be the extracellular compartment of the CNS and to not be related to the patient's body surface area (17). The volume of distribution increases steadily during the first 3 years of life, and it remains stable, except possibly for an increase during adolescence (17). The CSF clearance and elimination rate are relatively independent of age, except possibly during the first year of life. Exceptions to the aforementioned changes are pathological conditions such as communicating hydrocephalus and active CNS leukemia (18). Active CNS leukemia impairs the efflux of MTX from the CSF in children and adolescents, whether the drug is administered IT, intravenously, or orally. In children and adolescents with ALL, CSF and plasma MTX monitoring can help diagnose subclinical CNS leukemia and possibly predict CNS relapse (19).

Infants have higher MTX concentrations in the CNS than older patients, whether the drug is administered IT or systemically. When dosed on the basis of body surface area, IT MTX results in low drug levels in the CSF in young children, intermediate levels in adolescents, and high levels in adults; acute toxicity (arachnoiditis) is directly proportional to the CSF drug levels and to patient age when body-surface-area dosing is used (20). IT MTX dosing based on the extracellular-fluid volume of the CNS results in more uniform CSF MTX concentrations than dosing

based on the body surface area (20). When applied to children with ALL, the CNS volume-of-distribution dosing method is more effective than the body-surface-area method in preventing CNS relapse in infants and children (21). If IT MTX dosing during induction and consolidation therapy for ALL is administered according to the volume-of-distribution method, infants and children with an intermediate prognosis do not benefit from IT MTX during maintenance therapy, whereas adolescents do (22). Simulating CNS MTX concentrations after IT MTX with intravenous infusions of very high doses of MTX (33 gm/m^2/24 hr), in conjunction with high-dose leucovorin rescue, can replace both CNS irradiation and IT MTX as a preventative treatment for CNS relapse (23).

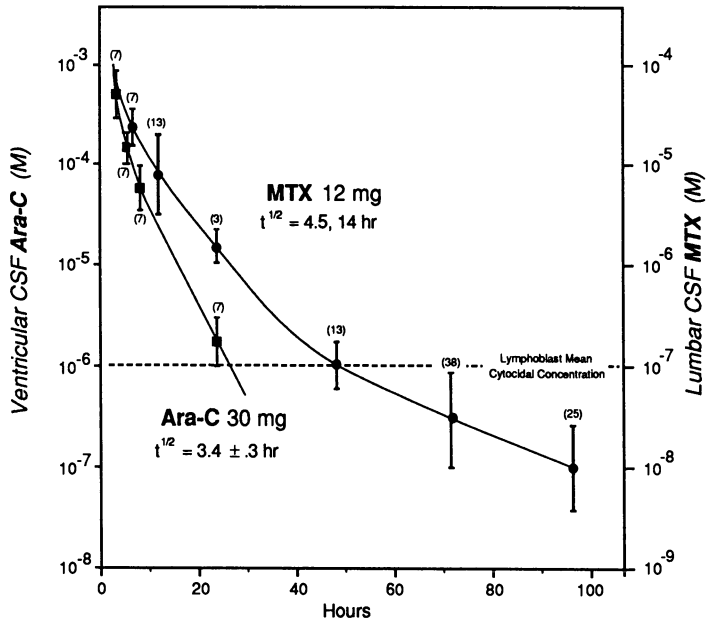

Figure 2. Cerebrospinal-fluid (CSF) concentrations after bolus administration of methotrexate (MTX) and cytarabine (Ara-C). Vertical bars designate ± 1 SD and values in parentheses indicate the number of patients studied. The methotrexate data is taken from Bleyer *et al.* (25) and the cytarabine data is derived from Zimm *et al.* (24).

In the CSF, cytarabine declines rapidly in a biphasic pattern with half-lives of <0.5 and ~3.4 hours, such that therapeutic levels generally persist for less than 24 hours (Fig. 2) (24). In contrast to systemic administration of cytarabine, in which cytarabine is rapidly eliminated from the systemic circulation by biotransformation to the inactive metabolite uracil arabinoside, there is little conversion of cytarabine to uracil arabinoside in the CSF following IT administration. In CSF, the ratio of uracil arabinoside to cytarabine is very low (24), a finding that is consistent with the very low levels of cytidine deaminase present in the brain and CSF (25). Plasma levels after IT cytarabine in humans do not exceed 1 μM, the minimum cytotoxic concentration of cytarabine *in vitro*, reflecting rapid clearance from the systemic circulation. Thus, little if any systemic toxicity occurs after IT cytarabine administration, an advantage over IT

MTX therapy in that the latter leads to a systemic exposure that is greater than the effect of the same dose administered systemically (intravenously, intramuscularly, subcutaneously, or orally) (26).

IT-administered MTX, on the other hand, disappears from the CSF in a triphasic pattern, with half-lives of approximately <1, 4.5 and 14 hours (Fig. 2) (27). Therapeutic MTX levels are maintained in the CSF for 2–7 days after standard IT doses of the antifolate, depending on the concentration required for an antitumor effect. Thus, IT cytarabine requires repeated administration to achieve the same effect as a single dose of IT MTX, and it is generally used as a second–line agent or in combination with MTX and hydrocortisone ("triple IT chemotherapy"). Two trials have compared the combination of IT MTX, cytarabine, and hydrocortisone with a combination in which the hydrocortisone was deleted (28,29). Neither showed any additional therapeutic benefit from the cytarabine–containing regimen. In addition, cytarabine appears to be a radiosensitizer of normal neural tissue (30), which does not appear to be the case for MTX (31).

ACKNOWLEDGMENTS

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TREATMENT OF ACUTE NONLYMPHOBLASTIC LEUKEMIA IN THE CHILDREN'S CANCER AND LEUKEMIA STUDY GROUP

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ABSTRACT

A total of 114 evaluable children with acute nonlymphoblastic leukemia (FAB categories: M1, M2, M4, M5) entered into this study for 8 years. Of the 114 patients, 82 (71.9%) achieved complete remission (CR). There was no difference in the CR rate among five different protocols proposed by CCLSG. The rates of continuous complete remission (CCR) at 20 months were 43.0 ± 10.7% (mean ± SE), 23.4 ± 11.0%, and 65.8 ± 10.6% in Regimen I, Regimen II, and ANLL-861, respectively. The curves of CCR showed a plateau after 20 months, so that few cases of long-term remitters might relapse after 2 years. The primary CNS involvement did not affect the CCR duration. Sixteen patients have completed the chemotherapy, and 15 of them have remained in remission for 33–104 months after diagnosis. The incidence of CNS involvement was 9.6% (11/114). CNS involvement frequently occurred in patients with M4 and M5. The CCR duration in patients with monocytic leukemia (M5) was significantly shorter ($p < 0.01$) than in patients with M1 or M2. Further strategy for the patients, including bone marrow transplantation, should be changed according to the ANLL subtype.

PATIENTS AND METHODS

During the period of this study (February 1981 to June 1989), previously untreated patients with ANLL were admitted to this study if their ANLL met the criteria (M1, M2, M4, M5) of the French-American-British (Fab) classification and if they were aged under 15 years. The 114 evaluable patients consisted of 59 (51.8%) males and 55 (48.2%) females. Their mean age was 8 years 3 months.

Protocol Regimens

Between February 1981 and October 1984, patients were randomized to protocol ANLL-827: regimen I (neo-MINI-COAAP therapy) or regimen II (BH-AC·DMP therapy). In regimen I, remission was induced with adriamycin (ADR), cyclophosphamide (CPM), cytosine arabinoside (ARA-C), vincristine (VCR), and prednisolone (PDN). Maintenance treatment consisted of ADR, CPM, ARA-C, VCR, PDN, and 6-mercaptopurine (6MP) every 4 weeks for 3 years, as shown in Fig. 1.

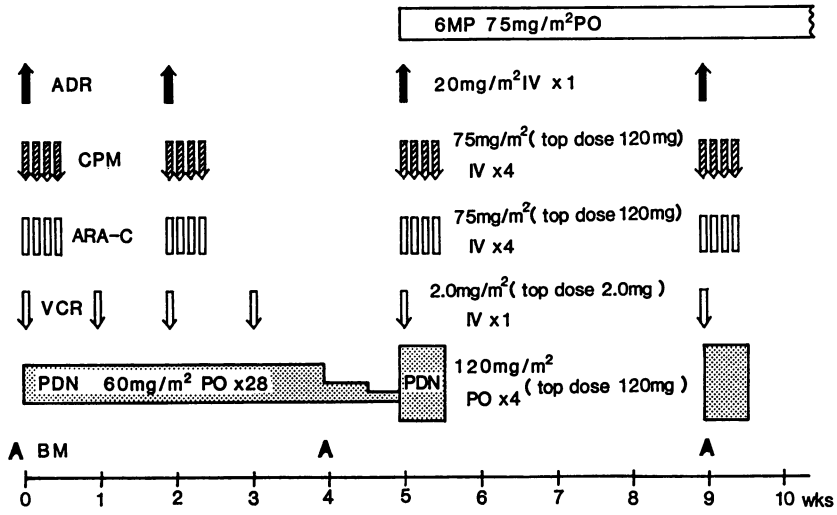

Figure 1. ANLL-827 protocol: Regimen I (neo MINI-COAAP therapy) for ANLL in children.

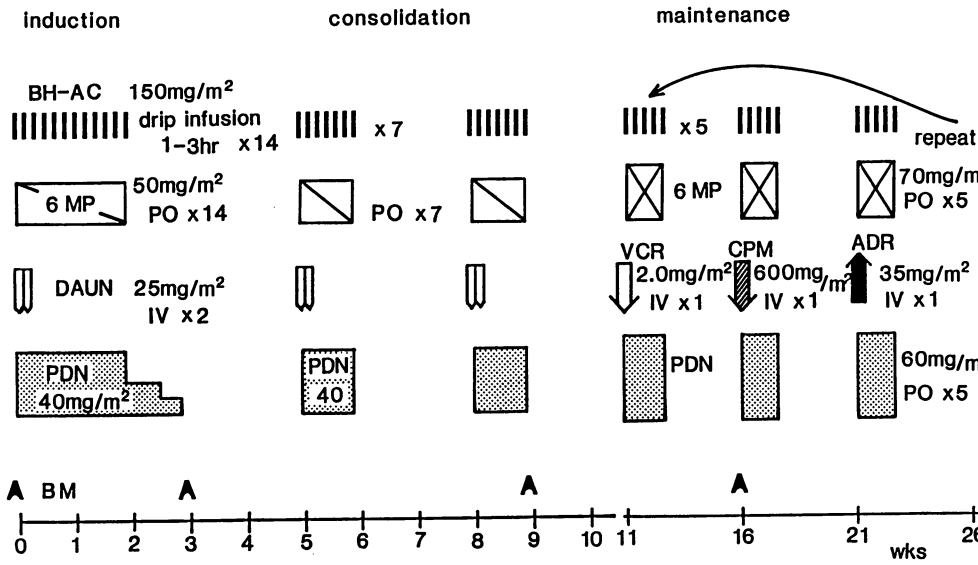

Figure 2. ANLL-827 protocol: Regimen II (BH-AC.DMP therapy) for ANLL in children.

Induction and consolidation therapy in regimen II consisted of BH-AC, 6MP, daunorubicin (DAUN), and PDN. BH-AC (N^4-behenoyl-1-β-D-arabinofuranosylcytosine), which was synthesized by a Japanese drug company and has been found to possess a strong antitumor activity (1). Although the exact antitumor mechanism of BH-AC has not been fully elucidated, its activation has been presumed to occur by metabolization to ARA-C. In maintenance therapy, VCR and/or CPM and/or ADR were added instead of DAUN, as shown in Fig. 2.

Between November 1984 and December 1987, patients were treated with protocol ANLL-861, combined therapy of regimen I and II, for 3 years (Fig. 3). In this protocol, remission was induced with regimen I and consolidation was done with regimen II. Aclarubicin was added instead of CPM in the regimen–II phase of maintenance (Fig. 3).

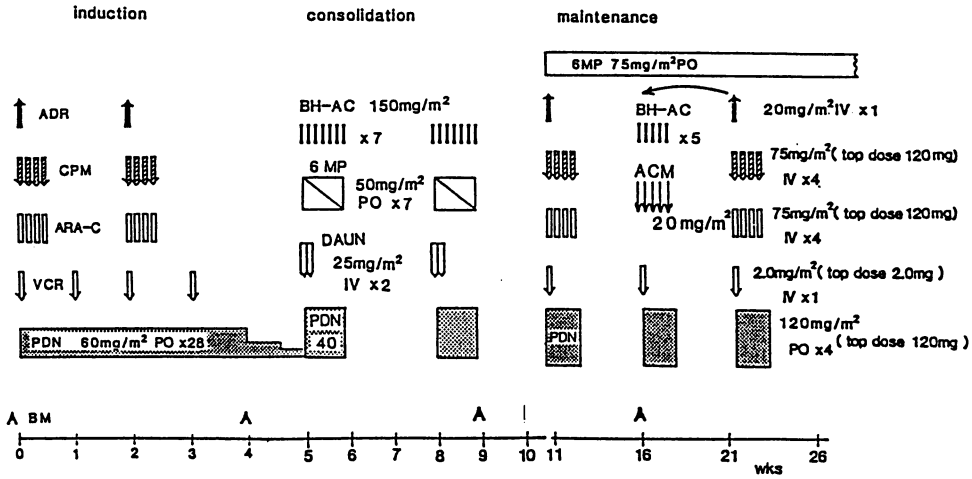

Figure 3. ANLL-861 or ANLL-pilot protocol for M1 or M2.

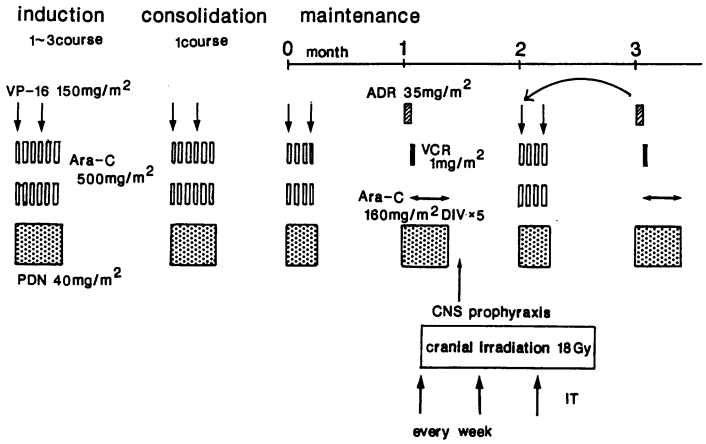

Figure 4. ANLL-Mo-pilot protocol for M4 or M5.

After January 1988, patients with acute myelocytic leukemia (FAB categories: M1, M2) were treated with protocol ANLL-pilot (same as ANLL-861) and patients with acute monocytic leukemia (Fab categories: M4, M5) were treated with a new protocol ANLL-Mo-pilot (Fig. 4). According to our previous data (2) and reports by others (2,3), the monocytic subtype was associated with a significantly shorter duration of remission and a higher incidence of central nervous system (CNS) disease. We began our study using etoposide, VP-16-213 (VP-16) and included CNS prophylaxis in patients with M4 and/or M5 (Fig. 4).

Statistical Evaluation

The duration of survival and remission was measured from the time of initial therapy. The probability of staying in remission or being alive was calculated according to the Kaplan-Meier method. Remission and survival curves were compared by the use of the log-rank test. To test the influence of factors on survival, the variables such as sex, age, Fab subtype, WBC, hemoglobin value and organomegaly were used as covariants in a proportional hazard model. Statistical calculations were done with the SAS sStatistical analysis system) program.

RESULTS

Induction of Remission

Detailed results of remission induction therapy for the 114 evaluable patients are shown in Tables 1 and 2. Of the 114 children, 82 (71.9%) achieved complete remission (CR). Among the 82 complete responders, there has been one withdrawal because of refusal of further therapies. There was no difference in CR rate among the five protocols.

Table 1. Outcome of patients with ANLL in CCLSG (1981-1989).

Protocol	CR/case	rate (%)	BM relapse	CNS leukemia	death
Regimen I	23/29	(79.3)	13	1	20
Regimen II	16/20	(80.0)	13	3	17
ANLL-861	25/39	(64.1)	8	5	21
ANLL-pilot	13/19	(68.4)	3	2	4
ANLL-Mo-pilot	5/7	(71.4)	2	0	4
Total	**82/114**	**(71.9)**	**39**	**11 (9.6%)**	**66 (57.9%)**

Of the 114 patients evaluated, 31(27.2%) were classified as M1, 53 (46.5%) M2, 17 (14.9%) M4, and 13 (11.4%) M5. The CR rates of M1, M2, M4, and M5 were 61.3% (19/31), 85.0% (45/53), 47.1% (8/17), and 76.9% (10/13), respectively, as presented in Table 2. The CR rates did not differ significantly among the protocols and morphological subtypes of ANLL (see Table 2).

Table 2. Outcome of CR rates according to protocols and FAB subtypes.

FAB	Regimen -I	Regimen -II	Protocol ANLL -861	ANLL -pilot	ANLL Mo-pilot	Total	CR rate (%)
M1	5/8	2/2	6/12	6/9		19/31	(61.3)
M2	15/16	9/11	14/16	7/10		45/53	(85.0)
M4	1/3	1/2	4/9		2/3	8/17	(47.1)
M5	2/2	4/5	1/2		3/4	10/13	(76.9)
total	23/29	16/20	25/39	13/19	5/7	82/114	(71.9)
CR rate	(79.3%)	(80.0%)	(64.1%)	(68.4%)	(71.4%)	(71.9%)	

Remission Duration and Survival

The probability of event–free survival according to protocols at 3 years was 24.1 ± 8.0%, 15.0 ± 8.0%, and 33.7 ± 8.0% in regimen I, regimen II, and ANLL-861, respectively (Fig. 5). The rates of continuous complete remission (CCR) at 20 months were 43.0 ± 10.7% (mean ± SE), 23.4 ± 11.0%, and 65.8 ± 10.6% in regimen I, regimen II, and ANLL-861, respectively. However, there was no statistical difference in the CCR rate among the protocol regimens. The curves of CCR show a plateau after 20 months, so that few cases of long-term remitters may relapse after 2 years. The median durations of CCR according to protocols were 11.5 months, 13.7 months, 41.1 months, and 6.2 months in regimen I, regimen II, ANLL-861, and ANLL-Mo-pilot, respectively. The probability of CCR at 13 months in the ANLL-pilot protocol was 57.3 ± 17.0%.

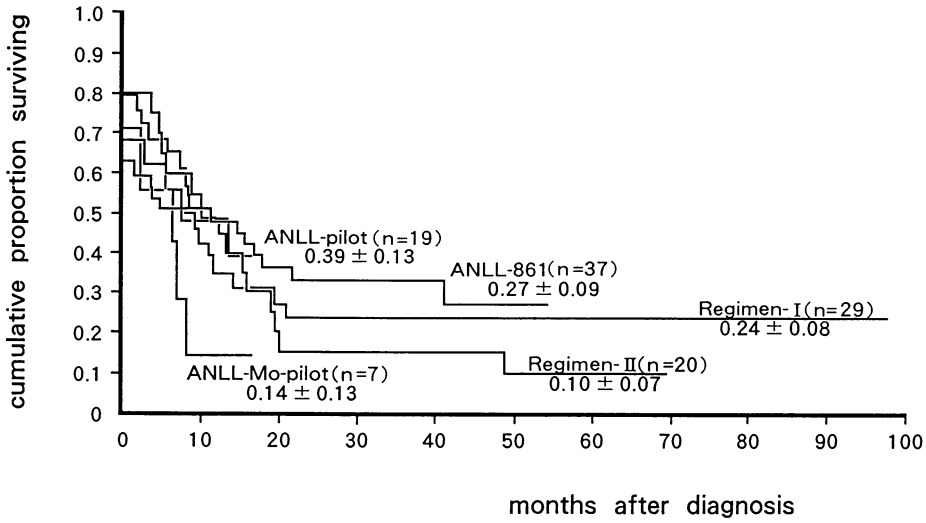

Figure 5. Probability of event-free survival among 112 patients with ANLL (M1, M2, M4, M5) according to five protocol regimens.

As is evident, the number of monocytic leukemia patients is too small to allow a definitive comparison of the CR rate and the overall duration of remission. The probabilities of event-free survival according to Fab subtypes at 5 years were 32.1 ± 7.6% and 15.2 ± 7.7% in M2 and in M1, respectively. The probability of event-free survival of M4 at 3 years was 17.7 ± 9.3% (Fig. 6). The CR duration in patients with monocytic leukemia (M4, M5) was significantly shorter ($p < 0.01$) than that in patients with M1 or M2. The CR rate of ANLL-Mo-pilot using VP-16 was higher than those of other protocols in monocytic leukemia, while there was no difference in the CCR rate. Therefore, we are planning to modify the ANLL-Mo-pilot protocol.

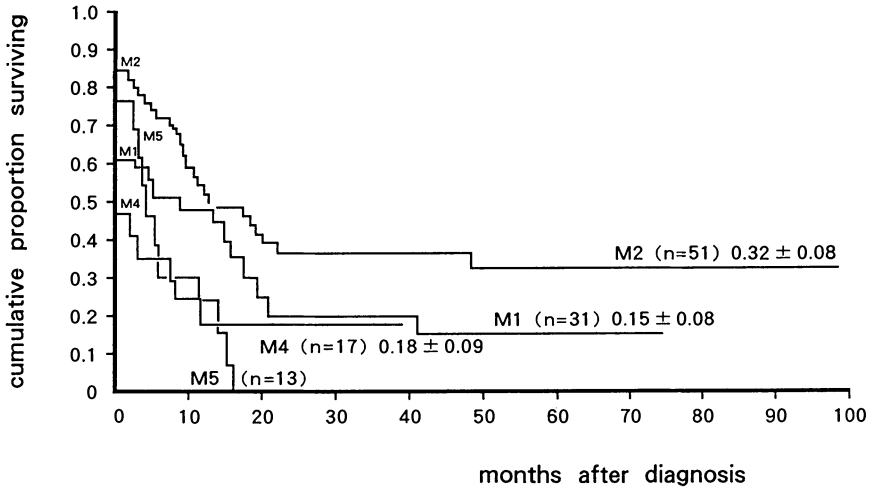

Figure 6. Probability of event-free survival according to Fab subtype.

The median values for overall survival according to the Fab subtype were 19.3, 33.6, 13.0, and 24.7 months in M1, M2, M4, and M5, respectively. The probabilities of overall survival at 3 years among the subtypes were 30.5 ± 10.4%, 47.1 ± 8.0%, 11.8 ± 10.1%, and 42.3 ± 14.3% in M1, M2, M4, and M5, respectively.

In regimen I, regimen II, ANLL-861, and ANLL-Mo-pilot, the median overall survival figures were 24.8, 31.6, 23.0, and 8.9 months.

The probability of an event-free survival of M2 according to the protocol at 3 years was 31.3 ± 11.6%, 27.3 ± 13.4%, and 47.6 ± 11.7% in regimen I, regimen II, and ANLL-861+pilot, respectively (Fig. 7). The durations of 50% CCR in M2 were 28.9, 33.6, and 38.5 months in regimen I, regimen II, and ANLL-861 + pilot, respectively. There was no difference in the 50% CCR and event-free survival of M2 among the protocol regimens.

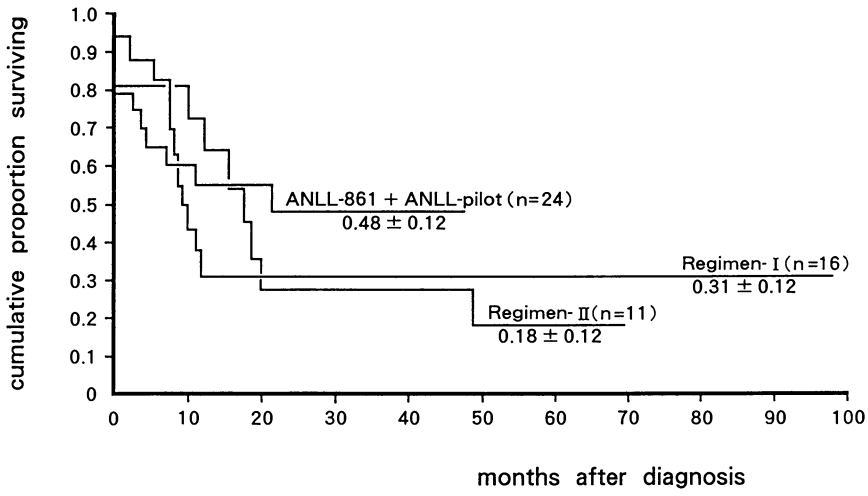

Figure 7. Probability of event-free survival of M2 according to five protocol-regimens.

Five patients died of infection during remission (two of sepsis; two of fungal infection; one of cytomegalovirus infection), and a total of 39 patients had a bone marrow relapse (Table 2).

Eleven of the 114 patients (9.6%) relapsed in CNS. Three of the 11 (2 of 53 patients with M2, one of 13 patients with M5) had a primary CNS relapse. The remaining eight patients (M1:3, M2:1, M4:2, M5a:2) had CNS leukemia after BM relapse. CNS involvement frequently occurred in patients with M4 and M5.

Sixteen patients have completed the therapy and only one of them has relapsed 11 months later. Fifteen of 16 are alive without evidence of disease 33–104 months (mean, 5 years 2 months) after diagnosis. None of these patients have developed CNS disease, although prophylactic cranial irradiation was not employed. Only one of 16 cases developed cardiomyopathy.

Median survivals in 81 nonresponders and in 31 complete responders to therapy were 12.3 ± 6.4 months and 33.6 ± 3.9 months, respectively, and were significantly different ($p = 0.003$).

Four of 114 patients were treated with BMT. Of the four patients, one with M2 received BMT during the first remission, one with M2 received it during the second remission, and the other two with M1 or M5b were nonresponders to these protocols.

Two patients with M2 remain in CR for 1 year and for 4 years each after BMT. Of the other two patients (Ml and M5b) who relapsed, the one with M5b is still alive.

Prognostic Factors

Covariance analysis of the influence of several factors on survival showed that both age and responsiveness significantly affected the survival period ($p < 0.01$). Furthermore, WBC and M5 significantly affected the remission duration ($p < 0.01$).

