Molecular Genetics and Therapy of Leukemia

Cancer Treatment and Research

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Introduction

Leukemia is a malignant disease that continues to play a cutting-edge role in the discovery of new basic knowledge about the malignant process and about the potential for controlling these malignant diseases. The thirteen contributions in this volume are entirely unique and represent new areas of important discoveries since the publication of *Leukemia: Advances in Research and Treatment* in 1993.

The first chapter describes the identification and cloning of the gene located at the breakpoint on chromosome 16 in the unique form of acute myeloblastic leukemia known as inversion 16 disease. This disease is the most sensitive to currently available cytotoxic chemotherapy and has the best prognosis of any form of acute myeloblastic leukemia in man. Dr. Siciliano and his coworkers pioneered in discovering the molecular basis for this disorder, which has just recently been elucidated. The second chapter is a presentation by Dr. Nagarajan of the important diseases associated with deletions of the long arm of chromosome 5. These leukemias are growing in importance because they represent a high proportion of the secondary — that is, treatment-induced — leukemias and are associated with a particularly poor prognosis. Dr. Nagarajan is one of the leading contributors to the localization of the essential deletion and the identification of its potential role in the control of hematopoiesis. In the third chapter, Dr. John Reed and coworkers, who have made substantial contributions to our growing knowledge about the bcl-2 gene, update the role of this important gene in regulating and controlling the processes of cell death. In chapter 4, Drs. Downing and Look summarize information about the 11q23 breakpoint leukemias that have become prominent following therapy with topoisomerase II binding chemotherapeutic agents, providing an important insight into the mechanisms of leukemogenesis in man. In chapter 5, Dr. Gewirtz describes an original and relatively unique approach to gene therapy, that is, regulating the myb oncogene expression as an approach to controlling the expression of the essential bcr/abl neogene in patients with chronic granulocytic leukemia. In the sixth chapter, we have an excellent review by Drs. Keith and Russell of the growing importance of the p53 suppressor gene and oncogene in leukemia.

In chapter 7, one of the pioneers in the field of cord blood hematopoietic

stem cells, Dr. Broxmeyer, summarizes this important and unique approach to replacement of hematopoietic stem cells in man. In the eighth chapter, Drs. Roberts, Estrov, Kitchingman, and Zipf update the mechanisms for detection of minimal residual disease in acute lymphoblastic leukemia, which are proving to be quite important in devising novel strategies for curative therapy in this disease. In chapter 9, Drs. Seymour and Kurzrock provide new information about cytokines and their role in the control of malignancy. In the tenth chapter, Drs. van Besien and Giralt summarize the current status of autologous marrow transplantation in the control of malignant diseases. In chapter 11, Dr. Pagliaro updates the information on the important function of the retinoblastoma gene in malignancy. In the twelfth chapter, Drs. Giralt and van Besien summarize the approach to patients who relapse after allogeneic bone marrow transplant, which provides unique opportunities for innovative approaches in the clinic. And finally, in chapter 13, Drs. Cortes, Kantarjian, and Freireich summarize the current status of chemotherapy of acute lymphoblastic leukemia in adults, which has moved adult acute lymphoblastic leukemia closer to the highly significant effects of therapy that are observed in childhood acute lymphoblastic leukemia.

In summary, this volume presents, in a multiauthored text, the contributions of leading investigators and scientific contributors to the topics being updated here. This volume complements *Leukemia: Advances in Research and Treatment*, published in 1993, by including new topics that have moved quickly over the last several years. The editors are aware that a volume such as this cannot be comprehensive; Many important contributors to leukemia research and treatment advances have not been included. These deficiencies are clearly ascribable to the editors. The authors who have contributed to this volume have created an important update of ongoing research in this field.

Molecular Genetics and Therapy of Leukemia

1. Molecular genetics of inversion 16 leukemia: implications for leukemogenesis

David F. Claxton, Paula Marlton, and Michael J. Siciliano

1. Introduction

The M4Eo subtype of acute myeloid leukemia (AML) has distinctive bone marrow morphology characterized by the presence of dysplastic eosinophils with abnormal granulation. This distinct subtype of acute myelomonocytic leukemia (AMML or AML-M4) is predictive of a more favorable outcome with longer remission duration and survival following appropriate chemotherapy [1,2]. The original descriptions of this entity were reported in association with the chromosome 16 abnormalities inversion(16)(p13;q22), translocation (16;16)(p13;q22), and deletion (16)(q22) [1,3,4], referred to hereafter as inv(16), t(16;16), and del(16q), respectively. Thus a cytogenetic–clinicopathologic association was described between M4Eo and these chromosomal abnormalities. The association is not exclusive, however, with many examples of inv(16) now described in the setting of other AML subtypes such as M2 and M5 as well as myelodysplastic syndrome [5,6]. Some important recent work has been the cloning of the breakpoints associated with inv(16) and t(16;16) and the identification of the genes associated with those chromosome alterations. The steps of that process will be reviewed here, as will the development of molecular genetic tools and procedures which that work has made available to diagnostics. We will then consider the biological and clinical implications of a deletion that has been shown to have occurred in some inv(16) patients. Finally, we will discuss the molecular genetic findings in relation to models for understanding the mechanism of this disease and what those possible mechanisms imply with respect to the understanding of leukemia and carcinogenesis in a wider sense.

2. Cloning of chromosome 16 breakpoints associated with AML-M4Eo

Inv(16) is recognized cytogenetically as a pericentric inversion of one chromosome 16 with breakpoints at 16p13 and 16q22. Molecular genetic inroads into understanding the genetic consequences of this cytogenetic event began with the identification of a genomic clone containing the sequences of the p-arm

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breakpoint associated with the inversion. Cosmid clones, containing between 30 kb and 50 kb of genomic DNA, were mapped into the p13 region of chromosome 16 and were used [7,8] as probes for fluorescent in situ hybridization (FISH). If such a probe was from a location distal to the breakpoint, it would produce a fluorescent spot on the p-arm of the inversion chromosome, whereas probes from clones mapping proximal to the breakpoint would identify the sequences on the q-arm of the inversion chromosome. What was being sought was a clone that, when used as a probe, produced a signal split between the p- and q-arms, indicating that it contained the breakpoints. The discovery of such a clone was facilitated by the identification of cosmid 35B11, identified as being proximal to the breakpoint by the above criteria. When a unique sequence from that cosmid was used as a probe on a pulsed field gel in which macrorestriction fragments from the DNA of inv(16) and normal cells were separated, novel 240-kb *Sac*II macrorestriction fragments were identified from the DNAs of only the inv(16) cells [9]. These data suggested that 35B11 was within 240 kb of the inv(16) p-arm breakpoint. Sequences from 35B11 (and flanking contiguous cosmids) were then used to identify very much larger genomic segments that were cloned into yeast artificial chromosomes (YACs). It was likely that such larger genomic clones would contain the breakpoint. Four YACs so identified had human inserts sized 100, 300, 550, and 780 kb, respectively. The three largest indeed contained the inv(16) p-arm breakpoint, as indicated by the fact that the FISH probe made from them was split to give a signal on the p- and q-arms of the inversion 16 chromosome in patient material [9].

So, the p-arm breakpoint had been cloned, but only in YACs containing very large pieces of DNA. The next step was to narrow down the cloned human DNA to include only the breakpoint and just enough flanking sequence to accomplish two key tasks — to identify a genomic clone that crossed the q-arm breakpoint, and to use both these p-arm and q-arm genomic clones to isolate the genes (in the form of cDNAs) on the respective arms that became rearranged as a result of the inversion. Thus cosmid-size clones containing DNA that crossed the p-arm breakpoint were sought by screening a human chromosome-16 cosmid library (from Los Alamos National Laboratory) with DNA from all four YACs. Cosmids identified by all three YACs that crossed the breakpoint, but not identified by the YAC that did not, were chosen. The very first such cosmid so identified (16C3) contained the breakpoint, as indicated by the fact that, when it was used as a FISH probe, signal was produced on both the p and q-arms of the inv(16) chromosome [10]. Cosmid 16C3 was then used to leap to the q-arm breakpoint. In order to do so, a fragment of it that contained purely unique sequence (16C3e) was isolated and then used to screen a genomic cosmid library that had been made from the DNA of inv(16) leukemia cells. Some cosmids from that library would be from the normal chromosome 16 and others from the inv(16). To distinguish them, restriction digests of clones identified by 16C3e were compared to digests of 16C3 itself. Unique fragments present in the former were likely to be from the

q-arm and present in the cosmid as a result of the inversion. Such a unique *Hind*III fragment (0.7 kb) was identified in one cosmid. The verification that it contained sequences from both the p- and q-arms in a FISH experiment was that signals were produced on both arms of *both* chromosome 16s in *normal* metaphase cells. The telltale *Hind*III fragment itself was then used as a probe (once again on the normal chromosome-16 cosmid library from Los Alamos). This allowed the identification of cosmid clones containing sequences that crossed the q-arm inv(16) breakpoint. Two such overlapping q-arm cosmids were isolated and verified as crossing the breakpoint in FISH experiments in which they were used as a probe — they produced a split signal on the inversion chromosome [10].

3. Identification of the genes affected by the rearrangements

With cosmids now available that spanned both the p-arm or q-arm breakpoints, human expression cDNA libraries were screened to isolate the genes which were at the breakpoints and therefore were very likely rearranged by the inversion event. Unique sequence probes from those cosmids were used for the screening to identify the p- and q-arm genes, respectively [10]. The p-arm breakpoint gene was isolated in this way. Sequencing showed that it coded for a smooth muscle myosin heavy chain gene named *MYH11*. The q-arm cosmid probe identified cDNA clones that showed a high degree of sequence similarity to a newly described mouse gene coding for a DNA-binding transcription factor core binding factor beta (CBF beta) [11]. The human gene was named *CBFB*. Mapping of the p- and q-arm cosmids indicated that the inversion breakpoints of each gene fell in introns [10]. Since the breakpoints were located in introns of both the p- and q-arm genes, a fusion transcript could be theoretically generated in the AML cells. Identification of such a transcript would surely verify the molecular genetic consequences of the cytogenetic event, and perhaps provide an insight into the basis of leukemogenesis of this malignancy.

To determine if a fusion transcript was produced in the leukemic cells, PCR primers were designed from the middle of the *CBFB* coding sequence and the 3' region of *MYH11* coding sequence. Reverse transcription was conducted on the total RNA from inv(16) leukemia cells derived from six patients to make cDNA from the RNA message produced in the cells. If a fusion transcript was produced and converted to cDNA by the reverse transcription process, then PCR of that cDNA (RT/PCR), using the primers described above, should produce a product. Product was generated from all six samples while no product was ever obtained using RNA from non-inv(16) cells [10]. This approach was extended by Claxton et al. [12] to a total of 28 viably preserved cell RNAs from inv(16) patients and seven cases with the related translocation t(16;16)(p13;q22). In all the typical inv(16) cases and, importantly in all seven translocations samples, fusion products were obtained that were composed of

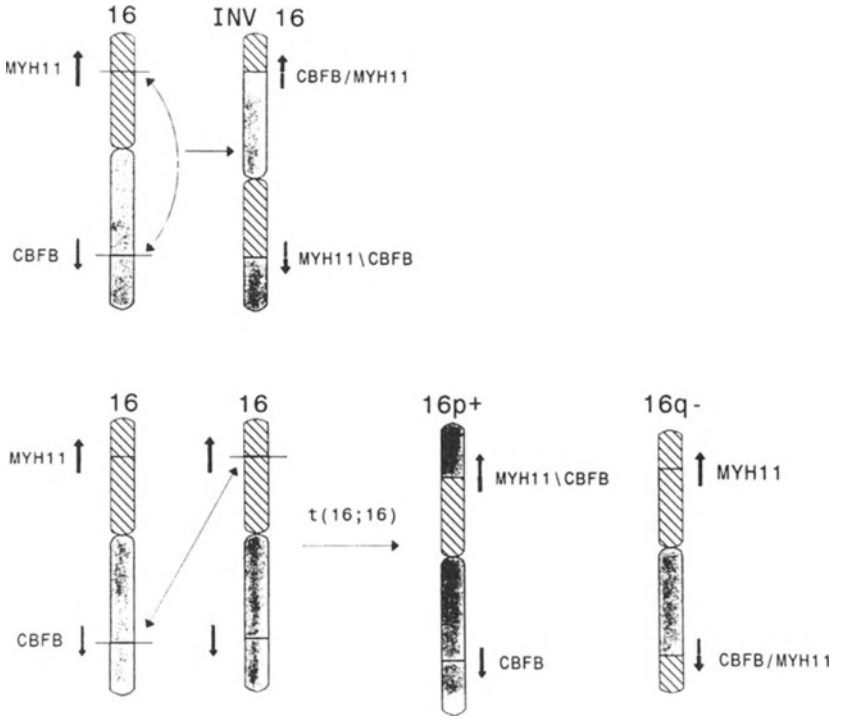

Figure 1. Schematic view of the consequences of inv(16) and t(16;16). The inv(16), shown at the top of the figure, fuses most of the *CBFB* coding sequence encoded at 16q22 upstream of the 3′ end of *MYH11* on 16p13 of the same chromosome. In t(16;16) (shown at the bottom), breakpoints on different chromosome 16 homologues translocate the distal p-arm region of the first homologue to the distal q-arm region of the second while the distal q-arm region of the second homologue is translocated back to the distal p-arm region of the first. This results in a chimeric *CBFB/MYH11* gene similar to that in inv(16) cases. Although the reciprocal fusion gene *MYH11/CBFB* should also be generated, deletion of *MYH11* sequences upstream of the breakpoint appears to be present in some cases that are clinically indistinguishable from those without the deletion. This suggests that the *CBFB/MYH11* chimera is the operative factor in the disease. (From Claxton et al. [12].)

upstream *CBFB* sequences and downstream *MYH11* sequences. No such products were obtained from 10 cases of AML not showing chromosome 16 alterations, nor in one inv(16)(p13q24) (i.e., a variant q-arm breakpoint). These data also indicated that the molecular consequences of the t(16;16) were similar to those of inv(16). Figure 1 illustrates the cytogenetic and molecular genetic events of inv(16) and t(16;16).

4. Diagnostic applications

The cloning and identification of the genes associated with the breakpoints in inv(16) produced reagents and procedures that are of great assistance in

diagnosing and monitoring the disease. Unique sequence in the region of the breakpoint can be used in Southern blot analysis of DNA from patient material to identify rearrangement fragments indicative of an inversion or translocation. For example, 16C3e from the distal side of the p-arm breakpoint [13] readily detects such rearrangements in cells from inv(16) patients but not in the cells of patients with other leukemias [10,13,14]. As explained more fully below, there is a subclass of inv(16) patients with a deletion proximal to the site of the p-arm breakpoint [inv(16)/del] [10,13–15]. Using a probe just proximal to the p-arm breakpoint (e.g., NE5A in Marlton et al. [13]), one can identify inversion patients that have the deletion — such a probe does not identify a rearrangement fragment in deletion patients. Therefore, by combining the use of probes both distal and proximal to the p-arm breakpoint in Southern analyses, one can determine the presence of the inversion or translocation and whether or not that primary event is associated with deletion.

Inversions can also be readily identified by FISH using robust probes that overlap inversion breakpoints. The inversion chromosome readily stands out in such analyses, since the signal is split between the p- and q-arms of the inv(16) chromosome. We define a robust probe as one being large enough so that its signal can be readily identified in even the poorest of metaphases. The best and most consistent probe available for this purpose, as described in Liu et al. [9], has been generated by inter-Alu-PCR [16] from a human–Chinese hamster ovary somatic cell hybrid (3–30) containing most of the p-arm of human chromosome 16. The larger YACs described above that contain sequences crossing the p-arm breakpoint have also proven to be excellent probes for diagnosis [9]. The deletion associated with some inversion cases has been very readily identified by using a FISH probe made from the cosmid contig containing cosmid 35B11. Since those sequences are deleted in the inv(16)/del patients, only a single signal (from the normal chromosome 16 present in the cells) is seen in either metaphase or interphase cells from such patients [13]. Others have used YACs [14] or the cDNA of the large multidrug resistance protein (*MRP*) gene [15], known to be deleted in some patients, as a probe. The FISH methods are quite unambiguous and have the advantage of being applicable directly to cytogenetic specimens, requiring no DNA or RNA extraction. Until an adequate FISH probe is developed for detecting the inversion in interphase cells, the usefulness of FISH in the detection of minimal residual disease (MRD) will be severely limited.

The development of sensitive RT-PCR assays, as described above, for the CBFB-MYH11 [10,12,17] transcript holds great promise for the monitoring of patients with the translocation during remission. The assay was shown to be very sensitive by detecting chimeric transcript in as little as 20pg of total cellular RNA — a quantity equivalent to the 2–5 cells level. Therefore, the technology used to develop an understanding of the molecular genetic consequences of the chromosome rearrangements also provided a sensitive method for detecting MRD in patients being treated for this disease. Sequencing of the RT-PCR products revealed that they were amplified from in-frame fusion

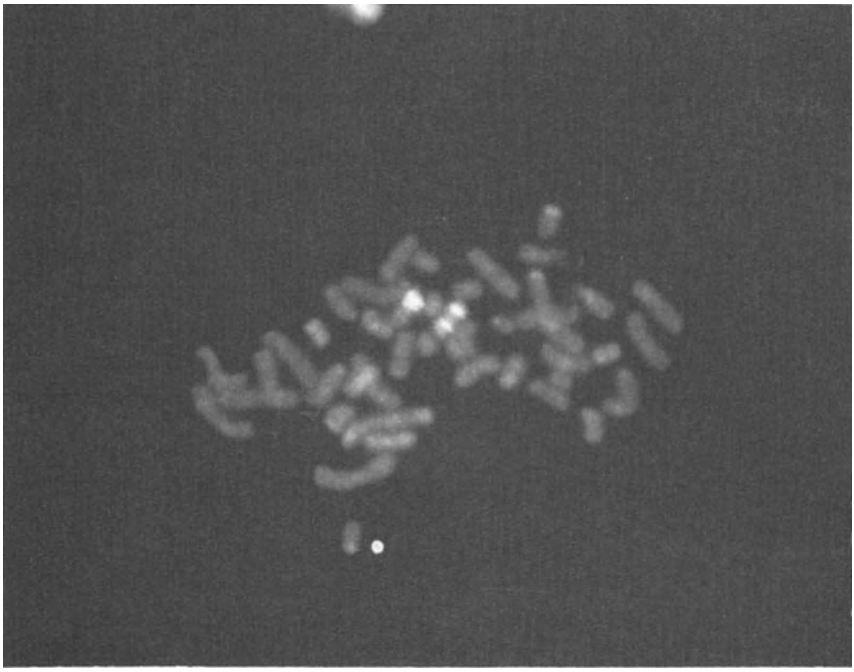

Figure 2. FISH on a bone marrow cell from a patient with AML-M4Eo using inter-Alu-PCR probe from an interspecific somatic cell hybrid (3–30) containing only the p-arm of human chromosome 16. Note that the p-arm of one chromosome 16 is brightly labeled, while on the other the signal is obviously split between the p- and q-arms. The inversion is clearly visible in this less-than-ideal metaphase.

gene transcripts in which the *CBFB* breakpoints were all located close to the 3′ end of the coding region, with only the last 17 of the 182 amino acids deleted from the resultant putative chimeric protein. Liu et al. [10] identified three different breakpoints in the *MYH11* coding region, and a fourth was identified in the expanded set of patients seen by Claxton et al. [12]. Therefore, four different classes of breakpoints have been identified — A, B, C, and D — relative to the position of the breakpoints at *MYH11* cDNA base-pair positions 1,921, 1,528, 1,201, and 994, respectively. The great majority of patients — 29 — had type A rearrangements, while four had type C, two type D, and one type B. Six of the translocation patients had type A and one type D. Similar results have been reported recently from France [17], where 20 of 22 inv(16) patients produced fusion transcript type A, one had type C, and one type D. There appears to be no correlation of fusion type with any clinical or cytogenetic parameters in any of these studies.

Though inv(16) patients have a relatively good prognosis with appropriate antileukemic therapy, they nonetheless relapse in substantial numbers. Experience by other authors [17] and ourselves is that the sensitive detection of

small numbers of inv(16) cells admixed with large numbers of normal cells requires two rounds of PCR. This kind of 'nested' amplification, while very sensitive, has the limitation of increased risk of false-positive amplification. It is possible that additional technical changes will need to be made before this amplification strategy yields clinically reliable data. The importance of identifying relapse early is certainly more than academic [18]. Since many individuals with inv(16) are young, patients who may be reliably predicted to relapse might be offered related or even matched unrelated allogeneic bone marrow transplantation prior to relapse. The early application of transplantation during subclinical disease would very likely improve the outcome for these patients.

5. Biological and clinical implications of inv(16)-associated deletion

As indicated above, in a subset of inv(16) AML patients, a deletion from the p-arm of chromosome 16 has been identified in association with the inversion [inv(16)/del]. The deletion was originally observed while mapping cosmids relative to the p-arm inversion breakpoint cluster (p-ibc) by FISH on patient metaphases [10]. One cosmid, 35B11, which, as mentioned above, mapped to within 240kb proximal of the p-ibc, had generated signal on each of the chromosome 16 homologues in most patients; however, in one case, signal was present only on the normal chromosome and on not on the inversion chromosome, indicating the deletion of sequences recognized by this cosmid. Deletions proximal to the p-arm breakpoints were similarly noted by two other groups studying the molecular genetics of inv(16) leukemia [14,15].

The frequency, characteristics, and biologic implications of the deletions were further defined [13]. Thirty-eight patients with inv(16) and four patients with the related t(16:16) were analyzed by FISH using cosmid and cosmid contig probes previously mapped as proximal to the 16p-ibc [13]. Sixteen percent of the inv(16) patients demonstrated deletion of at least 120kb from the inversion chromosome. Other studies have found 1 of 17 [14] and 5 of 13 [15] case with deletions, giving a cumulated frequency of 18%. Additional FISH studies utilizing cosmid and YAC probes that spanned the p-ibc allowed refinement of the estimated size of these proximal deletions to between 160 and 350kb [13]. Further characterization of the deletions was achieved in Southern hybridization analysis of genomic DNA from inv(16) patient samples. Utilizing probes from either side of the p-ibc, the deletion was demonstrated to within 10kb centromeric of and therefore presumably up to the p-ibc. Thus the deletions appear to occur as part of the same molecular process giving rise to the inversion.

Since the *MYH11* gene on the 16p is interrupted by the inversion, we presumed that upstream sequences of this gene would be absent in inv(16)/del patients. Sequence analysis of probes identifying deleted regions in the above experiments demonstrated *MYHII* coding elements. This confirmed the hy-

pothesis that upstream elements of *MYHII* are deleted in the inv(16)/del cases. The RT-PCR assay described above detects the transcript generated by the 5′-*CBFB/MYHII*-3′ fusion gene located on 16p as a result of the inversion. Since the deletions encompass only sequences proximal to the p-ibc, the 3′-*MYHII* region should be intact and the fusion gene preserved. As expected, in the inv(16)/del patients studied by RT-PCR, the identical transcript was detected as in inv(16) patients without the deletion.

Theoretically, the inversion of chromosome 16 could generate two fusion genes: 5′-*CBFB/MYHII*-3′ on the p-arm and 3′-*MYHII/CBFB*-5′ on the q-arm. The work described above showed that the 5′-*MYHII* region is substantially removed in inv(16)/del patients, and therefore this portion of the q-arm hybrid gene would be truncated, if not entirely abolished. Since inv(16) patients with and without deletions have indistinguishable clinical characteristics at diagnosis, we conclude that the 5′-*CBFB/MYHII*-3′ transcript rather than its reciprocal (which has not been identified in the small number of patients studied) is the critical leukemogenic factor for the inv(16) leukemia.

There are several other examples of leukemia-related translocations in which deletions have been noted. One third of 11q23 translocations are associated with truncation of the *MLL* gene [19,20]; *AML1* deletions have been identified in the t(8;21) [21]; and deletions of 3′*BCR* are well described in chronic myeloid leukemia (CML) [22]. As with inv(16), many translocations generate two possible fusion genes, of which only one appears to be critical. *BCR-ABL* and *ABL-BCR* transcripts have been identified in CML, with the former well established as critical for leukemogenesis [23]. Similarly, both *PML-RARA* and *RARA-PML* transcripts have been demonstrated, with only the former retaining the major functional domains of both genes and critical leukemogenic activity [24,25]. Thus the molecular features of inv(16) appear to correlate well with other established leukemia-associated molecular findings, perhaps implicating common mechanisms in the development of the underlying chromosomal anomalies.

The size estimates of the p-arm deletions clearly indicate that, apart from upstream segments of *MYH11*, additional DNA elements are removed. Recently, the newly described multidrug resistance protein gene (*MRP*) has been mapped to the short arm of chromosome 16 proximal to the p-ibc [15,26]. Kuss et al. studied this gene in inv(16) patients and found that it was deleted in 5 of 13 cases [15]. Intriguingly, those patients with only one copy of *MRP* had a better outcome, with more favorable relapse-free survival than those with two copies. In our study, we have been unable to discern any differences in outcome between deletion and nondeletion patients [13]. We did not study *MRP* specifically; however, in all likelihood this gene is deleted from the inv(16)/del case we described. Patient numbers in both studies are inevitably small, given the rarity of this leukemia; therefore, the credibility of the provocative notion that *MRP* loss may improve chemosensitivity and thus outcome remains to be confirmed. The result is particularly puzzling when one considers that the

superior outcome of inv(16) patients in general compared to other AML patients has been attributed to sensitivity of the leukemia cells to the chemotherapeutic agent cytosine arabinoside [27]. Since *MRP* gene products have not been shown to confer resistance to this agent, it is difficult to comprehend why the loss of one copy of *MRP* should be advantageous, should such increased survival be validated.

The original descriptions of M4Eo included not only inv(16) and t(16;16) patients but also cases with del(16q) [1,3,4]. The distinct molecular genetics of inv(16) and t(16;16), in which a chimeric gene is generated and transcribed, render it unlikely that del(16q), in which no similar hybrid gene is expected, would have the same molecular consequences. Thus it seemed probable on this basis that del(16q) would be associated with a different clinical phenotype, in keeping with the recent observations of other clinical investigators [28,29]. Del(16q) may therefore not be appropriately grouped together with the other chromosome 16 abnormalities indicative of more favorable outcome. A detailed clinical analysis of the outcome of leukemia patients with abnormalities of chromosome 16 including inv(16), t(16;16), and del(16q) was conducted [30]. The definitive conclusion was that del(16q) patients did not share the favorable outcome of inv(16)/t(16;16) patients; indeed, they fared no better than other M4 patients without chromosome 16 abnormalities. The del(16q) patients were also less likely to have eosinophilia at diagnosis and did not share the same propensity for CNS relapse that has been shown for the inv(16) patients [31]. These clinical differences are not surprising, given the unique molecular biology of inv(16)/t(16;16), and indicate that del(16q) patients should no longer be considered as a part of this group.

We were also interested to determine at the clinical level whether inv(16) is a dominant molecular event in leukemia or whether the additional cytogenetic aberrations that accompany this abnormality in 50% of cases could alter the biology of the disorder. The most frequent additional abnormalities accompanying inv(16) were trisomy 22, trisomy 8, and trisomy 21. A comparison of outcome in terms of both survival and remission duration revealed no differences between patients with inv(16) alone and inv(16) plus other abnormalities. This suggests the molecular consequences of the inversion are dominant and critical for the specific inv(16) leukemia phenotype even in the presence of other molecular genetic influences.

6. CBFB-MYH11: Implications for leukemogenesis

The roles of the family of genes associated with the core binding factor (CBF) in normal myeloid differentiation will now be discussed, as will the structure and function of *MYH11*, the myosin heavy chain gene involved in the chimeric gene product with CBF beta, in order to consider various models of how their perturbation can lead to leukemogenesis.

6.1. *The CBF transcription factor*

As described above, the 16q breakpoints are found within a gene designated *CBFB* — a gene encoding the human homologue of the beta subunit of the murine transcription factor, core binding factor (CBF). A series of three genes encodes alpha polypeptide subunits, which may heterodimerize with the beta subunit to form CBF. The role of this important transcription factor will be discussed further below. However, the reader should first be aware that there have been extensive nomenclature changes to the genes that encode the various CBF subunits in humans and mice. The attempt has been to simplify and to make the nomenclature consistent across species (see table 1 for an updated listing of CBF subunit genes in humans and mice). The new nomenclature will be used here.

The rearrangement of the *CBFB* gene from the q-arm of the 16 is viewed as the most striking finding from the molecular genetic studies. This is because the encoded polypeptide, CBF beta, interacts with a CBF alpha subunit encoded by a gene on human chromosome 21 (*CBFA2*, formerly *AML1*; see table 1). Remarkably, this gene has been shown to be rearranged by the 8;21 translocation leading to AML-M2[11]. Therefore, taken together with the data indicating that the rearrangement of the gene for the beta subunit leads to inv(16) leukemia, alterations of either of the subunits of CBF have been shown to be implicated in leukemogenesis.

The murine form of CBF beta (formerly referred to as Pebp2β) has been isolated and the gene cloned by two groups studying viral promotor binding peptides [11,32]. It appears to have important activity in regulating normal hematopoietic differentiation. CBF binds to and activates the transcriptional enhancers of the Moloney murine leukemia virus and the polyoma virus [33,34]. DNA contact to these and various eukaryotic regulatory sequences appears to be via the alpha subunit [35]. The beta subunit appears to act to increase the affinity of the alpha subunit for the 'core' consensus binding

Table 1. Updated symbols and chromosomal locations (Chr.) for the genes encoding the protein subunits associated with the CBF transcription factor in humans[a] and mice[b]

Human genes			Mouse genes		
New symbol	Replaced symbol	Chr.	New symbol	Replaced symbol	Chr.
CBFA1	*AML3*	6p21	*Cbfa1*	*Pebp2αa*	17
CBFA2	*AML1*	21q22	*Cbfa2*	*Pebp2αb*	16
CBFA3	*AML2*	1p36	*Cbfa3*	*Pebp2αc*	4
CBFB	*CBFB*	16q22	*Cbfb*	*Pebp2β*	8

[a] Genome Data Base (GDB), World Wide Web (URL:http://gdbwww.gdb.org), February, 1995.
[b] Mouse Genome Database (MGD), World Wide Web (URL:http://www.informatics.jax.org), February, 1995.

sequence TGT(C)GGT, and may also have undescribed interactions with other peptides. As indicated above, alpha subunits are encoded by one of at least three genes in both mice and humans (table 1). The predicted DNA binding domain of these factors is highly conserved among them in both species and highly homologous to the developmentally important Drosophila *runt* gene DNA binding domain. This gene family has been reviewed elsewhere [36].

Several of the alpha-factor cDNAs have only recently been identified, cloned, and chromosomally assigned in both humans and mice [35,37–39], and biological studies are ongoing. The best studied are the interspecies homologues, murine *Cbfa2* [40] and human *CBFA2* [37,41] (formerly *AML1*). These genes give rise to multiple products of differing lengths translated from alternatively spliced transcripts [37,40]. The predicted sequence of the longest open reading frame (ORF) among the published cDNAs consists of an N-terminal domain, a *runt* homology domain (DNA binding), a 62-amino-acid alternatively spliced sequence, and a serine- and proline-rich C-terminal domain critical for transcriptional activation function. The variably spliced central segment appears to confer differences in the transactivation (activation of gene transcription) function. Northern and cloning studies suggest that at least four alternatively spliced species of Cbfa2 are transcribed but that their relative proportions vary dramatically from cell type to cell type. It seems very likely that these differences in alternative splicing are regulated with differentiation and have functional significance. Expression of both the human and mouse genes appears to be regulated in association with differentiation or proliferation in certain cell types. For example, transformation of NIH3T3 cells with H-ras causes marked increase in both message expression [40] and binding of cellular factor to the core consensus site [42]. Recently, it was found that denervated muscle expresses much increased quantities of CBF alpha-2 transcript [43].

Cbf beta (the murine homologue of CBF beta) messages appear to be transcribed from a single gene, and although it is ubiquitously expressed in eukaryotic tissues [11], its levels of expression vary dramatically. The thymus expresses large amounts [11]. Alternative splicing is also operative for this gene, and splicing varies from tissue to tissue. Some splice variants of Cbf beta cooperate with the alpha subunit in DNA binding, while others do not [32]. Thus for this gene, as for the alpha-subunit genes, alternative splicing may be physiologically regulated so as to yield peptides of variable activity.

Eukaryotic transcriptional regulatory domains that appear to interact physiologically with CBF include a number of control elements for genes presumed to be involved in hematopoietic differentiation. The best established of these are listed in table 2. Of particular note and relevance among these are the myeloperoxidase (*MPO*) and neutrophil elastase (*NE*) genes, both transcribed during the late myeloblast and early promyelocyte stage of neutrophil differentiation. The potential role in leukemogenesis of genes transcribed at this stage of differentiation will be discussed below.

Table 2. Eukaryotic transcriptional regulatory domains likely controlled by CBF

Gene	Comment
TCRB	CBF binds to and activates enhancer [11,44]
TCRG	'Core' site is critical to enhancer activity [45]
TCRD	'Core' site is critical to enhancer activity [45]
CSF2	'Core' site increases promotor activity [46]
MPO	Promotor binds and is regulated by CBF [47,48]
NE	Promotor binds and is regulated by CBF [49]
CSFIR	Promoter binds and is regulated by CBF [50]

6.2. MYH11 gene product

MYH11 encodes the smooth-muscle-specific myosin heavy chain (therefore, the protein is also referred to as SMMHC) [10,51]. As with other myosin heavy chains, this chain consists of a head portion with ATPase activity that interacts with myosin light chains and actin, and a long tail that homodimerizes via its extended 'coiled coil' motif. The portion of the gene that is fused in frame to *CBFB* in inv(16) encodes the homodimerizing tail. Although there are no experimental data to address the issue, this tail might be predicted to mediate homodimerization of the CBFB-MYH11 protein in leukemic cells.

6.3. Models of CBFB-MYH11-mediated leukemogenesis

In considering possible modes of transformation mediated by this protein, it is helpful to consider data available for other leukemia-related transcription factor chimeric genes. In the case of the t(15;17) seen in acute promyelocytic leukemia, the *PML* gene at 15q22 is fused upstream of the *RARA* gene at 17q21. The resultant PML-RARα peptide has binding and transactivating activity for retinoic-acid-responsive elements. Expression of the chimeric cDNA inhibits differentiation in U937 cells [52] and prevents apoptosis of factor-dependent TF-1 cells after factor withdrawal [53]. The role of the RARα moiety in these effects is unclear. It is, however, certain that macromolecular structures normally containing PML protein and recently named *PODS* are disrupted by the expression of PML-RARα [54,55]. Treatment of transfected cells with retinoic acid restores the POD pattern to normal. The PML protein was recently shown to have growth-suppressive effects in cotransfection experiments with oncogenes [56]. Thus for this translocation, it may be postulated that the PML-RARα fusion protein expression has a transdominant effect in suppressing the normal function of PML. It must be noted that negative effects on the differentiation-inducing activity of RARα are also possible but are yet to be confirmed. This possibility appears more likely when it is considered that other translocations — the t(11;17) [57] and the t(5:17) — fuse different genes to *RARA* and yet are associated with a similar APL phenotype.

As indicated above, t(8;21) seen in AML subtype M2, results in fusion of the *CBFA2* at 21q22 upstream sequences to most of the coding sequences of a novel gene *ETO* at 8q22. Antisense inhibition of expression of this fusion gene causes differentiation and growth inhibition in CBFA2-ETO-expressing cells [58]. Recent evidence suggests that the fusion peptide CBFA2-ETO may transdominantly inhibit the normal function of the CBFA2 protein in transactivation of the *CSF2* (formerly *GM-CSF*) promotor [46]. Though the physiological relevance of this observation may be brought into question (i.e., inhibition of *CSF2* transcription seems unlikely as a route to leukemogenesis), this model provides data for inhibition of CBFA2 transcriptional activation of genes by CBFA2-ETO.

CBF, and the genes apparently regulated by this factor, would seem to function in mediating or regulating differentiation in hematopoietic (and presumably other) tissues. Thus it is reasonable, particularly given the precedents mentioned above for other translocations, to postulate that CBFB-MYH11 causes transdominant negative inhibition of CBF effect. CBFB-MYH11 expression has, in fact, been shown to inhibit transactivation of the Moloney murine leukemia virus LTR when compared to wild type CBFB [58]. In figure 3, three possible mechanisms by which this might occur are presented based upon a postulated normal complex of CBF and possible other peptides to its cognate DNA sequence. In scenario 1, the CBFB-MYH11 homodimerizes via the MYH11 tail and binds free CBF alpha-2 protein, preventing it from intercalating with DNA; in scenario 2, the CBFB-MYH11 causes a conformational change after DNA binding and thus prevents transactivation; and in scenario 3, other factors (as yet unidentified polypeptides) are prevented from interacting with the CBF-DNA complex, inhibiting the transactivation function.

There are some preliminary data on either side of the first scenario. It has been observed that expression of CBFB-MYH11 appears to diminish the binding of endogenous CBF alpha-2 protein to the consensus sequence [59] — perhaps because homodimerization of CBF beta subunits makes fewer available for heterodimerization with alpha subunits for DNA interaction. On the other side, recent reports suggest that the CBFB-MYH11 product retains the ability to bind to the 'core' DNA site cooperatively with the alpha factor [60,61], but the mechanism of inhibition of factor function remains to be elucidated.

If the CBFB-MYH11 fusion peptide inhibits the normal CBF function, how does this causes leukemogenesis? The observation that the *MPO* and *NE* genes transcribed at the promyelocyte stage are positively regulated by this factor suggests that additional, potentially as yet undiscovered genes might be inhibited at this point in differentiation. If a gene responsible for ongoing differentiation were blocked, proliferation would be predicted. As cells at this stage of maturation are rapidly dividing, a mutation preventing their concomitant differentiation would be expected to result in a massive accumulation of clonal blasts — i.e., leukemia. It has been shown recently [60] that

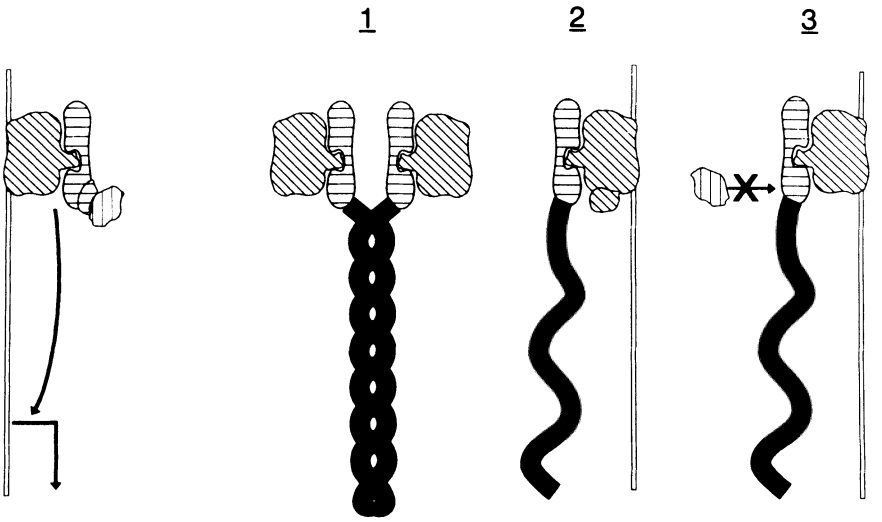

Figure 3. Schematic view of potential mechanisms of dominant negative inhibition of CBF action by the chimeric CBF beta subunit (CBFB-MYH11). At the left is shown the authors' conception of normal CBF at a DNA (long double vertical line) transcriptional control site. The alpha subunit (diagonally cross-hatched) binds to the DNA with the beta subunit (horizontally cross-hatched) bound to it. As yet undefined additional factors (vertically cross-hatched) may also bind to the complex. This results in the activation of transcription at a nearby gene (arrows). Scenarios 1 to 3 (to the right) depict possible molecular interactions of the chimeric beta subunit (shown as a long, helical, solid MYH11 tail attached to the horizontally crosshatched beta subunit) that might perturb the normal interaction of the transcription factor. In scenario 1, the MYH11 tails homodimerize and bind to the alpha subunits, preventing them from interacting with the DNA. In scenario 2, the MYH11 tail creates a conformational problem, blocking transactivation, and in scenario 3, the MYH11 tail prevents interaction of the additional factors required for transactivation.

overexpression of CBF-MYH11 transforms NIH3T3 cells. Characterization of the genes whose transcriptional regulation is modified as a result of expression of the chimeric subunit and the relationship of those perturbations to the disease process remain to be understood.

As described above, the involvement of the two subunits of CBF in different leukemia-associated translocations would appear to have considerable implications for our understanding of normal and leukemic cell growth and differentiation. That this factor is an important control element in these processes is beyond question. All human cell lines tested to date express the CBF beta and CBF alpha-2 transcripts (D. Claxton, unpublished data). It must be noted, however, that translocations of 16q22 and 21q22 are seen only in a very small minority of leukemias and have not been identified with any frequency in other malignancies [62]. The role of this system in these other human neoplasms is thus a major question for the coming years.

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2. Deletion of chromosome 5 (5q-) in leukemia: current knowledge

Lalitha Nagarajan

1. Introduction

Chromosomal abnormalities associated with distinct subsets of human leukemia suggest a link between specific genetic alterations and clinical outcome. A tight correlation between the cytogenetic anomaly and the progression of the disease indicates a direct cause and effect relationship between the genotype and the disease phenotype. In recent years, molecular cloning of genes residing at chromosomal translocation breakpoints of t(15:17), t(8:21), and inv(16), inv(3) has led to an understanding of the wide phenotypic variations observed in acute myelogenous leukemia.

Acquired interstitial loss of the long arm of chromosome 5 (5q- chromosome) is seen in patients with a range of myeloid disorders. Although the limits of these deletions vary from patient to patient, the chromosomal breakpoints appears to be identical in all of the dysplastic cells of a given patient. The presence of a region of overlap that is consistently deleted in all of these patients suggests that physical loss of an important gene from the 5q- chromosome coupled with a mutation of the second allele on the remaining 'normal' chromosome 5 results in total inactivation of this key regulatory gene — a recessive mechanism that originally led to Knudson's postulation of the existence of tumor suppressor genes in the familial retinoblastoma model and that has been proven in recent years by the isolation of candidate genes [1,2]. Unlike retinoblastoma, the median age of onset of 5q- chromosome is above 50 years of age, suggesting that both the deletion and mutation found in the dysplastic clone are acquired somatically.

The 5q- chromosome was first reported as early as 1974 [3]; nonetheless, molecular studies of this anomaly have been impeded by (1) a paucity of highly polymorphic loci on the distal long arm of chromosome 5; (2) a lack of material, particularly in the cases of myelodysplasia; (3) the technical limitations in separating the affected cells from the normal populations; and (4) the largeness of the deleted segment.

The cytogenetic identification of the 5q- chromosome as an acquired interstitial deletion was originally described as the sole anomaly in three women with macrocytic refractory anemia [3]. This description was followed by subse-

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quent reports that revealed the loss of an entire chromosome 5 or a part of the long arm of chromosome 5, in patients with de novo and therapy-induced myelodysplasia (de novo and t-MDS) or acute myelogenous leukemia (AML) [4]. The frequent association of chromosome 5 anomalies in the t-MDS/AML patients raised a possible role for alkylating agents used in the treatment of solid tumors, in the evolution of the hematopoietic disorder (reviewed in [5–9]).

An evaluation of the prognosis of the transfusion-dependent refractory anemia (RA) or 5q- syndrome, typically seen in elderly women, suggests that it is a clinical entity, readily distinguishable from the constellation of other preleukemic myelodysplastic disorders and from acute myelogenous leukemia. The RA patients present with macrocytic anemia with monolobulated megakaryocytes and normal neutrophil counts, and 5q- chromosome is the only detectable cytogenetic anomaly [8]. In contrast to the preleukemic cases, the disease manifestation ranges from RA with ringed sideroblasts to RA with excess blasts (RAEB) and RAEB in transformation (RAEBT). The preleukemic MDS patients show trilineage involvement; susceptibility to frequent infections is indicative of dysfunctional myelopoiesis. Presence of 5q- chromosome in RAEB and RAEBT correlates with poorer prognosis than with AMLs with diploid karyotype [10,11].

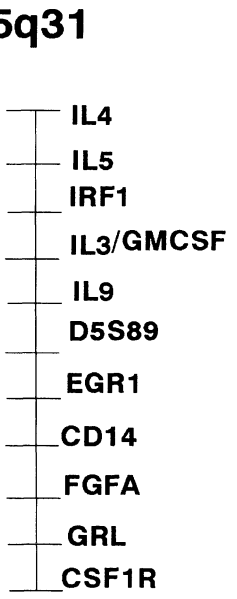

Figure 1. Consensus map of the genes of human chromosome 5q31. Genes and loci are denoted on the right. IL4, interleukin 4; IL5, interleukin 5; IRF-1, interferon response factor 1; IL3, interleukin 3; GMCSF, granulocyte macrophage colony-stimulating factor; IL9, interleukin 9; D5S89, a polymorphic DNA fragment rearranged in the AML cell line HL60; EGR1, early growth response gene 1; CD14, monocyte antigen; FGF A, acidic fibroblast growth factor; GRL, glucocorticoid receptor; CSF1R, colony stimulating factor 1 receptor.

Thus, the gross differences between the 5q- syndrome RA and MDS/AML suggest that these conditions could result from different genetic lesions. Therefore, for the purpose of this chapter, the RA (5q- syndrome) is not included in the category of MDS.[1]

2. Cytogenetic delineation of 5q- chromosome

The 5q- chromosome of RA patients cannot be cytogenetically discriminated from those of MDS and AML cases. A retrospective cytogenetic study of patients with interstitial deletions of chromosome 5 (including RA and MDS/AML) identified at least four types of cytogenetically identifiable deletions: (1) 5q13–33, (2) 5q31–35, (3) 5q22–33, and (4) 5q13–35. In physical terms, these are large deletions; since the long arm of chromosome 5 contains 4.5% of the haploid genome, these interstitial deletions would result in the loss of 0.5% to 1.5% of the genome of a diploid cell. Using high-resolution banding techniques, 5q31 has been identified as the most common cytogenetically deleted segment in 91 out of 93 patients with 5q deletion [12]. This segment corresponds to 17 Mb of DNA. As shown in figure 1, a unique and striking cluster of genes, which govern normal hematolymphoid growth and differentiation, has been fine mapped to reside within a few megabases of each other on band 5q31 [13,14].

3. Molecular delineation of the critical region

Is the smallest region of overlap (SRO) common to both the 5q- syndrome and MDS/AML, or is there more than a single critical gene on 5q31? Recent developments in recombinant DNA techniques have allowed considerable progress in the molecular definition of the minimal region of overlap. Fluorescence in situ hybridization (FISH), yeast artificial chromosome (YAC) cloning, dinucleotide polymorphism analyses by polymerase chain reaction (PCR), and irradiation-reduced hybrid mapping techniques have facilitated delineation of the smallest region of loss in molecular terms. These investigations have benefited a great deal from the wealth of information and reagents available from the human genome mapping efforts.

Our studies have focused on a unique DNA fragment, *D5S89*, which we isolated due to its structural rearrangement in the AML cell line HL60 [15]. Loss-of-heterozygosity analyses employing restriction fragment length polymorphisms and the recently developed dinucleotide polymorphism analyses demonstrate invariant loss of a *D5S89* or flanking locus from the 5q- chromosome in less than 20 AML and MDS patients examined [15–18]. Le Beau and coworkers performed metaphase FISH on 17 cases (16 cases of MDS/AML and 1 RA) with overlapping 5q31 deletions using a panel of 5q31 probes and determined that the loci telomeric to *IL9* and centromeric to the anonymous

locus *D5S166* reside within the smallest region of overlap; these results imply that the *D5S89* and *EGRI* loci are within the critical region [19].

A survey by Boultwood et al. of patients with 5q- syndrome RA identified two patients with uncharacteristically small interstitial deletions (del. 5q31–33) [20]. The smallest region of loss resides between the *GRL* gene and the *NKSF1* gene, which is telomeric to *CSF1R*, with apparent retention of all the centromeric and telomeric loci [20]. A case of t-MDS with impaired neutrophil maturation and del 5q(11–31) was studied in detail by us [21]. FISH of three 5q31-specific probes to the metaphases demonstrated that the interstitial deletion on the 5q- chromosome is not contiguous. The 5q- chromosome has lost the *D5S89* and *CSF1R* loci while retaining some of the in-between sequences, which were derived from a radiation hybrid spanning *D5S89* and *EGR1*.

Therefore, the 5q31 segment between *IL9-NKSF1* could harbor two critical genes (figure 2). The distance between *IL9* and *CSF1R* genes is estimated to be 7 Mb by radiation hybrid mapping [13,14], whereas interphase FISH assessments are less than 5 Mb [22].

4. Biology of RA and MDS and AML

The neutrophil maturation pathway of patients with 5q- syndrome RA is apparently normal. Using a PCR-based analysis to detect allele loss for specific 5q loci, we have determined that the peripheral blood neutrophils of patients with 5q- syndrome RA are derived entirely from the 5q- clone [18]. Other studies have identified expression of the monocyte antigen CD14 on the surface of the granulocytes of RA cases, suggesting that the myelomonocytic differentiation of these cases is not blatantly influenced [23]. Thus, the 5q- clone defective in erythroid and megakaryocytic maturation suppresses the differentiation of neutrophils from the diploid population of the marrow (figure 3A).

In contrast, as shown in figure 3B, in preleukemic t-MDS, the bone marrow reveals anomalies of all the lineages, with erythroid hyperplasia, megaloblastoid features, and an increase in immature granulocytes [24].

5. Candidate tumor suppressor gene from the smallest region of overlap

5.1. Interleukin genes

Although the interleukins *IL4*, *IL5*, *IL3*, *GMCSF*, *IL9*, and *IL13* map to the frequently deleted 5q31 region, hemizygosity of these genes is not likely to be crucial, since their expression will remain unaltered due to T-cells and endothelial cells, which do not characteristically exhibit the 5q- deletion.

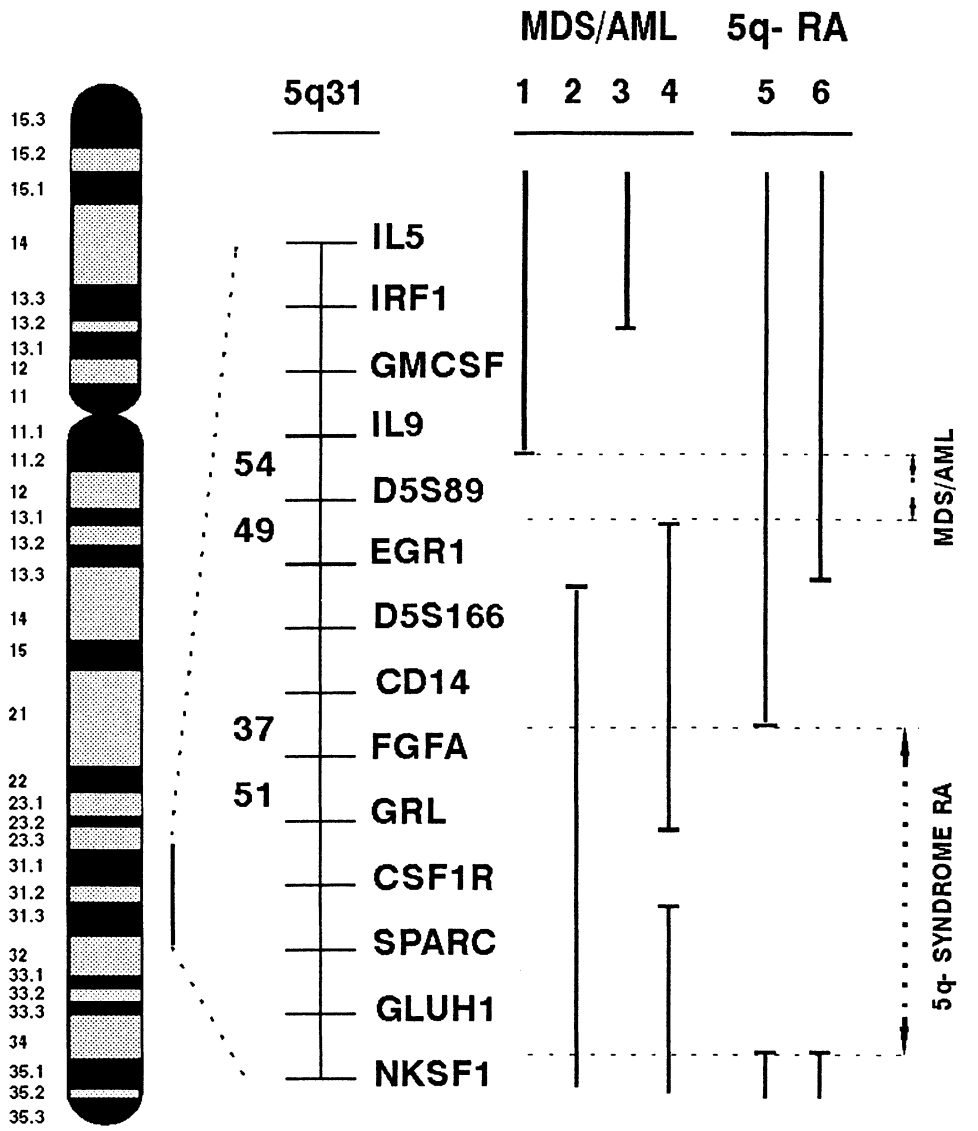

Figure 2. Molecular delineation of the RA and MDS/AML loci. The order of genes is as reported elsewhere. Genes and loci are as denoted in figure 1. SPARC, osteonectin; GLUH1, glutamate receptor; NKSF1, *IL12* subunit. The distances between IL9/D5S89/EGR1 and CD14/FGFA/GRL are denoted in centiray (cR) units (1 cR ≈ 34 kbp). Patients characterized as retaining proximal and distal loci within the minimal region overlap have been previously described. Patients 1 and 2 were described by Le Beau et al. [19], patients 3 and 4 by Nagarajan et al. [17,21], and patients 5 and 6 by Boultwood et al. [20]. Lines denote loci retained.

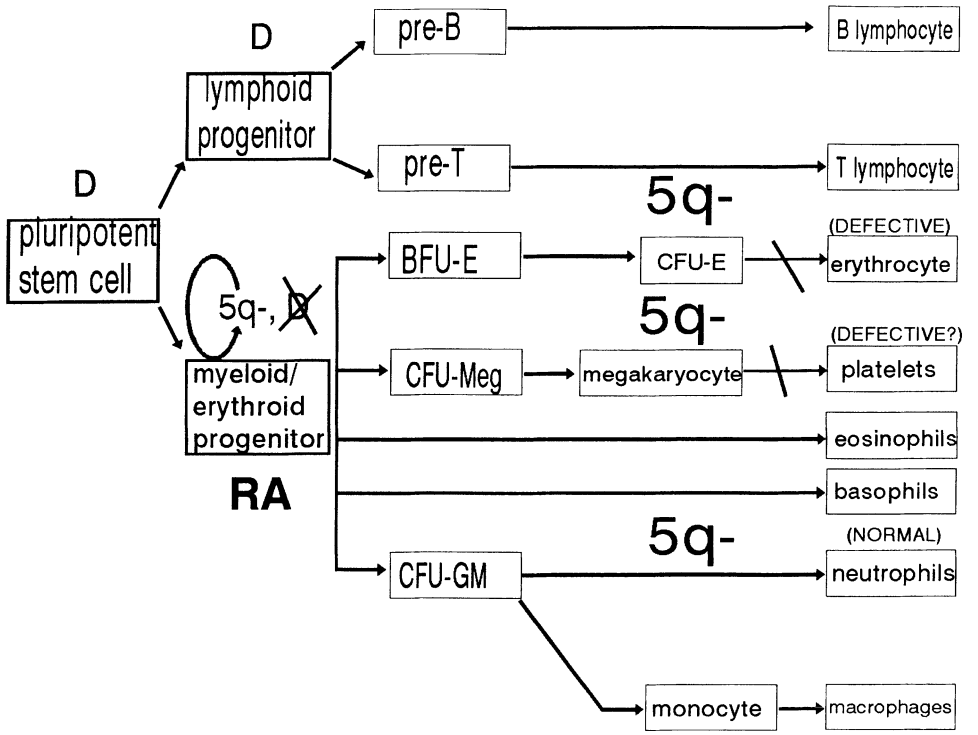

Figure 3. Model depicting suppression of normal hematopoiesis in the 5q- syndrome RA and MDS/AML with 5q- chromosome.
(**A**). The 5q- deletion occurs in the myeloid erythroid progenitor, which suppresses the normal diploid cells. Loss of the 5q31 gene impairs erythroid and megakaryocytic maturation, leading to refractory anemia (5q- syndrome). Note that the neutrophils derived from the 5q- clone are apparently normal. D, diploid progenitors.
(**B**). The 5q- deletion occurs in the myeloid erythroid progenitor, which suppresses the normal diploid cells. Loss of the 5q31 gene impairs erythroid, megakaryocytic, and granulocytic maturation with elevated blasts. D, diploid progenitors.

5.2. The Wilm's tumor suppressor gene homologue early growth response gene EGR1

An early growth response transcription factor (*EGR1*) encoding a Zn finger protein is within the smallest region of overlap in most cases. An intensive search for mutations and structural rearrangements of the *EGR1* gene has provided evidence for lack of genetic alterations in the remaining allele of *EGR1* in 5q- cases [25].

5.3. Interferon response factor I (IRF1)

IRF1 is thought to play an immediate early response role in *IL6*-mediated myeloid differentiation [26]. Overexpression of the DNA binding protein,

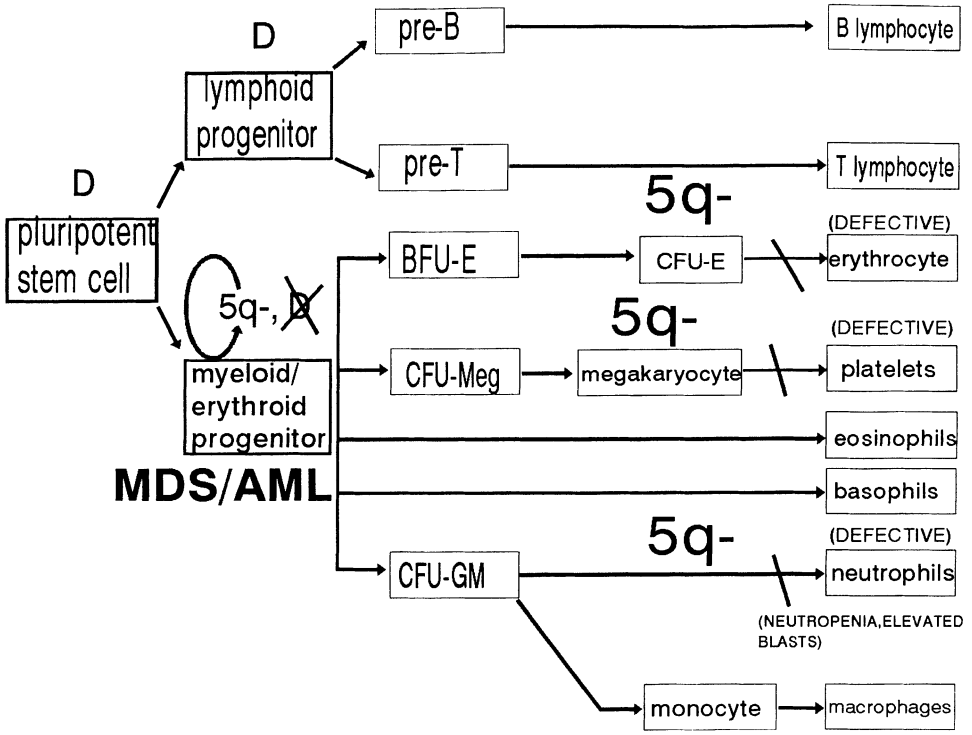

Figure 3. (continued)

interferon response factor 2 (*IRF2*), changes the stoichiometry between *IRF2* and *IRF1*; the altered ratio is believed to be responsible for transformation of the NIH 3T3 fibroblasts [27]. Although earlier studies suggested loss of an *IRF1* allele in a total of 13 patients (RA, MDS/AML) with 5q- chromosome [28], recent findings demonstrate that both alleles of *IRF1* may be retained in some cases of RA and MDS/AML, raising the possibility that *IRF1* is not a candidate gene [17,29]. At present, there is no experimental evidence on decreased expression or mutation of the remaining *IRF1* allele in the 5q- cells of AML and MDS cases to support the role of *IRF1* in neoplastic transformation.

5.4. Colony-stimulating factor I receptor

Initial reports on the loss of both alleles of the CSF1R gene in a subset of the dysplastic cells of a 5q-RA patient as detected by in situ hybridization of radiolabeled *CSF1R* [30] probe have not been demonstrated conclusively by Southern blotting data of sorted populations of 5q- cells. In normal hematopoiesis, *GMCSF* and *CSF1* exhibit functional overlap. Therefore, the

GMCSF may mediate differentiation through its receptor on cells that lack *CSF1*.

6. Conclusions

Recent studies have suggested that, cytogenetically, the interstitial 5q deletion appears to fall into at least two categories: (1) AML, MDS, and RA patients who have large contiguous breaks; and (2) t-MDS patients who have noncontiguous breaks. There is no direct evidence for any of the previously cloned genes being the candidate gene inactivated in 5q- patients. The wide phenotypic differences seen in MDS/AML patients with the 5q- chromosome raise the possibility that more than one critical gene resides within band 5q31 or that a single gene with multiple mutations could be responsible for the different phenotypic manifestations of 5q- chromosome.

The molecular analyses indicate that the proximal MDS/AML tumor suppressor gene encoded in the *D5S89* locus governs neutrophil/monocyte growth and differentiation; loss of function of this locus plays a role in MDS and AML with poor prognosis. A subset of MDS/AML patients carries mutations of this gene, whereas the 5q- syndrome RA patients carry an inactivated telomeric gene (figure 4). A third group of patients may exhibit mutations in both loci.

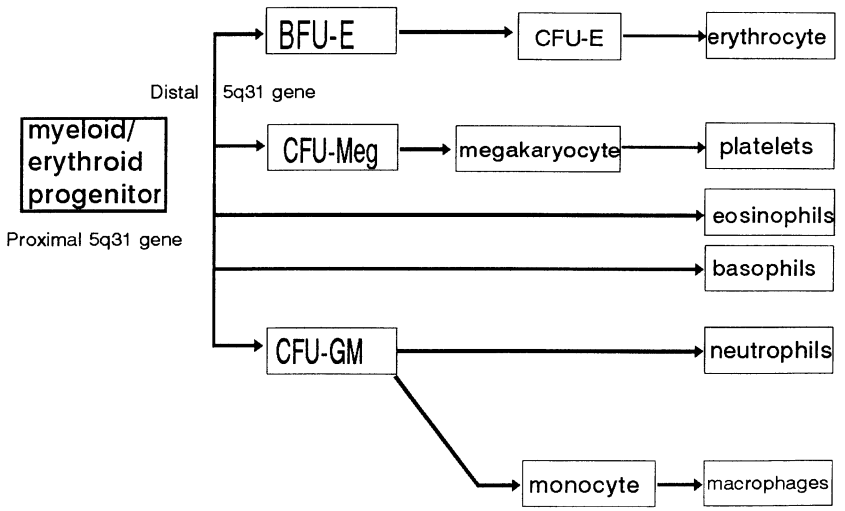

Figure 4. Model for the role of two 5q31 regulatory genes that govern hematopoiesis. The 5q- deletion occurs in the myeloid/erythroid progenitor. Loss of the distal 5q31 gene in the *CSF1R* locus impairs erythroid and megakaryocytic maturation, leading to refractory anemia (5q- syndrome). Loss of the proximal gene in the *D5S89* region affects granulocytic growth and differentiation, resulting in MDS/AML.

Patients with chronic myelomonocytic leukemia (CMMoL), classified as a distinct subset of MDS, carry a specific 5:12 chromosomal translocation resulting in gene fusion between the protein kinase domain of the platelet-derived growth factor receptor and a novel helix-loop helix transcription factor [31]. Likewise, the 5q-syndrome RA patients may harbor a unique molecular lesion that is different from the MDS/AML.