DISCUSSION

About 72% of patients with ANLL (Fab subtype: M1, M2, M4, M5) in this study entered complete remission. This result is consistent with those of other reports (2-6). The probability of CCR was 23–66% at 20 months, in particular that in M2 patients was 38–65% at 3 years. Furthermore, the curves of CCR showed a plateau after 20 months. The primary CNS involvement did not affect the CCR duration. The period of chemotherapy in ANLL-pilot (same as ANLL-861) was shortened from 3 to 2 years without aggressive CNS prophylaxis.

A recently completed study that compared 8-monthly courses of maintenance therapy with 36-monthly courses demonstrated that, while the relapse rate transiently increased for the patients in whom therapy was stopped at 8 months, the continuation of therapy for 28 additional months had no effect on the proportion of long-term survivors (7).

Patients with monocytic leukemia had a statistically shorter duration of remission and a high incidence of CNS involvement (3).

Bone marrow allografting in the first remission is induced, and approximately 70% are long-term survivors in patients under 20 years of age (8,9). Several recent reports have described the use of autologous BMT in patients in the first remission, with or without purging of the marrow of leukemic cells (7).

Therefore, the strategy including bone marrow transplantation should be changed according to the ANLL subtype. We started a new protocol of ANLL-Mo-pilot using VP-16 with CNS prophylaxis. However, the results were not different from those of the previous protocols.

In conclusion, the results of our group study are very encouraging and indicate that many patients with ANLL may hopefully be cured by chemotherapy.

ACKNOWLEDGMENTS

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TREATMENT OF CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA: THE RESULTS OF THE TOKYO CHILDREN'S CANCER STUDY GROUP L84-11 TREATMENT PROTOCOL STUDY

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ABSTRACT

The Tokyo Children's Cancer Study Group protocol (TCCSG L84-11), a randomized trial, was designed to evaluate moderate dose methotrexate (MD-MTX) therapy plus late skull irradiation and intensified central nervous system (CNS) prophylaxis by triple intrathecal (IT) therapy. From 1984 to 1988, children with newly diagnosed acute lymphoblastic leukemia (ALL), except for B-ALL, were enrolled in this protocol. Patients at high risk had one or more of the following risk factors: age below 2 or above 7 years, white cell count of 20,000/μl or more. Patients at extremely high risk had the following risk factors: T-cell phenotype, white-cell count of 100,000/μl or more, or mediastinal mass. Other patients were in the standard risk group. The standard risk (S) regimen consisted of two arms (S-1, S-2). For the remission induction, conventional VPL therapy was carried out. After the remission, triple IT therapy and 18 Gy radiation were given to S-1, and MD-MTX, late radiation (24 weeks later) and maintenance therapy was the standard form. The therapy was terminated after 3.5 years. The high–risk (H) regimen consisted of two arms (H-1, H-2). The outline of this regimen was similar to that of the S regimen. In the H regimen, cyclophosphamide, anthracyclines, BH-AC, cytosine arabinoside, and l-asparaginase were administered for cyclic intensification therapy. The extremely high risk

regimen (Hex) was a modified protocol of BFM-ALL83. There were 471 eligible patients. The remission rates were 97–100%. The patients with relapsed disease were 9/93 (S-1), 8/92 (S-2), 21/127 (H-1), 20/113 (H-2), and 8/46 (Hex). Event = free survival rates (EFS) were 80%, 66–74% and 69% in the S-, H-, and Hex regimens, respectively. In each group, no significant difference in EFS was demonstrated between the two arms (S-1 vs. S-2, and H-1 vs. H-2). The most common site of relapse was the bone marrow (49/66, 74%). Patients with CNS relapse were 5/93 (S-1), 2/92 (S-2), 1/127 (H-1), 0/113 (H-2), and 2/46 (Hex). The unexpected sequela of this protocol was the severe neurotoxicity that accumulated in the H-2 subgroup. Although the follow–up period is short, these preliminary results indicate that this protocol apparently reduces the CNS relapse rate, especially in the high–risk group. However, further modification of CNS prophylaxis is necessary to avoid neurological sequela.

BACKGROUND AND PURPOSE

During the past decade, the continuous and aggressive efforts by several cancer study groups in Japan have contributed to an improvement in the cure rate of childhood acute lymphoblastic leukemia (ALL) (1,2). In this report, the recent results of an ALL study performed by the Tokyo Children's Cancer Study Group (TCCSG) are reported.

This group was organized in 1969 and consists of 31 medical institutes in the vicinity of Tokyo. During the past 15 years, 11 treatment protocols for ALL have been studied.

Previous studies indicated that event–free survival (EFS) at 5 years was 50–60% among a standard risk group and approximately 30% among a high-risk group, who also had a central nervous system (CNS) relapse rate of over 20%. Poor prognostic factors, detected from one of the previous studies, were age (younger than 2 years and older than 7 years) and initial white blood cell count (WBC, greater than 20,000/μl).

In this paper, we report on the results of the recent protocol, TCCSG L84-11. This protocol was carried out from June 1984 through February 1989. The purpose of the L84-11 protocol was to reduce the overall relapse rate, particularly CNS relapse. In order to reduce the relapse rate, intensive chemotherapy was carried out. The additional therapy or agents that were added to the previous regimen were moderate-dose methotrexate (MTX) therapy at the early phase of remission, triple intrathecal (IT) therapy as an intensive CNS prophylaxis, intensification with multiagents during the remission phase, and late intensification with moderate dose MTX. In addition, an intensive treatment regimen for ALL with poor prognosis was used as a pilot arm. This group included ALL with the T-cell phenotype, mediastinal mass, and/or an initial WBC of over 100,000/μl.

MATERIALS AND METHODS

Five hundred and fourteen patients under 16 years of age with ALL were divided into three groups based on the risk factors established earlier by the TCCSG

study: standard-risk group, high-risk group, and extremely high-risk group (Table 1). Patients with B-cell type ALL (L3), in which the blasts expressed surface immunoglobulin, and patients with CNS involvement at onset were excluded from this study. Infants with ALL were also excluded from this study.

Table 1. Stratification of patients with risk factors.

	AGE (y/o)	WBC
Standard Risk Group (S-1, S-2)	≧2, <7	<20,000/μl
High Risk Group (H-1, H-2)	<2, ≧7	≧20,000/μl
Extremely High Risk Group (HEX)		≧100,000/μl T-ALL Mediastinal mass

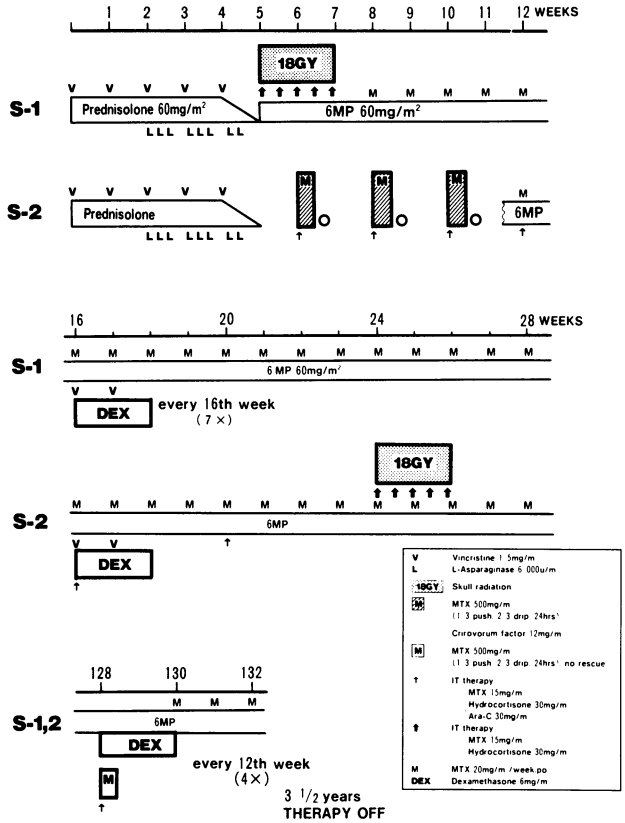

Figure 1. The standard risk regimen of TCCSG L84-11 protocol.

The standard risk group consisted of 2–7-year-old patients whose initial WBC was below 20,000/μl and the high-risk group consisted of patients either younger than 2 years, (except infants) or older than 7 years, or with an initial WBC greater than 20,000/μl. T-cell–type ALL, ALL presenting with a mediastinal mass, or ALL with a high initial WBC of over 100,000/μl were included in the extremely high risk group (Hex).

The standard-risk regimen (Fig. 1) was divided into two groups, S-1 and S-2. The patients with standard risks were randomized into these two subgroups. Vincristine (VCR), prednisolone, and 1-asparaginase were used for remission induction. After the remission was confirmed, skull radiation of the 18 Gy dose and triple IT therapy were started in the S-1 subgroup. The agents used in triple IT therapy were MTX at 15 mg/m^2, hydrocortisone at 30 mg/m^2, and cytosine arabinoside at 30 mg/m^2. In the S-2 subgroup, moderate-dose MTX therapy with leucovorin rescue was performed three times between the sixth and tenth weeks and skull radiation and triple IT therapy were performed in the 24th week. As an intensification therapy, VCR and dexamethasone (DEX) were administered every 16 weeks, seven times in total. They were started from the 16th week in the S-1 subgroup and from the 32nd week in the S-2 subgroup. Six-mercaptopurine and weekly MTX were orally administered as maintenance agents. In both groups, from the 128th week, a late intensification therapy using moderate dose MTX was started every 12 weeks. The therapy was terminated after 3½ years.

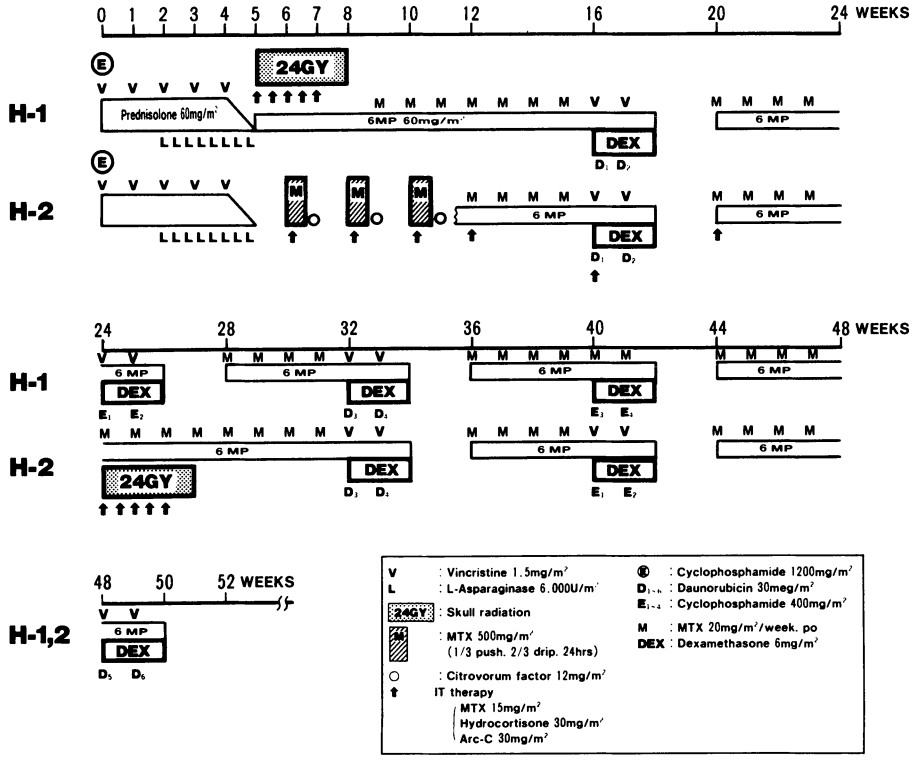

Figure 2. The first year of tratment of the high-risk regimen of TCCSG L84-11 protocol.

The schema of the first year treatment of the high–risk regimen is shown in Fig. 2. The high–risk patients were randomly divided into two groups, H-1 and H-2. The outline of this regimen was similar to that of the standard risk regimen. However, the major differences between the two in the first year were that in the high risk regimen, high-dose cyclophosphamide at 1200 mg/m^2 was administered on the first day of treatment, daunorubicin or cyclophosphamide was given in addition to VCR and DEX in the intensification phase, and the intensification interval was shortened to 8 weeks. The schema of the second- and third-year treatment for the high risk regimen is shown in Fig. 3.

The second-year treatment consisted of cyclic intensification of BH-AC plus aclacinomycin, l-asparaginase, and low dose MTX. This cycle was repeated twice. The third-year treatment consisted of the cyclic administration of cyclophosphamide, cytosine arabinoside, and moderate-dose MTX. Maintenance therapy was similar to that of the standard-risk regimen.

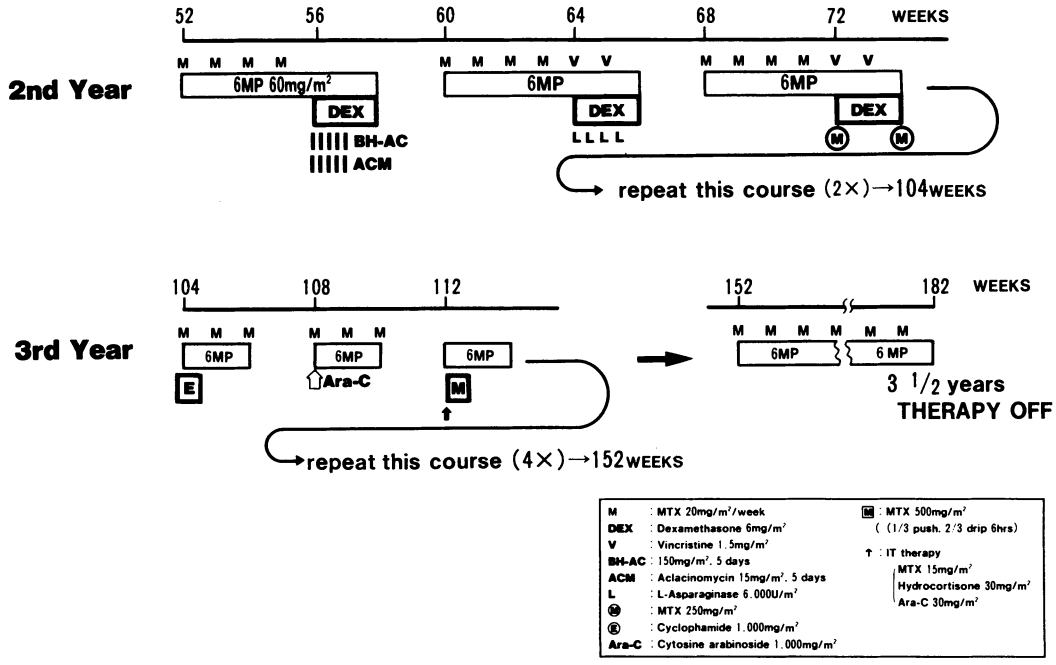

Figure 3. The second- and third-year treatment of the high risk regimen of TCCSG L84-11 protocol.

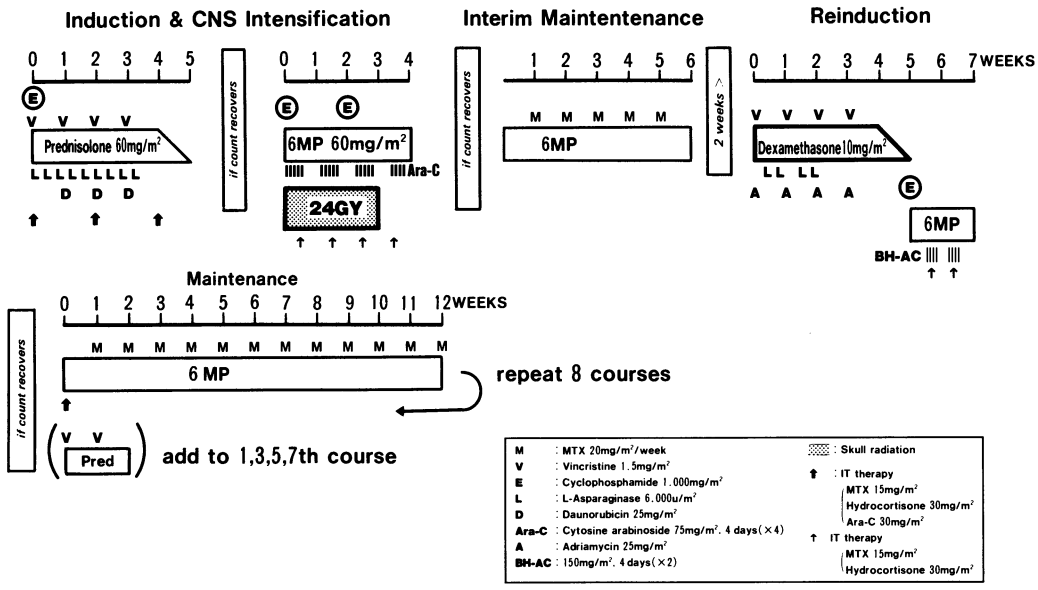

Figure 4. The extremely high-risk regimen (Hex).

The schema of the extremely high-risk regimen (Hex) is shown in Fig. 4. This Hex regimen was designed for ALL patients with poor prognosis. This pilot study was based on the concept and framework of the BFM-ALL83 protocol reported by the German group (3). The essential point of this regimen was the administration of multidrugs for remission induction and early-phase remission.

RESULTS

The results of the L84-11 protocol were analyzed in December, 1988. Five hundred and fourteen patients with ALL had been enrolled in this protocol. Statistical analysis was performed using the program package, SPSSX, by Dr. T. Saito from the National Children's Medical Research Center in Tokyo.

The overall results of the L84-11 protocol study are shown in Table 2. Four hundred and seventy one out of 514 enrolled patients were eligible for evaluation. In the standard-risk regimen, 93 and 92 patients were entered into S-1 and S-2 subgroups, respectively. In the high-risk regimen, 127 and 113 patients were entered into H-1 and H-2 subgroups, respectively, and in the Hex regimen, 46 out of 54 patients could be evaluated. The phenotype of 33 of these 46 eligible patients was T-cell type.

The remission induction rates were 97–100% in each regimen. A total of six patients were induction failures. In the standard risk-regimen, the patients with relapsed disease were nine and eight in subgroups S-1 and S-2, respectively and in the high-risk regimen, 21 and 20 in subgroups H-1 and H-2, respectively. In the Hex regimen,

Table 2. Overall treatment results of TCCSG L84-11 protocol.

	S1	S2	H1	H2	HEX	Total
No. of patient	93	92	127	113	46	471
Male/Female ratio	0.92	1.1	1.4	0.87	2.83	1.19
Age (Mo. ±SD)	50.9±16.7	49.9±17.9	87.5±47.2	95.5±46.0	99.5±47.1	
WBC (mean±SD×1000/μl)	6.5±4.3	7.1±5.1	26.1±31.7	23.5±32.9	118.7±98.9	
Complete remission	92	94	126	110	45	458
Induction failures	1	0	1	3	1	6
Remission induction rate	98.9 %	100 %	99.2 %	97.3 %	97.8 %	98.7 %
Continuous remission	81	76	99	78	32	366
Toxic death	2	2	2	6	4	16
Others*	0	6	5	7	1	19
Relapse	9	8	21	20	8	66
Bone marrow	4 (C1)	5	17	17 (C2)	6 (T1)	49
CNS	5	2	1	0	2	10
Testicle and ovary	0	1	3	3	0	13
Median f/u period (Mo.)	26	26	26	26	18	26
Max. f/u period (Mo.)	51	60+	60+	60+	57	60+
EFS** (%±SE)	80.5±6.5	80.6±7.0	74.7±5.8	66.5±6.3	69.3±7.5	74.4±3.0

(C) : accompanied with CNS relapse (T) : accompanied with testicular relapse
Others* include lost to follow up, unrelated accidental death and premature discontinuance of therapy.
Events**include induction failures, relapses and toxic death.

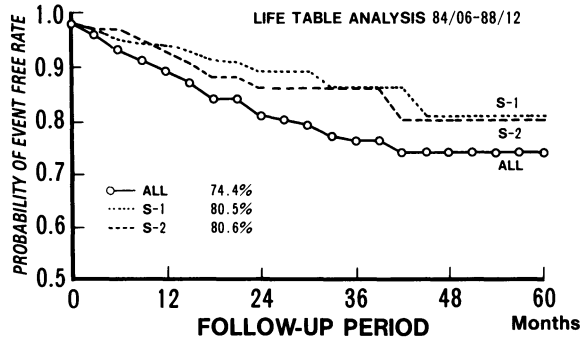

Figure 5. The results of the life table analysis of the standard-risk regimen of TCCSG L84-11 protocol.

eight patients had a relapse. Thus total relapse occurred in 66 patients, among whom bone marrow was the most common site of relapse, with 49 relapsed patients out of

66 cases (74%). Regarding the incidence of CNS relapse, it was unexpectedly low in all regimens, compared with the previous study, which had revealed that CNS relapse rate was greater than 20% in the high-risk group. In this study there was only one CNS relapse in the high-risk group.

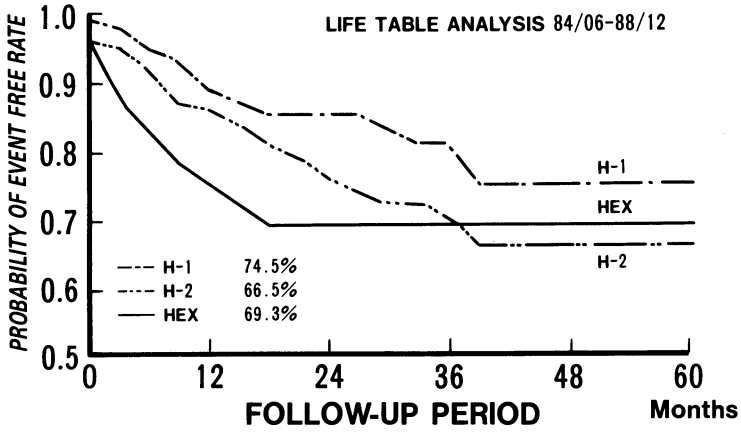

Figure 6. The results of the life table analysis of the high-risk and Hex regimens of the TCCSG L84-11 protocol.

Although the follow-up period (a median of 26 months) is still short for the determination of the EFS rate, the tentative results are more than satisfactory: The EFS rate is 80% in both the S-1 and S-2 subgroups, 66% and 74% in the H-1 and H-2 subgroups and 69% in the Hex regimen. The results of the life table analysis of the standard risk regimen revealed no statistical significance between S-1 and S-2 (Fig. 5). The results of the life table analysis of the high risk regimen and Hex regimen are shown in Fig. 6. There was no statistical significance between H-1, H-2, and the Hex group.

Table 3. The causes of death during remission.

	S 1	S 2	H 1	H 2	HEX	Total
Interstitial pneumonia	1	1	1	2		5
Sepsis	1	0	0	3	2	6
Pancreatitis		1				1
Myelopathy		0	1	1		2
Bleeding					2	3
TOTAL	2	2	2	6	4	16

Table 4. Major infections of TCCSG L84-11 protocol.

	S 1	S 2	H 1	H 2	HEX	TOTAL
Severe bacterial infection	4	6	14	10	9	43
Severe fungal infection	1	0	2	1	6	10
Interstitial pneumonia	2	2	5	1	1	11
Varicella infection	1	3	9	4	2	19
					TOTAL	83

The causes of death during complete remission are listed in Table 3. The major cause of death was severe infection, which occurred in 11 out of 16 patients (70%). The major complication with this protocol was infection (Table 4). A total of 83 events of life threatening infections were observed, however, the majority of these could be controlled by adequate supportive care.

Table 5. Drug-induced complications of the TCCSG L84-11 protocol.

	S 1	S 2	H 1	H 2	HEX	TOTAL
peripheral nerve palsy(VCR)	1	0	0	0	1	2
hemorragic cystitis (Cyclo)	0	1	0	0	1	2
shock, anaphylaxsis (l-asp)	0	0	0	1	1	2
thrombosis, bleeding (l-asp)	0	0	1	0	1	2
hypofibrinogenemia (l-asp)	11	5	24	14	6	60
pancreatitis (l-asp)	2	0	4	2	0	8
severe stomatitis (MTX)	2	5	1	2	1	11
severe liver dysfunction (MTX)	0	2	1	3	0	6
pigmentation (MTX)	0	4	0	3	0	7
cardiac toxicity	0	0	0	1	2	3
leukoencephalopathy	0	0	0	1	1	2
myelopathy (?)	2	1	3	9	1	16

The drug-induced complications observed in this study are listed in Table 5. Hypofibrinogenemia and pancreatitis due to l-asparaginase, and severe stomatitis and liver dysfunction due to MTX, were common complications. In addition to these complications, 16 patients with unexpected myelopathy were observed, as is shown in the bottom line of this table. In the majority of patients, the primary symptoms were paraplegia, gait disturbance, urinary and rectal incontinence, and respiratory failure. The majority of patients disclosed symptoms of myelopathy after 24 weeks. Severe respiratory failure followed by paraplegia was observed in 4 out of 16 patients. Two of these four patients died and the other two patients are still in the intensive

respiratory care unit. The incidence of this myelopathy in each regimen is shown in Table 6. A significant incidence is clearly demonstrated in the H-2 subgroup.

Table 6. Incidence of myelopathy.

Standard Risk		
S-1	2/88	(2.3%)
S-2	1/91	(1.0%)
High Risk		
H-1	3/125	(2.4%)
H-2	9/110	(8.1%)($P<0.05$)
Extremely High Risk		
HEX	1/53	(1.9%)

DISCUSSION AND CONCLUSIONS

Although the follow-up period of this protocol was short, the event-free survival was satisfactory and was better than we had expected. The CNS relapse rate was very low, especially in the high-risk group. It is supposed that the additional systemic chemotherapy and intensive CNS prophylaxis including triple IT therapy might reduce the occurrence of CNS relapse.

In our previous study, the prognoses of T-cell type ALL and ALL with extremely high initial WBC were poor. Therefore we created an extremely high-risk group for these patients and the Hex regimen was proposed. However, the results of the Hex regimen did not disclose any statistical significance when compared with those of the high-risk regimen. This finding confirms the fact that the reinforcement of chemotherapy changes the grade of risk factors of childhood ALL, as has been reported by BFM study groups (3,4).