Positional cloning and characterization of tumor suppressor genes have provided novel insights into normal cell growth and division. Frequently, these genes code for ubiquitously expressed negative regulators of cell growth. Isolation of the retinoblastoma gene allowed integration of signal transduction pathways and DNA synthesis, although the originally recognized phenotypic anomaly was inherited retinal tumors. The tumor suppressor genes thus far isolated are nuclear transcription factors, cell surface adhesion molecules, ras-GTPase regulatory proteins, and inhibitors of cell-cycle regulatory protein kinases. Loss of regulatory proteins that arrest cell-cycle progression and division appears to be a recurrent theme. The isolation and characterization of the 5q31 MDS/AML tumor suppressor gene may uncover novel cellular circuits, given the widespread role of tumor suppressors in normal cell growth and differentiation.

Acknowledgments

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Note

1. MDS: refractory anemia with ringed sideroblasts (RARS), refractory anemia with excess blasts (RAEB), refractory anemia with excess blasts in transformation (RAEBT).

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3. Bcl-2 family proteins and the regulation of programmed cell death in leukemia and lymphoma

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1. Introduction

Members of the *bcl-2* gene family play a central role in regulating the relative sensitivity and resistance of cells to a wide variety of apoptotic stimuli. The first member of this multigene family, *bcl-2*, was discovered by virtue of its involvement in the t(14;18) chromosomal translocations commonly found in non-Hodgkin's lymphomas [1–4]. Deregulation of the *bcl-2* gene either by translocations in B-cell lymphomas or through other mechanisms in several other types of cancer, including acute and chronic leukemias, contributes to neoplastic cell expansion by prolonging cell survival rather than by accelerating rates of cell division [5–8]. The Bcl-2 protein also can protect tumor cells from apoptosis induced by radiation and nearly all cytotoxic anticancer drugs [9–12], thus potentially contributing to treatment failures in patients with cancer [13–16]. Several additional homologues of *bcl-2* have recently been discovered in humans and other mammals, revealing the presence of a multigene family [17–20]. Moreover, homologues of *bcl-2* have been discovered in some DNA viruses, including Epstein–Barr virus (EBV), which plays a significant role in the pathogenesis of some types of non-Hodgkin's lymphomas and Hodgkin's disease [21]. Interestingly, some members of the *bcl-2* gene family function as inhibitors of cell death, similar to *bcl-2*, whereas others are promoters of apoptosis that oppose the actions of the Bcl-2 protein. Many of these Bcl-2 family proteins have the capacity to interact with each other through formation of homo- and heterotypic dimers [17,22], revealing an important role for protein–protein interactions in the orchastration of Bcl-2 family protein function and suggesting approaches to pharmacologically manipulating the physiological cell death pathway (see table 1).

2. Discovery of *bcl-2* at the breakpoints of t(14;18) translocations in lymphomas

Chromosomal translocations represent a general mechanism of proto-oncogene activation in human lymphomas and leukemias (reviewed in [23]).

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Table 1. Characteristics of known Bcl-2 interacting proteins

Protein	Description	Binding to other Bcl-2 homologues[a]	Function
Bax	Bcl-2 homologue	Bcl-X-L, Mcl-1, A1, Bad [not Bcl-X-S or Bad]	Death
Bcl-X-L	Bcl-2 homologue	Bcl-X-L, Mcl-1, Bcl-X-S, Bad	Survival
Bcl-X-S	Bcl-2 homologue [missing BD(b) and BD(c)]	Bcl-X-L	Death
Mcl-1	Bcl-2 homologue [PEST sequences]	Bcl-X-L, Bax	Survival [weak][b]
A1	Bcl-2 homologue [no TM domain]	Bcl-2, Bcl-X-L, Bax	Survival [weak][b]
Bak	Bcl-2 homologue	Bcl-X-L, E1b-19kDa [Not Bcl-2, Bax, Bcl-X-S]	Death[c]
Bad	Bcl-2 homologue [no TM domain; missing BD(a)]	Bcl-X-L [not Bax]	Death
BAG-1	Ubiquitin-like domain; acidic; no TM domain	Bcl-2, Bcl-X-L, Bcl-X-S	Survival
R-Ras	GTPase	N.T.	Death
Raf-1	Serine/threonine-protein kinase	N.T.	Survival
Nip-1	Phosphodiester homology; TM domain; PEST sequences	E1b-19kDa	Unknown
Nip-2	Ca^{2+}-binding motif homology; no TM domain; PEST sequences	E1b-19kDa	Unknown
Nip-3	Calbindin-D domain; TM domain; PEST sequences	E1b-19kDa	Unknown

[a] Only those interactions with other Bcl-2 family protein that have been experimentally documented are indicated. In many cases, binding to other members of the Bcl-2 protein family has not been tested.

[b] Anti-apoptotic activity may be weaker than for Bcl-2, based on gene transfer studies. Abbreviations: TM (transmembrane domain); N.T. (not tested).

[c] In some assays, Bak protected against cell death rather than promoting apoptosis.

In approximately 80% of neoplasms of B-cell origin, translocations involving one of the immunoglobulin (Ig) gene loci can be detected by routine cytogenetic techniques. In these translocations, typically a cellular proto-oncogene located on a different chromosome becomes fused in a *cis*-configuration with the Ig heavy-chain (IgH) locus on chromosome 14 or with one of the light-chain (IgL) loci on chromosomes 2 or 22. The result is that powerful transcriptional enhancer elements associated with the Ig gene loci exert their influence on the juxtaposed cellular proto-oncogene, thus deregulating its expression and causing continuously high levels of transcription of the involved oncogene.

The first discovered example of chromosomal translocations as a mechanism for activating a cellular proto-oncogene came from studies of Burkitt lymphomas, where the *c-myc* gene becomes involved in translocations with the IgH or IgL genes in nearly 100% of cases [23]. Using this as a paradigm, it was speculated by Croce and colleagues that other novel proto-oncogenes might similarly become activated by a mechanism involving chromosomal translocations. Among the more common translocations seen in B-cell malignancies are the t(11;14) typical of mantle cell lymphoma (also called intermediate differentiated lymphoma [IDL]) and the t(14;18) seen in most follicular lymphomas (also termed nodular poorly differentiated lymphoma [NPDL]). Thus the names *bcl-1* and *bcl-2* (for B-cell lymphoma-1 and 2) were coined for the genes that at that time were speculated to exist on chromosomes 11 and 14, respectively. Using probes derived from the IgH locus as a starting point, several independent groups of investigators then 'walked' across the t(14;18) breakpoints of non-Hodgkin's lymphomas and into the adjacent *bcl-2* gene sequences [1–4].

3. Structure and consequences of t(14;18) translocations in follicular lymphomas

Over 85% of follicular non-Hodgkin's lymphomas contain t(14;18) translocations, suggesting that this genetic alteration represents an early event in the pathogenesis of these malignancies that arise from germinal center B-cells [24,25]. DNA sequence analysis of the breakpoints of t(14;18) chromosomes has demonstrated that no two are identical [26]. Nevertheless, the breakpoints cluster into two regions on chromosome 18, with the major breakpoint cluster region (mbr) located within the 3′-untranslated region of the *bcl-2* gene and a less commonly involved minor cluster region (mcr) residing 3′- and completely downstream of the *bcl-2* transcriptional unit [27]. Thus, the breakpoints of t(14;18) chromosomes do not involve the coding regions of the *bcl-2* gene. Though speculative, it has been suggested that t(14;18) translocations may arise due to errors in the normal DNA recombination mechanisms involved in the cutting and splicing of gene segments in the IgH locus, where heavy-chain proteins are encoded in separate V, D, and J gene segments [1,28]. This speculation is based in part on the finding of sequences within or near *bcl-2* on chromosome 18 that resemble the classical heptamer/nonamer motifs flanking the V, D, and J gene segments, which presumably represent recognition elements for cellular recombinases. Other theories, however, have also been advanced in an effort to explain the origins of t(14;18) and related translocations in human cancers, particularly the idea that Chi-like motifs located within or near *bcl-2* may serve as DNA substrates for mediating illicit recombination events [29].

In this regard, lymphomas that contain t(14;18) translocations typically are widely disseminated and involve the bone marrow at the time of diagnosis [30],

which lends indirect support to the notion of an origin for these translocations due to errors in V, D, and J gene recombination, inasmuch as these gene rearrangements normally take place in the bone marrow at the pre-B-cell stage of B-cell differentiation. Regardless of the actual mechanisms responsible, the t(14;18) breakpoint can serve as a clonal marker for diagnosis and monitoring of patients with lymphomas, using the polymerase chain reaction (PCR) for detection of t(14;18)-containing cells at frequencies as low as 10^{-6} [30]. In this regard, PCR-based detection of malignant cells that harbor a t(14;18) has shown suggestions of prognostic utility, particularly for monitoring patients after receiving ablative therapy followed by autologous bone marrow transplantation [31].

Based on nuclear run-on transcription assays, t(14;18) translocations appear to chiefly deregulate *bcl-2* gene expression at the transcriptional level [32,33]. Although some t(14;18) breakpoints fall within the 3′-untranslated region of *bcl-2* and result in the production of *bcl-2*/IgH fusion transcripts, measurements of the turnover of normal and fusion transcripts suggest similar half-lives of approximately 2.5 to 3 hours under most circumstances [32–34]. In experiments where *bcl-2* 'minigenes' have been linked with IgH enhancer elements in plasmid constructs, high levels of *bcl-2* expression were obtained in B-cell lines, consistent with the notion that *cis*-acting regulatory elements located in the IgH locus are fundamentally responsible for the alterations in *bcl-2* gene expression seen in t(14;18)-containing B-cell lymphomas [35–37]. Hypomethylation of the promoter region of the *bcl-2* gene also occurs in the translocated but not the unrearranged *bcl-2* alleles of t(14;18)-containing lymphoma cell lines [35,38]. In addition, changes in the DNaseI hypersensitivity of specific sites in this region of the *bcl-2* gene have been reported, again consistent with the idea that transcriptional mechanisms play a major role in the deregulation of *bcl-2* gene expression caused by t(14;18) translocations [39].

The in vivo consequences of t(14;18) chromosomal translocations, where deregulation of *bcl-2* gene expression is concerned, can perhaps best be appreciated by comparisons of the patterns of Bcl-2 protein production in normal and neoplastic lymph nodes. In normal lymph nodes, Bcl-2 protein is found at high levels in the mantle zone region, a cuff of small dense lymphoid cells that surrounds the germinal center regions of secondary follicles. The mantle zone region is composed functionally of a population of mostly long-lived 'memory' B-cells with recirculating capacity. Little Bcl-2 immunoreactivity is found within the centers of the follicles, where the germinal-center B-cells reside — a population of cells that have recently encountered specific antigen and that are highly prone to apoptotic cell death [40–42]. These patterns of Bcl-2 immunoreactivity in normal nodes suggest that *bcl-2* gene expression is normally shut off as recirculating B-cells enter germinal centers and encounter antigens. Based on in vitro investigations using isolated germinal center B-cells, it has been suggested that those B-cells that successfully compete for antigen and that receive appropriate co-stimuli from helper T-cells are in-

duced to reexpress *bcl-2* and thus are spared from apoptosis and allowed to exit the germinal center and join either the pool of recirculating memory cells seen in the mantle zone or to differentiate into antibody-producing plasma cells [43]. In contrast to this distinct pattern of Bcl-2 immunoreactivity seen in normal nodes, immunostaining of follicular lymphoma specimens reveals strong Bcl-2 immunoreactivity in the germinal center compartment [44]. Presumably, the t(14;18)-containing lymphoma cells that take up residence in the germinal centers find the follicular regions of nodes a conducive environment for clonal expansion, where they enjoy a selective survival advantage relative to their normal counterparts.

The natural history of follicular lymphomas is consistent with *bcl-2*s function as a regulator of cell lifespan, as opposed to cell division. Follicular non-Hodgkin's lymphomas, for example, are considered low-grade tumors, with patients experiencing median survivals of 5 to 8 years, even if untreated, compared to only 1 to 2 years for high-grade lymphomas [45]. In these patients, there occurs a gradual accumulation of malignant cells, culminating inevitably in patient death, despite various attempts at therapeutic intervention. Typically, more than 99% of the malignant cells are resting G_0/G_1-phase cells, again in keeping with notion that the primary defect in these indolent B-cell tumors represents a selective survival advantage instead of an increased proliferative rate. Probably the best evidence that deregulation of *bcl-2* is indeed directly responsible for the characteristics of these tumors comes from transgenic mouse experiments in which the transgene consisted of a human *bcl-2* minigene linked to the IgH enhancer, thus mimicking the t(14;18) structure and resulting in high levels of Bcl-2 protein production specifically in B-cells in these animals. In these mice, follicular expansions of mature B-cells were observed in nodes and spleen, which were initially polyclonal in origin. The preponderance of these *bcl-2*-expressing B-cells were small resting G_0/G_1-phase cells, which exhibited markedly prolonged survival in vitro when explanted into cultures, compared to B-cells derived from transgene-negative littermate controls [7,8,46]. Thus, the characteristics of the lymphoproliferative disorder seen in *bcl-2*/Ig transgenic mice are highly reminescent of follicular lymphoma as it occurs in patients. Interestingly, in both patients with follicular lymphomas and in mice with *bcl-2*/Ig transgenes, transformation of the low-grade lesions to aggressive rapidly fatal tumors occurs frequently, and is often accompanied by the activation of additional cellular proto-oncogenes such as *c-myc* [47–49].

4. *BCL-2* gene expression in leukemias, Hodgkin's disease, and other types of cancer

In addition to its activation because of chromosomal translocations in B-cell lymphomas, high levels of *bcl-2* gene expression have been reported in a significant percentage of acute and chronic lymphocytic and nonlymphocytic

leukemias. Chronic lymphocytic leukemia (CLL), for example, is a low-grade B-cell malignancy that behaves clinically not unlike folllicular lymphoma and probably results primarily from a selective survival advantage, rather than an increase in proliferative rates in the malignant B-cells. Though translocations involving *bcl-2* occur only rarely in B-CLLs (probably 3%–4%), high levels of Bcl-2 protein are found in more than 70% of cases [38]. These levels of Bcl-2 protein were shown by comparisons to lymphoma cell lines to be just as high if not higher than those resulting from t(14;18) translocations. In the study of B-CLL from our laboratory, most cases were typical CD5+ cases of B-CLL. In contrast to CLL, however, normal CD5+ B-cells isolated from cord blood or peripheral blood have been reported to contain little if any *bcl-2* mRNA and no Bcl-2 protein [50,51]. Though it is difficult to exclude the possibility that CD5+ B-CLLs are representative of a rare subpopulation of normal B-cells in which very high levels of Bcl-2 protein represent a normal situation, it seems likely that *BCL-2* gene expression is dysregulated in these leukemias, thus accounting for the high levels of Bcl-2 protein observed. The precise molecular mechanisms involved remain unknown, but hypomethylation of both alleles of *bcl-2* in the promoter region and the first two exons has been described in B-CLLs [38].

Elevations in Bcl-2 protein levels have also been described in some acute leukemias. In acute myeloid leukemias (AMLs), for example, high percentages of Bcl-2-positive cells were detected by indirect immunofluorescence with flow-cytometric analysis in 30 of 82 cases (defined as more than 20% Bcl-2 positive cells) and correlated with poor response rates to chemotherapy and shorter overall survival [16]. Leukemic blasts with high percentages of Bcl-2 also survived longer in culture in the absence of growth factors, a finding that often defines a subgroup of patients with poor outcomes. Similarly, in a study of acute lymphoblastic leukemia (pre-B cell type), 100% of 16 cases evaluated by flow cytometry contained elevated levels of Bcl-2 protein compared to normal B-cell progenitors [52]. Leukemic cells with high levels of Bcl-2 also survived longer in culture, with the cells having the highest relative levels of Bcl-2 exhibiting a capacity to grow continuously in the absence of stromal cells.

The involvement of *BCL-2* in Hodgkin's disease has been controversial. Some groups have reported the presence of t(14;18)-containing cells by highly sensitive PCR methods, whereas others have failed to detect such cells [53–60]. Immunohistochemical analysis of Reed–Sternberg cells suggests that while Bcl-2 protein is present in many of these cells, the levels are not particularly high and thus probably do not reflect deregulated expression [53]. One potential explanation for these discrepant results may lie in recent observations showing that rare ($\leq 10^{-6}$) t(14;18)-containing cells can be detected by PCR in about one third to one half of normal lymph nodes [61,62]. DNA sequencing analysis has provided convincing evidence that these results do not reflect DNA contamination or PCR artifacts. Interestingly, the frequency of t(14;18)-

containing cells in peripheral blood and lymphoid organs increases with age, as does the risk of lymphoma [63]. It is possible, therefore, that the detection of t(14;18) translocations in some cases of Hodgkin's disease is a reflection of this phenomenon, rather than an indication of *bcl-2* gene activation in Reed–Sternberg cells. It appears, therefore, that t(14;18) translocations arise frequently in normal individuals but are insufficient to result in lymphoma in the majority of cases. Presumably this is because either (1) this translocation is by itself insufficient to fully activate the *bcl-2* gene, and other complementary mutations are required (such as point mutations, which tend to accumulate in translocated *bcl-2* gene because of the hypermutation mechanism associated with the immunoglobulin heavy-chain locus [64]); or (2) because other cooperating oncogenes must be activated or tumor suppressors inactivated.

Aberrant patterns and abnormally high levels of *bcl-2* gene expression have been reported in several types of solid tumors. Though the data are based largely on qualitative comparisons using immunohistochemical assays, it nevertheless appears that alterations in *bcl-2* expression may occur in as much as about half of all cancers, including about 90% of colorectal adenocarcinomas, 30% to 60% of prostate cancers, about 20% of squamous cell non-small cell lung cancers, about 60% of gastric cancers, and about 80% of undifferentiated nasopharnygeal cancers, as well as significant proportions of neuroblastomas, renal cancers, small cell lung cancers, and melanomas [15,16,38,65–72]. The details of how the patterns or levels of Bcl-2 protein production differ from normal cells vary among tumor types, with *bcl-2* deregulation occurring as a relatively early event in some tumors such as colorectal adenocarcinomas or relatively late in others such as prostate cancers [65,73,74]. In prostate, colorectal, and other solid tumors examined to date, no evidence of gross structural alterations in the *bcl-2* gene has been discovered by Southern blot analysis. Unlike the t(14;18) translocations seen in lymphomas, therefore, *trans*- rather than *cis*-regulatory mechanisms presumably are responsible for the alterations in *bcl-2* expression that occur in most types of nonlymphomatous cancer.

5. Loss of p53 tumor suppressor as a potential mechanism of *bcl-2* deregulation

One of the *cis*-regulatory mechanism that potentially may contribute to *bcl-2* deregulation in cancers is loss of the p53 tumor suppressor gene. The p53 gene becomes inactivated in over half of all human cancers. The protein encoded by this gene has at least two important actions with regards to its ability to function as a tumor suppressor. First, p53 induces cell-cycle arrest at the G_1/S-border [75]. Second, p53 can induce apoptosis in some types of cells [76,77]. In many cases, however, induction of p53 alone is insufficient to spontaneously

trigger apoptotic cell death but can markedly increase the sensitivity of tumor cells to apoptosis induced by radiation or DNA-damaging drugs [78,79]. The protein encoded by the p53 gene binds DNA and functions at least in part as a transcriptional regulator, acting as either an inhibitor or an inducer of gene expression, depending on the particular target gene. Although the mechanisms by which p53 downregulates the expression of particular target genes remain undetermined, its ability to upregulate gene expression has been associated with binding directly to specific DNA sequences having the consensus 5′-PuPuPuC(T/A)(A/T)GPyPyPy-3′ [80].

Data from our laboratory indicate that p53 can function as a downregulator of *bcl-2* expression, at least in the myeloblastic leukemia line where this was tested [81]. In transient cotransfection assays, wild-type p53 was shown to be capable of downregulating in a p53-deficient human lung cancer line H358 the expression of reporter gene plasmids that contained a 195-bp DNA fragment derived from the 5′-untranslated region (5′-UTR) of the *bcl-2* gene [82]. This p53-dependent negative response element (PNRE) functioned regardless of orientation and position, suggesting it has the characteristics of a transcriptional silencer. Immunoblot and immunhistochemical analysis of Bcl-2 protein levels in p53-deficient transgenic mice ('knock-outs') revealed elevated levels of Bcl-2 protein in some tissues, including spleen, thymus, and prostatic epithelium, compared to normal littermate control animals that retained both copies of their p53 genes [81]. However, loss of p53 did not detectably affect *bcl-2* expression in many tissues, implying that the extent to which basal levels of p53 influence *bcl-2* is highly tissue-specific. For example, *bcl-2* is not normally expressed in the liver, and in the absence of p53, *bcl-2* was still not expressed, implying the existance of p53-independent mechanisms for repression of *bcl-2* [81]. Indeed, a p53-independent negative regulatory element (NRE) has been described in the *bcl-2* gene [39,81].

The prediction of these observations is that in some but not all types of cancer, depending on the tissue of origin, loss of p53 will be associated with deregulation of *bcl-2* gene expression. The data available thus far, however, clearly indicate that regulation of *bcl-2* gene expression is complex, with multiple factors potentially providing input into the *bcl-2* gene promoter gene. Thus, loss of p53 as a single parameter may not necessarily correlate with elevations in *bcl-2* gene expression. It is unknown, however, what the in vivo influence of p53 is on *bcl-2* expression in the setting of chemotherapeutic drug- or radiation-induced DNA damage, which is known to upregulate p53 protein levels and p53 transcriptional activity [83,84]. In a human myeloblastic leukemia line with wild-type p53, for example, radiation and DNA-damaging drugs were shown to induce a marked decrease in *bcl-2* mRNA levels followed by apoptotic cell death [85]. Thus, while basal levels of p53 activity may be insufficient to significantly impact *bcl-2* gene expression in some types of cells, the elevated levels of p53 activity associated with genotoxic stress conceivably could be important as an in vivo mechanism for downregulating *bcl-2* and inducing apoptosis.

6. *bcl-2* and chemoresistance in cancer

Though *bcl-2* plays an important role in the origins of cancer, where it contributes to neoplastic cell expansion by delaying or preventing normal cell turnover due to programmed cell death, perhaps more important are the potential effects of overexpression of this gene on responses to therapy. Using gene transfer methods to overexpress *bcl-2* in leukemia and solid tumor cell lines that contained low levels of Bcl-2 protein, as well as antisense approaches to reduce the levels of Bcl-2 protein in t(14;18)-containing lymphoma cell lines that contained high levels of this protein, it has been shown that the levels of Bcl-2 protein correlate with relative sensitivity or resistance to a wide spectrum of chemotherapeutic drugs as well as γ-irradiation [9–11,16,18,86–96]. Bcl-2 has been experimentally shown to render cells more resistant to being killed by dexamethasone, cytosine arabinoside (Ara-C), methotrexate, cyclophosphamide, adriamycin daunomycin, 5-fluoro-deoxyuridine, 2-chloro-deoxyadenosine, fludarabine, taxol, etoposide (VP-16), camptothecin, nitrogen mustards, mitoxantrone, cisplatin, vincristine, and some retinoids. The extent to which gene transfer-mediated elevations in Bcl-2 protein levels provide protection from the cytotoxic effects of these drugs varies, depending on the particular drug and the cell line, but can be as much as four or more logs (10,000×) or as little as half a log (5×). When translated to clinical situations, however, even a fivefold increase in resistance may be highly significant, given that most attempts to employ so-called 'high-dose' aggressive chemotherapy involve a mere doubling of the concentrations of drugs.

The observation that Bcl-2 provides protection against such a wide variety of drugs that have markedly diverse mechanisms of action suggests that they all utilize the same final common pathway for ultimately inducing cell death and that Bcl-2 is a regulator of this pathway. Indeed, several studies have provided evidence that chemotherapeutic drugs, as well as γ-radiation, when administered in vitro to tumor cell lines induce cell death through mechanisms consistent with apoptosis as opposed to necrosis [97,98]. Furthermore, the data argue that despite the diversity of their biochemical mechanisms of action, all these drugs have in common the ability to active the programmed cell death pathway at some point that lies upstream of Bcl-2.

The drug resistance imparted to cancer cells by elevated levels of Bcl-2 protein differs from all other previously described forms of chemoresistance. Traditionally, pharmacologists have considered the chemoresistance problem in cancer in terms of four major issues: (1) problems with the delivery of a drug to the target, such as when a drug is metabolized to an inactive product or when the *mdr-1* gene product, P-glycoprotein, is overproduced in the plasma membrane of cancer cells and pumps drugs out of the cell; (2) modification of the drug target, an example of which is amplification of the gene for dihydrofolate reductase, which often occurs following exposure to methotrexate or loss of estrogen receptors in response to treatment with antiestrogens; (3) increased rates of repair of damage to DNA or other

structures; and (4) diminished rates of drug-induced damage to DNA or other macromolecules, as can occur for some drugs when glutathione levels are elevated in tumors. Bcl-2, in contrast, appears to act through a different mechanism. Studies from several laboratories [87,90,96], for example, have shown that Bcl-2 does not prevent entry of drugs into cells. Bcl-2 also does not alter the extent to which drugs induce damage to DNA or the rate at which cells repair damaged DNA. Furthermore, no effects have been found of Bcl-2 on nucleotide pools or rates of cell cycling, which represent additional variables that can influence the relative sensitivity of cells to anticancer drugs. Similarly, though Bcl-2 was reported to produce elevations in intracellular glutathione levels in one neural cell line [99], this has not been observed in several other tumor and leukemia lines, indicating that no consistent relation of Bcl-2 to this intracellular antioxidant exists ([87] and unpublished data).

It appears, therefore, that in the setting of Bcl-2 overproduction, drugs still enter cells and induce damage, but this damage is somehow ineffectively translated into signals for cell death. In fact, it has been shown that anticancer drugs can still induce cell-cycle arrest when Bcl-2 is present at high levels, but the cells typically fail to die or do so at markedly slower rates compared to control transfected cells [9,10,87,90]. Thus Bcl-2 can convert anticancer drugs from cytotoxic to cytostatic. Furthermore, when drugs are removed from cultures, a scenario that is analogous to the cessation of drugs that occurs clinically between cycles of chemotherapy, *bcl-2*-expressing cells can often reinitiate cell growth at higher rates than their control counterparts in clonogenic cell assays [10,90]. Similar effects have been reported for γ-irradiation, where again clonogenic assays indicate that *bcl-2* can be highly radioprotective [86]. Presumably, therefore, because they do not die as easily when exposed to drugs and radiation, cells with elevated levels of Bcl-2 protein are able to survive through the period of drug treatment or radiation and then repair damaged DNA and resume their proliferation either when drugs are withdrawn or after radiation. Taken together, these observations suggest that Bcl-2 defines a new category of drug- and radio-resistance gene, i.e., those that regulate the physiological cell death pathway.

6.1. Evidence for a role of bcl-2 *in chemoresistance in patients*

In addition to in vitro experiments, clinicocorrelative studies of *bcl-2* expression in cancer patients have suggested that *bcl-2* gene activation and high levels of Bcl-2 protein production may be important determinants of prognosis in at least some subgroups of patients. For example, in two studies of patients with non-Hodgkin's lymphomas (NHLs) having diffuse histology with a large cell component (DLCL), an association was found between *bcl-2* gene rearrangements and shorter survival, shorter disease-free survival (DFF), or failure to achieve a complete remission (CR) [13,14,100]. The data approached statistical significance ($p = 0.07$) in a third study of DLCL, but the median

survival in this case was short (two years), suggesting a need for longer follow-up [101]. Though *bcl-2* status was not of prognostic significance in five other reports involving patients with aggressive-histology NHL, in one study the combination of p53 and Bcl-2 immunostaining data defined a subgroup of patients at high risk for death [102]. Thus, as discussed above, the interplay between p53 and *bcl-2* gene regulation may have been a contributor to the particularly poor prognosis observed for these patients. Furthermore, in several of the studies where the correlation between *bcl-2* and survival did not reach statistical significance, there was a tendency of patients with evidence of *bcl-2* gene activation to relapse or die sooner. For example, the three-year survival for patients with *bcl-2*-positive tumors was only 45% as compared to 75% in a report by Romaguera et al. [103], and the time to treatment failure was shorter for patients with Bcl-2-positive tumors (48% vs. 11%) in a study by Jacobson et al. [104]. Similarly, survival at five years was shorter for patients with Bcl-2-positive DLCL (35% vs. 46%) in a report by Piris et al. [102] as well as in a study by Offit et al. [14]. In patients with follicular lymphomas, Yunis et al. reported a significant association between *bcl-2* gene rearrangements and both failure to achieve CR and reduced survival in cases where the histology included a large-cell component (FLCL) [13]. Conversely, in an analysis of patients with low-grade NHLs (follicular small-cleaved cell and follicular mixed cell), *bcl-2* status was not of prognostic significance [105]. One limitation of this study, however, was that the size of the *bcl-2*-negative group was small, since more than 85% of low-grade NHLs contain a t(14;18). Taken together, these data suggest a trend towards a clinically significant role for *bcl-2* gene activation and poor outcome in patients with lymphomas, particularly those that present as nodal (as opposed to extranodal) disease and where the histology includes a large cell component (DLCL; FLCL). Further studies involving larger groups of patients that received uniform treatment, however, are clearly required before any firm conclusions can be drawn as to the usefulness of Bcl-2 as a prognostic indicator when used either alone or in combination with other laboratory tests such as p53 immunostaining.

In addition to lymphomas, associations have been observed between *bcl-2* and poor responses to therapy in some groups of patients with leukemias or solid tumors [15,16,66,106,107]. However, in some studies, inverse correlations have been found between *bcl-2* and clinical outcome, with more aggressive tumors having lower levels of Bcl-2 protein relative to less aggressive ones [67,69]. In several of these studies, the treatment was primarily or even exclusively surgical, with only some patients receiving local regional radiotherapy and none treated with systemic chemotherapy. Thus, the relevance of these findings may be limited where the issue of *bcl-2* as a modulator of chemosensitivity is concerned, and instead may be reflective of a particular state of tumor cells differentiation during which *bcl-2* is expressed at higher levels. Alternatively, the loss of Bcl-2 during tumor progression could reflect deregulation of tumor cell growth to a point where the neoplastic cells are less

dependent on Bcl-2 because of an accumulation of defects in the expression and activities of genes that normally promote apoptosis such as p53 and certain proapoptotic homologues of Bcl-2 (discussed below).

6.2. Bcl-2 blocks both p53-dependent and p53-independent pathways for drug-induced apoptosis

As mentioned above, p53 can be a regulator of *bcl-2* gene expression. In addition, gene transfer studies have demonstrated that enforced production of Bcl-2 protein at high levels can partially or completely block apoptosis induced by p53 [108–110], suggesting a direct functional connection between p53 and its ability to both induce apoptosis and to downregulate *bcl-2* gene expression. Recently, a central role has emerged for p53 as a regulator of chemo- and radioresistance in tumors. For example. in vitro gene transfer studies have shown that cultured cell lines lacking functional p53 exhibit increased resistance to induction of apoptosis by multiple anticancer drugs and radiation [78,111]. Furthermore, p53 'knock-out' mice experience less radiation-induced apoptosis in the small intestine compared to normal littermate control animals [112,113]. In addition, thymocytes isolated from p53 knock-out mice have impaired apoptotic responses to induction of apoptosis by γ-irradiation and topoisomerase inhibitors relative to p53-expressing control animals [114,115]. Loss of p53 has also been associated with worse prognosis for patients with several types of cancers (for examples, see [116–118]).

It is therefore tempting to speculate that p53 and *bcl-2* may be functionally linked in a pathway that controls drug- and radiosensitivity. However, the situation is likely to be more complex, in that some types of anticancer drugs are able to induce apoptosis through mechanisms that are p53-independent and yet suppressible by Bcl-2. Studies with p53 knock-out mice, for example, have shown that while apoptosis induced in thymocytes by γ-radiation and DNA-damaging drugs is dependent on p53, apoptosis stimulated by glucocorticoids and calcium-ionophores is not [114,115]. In contrast, both of these pathways for cell death are blocked in thymocytes derived from transgenic mice that produce high levels of Bcl-2 protein in the thymus [93–95]. Moreover, even DNA-damaging drugs and radiation can induce apoptosis through p53-independent mechanisms in some types of cells. In contrast to in mature thymocytes, for example, apoptosis can be triggered in mature T-cells derived from p53 knock-out mice by radiation and chemotherapeutic drugs, despite the absence of p53 [119]. Again, apoptosis in these cells is also suppressible by Bcl-2. Thus, Bcl-2 appears to function at a point distal to the convergence of p53-independent and p53-dependent limbs of a final common pathway for drug- and radiation-induced apoptotic cell death.