The moderate-dose MTK therapy of the early phase of remission did not affect the incidence of testicular relapse, however, the observation period of relapse is as yet too short for evaluation.

One of the unexpected complications of this protocol was severe neurotoxicity. This peculiar complication was accumulated in the H-2 subgroup of the high-risk regimen. The treatment in this subgroup consisted of moderate-dose MTX therapy, triple IT therapy 12 times, and late skull radiation. Although the exact cause of this myelopathy was not determined, intensive CNS prophylaxis is a possible candidate.

This report is only the tentative analysis of the TCCSG study of childhood ALL and further follow-up is necessary for any final conclusion.

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BONE MARROW TRANSPLANTATION IN CHILDREN AND ADOLESCENTS WITH LEUKEMIA

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ABSTRACT

Results of HLA-identical sibling bone marrow transplants and autotransplants in children and adolescents with leukemia were analyzed and compared to chemotherapy. These data indicate that different therapies are most effective in different leukemias and remission states. Often, the most effective strategy was to combine modalities.

INTRODUCTION

Bone marrow transplantation, typically from an HLA-identical sibling, is effective in acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), and chronic myelogenous leukemia (CML) (1-5). Recently, favorable results were also reported with autotransplants (6-8). In this review, we consider how the results of transplants compare with chemotherapy and suggest optimal therapy strategies for children and adolescents with leukemia.

ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

First Remission

Since chemotherapy cures many children with ALL, bone marrow transplantation is usually reserved for those with high-risk leukemia. Here transplantation from an HLA-identical sibling results in about 60% leukemia-free survival (LFS), with a relapse rate of about 30% (Fig. 1). Autotransplants in this setting result in 30–60% LFS with

40–70% relapses. Chemotherapy in children with high-risk ALL results in 50–70% LFS with 30–50% relapses.

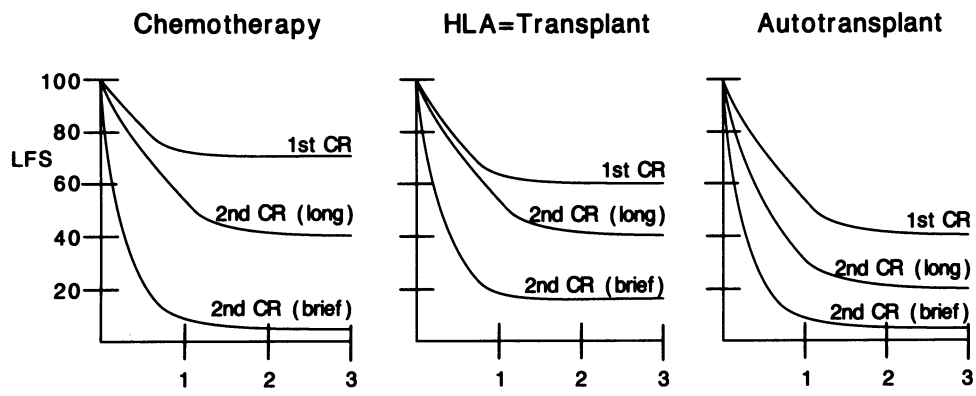

Figure 1. Results of HLA-identical transplants, autotransplants, and chemotherapy in children with ALL.

Second Remission

Results of HLA-identical sibling transplants in ALL in second remission are about 35% LFS and 50% relapse. Results depend on the duration of first remission with 35% LFS when first remission was >18 months and 30% when first remission was <18 months. Relapse rates are about 50% and 60%, respectively. The results of autotransplants in second remission are about 20% LFS, with about 70% relapses. Here again, the outcome correlates with a duration of first remission with LFS of 20–30% vs. <10%, respectively. Chemotherapy in second remission results in about 20% LFS with 30–40% and <10% LFS depending on whether first remission was greater or less than 18 months.

Advanced Leukemia

The results of HLA-identical transplants in advanced ALL are about 15% with relapse rates of about 75%. Autotransplants result in <10% LFS and chemotherapy in <5%.

Therapeutic Strategy

These data suggest that few if any children with ALL should receive a transplant in first remission. Possible exceptions are those with extremely high-risk leukemia. The approach to children failing initial chemotherapy depends on the duration of first remission: chemotherapy in those with a long first remission and transplants in those in whom first remission was brief. In most instances, results of HLA-identical transplants are comparable or superior to autotransplants. Children with advanced ALL cannot be cured with chemotherapy; transplants may rescue a few children.

First Remission

HLA-identical transplants result in about 60% LFS in children with AML in first remission; relapses are about 20% (Fig. 2). Results of autotransplants are less impressive with 30–40% LFS and 60–70% relapse. The results of chemotherapy in children with AML are controversial. Some studies report about 35% LFS (60% relapses), whereas others report 60% LFS (30% relapse). Several randomized trials report superior results with transplants.

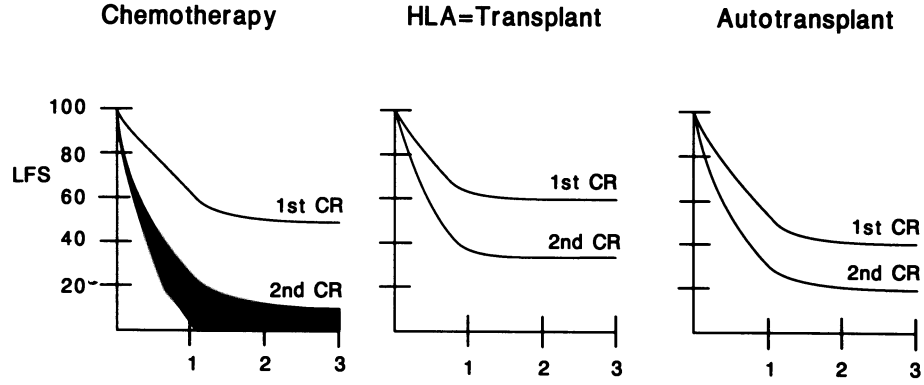

Figure 2. Results of HLA-identical transplants, autotransplants and chemotherapy in children with AML.

Second Remission

HLA-identical transplants result in about 40% LFS in second remission; the relapse rate is about 45%. Autotransplants result in 20–30% LFS with a relapse rate of 70–80%. Chemotherapy also results in low LFS, 0–20% in most series.

Advanced Leukemia

HLA-identical transplants in advanced AML result in about 20% LFS with about 70% relapses. Autotransplants result in <10% LFS and chemotherapy in <5% LFS. Relapse rates for the latter two therapies are >90%.

Therapeutic Strategy

There is controversy as to whether children with AML in first remission should receive an HLA-identical transplant or chemotherapy. Transplant results are clear, about 60% LFS, whereas chemotherapy results are variable, 35–60% LFS. Also, results of several randomized trials favor transplants. However, it is important to consider that a transplant in first relapse or second remission results in 40% LFS. These data suggest two alternative approaches in AML, a transplant in first remission or chemotherapy in first remission, reserving transplants for children who relapse. There are few data regarding autotransplants in AML in first remission, but results are probably not superior to these strategies.

In children who relapse, HLA-identical transplants and autotransplants are probably superior to chemotherapy, but allotransplants are more effective. The same conclusion applies to children with advanced leukemia.

CHRONIC MYELOGENOUS LEUKEMIA (CML)

CML is not curable with chemotherapy, interferon, or autotransplants. HLA-identical transplants result in about 70% LFS in the chronic phase, 40% in the accelerated phase, and 30% in the acute phase. Relapse rates are about 10%, 45%, and 45%, respectively. Since HLA-identical bone marrow transplantation offers the only possible cure of CML, it is the preferred therapy and should be performed in the chronic phase.

SUMMARY

The data reviewed indicate the efficacy of HLA-identical transplants, autotransplants, and chemotherapy in children with leukemia. All three therapies are effective. In some instances, transplant results, particularly HLA-identical transplants, are superior to chemotherapy. However, overall LFS is often highest when transplants are used to rescue children failing chemotherapy. Combined use of these modalities should result in long-term LFS in 60–70% of children with leukemia.

ACKNOWLEDGMENTS

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BONE MARROW TRANSPLANTATION IN CHILDREN AND QUALITY OF LIFE IN LONG-TERM SURVIVORS

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ABSTRACT

Allogeneic bone marrow transplantation (BMT) has been performed in 70 patients with hematological malignancies or other various diseases during the last seven years at Tokai University. The projected survival rates of HLA genotypically compatible marrow recipients were 100% for chronic myelogenous leukemia (N = 10), 81.5% for acute nonlymphocytic leukemia (N = 13), 37.6% for acute lymphocytic leukemia (N = 14), 40.0% for solid tumors (N = 5) and 100% for non-neoplastic diseases (N = 12). Survival rates of patients who received marrow grafts from donors other than HLA genotypically identical siblings were 18.8% for leukemia (N = 8) and 100% for non-neoplastic diseases (N = 6). The quality of life in pediatric long-term surviving marrow recipients has been good and acceptable in general. Profound common abnormalities among survivors are long-lasting hypogonadism due to radiation and subclinical impairment of lung function in the first several months post-BMT. About two thirds of children experienced a transient decrease in growth velocity in the immediate post-transplant period.

INTRODUCTION

Bone marrow transplantation has been successfully applied to the treatment of high risk patients with leukemia or some solid tumors, resulting in an increasing number of long-term survivors. These survivors could be at risk for developing a variety of late complications because of pre-transplant therapy, transplant conditioning, post-transplant immuno-suppressive treatment and graft-versus-host disease (GVHD).

We report our results in allogeneic BMT for children with leukemia or solid tumors and the quality of life in long-term surviving marrow recipients.

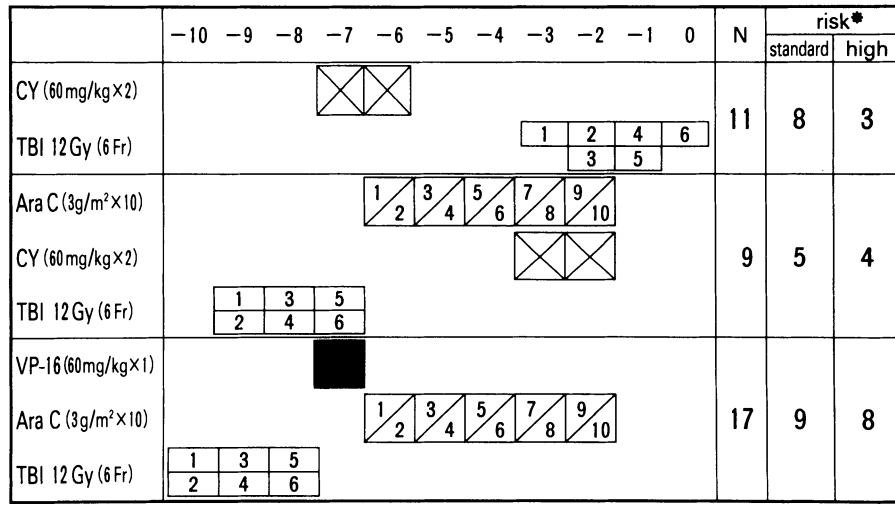
PATIENT POPULATION

Fifteen patients with acute non-lymphocytic leukemia (ANLL), 16 with acute lymphoblastic leukemia (ALL), 10 with the adult form of chronic myelogenous leukemia (CML), three with juvenile CML, four with non-Hodgkin's lymphoma (NHL) and one with neuroblastoma (NB) received a marrow graft from HLA-identical siblings (N = 42), HLA partially matched family members (N = 7) or an unrelated volunteer donor (N = 1) during the period between March 1982 and November 1989. Twenty other patients with aplastic anemia, immunodeficiency, or metabolic error were transplanted in the same period and were included in the analyses of the quality of life.

TRANSPLANTATION PROCEDURES

Preconditioning consisted of a high dose of cyclophosphamide (CY) and fractionated total body irradiation (TBI) in the first 11 patients, but this was changed into more intensified regimens including high-dose cytosine arabinoside (AraC) and/or etoposide (VP-16) with fractionated TBI in the later patients (Table 1).

Table 1. Three conditioning regimens for BMT in leukemia and lymphoma.



*standard:1st CR or CP
high:more advanced stages

Prevention of GVHD was attempted with methotrexate (MTX) alone, cyclosporine (CYA) alone, or CYA and short-term MTX.

Patients were isolated in laminar air flow (LAF) rooms and were given nonabsorbable antibiotics as gut decontamination during the neutropenic period. They were also given intravenous gamma-globulin preparations and acyclovir as prophylaxis of cytomegalovirus (CMV) pneumonia. Recombinant granulocyte colony-stimulating factor (rG-CSF), recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF), or human urinary macrophage-stimulating factor (huM-CSF, P-100) were

given to some patients in an attempt to hasten leukocyte recovery. Most patients received CMV negative blood products after transplantation.

FOLLOW-UP EVALUATION

Clinical evaluation included height, weight, physical examination, EKG, cardiac ECG, chest X-P, skull CT scan, EEG, ventilation tests, lung perfusion and/or ventilation scan, endocrinological studies, and a special check-up by dermatologists and rehabilitation specialists at various intervals.

RESULTS

Survival

Thirty-three of 49 patients with leukemia or solid tumors, and all of 20 patients with non-neoplastic diseases, are currently surviving +1 to +92 months post-transplant, with a median of 25 months (Fig. 1).

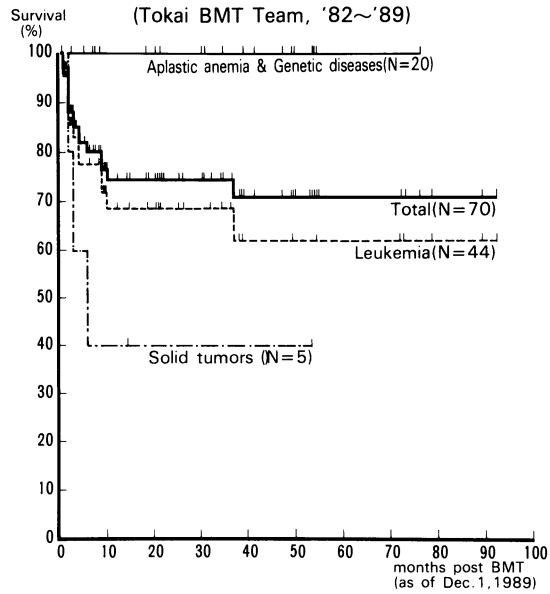


Figure 1. Survival of 70 allogeneic BMT recipients at Tokai University Hospital.

The type of leukemia, disease status at transplant, and histocompatibility matching between the donor and recipient gave significant differences to the outcome.

Projected survival rates of HLA genotypically identical marrow recipients were 100% for CML (N = 10), 81.5% for ANLL (N = 13), 37.6% for ALL (N = 14), and 40.0% for solid tumors (N = 5) (Fig. 2). The survival rate of patients with leukemia who received marrow from donors other than HLA genotypically identical siblings was

18.8% (Fig. 2).

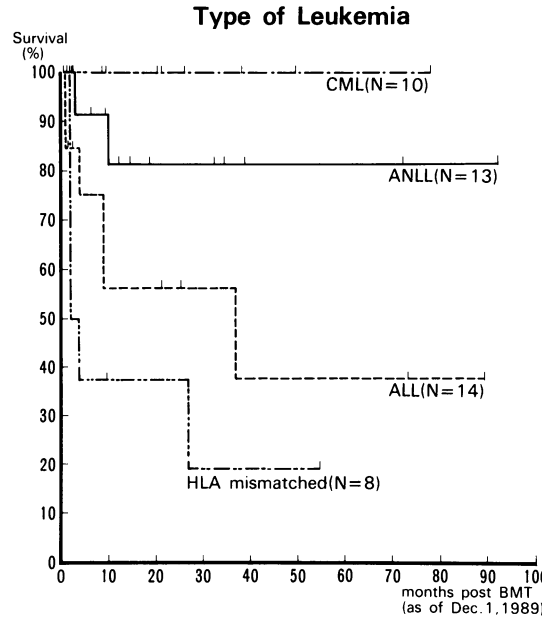


Figure 2. Survival rates of leukemic patients according to type of leukemia.

Patients who were transplanted during their first remissions or first chronic phase had a significantly better survival rate (85.6%) than those transplanted during later remissions (40.0%) or at relapse (28.6%) (Fig. 3).

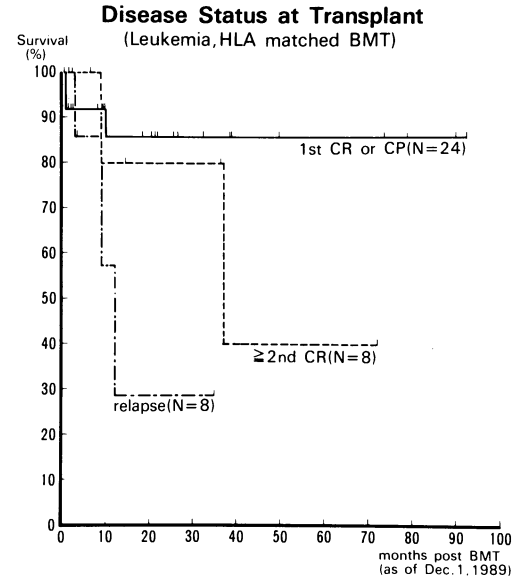


Figure 3. Survival rates of leukemic patients according to disease status at transplant.

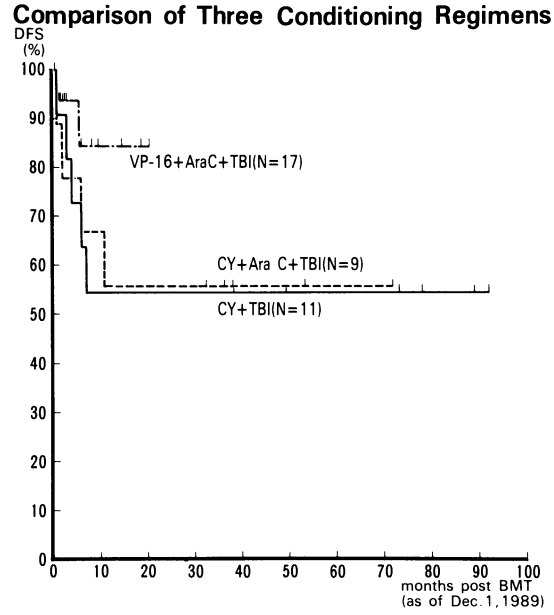


Figure 4. Disease free survival rates of leukemic patients conditioned with three different preparative regimens.

Three different conditioning regimens were compared. Although the VP-16+AraC+TBI regimen gave the best disease-free survival rate, the difference did not reach a statistical significance because of the short observation period of this regimen (Fig. 4).

Table 2. Incidence of infection after BMT. Tokai BMT Team, 1982–1989.

Causative agents	Period			
	Early (0–30 days)	Middle (31–100 days)	Late (≥100 days)	Total
Bacteria	2/65 (1)*	1/60	3/49	6/65 (1)
Fungus	1/65 (1)	0/60	0/49	1/65 (1)
Virus				
HSV stomatitis	0/65	0/60	1/49	1/65
VZV chikenpox	0/12	0/12	3/12	3/12
VZV H. zoster	0/53	12/46	14/38	26/53
CMV IP	0/65	3/60 (3)	0/49	3/65 (3)
CMV enteritis	0/65	1/60	1/49	2/49
Adeno cystitis	2/65	2/60	1/49	5/65
P. carinii	0/65	0/60	0/49	0/65
Total	5/65 (2)	19/60 (3)	23/49	47/65 (5)

* ( ) fatal cases.

Infection

The incidence of infections is summarized in Table 2. In the early period during the first 30 days post-BMT, there were two documented bacterial and one fungal infections, and two were fatal. CMV pneumonia developed in three patients with severe acute GVHD and became a cause of death in all three cases. Herpes zoster was the most common infection in the middle and late periods, but it did not become serious in any cases because of early treatment with acyclovir.

Growth

Fifteen out of 21 long-term surviving children showed a transient decrease in growth velocity after BMT. Recovery of growth tended to occur after 1–2 years if BMT was performed at younger ages, while the standard deviation scores of height in the older patients continued to decline for a longer period.

All eight patients with chronic GVHD experienced a regression in linear growth, whereas 7 out of 13 patients without chronic GVHD showed growth impairment. The growth spurt tended to occur after resolution of the chronic GVHD in younger patients.

Hormonal Studies

1. Growth hormone (GH)
   To examine the secretory response of GH to pharmacological stimuli, three different tests were performed separately. Three boys responded poorly in more than two tests, and two of these children showed a prolonged decrease in height velocity. The other 17 children tested showed normal responses.
2. Gonadal functions
   Luteinizing hormone (LH) and follicular stimulating hormone (FSH) showed over-reaction to an LH releasing hormone loading test in older children (≥ 10 years) who were given irradiation as conditioning for BMT. The response of testosterone to a human chorionic gonadotropin loading test remained normal in all patients tested.
3. Thyroid
   Basal thyroid functions and the responses of thyroid stimulating hormone (TSH) to a thyrotropin releasing hormone (TRH) test were normal in all patients tested.
4. Adrenal functions
   Responses of adrenocorticotropic hormone (ACTH) and cortisol to an insulin loading test were normal in all but one patient who was tested during prednisolone administration for chronic GVHD.

Lung Functions

1. Spirometry
   Subclinical impairment of ventilation was found in one third of children who could perform spirometry satisfactorily. Those with chronic GVHD had lower %VC (vital capacity) and $FEV_{1.0}$% (forced expiratory volume over 1 second)

than those without in the first 1 year after BMT. %VC and $FEV_{1.0}$% were also lower in those who showed defects in lung scans than in those who did not.

2. Lung scans

Ventilation and/or perfusion scans were performed in 29 patients and revealed abnormal defects in 12 patients. Although the causes of this abnormality in lung scans could not be identified, occlusion and narrowing of middle-sized pulmonary arteries were found in one patient who had idiopathic and steroid responsive interstitial pneumonitis.

Cardiac Functions

Seven leukemic patients had cardiac dysfunction expressed as decreased ejection fractions before conditioning. Cardiac failure progressed in three patients and became a cause of death in one patient. The ejection fraction gradually returned to the normal range in patients who survived conditioning for BMT.

Central Nervous System

Minimal to moderate abnormalities were already present on skull CT scans before BMT in a few patients. One patient with ALL who had had an intracranial hemorrhage during induction chemotherapy developed leukoencephalopathy 6 months post BMT.

EEG records showed transient low-voltage waves and occasional spikes in some patients, although none of them had clinical symptoms, except one patient with leukoencephalopathy, as stated above.

DISCUSSION

Remarkable progress has been made in the areas of both chemotherapy and bone marrow transplantation in the last two decades (1,2). Our present study agrees with many other reports regarding the clinical outcome of allogeneic BMT (3-5). We have been able to increase long-term survivors by reducing infectious complications and by reducing leukemic relapse.

Because of advances in chemotherapy, allogeneic BMT will be indicated for extremely high-risk leukemia patients, and relapse of leukemia will remain a difficulty to be solved. Many centers are trying to intensify the preparative regimens in order to ablate residual leukemic cells (6,7). We have used three different conditioning regimens, and high doses of VP-16 and AraC plus TBI gave the best disease-free survival rate, although a longer period of observation is needed. These two potent antileukemic agents are reported to be less immunosuppressive than cyclophosphamide if they are used alone (8), and about 10% of patients who were prepared with either VP-16 or AraC plus TBI failed to be engrafted (6,7). The rejection rate seems to be much less in our series, which must be due to the combined or synergistic effects of the two drugs.

Since we have to treat growing children, the quality of life (QOL) in the long-term survivors should be of great concern. We evaluated many aspects of QOL in our allogeneic marrow recipients and compared the results to other reports (9-13). Sanders *et al.* reported on the long-term follow-up of pediatric BMT recipients and

showed that a decrease in height velocity was observed in the patients who had chronic GVHD or had single-dose TBI (9). They also demonstrated that multiple endocrine abnormalities, such as hypothyroidism and gonadal failure, affected growth. Our observation of a high incidence of growth retardation in the patients with chronic GVHD agrees with their findings, but the degree and duration of retardation were less and was shorter in our patients, probably because we have used fractionated TBI since the beginning of the BMT project. We have not observed hypothyroidism or adrenal failure in our patients.

Infertility remains an irreversible side effect as long as TBI is used as conditioning (12,13). New and more potent preparative regimens that do not employ TBI should be developed for children and younger adults.

We conclude that allogeneic BMT can provide a high chance of disease free survival for patients with leukemia in whom chemotherapy failed to maintain remission and in whom only a small probability can be expected to survive by chemotherapy, and that the quality of life in our long-term surviving marrow recipients is good and acceptable in general.

ACKNOWLEDGMENTS

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AUTOLOGOUS BONE MARROW TRANSPLANTATION FOR CHILDREN WITH LEUKEMIA

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ABSTRACT

Seven patients with acute nonlymphocytic leukemia (ANLL) and three patients with acute lymphocytic leukemia (ALL) in first or second complete remission were treated by high-dose cyclophosphamide and total body irradiation followed by transfusion of autologous bone marrow. In eight cases, marrow cells were treated *ex vivo* by 4-hydroperoxycyclophosphamide (4-HC). Engraftment occurred in all 10 patients. One ANLL patient who received unpurged marrow relapsed at day 393, however, nine others remain in unmaintained complete remission with a median follow-up of 448 days post-transplant (range: 57+ to 1651+ days). These preliminary results suggest that 4-HC purged autologous bone marrow transplantation is effective for children with ANLL or ALL in first or second remission who have no human leukocyte antigen matched donor.

INTRODUCTION

Allogeneic bone marrow transplantation has proven curative for children with leukemia who were considered to have a poor prognosis by conventional chemotherapy. However, allografting is a feasible proposition for only a small proportion of patients who have a human leukocyte antigen (HLA)-compatible donor. Autologous bone marrow transplantation (ABMT) is, in contrast, available to all patients. We report our experiences with intensive cytotoxic therapy followed by ABMT in 10 patients with acute leukemia. They were entered into our ABMT protocol between May 1985 and September 1989.