One interesting aspect of the functional interaction between p53 and Bcl-2, however, concerns recent findings suggesting that overexpression of Bcl-2 can prevent p53 from acting as a repressor of some promoters that lack consensus p53-binding sites, while simultaneously having no effect on the ability of p53 to

trans-activate other types of promoters that contain typical 10-bp p53 binding-site motifs [120,121]. How the Bcl-2 protein can prevent p53 from fulfilling its role as a *trans*-repressor without influencing its actions as a *trans*-activator remains enigmatic from the biochemical standpoint, but suggests that p53's dual role as both an inducer of apoptosis and a blocker of cell-cycle progression may be linked to inhibition of gene expression in the case of the former and to the induction of gene expression in the latter. This model is consistent with data showing that gene transfer-mediated elevations in Bcl-2 protein block p53-induced apoptosis without impairing p53-induced cell-cycle arrest [108–110] and with the observation that p53 appears to inhibit cell proliferation primarily by inducing the expression of an inhibitor of cyclin-dependent kinases, p21-Waf-1/Cdi-1/Sdi-1.

7. *bcl-2* regulates a distal event in an evolutionarily conserved pathway for cell death

In addition to rendering tumor cells relatively more resistant to induction of apoptosis by chemotherapeutic drugs and radiation, the Bcl-2 protein can also provide protection against a broad range of stimuli and insults that trigger the physiological cell death pathway. Most of these data are derived from gene transfer studies in which cells were stably transfected with a *bcl-2* expression vector, versus a control vector, and were then challenged in various ways that are known to result in apoptotic cell death. For example, in hemopoietic and lymphoid cells, gene transfer-mediated elevations in Bcl-2 protein levels have been shown to prolong survival markedly when cells are placed into cultures without growth factors [5,6,122–124]. These Bcl-2 transfected cells still undergo cell-cycle arrest in the G_0/G_1 phase in the absence of growth factors, indicating that Bcl-2 does not render cells factor-independent for growth but rather specifically prolongs survival without simultaneously simulating mitogenesis. Similarly, microinjection of *bcl-2*-expression plasmids into sympathetic neurons strikingly delays apoptotic cell death caused by nerve growth factor (NGF) deprivation [125]. Interestingly, Bcl-2 also can protect sensory neurons from death induced by withdrawal of NGF, brain-derived neurtrophic factor (BNDF), or neurotrophin-3 (NT-3) but not ciliary neurons from ciliary neurotrophic factor (CNTF) deprivation [126]. Bcl-2 also provides protection against apoptosis induced in neuronal cell lines by L-glutamate, a excitotoxic neurotransmitter thought to play an important role in stroke [127,128]. Cell death induced by free radicals, drugs that generate free radicals in cells, and agents that interfere with glutathione synthesis in cells is also opposed by Bcl-2 though if very high concentrations of these agents are employed the cytoprotective effects of Bcl-2 can be overwhelmed [99,100,129]. In this case, however, the cell death typically is necrotic rather than apoptotic.

TGF-β (transforming growth factor-β) induces apoptosis in some types of cells and functions essentially as a tumor suppressor in epithelial and hemopoi-

etic tissues. In a myeloblastic leukemia line, TGF-β downregulated *bcl-2* gene expression and induced apoptosis through a mechanism that was completely suppressible by transfection with a *bcl-2* expression vector [130]. Interestingly, TGF-β-mediated cell cycle arrest was not blocked by Bcl-2. In addition, cytokines that induce cell death, such as tumor necrosis factor (TNF) and Fas-ligand, have also been shown to utilize Bcl-2-suppressible pathways to mediate their cytotoxic actions [131–133], though in some types of cells Bcl-2 provides little or no protection, for reasons that will be discussed below [134,135]. Similarly, cell death induced by cytolytic T-cells (CTLs) can be paritally blocked by overproduction of Bcl-2 protein in target cells [136,137]. CTLs, however, have at their disposal a variety of mechanisms for killing target cells, some of which involve apoptosis (TNF, Fas-Ligand, proteases, ATP) and others necrosis (perforin), and thus Bcl-2 does not protect in all instances [95,138]. Moreover, gene transfer studies have documented that Bcl-2 can protect cells against apoptosis induced by serine proteases derived from the cytotoxic granules of CTLs [139], as well as cysteine proteases of the ICE (interleukin-1-β converting enzyme) family [140–142]. Bcl-2 also increases resistance to cell death induced by heat shock [143], as well as by calcium ionophores [93–95, 129] and even some types of viruses [144,145]. With regards to viruses, for example, the Tax protein of HIV has been shown to induce apoptosis via a Bcl-2-suppressible mechanism [145]. In addition, Bcl-2 has been demonstrated to suppress the pro-apoptotic effects of the adenovirus E1a protein, which renders cells more sensitive to induction of apoptosis by serum withdrawal, DNA-damaging drugs, and radiation [146].

In addition to viral oncogenes such as E1a, apoptosis induced by certain cellular oncogenes including c-Myc, c-Myb, and R-Ras can be blocked by Bcl-2 [130,147–149]. In the case of c-Myc, for example, it has been shown that Myc simultaneously stimulates cellular pathways for both cell proliferation and apoptosis [150,151]. The apoptotic effects of Myc can be suppressed by supplying cells with appropriate growth factors that generate survival signals. In that absence of growth factors, however, Myc-transfected cells undergo rapid apoptotic cells death via a mechanism that is completely suppressible by Bcl-2 [147,148]. This cooperation between Myc and Bcl-2 may explain why low-grade lymphomas that contain a t(14;18) involving *bcl-2* take on an aggressive, rapidly fatal phenotype when a subsequent t(8;14) translocation occurs that activates the *c-myc* gene [47,48]. It has been argued that the dual role of Myc as both an inducer of mitogenesis and apoptosis helps to build additional controls into cell growth regulation, thus cordinating extracellular stimuli with intracellular gene expression. In addition, however, these observations imply that tumor cells may be more dependent on genes such as *bcl-2* for their survival, thus offering hope that if the means for pharmacologically inhibiting *bcl-2* function were developed, tumor cells would be rendered relatively more vulnerable to apoptosis compared with normal cells. In this regard, enforced production of high levels of Bcl-2 protein through gene transfer manipulations has also been shown to protect cells from apoptosis induced by loss of attach-

ment to excellular matrix proteins mediated by certain integrins [132]. It is conceivable, therefore, that the deregulated expression of *bcl-2* contributes in at least some types of cancers to the acquisition of anchorage-independent growth, local invasiveness, and metastatic properties. This dependence on *bcl-2* for survival in the absence of appropriate cell attachment signals may therefore again render tumor cells more dependent on *bcl-2* than normal cells, which retain their appropriate attachments to other cells and excellular matrix.

The broad range of stimuli against which Bcl-2 can confer protection suggests that the Bcl-2 protein functions at a distal point in what may represent a final common pathway for apoptotic cell death. Thus, despite the various upstream 'signals' that are generated by these stimuli, eventually they must utilize the same mechanisms to ultimately kill cells, since Bcl-2 can provide protection from all of them. Nearly all of the cell death-inducing stimuli mentioned above have been shown to trigger apoptosis, as opposed to necrosis. In addition, however, Bcl-2 has also been reported to provide protection even in one model of necrotic cell death [99]. Furthermore, elements of the cell death pathway regulated by Bcl-2 appear to be well conserved throughout evolution, in that the human Bcl-2 protein has been shown to block cell death when expressed in insect cells, nematodes, and even yeast under some circumstances [21,22,99,152,153].

Though Bcl-2 clearly can have profound effects on the relative sensitivity of cells to apoptosis induction by a wide variety of insults and stimuli, most data argue that Bcl-2 is not absolutely required for cell survival. In experiments where antisense techniques were used to achieve reductions in Bcl-2 protein levels, for example, spontaneous cell death did not result, though the cells were markedly more sensitive to induction of death by growth factor deprivation and chemotherapeutic drugs [91,154]. Similarly, in thymocytes derived from *bcl-2* knock-out mice, rates of spontaneous cell death were not appreciably different for *bcl-2*-deficient and normal cells, but absence of *bcl-2* was correlated with greater sensitivity to apoptosis induced by glucocorticoids and radiation [155,156]. In fact, the relative normalcy of *bcl-2* knock-out mice argues persuasively that Bcl-2 is not necessarily required for cell survival. Thus, Bcl-2's effects on cell death pathways may be more analogous to the volume knob on a radio than the on–off button. Bcl-2 does not turn on a cell survival pathway or turn off a cell death pathway, but rather adjusts the magnitude of cell death 'signals' so that either cell-survival signals are amplified or cell-death signals are squelched.

Despite the broad significance of Bcl-2 for regulation of cell death, some scenarios have been reported in which gene transfer-mediated elevations in Bcl-2 protein levels have failed to protect against cell death [93,94,134, 135,138]. Furthermore, in some cases, the cell death process was clearly consistent with apoptosis as opposed to necrosis, such as with antigen receptor-induced apoptosis in some B- and T-lymphocyte cell lines [157,158]. Although these data have often been used to argue for the existance of *bcl-2*-independent pathways that regulate apoptosis, it is also possible that the mechanisms

involved in cell death induction did indeed involve the *bcl-2* pathway but that the mere overproduction of the Bcl-2 protein was insufficient to provide protection for a variety reasons, including absence of partner proteins that Bcl-2 may require to fulfill its mission as a cell death blocker, presence of high levels of proteins that inhibit Bcl-2, or stimulation of posttranslational modifications of the Bcl-2 protein that impair its function.

8. The Bcl-2 protein: possible mechanisms of action

The predicted amino-acid sequence of the Bcl-2 protein has failed to provide any clues about the biochemical mechanism by which this protein blocks cell death. In humans, mice, rats, and chickens, the protein has a molecular mass of about 25 to 26 kDa and contains a stretch of hydrophobic amino acids near its C-terminus that constitutes a transmembrane domain [159]. The intracellular membranes into which Bcl-2 inserts are strikingly unusual compared to other known proteins. A combination of subcellular fractionation, immunofluorescence confocal, laser-scanning, and electron microscopic methods have provided conclusive evidence that Bcl-2 is associated with mitochondria, specifically the outer mitochondrial membrane, as opposed to the inner membrane where many of the steps of oxidative phosphorylation occur [160–164]. Consistent with the absence of Bcl-2 from the inner membrane, it has been shown by use of mutant cells lacking mitochondrial DNA that absence of a complete respiratory chain does not interfere with ability of Bcl-2 to block apoptosis [165]. Bcl-2 immunoreactivity in the outer membrane of mitochondria is not uniformly distributed, but rather is patchy in its distribution — a property suggestive of proteins that associate with the mitochondrial junctional complexes, where the inner and outer membranes come into contact and where various transport phenomenon occur. In addition to the mitochondrial outer membrane, much of the Bcl-2 protein is found in the nuclear envelope. Similar to the situation with mitochondria, electron microscopic data suggest that the Bcl-2 protein is nonuniformly distributed in the nuclear envelope in a punctate pattern that is reminiscent of nuclear pore complexes where the inner and outer nuclear membranes come into contact and where transport between the nucleus and cytosol of proteins, RNA, and possibly ions occurs. Bcl-2 is also found in at least parts of the endoplasmic reticulum.

Although the functional significance of the unusual intracellular distribution of the Bcl-2 protein remains unclear, the possible association of Bcl-2 with mitochondrial junctional complexes (MJCs) and nuclear pore complexes (NPCs) is of particular interest. The nucleus and mitochondria have several features in common, including the fact that both contain DNA. Both the nucleus and mitochondria are also the only intracellular organelles that have a two-membrane system, an outer and an inner membrane. The MJCs and NPCs where these membranes come into contact are the sites of transport of macromolecules and possibly some ions into and out of these organelles.

Undoubtedly, the MJCs and NPCs are also critical structures for maintaining the integrity of mitochondria and nuclei, and disruptions of these multiprotein complexes would presumably compromise the structure and function of these essential organelles. In support of a role for Bcl-2 in regulating some aspect of protein transport in the nuclear envelope, reduced ratios of nuclear to cytosolic cdc-2 and cdk-2 kinase have been detected in HeLa cells transfected with *bcl-2* expression plasmids [166]. In addition, translocation of the p53 protein from cytosol into the nucleus was reported to be prevented by cotransfection of a mouse erythroleukemia line with the combination of *bcl-2* and *c-myc* expression vectors [109]. Conversely, Bcl-2 was able to block p53-induced apoptosis in a *v-myc*-transformed T-cell lymphoma without disturbing p53 translocation into the nucleus [108], suggesting that interference with transport of p53 is not a consistent observation among different tumor lines that simultaneously overproduce Bcl-2 and Myc oncoproteins. Gene transfer-mediated elevations in Bcl-2 protein also do not interfere with nuclear accumulation of p53 in other cell lines where *c-myc* is not overexpressed [110,167,168]. Moreover, Bcl-2 has been shown to protect the cytoplasm of enucleated cells from 'apoptosis,' suggesting that the presence of a nucleus is not essential for Bcl-2 action [169]. A role for Bcl-2 in regulating protein transport in mitochondria, however, has not been explored.

Another possible functional implication of the intracellular locations of the Bcl-2 protein is suggested by data showing that Bcl-2 can influence intracellular Ca^{2+} homeostasis [170,171]. For example, in an IL-3-dependent hemopoietic cell line 32D, a striking loss of Ca^{2+} from the endoplasmic reticulum (ER) was seen in control cells prior to apoptosis induction by growth factor withdrawal, whereas ER pools of Ca^{2+} were maintained in the normal range in cells overproducing Bcl-2. Conversely, estimates of mitochondrial Ca^{2+} pools suggested that elevations occur in the amounts of releasable Ca^{2+} in mitochondria and that Bcl-2 prevents the accumulation of Ca^{2+} in this organelle [170]. A functional connection between dysregulation of intracellular Ca^{2+} and apoptosis has been well established by experimentation involving use of Ca^{2+}-ionophores and other agents, including the observation that apoptosis is induced by thapsigargin — a drug that poisons the Ca^{2+}-ATPase of the ER and results in massive loss of Ca^{2+} from this organelle [172]. Similarly, gene transfer-mediated elevations in calbindin-D, a Ca^{2+} binding protein that resides in the lumen of the ER, have been shown to delay the onset of apoptosis in a glucocorticoid-treated lymphoid cell line, suggesting that increasing the ability of the ER to sequester Ca^{2+} protects against apoptosis [173]. In this regard, the rate of efflux of Ca^{2+} from the ER was shown to be substantially reduced in Bcl-2-transfected WEH17.1 T-cell lymphoma cells compared to controls when treated with thapsigargin [171]. The presence of Bcl-2 in nuclear and ER membranes, therefore, may have some relevance to the fact that most of the Ca^{2+} in cells is sequestered in the lumen of the ER and, by extension, the space between the inner and outer nuclear membranes. Furthermore, in most types of cells, the mitochondria represent the next largest intracellular storage site

for Ca^{2+}, again suggesting that Bcl-2 is at least located in the right places to function either directly or indirectly as a regulator of intracellular Ca^{2+} homeostasis. Perhaps relevant to a role for mitochondria in Ca^{2+} sequestration during apoptosis, mitochondria were reported to be absolutely required for the apoptosis-like nuclear disintegration seen in a cell-free assay in which 'apoptotic' cytosolic extracts were mixed with nuclei; and addition of Ca^{2+} ionophores blocked nuclear destruction in this system [174].

It has also been suggested that Bcl-2 may function in an antioxidant pathway, based on the findings that (1) Bcl-2 prevents induction of apoptotic and (in some cases) necrotic cell death induced by agents that either result in oxygen free-radical production or that deplete intracellular glutathione; (2) overexpression of certain antioxidant enzymes, such as forms of superoxide dismutase (SOD) or glutathione peroxidase, can also render cells more resistant to induction of cell death analogous to Bcl-2; and (3) Bcl-2 prevents the accumulation of lipid peroxides, suggesting that Bcl-2 somehow nullifies damage to membranes by reactive oxygen species [99,100]. The relevance of these findings to the intracellular locations of the Bcl-2 protein could be that mitochondrial, ER, and plasma membranes are the major sites of free-radical generation in cells. Additional evidence supporting a possible role for a redox mechanism for Bcl-2 comes from studies of SOD-deficient yeast, where expression of the human Bcl-2 protein was shown to restore growth under aerobic conditions [99]. Also, in *bcl-2* knock-out mice, hypopigmentation of coat hairs occurs during the second hair follicle cycle — a finding that has been speculated to reflect a defect in one of the redox-dependent steps of melanin synthesis in melanocytes [156,175]. However, in a cell-free system for 'apoptosis' in which Bcl-2 can function to prevent nuclear breakdown and DNA degradation, chemicals that modulate redox conditions had no significant effects on either induction of apoptotic-like changes in nuclei or the ability of Bcl-2 to function [174]. Also, Bcl-2 is able to block apoptosis induced by staurosporine and anti-Fas antibodies in fibroblasts grown under anaerobic conditions, arguing against a requirement for reactive oxygen specifics [176], though these observations do not exclude a role for redox-sensitive, thiol-based chemical reactions. At present, however, no data have been obtained that directly link Bcl-2 to the regulation of antioxidant pathways or any other particular mechanism such as Ca^{2+} or protein transport.

9. *bcl-2* homologues and interacting proteins

In the absence of a clear biochemical function for the Bcl-2 protein, a number of groups have searched for proteins that interact with Bcl-2 in the hope that the predicted amino-acid sequences of these Bcl-2-interacting proteins would provide insights into the mechanism of action of Bcl-2. Using a variety of interaction cloning techniques, as well as protein purification and sequencing,

several proteins have now been identified that are capable of specifically binding to or at least coimmuno-precipitating with Bcl-2. One class of Bcl-2-interaction proteins represents homologues of Bcl-2, which can form heterotypic dimers with Bcl-2 as well as homotypic dimers with themselves in some cases. A second class of Bcl-2-binding proteins can be defined as protein that do not share homology with Bcl-2. Knowledge about these protein–protein interactions is beginning to provide insights into the molecular details of how the Bcl-2 protein functions.

9.1. Homologues

At present, six mammalian homologues of Bcl-2 have been reported, including Bax, Bcl-X, Mcl-1, A1, Bad, and Bak [17–20,177–180]. Some of these proteins have additional forms that arise through alternative splicing mechanisms, the most interesting to date of which are the long and short forms of Bcl-X. The Bcl-X-L and Bcl-X-S proteins have opposing functions, with Bcl-X-L functioning as a blocker of cell death analogous to Bcl-2 and the Bcl-X-S protein acting as an antagonist of Bcl-2 that accelerates apoptotic cell death [3]. In addition to Bcl-X-S, some of the other Bcl-2-like proteins have been shown to function as inducers of rather than protectors from cell death, including Bax, Bad, and Bak [17,177–180]. Conversely, the Mcl-1, and A1 proteins function as cell death blockers, though perhaps less efficiently than Bcl-2 [our unpublished observations]. In addition to mammalian homologues, several homologues of Bcl-2 have been described in viruses, including the E1b-19kDa protein of adenovirus, the BHRF-1 protein of Epstein Barr virus (EBV) and the LMWS-HL open reading frame found in the African swine fever virus [176,181–183]. Both E1b and BHRF-1 function as blockers of cell death, whereas the properties of the LMWS-HL protein have yet to be reported. A homologue of *bcl-2* has also been discovered in the nematode, *C. Elegans*, which functions as a blocker of cell death and has been termed *ced-9* [21].

Sequence alignments have identified the presence of three conserved domains, termed Bcl-2 domains (BD) a, b, and c [184]. As shown, most members of this family contain a stretch of hydrophobic amino acids at their C-terminus that presumably allows for posttranslational insertion into membranes. The Bad and A1 proteins, however, lack any obvious transmembrane domains. In addition, alternatively spliced versions of Bcl-2, Bcl-X-L, and Bax have been described that do not contain membrane-anchoring sequences [2,17,185]. For the most part, however, these splicing variants are relatively less abundant than the 'full-length' forms, and their functions are not well studied to date. In studies where truncation mutants of Bcl-2 have been prepared that lack the transmembrane domain, function as a blocker of apoptosis in lymphokine-dependent hemopoietic cells was shown to be impaired relative to the wild-type Bcl-2 protein, but not completely absent [100,186]. Similar results were obtained for an epidermal cancer cell line, where apoptosis was induced by

E1b-19-kDa-deficient adenovirus [187]. Moreover, replacement of the transmembrane domain of Bcl-2 with heterologous membrane targeting sequences derived from either the IL-2 receptor or a mitochondrial outer membrane protein Mas70p restored the antiapoptotic function of these truncation mutants to nearly normal levels [186,187]. Conversely, in TNF-treated L929 fibroblasts and NGF-deprived sympathetic neurons, inhibition of cell death by transmembrane-deficient versions of Bcl-2 was essentially comparable to the wild-type Bcl-2 protein, suggesting that Bcl-2 need not necessarily be targeted to membranes [188]. Given that Bcl-2 can form homotypic dimers with itself as well as heterotypic dimers with several of its homologues [22], it remains possible that transmembrane-deficient versions of Bcl-2 were nevertheless able to localize at least in part to the usual membrane sites through protein–protein interactions. In this regard, subcellular localization studies of the Bcl-X-L, Bcl-X-S, and Mcl-1 proteins suggest that these proteins reside at the same or similar membrane sites as Bcl-2 [185,189]. Also, though only examined at the level of conventional light microscopy, the intracellular immunostaining patterns of antibodies specific for Bax, Bcl-X, and Mcl-1 are very similar to Bcl-2 in that punctate immunostaining of cytosolic structures resembling mitochondria is seen, as well as nuclear and perinuclear membranes in some cells [190–192]. Likewise, electron microscopic analysis of the subcellular localization of the BHRF-1 protein from EBV suggests association with the outer mitochondrial membrane and other sites typically occupied by Bcl-2 [193]. The 19-kDa E1b protein also has a similar localization, but appears to reside more so in nuclear and perinuclear membranes than mitochondrial membranes [194].

Though some members of the Bcl-2 protein family were discovered by virtue of their ability to bind to Bcl-2, this is not the case for the majority of these proteins. Thus, to date, it is unknown whether all the homologues of Bcl-2 can form heterotypic dimers with the Bcl-2 protein. Nevertheless, several homologues have been investigated in this regard, mostly by use of yeast two-hybrid assays, though in some cases in vitro bindings studies using recombinant fusion proteins or coimmunoprecipitation experiments involving mammalian cells have also been performed. These studies suggest that Bcl-2 can form heterodimers with the Bax, Bcl-X-L, Bcl-X-S, Mcl-1, and Bad proteins [17,22,195,196]. In those cases tested, the Bcl-X-L protein appears to have similar binding characteristics and has been shown to interact specifically with Bax, Bcl-X-S, Mcl-1, Bad, Bak, and itself, in addition to Bcl-2 [22,195]. This result is not entirely surprising, since the Bcl-2 and Bcl-X-L proteins are 47% identical in their amino acid sequences [18]. Essentially nothing is known at present about the affinities of these protein–protein interactions. Furthermore, though it is convenient to think of these protein interactions as homo- and heterotypic dimers, their stoichiometry remains undetermined to date.

Investigations of the domains within Bcl-2-family proteins required for homo- and heterotypic dimer formation have thus far been consistent with an antiparallel or head-to-tail arrangement, wherein structures that are present

within about the first 80 amino acids of Bcl-2 appear to interact with structures present within the carboxyl portions of the protein [22]. For example, it was shown by use of yeast two-hybrid assays that amino acids 1–81 of the human Bcl-2 protein can mediate interactions with amino acids 83–218, whereas the 83–218 fragment cannot homodimerize with itself. Either the 1–81 nor the 83–218 fragment of Bcl-2 appears to be sufficient for interactions with Bcl-X-L and Mcl-1, but other Bcl-2 family proteins have not been tested in this regard. The BD(a), BD(b), and BD(c) domains of Bcl-2 all appear to be required for Bcl-2 to retain its cell-death blocking function [132,197,198]. Of particular note, it has been shown in a lymphokine-dependent hemopoietic cell line that mutations in the BD(b) and BD(c) domains of Bcl-2 (also termed BH1 and BH2 domains) are required for Bcl-2 to coimmunoprecipitate with Bax but do not impair associations with wild-type Bcl-2 protein [197]. These mutant forms of Bcl-2 that fail to bind to Bax are also functionally deficient with reference to blocking of cell death caused by lymphokine deprivation. These results have been interpreted as evidence that Bcl-2 must be able to bind to Bax in order to function. Since gene transfer-mediated elevations in Bax protein levels accelerate the rate of cell death caused by growth factor withdrawal, whereas Bcl-2 has the opposite effect, it has been argued that the ratio of Bax and Bcl-2 proteins determines the relative sensitivity of cells to apoptosis [17]. Furthermore, the mutagenesis studies suggest that the Bcl-2/Bax interaction defines a critical aspect of this regulation of susceptibility to apoptotic cell death. However, it is probably necessary for Bcl-2 to bind to other proteins in addition to Bax in order to fulfill its mission as a suppressor of apoptosis, since some N-terminal truncation mutants of Bcl-2 and internal deletion mutants lacking the BD(a) domain are able to bind Bax but fail to suppress cell death [199–201].

When expressed in yeast (*S. cerevisiae*), the Bax protein confers a lethal phenotype that can be specifically neutralized by coexpression of Bcl-2, Bcl-X-L, or Mcl-1 [22]. For suppression of Bax-induced death in yeast, it is not necessary that the transmembrane domains of the Bcl-2, Bcl-X-L, and Mcl-1 protein be included [22]. Mutant versions of Bcl-2 that fail to bind to Bax also fail to suppress Bax-mediated cytotoxicity in yeast [22,198]. These observations suggest that elements of the Bax/Bcl-2 pathway may be conserved even in single-cell eukaryotic organisms and raise the possibility of applying yeast genetics approaches to delineation of some of the downstream effectors or even upstream activators involved in the physiological cell death pathway. It is not immediately obvious why single cell organisms such as yeast might have mechanisms for committing suicide, unlike multicellular organisms where altruistic cell death can be easily reconciled with the greater goal of protection of the whole organism. One idea, however, is that yeast may use suicide as a means of minimizing the deleterious effects of viruses, so that viral production would be limited and the likelihood of infection of all progeny of a given yeast cell reduced. Elements of this cell death pathway could then have been transfered to multicellular eukaryotes, where again it would have served the

organism when confronted with viruses. Clearly, however, the finding of cell death-blocking homologues of Bcl-2 in viruses suggests that some viruses have 'learned' to subvert the suicide defense mechanism, a finding that may also be relevant to the issue of viral latency whereby viral genomes can ensure their longevity by piggybacking on the chromosomes of host cells and then resume their replication at opportune times. It may also be relevant in this regard that genes encoded within the EBV virus also appear to be able to induce expression of the endogenous *bcl-2* gene [202–204].

In contast to Bax, the cell death-enhancing protein Bcl-X-S is not lethal when expressed in yeast, at least when lacking its transmembrane domain, which is the only way that the function of this protein in yeast has been tested thus far [22]. This observation has suggested that Bcl-X-S may enhance cell death by a different mechanism than Bax. Because of an alternative splicing event, the Bcl-X-S proteins is missing a well-conserved region that includes the BD(b) and BD(c) domains [18]. Its ability to interact with Bcl-2 and Bcl-X-L thus appears to be dependent on preservation of the NH_2-terminal portion of the molecule where the BD(a) region resides. Interestingly, the interaction of Bcl-X-S with either Bcl-2 or Bcl-X-L in two-hybrid assays has been reported to be significantly stronger than dimerization of Bcl-2 and Bcl-X-L with themselves or each other [22]; though certainly such assays are far from quantitative. When taken together with the evidence that Bcl-2/Bax interactions may be particularly important for Bcl-2 to function as a cell death inhibitor, these observations suggest that Bcl-X-S may antagonize Bcl-2 by binding to it and thus preventing Bcl-2 from forming heterodimers with Bax. This would then leave Bax unopposed to increase the sensitivity of cells to apoptotic stimuli. A similar mechanism appears to apply for the Bad protein. This homologue of Bcl-2 is composed essentially of only the carboxyl portions of Bcl-2, including domains with homology to the BD(b) and BD(c) regions, but lacks the NH_2-terminal sequences where BD(a) resides and also lacks a transmembrane domain. Nevertheless, Bad can bind to Bcl-2 and Bcl-X-L and neutralize their anti-cell death activities in mammalian cells. Interestingly, Bad appears to be relatively specific for Bcl-X-L in that it coimmunoprecipitates with Bcl-X-L much more efficiently than with Bcl-2 and also is considerably more effective at negating Bcl-X-L function than Bcl-2 [195]. Similarly, the cell death promoter Bak also appears to bind preferrentially to Bcl-X-L and interacts only weakly if at all with Bcl-2 [178], lending further support to the idea that the structural features of some of these homo- and heterodimerizations among Bcl-2 family proteins are sufficiently different that isoform specificity can occur. Interestingly, the Bak protein was also reported to function as a protector from, rather than promoter of, cell death under some circumstances [180]. This observation suggests that the functional repurcussions of interactions of Bak with various Bcl-2 family proteins are likely to be complex, and implies that for proper regulation of cell survival, a delicate balance of appropriate homo- and heterodimers must be maintained.

9.2. Other Bcl-2-binding proteins

At least six other proteins have been reported that can bind either directly or indirectly to Bcl-2. These include BAG-1, R-Ras, Raf-1, Nip-1, Nip-2, and Nip-3 [137,205–207]. At present, it remains unknown whether the interaction of Bcl-2 with any of these proteins is essential for its function as a blocker of apoptosis.

The BAG-1 protein is 219 amino acids in length in mice and is acidic in nature (pH 4.18) [137]. A domain within BAG-1 has as much as 50% amino-acid sequence identity with some ubiquitin and ubiqutin-like proteins, raising the possibility of a connection to protease pathways for protein degradation. Downstream of this ubiquitin-like domain is a region that, based on computer predictions, may assume a mostly α-helical conformation, with some of the helices being amphipathic and thus good candidates for participation in binding with other proteins via coiled-coil-type interactions. Otherwise, however, the predicted primary sequence of the BAG-1 protein reveals no clues as to its potential biochemical activities. In gene transfer studies, BAG-1 was shown to have anti-cell death activity — thus its name, 'Bcl-2-associated AthanoGene-1' (BAG-1) — and synergized with Bcl-2 in preventing cell death induced by apoptotic stimuli for which neither BAG-1 nor Bcl-2 alone was particularly effective, specifically anti-Fas antibody and cytolytic T-cells (CTLs).

The significance of these observations lies in the controversy over whether Fas and CTLs induce cell death through Bcl-2-dependent or -independent mechanisms [95,133,135,136,138]. Previously, some investigators had speculated that Fas and CTLs may activate the cell death pathway through a Bcl-2-independent mechanism, because gene transfer-mediated elevations in Bcl-2 protein levels were by themselves insufficient to provide protection [95,135,138]. The BAG-1 findings demonstrate that these stimuli do indeed kill cells through a Bcl-2-dependent pathway, but indicate that for Bcl-2 to block cell death under these circumstances, adequate levels of an additional partner protein (BAG-1) must be maintained. It remains to be determined whether the combination of elevated levels of BAG-1 and Bcl-2 can also abrogate the apoptotic effects of other types of stimuli that have been reported to involve Bcl-2-independent mechanisms, such as cross-linking of surface receptors for antigen on lymphoid precursors [157,158,208]. In this regard, the observation that Bcl-X-L protects WEH1231 B-cell lymphoma cells from anti-lg-induced apoptosis, whereas Bcl-2 fails to do so [208], begs the question of whether homologues of BAG-1 may exist that preferentially interact with Bcl-X-L as opposed to Bcl-2. Alternatively, such results could potentially be explained by high levels of a Bcl-2-specific inhibitor, possibly a protein that functions in a converse manner to the Bad protein, which is relatively specific for Bcl-X-L.

9.2.1. R-Ras and Raf-1. Two potential signal-transducing proteins have been identified that interact directly or indirectly with Bcl-2: the GTPase R-Ras and

the serine/threonine-kinase Raf-1 [205,206]. R-Ras is a 23-kDa member of the Ras family of small-molecular-weight GTPases. Mutant versions of R-Ras that constitutively bind GTP and that therefore are chronically in an active conformation (such as a mutant with glycine-to-valine substitution at position 38) induce anchorage-independent growth in NIH-3T3 cells and render these cells highly tumorigenic in nude mice, but unlike their p21-Ha-Ras counterparts fail to induce morphological transformation [208]. Like Ha-Ras, the R-Ras protein binds to Raf-1 kinase in a GTP-dependent fashion via its effector domain [209,210].

R-Ras was identified as a Bcl-2-interacting protein during yeast two-hybrid screening of cDNA libraries [205]. This protein was also reported to coimmunoprecipitate with Bcl-2 from *bcl-2*-transfected HeLa cells. However, Bcl-2 and R-Ras could not be coimmunoprecipitated from 32D hemopoietic cells in which both the Bcl-2 and R-Ras proteins were overexpressed by gene cotransfection, nor from Sf9 cells coinfected with recombinant Bcl-2 and R-Ras baculoviruses [208]. Under these same conditions, however, Bcl-2 could be readily coimmunoprecipitated with Bax, and R-Ras coimmunoprecipitated with Raf-1 kinase. Thus, if R-Ras does associate with Bcl-2, it presumably does so with lower affinity, lower stoichiometry, or more transiently than some other proteins.

In both 32D cells and NIH-3T3 cells, R-Ras (V38) significantly accelerates the rate of apoptotic cell death caused by growth factor withdrawal [211]. Furthermore, coexpression of Bcl-2 completely nullifies this effect of R-Ras. Bcl-2 protein, however, had no effect on R-Ras GTPase activity in vitro, suggesting that it does not function as a GTPase-activating protein (GAP) for R-Ras. Because no GDP exchange proteins for R-Ras have been discovered thus far, it has not been possible to test the idea that Bcl-2 might oppose R-Ras by blocking its loading with GTP. Thus, it remains possible that Bcl-2 acts as an inhibitor of R-Ras exchange proteins. However, examination of the ratio of GTP/GDP associated with R-Ras in 32D cells demonstrated that Bcl-2 does not appear to alter guanine nucleotide binding by R-Ras in cells. Furthermore, the observation that Bcl-2 blocks the effects on cell death of even the constitutively active R-Ras(V38) protein argues that Bcl-2 functions downstream of R-Ras. One idea, then, is that Bcl-2 somehow interferes with the interaction of R-Ras with an effector protein, such as Raf-1. In this regard, it was shown that Bcl-2 does not block the ability of R-Ras(V38) to bind to and induce activation of Raf-1 kinase in Sf9 cells [211]. However, at least three other effector proteins, in addition to Raf-1, have been described for Ha-Ras. Consequently, the notion of Bcl-2 preventing R-Ras from interacting with a downstream effector remains possible, but Raf-1 kinase would appear not to represent that effector protein. It is also unlikely that R-Ras accelerates rates of cell death in the setting of growth factor withdrawal by using Bax as a downstream effector, since Bax could not be coimmunoprecipitated with R-Ras or R-Ras(V38) in transfected 32D cells that contained high levels of these proteins [211].

Interestingly, although it has been difficult in some types of cells to coimmunoprecipitate R-Ras and Bcl-2, this is not the case for Raf-1 and Bcl-2. Both in transfected 32D cells that coexpressed Bcl-2 and a truncated version of Raf-1 that retained essentially only the catalytic domain and in Sf9 insect cells coinfected with Bcl-2 and Raf-1 baculoviruses, Bcl-2 and Raf-1 were reported to coimmunoprecipitate with reasonably high stoichiometry (5%–30%) under the same conditions where no detectable association of R-Ras and Bcl-2 could be found. In addition to a potential physical interaction, Bcl-2 and Raf-1 have been shown to interact functionally, in that coexpression of Bcl-2 and a constitutively active version of Raf-1 in 32D cells resulted in synergistic prolongation of survival in the absence of lymphokines, compared to cells transfected with either Bcl-2 or Raf-1 alone [206]. However, the ability of Raf-1 and Bcl-2 to coimmunoprecipitate does not necessarily imply direct binding of these proteins, Mapping studies indicate that the C-terminal half of the Raf-1 kinase where the catalytic domain resides is sufficient for coimmunoprecipitation with Bcl-2. In contrast, sequences located in the NH_2-terminal end of Raf-1 are directly involved in binding to Ras proteins [206,209,210]. Though mapping to the catalytic domain, kinase activity appears not to be necessary for Raf-1 coimmunoprecipitation with Bcl-2, based on experiments performed using a point-mutant form of Raf-1 with a disrupted ATP-binding site [195].

The observations showing that Raf-1 can cooperate with Bcl-2 to protect cells from apoptosis seem paradoxical when one considers that R-Ras accelerates cell death and yet can activate Raf-1. The apparent solution to this dilemma lies in experimental evidence suggesting that Raf-1 enters into separate independent complexes with R-Ras and Bcl-2, such that every little ($<1\%$) of the Bcl-2 or R-Ras in cells can be found in a three-way complex that simultaneously involves Bcl-2, R-Ras, and Raf-1. Thus, Bcl-2/Raf-1 and R-Ras/Raf-1 represent largely independent protein complexes and presumably have distinct functions. Though the intracellular locations of the R-Ras protein have yet to be defined, one further potential implication is that Bcl-2 and R-Ras may also target Raf-1 to different membranes in cells and thus influence the protein substrates with which it in comes into contact. Further work is required, however, before the significance of these protein–protein interactions for modulation of Bcl-2 function are understood. In this regard, Bcl-2 appears not to be a substrate of Raf-1, but the possibility of phosphorylation of Bax, other Bcl-2 family proteins, or other Bcl-2-interacting proteins has not been explored. Interestingly antibodies directed against the EBV homologue of Bcl-2, BHRF-1, have been reported to coimmunoprecipitate a poorly characterized serine/threonine-kinase activity [212], but it remains to be determined whether this is Raf-1.

9.2.2. Nip-1, Nip-2, and Nip-3. Using the E1b-19-kDa protein as a bait, cDNAs were cloned that encode three separate proteins that can bind to both E1b and Bcl-2 [207]. All of the 19-kDa-interacting proteins (Nips) were shown

to coimmunoprecipitate with E1b and Bcl-2, but not with mutants of E1b-19kDa that lack antiapoptotic function. Immunofluorescence microscopy also suggests that the Nip proteins reside in similar locations in cells to E1b-19kDa and Bcl-2. The Nip-1 protein is 228 amino acids in length and has a transmembrane domain located at its C-terminus, similar to the topology of the Bcl-2 and E1b-19kDa proteins. A region within the Nip-1 protein has homology to the catalytic domain of some phosphodiesterases. Nip-2 is predicted to be 315 amino acids in length and contains potential Ca^{2+}-binding sites as well as a region with homology to noncatalytic portions of the Rho-GAP protein. Though it lacks a hydrophobic anchor, Nip-2 appears to localize mostly to the nuclear envelope and ER, similar to E1b-19kDa. Nip-3 is a 194-amino-acid protein with homology to calbindin-D, but some splice variants may lack the calbindin-D domain. Nip-3 contains a transmembrane domain near its C-terminus. Of the Nip proteins, the intracellular distribution of Nip-3 is most reminiscent of the mitochondrial pattern seen for Bcl-2 but changes to a predominantly nuclear membrane and perinuclear membrane pattern upon transfection of cells with E1b-19kDa. All the Nip proteins contain PEST sequences, implying that their levels are regulated by protein degradation mechanisms. The function of the Nip proteins has yet to be reported.

10. Expression of Bcl-2-family proteins in normal tissues and cancers

The in vivo patterns of Bcl-2, Bax, Bcl-X, and Mcl-1 protein production have been determined by immunohistochemical means in most normal adult tissues in either humans, mice, or both [73,190,191,213,214]. In addition, the developmental patterns of *bcl-2* expression have been examined in fetal tissues from humans and mice, revealing that *bcl-2* expression is more widespread in the embryo than in adult tissues [215–217]. From these results, a few general conclusions can be reached. First, expression of all these *bcl-2* family genes occurs in a wide variety of adult tissues in vivo. Second, the regulation of the expression of *bcl-2*, *bax*, *bcl-X*, and *mcl-1* is highly tissue-specific and, moreover, varies depending on the stage of differentiation or activation of the cells in many cases. Third, in some instances the patterns of expression of these genes correlate with the in vivo regulation of programmed cell death in a way that coincides with the known function of the proteins. In other cases, however, there is no obvious correlation or even an inverse correlation with the known function of some Bcl-2-like proteins and cell death regulation in vivo. In these cases, however, the issue of protein–protein interactions must be taken into consideration, since various homo- and heterotypic interactions among these proteins modulate their functions. Fourth, the highly tissue-specific regulation of these genes clearly dictates which of the various members of this multigene family will be expressed in any particular cell at any given time, thus influencing the repetoire of Bcl-2-like proteins that are available to participate in homo- and heterotypic dimer formation.

A particularly striking example of differntial tissue-specific regulation of expression of *bcl-2* family genes comes from immunohistochemical analysis of lymph nodes. As discussed above, Bcl-2 protein is normally found at high levels in the long-lived mantle zone lymphocytes that surround the germinal centers of secondary lymphoid follicles. In contrast, very little Bcl-2 immunostaining is typically seen in germinal center B-cells. In contrast, follicular center lymphocytes are strongly Mcl-1 positive, while mantle zone lymphocytes are completely Mcl-1 negative [191]. The implication, therefore, is that while Bcl-2 and Mcl-1 have been shown to be physically capable of interacting [22], the differential regulation of these members of the *bcl-2* gene family precludes such interactions, at least in most normal peripheral B-cells. In contrast to Bcl-2 and Mcl-1, Bax immunostaining is found in most lymphocytes in nodes, whereas Bcl-X immunoreactivity was limited to lymphoblastic cells in the interfollicular regions of node (probably activated T-cells) and plasma cells [190,213]. These immunostaining results derived from lymphoid tissues, therefore, provide another example of how the strikingly different regulation of the expression of *bcl-2*-related genes ultimately determines which of the various Bcl-2 family proteins are available within any given cell to participate in homo- and heterotypic dimer formation.

Though little has been published thus far about the regulation of *bax*, *bcl-x*, and other *bcl-2* homologues in human cancers, preliminary observations suggest that alterations in the expression of some of these genes may occur in tumors. For example, elevated levels of Bcl-X-L protein have been detected in several cases of acute leukemia in association with transformation to a drug-resistant phenotype, and reductions in Bax protein levels have been observed in breast and ovarian cancers, as well as in chronic lymphocytic leukemia specimens [51,214–216]. In addition, high levels of Bcl-X and Mcl-1 immunoreactivity have also been found in the RS cells of most cases of Hodgkin's disease [191,217]. In a study of women with metastatic breast cancer, reductions in Bax were observed in the infiltrating tumor cells in about 35% of cases and correlated with poor responses to combination chemotherapy, faster time to tumor progression, and shorter overall survival [218]. Further analysis of the expression of the various *bcl-2* family genes, however, is required to delineate the mechanisms responsible for and the prognostic significance of alterations in their expression in specific types of human cancer.

11. Tumor suppressor p53 is a direct transcriptional regulator of *bax* gene expression

Recently, the promoter region of the human *bax* gene has been cloned, revealing the presence of four sites with homology to the consensus sequence for p53 binding located upstream of a TATAA box and the transcription start site [219]. In reporter gene assays, p53 was reported to strongly *trans*-activate the *bax* promoter. The p53 responsive element was mapped to a 39-bp region that

included the four p53-binding site consensus sequences. Furthermore, p53 protein was demonstrated by gel-retardation assays to be capable of binding in a specific fashion to double-strand oligonucleotides containing this 39-bp sequence derived from the *bax* promoter. In addition, in a p53-deficient myeloblastic leukemia line, a temperature-sensitive version of p53 was shown to induce marked elevations in *bax* mRNA levels and Bax protein in a conditional fashion [81]. In these same experiments, p53 inhibited expression of *bcl-2*, as discussed above [81]. Finally, in p53 knock-out mice, reductions in steady-state levels of Bax protein were detected in some tissues by immunoblotting or immunohistochemical assays [81].

Taken together, these findings suggest a potential mechanism by which p53 may control the sensitivity of cells to apoptosis induced by radiation, DNA-damaging chemotherapeutic drugs, and even growth factor deprivation [78,111,220]. It has been shown, for example, that DNA damage induced by drugs or radiation results in elevations in p53 protein and p53 transcriptional activity [83,84]. These elevations in p53 would be expected to induce an increase in Bax protein production and simultaneously a reduction in Bcl-2 protein synthesis. In doing so, p53 would shift the Bcl-2/Bax ratio into a condition of Bax excess and thus place the cell at increases risk of apoptosis.

Several caveats about this model for p53-mediated control of sensitivity to apoptosis deserve discussion. First, the transcriptional and posttranscriptional inputs into the *bax* and *bcl-2* gene are likely to be complex. Therefore, while p53 is one potential regulator, clearly other *trans*-acting factors also exist that make contributions to the final net output from the *bax* and *bcl-2* genes. Two questions that must be pursued in the future, therefore, are these: (1) in which tissues does p53 play a dominant role as a regulator of *bcl-2* and *bax*, and (2) in which types of cancer does loss of p53 lead to elevations in Bcl-2, decreases in Bax, or both? A second caveat is that these observed effects of p53 on *bcl-2* and *bax* gene expression, while correlating with the apoptotic behavior of cells, do not necessarily explain the mechanism by which p53 increases the sensitivity of cells to apoptotic stimuli. Certainly, the gene transfer experiments showing that enforced production of high levels of Bcl-2 protein can partially or completely block p53-induced apoptosis in leukemia and lymphoma cell lines argue in favor of this hypothesis [108–110,119,167], but they do not prove it. In this regard, it has also been reported that p53 can induce apoptosis even in the presence of cycloheximide or actinomycin-D, at least in a large-T antigen-induced pituitary tumor line when subjected to UV-radiation [79]. In some cells, therefore, it would appear that p53-mediated elevations in *bax* gene expression are not necessarily required for p53-induced apoptosis. However, these findings do not exclude a potentially important functional role for p53-induced decreases in *bcl-2* gene expression. Furthermore, in some types of cells, the steady-state levels of Bax protein may already be sufficiently high that a further p53-mediated increase is not required. In this regard, it has been reported that enforced production of high levels of Bcl-2 does not block the ability of p53 to *trans*-activate reporter gene constructs that

contain typical p53 binding sites, but does interfere with *trans*-repression mediated by p53 [168]. In fact, when cotransfected with expression plasmids producing either Bcl-2 or E1b-19kDa, p53 paradoxically acted as an up-regulator of the expression of some reporter gene constructs that are normally repressed by p53. Finally, p53 does not induce apoptosis in all those types of cells in which it can induce cell-cycle arrest. This is particularly true when comparing normal and transformed cells, since the latter are often markedly more sensitive to p53-dependent apoptosis induced by radiation, DNA-damaging drugs, and growth factor deprivation [111,114,221]. It will be of interest under these circumstances to compare the effects of p53 on the expression of *bax* and *bcl-2* in search of correlations with susceptibility to apoptosis.

Though speculative, the connection between p53 and the regulation of *bax* gene expression may provide insights into the clinical behavior of some types of cancer. For example, the ability of p53 to induce expression of *bax* may explain the somewhat paradoxical observation that patients with follicular lymphomas typically respond well to therapy at least initially, despite the high levels of Bcl-2 in these neoplasms caused by t(14;18) translocations. In this regard, although relapse occurs almost invariably, many patients can be induced into partial or complete clinical remissions a few times, often by use of the same drug regimen. Eventually, however, most patients experience transformation of their disease to an unresponsive state. The ability of follicular lymphoma patients to respond initially could theoretically be explained by induction of increases in p53 protein levels and transcriptional activity as a result of drug-induced damage to DNA, followed by elevations in Bak production. Eventually, this p53–Bax pathway for induction of apoptosis may fail due to loss of p53 expression or function, mutations in the *bax* gene, or other mechanisms resulting in a nonresponsive state in which the ratio of Bcl-2 to Bax remains high and the tumor cells thus fail to undergo apoptotic cell death. Consistent with this idea, p53 gene mutations have previously been associated with histological transformation of follicular lymphomas [222,223]. In addition, a recent comparison of several human lymphoma lines, some with wild-type and others with inactive p53, revealed induction of *bax* and apoptosis upon radiation or exposure to DNA-damaging drugs in lymphoma lines that contained wild-type p53 but not in lines in which p53 function had been lost [85]. Interestingly, analysis of several solid tumor lines indicated that despite the presence of intact, function p53, most of these fail to induce *bax* and also do not undergo apoptosis in response to x-radiation, whereas p21-Waf-1 is induced and cell cycle arrest does occur [85]. The implication therefore is that x-radiation and at least some types of drug-induced DNA damage appear to be only cytostatic and not cytotoxic in solid tumors despite the presence of p53, possibly because of a failure of p53 to induce expression of *bax* in these cells. Understanding more about why p53 fails to induce expression of *bax* in some types of tumors may therefore provide new insights that could be used to improve the treatment of cancer.

12. Conclusions and future directions

The discovery of *bcl-2* and its homologues has revealed a critical point in the regulation of the physiological cell death pathway. Alterations in the expression of *bcl-2* have been found in the majority of human cancers. Moreover, Bcl-2 and its homologues contribute significantly to the regulation of the relative resistance of tumor cells to apoptosis induced by radiation and essentially all chemotherapeutic drugs. With improved understanding of the mechanisms that regulate the expression of *bcl-2* family genes in normal and malignant cells, as well as with advances in our knowledge of the molecular details of how Bcl-2 and its various interacting proteins function biochemically, it should eventually become possible to develop novel approaches to the treatment of cancer. Among the approaches that deserve attention are attempts to use biological response modifiers to alter the ratios of pro- and anti- apoptotic Bcl-2 family proteins, thus putting tumor cells into a more vulnerable state with reference to apoptosis induction by currently available anticancer drugs. In this regard, a wide variety of receptors for cytokines and lymphokines, as well as phorbol esters and some retinoids, have been reported to either positively or negatively regulate signal transduction pathways that control *bcl-2* gene expression, including IL-2, IL-3, IL-4, IL-6, IL-10, TGF-β, TNF, kit-ligand, and CD40 [34,43,89,130,224–228]. For the most part, however, it remains to be established what the effects of these biological agents are on the expression of *bax* and other members of the *bcl-2* gene family — a critical issue if one is to rationally and predictably alter the therapeutic responses of cancers. Second, antisense oligonucleotides have been applied against *bcl-2* in vitro and more recently have even been used successfully in an animal model [91,229–231]. Tumor-to-tumor variability in the uptake and intracellular compartmentalization of these agents, however, remains a significant obstacle at present. Finally, the physical interactions among Bcl-2 family proteins and other Bcl-2 interacting proteins can served as targets for attempts to develop new small-molecule drugs that block these protein–protein interactions and thus promote cancer cell death. More investigations of the functional significance of these protein interactions and improved knowledge of some of the structural details are required if progress is to made on this front. Taken together, however, these and other approaches raise hopes that improved treatments of lymphomas, leukemias, and other cancers will eventually arise from investigations of the *bcl-2* gene family.

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4. MLL fusion genes in the 11q23 acute leukemias

James R. Downing and A. Thomas Look

1. Introduction

Remarkable progress has been made over the last five years in defining the molecular pathogenesis of the acute leukemias. Of the many leukemia-specific cytogenetic abnormalities that have been identified to date, structural lesions within the 11q23 chromosomal band are among the most common [1–4]. Translocations, deletions, and inversions within 11q23 have been detected in a variety of hematopoietic malignancies, including 7%–10% of the acute lymphoblastic leukemias (ALLs), 5%–6% of the acute myeloblastic leukemias (AMLs), and as many as 85% of secondary leukemias occurring in patients treated with topoisomerase II inhibitors [2,3,5–8]. Incidence rates of 11q23 translocations are strikingly high in infants (80% of ALL cases and 45% of AML), making these rearrangements the most common cytogenetic abnormalities in acute leukemias of this age group [9–11]. A number of different reciprocal chromosome bands have been shown to participate in balanced 11q23 translocations, including 1p32, 1q21, 2p21, 4q21, 5q31, 6q27, 7p15, 9p22, 10q12, 15q15, 16p13, 17q21, 19p13, and Xq13, with the most common translocations being t(4;11)(q21;q23), t(9;11)(p22;q23), and t(11;19)(q23;p13) [1]. Although some of these rearrangements are found almost exclusively in ALL (e.g., t(4;11)) or AML (e.g., t(9;11)), the majority have been observed in both myeloid and lymphoid leukemias, suggesting transformation of a pluripotent progenitor cell [1,12–16].

Rearrangements of 11q23 are associated with distinct clinical features in both ALL and AML. Affected patients with lymphoblastic leukemia typically present with hyperleukocytosis, hepatosplenomegaly, and a high incidence of central nervous system leukemia, and they have an overall poor prognosis when compared to that of patients whose leukemic cells lack this cytogenetic finding [3,9–12,16]. In addition, the lymphoblasts from these cases typically lack expression of the common ALL antigen (CALLA, or CD10), express the B-cell antigen CD19, and coexpress several myeloid-associated antigens including CD15 and CDw65, suggesting features of mixed lineage differentiation [14–18]. Although identified in the leukemic lymphoblasts of over 80% of infants less than six months of age, the frequency of 11q23 rearrangements

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decreases continuously throughout life, accounting for less than 3% of adult ALL cases [19–21]. However, regardless of the patient's age, identification of an 11q23 translocation in ALL appears to predict a poor response to conventional therapy.

In cases of de novo and secondary AML with an 11q23 rearrangement, the leukemic blasts typically have features of monocytic differentiation and are subclassified as myelomonocytic (M4) or monocytic (M5) according to the French–American–British (FAB) classification scheme [5,6,22]. In addition, the blasts often express both myeloid and lymphoid-associated antigens, indicating a mixed lineage pattern of differentiation in these leukemias as well. However, in contrast to findings in ALL, the presence of 11q23 translocations in either de novo pediatric or adult AML does not appear to confer an adverse prognosis [23,24].

The distinctive biologic and clinicopathologic features of acute leukemias with 11q23 abnormalities suggest that these rearrangements alter genes whose products provide critical functions in the normal growth and differentiation of multipotent progenitors that give rise to both the myeloid and lymphoid lineages. Tests of this prediction were recently made possible by cloning of the gene on chromosome 11 that is targeted by 11q23 rearrangements [25–31], referred to in this chapter as *MLL* for *M*ixed *L*ineage *L*eukemia, but also known as *HRX* (28), *Htrxl* (30), and *ALL-1* [29]. The *MLL* gene shows homology to the *Drosophila trithorax* gene, which acts as a 'master' regulator of processes vital to the development of this organism. In this chapter, we summarize current understanding of the molecular biology of 11q23 translocations and the roles of the human trithorax homologue in leukemogenesis.

2. Molecular cloning of the 11q23 target gene

Positional cloning approaches were used to isolate the gene on chromosome 11 that is disrupted by leukemia-associated 11q23 translocations [25–30]. These studies made it clear that the diverse array of 11q23 translocations target a single transcriptional unit encoded by a large gene, *MLL*, containing 21 exons dispersed over 100 kb of genomic DNA (figure 1). Sequence analysis of 11 kb of overlapping *MLL* cDNA clones revealed a single open reading frame encoding a 3,972-amino-acid protein with a molecular mass of 431 kDa. MLL contains three regions of homology to the product of the *Drosophila trithorax* gene, including two central zinc-finger domains and a 220-amino-acid C-terminal region exhibiting 82% similarity and 61% identity (figure 1) [28,29]. Also identified were three so-called A–T hook motifs in the N-terminus that encode structural domains initially identified in the high-mobility-group nuclear proteins, which appear to mediate binding to the minor groove of DNA. Lastly, a region of 47 amino acids localized between the A–T hook

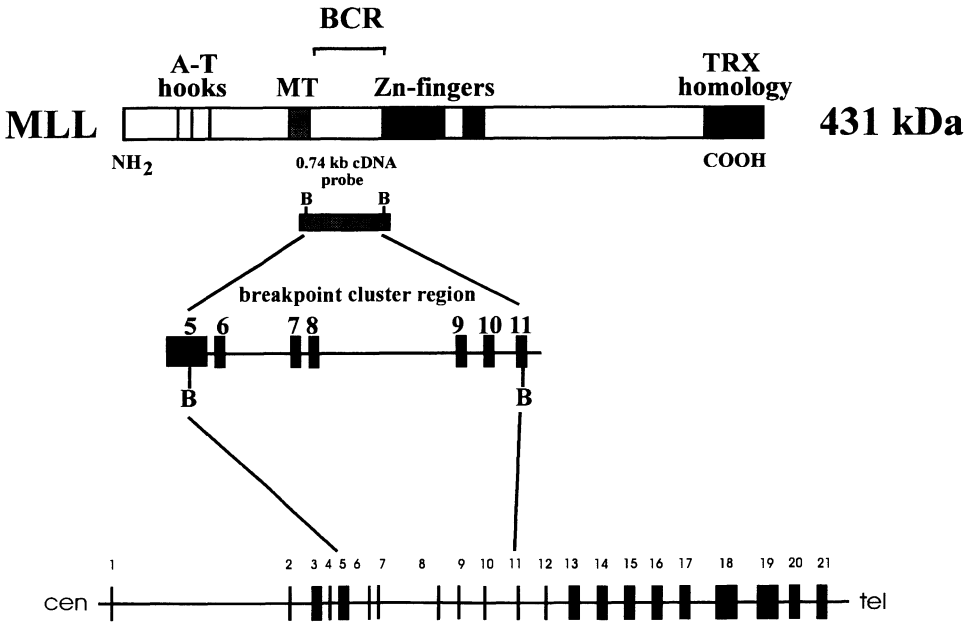


Figure 1. Schematic representation of the MLL protein. The black-filled regions are domains of high homology to the *Drosophila* gene *Trithorax* (TRX homology) and include two zinc-finger (Zn-finger) domains and a C-terminal region. The position of the A–T hooks and methyltransferase (MT) domains are indicated, along with the location within the protein of the 11q23 breakpoint cluster region (BCR). The portion of MLL encoded by the 0.744-kb cDNA probe is illustrated, along with the breakpoint cluster region and the entire genomic organization of the *MLL* gene and its orientation relative to the centromere (cen) and telomere (tel).

and the zinc-finger domains, was noted to have 76% similarity and 51% identity to the noncatalytic domains of human DNA-methyltransferases, enzymes that produce fully methylated double-stranded DNA from a hemimethylated substrate [32,33]. Taken together, these structural features of MLL suggest that its functions are likely to be mediated by direct physical interaction with DNA.

The similarity of MLL to the *Drosophila trithorax* gene may provide clues to the normal physiologic functions of MLL. *Trithorax* encodes a putative zinc-finger transcription factor that regulates the spatially restricted expression of the *Drosophila antennapedia* and *bithorax* homeotic gene complexes, and thus plays a pivotal role in segmental determination during *Drosophila* development [34,35]. The trithorax protein activates the transcription of multiple genes in these complexes, possibly through direct interactions with *cis* regulatory sequences. The homology of MLL to trithorax in its zinc-finger domains suggests that it may regulate the transcription of genes important in normal hematopoietic development. The significance of the C-terminal homology between trithorax and MLL remains to be defined; however, it has

recently been shown that this region shares homology with another *Drosophila* gene, *enhancer of zeste*, which is also involved in the control of the *antennapedia* and *bithorax* complexes [36]. Genetic analysis suggests that *enhancer of zeste* collaborates with *trithorax*, indicating that this C-terminal region may be involved in functional interactions between the protein products of these genes. Identification of mammalian proteins that interact with this region of MLL should provide additional insight into the intracellular signaling pathways that it regulates.

Besides its role in development, *trithorax* is also expressed in many adult fly tissues, suggesting a more generalized function in differentiated cells. Similarly, *MLL* is widely expressed in adult mammalian tissues, with differential expression of three alternatively spliced transcripts (14.5, 12.0, and 11.5 kb) in various tissues [32,33]. The 12.0-kb mRNA encodes a protein that lacks the A–T hook domains, and thus would be predicted to have an altered ability to interact with DNA [33]. Thus, control of the ratio of the different MLL isoforms may be a critical regulatory mechanism.

Because of the exceptionally large size of the *MLL* mRNA and its encoded protein, only limited studies of the normal roles of mammalian MLL have so far been possible. Recent analysis has shown that the A–T hook domains of MLL bind cruciform DNA and that binding specificity is dependent on the three-dimensional structure and not the specific DNA sequence [37]. In addition, transcriptional assays using constructs that fuse the GAL4 DNA-binding domain to portions of MLL have identified a repression domain between the A–T hooks and zinc fingers, as well as a strong activation domain located 3′ to the zinc-finger domains [37]. The repression domain appears to partialy overlap the region of homology with DNA methyltransferases. These data lend further support to the concept that MLL functions either through direct interaction with DNA or in a transcriptional regulatory complex with other DNA-binding proteins. Studies are in progress in a number of different laboratories to define the normal developmental role of MLL through targeted gene disruption and inappropriate expression of MLL in murine tissues.

3. Structure of *MLL* fusion products

In 11q23 translocations, breakpoints are localized to an 8.5-kb breakpoint cluster region positioned between exons 5 and 11 in *MLL* (figure 1) [27–29,38–40). Although theoretically these translocations could result in the formation of chimeric *MLL* genes on both derivative chromosomes, cytogenetic analysis has implicated the der(11), and expression studies have demonstrated that only the der(11)-encoded mRNA is consistently detected [41–43]. The der(11)-derived product consists of the N-terminal portion of MLL fused in frame to variable coding regions of the genes located on the partner chromosome. As a result of these translocations, the N-terminal portion of MLL, including the A–T hooks, methyltransferase domains, and a portion of the

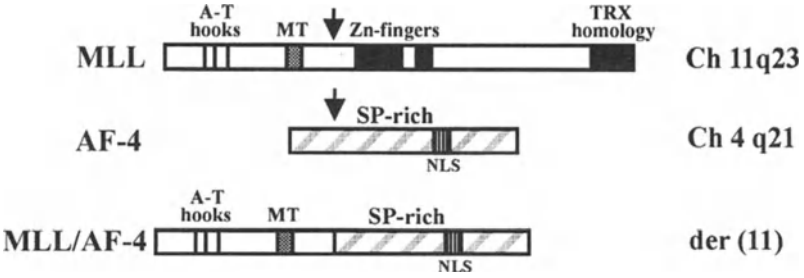
putative repression domain, is fused to coding sequences from the genes on the reciprocal chromosomes (figures 1 and 2A) [28,29]. Thus, the consistent pattern of separation of the N-terminal structural domains of MLL from C-terminal coding sequences that are not included in the der(11) product creates chimeric proteins that may have altered biologic activities.

In the der(11)-encoded chimeric product resulting from the (4;11) translocation, the N-terminal portion of *MLL* is fused to a portion of the *AF-4* gene (also called *FEL*) from chromosome 4q21(29,33,44). *AF-4* encodes a 140-kDa protein that lacks significant homology to proteins of known function but that is rich in serine/proline (16%/11%) and has two consensus nuclear localization signals. The translocation fuses this portion of *AF-4*, including its nuclear localization signals, to N-terminal amino acids from *MLL* (figure 2A) [29,33,44].