PATIENTS

Patient characteristics are shown in Table 1. Ten patients with acute leukemia were included in this study. Their median age was 10 years (range, 5–18), with four females and six males. Seven patients had acute nonlymphocytic leukemia (ANLL) and three had acute lymphocytic leukemia (ALL). The subtypes of ANLL and ALL,

Table 1. Patient characteristics. NCCH 1989.

Total	10	
Age (years)		
Median	10	
Range	5–18	
Sex		
Female	4	
Male	6	
Diagnosis		
ANLL	7	
M1		4
M2		1
M4		1
M5		1
ALL L1	3	

according to the FAB classification, were as follows: M1 (four patients), M2 (one), M4 (one), M5 (one) and L1 (three). At the time of marrow collection and transplantation, two of the seven ANLL patients were in their second complete remission (CR) and the others were in their first CR. Out of three ALL patients, two who belonged in the high-risk group (initial leukocyte count $>1 \times 10^{11}$/liter, or age >10 years) were in their first remission; the other who belonged in the standard risk group was in his second remission (Table 2). No patients had active extramedullary leukemia at the time of ABMT.

Informed consent was obtained from all patients or their parents.

Table 2. Remission status at ABMT. NCCH, 1989.

		1st CR	2nd CR	Total
ANLL	M1	2	2	4
	M2	1	0	1
	M4	1	0	1
	M5	1	0	1
ALL	L1	2	1	3

METHODS

Patients underwent marrow collection over a median interval of 7 months (range, 0.5–24) after entering their first or second remission. A total volume of 10–15 ml/kg of patient body weight of bone marrow was collected from the anterior and posterior iliac crests, in each patient, under general anesthesia.

In 8 of 10 patients, the harvested marrow was processed *in vitro*, purging to eliminate the contaminated leukemic cells. Approximately 80% of each of the collected

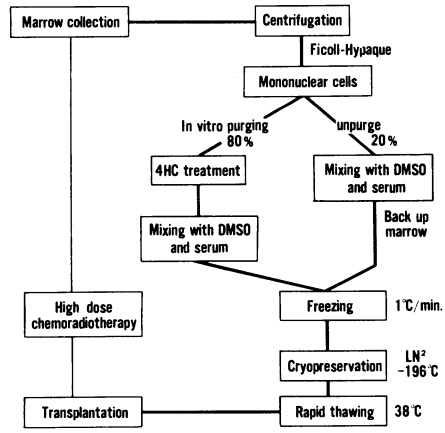

Figure 1. Method of autologous bone marrow transplantation. NCCH 1989.

marrow was centrifuged with Ficoll-Hypaque solution and treated *ex vivo* with 4-hydroperoxycyclophosphamide (4HC) (1), which is the active form of cyclophosphamide (CY) at a concentration of 40–60 μg per ml with 37°C and 30 minutes incubation, and under those conditions more than 99% of the measurable CFU-GM was inhibited, but more primitive stem cells were kept in long-term culture. The mean number of nucleated marrow cells was 3.1 x 10^8 per kg (range, 0.5 to 5.1 x 10^8). The remainder of the collected marrow-cell suspension was reserved for infusion in the event that engraftment with the treated autologous-marrow inoculum failed. The purged and unpurged marrow-cell fractions thus obtained were separately resuspended in 20% autologous or irradiated cross-matched serum from a family member, 10% dimethylsulfoxide (DMSO), and RPMI-1640 tissue culture medium at a concentration of 2–5 x 10^7 cells per ml, and 70-ml aliquots of the cell suspension were placed in polyolefin bags (Del-Med). The bags were frozen in a controlled-rate freezer (Cryo-Med) at -1°C per minute to a temperature of -100°C and then stored in a liquid-nitrogen freezer at -196°C.

At the time of transplantation, each bag was thawed rapidly in a 37°C water bath, and the thawed cell suspension was infused through a central venous catheter at a rate of 10–15 ml/min with no attempt to remove DMSO.

PREPARATIVE REGIMENS

The preparative regimens employed in these patients were identical to those used for allogeneic-marrow transplantation at our institution. All patients received CY 60 mg/kg x 2 (along with MESNA: 120% of the dose of CY), followed by fractionated total body irradiation (F-TBI) at 2 Gy x 6 at a dose rate of 5 cGy/min without lung shielding.

SUPPORTIVE CARE

All patients were nursed in laminar-air flow rooms (LAFR) with high-efficiency

particulate air (HEPA) filtration systems. Intestinal decontamination with oral non-absorbable antibiotics (polymyxin B, vancomycin, and nystatin) and sterilized food service were started from 2 weeks before transplantation. Double lumen central venous catheters were placed in all patients after isolation in LAFR for the administration of fluids, high-dose CY, MESNA, and blood products. At the time of F-TBI, a bio-clean wagon equipped with a HEPA filter was used for keeping the patient in the same condition as LAFR. Isolation procedures were discontinued when the absolute neutrophil counts consistently exceeded 0.5 x 10^9/l.

All blood products were irradiated with 15 Gy before infusion to prevent possible graft-versus-host reactions.

ENGRAFTMENT AND HEMATOLOGICAL RECONSTITUTION

As shown in Table 3, all but two patients received 4-HC purged marrow. With patient No. 1 initially we intended to carry out *in vivo* purging by double transplantation, but after the first transplantation her general condition was too poor to do the second; and with patient No. 2 the initial 4-HC purged marrow was not taken and his unpurged back-up marrow was reinfused three weeks after the first transplantation.

Table 3. Hematological reconstitution and disease-free survival after autologous bone marrow transplantation in children with acute leukemia. NCCH, November, 1989.

No.	Age/Sex	Diagnosis	4HC Tx	No. of cells infused ($\times 10^8$/kg)	Time to attein(days) ANC >0.5×10^9/l	Plt >50×10^9/l	Disease-free survival (days)
1	7/F	ANLL(M1)	NO	2.0	24	62	1651+
2	15/M	ANLL(M1)	NO	0.5	46	>393	393
3	18/M	ALL (L1)	YES	0.3	37	99	1352+
4	11/F	ANLL(M5)	YES	0.9	51	51	1254+
5	5/M	ANLL(M4)	YES	0.6	57	120	917+
6	16/M	ANLL(M1)	YES	1.2	41	115	484+
7	10/M	ANLL(M1)	YES	1.4	40	49	413+
8	6/F	ALL (L1)	YES	6.0	17	26	293+
9	7/M	ALL (L1)	YES	2.2	26	26	252+
10	14/M	ANLL(M2)	YES	5.0	43	NE	57+
		Median		1.3	41	62	448+

The median cell dose at transplant was 1.3 x 10^8 nucleated cells/kg, with a range of 0.3–60 x 10^8/kg. All 10 patients engrafted, and the median time required to attain an absolute neutrophil count in excess of 0.5 x 10^9/l was 41 days (range, 17–57) after transplantation. One patient (No. 2) had recovery of leukocyte counts but had persistent thrombocytopenia at the time of leukemic relapse 393 days after transplantation, and another patient (No. 10) still required periodic platelet support at day 57. In the remaining eight patients, the median time required to attain a platelet count exceeding 50 x 10^9/l was 56 days (range, 26–120) after transplantation.

As noted in other published data (2), neutrophil and platelet recovery in our series was also more prolonged than that of allogeneic BMT, but in eight patients satisfactory hematological recovery was obtained.

Table 4. Nonhematological toxicity in 10 children with acute leukemia following ABMT. NCCH, 1989.

	No.of pt. (%)
Early complications	
Nausea & vomiting	10 (100)
Mucositis	9 (90)
Mild elevation of GPT	8 (80)
Unkown fever	4 (40)
Allergic reaction	3 (30)
Hemorrhagic cystitis	0 (0)
Interstitial pneumonitis	0 (0)
Veno-occulsive disease	0 (0)
Late complications	
Herpes zoster	6 (60)
Pneumococcal meningitis	1 (10)

NONHEMATOLOGICAL TOXICITY

Nonhematological complications are shown in Table 4. Nausea and vomiting were observed in all patients and moderate oral mucositis in nine patients. Mild elevation of liver transaminase (up to 3–5 times of normal limits) occurred in eight patients, and a fever of unknown origin occurred during myelosuppression in four. At the time of reinfusion, allergic reaction, which seemed to be due to DMSO, was observed in three patients (two had transient fever and severe headache, and one suffered temporary hypotension), but no patients developed hemorrhagic cystitis, venoocclusive disease, or serious infection, especially interstitial pneumonitis. Although several months after transplantation, six patients suffered Herpes zoster, and one patient developed a pneumococcal meningitis, they were also not grave. As a whole the side effects following autologous BMT were milder than those following allogeneic BMT.

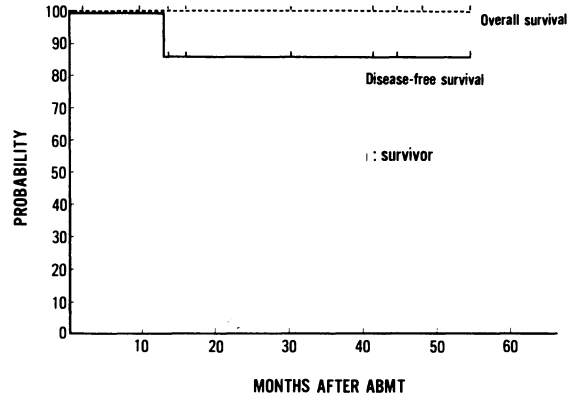

Figure 2. Survival analysis in 10 children with acute leukemia following autologous bone marrow transplantation. NCCH, November 1989.

LEUKEMIC RELAPSE AND DISEASE-FREE SURVIVAL

Hematological leukemic relapse occurred in one patient with ANLL (No.2) 12 months after the unpurged marrow transplantation; however, others, including three ALL patients, are maintained complete remission, with a median follow-up of 448 days (range, 57–1,651 days). Their performance status is 100%. Three patients have remained in CR for more than 3 years.

The Kaplan-Meier estimate is that a total of 10 patients showed 100% overall survival and an 83% probability of disease-free survival, with a plateau extending from 12 to 52 months (Fig. 2).

Comparing this disease-free survival curve with that of allogeneic BMT for acute leukemia performed in this hospital, there is no significant difference between them.

DISCUSSION

High-dose chemotherapy with total body irradiation followed by allogeneic bone marrow transplantation from HLA-identical, mixed-lymphocyte culture negative donors has proved to be curative according to reports from several BMT centers (3,4). However, allogeneic bone marrow transplantation is limited by the scarcity of suitable donors: only 30% of our leukemia patients have HLA-identical donors. As an alternative to allogeneic bone marrow transplantation, we have transplanted the leukemic patient's own cryopreserved remission bone marrow after high-dose chemotherapy and irradiation.

In this series, the initial two ANLL patients (No. 1 and 2) in their first CR received unpurged marrow transplantation, which was successful in one patient but not in another: the former has been in complete remission more than 52 months after ABMT, but the latter relapsed. The distinct difference between the two transplantations is the time interval between remission and marrow collection (7 vs. 0.5 months). In the latter case, it is suspected that the time was too short to achieve a deep complete remission, and contaminated residual viable leukemic cells in cryopreserved marrow might have been reinfused.

Generally, it is anticipated that the risk of relapse is greater after autologous BMT than after allogeneic BMT because of such possible reinfusion of malignant cells and the lack of a so called graft-versus-leukemia effect, therefore we decided to do *in vitro* purging by 4-HC based on Körbling's method (5) for other patients. But, even using the purged marrow, there remained the possibility of some relapse due to insufficient elimination of contaminated cells. Fortunately, none who received 4-HC treated marrow have relapsed in this series.

Overall the disease-free survival of 83% obtained in this series is similar to that obtained with allogeneic transplantation for childhood acute leukemia in our institution.

In conclusion, although the number of patients is small and the follow-up time is not enough, these results are highly encouraging in treating children with refractory leukemia. Moreover, children with acute nonlymphocytic leukemia and even acute lymphocytic leukemia in their first or second remission who have no HLA-identical donor have a chance of cure by receiving 4-HC purged autologous bone marrow transplantation.

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PERIPHERAL BLOOD STEM CELL AUTOGRAFTS IN CHILDREN WITH ACUTE LEUKEMIA AND LYMPHOMA

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ABSTRACT

There is considerable interest in the use of peripheral blood hematopoietic stem cells (PBSC) in autograft settings (PBSCT), and in this paper, a clinical and laboratory experience of PBSCT at a single institute is presented. Twenty-eight children with various types of cancer underwent a total of 90 leukaphereses to collect PBSC and 17 of them subsequently received marrow-ablative therapy and autografts with such collected and stored PBSC. We found that frozen-thawed progenitor dose is important in determining the rate of hematopoietic recovery and that cytoreductive regimens without TBI used in the study are effective against leukemia in the current standards. Although it is not known yet whether PBSCT is better than autologous bone marrow transplantation to prolong the remission interval, and ultimately the cure, the preliminary clinical data justify the incorporation of PBSCT in the design of a pilot therapeutic protocol for the salvage treatment of refractory acute leukemias/lymphoma. Not all collections from patients yield a sufficient number of progenitors for a safe autograft, but the value of preleukapheresis administration of G-CSF to such patients to expand the stem cell pool awaits proof.

INTRODUCTION

Allogeneic bone marrow transplantation (BMT) is accepted as an effective treatment for a number of malignant disorders, but this remains severely restricted by age and the availability of an HLA identical donor. Furthermore, patients transplanted continue to experience a 30–40% likelihood of mortality within the initial 12 months after BMT. The occurrence of graft-versus-host disease (GVHD) has been a major limitation to the success of allogeneic BMT. The use of autologous marrow has the obvious advantage of lower early morbidity and mortality, avoiding GVHD, but a major disadvantage of its use is the high probability that stored remission marrow from patients will contain residual leukemia cells.

As an alternative to BMT, autografts with peripheral blood stem cells (PBSCT) have become an accepted treatment modality to restore the hematopoiesis after

marrow ablative therapy (1-4). Although PBSCT is being used increasingly for the treatment of adult malignant disorders, there has been little published on the detailed evaluation of the effectiveness of this procedure in children (5-7). This report summarizes the clinical and laboratory experience of blood stem cell harvest and PBSCT with 17 children at the University of Tokushima.

PERIPHERAL BLOOD STEM CELL AUTOGRAFT (PBSCT)

The number of hematopoietic stem cells (SC) is low in the peripheral blood, but in the recovery phase of chemotherapy they leave the meshwork of marrow stromal cells and go through the sinus endothelium into the bloodstream. A large number of peripheral blood stem cells (PBSC) can be collected by appropriately timed leukapheresis during this phase of marrow recovery, and they can be cryopreserved without losing their ability to reconstitute hematopoiesis. Such PBSC have been increasingly used as an alternative source of hematopoietic reconstitution in autograft settings after high-dose chemoradiotherapy. Although PBSC are shown to have different growth and functional characteristics to marrow progenitors (8), the effectiveness of PBSCT in reconstituting hematopoiesis after marrow-ablative therapy has been supported by both *in vitro* (9) and *in vivo* studies (1-7).

The relative merits of PBSC and bone marrow require study, but PBSC is readily harvested without the use of anesthesia and without the discomfort involved in multiple bone marrow aspirations, and PBSC restore hematopoietic (1–7) and immune function more rapidly than marrow progenitors do (10). The possibility of contamination of the PBSC with tumor cells has not yet been defined.

So far, however, recent experience in PBSCT has been concentrated on adult patients, with only a few pediatric patients studied. Problems associated with the leukapheresis procedure in small children are access to blood and tolerance of an extracorporal circulation, as well as postleukapheresis cytopenia (11). There is a notion that children are at a greater risk than adult patients for significant cardiovascular morbidity due to the rapid blood volume changes during leukapheresis. Recently, however, we have developed a suitable protocol of PBSCT and demonstrated that PBSCT can be safely performed in children (5,6).

COLLECTION OF PBSC BY LEUKAPHERESIS

The optimal conditions for harvesting PBSC from children must be established before PBSCT can be widely used. We found that, in children after cytoreductive chemotherapy, the number of PBSC increased up to 50-fold (5) and that in a large number of children with cancer, harvesting of PBSC is a safe and reliable procedure with a low incidence of serious morbidity (6). There is wide variation in the techniques used for collecting PBSC, but our group, as well as others (1), found that the use of a computer-assisted automated continuous cell separator, such as the Fenwall CS 3000, is far more effective than other intermittent or manual-drive machines.

Thirty-three consecutive patients were referred to the University Hospital of Tokushima for PBSC collection, and a total of 90 leukaphereses were performed in 28 patients (mean, 3.4 $\pm$ 2.1 times per patient). Five patients were removed from the

study because of failure to induce remission (4), and therapy-induced liver failure (1). Two to 3 weeks after completion of myelosuppressive chemotherapy, when both the WBC and platelet counts were rising rapidly, the collection of blood mononuclear cells was started using a continuous-flow blood cell separator (CS-3000, Fenwall Laboratories, Dearfield, IL). The number of circulating CFU-GM is maximal in early remission, but we believe that cells harvested after a minimum of one course of consolidation therapy are less likely to be contaminated with tumor cells.

A mean volume of 180 ml/kg body weight of blood was processed during each leukapheresis, and a mean of 5.1 ± 4.0 x 10^8 mononuclear cells/kg (range 0.5–15.1 x 10^8/kg) was collected and stored per patient. The morbidity related to PBSC harvest was small, although reversible cyanosis or hypotensive episodes developed in two infants and they recovered rapidly with no therapy. Transient decreases in the platelet counts developed in all patients, but three children showed sustained cytopenia for more than a week after collection of PBSC. Two of them did not recover at all and underwent emergent PBSCT with a successful reconstitution of hematopoiesis (11).

Table 1. Collection of the peripheral blood hematopoietic stem cells from 28 children with various types of cancer.

Age/ Sex	B.W. (kg)	Diagnosis	Stage	Interval*	Number of leukaphresis	Cell (x 10^9)	CFU-GM (x 10^4/kg b.w.)	CFU-Mix
8/M	22	ANLL	1st CR	36	5	33.2	272	50
3/F	13	T-ALL	1st CR	2.5	2	14.3	203	20
3/F	12	ANLL	2nd CR	19	4	18.0	200	3
1/M	9	ALL	1st CR	2.5	1	3.5	180	11
5/F	14	NB	1st CR	2	4	4.2	55	16
13/F	37	B-NHL	1st CR	2	3	33.5	54.3	0.9
10/M	50	ALL	2nd CR	26	2	38.0	46.4	8.4
5/F	18	T-NHL	2nd CR	8	3	3.2	35.6	1.4
5/F	21	ANLL	1st CR	4	2	6.3	34.6	1.7
10/M	30	ALL	2nd CR	16	2	7.0	33.0	6.2
12/M	45	T-NHL	1st CR	5	4	20.8	32	3.9
1/F	9	ANLL	1st CR	7	2	4.6	30.0	2.9
12/M	34	ANLL	1st CR	2	3	24.2	30.0	1.4
2/F	11	NB at 1st relapse		14	2	4.7	29	2.1
15/M	50	T-ALL	1st CR	12	8	22.1	22.5	2.4
12/M	44	ALL	2nd CR	46	3	16.0	19.5	5
4/M	15	ALL	2nd CR	16	1	2.6	16.3	7.4
8/M	25	Hybrid LK	2nd CR	24	3	14.4	12.3	1.4
12/F	35	Hybrid LK	6th CR	30	4	20.5	12.2	0.5
10/F	35	ALL	4th CR	66	3	19.2	9.1	0.6
10/F	33	ANLL	1st CR	5	3	11.8	2.8	0.2
10/M	34	ALL	2nd CR	11	10	13.2	2.2	0.2
9/M	20	RB at 2nd relapse		16	1	1.6	2.1	0
13/F	43	ALL	1st CR	3	5	21.0	1.5	0.6
11/F	50	ALL	3rd CR	60	5	12.8	0.9	0.1
17/F	55	ALL	1st CR	36	2	6.1	0.9	0
8/F	25	NB	1st CR	6	2	3.2	0.6	0
11/M	36	ALL	2nd CR	48	1	6.0	0	0

ALL = acute lymphoblastic leukemia; T-ALL = T-cell type ALL; ANLL = acute nonlymphoblastic leukemia; NHL = non-Hodgkin's lymphoma; B-NHL = B-cell type NHL; NB = neuroblastoma; RB = retinoblastoma; CR = complete remission.

There was a wide range in progenitor yields from patient to patient, and the intensity as well as types of drugs used in the preceding chemotherapy will determine the yield of progenitors (Table 1). The number of CFU-GM obtained ranged from 0 to 272 x 10^4/kg with a mean of 4.8 x 10^5/kg. A threshold of 3 x 10^5 CFU-GM/kg was collected in 13 of 33 referred patients (39%) by a mean of 2.8 (1 to 5) leukaphereses, and 19 patients (58%) cleared the minimum requirement of 1 x 10^5 CFU-GM/kg by a mean of 3.0 (1–8) leukaphereses.

NEED FOR FRACTIONATION BEFORE STORAGE

Large numbers of platelets and RBC, as well as lymphocytes and SC, are obtained by leukapheresis. The removal of platelet and platelet-induced cell clumps from the graft is especially important for small children, because they have only a limited respiratory capacity. When cells obtained by leukapheresis are not fractionated, other serious problems are hemolysis during thawing of the graft and the amount of DMSO infused into the children. Contrary to marrow graft, PBSC graft contains a large number of monocytes, which easily aggregate after thawing, and hence, application of cell washing procedures before transfusion to remove DMSO and hemolysis products is practically impossible. Hence, we have developed a simple and efficient separation and storage protocol for PBSC (6). Even after this separation procedure, our patients received 232 ± 147 ml of graft containing 0.8 ± 0.4 ml/kg b.w. of DMSO, and the toxicity associated with graft infusion still remains a major concern (Table 2).

Table 2. Toxicity related to the PBSC graft infusions observed in 17 children at the University Hospital of Tokushima.

Toxicity	Number of patients suffered
Red urine	17
Nausea	13
Vomiting	10
Severe headache	6
Abdominal pain	2
Cyanosis	2
Dyspnea	1
Confusion	1

USE OF RECOMBINANT G-CSF IN PBSCT

The major limitation of PBSCT is the inability for collection of a sufficient amount of stem cells when patients were heavily treated before leukapheresis. Five patients (three, ALL; two, ANLL) who had a low yield of blood CFU-GM received an infusion of recombinant G-CSF before leukapheresis (300–1000 $\mu g/m^2$ daily x 7 days) to expand the SC pool and this led to a 1.5– to 53.5-fold (15- ± 22-fold) increase in the CFU-GM number. The number of CFU-mix and BFU-E also increased. Three of them underwent PBSCT with the graft containing such collected cells; the speed of

myeloid engraftment appeared to be enhanced, but no proof was obtained. None of these five studied patients has so far developed a recurrence of leukemia after a minimum follow-up of 6 months. G-CSF stimulates and expands the hematopoietic progenitors by driving the differentiation of immature SC at the expense of the SC pool. The value of this procedure needs clarification.

TRANSPLANTED PATIENT CHARACTERISTICS

The length of survival and the ultimate cure rate in children with leukemias or non-Hodgkin's lymphoma (NHL) have improved but there is increasing evidence that patients who fail a first-line combination chemotherapy have a grave prognosis with further currently available therapy and that cure is extremely unlikely. The treatment of such patients remains a difficult challenge in oncology.

So far 17 children (age, 1–13 years old) have undergone PBSCT in our institute. Eleven patients had acute lymphoblastic leukemia (ALL) or NHL and three had acute nonlymphoblastic leukemia (ANLL); of which nine were associated with very high-risk features. The criteria used to define a very high-risk prognosis include: relapse within 18 months of first-line therapy (5), multiple relapse (2), and primary resistance to induction therapy (2). Two other patients had advanced neuroblastoma and one had disseminated relapsed retinoblastoma. No patients had HLA-compatible marrow donors, and the patient characteristics and clinical results after PBSCT are shown in Table 3.

Table 3. Characteristics of the 17 patients and clinical courses after treatment with high-dose chemotherapy and autologous peripheral blood stem cell rescue. All patients were treated at the Department of Pediatrics, University Hospital of Tokushima.

No.	Regimen	Age/ Sex	Diagnosis	Clinical features and risks	Status at PBSCT	Interval (mo) from Dx/Relapse to PBSCT	Response duration (mo) and outcome
001	MCNU,etc.	5/F	T-NHL	Relapse at 8 mo of Tx.	2nd CR	2	2, DOD at 11
002	Bu + CY	13/F	ANLL	Induction failure	1st CR	7	11, DOD at 11
003	MCNU,etc.	3/F	T-ALL	Induction failure	1st CR	4	24+, DFS
004	MCNU/Bu	11/F	ALL	Multiple relapse	3rd relapse	-	3, Bleeding
005	MCNU/Bu	12/M	T-ALL	Huge mediastinal tumor	1st CR	8	21+, DFS
006	PAM,etc.	2/F	NB	Stage IV, Relapse	2nd CR	3	1, Died of Shock
007	MCNU/Bu	10/M	ALL	CNS + BM + testes relapse at 4mo of Tx.	2nd CR	19	18+, DFS
008	PAM,etc.	5/F	NB	Stage IV	1st CR	6	15+, DFS
009	MVAC	3/F	ANLL(M5)	Relapse at 15 mo of Tx. Resisted to reinduction	2nd CR	8	4, on relapse
010	MVAC	0.8/M	ALL	Infant, Ia(+), CALLA(-) WBC>100x10^9/l	1st CR	4	7, DOD at 9
011	MVAC	12/F	ALL	Chronic CNS leukemia	4th CR	4	7, CNS Relapse
012	MVAC	10/M	ALL	Relapse at 15 mo of Tx.	2nd CR	13	+8, DFS
013	MVAC	13/F	B-NHL	MOI (liver,kidney,ovary)	1st CR	4	2, B-cell ALL
014	MVAC	12/M	ANLL(M5)	Heart failure in induction	1st CR	4	7+, DFS
015	PAM,etc.	10/M	RB	Relapse, Dissemination	3rd relapse	-	4, DOD
016	MVAC	13/M	ALL	Relapse after Off. Tx.	2nd CR	4	3, BM relapse
017	MVAC	8/M	ALL-ANLL	Hybrid leukemia Relapse at 21 mo of Tx.	2nd CR	6	2+, DFS

To clarify the effectiveness of PBSCT, our initial study was made on such poor-risk patients. By using this setting, the problem of patient selection bias can be minimized and a clinical advantage of PBSCT becomes apparent more easily.

NEW APPROACHES TO CYTOREDUCTIVE REGIMEN

The long-lasting debilitating effects of total body irradiation (TBI) are one of our primary concerns in young children. In view of the importance of combined chemotherapy not involving TBI, we devised nitrosourea-based high-dose regimens without TBI. Twelve of 13 patients with leukemias/NHL received MCNU-based chemotherapy, such as the MVAC-regimen (6), and the remaining patient received busulfan/CY. The patients with neuroblastoma or retinoblastoma were treated with melphalan-based regimens (5,6). The patients were nursed in single-bed rooms with a laminar air flow facility, but received no gut sterilization with non-absorbable antibiotics. All patients tolerated the regimens well.

HEMATOLOGICAL ENGRAFTMENT

After PBSCT, hematological engraftment was not documented in three patients who, respectively, received 0.7, 0.9, and 2.1 x 10^4 CFU-GM/kg. In 12 patients who received more than 1 x 10^5 CFU-GM/kg, the mean number of days required to achieve a granulocyte count of 0.5 x 10^9/l was 10.5 (6–16) and a platelet count of 50 x 10^9/l was reached between days 9 and 46 (11 ± 10.2 days). Patients who did not receive G-CSF pre- or postleukapheresis were analyzed and we have found that the number of CFU-GM infused per kilogram of the patients' body weight was significantly related to the time to the recovery of granulopoiesis by a linear regression analysis ($p < 0.05$, $r = 0.6$), as shown in Fig. 1, but not of other lineages. Thus, the minimum number of blood CFU-GM resulting in a successful graft in cancer children was 1 x 10^5/kg and a threshold assuring a safer autograft is 3 x 10^5/kg. This is in accordance with the reports from other centers (12).

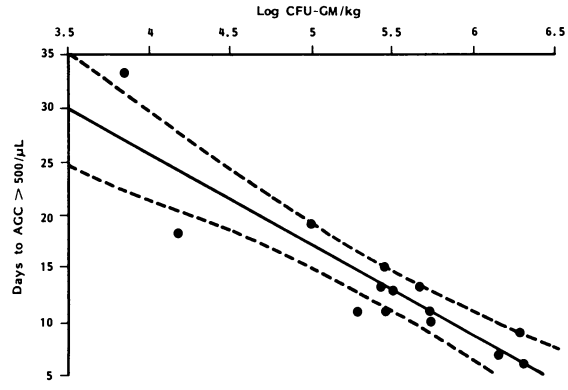

Figure 1. The number of CFU-GM infused per kilogram of the patients' body weight was significantly related to the time to the recovery of granulopoiesis by a linear regression analysis ($p < 0.05$, $r = 0.6$).

IMMUNOLOGICAL RECONSTITUTION AFTER PBSCT

In both autologous and allogeneic grafts, a long-lasting immunological deficiency was observed in the majority of BMT recipients. PBSC grafts contain larger numbers of lymphoid cells than bone marrow grafts, and consequently rapid recovery of immunological function is expected. In this study we have been monitoring factors including phenotypes, T-cell proliferative ability on stimulation, immunoglobulin productive ability of the B cell, capacity to secrete cytokines by lymphocytes, and NK-cell and LAK-cell reconstitution. Data are limited but appear to support the notion that immunological recovery is quicker after PBSCT than BMT.

ANTI-CANCER EFFECT

The study was updated as of December 1, 1989. The follow-up period is too short and the number of patients is too small, but the preliminary data have so far been encouraging: 6 of the 14 patients with leukemias or NHL are alive and well in unmaintained clinical remission during a median follow-up of 8 months (range, 2–24 months) after PBSCT. One of the two with neuroblastoma is surviving disease free for 15 months. Therapy-related death developed in two patients who underwent PBSCT at relapse. One developed local recurrence of NHL at 2 months. One with chronic CNS leukemia recurred in the CNS 7 months later and five developed systemic leukemia relapse 2, 3, 4, 7, and 11 months after PBSCT. The actual disease-free survival after PBSCT for eight patients with very high-risk leukemias/NHL and treated with MCNU-based high-dose chemotherapy without TBI is shown in Fig. 2. A follow-up study on the growth and endocrine function of children after high-dose chemotherapy without TBI and PBSCT is currently under investigation.

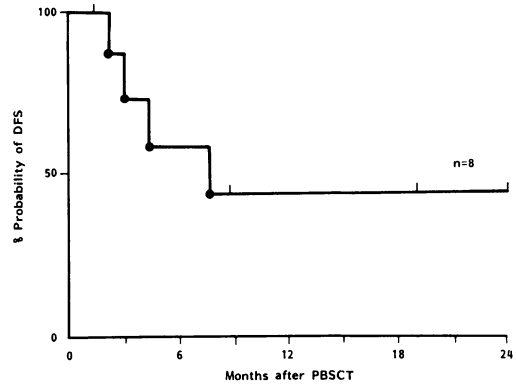

Figure 2. Actual disease-free survival after PBSCT for eight patients with very high-risk leukemias/NHL and treated with MCNU-based high-dose chemotherapy without TBI.

DISCUSSION

Dose-intensive therapies and PBSCT is still in its infancy, but our experience suggests that the collection of PBSC is a practical clinical procedure in children, and

the age or body size of patients is not a primary limitation for leukapheresis. Our clinical trials were conducted on patients considered to be at high risk of relapse or with advanced resistant cancer, but morbidity and mortality associated with this procedure was small compared to those with allogeneic BMT. The risks of life-threatening infections increase as cytopenia persists following BMT. We did not use a protective environment, but both the absence of GVHD and rapid recovery of granulopoiesis make PBSCT far safer than allogeneic BMT. In our study without leukemic purging procedures, the pattern of recurrence suggests that stem cell grafts obtained by our procedure are not appreciably contaminated with residual clonagenic leukemic cells. Although it is too new to yield any conclusion, our study suggests that PBSCT offers a roughly equivalent potential to allogeneic or autologous BMT for curing children relapsing from leukemias/NHL.

The indications for PBSCT have not yet been defined. In this study patients are eligible for PBSCT because of their poor prognostic features, and the preliminary results may not extend to other patients at lower risk of relapse. However, merely curing children with cancer may no longer be adequate and the final goal of our study is to increase the rate of cure while diminishing the frequency and severity of the late effects of therapy. From this point of view, PBSCT is likely to be most effective in patients with a good performance status in remission, when used as a primary upfront therapeutic regimen, with the aim of intensifying therapy and shortening treatment. Our data provide a basis for the prospective study of such strategies with a larger number of patients at an earlier stage of their disease. However, stem cells appear to have a limited proliferative capacity and are not able to divide indefinitely. Decreased stem cell content and self-renewal capacity in the recipient, that is donor cell dose dependent, have been reported after BMT (13). To help define the role of current therapeutic options - which include intensive chemotherapy, allogeneic and autologous BMT, and PBSCT in children with relapsed leukemia or in children with high-risk leukemia/NHL in first CR - a randomized clinical trial is needed.

When relapse occurs after PBSCT, it is not possible to determine whether this is caused by residual leukemic cells in the patient or leukemic cells in the transplanted graft. As so far no data have been available as to how many leukemic cells are essential to be clonagenic and to induce the recurrence of leukemia upon reinfusion into patients, there is no clear solution to this problem. The mere presence of malignant cells in the circulation per se does not preclude the possibility of performing PBSCT, and so the efficacy of the leukemic-cell purging procedure must be documented before incorporation into the PBSCT protocol. The final resolution must rely on a clinical study on a large scale.

FUTURE PROSPECTS

PBSCT is a safe and effective treatment modality in childhood cancer, but only a few patients may become long-term survivors with currently available pre-transplant conditioning regimens. We may have reached the limitation of very intensive treatment. Incorporation of two-step PBSCT with different conditioning regimens into the therapeutic protocol is our future interest to improve the clinical results. There has been a considerable interest in the *ex vivo* use of cytokines such as IL-3 and IL-6 for the induction of proliferation of stem cells and the consequent enhancement of

hematopoietic reconstitution in patients with a low progenitor yield. The augmenting effect of these cytokines on immune function may be of value in delivering resistance to post-transplant cancer recurrence and the need for research is urgent.

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VARIED PROBLEMS ASSOCIATED WITH IMMUNOCOMPROMISED HOSTS

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ABSTRACT

We investigated 332 children with malignancy who were admitted to our University Hospital from 1966 to 1985 and examined the annual changes in the main cause of death in 64 autopsied children with leukemia. It was found that, recently, infection has been the most important factor and the main cause, but the increase in fungal infection is also drastic. Considering this situation, we introduced an oral combined administration of high-dose amphotericin B syrup and trimethoprim-sulfa-methoxazole for the prophylaxis of infections at the time of remission induction, and this regimen was found to be effective for protection against bacterial and mycotic infection in children whose neutropenia was in the range of 1000/μl to 100/μl. We also studied the effects of the nutritional condition on the immunological function of children with leukemia undergoing intensive chemotherapy. We found that the concentration of total serum protein tends to decrease after the induction therapy, and there were decreases of albumin, prealbumin, and transferrin, in particular. The changes were in accordance with the decrease in immunoglobulin. Lentinan, the immunomodulator, was intravenously administered every 2 weeks to the children to whom intensification chemotherapy was repeatedly given, and changes in the subpopulation of peripheral lymphocytes were followed. Restorations of PHA responsiveness and a population of killer T cells were found in children receiving Lentinan.

INTRODUCTION

The treatment of childhood leukemia has made a great deal of progress over the last 30 years. These advances have been achieved in large part by the use of intensive multimodality treatment regimens. However, this intensive therapy causes myelosuppression and immune deficiency states in the patients, and consequently there

have been many patients who have died of hemorrhages and severe infections (1). It cannot be overemphasized that the recent good results with intensive chemotherapy in the treatment of children with acute leukemia have been obtained in cooperation with the progress of supportive therapy.

The management of patients with bleeding manifestations has improved remarkably, because of the introduction not only of platelet transfusions for the thrombocytopenia frequently encountered in leukemic patients, but also the supplementary and prophylactic treatment of coagulation disorders in association with the recent growing understanding of the pathogenesis of DIC.

At present, the most important problems are the management of patients with severe infections who are in an immunocompromised state due to myelosuppression in leukemic patients undergoing intensive chemotherapy.

We present in this symposium first the historical changes in the causes of death of children with acute leukemia whom we have treated and then explain the various management programs on immunocompromised children that we have tried.

HISTORICAL CHANGES IN THE MAIN CAUSE OF DEATH IN CHILDREN WITH ACUTE LEUKEMIA

Three hundred and thirty two children with malignancy were admitted to our University Hospital from 1966 to 1985. Of these patients, 169 (50.9%) had leukemia. Autopsies were performed on 97 children with malignancy and 64 of them were leukemic. Our colleagues, Dr. Kato *et al.*, investigated the main cause of death, in these patients with acute leukemia, using autopsy findings, and classified them as tumor death, bleeding, and infection (2).

In order to clarify the relationship between treatment modality and historical changes in the cause of death, the total study period from 1966 to 1985 was divided into three sections; the period before 1971; the period from 1972 to 1978, when platelet transfusions started to be used and daunomycin and cytosine arabinoside were introduced to protocol studies of TCLSG; and the last period, from 1979 to 1985, when prophylactic irradiation for CNS leukemia was routinely used and prophylactic control of infection using antibiotics at remission induction was started.

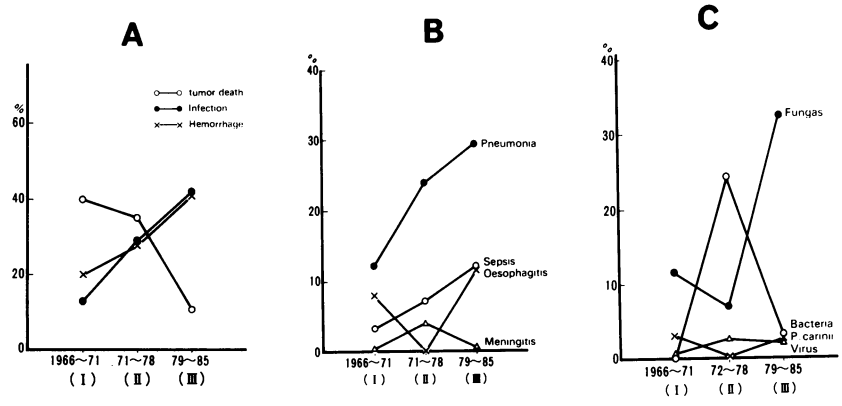

Figure 1. Changes in (A) main cause of death diagnosed by autopsy, (B) kinds of infections, and (C) pathogens of infections of children with leukemia.

The changes in the main causes of death of children with acute leukemia for each period are shown in Fig. 1A. Tumor deaths as the main cause have remarkably decreased from year to year, and instead death due to bleeding and infections has increased recently. Fig. 1B shows changes in the kinds of infection in children with malignancy for each period. Recently, the number of pneumonia and sepsis occurrences has increased remarkably and infection of the esophagus, which may be due to fungus, also increased in the last period. As for changes in each period with respect to pathogens of infection, the number of patients who died of bacterial infection increased in the second period, probably because of the introduction of more intensive chemotherapy after 1972 than had been used before (Fig. 1C). Finally, in the last period, when infection control using antibiotics was introduced, the number of bacterial infections reduced remarkably, and instead fungal infection increased drastically. The remainder were a small number of *P. carinii* and viral infections.

The above-mentioned changes in the main causes of death of children with acute leukemia suggest to us the importance of the control of infections in the management of these children. In fact, here lies the point, in that the infections at remission induction or maintenance often result in death of the patients, and infections remarkably prolong the treatment period, even if the patients do survive, and they may cause a relapse later. Because of this, many institutions have recently introduced infection control using a protected environment and prophylactic antibiotics during remission induction (3,4).

Table 1. Methods for the prevention of infections.

1.	Room and equipments		
	Formalin gas (24 hours)		
2.	Alimentary tract		
	(1)	Polymyxin B:	1,500,000~3,000,000 U/day
	(2)	Kanamycin syrup:	30 mg/kg/day
	(3)	Amphotericin B syrup:	200 mg/day
3.	Respiratory tract		
	Inhalation with		
	(1)	Kanamycin:	10 mg/day in 5% dex. sol.
		Amphotericin B:	1 mg/day in 5% dex. sol.
4.	Oral cavity		
	Gurgling with		
	(1)	Amphotericin B sol.	50 mg in 5% dex. sol.
5.	Skin and mucous membrane		
	Cleaning with 5% chlorhexidine cream		
6.	Foods		
	1400 W 30 sec. by electric range		

PROPHYLACTIC CONTROL OF INFECTION USING A COMBINATION OF ANTIBIOTICS

At the beginning of the second period we used a room sterilized with formalin gas for protected isolation of patients who were presumed to become granulocytopenic for a long period during remission induction (5), and later this was changed to a laminar air flow room using HEPA (high-efficiency particulate air). The patient's body

was kept clean by antiseptic solution every day; they were given sterilized foods and prophylactic antibiotics were administered as shown in Table 1. We used oral administration of polymyxin B, kanamycin, and amphotericin B syrup for the sterilization of the alimentary tract and inhalation of an injection solution of kanamycin and amphotericin B for the respiratory tract (5).

Table 2. Relationship between neutropenia and infection rate.

Neutropenia(/uL)	Rate of infection days	Prophylactic	Control
N≧1000	TDHS (days)	164	643
	TDI (days)	12	50
	RDI (%)	7.3	7.8
1000>N≧500	TDHS (days)	97	174
	TDI (days)	2	40
	RDI (%)	4.1	40.0
500>N≧100	TDHS (days)	284	167
	TDI (days)	29	76
	RDI (%)	10.2	45.5
100>N	TDHS (days)	360	224
	TDI (days)	163	168
	RDI (%)	45.3	75.0

(TDHS : total days of hospital days, TDI : total days with infection, RDI : rate of days with infection)

Table 2 shows the relationship between the degree of neutropenia and febrile days due to infections. The rate of infection in the prophylactic group, neutropenic periods from 1000 to 100/μl of granulocyte counts, was significantly reduced compared with a control group. Moreover, the advantages of the introduction of prophylactic antibiotics were useful not only to control the infections themselves, but also to improve the rate of remission induction. For example, we have often experienced such intractable patients with acute leukemia that we could not eliminate leukemic blasts completely from bone marrow during the induction therapy, but we could not continue to use anticancer drugs, because of severe neutropenia. However, we could get complete remission for these intractable patients by continuing the intensive chemotherapy, despite severe neutropenia, in the security that we would be able to protect them from infections using prophylactic antibiotics and a protected environment (5). Thereafter, this trial has been promoted by further studies of the research group supported by the Ministry of Health and Welfare of Japan (6).

Recently, we have used a combination of trimethoprim-sulfamethoxazol and amphotericin B based on the theory of selective antimicrobial modulation instead of a combination of the previously mentioned three drugs, and we found that this new protocol, using the latter regimen, gave similar results. Subsequent to this, we thought that this regimen was not enough to control fungal infections, which have remarkably increased as the pathogens during remission induction. So we studied the prophylactic effect of a large dose of per oral amphotericin B syrup for the fungal infection, as shown in the following regimen.

Patients were randomly allocated to one of three prophylactic regimens: group A, AMPH 100 mg/kg/day; group B, AMPH 50 mg/kg/day; group C, AMPH 50

mg/kg/day and the inhalation of AMPH and kanamycin. In addition, each regimen included oral trimethoprim-sulfamethoxazol (TMP-SMZ), 0.1 tablet/kg/day (7).

The serum concentration of AMPH at the oral dosage of 50 mg/kg/day and 100 mg/kg/day reached the maximum levels of 0.097 μg/ml and 0.113/ml, respectively, from 7 days after the administration and then persisted in the same concentration. These steady states were over the level of the MIC90 for *Candida albicans* of AMPH (0.09 μg/ml). The treatment started 1 week before an expected neutropenia of <500/μl and ended when the neutrophile count was >500/μl. Patients were cared for with or without protected environments and sterile foods. Owing to the difficulties in diagnosis of the mycotic infection, we used the criteria shown in Table 3.

Table 3. Criteria for the estimation of the prophylactic effects.

	Clinical effect	
	Effective	Ineffective
	1) No fever 2) Fever fell to normal within 5 days dy administration of antibacterial antibiotics (including the case of removal of fever within 5 days by change of antibacterial antibiotics or additional dosing)	1) Fever was not alleviated despite administration of antibacterial antibiotics, or change of the drug, or additional dosing
Mycotic count* Mycological effect		
Decreased Eliminated Negative unchanged — Effective	Effective	Ineffective
Positive unchanged Increased Fungal replacement — Ineffective	Ineffective	Ineffective
Unknown — Unknown (including cases not tested)	Unknown	Ineffective

* Cultered sample
Blood, Urine, Sputum

▨ : Overall effect

Table 4. Ratio of febrile days to the total days during prophylaxis by neutrophile level.

Neutrophil (/μℓ)	Prophylactic Regimen		
	A (n=24) 1484*	B (n=58) 3009*	C (n=35) 2019*
>1.000 %	2.6	3.1	3.2
1.000—500 %	2.7	3.5	3.6
500—100 %	4.2	9.9	3.4
	**	***	
100—0 %	40.3	26.2	24.3

n : Number of patients * : Days on study, dys.
χ^2 : **P<0.01 ***P<0.001

The clinical, mycological and overall efficacy rates were superior in both groups A and C compared to group B. Also, as shown in Table 4, the ratio of febrile days to total days of study was significantly lower in both groups A and C compared with B in the neutrophile count range 500 > N > 100. The comparison of frequency of infections during prophylaxis between each group revealed that there was no difference

in the frequency of sepsis among the three groups, but in terms of the type of infection, no skin infections and digestive tract infections occurred in group A and minor infections were most frequent in B. No disseminated fungal infections were observed, but we observed extensive local infection caused by *Candida albicans* in one patient in group B and by *Aspergillus* in group C. As a whole, the lowest episode of documented infection was found in group A.

From these findings, we presume that this kind of prophylactic regimen can protect children with leukemia who become neutropenic by intensive chemotherapy from fungal infection, but it seems that an endogenous bacterial infection cannot be prevented with this prophylactic regimen if a patient's neutrophile count falls below 100/μl. More effort must be made to explore how we can control the infections of such patients who are severely neutropenic below 100/μl.

CHILDREN WITH LEUKEMIA AND THEIR NUTRITIONAL STATUS

The nutritional status of cancer patients is a complex problem. Intensive chemotherapy or irradiation itself is most striking for its transient but significant effect on appetite and tolerance of oral alimentation, resulting in the lowering of the nutritional state. On the other hand, the appetites of children with acute leukemia are stimulated by adrenocorticoid therapy and they often become obese.

There are few reports as to whether poor intake of food influences the condition of children with leukemia. Our colleagues, Dr. Kamiya *et al.*, attempted to assess nutritional status by studying the changes in the nutritional and immunological parameters of patients undergoing intensive chemotherapy and CNS irradiation (8). The subjects of this study were nine children with acute lymphoblastic leukemia treated by the tenth protocol of TCLSG, eleven children with ALL treated by the eleventh protocol of TCLSG, and three children with ALL. The protocol of ALL consisted of prednisolone, vincristine, L-asparaginase, and CNS irradiation. On the other hand, the protocol of ANLL consisted of adriamycin, cytosine arabinoside, prednisolone, and vincristine.

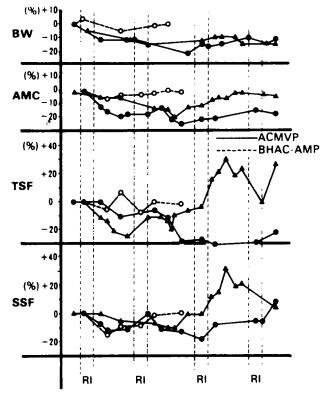

Figure 2. Change of anthropometric value during remission induction of children with ANLL.

Anthropometric values were obtained for body weight, Kaup's index, triceps and subscapular skinfold thickness, and arm muscle circumference, and intake of energy was also calculated. Body weight, Kaup's index, and skinfold thickness increased in the children with ALL who were treated by the protocol including corticosteroid hormone, and their energy and protein intake also increased. On the other hand, the children with ANLL showed a decrease in the above-mentioned anthropometric values after every course of remission induction as a whole, except for one case who had received adrenocorticosteroid therapy after a second course of remission induction because of therapy for peripheral neuritis (Fig. 2).

Changes in albumin, prealbumin, and transferrin as nutritional parameters and IgG, IgA, IgM, C3, and C4 as immunological parameters during remission induction were also studied (Fig. 3).

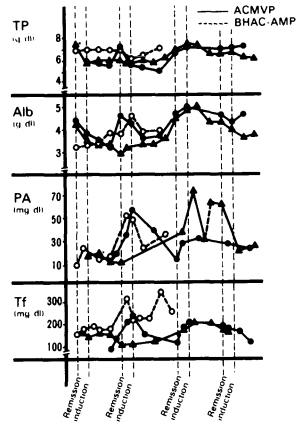

Figure 3. Changes of serum protein during remission induction of children with acute leukemia (ANLL).

It is said that protein such as albumin has a long half-life and reflects a decrease of protein in the body. On the other hand, the half-lives of transferrin and prealbumin are short and their values change in the protein intake of food. Figure 3 shows the decrease of the concentration of total protein and albumin just after remission induction and recovery before next induction. The changes in prealbumin were more remarkable, as this protein was influenced by the direct intake of food (9). These changes were found in the children with leukemia, regardless of the subtype, but more remarkably in the children with ANLL. The immunoglobulin level decreased definitely as the courses of chemotherapy were repeated, but no apparent changes were observed for the level of serum complement, C3 and C4 (9). Previous studies have demonstrated that the values of immunoglobulin and complement are subject not to the influence of the intake of nutrient but to infection. Moreover, it is said that the disturbance of nutrition exerts a bad influence upon cellular immunity rather than humoral immunity. So, we think that studies on the effect of nutrition on cellular immunity should be examined in the future.

The exact indication for supplemental alimentation by either central venous or enteral hyperalimentation remains unclear (10). At present, hyperalimentation has not yet been shown to improve the survival of cancer patients in any randomized controlled

study. But recently, in a prospective, double-blind, randomized trial comparing standard TPN to a branch-chain modified amino acid TPN (BC-TPN) solution, patients in the latter case had improved nitrogen retention, reversal of skin test allergy, elevation of absolute lymphocyte count, and improved transferrin levels when compared to those receiving the standard TPN (11). In the future, a similar comparative study should be planned in leukemic patients undergoing intensive chemotherapy.

EFFECT OF LENTINAN ON THE LYMPHOCYTE FUNCTION OF CHILDREN WITH ACUTE LEUKEMIA UNDERGOING MAINTENANCE AND INTENSIFICATION THERAPY

As for treatment of leukemia, once patients are induced to complete remission, the control of relapse is the most essential goal, and therefore intensive chemotherapy has been introduced in patients even during complete remission. Consequently, it has been reported that they are immunocompromised hosts and, in fact, a significant number of children have died of severe infection, even in remission. We were interested in whether these immunocompromised children with acute leukemia during maintenance therapy could be immunologically restored with such drugs as immunomodulators.

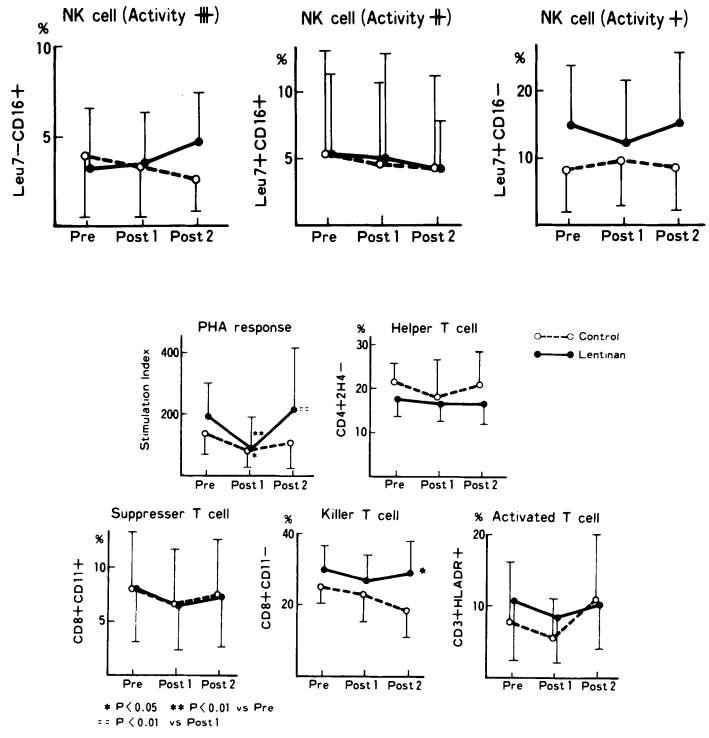

Figure 4. Changes of immunological parameter of pre- and post-intensification of children with acute leukemia who are placed on a regimen of Lentinan (2).