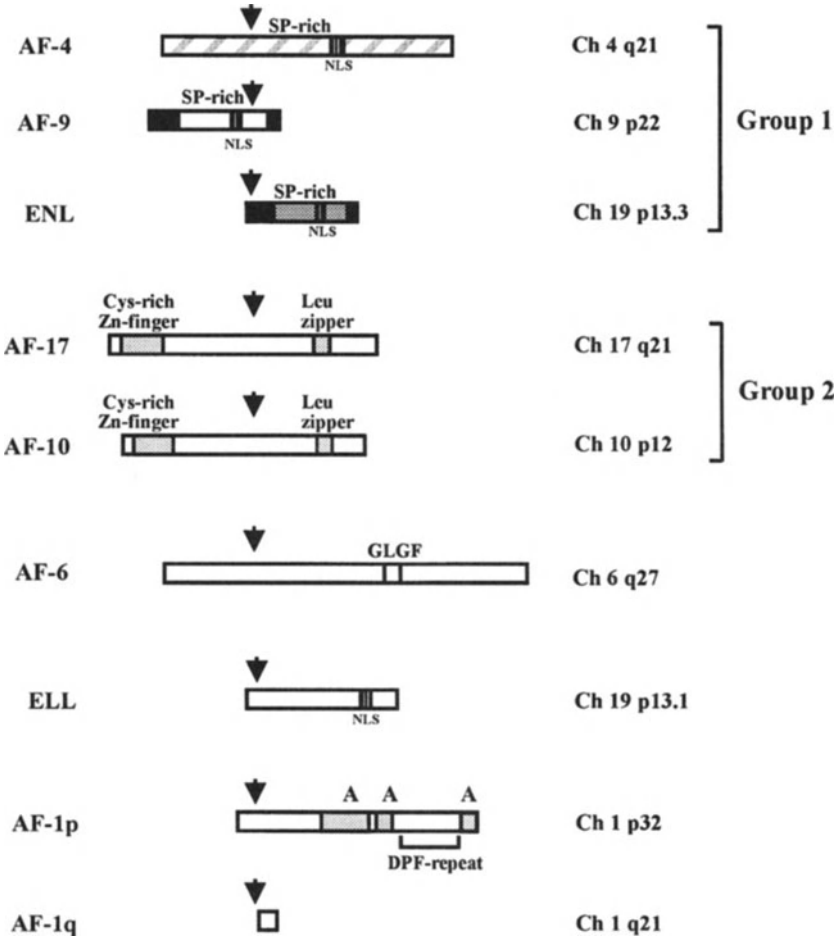
The marked diversity of the reciprocal products fused to *MLL* by the 11q23 translocations raised the possibility that common structural motifs would be identified in the various translocation partners, which would directly contribute to the formation of an active transforming protein. Sequence analysis of several der(11)-encoded chimeric products, including *MLL/ENL* and *MLL/AF9* produced by the t(11;19) and t(9;11) translocations, respectively, showed a high level of sequence homology with each other and with *AF-4*, providing support for this hypothesis (figure 2B) [28,29,45–47]. Each of these proteins is characterized by a high content of serine and proline residues typical of transcriptional activation domains and contains nuclear targeting signals. In addition, *ENL* and *AF-9* share 56% identity in their 140 N-terminal amino acids and 82% identity in their 67 C-terminal amino acids, suggesting that they have similar biologic functions [46]. Recent studies have identified a transcriptional activation domain in *ENL* that is localized in its C-terminal region of homology with *AF-9* [45]. Moreover, a fourth fusion partner, *AF-X1* rearranged by the (X;11) translocation, encodes a protein rich in serine/proline amino acids that has recently been shown to be a member of the Forkhead family of transcription factors [48,49]. Taken together, these studies suggest that some of the fusion partners of the MLL protein contribute domains with shared structural features, which replace the C-terminal sequences of normal MLL and result in oncogenic activation of the resultant chimeric proteins.

More recent sequence analysis of additional chimeric products suggests that they may act through alternative mechanisms. Either complete or partial cDNA sequences have been obtained for over 12 different chimeric products [28,29,46,48,50–56]. Significant homology has not been observed between these newly identified partner genes and those in group 1 (figure 2B). Two genes, *AF-17* and *AF-10*, involved in the (11;17) and (10;11) translocations, have homology to each other and appear to be members of a new class of transcription factors [51,52]. By contrast, the other partner genes characterized to date lack regions of significant sequence similarity. Moreover, the *AF-1q* gene of the (1;11)(q21;q23) translocation encodes a small 9-kDa protein

A. t(4;11) translocation



B. *MLL* translocation partners



that lacks homology to any other proteins in protein sequence backs [53]. The marked sequence diversity of the partner genes and the minimal contribution of AF-1q to the chimeric product in the t(1;11) is most consistent with a truncation mechanism that alters or inactivates activities encoded by full-length MLL, rather than the generation of chimeras with gain-of-function oncogenic roles.

In support of this interpretation is the recent identification of partial internal duplications of *MLL* in several cases of AML that lacked typical 11q23 translocations [57–59]. Internal duplications were detected in 2 of 19 patients with normal karyotypes, as well as 3 of 4 patients with trisomy 11 [59]. Although the extent of the duplicated region varied in these cases, a portion of the N-terminus was internally duplicated in each (figure 3). The duplicated region includes either the methyltransferase domain alone or both the A–T hook and methyltransferase domains. The partially duplicated *MLL* gene is transcribed into mRNA capable of encoding an in-frame fusion protein. In each fusion product, the N-terminal A–T hook and methyltransferase domains are separated from the zinc-finger domains by a variable amount of duplicated N-terminal sequence. Characterization of these novel *MLL* gene rearrangements has suggested that functional domains within MLL are critical to leukemogenesis on their own, and that sequences provided by fusion partners may in some cases serve to dissociate MLL N-terminal domains from regulatory regions located in C-terminal amino acids. How disruption of the normal MLL structure leads to transforming activity remains to be defined. However, recent identification of transcriptional repression and activation domains in the N- and C-terminal portions of the molecule, respectively, suggests that MLL structural alterations may affect the ability of the protein to regulate gene expression in hematopoietic stem cells.

4. Genetic mechanisms involved in *MLL* recombination: insights into pathogenesis

Several unique clinical features of leukemias with 11q23 translocations suggest that rearrangements of *MLL* occur in primitive hematopoietic stem cells.

Figure 2. (**A**) Schematic representation of the MLL and AF-4 proteins and the der(11)-encoded MLL/AF-4 chimeric protein. The location within AF-4 of the serine-proline (SP)-rich region and nuclear localization signal (NLS) are indicated. The position of the breakpoints in MLL and AF-4 are indicated by the arrows. (**B**) MLL translocation partners. The position of the characterized breakpoints in each partner protein is indicated by the arrows. Group 1 members are SP-rich proteins that contain NLS and include AF-4, AF-9, and ENL. AF-17 and AF-10, which constitute group 2, compose a novel family of transcription factors that contain a cysterine (cys)-rich zinc (Zn)-finger domain and a leucine (leu) zipper. AF-6 contain a central domain characterized by the presence of a glycine-leucine-glycine-phenylalanine (GLGF) motif. AF-1p contains three acidic (A) domains along with an amino-acid repeat motif consisting of aspartic acid–proline–phenylalanine (DPF).

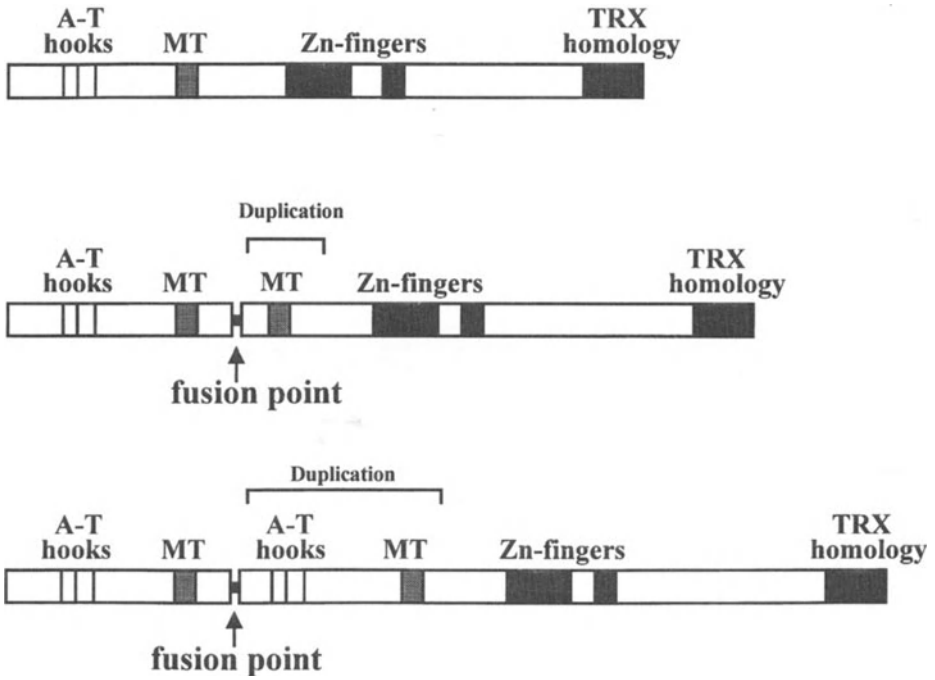


Figure 3. Schematic representation of AML-associated MLL internal duplications. The extent of the duplicated region is noted, along with the position of the fusion points.

First, both AMLs and ALLs with 11q23 translocations have features of biphenotypic differentiation, suggesting transformation of a cell that has not made a lineage commitment [3,12,60]. Second, 11q23 rearrangements are also frequent among leukemias that display so-called lineage infidelity, defined as the aberrant expression of phenotypic markers that are normally restricted to different blood cell lineages [61]. Third, different immunoglobulin gene rearrangements have been identified in leukemic clones from twins who harbor identical *MLL* rearrangements, suggesting transformation of a primitive stem cell prior to completion of immunoglobulin gene rearrangement [62,63]. The stem cell origin of these leukemias would predict a refractory subpopulation of blasts, thus explaining in part the poor responses of such cases to chemotherapy.

The B-cell precursor origin of some of the 11q23 acute leukemias has led to speculation that aberrant immunoglobulin gene rearrangements may be responsible for a certain fraction of *MLL* translocations. Sequence analysis of several *MLL* translocations, including those from cases with the t(4;11) and t(9;11), demonstrated heptamer-like sequences flanking the breakpoints on both chromosome 11 and the reciprocal chromosomes, consistent with a causative role for the V–D–J recombinase [40,64,65]. Moreover, extra N-region

nucleotides not contributed by either partner chromosome were identified at the translocation junction, indicating that the enzyme terminal deoxynucleotidyl transferase was active at the time the translocation took place. Although these data support a role of aberrant immunoglobulin recombinase activity in the generation of some 11q23 translocations, sequence analysis of other chimeric *MLL* genes has failed to identify heptamer- or nanomer-like sequences at the breakpoints [40,55]. Thus, although this mechanism may be responsible for some 11q23 translocations, it does not appear to explain chromosome rearrangements in the majority of cases.

A second potential mechanism of recombination has been suggested by the identification of breakpoints within *Alu*-repeats in some balanced 11q23 translocations [40]. In addition, *Alu*-repeats were identified at the point of recombination in several cases with internal *MLL* duplications [59]. The position of the breakpoints within these sequences suggests that *Alu*-mediated homologous recombination may be responsible for some of these rearrangements. Although a high density of *Alu*-repeats was apparent from sequence analysis of the entire 8.3-kb *MLL* breakpoint cluster region, they were missing in several introns with documented breakpoints [40]. Moreover, *Alu*-repeats have been identified in only a minority of breakpoint regions affecting the reciprocal gene. Thus, *Alu*-mediated homologous recombination also appears to explain some, but not all, instances of *MLL* translocation.

A third mechanism of genetic recombination was suggested from the frequent identification of *MLL* rearrangements in secondary AMLs arising after prior therapy with topoisomerase II inhibitors [5–8]. Chemotherapeutic agents that inhibit topoisomerase II include those in both the epipodophyllotoxin and anthracycline classes of drugs. In contrast to secondary AMLs induced by alkylating agents, cases of AML linked to these agents tend to appear within 6 to 60 months (median, 30 months) after diagnosis of the primary malignancy and to lack myelodysplastic phases. In addition, like de novo AML cases containing 11q23 translocations, these AMLs typically have monoblastic or myelomonoblastic morphology [5,6,8,23,66,67]. The preponderance of AML over early B ALL in secondary leukemia cases is in marked contrast to de novo 11q23 leukemias, and suggests that the target cell is a myeloid progenitor cell stimulated to enter cell division by chemotherapy-induced neutropenia.

Topoisomerase II catalyzes a two-step reaction consisting first of double-stranded DNA cleavage and second of strand relaxation and religation [68]. Both the epipodophyllotoxins and the anthracyclines appear to stabilize the DNA-topoisomerase II complex after cleavage, resulting in the accumulation of double-strand DNA breaks, which have been implicated in aberrant nonhomologous recombination [69,70]. Analysis of breakpoint sequences from several 11q23 translocations has identified topoisomerase II consensus binding sites adjacent to the chromosomal breakpoints [65]. Moreover, within the breakpoint cluster region of *MLL*, one consensus topoisomerase II binding site and 11 sites containing only a single base-pair mismatch were identified

[40]. However, it appears that this mechanism is only responsible for a subset of cases because many *MLL* junctions in secondary AML lack adjacent topoisomerase II-binding sites [40].

How could epipodophyllotoxins induce AML with characteristic MLL fusion proteins? The close association between treatment with topoisomerase II inhibitors and the formation of MLL chimeric products, as well as the rapid onset of these secondary leukemias, suggests a collaborative mechanism in which both the drug and fusion protein act synergistically to accelerate the multistep process leading to AML. A possible mechanism incorporating the known effects of the epipodophyllotoxins on cell-cycle progression and topoisomerase II activity is shown in figure 4. According to this model, epipodophyllotoxin treatment arrests cycling myeloid progenitors in the G2 phase, with most normal myeloid progenitors subsequently targeted to undergo apoptosis. As a result of these effects on myeloid stem cells, the patient develops neutropenia, causing growth factor production and the proliferation of pluripotent hematopoietic progenitors. The blockade of cells in G2 is not absolute, however, and some cells that are capable of self-renewal survive with double-strand DNA breaks at the sites of topoisomerase II integration. During subsequent repair processes, some of these breaks are joined by nonhomologous recombination, which produces chromosomal rearrangements. The myeloid progenitors with 11q23 translocations that occur secondary to this process begin to overgrow because of a proliferative advantage conferred by the hybrid MLL protein. In addition, the MLL fusion proteins may also specifically relax cell-cycle checkpoints normally activated by the

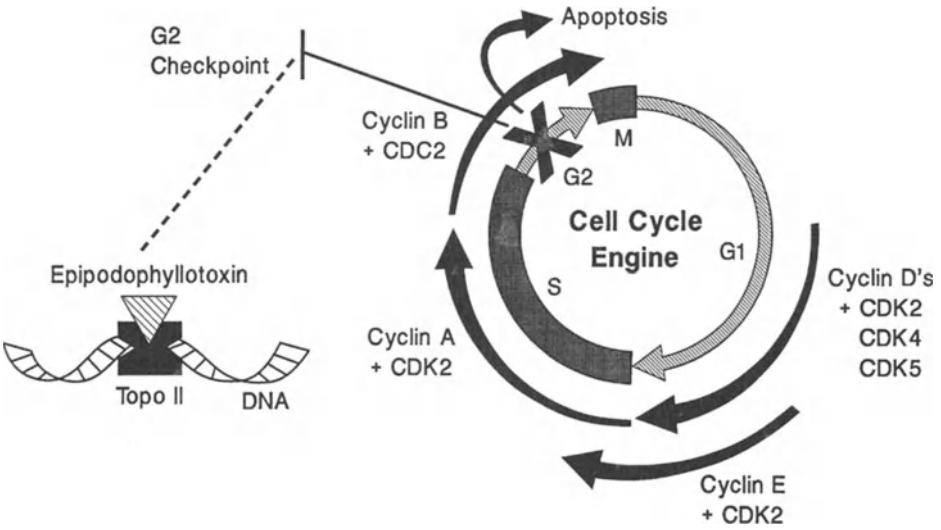


Figure 4. A model of the mechanisms linking epipodophyllotoxin therapy, cell-cycle progression of myeloid progenitor cells, and MLL fusion proteins in the origins of therapy-induced acute myeloid leukemias (see text for explanation).

presence of the integrated topoisomerase II:drug complex. This could lead to attenuated apoptosis and increased survival of cells with 11q23 rearrangements. Such cells would then enter mitosis harboring integrated topoisomerase II complexes. As a result, the MLL fusion protein could actually contribute to increased genetic damage at other loci in the context of repetitive epipodophyllotoxin treatment, leading to the acquisition of additional genetic lesions in the expanding 11q23 positive clone and the rapid progression of a multistep process culminating in overt AML.

Thus no single mechanism appears to account for all the *MLL* gene rearrangements identified to date; rather, a variety of different recombination mechanisms appear to be involved in the generation of *MLL* fusion genes and internal gene duplications. The clustering of breaks within the small 8.5-kb breakpoint cluster region in *MLL* suggests that this region may be predisposed to rearrangements by its high content of sequences implicated in genomic recombination, including recombinase accessible heptamers and nanomers, *Alu*-repeats, and topoisomerase II-binding sites. In addition, the chromatin structure of this region may predispose to a high frequency of recombination. However, the biologic properties of the resultant fusion proteins and the selective advantage they confer upon the leukemic clones that express them undoubtedly also contribute to the clustering of breakpoints within this region.

The exceedingly high frequency of 11q23 translocations among infant leukemias also has implications for potential mechanisms leading to gene rearrangement and the biologic activity of the resultant chimeric proteins. A number of pairs of infant twins have been characterized who have identical *MLL* gene rearrangements [62,63]. In some cases, the twins had identical immunoglobulin (Ig) gene rearrangements, consistent with transformation of a common progenitor cell that had completed V–D–J recombination. In contrast, in other cases the twins had different Ig rearrangements, suggesting that transformation occurred before or during Ig gene recombination and that the leukemic clones had subsequently evolved independently. Regardless of the Ig gene findings, however, the identification of identical *MLL* rearrangements in each twin provides unequivocal proof that the leukemic clone arose in one infant and then underwent interplacental metastasis to the sibling. The documentation of intra utero *MLL* rearrangements, the high frequency of 11q23 translocations in infant leukemias (approaching 80% of ALLs and 50% of AMLs), and the progressive decrease in the frequency of these chimeric oncoproteins with increasing age suggest that certain pluripotent progenitors are in a proliferative state in infancy that renders them uniquely susceptible to transformation by chimeric MLL oncoproteins. The reason for this susceptibility remains to be defined, but may be related to patterns of gene expression or even epigenetic changes in *MLL* chromatin configuration that are found in subsets of progenitors at restricted stages of development. Presumably, myeloid progenitors similarly susceptible to the transforming effects of chimeric MLL proteins, or prone to productive MLL rearrangements, are reactivated in

patients undergoing therapy with epipodophyllotoxins, accounting for the rapid onset of secondary AML in children and adults treated with these agents. Moreover, the short latency period between *MLL* rearrangement and overt leukemia following 11q23 translocations in infants and after epipodophyllotoxin therapy suggests that alterations in *MLL* are either sufficient by themselves for leukemia induction or that they predispose a preleukemic cell to secondary mutations necessary for the development of a fully transformed phenotype (as outlined above). The linkage of topoisomerase II inhibitors to *MLL* gene rearrangements, along with the definitive identification of intra utero *MLL* translocations, suggests that careful epidemiologic studies may provide critical insights into the identification of leukemogenic agents responsible for these infant leukemias.

5. Molecular diagnosis of 11q23 rearrangements

Because 11q23 translocations and the chimeric products they generate define high-risk clinical subgroups of patients, the accurate identification of leukemias harboring these molecular abnormalities is essential. The tight clustering of breakpoints within the *MLL* gene has resulted in the ability to detect *MLL* rearrangements by probing Southern blots of *Bam*HI-restricted genomic DNA with a 0.75 *Bam*HI cDNA restriction fragment that contains portions of exons 5 through 11 (figure 1) [24,38,39,71,72]. In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) assays have been developed for the detection of the der(11)-derived chimeric messages encoded by the t(4;11), t(6;11), t(9;11), and t(11;19) [42,48,73–75]. The recent development of multiplex RT-PCR assays for the detection of these translocations in a single-step reaction has greatly simplified the application of these tests in the clinical setting (figure 5) [76]. Moreover, PT-PCR assays for *MLL* chimeric messages are able to detect the fusion transcripts at a sensitivity of greater than one leukemic cell in 1×10^5 normal cells [74]. Thus, RT-PCR assays for *MLL* chimeric transcripts appear to be ideal for both the routine diagnosis and monitoring of the responses to therapy in individual patients. In addition, recently developed interphase fluorescence in situ hybridization (FISH)-based assays may provide an alternative approach for the rapid detection of acute leukemias with 11q23 translocations.

The recent application of these molecular genetic approaches to the characterization of a large number of infant leukemias has revealed that up to 30% of *MLL* rearrangements are missed by classic cytogenetic studies [39,77]. Moreover, infants with *MLL* rearrangements were found to have a significantly poorer event-free survival when compared to infants that lacked this molecular genetic lesion [39,77,78]. Thus, in infants, as in older children and adults [21,79], molecular analysis is critical for the assignment of patients with hybrid MLL genes to bone marrow transplant or other therapy appropriate for their high risk of treatment failure. In addition, it has become increasingly

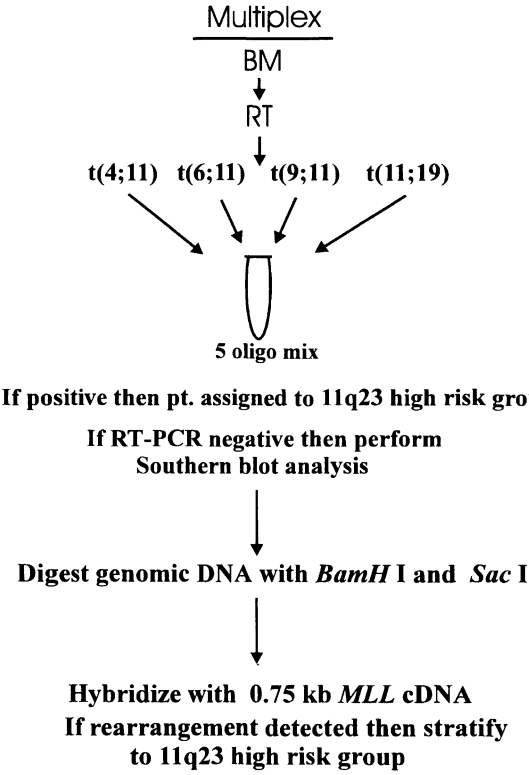


Figure 5. Flow diagram illustrating the sequential application of molecular assays for the evaluation of a clinical sample for rearrangements in the *MLL* gene. RNA is extracted from a diagnostic bone marrow aspirate (BM), reverse transcribed (RT), and analyzed in a multiplex RT-PCR assay for the presence of one of the chimeric transcripts encoded by the four most common 11q23 translocations. If the RT-PCR assay is negative, genomic DNA is extracted from the BM sample and analyzed by Southern blot analysis for evidence of *MLL* rearrangements.

clear that this group does not completely coincide with the one defined by cytogenetically identified 11q23 structural abnormalities. For example, the majority of 11q23 deletions and inversions do not involve the *MLL* gene, but rather affect other distinct genetic loci [79]. Also, the *RCK* and *PLZF* genes are located in chromosome band 11q23 and are targeted by leukemia-associated translocations that do not involve MLL, namely, the t(1;14)(q23;q32) and t(11;17)(q23;q21) [80,81]. In marked contrast to lymphoid leukemias with *MLL* rearrangements, those carrying 11q23 structural abnormalities that do not affect the *MLL* gene have a favorable prognosis [39,77–79]. Thus, accurate risk stratification requires molecular identification of *MLL* gene rearrangements, not simply cytogenetic identification of a rearranged 11q23 band.

The best approach to identify acute leukemias with *MLL* rearrangements is

controversial and may change with the development of newer molecular-based assays for their detection. At present, use of the RT-PCR multiplex assay to detect fusion transcripts resulting from the four most common translocations allows detection of greater than 80%–90% of the cases with *MLL* gene rearrangements. The remaining 11q23 translocations, however, involve as many as 16 different chromosomes in reciprocal fusions, and only a proportion of the genes altered by these translocations have been cloned and sequenced. Thus, in the absence of RT-PCR methods for detection of these rare 11q23 translocations, *MLL* gene rearrangements will need to be identified by Southern blot analysis of genomic DNAs digested with two separate restriction endonucleases (*Bam*HI and *SacI*), and hybridized with an exon 5–12 *MLL* cDNA probe (figure 5). Alternatively, a FISH-based assay could be used to detect the majority of *MLL* rearrangements missed by the multiplex assay; however, internal duplications of *MLL* would be missed using the FISH approach.

6. Future directions

The recent explosion of knowledge in molecular biology has implicated specific gene rearrangements and aberrantly expressed fusion proteins in the pathogenesis of acute leukemias harboring 11q23 chromosomal translocations. The high frequency of these abnormalities in the acute leukemias of infants and in patients undergoing epipodophyllotoxin chemotherapy lend urgency to efforts to understand how these chimeric proteins contribute to malignant transformation. In addition, the diversity of the consequences of these rearrangements at the molecular level, with more than 16 different chimeric proteins involving the MLL gene characterized or in the process of being characterized, implies a central role for the MLL protein in normal and malignant hematopoietic stem cell biology.

As the era of gene discovery and sequence comparisons draws to a close, the more difficult work of elucidating the role of MLL in development and malignancy should assume highest priority. Although structural similarities between MLL and the *Drosophila* trithorax developmental regulatory protein are intriguing, they have not led so far to an understanding of the signaling pathways normally regulated by MLL. Such insight will come from biochemical analysis of the MLL protein and targeted disruption of its gene in murine systems — research that should benefit from new information emerging from the more genetically tractable analysis of the roles of *trithorax* and *enhancer of zeste* in *Drosophila*. Similarly, both biochemical analysis and mechanistic studies in model systems will be needed to establish the roles of the diverse MLL chimeras in the genesis of human acute lymphoid and myeloid leukemias. Although certain structural features of the partner proteins are intriguing, they each represent novel proteins discovered first as a result of their involvement in oncogenic chimeras with MLL, so information on their normal roles

that might predict mechanisms active in leukemogenesis is presently unavailable. The most that can be said at this time is that the 11q23 rearrangements uniformly result in dominantly acting hybrid proteins that consistently include a relatively small N-terminal region of the MLL protein. Although expression of these fusion proteins is linked to leukomogenesis, it cannot be stated for certain whether they act as gain-of-function transforming oncogenes or as dominant interfering or dominant negative proteins, opposing the normal actions of MLL or other components of a larger complex of proteins that regulate critical aspects of hematopoietic stem cell development. One can be assured, however, based on the large number of intriguing new proteins that have been discovered in these fusions, that the next few years will yield important and at present unpredictable insights into both leukemogenesis and normal embryologic and hematopoietic stem cell biology.

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5. The c-myb proto-oncogene: a novel target for human gene therapy

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1. Introduction

The hypothesis that viruses could cause cancer was initially viewed with great skepticism but is now firmly accepted. Using recombinant technology, it has been proven beyond any doubt that retroviruses in particular contain within their genomes transforming genes capable of inducing neoplastic transformation [1,2]. These viral oncogenes have (v-onc) been shown to be derived from highly conserved, normal cellular genes that were almost certainly incorporated into the viral genome during its transit through the host cell. The normal cellular counterparts of the v-onc genes are termed proto-oncogenes. Proto-oncogenes in turn have been shown to be intimately involved in the processes of cell proliferation and differentiation. They encode, for example, proteins that function as growth factors or growth factor receptors [3,4], signal transducing proteins [5], and a large number of proteins with transcriptional activity [6,9]. Accordingly, it is not difficult to imagine situations where v-onc (1) amplification [10], (2) mutation [11], (3) translocation leading to structural alteration [12], or (4) change in transcriptional regulation might either lead to, or be associated with, induction of a malignant phenotype in the cell in which these changes occurred [1,13].

Recent years have also seen the advent of innovative technologies for disrupting gene expression quite specifically. All rely on some type of nucleotide sequence recognition for specificity but differ in where and how they perturb the flow of genetic information (figure 1). Inhibition at the level of transcription can be accomplished by two different methods. One that is widely employed is the process of homologous recombination [14]. In a typical system, a plasmid vector is constructed for gene targeting that contains a selectable gene flanked by sequences complementary to the region of interest in the genomic DNA. The vector is then introduced into a cell where, during the course of cell division, the targeting vector and the complementary portion of genomic DNA undergo a cross-over event that leads to exchange of the genetic material. These exchanges result in insertion of the selectable gene into the targeted genomic DNA, with consequent destruction of the target gene. Targeting is carried out so that upstream sequence is left intact, hope-

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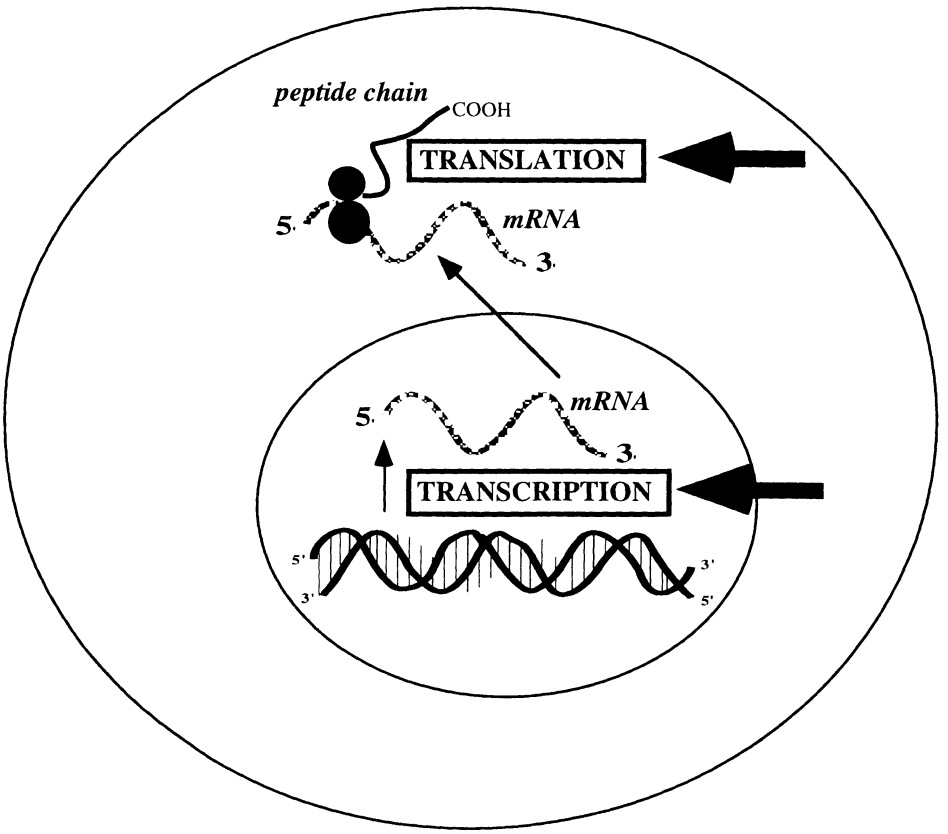

Figure 1. Inhibition of gene function may be accomplished by physical destruction of the target gene itself (homologous recombination). It may also be accomplished by interference with expression at the transcriptional level (triplex formation) or at the translational level (antisense strategies). See text for details.

fully allowing the altered gene to be expressed in a spatially and temporally correct manner. The effect of expressing the mutated, dysfunctional gene in a developing animal may then be discerned. This method, though quite effective at generating heterozygous or homozygous loss of function mutants, is hampered by that fact that it is nonetheless very inefficient, time-consuming, and expensive. An alternative 'antigene' strategy can be effected by targeting polypurine–polypyrimidine sequences within or flanking the gene that one wishes to disrupt with a complementary oligodeoxynucleotide [15]. This leads to so-called 'triplex' formation in the major groove of the DNA helix via Hoogsten bond formation. The DNA helix cannot be opened in the triplexed area, thereby preventing transcription of the target gene.

Perturbation of gene function at the posttranscriptional level can also be effected by impairing utilization of RNA. This is the so-called 'antisense' strategy [16]. The technique relies on either introducing or expressing in the

target cell a nucleotide sequence that is the reverse complement (antisense) of the mRNA one is trying to disrupt. Hybridization between the target and the exogenous nucleotide sequence inhibits the target's function and may lead to its destruction. A variation of this technique relies on complementary base pairing to target a catalytic RNA molecule, or ribozyme [17]. In contrast to triplex-forming oligonucleotides, antisense compounds do not have the same nucleotide sequence constraints that govern their ability to hybridize with an mRNA molecule. In contrast to ribozymes, they do not require viral vectors for entry into cells. Accordingly, they are easier to design and perhaps to utilize for disease therapy.

Antisense DNA molecules have already been used to block replication of several types of viruses, including HIV [18], and to block synthesis of a diverse array of proteins, including those encoded by cell-cycle-regulated genes [19], cell adhesion proteins [20], growth factors and their receptors [21,22], and elements of the signal transduction apparatus [23]. Nevertheless, in addition to the utility of this method for answering basic biologic questions, many groups, including our own, believe that the method has translational applications as well. This chapter reviews why we believe this to be true and uses our studies targeting the c-myb protooncogene for illustrative purposes.