These studies attempted to observe changes of lymphocyte subpopulations in

patients undergoing chemotherapy and receiving Lentinan. It has been reported that Lentinan could restore PHA-induced blastic and immunoglobulin production of the lymphocytes from cancer patients in whom they were originally shown to be decreased (12). Thus, we examined whether the immune status of children undergoing intensification and remission may be modulated by the administration of Lentinan. Two milligrams of Lentinan were administered intravenously every 2 weeks to the children with ALL to whom intensification was repeatedly given by the TCLSG eleventh protocol.

We measured the PHA blastogenesis, the percentage of helper T cell (CD4+2H4-), suppressor T cell (CD8+CD11+), Killer T cell (CD8+CD11-), activated T cell (CD3+HLAD R+), and NK cell (Leu7-CD16+, Leu7+CD16+, Leu7-CD16-) at the time just before (pre), after the intensification (post 1), after several administrations of Lentinan, and just before next intensification (post 2). These values in the patients were compared with those of control patients who were treated under the same protocol. Figure 4 shows that PHA responsiveness was demonstrated to be significantly increased at the time of post 1 and post 2. Killer T cells were also found to be significantly increased as compared with the control group after the administration of Lentinan for 8–10 weeks. It is necessary to be careful when interpreting and drawing a conclusion from this phenomenon but these preliminary studies may suggest that Lentinan may have some immunomodulation for children with ALL. A similar comparative study is currently underway at our research group.

CONCLUSION

Advances in the therapy of leukemia have been obtained by introduction of very intensive chemotherapeutic schedules that are highly myelosuppressive and are otherwise toxic. Consequently, knowledge and skill for supportive therapy are required to treat children with acute leukemia who are immunocompromised. Further efforts should be made to create an immunological reconstitution for these children.

ACKNOWLEDGMENTS

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IV. The Total Care and the Patient Support System for Children with Leukemia

23

ACTIVITIES OF THE CHILDREN'S CANCER ASSOCIATION OF JAPAN

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ABSTRACT

The Children's Cancer Association of Japan was founded 20 years ago by parents who had lost their children to cancer, with crucial support from the late Mr. Taketomi Mori and the late Dr. Taro Takemi. Since then, the association has been providing total support for families and children with cancer in an attempt to improve their quality of life. The association's lobbying activities are essential for the public funding of medical research and the treatment of childhood cancer.

HISTORY AND OBJECTIVES OF THE ASSOCIATION

A group of parents whose children became victims of cancer or leukemia founded the Children's Cancer Association of Japan in October 1968. Their purpose was, and is, to provide every child with cancer with the best medical care and the best available medical facilities, regardless of the parents' social status or income, even though the service was too late to be available to their own children. The objectives of the association extend further: to provide needed counselling for the problems associated with medical care, to provide social and moral support for children and their families, and to help cured children to adapt to society. All activities of the association are focused on the above objectives.

The following is a brief history that should help in the understanding of our current activities. About 20 years ago, cancer was considered to be a problem of elderly people, and the fact that children might develop cancer was hardly recognized in Japan. In those days, the diagnosis of cancer amounted to a death sentence for children. Young parents of sick children were helpless in their fight against the disease. Despite their all-out efforts to save the children, the end results were the same: death of the children and large debts for medical expenses. Mental and financial difficulties resulting from the children's deaths often ruined the families, causing separation, or even suicide of parents, in addition to the loss of the children.

No organization was available in those days to help those families who had children with cancer. Recognizing this fact, NHK (Nihon Hoso Kyokai; Japanese National Broadcasting Corporation) launched a campaign on childhood cancer as an important social problem which triggered larger campaigns by the general news media. The late Mr. Taketomo Mori, president of Fukoku Life Insurance Company,

announced that he would donate one billion yen over 10 years, 100 million yen a year for 10 years, to the Japanese Ministry of Health and Welfare to help children with cancer. The late Dr. Taro Takemi, then the president of the Japanese Medical Association, gave his support in the organizing of the Children's Cancer Association of Japan through his influence on governmental organizations. Doctors and layman joined in the efforts.

Subsequently, the association was identified by the Japanese government as one of the organizations that make significant contributions to society. This recognition was achieved by the strong desire of the members of the association, as well as strong help from the late Dr. Takemi.

Following its establishment 20 years ago, the association played a crucial role in the governmental decision to defray the cost of treatment for children's cancer by using public funds. The association took the initiative and organized a joint campaign by the Japan Pediatric Society, the Japanese Society for Pediatric Surgeons, and the Japanese Medical Association, targeting the Japanese government to consider paying the cost of treatment for children with cancer. The decision by the government to fund research on children's cancer, as well as treatment for children, had a great impact on subsequent medical progress and relieved families of children with cancer from the financial burden and fears of being unable to pay for the best medical care for their children.

Moreover, the association's activities helped sick children and their families, and doctors and nurses, to work together as a team to fight against cancer, and also helped with the medical and social problems of the family arising from having children with cancer. The strong tie between the association and the medical workers was essential in establishing the Japanese Study Group for Children's Cancer on December 4, 1984. This study group incorporated those in the fields of pediatrics, pediatric surgery, and others involved in the care and treatment of children with cancer. Members of the study group have a strong commitment to the medical and social aspects of children's cancer, and Children's Cancer Association of Japan will continue to support the activities of the study group.

Board Committee; Chairman, Executive Directors and Directors
Auditors
Councillors
Medical and Scientific Advisory Panel
Committee for Registration System for Children with Cancer
    Local Centers at Hokkaido, Tohoku, Kantoh, Sinetsu, Kinki, Chugoku and Kyushu
Supportive members
Special supportive members
Administration
    Manager and staff
    Case worker
Medical Case Advisor
Financial Aid Committee

The Association has branch offices at Hokkaido, Niigata, Saitama, Nagano, Shizuoka, Aichi, Kansai, Kyushu

Figure 1. Organization of the Children's Cancer Association of Japan.

ORGANIZATION AND ACTIVITIES OF THE ASSOCIATION

Figure 1 shows the organization of the Children's Cancer Association of Japan. The members are (a) families of children who are currently being treated for cancer, (b) families who have lost their children, and (c) those who support the activities of the association. These members pay annual dues of 2000 yen or more. Affiliate members of the association include individuals, organizations, and private companies who support the association by contributing annual membership dues of 10,000 yen or more.

The activities of the association are primarily based on counselling and case work. These activities include a peer support system for parents and communication services.

1. Counselling of parents and children with cancer by case workers and medical doctors and scheduled counselling service at the main and branch offices of the Association (Fig. 1). All problems associated with children with cancer are addressed in these counselling sessions.
2. Providing social care and services. Recent success in the treatment of childhood cancer has created new problems for long-term surviving children. Emotional and social problems in family, school, and society, and also physical disability resulting from the medical treatment are addressed through the unique care service of the association.
3. Eliminating the discrimination against children with cancer and their families. In the Japanese community, cancer has often been linked with death, and children with cancer may be discriminated against in schools and other social environments. The association provides moral support for these children and their families, and attempts to improve the attitude of the public.
4. Helping families who have dying children or those who have recently lost their children to cancer, with warm care, and providing necessary assistance.
5. Communication services. (a) Providing information on children's cancer and advocating the necessity for early diagnosis and early treatment. The association helps families of children with a suspected diagnosis of cancer, including the selection of speciality hospitals, and also provides answers to questions from the families regarding medical care and specific medical facilities. (b) The association's newsletter, *Nozomi* (*Hope*), is regarded as an extremely useful means of total care service by the members of the association and related organizations. (c) Publication of the journal children's cancer. This scientific journal publishes papers by young members of the Japanese Study Group for Children's Cancer. The association provides the financial support for its publication.

THE FIGHT AGAINST CHILDREN'S CANCER

During the last decade, the number of deaths of children resulting from cancer and leukemia decreased significantly in Japan (Table 1). For example, a total of 1369 children died in 1987 compared to 1778 in 1979. During the same time period, the number of children treated for cancer under the National Medical Aid Program in Japan increased from 11,179 to 18,513 (Table 2). These trends indicate substantial

improvements in the treatment of children's cancer.

TABLE 1. The number of children who have died from cancer in Japan.

Year	Age of children 0–4	5–9	10–14	15–19	Total
1967	649 (7.7)	384 (4.9)	363 (4.5)	633 (6.1)	2,029
1979	513 (5.8)	472 (4.8)	352 (4.0)	441 (5.5)	1,778
1984	337 (4.5)	341 (3.9)	364 (3.7)	428 (4.9)	1,470
1985	295 (3.9)	340 (4.0)	388 (3.9)	447 (5.0)	1,470
1986	310 (4.3)	315 (3.9)	359 (3.6)	392 (4.1)	1,376
1987	298 (4.2)	268 (4.2)	362 (3.8)	441 (4.6)	1,369

Numbers in parentheses: Number of children have died from cancer per 100,000.

TABLE 2. The number of children treated under the National Medical Aid Program Service.

Year	In hospital	Ambulatory	Total
1972	3,706	-	3,706
1979	6,039	5,140	11,179
1984	7,680	7,316	15,005
1985	8,106	8,095	16,201
1986	8,394	9,292	17,686
1987	9,019	9,494	18,513

Source: Ministry of Health and Welfare, Japan

TABLE 3. The balance sheet of the Children's Cancer Association of Japan during the period beginning in 1968 and ending in 1988 and the budget for 1989.

	1968-1988	1989 budget
Income	(units, 10,000 yen)	
Public donations and subscriptions	148,570	6,820
Donations by supporting members	9,350	990
Donations by special supporting members	5,850	1,000
Subsidy from Japan Shipbuilding Industry Foundation	5,850	1,000
Interest	45,310	2,600
Total	**209,080**	**11,410**
Expenditure		
Financial aid to the children and their families	27,130	420
Financial support for medical research	33,260	1,730
Cost of registration of children with cancer	18,070	1,300
Case working, publications, investigations, etc.	40,810	2,760
Fund raising and administration costs	36,290	2,730
Symposium commemorating the 20th anniversary		2,860
Total	**155,560**	**11,800**

FINANCIAL ASPECTS

Table 3 shows the sources of income of the Children's Cancer Association of Japan and the major expenditures. The major source of income is donations from the public, and contributions by the members of the association, and interest on funds. Major expenditures are divided roughly equally into (a) financial aid to the children and their families; (b) financial support for medical research and development; (c) the cost of registration of children with cancer; (d) case working, publications, and investigations; and (e) fund raising and administrative costs.

SUMMARY AND CONCLUSIONS

Recent developments in medical technologies for the treatment and care of children's cancer have been remarkable. In particular, public funding of the treatment has greatly advanced progress in these fields. Many children with cancer have been successfully treated and have entered adult life. Despite these successes, however, the goal declared 10 years ago at the time of the First International Symposium on Children's Cancer, that the deaths from children's cancer should be reduced by 50% during the next decade, has not been achieved. In many cases, we still lose children to cancer. The total care of children with cancer remains an essential issue. The association has the task of promoting total care, and also of helping surviving children and their families to normalize their family and social lives. These aspects are extremely important for the quality of life for affected children and their families. Further studies are needed in these areas.

CANDLELIGHTERS: PARENTS AND CAREGIVERS, FRIENDS AND ALLIES, WORKING TOGETHER FOR LIFE

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ABSTRACT

The medical and psychosocial successes that are well documented in childhood cancer arise from the unique collaborative relationship between pediatric cancer families and their caregivers. This relationship extends beyond the caring partnership in the delivery of treatment. The medical care team serves as advocate and ombudsman for the patient and family in meeting economic, education, and employment problems, and in addressing discrimination, where found, against childhood cancer survivors. Medical caregivers are assisted in their dual role by parent peer support groups within the Candlelighters Childhood Cancer Foundation network. These groups provide information, advocacy, education, and assistance. Through the friendship network created by participation in parent peer support groups, families receive: (a) empowerment to feel equal to the tasks set to them by the experience of having a child with cancer; (b) confidence that they can find solutions to problems of everyday living; (c) reinforcement in respect for their medical care team; and (d) the opportunity to advocate for the social and political changes needed to ensure the availability of the life-saving technology needed for their child as well as changes needed to combat discrimination against themselves and their children while in treatment or after cure.

INTRODUCTION

In 1950, 1% of our children with cancer survived; in 1986, over 61% of our children with a cancer diagnosis will survive their disease (1); in 1990, 1 in every 1000 20 year-olds will be a long-term survivor of childhood cancer (2).

We have obviously come a long way. Cancer is now a chronic, as opposed to a life-threatening, disease for the majority of our children. However, saving a child from death is a hollow victory if the survivor cannot have a life and a livelihood.

This paper will focus on the unique aspects of the partnership among caregiver and family, fostered and enhanced by the parent peer support group structure and its place in the continuum of caring.

THE PARENT PEER SUPPORT GROUP: A PROVEN ADJUNCT TO EFFECTIVE MEDICAL TREATMENT

Parents as the Most Significant Unit of Psychological Support

Professionals engaged in the psychosocial support of children and adolescents with cancer agree that childhood cancer is a family disease and the family unit is the most significant psychosocial caregiver (3–7). Studies correlate the child or adolescent patient's favorable adjustment to the treatment and rehabilitation process with the positive or negative profile of social supports, understanding, open communication, and the ability of the parent/family structure to provide strong emotional and educational reinforcement (3–6).

Experiential parent peer self-help groups are recognized as an important part of the social support system (8,9) which has demonstrated its significance in enhancing the education, understanding and empowerment of the family in meeting the challenges posed by cancer in a child (10–16).

Candlelighters as an Experimental System

The Candlelighters Childhood Cancer Foundation (CCCF) is a nonprofit, educational organization. Its major support is from a cancer control grant from the American Cancer Society. CCCF links over 300 parent groups within the United States, 30 groups in Canada, and 128 groups and contacts in other parts of the world, providing experiential sharing to families of children with cancer.

At its inception with three groups in 1970, Candlelighters was not an experiential peer support organization. Our initial purpose was advocacy, specifically to provide support to our medical caregivers by lobbying for increased appropriations for research and clinical trials. After our first successful foray in the halls of Congress, we realized that we had had the joyous experience of socializing with peers that countered the isolating effect of our child's disease. We had selfishly backed into peer support.

Sharing through this friendship network provides a range of examples of how people in the same situation successfully manage their lives: jobs, insurance, vocations, education, grandparents, friends, teachers, neighbors, siblings, and the ill child. It empowers participating parents to believe that solutions are achievable; life can still hold fun, discipline isn't a "No, no." Kids do live, grow up, get pregnant and provide you with grandkids; some of our children will die but their process of "living with their cancer" can enrich all the lives they touch (8,14,15).

One of the most compelling characteristics of the parent peer support group and network lies in the quality and motivation of the families who join such networks. From the beginning of the application of the peer support group concept to pediatric cancer families by Candlelighters almost 20 years ago, it was evident that the families who reached out beyond themselves to meet and work together were there because they wanted to be, not because they needed to be.

Those families who could share with others and form plans of action to deal with social and political problems impeding their children's cure obviously had the skills and resources to beat their problems all by themselves. Their joinder into a self help group network was motivated by a joy in socialization with others of common experience, their interest in advocacy, and their recognition that they "owed" their

affected peers who couldn't reach out and help themselves an obligation to be sure that they had done all they could to make sure resources were in place wherever and whenever those families needed them to help solve their practical problems.

For some families these "resources" available outside the peer support group meeting concept have included the opportunity to: (a) talk with other families anonymously through a hotline program; (b) receive newsletters that permit them to share vicariously in anticipating problems, sharing solutions, and understanding their possibilities and their child's; (c) have access to a "parent consultant" as part of the medical team; and (d) receive specialized bibliographies and materials.

CCCF fosters this continuum of experiential sharing through: (a) newsletters for groups, families, the professional care team, and youth patients and siblings, and other publications such as a parent group organization handbook, a bone marrow transplantation handbook (under revision), a visitor training program manual, and an annotated bibliography in English and Spanish of reviewed publications useful to parents, children, and caregivers; (b) leadership, group development, and advocacy training workshops and consultation; (c) pen pals linking families and patients who want to talk to someone who has overcome the problem they are currently facing; and (d) second opinions from a panel of medical volunteers at major pediatric cancer centers.

The key to CCCF success lies in its flexibility. CCCF recognizes that the experts in what families need locally are local families. Each group takes on the special fingerprints and profile of the parents and collaborative professionals networking together to bring a parent group into existence. Geography, economics, ethnicity – all are accommodated under this approach to organization and networking.

Advocacy: The Reinforcement of Empowerment

Empowerment, confidence, and friendship are the end products of participation in peer support groups. Peer support is the glue that holds families together, awakens them to their common interest, and makes possible the next form of parental expression – advocacy – the "teeth" or "enforcement" arm of empowerment.

Some of the "advocacy" programs available through CCCF nationally include:

1. **Insurance Ombudsman Project**

   Families/medical care providers provide CCCF with the diagnosis and proposed treatment, a copy of the definitions and exclusion language of the insurance policy, and letters or content of oral denials of coverage giving the bases for denial. CCCF, using volunteer legal and medical expertise, (a) prepares a patient profile of benefit for the procedures, matches it with the insurance contract language, and provides families and caregivers with relevant peer review, study report and AMA, USPDI materials to support insurance coverage; (b) drafts a "suggested" response letter fitting this data into the insurance contract's language; (c) arranges support letters from other institutions doing the procedure for the stated condition as backup for the treating pediatric oncologist; and if necessary, (d) sends a letter from CCCF stating our experience with the acceptance of and reimbursement for this procedure.

2. **Ombudsman program on employment and insurance discrimination**

This involves: (a) mediating, providing advice and arranging where necessary for local intervention by legal or medical personnel to attempt to resolve unfair discrimination in parental leave, employment, and insurance against families with children in treatment; (b) addresses the inability of our childhood survivors to get life and health insurance; (c) helps our children who are eligible to apply for waivers to enter the services, get ROTC scholarships, or enter the reserves or service academies; and (d) counsels families on how to get insurance for their surviving children.

3. **Ombudsman program on questionable and unproven remedies**

Primarily at the request of medical care team members, and less often families, and with the help of medical and legal volunteers, provides information and individualized scientific reviews critiquing "weird" and dubious remedies so that physicians can counsel their families more effectively in avoiding nonproductive therapies and in choosing productive therapies.

4. **Education Ombudsman Program**

CCCF has a cadre of volunteers who provide assistance in procuring appropriate assessments relating to our children with treatment related learning disabilities.

Profile and Evaluation of the Experiential Self-Help Group

The significance of parent peer support groups in the socialization, empowerment, and support of the family faced with a child or adolescent with cancer was studied extensively and reported on in the Chesler and Barbarian book, *Childhood Cancer and the Family: Meeting the Challenges of Stress and Support* (10–16). Professor Chesler's study of 48 groups in the CCCF network found that:
(a) experiential self-help groups act as a buffer against the stresses experienced by families of children with cancer, helping to prevent or offset some of the problems brought about by psychosocial stresses; (10,14–16) and (b) the empowerment skills learned through experiential peer support groups assist families in serving as advocates for and promoting the wellness of their cancer child, as well as in supporting the integrity and harmony of the family structure (10,13).

Further, and of particular relevance to medical professionals considering fostering the development of self-help groups in their hospitals, Drs. Chesler and Barbarin (10) found that participation in a self-help group did not encourage negative attitudes toward the treatment and doctors, intrude on physician-patient relationships, or increase parents' emotional burdens without professional staff controls to correct misinformation and prevent "inappropriate" exchanges. Their research confirmed that parents who actively participated in self-help groups were more positive toward the medical staff than parents who were not active and that group participation was motivated by a need for support and for information that was not otherwise available (10).

Parent/Caregiver Collaboration – Advocates for Each Other

The dictionary defines an advocate as one who pleads the cause of another. Advocacy can be a nurturing parent seeking information on how to support a child in treatment and through the illness, group efforts towards changing policy or procedures in the treatment of children, individual court cases brought to protect a child's right to adequate treatment, lobbying for legislation to ensure medical care for children with cancer, lobbying to change insurance company policy precluding payment for participation in clinical trials, or expanding the funds to pay for care for the socioeconomically disadvantaged.

Allies in informing, supporting, and empowering the family to advocate effectively for their children include the medical, psychosocial, and education teams, as well as experiential parent peer support groups, which both reinforce parental competence and control and open the avenues for advocacy.

The medical care team's role as allied advocates focuses on the presentation of the best medical options to the family, the surveillance of the delivery of care, the provision of professional psychosocial support, and aid in tapping community resources as needed (13). In its best form the medical care team serves as an ombudsman for the child both during and after treatment, for example, in outreach programs to school teachers, nurses, and students to enhance the opportunities for their patients to receive the best in education (17); in provision of arguments to support insurance reimbursement for necessary treatment (18,19), or the documentation of cure needed for admission on waivers to the armed forces (20).

Family Physician/Pediatrician: Special Advocate

Another medical caregiver whose special relationship with the affected family impacts on facing the practical problems of medical management at home, adherence to stringent protocols, and quality of life is the family physician or pediatrician. This caregiver usually was the one who spotted the condition in the first place and referred the family into center-related treatment.

The primary care physician acts as a *gatekeeper* to curative or controlling care and enhances the specialists in many ways, including (a) reinforcement of suggested referrals; (b) keeping involved in the cancer care of the patient by continuing with well care and surveillance; (c) being available to help the family monitor for complications and side effects; (d) serving as a home-base medical ombudsman in sorting through complicated consent forms and medical issues.

The primary care physician also functions as *advocate and friend*: (a) working with the parents to dispel misconceptions in the community about what cancer does to this child's abilities in school or on the soccer field; (b) working with the families and child on special education needs; (c) discussing insurance reimbursement issues and employment issues (medical and parental leave; job opportunities for the child coming off treatment); (d) keeping the sense of normalcy and integration that flows from continued involvement in the cancer child's care.

Finally, the primary care physician may be the *key to keeping the affected child on the path of productive treatment* by (a) developing a collegial, collaborative dialogue with families to keep them from turning to "quack" remedies and (b) thinking through participation in controlled clinical trials for families who have exhausted other therapies.

Today, when more than two thirds of our children with cancer are expected to survive, families, caregivers, and peer support groups need to prepare our children for life after cure. This includes:

Taking a Close Look at the Realities of Survivorship
- What do our cured children expect?
- What are they becoming/can they become?
- What are their strengths and their weaknesses?

Identifying and Networking to Remove Obstacles to Full Integration into Society:

Understanding, anticipating, and providing practical solutions to:
- Insurance-medical, health, employment, and education problems
- The perceptions of family, self, others and society – affect on self esteem and self worth
- The need for long term medical surveillance

Surveys conducted by local Candlelighters network groups and a formal study by Mark Chesler at the University of Michigan have documented the positive and negative fears that our survivors say they have.

Negative experiences recorded include: long-term medical physical affects of cancer; insurance discrimination (life, health, and disability for the cured child), for example, inability to increase/improve benefits after diagnosis or to obtain new medical or life policies; exclusion from parents' new policies; limitations on payments for prostheses; school obstacles, for example, not being permitted to participate in physical education, placement in slow learners classes, dropping out of high school due to poor grades or due to absenteeism and learning disabilities; and discrimination in employment (21–26).

The universal problem of denial of access by our survivors to making the military a career has been substantially resolved by the adoption in March of 1986 by the Department of Defense of new guidelines for the admission of childhood cancer survivors (20). These guidelines provide that individuals who have a history of childhood cancer who have not received any surgical or medical cancer therapy for 5 years and are free of cancer will be considered, on a case-by-case basis, fit for acceptance into the Armed Forces (20).

Unfortunately, the military appears to be trying to reneg on their regulations by, in our opinion, trumping up reasons to discharge our cured children after induction. For example, if six people dropped from heat prostration after a 10 mile run, an attempt would be made to attribute the childhood cancer survivor's prostration to asthma related to cancer treatment.

TEACHING OUR SURVIVORS TO CARE AND ADVOCATE FOR EACH OTHER

As we have demonstrated, participation in experiential self-help groups provided

empowerment tools and training to parent participants (10,11,27,28). These are necessary skills for our new survivors of childhood cancer. But do they want them and how do they achieve them?

Interim analysis of a survey in progress by Chesler established a desire to be involved with other survivors and a willingness to serve as "buddies" and "advocates."

CCCF believes that a national collaboration between our group and late effects clinics is the first step toward establishing an advocacy and support network for our survivors. Some steps in such a collaboration would include the identification of survivors who (a) wish to serve as contact persons for the establishment of local groups; (b) are established in academia, jobs and professions to serve as "buddies" for new survivors exploring job, and education options; (c) could participate in development of a national publication to serve their interests; and (d) could advise and assist in the preparation and dissemination of a specialized survivors manual, which will be updated as our experience grows (29,30).

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25

WHO HELPS FAMILIES OF CHILDREN WITH CANCER? PARENT AND PATIENT SUPPORT IN THE U.K.

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ABSTRACT

Pediatric cancer is a "family" disease because the child's illness has major personal and social repercussions on parents, brothers and sisters, grandparents, and the extended family. Some of the measures described in this article, adopted in the U.K. to counter these difficulties, may be useful in other countries/cultures; others may not. We have found that our symptom care team and, where appropiate shared care, have been especially helpful to our families. Charities are a *major* source of financial support.

INTRODUCTION

In the U.K. National Health Service (NHS) there are three tiers of patient care. Each individual/family chooses a *primary care team* (generally a family practitioner plus community nurses) located less than 10 km from their home, and they also have access to the *general/local pediatric unit* (usually less than 50 km from home) and to tertiary referral centers for the management of more complex disorders. As in Japan and other countries where pediatric oncology is now an established subspeciality, most (currently 85%) of U.K. children with cancer are referred to a tertiary treatment center. Our hospital, together with the units at St. Bartholomew's Hospital and the Royal Marsden Hospital, caters for the needs of the nine to ten million population of the Thames regions (greater London and southeast England).

The advantages of tertiary referral are widely acknowledged: (a) the multidisciplinary teams at these centers have the experience and facilities to provide the intensive medical and psychosocial support that families need; (b) survival is better and long-term morbidity is less than when patients are treated inexpertly; and (c) it makes economic sense to concentrate scarce resources. However, there are also disadvantages: (a) families are far from home and friends; (b) long, tedious, and repeated journeys are expensive and exhausting; and (c) the family tends to become dependent on the tertiary center, at the expense of the primary care team and the

local pediatric unit. In our department we try to attain "the best of both worlds" by emphasizing shared care (Fig. 1) wherever possible. In practice this means that much of the continuing chemotherapy is supervised from and administered by staff at the local pediatric unit, under the direction of a specified consultant. Of course, shared care only works if the local unit is committed to the scheme, and confident in what it does; In a few instances shared care just does not work and all treatment is carried out at our hospital.

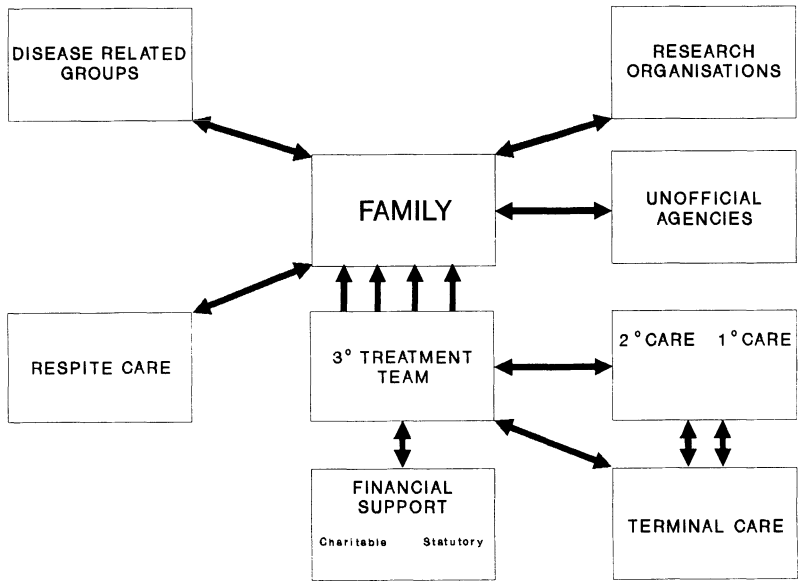

Figure 1. Who helps families of children with cancer? UK 1989.

A number of "external" agencies interact to provide the extra practical and moral support that parents need if their family is to survive, relatively intact, the experience of having a child with a shocking, debilitating, and life-threatening disease (Fig. 1). Most of the agencies have emerged over the past few years, reflecting increased awareness that medical and nursing treatment is simply not enough. There is no organizational "suprastructure"; the individual agencies usually operate through one of the 20 UK pediatric oncology centers, according to need.

AGENCIES SUPPORTING FAMILIES

For most parents, the time of diagnosis is the hardest of all. They are stunned by the news that their child has cancer and are unable to react normally to the practical differences the diagnosis inevitably makes in their lifestyle. They also need urgent moral support, to help them sustain their child – and the rest of their family – through the crisis. Life-threatening treatment complications and, of course, disease

relapse are other times of major stress. Terminal disease is most often managed at home. The child's initial major concerns are (a) fear of separation from parents and (b) the unpleasantness of the disease and the side effects of its treatment. His or her attitudes are age dependent and vary throughout the illness.

At the **tertiary referral center**, the family meets the doctors and nurses who will carry out the child's treatment, as well as a play therapist, a teacher, and, in some cases a consultant psychologist. They are also introduced to a) the medical social worker, funded by a charity called the Malcolm Sargent Cancer Fund for Children, and (b) a member of our *symptom care team* (either a doctor or senior nurse), funded by another charity – the Rupert Foundation.

The Malcolm Sargent Fund provides financial help for the extra expenses incurred by parents because of the child's illness and treatment, e.g., accommodation at or near the hospital or at home. The medical social worker can help parents discuss the way they feel about the child's illness and treatment, and the effects upon the family of the inevitable stresses and strains. Any problems experienced by siblings or other members of the extended family may also need discussion with the medical social worker, who can arrange counselling or act as a liaison with appropriate helping agencies. It is important to recognize that the medical social worker is the only individual in the treatment team who is there specially to help *parents*, rather than children.

The principal role of the symptom care team nurse/doctor, at this stage of the illness, is to provide continuity between our pediatric oncology center and the referring pediatric unit, general practitioner, community nursing services, and school. Each member of the team has a car, equipped with a radio telephone, and is prepared to travel to the child's home to make contact with local agencies.

Two other charities provide *financial help*: the Family Fund, supported by the Rowntree Trust, provides financial assistance where a specific facility is lacking in the home; for example, a family without a washing machine will have difficulty in coping with post-chemotherapy emesis and a grant for purchasing a machine may be available through this fund. The Family Holiday Association, yet another charity, help the families to take much needed breaks later in treatment. Some financial help is also available direct from government: (a) the *Attendance Allowance* is payable if the patient needs more care, from his parent, than a healthy child of similar age. (b) A mobility allowance may be payable to the families of over 5's who cannot walk. (c) An invalid care allowance may be available to a parent who is unable to work because of the child's illness. Each of these government grants is reassessed annually.

Two types of *respite care* are available. When their child is in the hospital, parents may be able to stay at one of the Homes from Home provided by the Sick Children's Trust, which, like the Ronald McDonald houses in the United States and elsewhere, aims to accommodate the whole family. This arrangement is a particular advantage when the patient's stay is protracted or multiple admissions are needed. Besides accommodation, the trust aims to provide a "respite" for fatigued and worried parents. A shared bottle of wine in the home's sitting room, in the evening, with other families may be more therapeutic than several talks with the doctor! The Family

Holiday Association and the CLIC (Cancer and Leukemia In Children) charity provide free holiday accommodation for families who need a break but cannot afford one. Several *disease-related groups* now exist in the United Kingdom including the Neuroblastoma Society (see Fig. 2), the Retinoblastoma Society and the Marcus Nunn Histiocytosis Association. They (a) raise money for research, (b) produce information booklets giving details of specific types of children's cancer, and (c) offer telephone contacts with parents who have been through similar experiences. Not all parents want or need these kinds of contact, but sometimes strong and supportive relationships develop. Information booklets are also available via the major research charities, e.g., Leukemia Research Fund and through the U.K. Children's Cancer Study Group (UKLCSG).

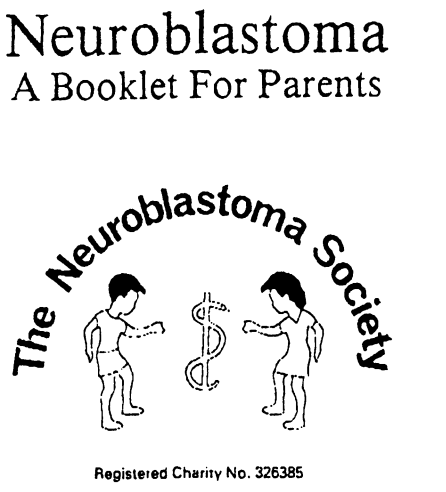

Fig. 2. A booklet for parents: Neuroblastoma Society.

The main *research organizations* in the United Kingdom are the Leukemia Research Fund, the Imperial Cancer Research Fund, the Cancer Research Campaign, and the Medical Research Council. Their main role is to fund the vast majority of basic cancer research in the United Kingdom but they also provide extra doctors (research fellows) and nurses (e.g., for bone marrow transplantation), and the Cancer

Research Campaign supports the UKCCSG office and data center in Leicester. They also provide literature for parents about the different kinds of child cancers.

Almost all families become involved with *unofficial agencies*. We know that at least 70% of our families use some form of "alternative medicine," e.g., special diets and vitamins or "spiritualists." These contacts are not discouraged, but we ask parents to be open with us so that potentially harmful regimes can be avoided. The media, especially newspapers, may also become involved.

The symptom care team plays a major role in *terminal care*. The frequency of home visits and telephone calls increases, and the team make a particular effort to involve the family practitioner and local nursing personnel. There are one or two children's hospices in the country, which provide invaluable respite care facilities for some families, but they are very rarely used in terminal disease, the parents' preference being to manage at home if at all possible. Sometimes, nurses trained in the terminal care of adults (via the MacMillan Fund or Marie Curie Fund) are involved, but they usually seek guidance from our team. Because of his or her intensive work with children who are terminally ill, the symptom care team nurse will usually be able to initiate some bereavement work with a family. The symptom care team and the medical social workers run a group at the hospital offering follow-up support to bereaved parents. There are also national organizations specializing in bereavement work, e.g., Compassionate Friends.

In summary, there is a wide range of potential support for parents. The medical social worker and the symptom care team nurse/doctor are central figures and work closely with the treatment team. Not all agencies are, of course, needed for each case, but the variety available means that most contingencies can be covered.

CARE OF THE CHILD AND FAMILY

Our hospital caters mainly for young children (the median age of our patients is around 4 years). There is, therefore, a great emphasis, in the ward and in outpatients, on play therapy, with regular advice from a senior staff psychologist. Play therapists and intravenous team nurses work closely with the psychologist to try to help children overcome needle phobia. For older children, programmed school lessons are an important part of the day.

Although there is often resistance to the idea, most parents eventually agree that it is better if the diagnosis is revealed to their child and openly discussed. Of course, children of different ages require different levels of explanation (the word *cancer* doesn't mean much to a 3 year old!). We feel that the pluses of this policy far outweigh the minuses. The biggest advantage is that all concerned with the child's care, particularly the family, can be open and honest with him or her. Deception, and the ensuing distrust, is one of the greatest "enemies" of effective care. The more the children know, the more they are able to take part in their own treatment. Children of 5+ years often help with their own care, especially mouth care and indwelling catheters. Because of a more liberal attitude to hairstyles these days, alopecia does not

cause the anguish that it used to, but a wide range of wigs is available through the NHS. When the child is in hospital, visits from siblings and friends (school friends, members of cub scouts and brownie guide packs) are welcomed where practicable to try to reduce the child's sense of isolation from the "normal world." Parents are encouraged to bring in familiar toys and books, and to work with the play leader and teacher to entertain their children. We also encourage parents to import familiar items of bedding, e.g., duvets and colorful pillows. It is never possible to "normalize" the life of a child with cancer on treatment, but it is valuable to try to inject homelike familiarity into the child's surroundings. It is particularly important to maintain a routine that the child can recognize. Otherwise the pain and uncertainty of hospital admission are aggravated by boredom and inactivity.

The first priority when the child enters the hospital is the confirmation of the diagnosis. Then the family start their "journey through the disease." First, the cancer subtype and stage are established and a treatment plan is formed. Parents are shocked and bewildered by all that is going on and need several prolonged discussions, often going over the same information two or three times. For continuity, and so that parents feel they have alternative sources of information (especially when they think the doctor may be "too busy" to talk), it is vital that these meetings are attended either by the medical social worker or a senior nurse. Most parents need reassurance that (a) there really *is* a chance of cure; (b) that the disease wasn't their fault; (c) that any delay in diagnosis is unlikely to have influenced the prognosis; (d) that pain can be controlled; and (e) that they are being "told the truth." We stress that the family will (a) be fully informed and consulted at all stages of the child's treatment; (b) that practical, e.g., financial, help is available through the medical social worker and the symptom care team and (c) that, for everyone's sake, it is best not to conceal the diagnosis from the child, siblings, family, and friends. As treatment progresses, and the parents can see symptomatic improvement, they start to absorb more information via repeated conversations with staff, the literature they are provided with, and contacts with other parents on the ward. Informal weekly "tea meetings" for parents are held that are also attended by the play therapist and the i.v. therapist. These meetings provide an emotional outlet for parents, an opportunity to share their knowledge, and sometimes an opportunity to question the rationale or style of nursing or medical practice on the ward. More formal parent groups have met away from the ward under the joint chairmanship of the medical social worker and a senior psychologist.

When the child is discharged from the hospital to continue care as an outpatient parents often feel "insecure." They do not yet trust their primary care team and need reassurance that they can ring the ward or the doctor on duty at any time for advice. A member of the symptom care team allocates herself to the family and takes the initiative with telephone contact. She will also try to visit the home within a week or two of the diagnosis. These visits are invaluable for (a) assessing just what facilities are available, (b) to enable parents to talk in more relaxed circumstances than are achievable in the hospital, and (c) to link up with the family practitioner and community nurse. By spending time talking to parents in their home, in more relaxed circumstances than are ever achievable in the hospital, team members can identify

parents' real worries and help to start counteracting them. The visits are followed up by frequent phone calls (with a member of the team available 24 hours per day, every day) and by repeat visits.

If the child remains in remission and comes off treatment, a lot of the "old worries" resurface. The possibility of disease relapse, without the "insurance" of treatment, is the principle concern, and the family want to know that they will continue to have regular contact with the treatment teams. Once again, the symptom care team member can be pivotal in providing reassurance. As time goes by, the fear of disease relapse recedes and parents start to worry more about "late effects" of treatment, especially puberty, sexuality, fertility, and the function of the organs that they have been specifically warned might be damaged by chemotherapy and/or radiotherapy. They also need reassurance that their younger children will not be affected by cancer: Sometimes, parents are only reassured if these siblings are clinically examined just before the age that the patient was diagnosed. In some cases, where concern is very acute, we will carry out abdominal ultrasound or urinary VMA/HVA measurements.

The parents of the 30–40% of children who relapse say that this phase of the illness is "even worse" than the time of diagnosis. At this time, the most important single question is, "Is cure, or extended survival, still possible?" Again joint discussions with doctors and the social worker/nurse to whom the family are closest are crucial and may need to be repeated. If medical cure is considered possible, a "curative intent" treatment plan is established and followed. If the likelihood of cure is considered to be very low, the team and family need to decide whether to embark on experimental (phase III) chemotherapy with a possible benefit to that patient, in terms of a remission of moderate length, or ought to stop anti-cancer treatment and focus on "symptom care." This time, families will often want to seek second opinions, either within the United Kingdom or abroad. It is rarely necessary for the child to travel, and we invariably willingly agree to send the case records, scans, and pathology slides. Appearing "obstructive" at this time may undo a great deal of the good that has been achieved in terms of our relationship with family earlier in the illness.

The symptom care team plays a major role in the management of terminal disease. The frequency of home visits and telephone calls increases, and the team make a particular effort to involve the family practitioner and local nursing personnel, including nurses specializing in terminal care (see above). There are two children's hospices in the country, but they are very rarely used in terminal disease, as parents prefer to manage at home if at all possible (the hospices' main role is to provide respite care for some families during the illness). We always try to send flowers to the child's funeral and often a member of the team will be present. Because of her intensive work with children who are terminally ill, the symptom care team nurse will usually be able to initiate some bereavement work with the family. She also encourages parents to return, with their other children if they wish, to our hospital to talk over any continuing concerns. They need, principally, to be reassured that "everything possible was done", that the disease "wasn't their fault," and that, in most cases, their other children are not at risk of cancer.

For the first few years after the child's death we try to remember to send cards

at Christmas (because this is the major family occasion in the United Kingdom) and on the anniversary of the child's death.

It is important to end on an optimistic note! Two thirds of children are cured of their cancer these days, and the number is constantly increasing. It is the responsibility of the doctors at the tertiary centers not only to pursue a higher cure rate, but to make sure their patients are "cured at least cost," i.e., cured with a minimum of unwanted sequelae from treatment and its complications. In particular, there is current effort in our center to exclude (a) alkylating agents and (b) radiation therapy wherever possible from treatment. Less toxic analogues of currently available drugs, e.g., carboplatin for cis-platinum and epi-adriamycin for adriamycin, will hopefully give equally good survival with lesser risks of renal, hearing, and cardiac damage. Better informed parents can validly question their child's treatment and deserve deliberate explanations of the reasons for each treatment modality. Denial of parent's rights, in this respect, is much more likely to hinder than help in the child's management.

When children reach the age of 14–15 they are transferred to long-term follow-up clinics where "late effects" can be properly assessed in a nonpediatric setting. These clinics are often carried out with specialists in endocrinology and reproductive medicine and orthopedic surgeons and plastic surgeons may be attached to the clinics. Because of their previous history of cancer, patients may encounter problems with employment and life insurance. In the United Kingdom, patients who are over 10 years off treatment for acute leukemia, Wilms' tumor, and neuroblastoma are now regarded as "standard risks" for life insurance, but independent assessment by a reassurance company may be necessary. Often, a forceful letter from the doctor in charge of the long-term follow up clinic is all that is needed to overcome prejudice of this sort.

SUMMARY AND CONCLUSIONS

There is a wide range of potential support for the child with cancer and his or her family. Besides the medical and treatment teams, the medical social worker, the symptom care team nurse/doctor, and the play specialist are especially important in our center. Help from the various "support agencies" can be coordinated by members of the team according to the needs of the individual family. The journey through the illness is a difficult one. If the child is cured there is, naturally, cause for great rejoicing. Even if the child dies, however, there has almost always been a good quality remission, and it is now extremely rare for parents to regret that therapy was started. Whether the child lives or dies, the parents invariably comment that "their family will never be the same again." Superficially, this comment may convey the impression that they have been shattered by the experience, but that is by no means always the case. They more often mean that they have developed different priorities in life, especially concern and compassion for others who are even less fortunate than themselves.

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THE RONALD MCDONALD HOUSE PROGRAM IN THE UNITED STATES

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ABSTRACT

The Ronald McDonald House program at present consists of 125 houses in the United States and around the world that are situated near children's hospitals or large general hospitals with a significant pediatric population. They are owned and operated by individual charity organizations, but they are associated with each other by the Ronald McDonald Children's Charities program. The families pay a modest contribution and the remainder of the funds are raised by volunteer organizations. One of the great assets of such a house is the interaction between the families, who provide a significant amount of support for each other.

The Ronald McDonald House program consists of a variety of houses situated near children's hospitals that provide a place for parents to stay with or without their children. They were designed to provide a "home away from home," and indeed they do just that.

The idea originated in Philadelphia in 1974 and in the 15 years since the first house opened, a total of 125 are now operating all over the world. At the old Children's Hospital of Philadelphia, we had nowhere nearby for families to spend the night, so they slept on chairs in the rooms and benches in the corridors. Sometimes it is appropriate for parents to spend the night beside their sick child, but often it is important for them to get away and have a good night's rest in a proper bed. Obviously many can return to their own home, but others live too far away. So when the hospital was planning to move to a new site, we asked if we could find a house within walking distance to make a temporary home for our families. Once the idea was born, it grew rapidly and captured the imaginations of a widely disparate group of people. The oncology staff knew what they wanted and found a property. A football player whose daughter had leukemia involved the whole football team in a fund-raising project. The Philadelphia McDonald's Cooperative sold Shamrock shakes to help pay for the house. Finally, a father of a newly diagnosed patient with leukemia, who is in the construction business, renovated and furnished the house. The house was named the Ronald McDonald House because of the warm friendly feelings kids get from Ronald McDonald. Somehow it makes them feel better to know that they and/or their families will be staying at Ronald's house. The house was such a success that the

McDonald's advertising agency in Philadelphia discussed the concept with the McDonald's Corporation. They believed it was a project that they should support, and the next house was opened in Chicago about 18 months later.

As has already been stated, since the beginning in 1974 a total of 125 houses have been opened, the last two in November, 1990. In addition to those in the United States, there are houses in Canada, the Netherlands, Australia, West Germany, and Austria. There are plans underway for houses in Paris, London, Japan, New Zealand, and other sites around the world. These houses have more than 1800 bedrooms, serving 450,000 family members every year, for a total of 1.5 million since the inception of the program. More than 10,000 volunteers take part in the house activities. They are board members, hospital staff, local McDonald's owner-operators, and many teenagers and university students and parents.

As the programs developed, it became obvious that they provided much more than inexpensive and convenient lodging. To some extent they did provide a home away from home, but they also provided other benefits that could not be found in other homes – a caring and attentive staff that gives practical and psychological support and the presence of other parents facing problems similar to theirs. I think we envisioned the value of the attentive staff, but we underestimated the incalculable benefit of the bonds that develop between families.

Let me go into these two facets of the Ronald McDonald House program in a little more detail. The staffing varies from house to house depending upon the vision of the board, the needs of the hospital, and the strengths and attributes of the volunteers. The most important member is the house manager, because he or she provides a constant presence. This person or couple must be carefully chosen. They come from many different backgrounds, such as social work, nursing, teaching, or the church. In Philadelphia we have had only two house managers in the 15 years our house has existed, which is probably a record. The first was a technician who worked with me in the hospital and had a strong Christian background of service, and the second was an oncology nurse. House managers are of widely different ages, married or single, with or without children. We have always felt that having a family with young children living in the house is a great asset. In most cases, the house parents live in, occupying a private apartment within the building. It is an intensely demanding job, taking a heavy emotional toll, so that it is essential to provide proper relief, such as time off during the day and the weekend. Currently, our house manager, who has four children, has 7 hours off during the afternoon and every weekend, thus she works a 17 hour day 5 days a week. Of course, some of these hours are spent sleeping. Her husband provides valuable back-up support.

How the house is staffed varies with the size, talents, and dedication of the volunteers and its organization. For example, in some all the cleaning and maintenance are done by the parents and volunteers. In ours, they are done professionally. There is a certain amount of office work and bookkeeping that are necessary. Each house has a kitchen and eating facilities where families make their own meals. In ours, we provide free all the staples, such as milk, orange juice, cereal, coffee, tea, and sugar, and a large freezer is stocked with donated frozen meals. In addition, several evenings a week a volunteer comes in and cooks dinner, which is kept ready for the parents' return. This creates a welcoming gesture and encourages them to eat together as a family.

Anyone starting the initial phase of a McDonald House must give much thought

to the core of volunteers, because it is by and large on them the success of the house will depend. They do everything from fund raising to providing relief for the managers to washing the kitchen floor. Each house requires many hours of volunteer time a day. The phones must be answered, the office attended to, families checked in and out, people driven to the hospital, siblings at the house supervised, the house manager relieved, letters answered, maintenance dealt with, and the cleaning organized. Volunteers must be carefully recruited, interviewed, and trained. They too get involved with families and must be prepared to deal at times with parents' grief and anger. They come from all walks of life and have different motivations. Many know of a family who stayed at the house or are related to a patient. Some are students in nearby college or universities. Some are older citizens who want to continue to serve others in an active way. Some are leaders in the community who contribute by their contacts and are able to raise funds and donated services. Hospital staff frequently find it a wonderful change of pace to work at the house in a more relaxed environment than, for example, the neonatal intensive care unit. Some service organizations whose members are expected to serve the community will adopt the house as one of their charities and the members spend time there or raise funds on its behalf.

A very important aspect of the house is its financial stability. The McDonald Corporation does not provide all the funds to build or maintain the houses. They do, however, provide a start-up grant and programs, resources, and expertise that may not be available or affordable to the houses. With the help of its national public relations agency, McDonald's provides resource guidelines for fund raising, volunteerism, operations, and construction. They create brochures and videotapes to help volunteers tell the Ronald McDonald House story.

They also plan and host an international conference, to which the manager and president from each house is invited. People who attend these meetings say they are invaluable both in content and for the opportunity to share their experiences with each other. They write and distribute a national newsletter, create publicity opportunities for the houses and coordinate national donor programs. The DuPont Company, for example, donates carpeting to each of the houses needing it, and Apple computer provides each of the houses with a computer.

On the local level, McDonald's owner/operators serve on the board of directors and are responsible for assisting in fund raising for the house. Additional funds to support the house are raised by members of the board of directors and come from many sources. Sports teams get involved, local civic and community organizations, and even local celebrities lend a hand. The amount needed to build a house varies with the size and location. The original Philadelphia house cost $34,000 to buy and about the same amount to renovate. The house opening that I attended last month cost over two million dollars, and the house being built in New York City is going to cost many millions.

Once the house is built, it must have a sound operating basis. Most houses charge between 5–15 dollars a night per room; if families are unable to afford that modest rate, and the fee is waived. Our operating budget is about a quarter of a million dollars, with 15% coming from room rents, one quarter from the local McDonald Coop, and the remaining 60% from funds raised in the community. In addition, there are many hours of volunteer time and donated services. Recently we mounted a very successful capital fund-raising campaign to carry out many major repairs, redecorating and refurnishing. The funds raised by volunteers were matched

dollar for dollar by the local McDonald Coop.

Finally, I come to the tone of the house and the interactions between the families. The shared bond of the hospitalized child breaks down the usual reticence between strangers. In the normal world, parents have healthy children, and a family dealing with a serious illness is an anomaly. At the Ronald McDonald House everyone is in the same boat, and there is no need to hide one's anxieties. It becomes natural to ask somebody why they are there and to share their own reasons. Families staying more than a few days develop significant friendships and come to rely on each other for support. We have many families from outside the United States, speaking different languages, and the American families make a real effort to communicate with them and help them to understand the routine. When one family tells another that they understand what they are going through, those are not empty words. They often have really shared the same experience.

To close, let me say that hospitals associated with a Ronald McDonald House are extraordinarily fortunate. It provides a wonderful service for families that is far beyond an inexpensive, convenient place to stay. To be successful it needs a carefully selected house manager, a large core of volunteers, an effective board of managers who are truly involved and not figureheads, and a stable financial structure. When it functions well, it truly becomes a home away from home.