2. The c-myb proto-oncogene

Of the genes that we have targeted for disruption using the antisense ODN strategy [19,22,24], one that has been of particular scientific interest in our laboratory, and one where therapeutically motivated disruptions are now in clinical trial, is the c-myb gene [25]. C-myb is the normal cellular homologue of v-myb, the transforming oncogene of the avian myeloblastosis virus (AMV) and avian leukemia virus E26. It is a member of a family composed of at least two other highly homologous genes designated A-myb and B-myb. The molecular and cell biology of the Myb-family genes and their encoded proteins has recently been reviewed in detail by Lyon et al. [26].

Located on chromosome 6q in humans, c-myb's predominant transcript encodes an approximately 72-kDa protein (Myb) [25,26]. Myb protein consists of three primary functional regions [27] (figure 2). At the NH_2 terminus is a DNA binding domain that recognizes the core consensus sequence 5′-pyAAC(G/Py)G-3′ [9]. This region consists of three imperfect repeats consisting of 51–52 amino acids that have been designated R1, R2, and R3, respectively. R2 and R3 have been shown to be absolutely, required for DNA binding, while the role of R1 is less clear; it may contribute to the stability of the DNA–protein interaction, but it does not appear to be required for binding. Within each repeat are three perfectly conserved tryptophan residues. Together they form a cluster in the hydrophobic core of the protein that maintains the DNA binding helix-turn-helix structure.

The midportion of the Myb protein contains an acidic transcriptional acti-

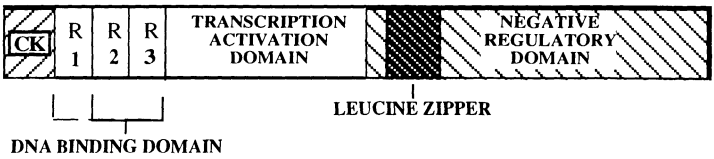

Figure 2. Functional map of the c-myb protein indicating the DNA binding, transcriptional activation, and repressor domains. The location of the putative leucine zipper-like structure is also indicated.

vating domain. In concert with the DNA binding portion of the protein, these two regions are sufficient to *trans*-activate promoters containing Myb binding sites. An immediately adjacent downstream region of the protein also appears to play a role in the activation of some targets, e.g., c-myc [28]. This same region, independent of the DNA binding domain, has also been reported capable of transactivating certain targets such as the heat shock protein [29] and DNA polymerase alpha [30]. Finally, it has also been reported that some Myb transactivation targets require an interaction between Myb and other transcription factors, such as the nuclear factor-m (NF-M) [31], and the cat enhancer binding protein (C/EBP) [32]. It is therefore likely that some of Myb's transcriptional activating properties may be dependent on the protein partners with which it interacts. This, of course, appears to be true of many transcriptional factors.

Myb also contains a negative regulatory domain that has been localized to the carboxy terminus. Interestingly, the carboxy terminus is deleted in v-myb, and this has been thought to contribute to v-myb's transforming ability. Recently, a putative leucine zipper structure was described within the amino terminal portion of Myb's carboxy terminal domain [33]. Leucine zippers, such as those found in the transcription factors Jun, Fos, and Myc, are thought to facilitate the protein–protein interactions that permit heterodimerization of DNA binding proteins. As noted above, such dimerization is thought to play a key role in regulating the transcriptional activity of these factors. A Myb dimerizing binding partner has yet to be identified, but Myb–Myb homodimerization, which likely occurs through its leucine zipper, does lead to loss of DNA binding and *trans*-activation ability [34]. Accordingly, one could reasonably postulate that Myb-driven gene *trans*-activation might be regulated by the binding of additional protein partners in the leucine zipper domain of the carboxy terminus of the protein (33). Alternatively, as was true for the *trans*-activating functions of the protein, repressor activity might also require the interaction of Myb with other proteins.

Can one reasonably postulate that aberrant c-myb expression or Myb function might play a role in carcinogenesis? Amplification of c-myb in acute myelogenous leukemia (AML) and c-myb overexpression in $6q^-$ syndrome has been reported [35], suggesting that altered c-myb expression may indeed play a role in leukemogenesis. The exact nature of this role remains to be

defined. One potential mechanism is through Myb's ability to regulate hematopoietic cell proliferation. Indeed, regulating progression through the G_1/S gate of the cell cycle seems to be a key function of c-myb [36]. The mechanism whereby c-myb regulates cell proliferation is uncertain but might well relate to its role in regulating a number of important cell-cycle genes, including c-myc [28], PCNA [37], and cdc2 [38]. Myb may also play a role in regulating differentiation [39]. Evidence to support this hypothesis includes the observations that as primitive hematopoietic cells mature c-myb expression declines [40], and that constitutive expression of c-myb usually [41], but not always [42], inhibits the ability of hematopoietic cells to undergo differentiation. More directly, c-myb has been shown to function as a transcription factor for several cellular genes, including the neutrophil granule protein mim-1 [43], CD4 [44], IGF-1 [45], and CD34 [46], and possibly other growth factors [47] or growth factor receptors such as c-kit [22]. Finally, although c-myb was once thought to be hematopoietic cell specific, it has become clear in recent years that c-myb is expressed in non-hematopoietic tumor tissues as well [48]. Such expression is often low level, but studies from our laboratory suggest that downregulation of c-myb under such circumstances can slow the growth of some malignant cell types, including malignant melanoma [48]. Accordingly, c-myb may also become a rational target in other malignant diseases, thereby increasing the scope and relevance of the studies to be proposed below.

3. Targeting the c-myb gene

Our laboratory has been using antisense oligodeoxynucleotides (AS ODN) since 1988 to understand the role of c-myb and other proto-oncogenes in normal and malignant human hematopoiesis. These investigations were initially designed to elucidate the role of Myb protein in regulating hematopoietic cell development. Because the results obtained from these studies had obvious clinical relevance, more translationally oriented studies were undertaken. These have now culminated in phase I clinical trials that are presently ongoing at the Hosptial of the University of Pennsylvania. The development of the c-myb targeted antisense oligodeoxynucleotide will be summarized below, along with a brief allusion to our initial clinical experience with this agent.

3.1. In Vitro experience in the hematopoietic cell system

3.1.1. Role of c-myb-encoded protein in normal human hematopoiesis. Attempts to exploit the c-myb gene as a therapeutic target for antisense oligodeoxynucleotides began as an outgrowth of studies seeking to define the role of Myb protein in regulating normal human hematopoiesis. During the course of these studies, it was determined that exposing normal bone marrow mononuclear cells (MNC) to c-myb antisense oligomers resulted in a decrease

in cloning efficiency and progenitor cell proliferation [49]. The effect was lineage indifferent, since c-myb antisense DNA inhibited granulocyte-macrophage colony forming units (CFU-GM), CFU-E (erythroid), and CFU-Meg (megakaryocyte). In contrast, c-myb sense oligomers had no consistent effect on hematopoietic colony formation when compared to growth in control cultures ($p = .778$, $p = .796$, $p = .375$ for CFU-GM, CFU-E, and CFU-Meg, respectively). Inhibition of colony formation was also dose related and sequence specific. If progenitor cells were exposed to 0.3, 1.4, 7, and 14 μM of c-myb oligodeoxynucleotides, the sense oligomers did not significantly effect colony formation, while the antisense oligomers at the above concentrations led to the formation of 21 ± 3, 17 ± 1, 10 ± 2, and 4 ± 2 megakaryocyte colonies, respectively [49]. Antisense oligodeoxynucleotides at concentrations of 1.4, 7, and 14 μM led to the formation of 775 ± 127, 515 ± 9, and 244 ± 25 myeloid colonies, respectively [49]. The requirement for Myb during normal hematopoiesis has also been confirmed in gene targeting experiments carried out using the technique of homologous recombination [50]. In other investigations, it was also determined that hematopoietic progenitor cells appeared to require Myb protein during specific stages of development [51], in particular when they were actively cycling.

3.1.2. Myb protein is also required for leukemic hematopoiesis. Since the c-myb antisense oligomers inhibited normal cell growth, we were also interested in determining their effect on leukemic cell growth. To address this question, we employed a variety of leukemic cell lines, including those of myeloid and lymphoid origin [52,53]. In addition, we also employed primary patient material [54].

We first detemined the effect of myb sense and antisense oligomers on the growth of HL-60, K562, KG-1, and KG-1a cell lines [9]. The antisense oligomer inhibited the proliferation of each leukemia cell line, although the effect was most pronounced on HL-60 cells. Specificity of this inhibition was demonstrated by the fact that the sense oligomer had no effect on cell proliferation, nor did 'antisense' sequences with 2 or 4 nucleotide mismatches. To determine whether the treatment with c-myb antisense modified cell-cycle distribution of HL-60 cells, we measured the DNA content in exponentially growing cells exposed to either sense or antisense myb oligomers. Control cells and cells treated with c-myb sense oligomers had twice the DNA content of HL-60 cells exposed to the antisense oligomers. The majority of these cells either appeared to reside in the G1 compartment or were blocked at the G1/S boundary.

To examine the effect of the c-myb oligomers on lymphoid cell growth, we employed a lymphoid leukemia cell line designated CCRF-CEM [52]. As noted earlier in the case of normal lymphocytes [36], the CCRF-CEM cells were extremely sensitive to the antiproliferative effects of the c-myb antisense DNA. When exposed to the sense oligomers, we found negligible effects on CEM cell growth in short-term suspension cultures. In contrast, exposure to c-

myb antisense DNA resulted in a daily decline in cell numbers. Compared to untreated controls, antisense DNA inhibited growth by about 2 logs. Growth reduction was not a cytostatic effect, since cell viability was reduced only by about 70% after exposure to the antisense oligomers, and CEM cell growth did not recover when cells were left in culture for an additional nine days. The c-myb antisense oligomers also appeared effective in inhibiting cell growth of primary patient material when derived from patients with both acute and chronic myeloid leukemias [54]. Colony inhibition was about 58%–95%, and as determined by elimination of bcr-abl expressing CML clones, apparently quite efficient.

3.1.3. Evidence that normal and leukemic progenitor cells rely differentially on c-myb function. In order to be useful as a therapeutic target, neoplastic cells, leukemic or otherwise, would have to be more dependent on Myb protein than their normal counterparts. To examine this critical issue in hematopoietic cells, we incubated phagocyte- and T-cell-depleted normal human marrow mononuclear cells (MNCs), human T-lymphocyte leukemia cell line blasts (CCRF-CEM), or 1:1 mixtures of these cells with sense or antisense oligodeoxynucleotides to codons 2–7 of human c-myb mRNA (table 1). Oligomers were added to liquid suspension cultures at time 0 and at time +18 hours. Control cultures were untreated. In controls, or in cultures to which 'high' doses of sense oligomers were added, CCRF-CEM proliferated rapidly, whereas MNC numbers and viability decreased by less than 10%. In contrast,

Table 1. Effect of c-myb oligomer exposure on colony/cluster formation by T-cell leukemia and normal bone marrow progenitor cells (MNC)

Cells plated	No. added	Oligomer/amt. added[a]	Colony/cluster
MNC	5×10^4/ml	None	24 ± 4
		Myb S (20; 5.0)	31 ± 4
		Myb AS (20; 5.0)	30 ± 6
T Leukemia	5×10^4/ml	None	TNTC[b]
		Myb S (20; 5.0)	TNTC
		Myb AS (20; 5.0)	1 ± 1
MNC + Leukemia	5×10^4/ml of each	None	TNTC
		Myb S (20; 5.0)	TNTC
		Myb AS (2; 0.5)	TNTC
		Myb AS (5; 1.0)	TNTC
		Myb AS (10; 2.5)	41 ± 5
		Myb AS (20; 5.0)	34 ± 1

[a] μg/ml added to the culture medium at time 0 and +18 hours, respectively.
[b] Too numerous to count (>1,000 colonies).
Note: Cells were exposed to oligomers for four days in suspension cultures, and then transferred to semisolid media as described in the methods section. After 12 days in culture, colonies and clusters were counted in paired dishes with an inverted microscope. Colony/cluster counts are presented as mean ± SD. (Adapted from reference #52.)

when CCRF-CEM were incubated for four days in c-myb antisense DNA, cultures contained $4.7 \pm 0.8 \times 10^4$ cells/ml (mean ± SD; n = 4) compared to $285 \pm 17 \times 10^4$/ml in controls. At the effective antisense dose, MNC were largely unaffected. After four days in culture, remaining cells were transferred to methylcellulose supplemented with recombinant hematopoietic growth factors. Myeloid colonies/clusters were enumerated at day 10 of culture inception. Depending on the cell number plated, control MNC formed from 31 ± 4 to 274 ± 18 colonies. In dishes containing equivalent numbers of untreated or sense-oligomers-exposed CCRF-CEM, colonies were too numerous to count (TNTC). When MNCs were mixed 1:1 with CCRF-CEM in antisense oligomer concentrations less than or equal to 5 μg/ml, only leukemic colonies could be identified by morphologic, histochemical, and immunochemical analysis. However, when antisense oligomer exposure was intensified, normal myeloid colonies could be found in the culture, while leukemic colonies could no longer be identified with certainty using the same analytic methods.

Finally, at the antisense DNA doses used in the above studies, AML blasts from 18 of 23 patients exhibited about a 75% decrease in colony and cluster formation compared to untreated or sense-oligomer-treated controls. When 1:1 mixing experiments were carried out with primary AML blasts and normal MNC, we were again able to preferentially eliminate AML blast colony formation while normal myeloid colonies continued to form [52]

3.1.4. Use of c-myb oligomers as bone marrow purging agents. The experiments described seemed to suggest that leukemic cell growth could be preferentially inhibited after exposure to c-myb antisense oligodeoxynucleotides. In thinking about a clinical use for this observation, application in the area of bone marrow transplantation seemed compelling. In this setting, exposure conditions were completely under the control of the investigators, and patient exposure to the material would be minimal. These conditions would make approval by regulatory agencies less difficult. We therefore determined whether the antisense oligomers could be utilized as ex vivo bone marrow purging agents [52,54]. To test this hypothesis, normal MNCs were mixed (1:1) with primary AML or CML blast cells and then exposed to the oligomers using a slightly modified protocol designed to test the feasibility of a more intensive antisense exposure. Towards this end, an additional oligomer dose (20 μg/ml) was given just prior to plating the cells in methylcellulose. In control growth-factor-stimulated cultures, leukemic cells formed 25.5 ± 3.5 (mean ± SD) colonies and 157 ± 8.5 clusters (per 2×10^5 cells plated). Exposure to c-myb sense oligomers did not significantly alter these numbers (19.5 ± 0.7 colonies and 140.5 ± 7.8 clusters; $p > .1$). In contrast, equivalent concentrations of antisense oligomers totally inhibited colony and cluster formation by the leukemic blasts. Colony formation was also inhibited in the plates containing normal MNCs, but only by about 50% in comparison to untreated control plates (control colony formation, 296 ± 40 per 2×10^5 cells plated; treated colony formation, 149 ± 15.5 per 2×10^5 cells).

3.1.5. Evaluation of the efficiency of an antisense purge. In antisense-treated cocultures of normal MNC and primary AML blast cells (1:1 ratio), only normal colonies could be identified. To assess the potential effectiveness of an antisense purge, we conducted similar coculture studies with CML cells obtained form patients in blast crisis and in chronic phase of their disease. Since these cells express the tumor-specific, and easily detected, bcr-abl fusion gene, it was an easy task to measure residual leukemic cells in oligonucleotide-treated cultures [52,54].

To carry out these studies, RNA was extracted from cells cloned in methylcellulose cultures after exposure to the highest c-myb antisense oligomer dose. The RNA was then reverse transcribed and the resulting cDNA amplified as described in the materials and methods section. For each patient studied, mRNA was also extracted from a comparable number of cells derived from untreated control colonies using the same technique. Eight cases were evaluated, and in each case bcr-abl expression as detected by RT-PCR correlated with colony growth in cell culture. In cases that were inhibited by exposure to c-myb antisense oligomers (7 of 11), bcr-abl expression was also greatly decreased or nondetectable.

These results suggested that bcr-abl-expressing CFUs might be substantially or entirely eliminated from a population of blood or marrow mononuclear cells by exposure to the antisense oligodeoxynucleotides. To explore this possibility further, replating experiments were carried out on samples from two patients. We hypothesized that if CFUs belonging to the malignant clone were present at the end of the original 12-day culture period, but not detectable due to failure to express bcr-abl, they might reexpress the message upon regrowth in fresh cultures. Accordingly, cells from these patients were exposed to oligomers and then plated into methylcellulose cultures formulated to favor growth of either CFU-GM or CFU-GEMM. As was found with the original specimens, untreated control cells and cells exposed to sense oligomers had RT-PCR-detectable bcr-abl transcripts. Those exposed to the c-myb antisense oligomers had none. One of the paired dishes from these cultures was then solubilized with fresh medium, and all cells contained therein were washed, disaggregate, and replated into fresh methylcellulose cultures *without* reexposing the cells to oligomers. After 14 days, CFU-GM and CFU-GEMM colony cells were again probed for bcr-abl expression. Control and sense-treated cells had RT-PCR-detectable mRNA, but none was found in the antisense-treated colonies. These results suggest that elimination of bcr-abl-expressing cells and CFUs was highly efficient and perhaps permanent.

Since the publication of these results, we have continued to evaluate potential patients using the above-described protocol. Samples have been obtained from patients with chronic myelogenous leukemia (CML), as well as polycythemia vera (PV). A mean ($\pm$ SE) decrease in CFU-GM colonies of 77 $\pm$ 13% was noted in 11 of 14 CML while the decrease in 3 of 3 PV was 96 $\pm$ 7%. c-myb antisense oligomers had a minimal (<20%) to moderate (50%) effect on normal CFU-GM colony formation. In all CML responders tested,

bcr-abl mRNA, which encodes a protein thought to be important in the pathogenesis of CML, was found to be downregulated by corresponding c-myb antisense ODN. These results appear to substantiate our belief that antisense oligodeoxynucleotides my find a place in the armamentarium of antileukemia drugs.

3.2. In vitro experience in nonhematopoietic cell neoplasms

3.2.1. c-myb is a target of unexpected utility in maligant melanoma. As noted above, c-myb is located on chromosome 6q22–23 in humans. Structural aberrations in this chromosomal location have also been linked to some human melanomas. Accordingly, we noted with interest a report that suggested that altered c-myb expression might play a role in the pathogenesis of maligant melanoma [55]. Accordingly, we targeted the c-myb gene in human melanoma cells with antisense ODN to learn more about the biologic importance of its expression and the therapeutic potential of disrupting its function. To this end, we screened five human melanoma cell lines (Hs294T, SK-MEL-37, A375, C32, WM39) for c-myb mRNA by Northern analysis. Total RNA form each cell line (20 μg) was blotted to nitrocellulose and then probed with a ^{32}P-labeled human c-myb cDNA. None of the lines gave a positive signal. However, when a sensitive RT-PCR was employed, a technique that effectively amplifies small quantities of mRNA, c-myb mRNA was unambiguously detected in all. To determine the biological significance of this low-level c-myb expression in the melanoma cells, we targeted c-myb mRNA in SK-MEL-37 and Hs294T cells with unmodified or P-ODN, as well as control DNA sequences. In Hs294T cells, exposure to c-myb control sequences had no statistically significant effect on cell proliferation. In contrast, the c-myb antisense DNA inhibited growth in a dose-responsive manner up to about 60% ($p <$ .001) of control cell values. Growth inhibition was accompanied by loss of RT-PCR-detectable c-myb, but not β-action mRNA (whose levels are constant in all cells), suggesting that growth inhibition was secondary to pertubation of c-myb expression. Visual examination of the cultures revealed some clue regarding the inhibitory mechanism. Hs294T cells appeared to undergo cytolysis after exposure to the c-myb antisense, suggesting that c-myb perturbation could be a lethal event in these cells. This contrasted with the effect observed on SK-MEL-37 cells, which appeared to undergo growth arrest with or without what appeared morphologically to represent differentiation towards a more mature phenotype.

3.2.2. Relationship between DNA dose, frequency of exposure, and inhibition of cell growth. We also examined cell growth inhibition as a function of oligodeoxynucleotide (ODN) concentration and frequency of exposure. When SK-MEL-37 were exposed to ODNs, the most important factor for achieving growth inhibition was initial exposure to high concentrations of material. For example, at concentrations less than or equal to 50 μg/ml, no effect on cell

growth was observed when the ODNs were added to cultures in divided doses of 20 μg/ml/day × 5 days, or 10 μg/ml/day × 5 days. In contrast, when cells were exposed to a single bolus of 50 μg/ml, cell growth was inhibited by about 25% in comparison to untreated controls. This relationship was even more apparent at higher doses. A single total ODN dose of 100 μg/ml inhibited growth much more significantly than the same total dose delivered in divided doses of 20 μg/ml/day × 5 doses. Even at doses up to 250 μg/ml, 50 μg/day for 5 days was not as effective as a total dose of 200 μg given as 100 μg/ml/day × 2 doses (50% vs 70% inhibition, respectively).

To determine if these results were influenced by possible degradation of unmodified ODN, we carried out similar experiments with Hs294T cells exposed to the more stable phosphorothioate-modified antisense oligomers. A similar but less strict relationship between extracellular concentration and inhibition of cell growth was again observed. That is, initial high concentrations were more effective than equivalent final concentrations built up by cumulative lower doses. Accordingly, it appears that for either type of compound, sufficient cellular uptake to inhibit the target gene is only achieved by initial exposure to some critical 'high' concentration of compound. Whether this relationship would hold for leukemic cells has not been formally examined, but we have no reason to believe that this would not be true for these cell types as well.

3.3. In vivo treatment models

The experience gained with in vitro culture systems suggested, but did not prove, that in vivo activity with the c-myb-targeted antisense molecule might be expected as well. To test this, we developed SCID mouse human chimeras bearing either human leukemia [56] or human melanoma [48]. This animal system has the obvious advantage of allowing acitivity against a human tumor to be determined in a setting simulating actual clinical use.

3.3.1. In vivo treatment of human leukemia in an SCID mouse model. We established human leukemia–SCID mouse chimeras with K562 cells and treated diseased animals with phosphorothioate-modified antisense oligodeoxynucleotides [56]. K562 cells express the c-myb proto-oncogene, which served as the target for the antisense DNA. They also express the tumor-specific bcr/abl oncogene, which was utilized to track the human cells in the mouse host. Once animals had detectable circulating leukemic blast cells, the mean (±SD) survival of untreated control mice was 6 ± 3 days. The survival of animals treated for 7 or 14 days with either sense or scrambled sequence c-myb oligodeoxynucleotides was not statistically different from the control animals. In distinct contrast, animals treated for similar lengths of time with c-myb antisense oligodeoxynucleotides survived at least 3.5 times longer than the various control animals (figure 3). In addition, animals receiving c-myb antisense DNA had significantly less disease at the two sites most fre-

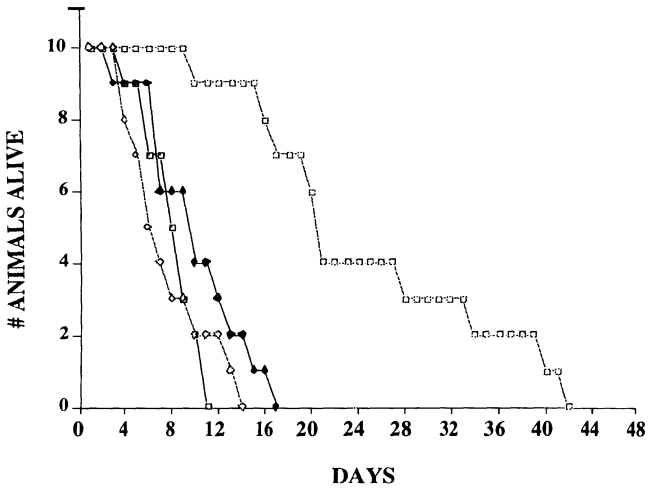

Figure 3. Survival curves of SCID–human chimeric animals transplanted with K562 chronic myelogenous leukemia cells. Animals received a 14-day infusion of oligomers at a dose of 100 μg/day. Legend: —□—, control; —•—, sense; —▪—, antisense; —◦—, scrambled. (Adapted from reference #56.)

quently manifesting leukemic cell infiltration, namely, the central nervous system and the ovary (figure 4). These results suggested that phosphorothioate-modified antisense DNA might be efficacious for the treatment of human leukemia in vivo and were an important component in justifying the clinical trials that have been activated at our institution.

3.3.2. In vivo treatment of human melanoma in an SCID mouse model — utility of c-myb as a target. To determine if the in vitro results discussed above were applicable in an in vivo system, we examined the effect of the c-myb antisense DNA on human melanoma cell growth in an SCID mouse model [48]. In the first of three types of experiments to assess this question, 41 mice were inoculated with Hs294T cells. When tumor nodules became palpable, animals were randomly assigned to receive no treatment (13 animals), or seven-day infusions (500 μg/day; 25 μg/g body weight) of c-myb sense (14 animals) or antisense (14 animals) phosphorothioate-modified oligodeoxynucleotides (P-ODN). Animals were examined daily for 40 days to determine the effects of the P-ODN on survival and tumor growth. The antisense P-ODA treatment significantly inhibited local tumor growth in comparison to the control and sense P-ODN-treated groups. In fact, until about day 35, calculated tumor weights in the antisense group were about 50% lower than the other groups. After this time, growth in the antisense-treated group recovered and essentially paralleled that seen in the control and sense-treated animals. Nevertheless, when the animals were sacrificed on day 40, tumors removed from the antisense-treated animals were significantly smaller ($p < .05$) than those in the control groups. The mean (±SD) weight of tumors taken from the animals in

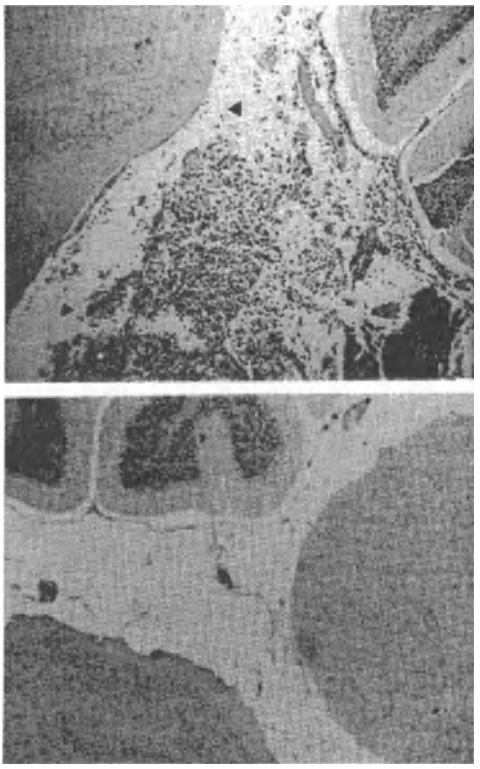

Figure 4. Composite photomicrographs (×400) of mouse brain obtained form chimeric SCID–human K562 leukemia-bearing mice treated with (A) sense, or (B) antisense phosphorothioate c-myb ODN. Note extensive meningeal and sub arachnoid infiltration with leukemic blasts in (A) and lack of involvement in (B). (Adapted from reference #56.)

the control, sense, and antisense groups was 3.5 ± 1.7 grams, 3.3 ± 1.2 grams, and 2.5 ± 0.5 grams, respectively.

We then examined the growth-inhibitory effects of the c-myb antisense P-ODN against a sub-clinical tumor burden. In this experiment, mice were subcutaneously innoculated with 2×10^6 Hs294T tumor cells. Three days later, animals were randomized to receive no treatment (9 mice) or a seven-day infusion (500 μg/day) of c-myb sense (8 mice) or antisense P-ODN (10 mice). Tumor growth was evaluated over a 65-day period. While no control animals were lost during this period, three sense and four antisense treated animals died of uncertain causes. In the remaining animals, inhibition of tumor growth in the antisense-treated group was again noted throughout the observation period and appeared to be greater than that observed in the first experiment. When sacrificed at 60 days after the pumps were implanted, mean (±SD) tumor weights of control, sense, and antisense groups were 4.5 ± 1.7 g, 4.0 ± 1.5 g, and 2.1 ± 1.2 g, respectively. The differences between these groups were

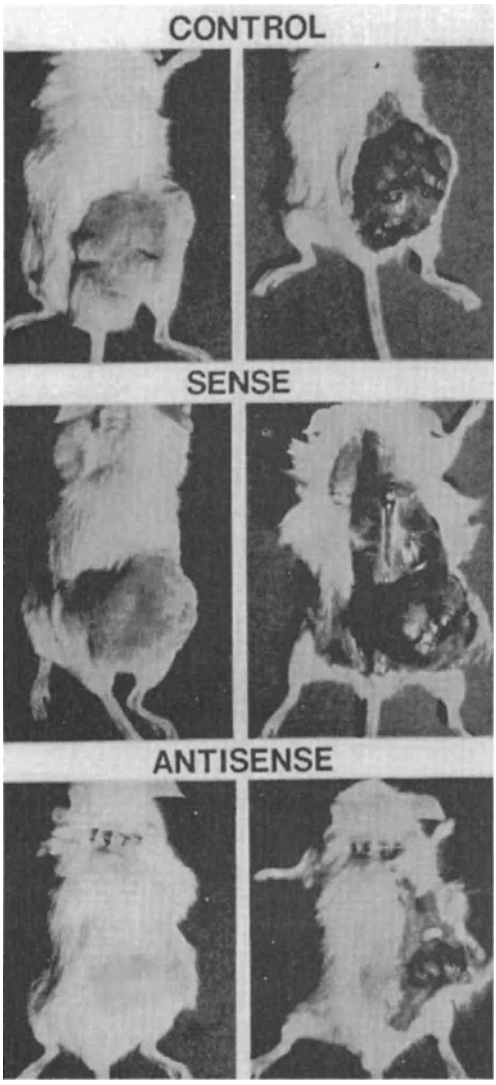

Figure 5. Representative photomicrographs of human melanomas in situ in animals treated with oligodeoxynucleotides targeted to the c-myb gene. Control and sense-treated animals are shown in the top and middle panels, in situ (left) and with the skin reflected to display the tumor (right). Corresponding views of animals treated with the antisense compound are shown in the bottom panel. (From reference #48.)

statistically significant ($p < .05$). Figure 5 illustrates typical tumors observed in these mice.

We lastly examined the effect of a repeat infusion on tumor growth. In this experiment, animals (10 per group) were again inoculated with 2×10^6 tumor cells. Three days later they were randomized to receive no treatment (control)

or an infusion of sense or antisense P-ODN (500 μg/day × 14 days). Sixteen days after the first infusion ended, a repeat infusion of identical dose and duration was begun. In this experiment, three control and one sense-treated animal died tumor-related deaths during the observation period. In the antisense-treated animals, tumor growth inhibition was more dramatic than in the previous experiments and persisted throughout the observation period. When animals were sacrificed 85 days after the first pump was implanted, mean ± SD of tumor weights of control ($n = 7$), sense ($n = 9$), and antisense ($n = 10$) groups were 3.0 ± 2.0 g, 1.7 ± 1.5 g, and 0.7 ± 0.5 g. The difference in tumor weights between the control and antisense-treated groups was highly significant ($p < .01$), as was the difference between the sense- and antisense-treated groups ($p < .05$). Though it appeared that tumor sizes differed between the control and sense-treated groups, the mean sizes were not of statistical significance ($p > .05$). It is important to note that in contrast to the experiments carried out with a lower total dose of P-ODN, none of the animals in the high dose, repeat infusion sense- or antisense-treated groups died before the experiment was terminated. These results suggest that toxicity of the P-ODN was an unlikely cause of animal deaths.

3.4. Pharmacokinetic/dynamic studies in the melanoma model

An important issue in all these studies is whether tumor responses can be shown to correlate with changes in the target gene (in this case, c-myb) expression. Towards this end, we first sought to satisfy ourselves that the oligomers were indeed entering the tumor tissue. To determine oligo uptake in tumor tissue and to correlate effects on c-myb expression with tumor growth, five animals with established tumors (~0.5 g) were infused with c-myb P-ODN (500 μg/day) for seven days. On days 7, 9, and 11 postinfusion, an animal was sacrificed and its tumor excised to determine tissue c-myb mRNA levels. As shown in figure 6, c-myb mRNA levels were measurably decreased (as normalized to β-actin), but not completely extinguished, in comparison to control expression. This decrement in c-myb expression persisted for approximately two days after the infusion finished but returned towards baseline thereafter.