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TOTAL CARE OF THE CHILD WITH CANCER

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ABSTRACT

Total care is the term used to encompass all types of treatment given to the child with cancer. It includes multimodal therapy, designed to achieve the maximum of cures with the minimum likelihood of late complications, physical support, such as antibiotics, blood products, and nutrition, as well as psychosocial support. The following article deals with the staff necessary to deal with psychosocial issues and the manner in which they work. The size of the staff varies with the number of patients treated, but includes pediatricians, psychologists, social workers, nurses, play therapists, educators, and chaplains. They are involved with the patient in varying degrees, depending upon the stage of the disease and the particular kinds of crises. These crises occur at the time of diagnosis, the initiation of treatment, the discontinuation of treatment, entry into adult life, relapse and death. Programs can be developed to make the maximum use of the staff time available.

INTRODUCTION

Total care is the term that Dr. Sidney Farber, my teacher, and Dr. Nishimura's, gave to the broadly based care that Dr. Farber prescribed for children with cancer and their families at the Children's Hospital of Boston. It was based on an awareness that the diagnosis of cancer in a child had a far-reaching impact on the patient, his or her family, and the environment in which they lived. The oncology staff was small in those days, but they attempted to cover the many areas that we now address with more sophistication.

Total care starts with multimodal therapy designed to achieve the maximum chance of cure with the minimum likelihood of late complications. Additional physical support includes antibiotics, blood products, and nutrition. I plan to go beyond these elements and to limit my remarks to the numerous ways in which psychosocial and economic issues can and should be addressed and resolved. The ancillary services offered are wideranging and vary with the age of the patient and the time course of the disease. The person who delivers the service depends upon the resources of the

institution and to some extent its philosophy. However, all the issues that I raise should be addressed by some member of the multidisciplinary team. The program described was developed at the Children's Hospital of Philadelphia over the years, and we are constantly trying to improve the support offered to families. The relevant staff has necessarily become large because our commitment to total care is sincere and deeply based.

THE TOTAL CARE STAFF

First let me describe the staff, and then the programs they provide and the variety of ways they are used according to the stage of disease. Table 1 shows the staff that form the basis of this program.

Table 1. Supportive Care Staff.

Nurses	
Inpatient	56
Outpatient	6
Home Care	2
Physician Assistant and Pediatric Nurse Practitioner	
Oncology	2
Neuro-oncology	2
Bone marrow transplant	2
Late effects	1
Technical Staff	
Pharmacist	2
Technicians	3
Support Staff	
Psychologist	1
Social Work	7
Educators	2
Play Therapist	1
Chaplain	1

A large number of staff, 89 in all, is necessary because of our large clinical service, but the various areas that they cover need to be addressed when planning any program of total care. The mixture may also vary depending upon the way responsibilities are assigned. For example, we have only one psychologist and seven social workers, whereas at the Children's Hospital of Los Angeles, they have more psychologists than social workers to cover many of the same psychosocial issues.

PARTICULAR TIMES OF CRISIS

Having described the team, let me focus on the course of the disease and point out the times of change or crisis when psychosocial needs are the greatest (Fig. 1). These are at diagnosis, initiation of treatment for remission induction and consolidation, and maintenance. The latter may require less support if all proceeds smoothly.

Discontinuing treatment can reawaken anxieties of relapse and these need to be dealt with. Of course relapse on or off treatment is always a crisis, as is the child's death, needless to say. Growing up as a cured cancer patient can have its own unforeseen difficulties and the members of the oncology team are the ones who are best able to detect them and to suggest solutions (Fig. 1).

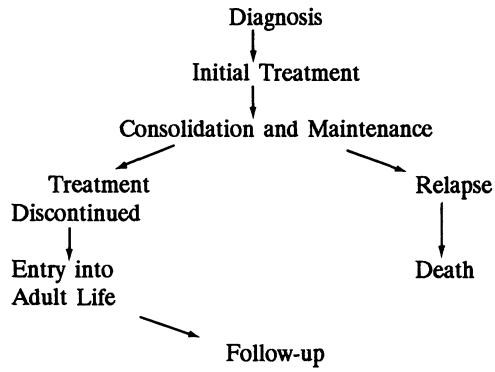

Figure 1. Critical points in the treatment of children with cancer.

The issues raised at these different times vary and not all the staff are called upon equally. I will go into more detail later about the way the different staff members contribute, but let me briefly list the people who are most involved at each time point. Each child has a responsible pediatric oncologist who follows him or her throughout the entire course. In addition the patient is assigned a social worker, and for inpatient care a primary nurse. Others of the staff are involved at the appropriate time.

Time of Diagnosis

Time does not permit me to go into great detail about how we present the diagnosis to the parents and patient. A formal conference is essential, which will permit at least a 1 hour discussion. Ideally, the team will consist of the responsible physician, the social worker assigned to the patient, and the primary nurse. Others may be present as observers, such as trainees, but not so many that the conference loses intimacy. The chaplain is involved around the time of diagnosis, depending upon the parents' beliefs, but is not usually present at the initial conference. The meeting should address what to tell the child and who should do the telling. We encourage that the truth about the disease be told, and the explanation will vary according to the age and understanding of the patient. Although the child should be provided with information about the disease and its treatment, he or she should not be present at the initial conference. Parents need the freedom to react to the news, express emotions, and raise questions. Usually the diagnosis is made while the patient is in the hospital, which also allows time for many additional meetings to reinforce the original discussion. During this period, the social worker and nurse also function as educators, explaining details about the disease and its treatment. Early discussions with the social worker also address the ways in which the illness is likely to affect the family, including possible social, emotional, and economic needs. The worker also discusses concerns related to

particular family members such as siblings and grandparents, and suggest ways parents can best support the patient.

Initial Treatment

The next period involves all of the primary treatment directed at cure. This is the period where the family learns to live with the disease and the disruption of treatment. The basic problems of living with cancer are the same for all, but the various issues depend in part upon the expected outcome of the disease. For example, a patient with a stage I Wilms tumor can usually be cured with minimal treatment and therefore relatively minor disruption to family life. The treatment for a child with AML, on the other hand, is so rigorous as to be life threatening in itself. Numerous hospitalizations are entailed and often ultimate failure. The issues also vary with the age of the patient, for example, the educator is only involved with school-aged children. Lastly, the effect on the family is also dependent in part upon the economic resources and the distance they live from the hospital. Financial issues have to be dealt with during this period and will vary from country to country. In the United States, there is no blanket governmental health insurance. The initial reaction of "I'll spend anything to save my child's life" becomes more realistic as the expenses of long-term treatment become known and family resources available to deal with them are faced.

The way parents manage the child during this stage greatly influences his or her future adjustment. If the child is encouraged to participate as much as possible in normal routines and activities, there is less risk of impaired self-esteem and self-confidence in later years.

Coming Off Therapy

The time when treatment is completed is usually one of excitement and joy, but it also can be a period of increased anxiety. The moment the diagnosis became known is relived with new fears that the treatment has been only controlling the disease, and that its interruption may lead to relapse. These concerns must be dealt with and a realistic level of optimism should be encouraged. Families should be reassured, and children encouraged to feel they have graduated and that it is a time for celebration. We have had many parties in the clinic where all interrupt what they are doing so that staff and other families can join in this time of triumph.

As survival time increases, patients are followed in the late effects clinic by a specially trained nurse. There, possible orthopedic, endocrinologic, oncogenic, and functional complications are watched for, and the appropriate referrals are made. For example, the educator may have to deal with disease-related or iatrogenic learning limitations. These are particularly common in children who had cranial irradiation for leukemia or brain tumors.

Entering Adult Life

The issues involved for the cured cancer patient vary with his or her abilities and goals. By and large, it has been shown that cured cancer patients have more optimistic views of life and a better sense of values. They feel they are special, as indeed they are. Since they have survived a life-threatening illness, they believe it is up

to them to use the life that they have been given to its fullest advantage. Despite their strong characters, they often meet obstacles to achieving their goals that are not encountered by their healthy peers. They may have physical or intellectual impairments; they may not be able to have children or a normal sexual life. Even when none of these problems exist, they may have difficulty getting employment or health and life insurance. I include the pediatrician again at this time specifically, because he or she may have to work on behalf of the patients to reassure employers and insurance companies that the patient is indeed cured and not a risk to their organizations.

Time of Relapse

Returning to the critical points of treatment illustrated in Fig. 1, the time of relapse obviously requires maximum supportive care. Here the staff of social worker, primary nurse, and home care nurse are all involved in helping the family face the tragedy. Many families have told us that the time of first relapse is more stressful than either the time of diagnosis or the actual death. Originally, the diagnosis is so shocking that it is difficult to conceive, and it is soon followed by the hope offered from treatment. It gives something positive on which to focus. But now with all the knowledge of the disease and the course of other patients that they have come to know, comes the realization that after all they have gone through the treatment has been unsuccessful and that almost certainly death will follow. Their hopes are dashed and they are cheated of the goal that they have been striving for. Anger is often an accompaniment to the grief. Again, the question arises, what should be shared with the patient. Many staff members become involved, and time for discussion must be formally allocated. The discussion will probably range over the past, to try to answer the question of what happened; the present, what to do now, and the future; how will we deal with the death and in what manner will it come? Recently, in a 3 day period in our clinic, we had five patients with acute lymphocytic leukemia in first remission who relapsed. You can imagine the hours involved in supporting these five children and their families. I should say here, as an aside, that attention must be paid to the support of the staff who live stressful lives themselves. We ask, "Who takes care of the caretakers?" and the answer is that we care for each other.

Time of Death and Afterwards

The time of death and the follow-up afterwards, strangely enough for the staff, can be rewarding. People ask us how can we take care of children with cancer who most likely will die? It is possible to get a feeling of satisfaction when you have helped a family and their child to meet death as well prepared as possible and to have walked that difficult and trying road in their company. We are not always successful, by any means, but at times we know we have become vital and cherished members of a particular family.

Again time does not permit me to go into details about the help needed around the time of death. The issues that have to be addressed are:

1. Continuation or discontinuation of treatment directed to prolongation of life
2. Use of supportive measures such as transfusions and antibiotics

3. Control of symptoms of pain and anxiety
4. Place of death, whether home or hospital
5. Nursing care needed

The siblings and their support need to be addressed also. The funeral plans, including its type and cost, are discussed at this time and also the need for an autopsy is brought up. If practical, visits from one or more members of the team are made to the home before and after death. If it is to take place in the hospital, then a clear definition of the goals of the hospitalization must be recorded and revised regularly. Conferences must be held with the inpatient staff, so they have the parents' wishes regarding resuscitation attempts clearly in mind. With such preparation, it is often possible to allow death to come with dignity and quiet in supportive surroundings chosen by the family.

Follow-up is important immediately in the first few days, both personally and by telephone. Later, depending upon the family's circumstances and strengths, the follow-up calls and visits can be less frequent, but continued until family health is restored.

ENRICHMENT PROGRAMS

Finally let me describe some of the programs we have devised to help us maximize the time spent in supportive care. Many of them involve educational programs and special groups addressing different issues, and are summarized in Table 2.

Table 2. Enrichment Programs.

Educational
School nurses and teachers
Parents, special parent groups
Patients
Hospice and home care nurses
Long-term Survivors
Supportive Care Groups
Parents of inpatients
Parents of newly diagnosed patients
Teenage patients
Siblings
Bereaved parents
Staff
Special Programs
Summer camp
Sibling camp
Hospice and home care
Celebration of life
Healing service

Educational Programs

One of the first programs we designed was for the teachers and school nurses of recently diagnosed patients. It is important for them to know about the disease, its treatment, and its effect on the patient. We have two such all-day programs a year and focus at one on the younger school children and at the other on high school students. The programs have proved very popular and are attended by many hundred teachers and nurses.

For parents we provide periodically a series of lectures in the evenings given by the staff. They address specific diseases such as leukemia, chemotherapy, radiation therapy, and learning problems. Special groups, such as the parents of the children with brain tumors, have a monthly meeting and organize their own program. Periodically, we arrange a Saturday program, inviting parents and patients. After one or two formal lectures, they break up into small discussion groups.

Supportive Care Groups

These groups can vary with the need. There is a regular weekly meeting for the parents of inpatients. Occasionally parents of children who have gone home arrange their clinic visit so that they can attend the inpatient meeting. Every few months an evening group meeting is arranged for parents of newly diagnosed patients. They usually meet for six sessions. A similar series is arranged for parents of children who have recently died. Intermittently we arrange special groups for teenage patients, siblings, and the inpatient staff. The staff meeting focuses on the children who have died and the staff's feeling of loss.

Special Programs

We have a variety of imaginative special programs. The summer camp for patients is a great success and is supported by the Ronald McDonald House. It requires a great deal of staff time in its planning and execution, and a large number of staff to run it. Recently we had a weekend camp for siblings of cancer patients and they enjoyed sharing their concerns and problems.

We have a very successful Christmas Party that several hundred family members attend. We also encourage birthday parties and celebrations when children come off treatment. These are held in the outpatient clinic.

HOME CARE PROGRAM

The home care program provides nursing and pharmacy support to give some forms of chemotherapy and antibiotics at home. This is popular with parents. We are currently doing an analysis of the costs involved to determine whether it is indeed cost effective. The hospice program supports the families of children who are no longer receiving active treatment. It is responsible for patients either at home or in the hospital. The majority of our children die at home. Each year we have a memorial service, which is very well attended by families. At the service the names of the children who have died are read out aloud by different staff members. A healing

service is offered to an occasional family. This provides a religious setting for prayer and a blessing for the patient and his family.

The foregoing is a description of the supportive care program and is but a summary of the many areas that have to be covered. Total care includes the medical treatment of the disease, together with physical and psychosocial support to try to minimize the impact cancer has on the patient and his or her family. Cure alone is not enough. The aim is to ensure that the child enters the future with all his or her physical and mental faculties intact.

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HOME CARE FOR CHILDREN WITH CANCER

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ABSTRACT

Children with leukemia from the time of remission to full recovery or death will more often reside in their own home than in the hospital. Competent professional nursing can provide the support needed to the family throughout the experience. While all periods of treatment will be covered, home care of the dying leukemic child will be emphasized. The feasibility of home care as an alternative to hospitalization for children dying of cancer was studied. The home care system was defined as nurse-directed with a consultant physician and did not entail extensive participation by other health professionals. Of 58 children cared for at home during the 2-year project, 79% died at home and 21% died in the hospital or en route to it. The findings, as shown by interview data, suggest that home care at the end stage of life is a viable alternative for children dying of cancer and for their families. Overall, the study has demonstrated that physicians will refer patients, and that children and parents agree to participate. It further demonstrated that home care nurses can be recruited and that they can secure needed equipment, supplies, and medications. No clear criteria have emerged that would rule out the home care alternative for any child dying of cancer.

From the 1985 data in the United States of the estimated numbers of new cancer and cancer death in children under the age of 15, of the 6550 new cases and 2175 deaths, there are approximately 2000 new cases of leukemia and 850 deaths from leukemia (1).

The following will examine some of the published and unpublished results of the five extramural funded studies of which I have been principal investigator. These studies are (a) Home Care for the Child with Cancer, (b) Impact of Childhood Cancer on Families, (c) Impact of Childhood Cancer on Chinese Families in Taiwan, (d) Longitudinal Follow-up of Childhood Cancer (the families involved in Home Care for the Child with Cancer), and (e) Longitudinal Follow-up of Childhood Cancer Families in Taiwan.

Home Care for the Child with Cancer, funded by the National Cancer Institute in 1976, was a feasibility study to determine whether it was advisable that children die

Table 1. Criteria for Referral to Study

1.	Patient was 17 years of age or younger.
2.	Patient had some form of cancer and was expected to die fairly soon as a consequence.
3.	No inpatient hospitalization-requiring procedures were planned

Table 2. Services Available for Family

1.	Nurse on call 24 hours, 7 days a week.
2.	Nurse available to help family deal with problem.
3.	Nurse available for home visit anytime.
4.	Child returning to hospital is always an option.
5.	Child's doctor may be called anytime.

Table 3. Two Factor Index of Social Position of Household

Hollingshead	Child died			
	At home		In hospital	
	n	%	n	%
I (Highest)	4	9	-	-
II	6	13	1	8
III	13	29	4	33
IV	20	44	7	58
V (Lowest)	2	4	0	-
Unknown	1			
Total	46	99	12	99

N = 58

at home with a health care system assisting the parents, rather than in the hospital (Table 1). In the United States during the 1970s, children who were dying from leukemia were admitted to the hospital to die. There was no hospice/home care at that time. During the periods of home care, a nurse, recruited from the community and trained by the project nurses, was available at all times for consultation or home visits (Table 2). Readmission to the hospital was always an option. Of the 58 children who died during the 2 year study, 46 died at home, 11 died in the hospital, and 1 died in an ambulance returning to the hospital (Tables 3 and 4). The mean duration of home care was 39 days, with a range of 1–256 days. Of the nurses involved, only one was unwilling to provide home care for a dying child again.

Evaluation of the project revealed that (a) home care provided a feasible alternative to hospitalization, (b) home care was satisfactory for families of diverse backgrounds, and (c) home care was considerably less expensive than hospitalization (2,3). Eighty percent of the families cared for their child at home through death. At the moment of death the majority of children gradually stopped breathing (Table 5).

Table 4. Reasons for return to the hospital for 12 children who received home care during phase 1 and who died in hospital.

Family	Reason(s) for return of child to hospital
1.	Father and siblings did not want child to die at home; died in ambulance en route to hospital.
2.	Child requested return home. Mother told physician and nurse that she didn't want child to die at home. Parents felt that some medical treatment might still help.
3.	Child and parents sought readmission to control sudden, severe pain. Mother also related inadequate rest, fear of the death event, and fear the siblings wouldn't be able to use their room again if child died in it.
4.	Mother said she planned on rehospitalization when child dying. Felt overburdened at home and more secure in hospital. Mother felt nurse did not offer enough assistance with physical care.
5.	Mother felt anxious, exhausted, overburdened, that home care was too much responsibility for her.
6.	Mom anxious, exhausted, concerned that she couldn't help quickly enough. Father felt that the child's presence in home was not good for siblings, nor himself.
7.	Mother felt anxious, overburdened and alone in caring for child at home, felt more secure in hospital. Could sleep at night knowing that nurses were responsible. Physician seen as encouraging hospitalization.
8.	Child developed respiratory distress. Child requested return to hospital. Family unable to reach nurse and felt lack of support from nurse.
9.	Child developed pain, requested return to hospital to establish pain control and to stay overnight. Died before discharge. Mother later reported fear of what death would look like.
10.	Mother planned death at home, child readmitted for transfusion when rectal bleeding began. Mother felt poor physician support prevented death at home.
11.	Father not accepting of death and cessation of chemotherapy. Family went to Mexico for Laetrile.
12.	Rehospitalized for control of status epilepticus. Mother said she wouldn't be able to stand seizures at home.

Table 5. Moment of Death for 46 Children Who Died at Home

	Number	(%)
Gradually quit breathing (2 also had bleeding)	32	69.6
Brief difficulty breathing (1 also had bleeding)	8	17.4
Seizure	3	6.5
Delirious, restless	3	6.5

Nurses in both urban and rural areas were able to provide 24-hour, 7-day coverage to the family, and physicians not only made appropriate referrals, but also became advocates for the program (6). Perhaps all families could have their child die at home if the health care system and its personnel were perceptive and supportive enough.

A 2-year follow-up of these 58 families, involving interview and administration of the symptoms checklist-90-revised (SCL-90) (5), revealed that the grieving process had continued throughout the 2 year interval, although the fathers reported a more rapid resolution of intense grief than did the mothers. Eighty-four percent of the parents believed that their child's death had not impaired their functional ability in normal daily activities. This was supported by the objective findings that although the psychological pattern of the parents were more symptomatic than the norm, the pattern was less atypical than that of diagnosed psychiatric outpatients, possibly revealing a lack of full resolution of grief (6). At the 2 year interview the parents were asked about home care as an option, and all of those who cared for their child at home said they would choose home care again. The majority of the parents whose child died in the hospital indicated that they would prefer home care again.

The impact of Childhood Cancer on American Families, funded by the American Cancer Society of both the California and Minnesota divisions, as well as by the St. Paul Foundation, followed families from the time of diagnosis over a 5 year period of time. Several themes were identified.

Unpreparedness

All parents indicated they had never considered the possibility of a serious illness. They had assumed the problem was minor. None of the parents were prepared to hear their child had cancer.

Need for Information

Many parents said that in the days following the diagnosis, time seemed to be greatly speeded up and that the excessive number of events occurring in rapid succession had made it difficult to comprehend or cope with all the information that was given to them. On the whole, however, parents were pleased by the physician's honesty at the time of diagnosis and early treatment, as well as by the manner in which information was given. Most of the families began to rely more frequently on the expertise and services of the medical center and to distrust the competence of their local physician. A few also began to question the local physician's ability to treat their other children for occasional illnesses or to understand the family's need to be hypervigilant. Growing dissatisfaction was especially apparent in families where the child was not doing as well as hoped for. Fear of coming home was especially problematic for mothers who had become dependent on the ready access of the health team members in the hospital. Relinquishing and reclaiming control was a pattern that was seen in most of the families. They would relinquish the control in the hospital but reclaim the control back in the home setting. Vulnerability became a new reality for most of the families. The shock of this rare disease and the fact this happened to them in their family made them sensitive to other areas of vulnerability. This sense of vulnerability leads to the next major theme identified, which was uncertainty (7).

Uncertainty

Cohen and Martinson(8) studied the conditions that create or increase cognitive awareness of uncertainty, the social interactions that occur around issues of uncertainty,

the strategies that parents use to manage the stress of uncertainty, and the consequences of living under conditions of sustained uncertainty. We learned that the survival rate for the child's cancer was the single most important piece of information given to the parents following the diagnosis. Surprisingly, we found that two individuals - a husband and a wife - can interpret the same percentage in exactly opposite ways. A 10–15% chance for survival was interpreted by one father to mean his daughter would either live or die; it was really a 50-50 proposition. The mother, however, interpreted the survival rate to mean that the child probably would die. No wonder this couple had communication difficulties. I thought we were doing a great job when both couples knew the same percentage, because a few years earlier this information was considered inappropriate. Clearly, however, it is not enough.

The siblings in this study, where the child with cancer was still living after 5 years since diagnosis, volunteered the information that they knew a person could die from cancer. In contrast with the siblings where the child had died, no siblings admitted knowing of this possibility until the very end, when the dying process made such knowledge obvious and unavoidable. Where the child was living, the children expressed no fears for their own well-being, and only one mentioned fear of leukemia. In contrast 75% of the siblings where the child had died reflected worry about getting cancer themselves and several also expressed fears for the safety of parents. Equally sobering was the pattern that became clear in response to the "three wishes," where 40% of the siblings where the child was living wished for the child to get well, while 85% of the siblings where the child with cancer had died wished for the return of the deceased sibling and/or a unified family.

The impact of Childhood Cancer on Chinese Families in Taiwan, funded by the National Science Council (Taiwan's equivalent of the NIH), extensively studied 75 families: 25 of the families had a child who was newly diagnosed with cancer; 25 had a child who had relapsed and was expected to die shortly; and 25 had a child with cancer who had already died. We studied all the newly diagnosed children within 6 months. Children were dying from leukemia who were routinely being saved in the United States, not from improper medical treatment but from insufficient funds to pay for medication and hospitalization (9). Within 5 years of diagnosis, all but one of the 14 children with leukemia had died. The research results led to the establishment of the Childhood Cancer Foundation in Taiwan, which this year is providing free chemotherapy to all newly diagnosed children with leukemia enrolling in the TPOG for

Table 6. Follow Up of 58 Families.

Participated/located	47
Left U.S.A.	1
Unable to locate	1
Information from sibling	2
Refused	7
Reasons:	
Too painful	3
Cancer in family	2
Not allowed by father	1
Not willing to discuss	1

Table 7. Three Styles of Grieving.

Getting Over It
a) Grief not intense
b) Death accepted as matter of fact
c) Had no effect on their lives now
d) Memories less vivid
Filling in the Emptiness
a) Keeping Busy (e.g. Candlelighters)
b) Substitute other problems/events (e.g. build new home)
Keeping the Connection
a) Emptiness is still there
b) Incentive to cherish other relationships
c) Treasure daily events as a special gift

the whole island. The foundation also has facilitated family support groups, provided educational materials, and is preparing specialist pediatric oncology nurses to provide education and follow-up to all children enrolled in the TPOG protocol.

Follow-up study of the 58 families 7–9 years after death (Table 6) led to the identification of three styles of grievers (Table 7). The three patterns identified were (a) "getting over it," (b) "filling the emptiness," and (c) "keeping the connections." "Getting over it" could be described as those who resolved their grief in a more traditional sense. Death was accepted as a matter of fact, and their grief was not described in an intense manner. They stated that the death did not have any effect on their current life and that their memories were less vivid of the child. "Filling the emptiness" was the second pattern of grieving. They became very busy with activities or other problems. Altruistic involvements were utilized, and it was very important for these individuals to use their experiences by helping others. "Keeping the connection" was the third pattern by which individuals described a continuing sense of their loss. These individuals integrated their pain and loss into their lives and, in contrast to the "getting over it" style, this group had vivid memories and stories that they did not want to forget. They cherished their recollections, and others around them became more precious because of the loss (10).

Utilizing the SCL-90, the individuals having difficulty at 2 years postdeath were the same individuals who had difficulties 7-9 years later. One implication is that we can identify the high-risk individuals at 2 years postdeath, work with these individuals, and see if changes are possible. The SCL-90 has been well received by numerous parents in both the West coast and the Midwest. The most significant finding is that individuals who continue to feel emptiness and pain long after the loss of a loved one can be assured that they are not alone.

In the Follow-up Study of the Chinese Families in Taiwan 7 years after diagnosis, 16 of the 25 families were interviewed, and with only three families we were unable to locate any information and do not know if the child is alive or dead. The four families who refused to be interviewed had considerably less schooling, and 2 of the 4 families believed it was bad luck to talk of the deceased child. One poor family whose child had lived only 3 months after diagnosis and did not have sufficient funds to continue treatment had taken over 4 years after the death of the child to repay the

money they had borrowed to treat the child for 4 months.

While progress is being made in supporting home care from the time of diagnosis through recovery and cure, or to the death and bereavement of families, there is a challenge to all of us today to ensure the highest quality of both medical and nursing care for the family. While hospice/home care has become more widely available in the United States, this is not true throughout the world where children are being treated for leukemia. Home care, to be of the highest quality, must be organized and must work in collaboration with the physician and nurses in the hospital. Home care requires a competent medical and nursing team who work with the family as an equal partner and who is most eager to learn.

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