Normalization of c-myb expression may have related to P-ODN concentration in tissue falling below a critical level. In support of this hypothesis, P-ODN levels in the tumor tissue decreased rapidly from levels estimated to be about 500 ng (per 50 μg extracted DNA) during the infusion to a level less than 50 ng, but greater than 10 ng (per 50 μg extracted DNA) on day 8, one day after the infusion finished. It should be pointed out, however, that while human c-myb is selectively suppressed by the antisense oligos, the PCR primers employed for detection will amplify both human and murine c-myb mRNA. Since murine blood and stromal elements gradually infiltrate the tumor, some of the c-myb mRNA detected could be contributed from this source. Regardless, it is clear that the downregulation of the target is transient, and this is expected.

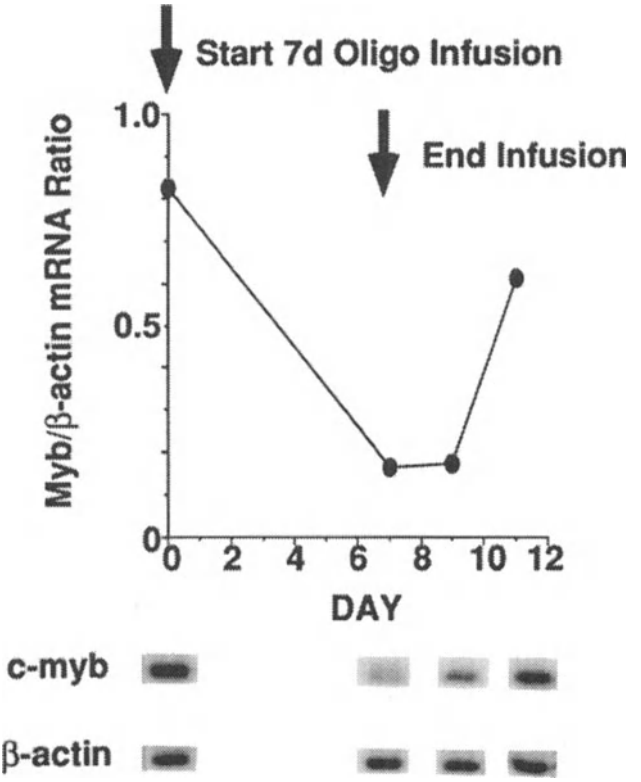

Figure 6. Effect of Myb-directed P-ODN on c-myb mRNA expression in human melanomas growing in SCID mice. P-ODNs were infused into mice (500 μg/day × 7 days) with approximately 1 g tumors. On days 7, 9, and 11, tumors were excised and total RNA was extracted for determination of c-myb mRNA levels. The relative amount of Myb mRNA was normalized to β-actin mRNA detected in the same sample. (From reference #48.)

An important goal of future studies will be to determine the length of time that target gene suppression is required for a useful clinical effect.

3.5. Initial experience with c-myb P-ODN as marrow purging agent

We recently initiated a clinical trial to evaluate the effectiveness of phosphorothioate-modified c-myb antisense ODN as a marrow purging agent in patients with chronic phase (CP) or accelerated phase (AP) CML [57]. Five patients have been treated thus far. Four did well, with engraftment (MNC >500) occurring between 11 and 18 days posttransplant. One patient failed to engraft with either purged or untreated back-up marrow and died on day +50 with pulmonary aspergillosis. Clinical and laboratory response, including cytogenetics and RT-PCR for bcr/abl gene expression, is being evaluated in all patients. Two CP patients and one AP patient have been more fully evaluated.

Both CP patients demonstrated pretransplant in vitro antisense ODN sensitivity, which was dose and sequence dependent. One CP patient (#1) also demonstrated unequivocal elimination of bcr/abl positive cells in the purged specimen, and was reconstituted with bcr/abl negative blood cells (figure 8). Patient #1 continued to manifest complete disappearance of the Ph+ chromosome and was bcr/abl negative by RT-PCR for several months posttransplant. Both CP patients remain in complete clinical and partial cytogenetic remission (~15% Ph^+ metaphases). The AP patient was unresponsive in vitro. Though in a clinical CR, his metaphases are 100% Ph^+. Though follow-up is short and patient numbers are small, we suggest that purging with phosphorothioate-modified c-myb antisense ODN is a safe and potentially useful procedure in CML patients without an allogeneic marrow donor.

4. Conclusions

The above discussion was meant to recapitulate how the c-myb gene was identified as a potential therapeutic target and how its utility was explored step wise using in vitro and in vivo models. A frequently asked question is, 'Why not target the bcr/abl gene with antisense DNA?' This target has the advantage of being tumor specific and is of clear importance in the pathogenesis of this disease. Nevertheless, recent studies suggest that the most primitive stem cells may not express bcr/abl mRNA [58]. Since CML is a stem cell disorder, bcr/abl mRNA may not be an appropriate target for these most primitive cells. Further, it is not certain that, once transformed, continuous expression of bcr/abl is required for maintenance of the malignant phenotype. Finally, since bcr/abl likely functions as a signal-transducing protein, and since the signaling apparatus in cells appear highly redundant, targeting bcr/abl may allow resistance to emerge. Other antisense targets, for example c-myb, may then turn out to be more effective in the long term.

It is straightforward that much of the translational aspects of this development are dependent on the 'antisense' approach to gene therapy. Though simple in theory and execution, antisense experiments may be far more difficult to conduct and interpret than the above discussion would indicate. The reasons for this are multifactorial but relate to an often frustrating inability to identify a suitable region of an mRNA to target, or to nonsequence-dependent effects on cell function. These problems in turn are likely to be related to mRNA secondary and tertiary structure and to the particular chemistry of the oligo employed, respectively. This does not mean, however, that carefully controlled experiments cannot be informative. Rather, the caveat here is not to confuse a biologic effect with an 'antisense effect,' though so-called nonspecific effects could obviously be advantageous if appropriately exploited. Whether c-myb will turn out to be a useful target in hematologic malignancies, or in the less obvious case of solid tumors, remains to be seen, but the question will be answered in the near future. If encouraging results are obtained, this

work will be all the more exciting, because at the same time that c-myb proves itself a useful target it will simultaneously open up a new approach to anti-cancer therapeutics.

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6. The role of p53 in malignancy

F.J. Keith and N.H. Russell

1. Introduction

Mutations in the p53 gene are currently the most common genetic lesion found in human cancers [1,2]. The p53 protein was initially discovered in coprecipitation studies with SV40 large T antigen [3], with which p53 forms a stable complex [4]. Initial studies linked mutations in p53 to malignant transformation and culture cell immortalization [5–7], and thus it was shown that the p53 gene was a tumor suppressor gene [8,9]. The p53 gene is expressed at low levels in normal cells of the body, with the highest levels being found in the testes, ovary, thymus, and spleen [10]. The levels of p53 protein rise due to an alteration in its half-life [11], in response to DNA damage by a variety of agents including irradiation, chemical carcinogens, and tumor viruses. The major function of these elevated levels of p53 protein is to prevent propogation of cells with damaged DNA and thus prevent the onset of cancer. It has been shown that wild-type p53 achieves this by causing G1 arrest of the cell cycle [12] and so allowing either repair of the damaged DNA or, in cases of high DNA damage, the onset of apoptosis [13]. Thus p53 acts as a 'guardian of the genome' [14] and is vital to maintenance of DNA integrity. It is therefore evident that mutations in such a crucial gene are disastrous for the cell, since further lesions in the genome are tolerated and the chance of malignant transformation increases [15]. As well as being a common event in cancer, mutations in p53 have been linked to aggressive disease, tumor progression, resistance to chemotherapy, and poor prognosis in a number of tumors [16–23]. This chapter aims to address the role of p53 mutations in cancers, with particular focus on the hematological malignancies, and discusses the clinical consequences of the loss of wild-type p53 function.

2. Structure of p53 gene and protein

The p53 gene is situated within 15–20kb of DNA on the short arm of chromosome 17 [24–26] at position 17p13.1 [27]. The gene has 11 exons, the first of which is noncoding and is situated 8–10kb upstream of exons 2–11. The

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remaining exons are spliced together to produce a 2.2–2.5 kb mRNA of 393 codons. The p53 gene is highly conserved across species, with 81% homology between the coding regions of human and murine DNA sequences [28]. There are five regions with more than 90% identity: I, codons 13–19; II, codons 117–142; III, codons 171–181; IV, codons 234–258; and V, codons 270–286 [29]. The main features of the coding region of the p53 gene and protein are illustrated in figure 1.

The p53 protein has also been conserved throughout evolution with 80% homology between murine and human proteins and 56% homology between Xenopus and human proteins [29]. The mapping of mutations to the sequence of the p53 protein has identified three main functional domains (see figure 1). The 40 acidic amino acids at the amino terminus of the protein function as a transcriptional activator in vitro [30]. The region from amino acid 91 to 301, which contains 86% of mutations and the highly conserved regions II–V [1,31], functions as a sequence-specific DNA binding domain [32]. The consensus sequence for the p53 specific binding site has been defined as 5′-Pu Pu Pu C A/T A/T G Py Py Py-3′ [33]. Several cellular proteins as well as the viral SV40 T antigen bind this central region of p53 [34]. In addition, two other cellular proteins that bind this region of wild-type but not mutant p53 have been identified [35]. The third functional region at the carboxyl terminus contains

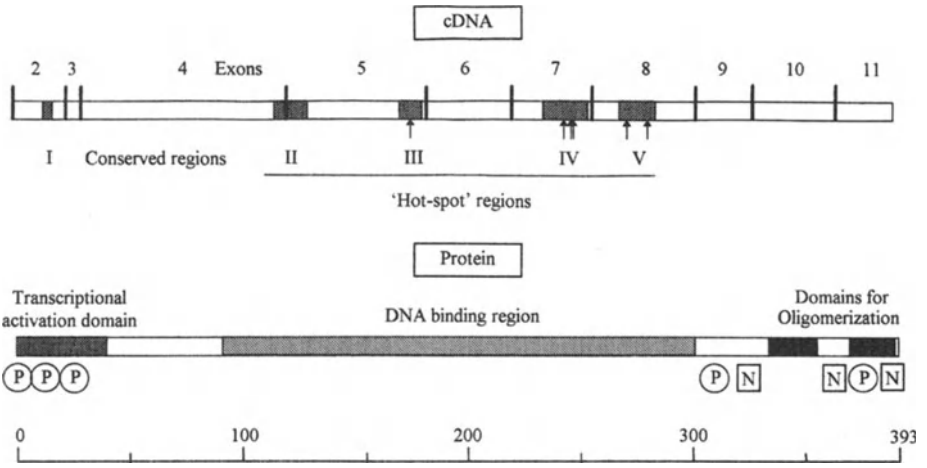

Figure 1. Structural and functional features of the human p53 gene and protein. The main features of the p53 gene and protein are indicated. The gene contains 11 exons, 10 of which encode for p53 and are illustrated spliced together. There are 5 highly conserved regions [29], four of which are the hot spot regions of gene mutations [1]. The six most common mutations are indicated by arrows: 175, 245, 248, 249, 273, and 282 [97,98]. The transcriptional activation domain, 1–40 [30], the DNA binding region, 91–301 [32], and the domains required for the formation of dimers, 334–356, or tetramers, 363–386 [146,147], of p53 protein are indicated. Phosphorylation sites for ds DNA protein kinase (PK) and casein kinase I PK are indicated at the N-terminus and those for cyclin-dependent kinase and casein kinase II are indicated at the C-terminus [148]. The nuclear localization signals [149] are also shown. A scale of codons or amino acids is shown below.

several functions, including a region required for the formation of tetramers [36], the nuclear localization signal necessary for p53 function [37], and a phosphorylation site for cyclin-dependent kinase [38] and casein kinase II. In addition, sites for phosphorylation by casein kinase I and double-stranded DNA protein kinase are situated at the amino terminus.

3. Function of p53 protein

Considerable information has emerged over the last few years concerning the function of wild-type p53 protein. The p53 protein is crucial for the regulated proliferation of cells in a number of different ways, having been implicated in the control of cell proliferation, the regulation of cell survival, and the regulation of differentiation. Introduction of wild-type p53 into cell lines that have lost endogenous p53 function can cause suppression of malignant transformation [8], defining wild-type p53 as a tumor suppressor gene. Subsequently in similar studies, expression of wild-type p53 was also shown to cause cell-cycle arrest [12] and apoptosis [13], as well as the suppression of cell proliferation. Thus regulation of cell proliferation and survival has emerged as an important function for wild-type p53 (figure 2). The p53 protein accumulates in the nucleus in response to DNA damage [39], resulting in cell-cycle arrest in G1 [12] and either DNA repair [39] or apoptosis [13]. Apoptosis or programmed cell death can be triggered in cells by a number of mechanisms, including withdrawal of trophic factors, radiation, cytotoxic drug exposure, and overexpression of viral or cellular proteins. Wild-type p53 has been implicated as an important mediator of apoptosis occurring via these diverse mechanisms.

Further insights into the function of p53 have been obtained from studies on p53 knock-out mice, which, though born healthy, develop a variety of tumors several months after birth [15], demonstrating that the protein does indeed have a tumor suppressor function. The fact that these mice develop normally in utero indicates that for the vast majority of cell divisions, normal p53 function is not critical. The answer to this apparent paradox lies in studies showing that high levels of p53 are induced by exposure of cells to agents that damage DNA [40] and that thymocytes from p53 knockout mice show remarkable resistance to apoptosis induced by irradiation [41,42]. These findings have led to the concept that the loss or mutational inactivation of p53 may allow the accumulation of diverse and potentially oncogenic mutations due to the replication of incompletely repaired DNA sequence in cells permitted to survive as a consequence of the loss of p53 triggered apoptosis (figure 2). These p53-deficient cells do not undergo the transient G1 arrest after irradiation exposure, where lesions in the DNA are normally repaired [43]. The loss of wild-type p53 function would therefore promote genomic instability and allow tumor development or progression as the consequence of the loss of wild-type p53-induced apoptosis. In the absence of p53, DNA-damaged cells are unable

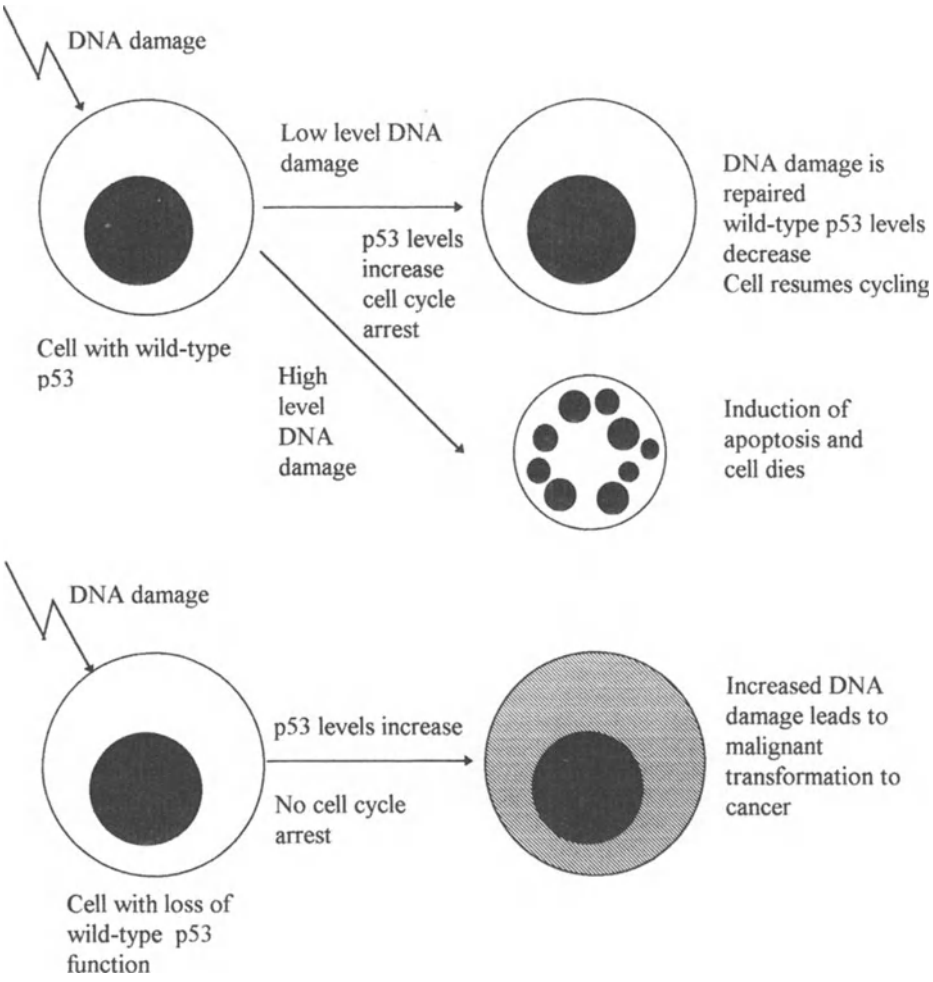

Figure 2. Model of p53 function. p53 is proposed to function as the 'guardian of the genome,' protecting the integrity of cellular DNA from damage [14]. Normal cells contain low levels of p53, which rise in response to DNA damage. Expression of wild-type p53 results in either cell-cycle arrest and repair of damaged DNA before resumption of cell cycling or the onset of apoptosis and suicide of the cell. Cells in which wild-type p53 function has been lost do not respond to further DNA damage and continue to cycle without repairing the damaged DNA, resulting in malignant transformation to cancer.

to trigger normal signals for apoptosis. The fact that many cytotoxic drugs including 5-fluorouracil, etoposide, and cytosine arabinoside have been shown to induce p53 to high levels [44] and to induce apoptosis via p53-dependent pathways [42,45] may explain the poor response to cytotoxic drug therapy that is characteristic of tumors with p53 mutations [16,45,46]. Of relevance here is the observation that even cells with one abnormal copy of the p53 gene exhibit

increased resistance to the induction of apoptosis compared to homozygous wild-type p53 cells [19].

3.1. Apoptosis, p53, and Bcl-2

Wild-type p53 functions to induce p53-dependent apoptosis in response to DNA damage. Normal hematopoetic cells from mice deficient for wild-type p53 were found to be more resistant to apoptosis induced by irradiation, heat shock, or low growth factor concentrations [19]. In addition, there was a dose–response relationship between the number of p53 wild-type alleles and the resistance to apoptosis. However, the resistance to apoptosis was not universal to all inducing agents, indicating that p53-independent apoptosis was still inducible. Apoptosis induced by wild-type p53 protein can be completely overcome by the expression of high levels of Bcl-2 [47–49]. When Bcl-2 was coexpressed with a temperature-sensitive p53 construct in a v-myc-induced murine T-cell lymphoma cell line, it was found that the cells were completely protected from apoptosis induced by the wild-type p53 conformation expressed at 32°C. Therefore, the growth-arresting signal of p53 is upstream of the apoptosis protection role of Bcl-2 [47]. In addition, the expression of human Bcl-2 in rat cells transformed with E1A also blocks apoptosis induced by wild-type p53 [48]. Cells transformed with E1A and the p53 (Val135) temperature-sensitive mutant were transfected to express the human Bcl-2 gene. At the permissive temperature where p53 was in the wild-type conformation, the cells did not apoptose and remained in a reversible growth-arrested state. The G1 specificity of the p53-dependent cell-cycle arrest was lost in the cells overexpressing Bcl-2 [48,49]. Thus it can be concluded that the function of p53 can be modified from inducer of apoptosis to inducer of growth arrest by the expression of Bcl-2. The action of Bcl-2 on p53 function is partially explained by experiments involving Bcl-2 and c-myc cotransfectants that resulted in the loss of nuclear translocation of the wild-type p53 protein during G1 of the cell cycle [49].

However, the interaction between p53 and Bcl-2 is complex. It has been shown that wild-type p53 can inhibit the expression of endogenous Bcl-2 [50]. A temperature-sensitive mutant of p53 was used to show that the expression of endogenous Bcl-2 decreased in the presence of wild-type p53 at the permissive temperature in a murine myeloblastic cell line. There was a concomitant increase in the expression of Bax, a dominant inhibitor protein of Bcl-2. The p53-dependent increase in expression of Bax has identified Bax as a p53 immediate early response gene [51]. This response is not found with all apoptotic inducers and may account for the more rapid kinetics of apoptosis found in response to p53 and the higher levels of Bcl-2 needed to fully inhibit p53-dependent apoptosis [51]. Mice deficient for p53 have an increased expression of Bcl-2 and decreased levels of Bax protein in tissues that normally express Bcl-2 [50]. In contrast, the expression of Bcl-2 in breast cancer cells

was shown to decrease in cells expressing mutant p53 [52]. However, further evidence for the regulation of Bcl-2 by p53 was provided by the identification of a functional p53-dependent negative response element in the bcl-2 gene [53]. In the light of these results, it has been predicted that the loss of p53 observed in many human tumors may be associated with increased expression of Bcl-2 and decreased expression of Bax [53]. However, in breast cancer cell lines, expression of mutant p53 and Bcl-2 were inversely correlated [52]. In conclusion, though, the importance of the regulation of Bcl-2 and Bax by p53 is underlined by the experiments of Wang and Chiou [47,48], which showed that apoptosis induced by p53 could be prevented by the overexpression of Bcl-2.

3.2. DNA binding and transcription factor

A major clue to the mechanism whereby p53 can inhibit cell proliferation, causing G1 arrest and allowing the cell to undergo either DNA repair or apoptosis, has recently been defined. P53 is known to be a DNA binding protein [54], a function that is lost by most mutant p53 proteins. In addition, the p53 protein activates transcription in vitro of genes containing a p53 response element [55], including the epidermal growth factor receptor [56]. However, a response element is not essential for regulation of transcription by p53 [57]. Several genes have been identified a transcriptionally regulated by p53, including c-fos, c-jun [58], mdm-2 [59–61], the apoptosis resistance gene bcl-2 [53], GADD-45, a DNA repair gene [43], and the cell-cycle control gene WAF1/CIP1/p21 [62]. WAF1 (wild-type p53 activated fragment) is a wild-type p53 inducible gene expressed in cells undergoing p53-dependent G1 arrest or during apoptosis in response to DNA damaging agents [63]. The encoded 21-kDa nuclear protein associates with several cyclin-dependent kinases (cdk) inhibiting their activity [62,64] and consequently cell-cycle progression. Thus cells deficient in wild-type p53 do not induce expression of active WAF1 after treatment with DNA-damaging agents, and so prevent G1 arrest and DNA repair [63]. Therefore, it appears that WAF1/CIP1 is a crucial downstream effector of p53 function. One of the other genes known to be regulated by wild-type p53 is the mdm-2 gene [65]. The levels of mdm-2 mRNA increase in response to wild-type p53 due to sequence-specific binding of p53 to DNA downstream of the mdm-2 gene and subsequent elevated transcription of the mdm-2 gene [65]. In addition, there is evidence for a further internal promoter within the mdm-2 gene that is recognized by p53 [65]. The mdm-2 protein is known to bind to p53 in either the wild-type or mutant conformation, inactivating the wild-type growth suppression activity of p53 [59]. Thus since mdm-2 is positively regulated by wild-type p53, which it can then bind and inactivate, the normal function of mdm-2 within cells may be to negatively regulate the activity of wild-type p53 [66], a function required for cell-cycle progression. The binding site for mdm-2 has been localized to amino acids 18–23 of p53 [67]. Deletion of this region abolishes interaction of p53 with mdm-2 without

loss of p53 transcriptional activation activity, indicating that mdm-2 is not involved with this aspect of wild-type p53 activity [68].

4. p53 function in normal and leukemic hematopoeisis

The wild-type p53 is an allosteric protein that has been demonstrated to exist in two conformations, recognized by different antibodies, as differential exposure of domains occurs in these conformational states [69,70]. The monoclonal antibody pAb1620 immunoprecipitates the human wild-type protein in the suppressor conformation [71], and the pAb240 recognizes wild-type p53 in the promotor or the mutant conformation [69,71] (figure 3). Conformational change of p53 appears to occur as part of cell-cycle activity, as exemplified by

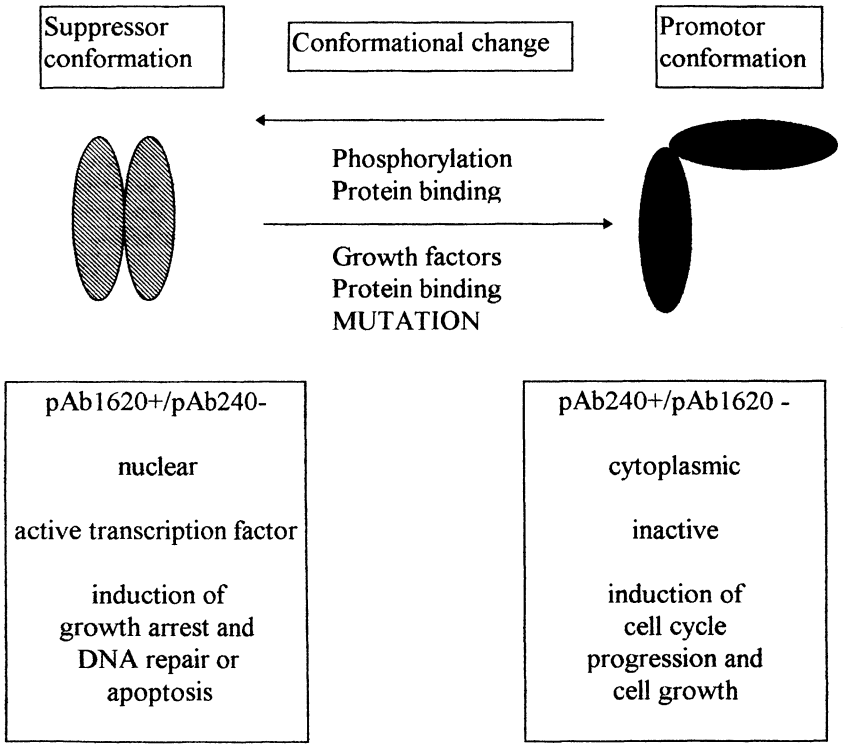

Figure 3. Conformation of p53. Wild-type p53 can exist in two conformations, suppressor and promoter, as defined by function and reactivity with monoclonal antibodies pAb1620 and pAb240 [150]. A summary of the properties and consequences of both conformations is listed. A conformational shift from promoter to suppressor can occur, possibly by phosphorylation or protein binding, leading to activation of p53 and cell growth arrest. Alternatively a conformational change in the opposite direction can occur by growth factors [72,78,81] and possibly protein binding. In addition mutation of p53 results in alteration of the conformation of p53 towards the promoter phenotype such that there is a loss of functional suppressor activity [69].

studies showing that following the addition of fresh medium to fibroblasts, the suppressor conformation was lost [72]. Also, the addition of mitogen to lymphocytes resulted in the expression of wild-type p53 with the mutant conformation (pAb240 positive) [73]. These data suggest that the tertiary structure of p53 is flexible and that the conformation associated with the suppressor function is modulated by factors that operate following growth factor stimulation. This is further demonstrated by the isolation of both murine and human p53 mutants, which are at least partially temperature sensitive for conformation between 30 and 37°C [74–76]. Zhang [76] characterized a temperature-sensitive human p53 mutant (p53-Ala143) exhibiting a conformation at 32.5°C recognized by pAB1620 and able to bind DNA, but at 37°C the protein was not detectable by pAb1620.

The conformation of wild-type p53 has been studied in human hematopoetic cells. Whereas light-density mononuclear cells expressed negligible amounts of p53, purified myeloblasts expressed p53 recognized by pAb240 in the mutant conformation [77], whereas wild-type p53 in the suppressor conformation could not be detected. Similar findings were reported by Zhang et al. [78], who found that 32 out of 37 AML samples expressed p53 in the mutant conformation whereas only three patients had point mutations of the p53 gene as detected by SSCP. These findings were extended by Zhu et al. [79], who demonstrated that the conformation of p53 in AML blasts was regulated by both exogenous and autocrine growth factors. AML blasts that secreted autocrine granulocyte-macrophage colony-stimulating factor maintained p53 in the mutant pAb240 positive conformation. Furthermore, deprivation of growth factors in factor-dependent leukemic cells resulted in a decrease of pAb240 expression and increased expression of pAb1620, which was associated with the onset of apoptosis, a process that could be prevented by the complete suppression of p53 expression by antisense oligonucleotides [79]. These data suggest that in AML blasts, wild-type p53 is maintained in the mutant conformation by either exogenous or autocrine growth factors. P53 in this conformation acts to acts to promote leukemic cell proliferation, to suppress apoptosis, and to promote the survival of the leukemic clone; therefore, despite the lack of mutation, the normal function of wild-type p53 is lost as the wild-type protein is locked in a mutant conformation. Interestingly, AML cells with the loss of wild-type p53 conformation (pAb240+) not only exhibit autonomous growth in culture but also express high levels of bcl-2 [80,81], which may in part be related to the loss of wild-type p53-induced suppression of bcl-2 expression [50].

The actual mechanism underlying the conformational change in p53 is not understood. One possible mechanism would be the induction, by growth factors, of proteins that bind to p53 and alter its conformation (figure 3). A number of cellular proteins are known to bind to wild-type p53 and inhibit its tumor suppressor activity; these include products of several tumor virus proteins including SV40 and papillomaviruses [82,83]. The mdm-2 protein, which binds to both wild-type and mutant p53, can also overcome the growth-

suppressive activity of p53 [84]. Recently, the mdm-2 mRNA has been reported to be overexpressed in over 50% of cases of various leukemias [85]. Other proteins may have a similar effect; for instance, c-abl protein has also been reported to bind to wild-type p53 and may arrest growth in a p53-dependent fashion [86]. Such protein–protein interactions could abolish wild-type p53 function and stabilize the mutant conformation, thereby contributing to leukemogenesis. These results suggest that a strategy to restore normal wild-type p53 function may be of therapeutic value in AML and other leukemias.

4.1. P53 and hematopoetic differentiation

Endogenous p53 also has a role in the regulation of cell maturation and differentiation in the hematopoetic system. The levels of endogenous p53 were found to be increased in normal mature lymphoid, granulocytic, and monocytic cells compared to the bone marrow progenitor cells, which did not express p53. Induction of maturation of a myeloblastic leukemia cell line also resulted in increased expression of p53 [40]. In addition, the constitutive expression of wild-type p53 in HL-60 promyelocytic leukemia cells induced differentiation of the cells along the granulocytic pathway [40]. Thus endogenous p53 is involved in hematopoetic cell maturation. A possible function for p53 is to arrest cell cycling during terminal differentiation of hematopoetic cells.

5. P53 mutations

P53 inactivation in human tumors is most frequently due to point mutations and loss of the remaining allele. The majority of somatic mutations cluster in exons 5–9, coding for evolutionary highly conserved domains [29].

5.1. Methods of detection of p53 mutations

Several techniques have been employed to detect p53 mutations in cancers. Southern blotting was the original method used to screen for mutations; however, this method is not very sensitive to point mutations and can generally only detect larger gene lesions.

A simple and rapid method for screening for p53 mutations used in many clinical laboratories is immunohistochemistry. The wild-type p53 protein has a half-life of 10–20 minutes in normal cells and so does not normally accumulate to levels detectable by immunohistochemistry of tissue sections. However, the mutated p53 protein has a longer half-life and can be detected by antibodies. Thus a positive result for detection of p53 by immunohistochemistry in solid tumors is indicative of a p53 mutation [1,87]. However, false-positive results can be obtained if wild-type p53 is stabilized by binding of cellular or viral

proteins, e.g., SV40 large-T antigen, resulting in an increased half-life and detection by immunohistochemistry. Also, false negatives may occur in the presence of chain-terminating mutations, leading to absent or reduced levels of an unstable protein. In addition, this technique does not reveal any information about the nature of the mutation involved.

The advent of PCR (polymerase chain reaction) technology has established PCR-SSCP (single-strand conformation polymorphism) as the most effective method for screening for the point mutations common in p53 [88,89]. This method takes advantage of the differing behavior during gel electrophoresis of a 100–400-bp region of amplified DNA containing a mutation compared to the corresponding wild-type region. However, mutations altering the single-stranded conformation do not necessarily result in an altered amino acid sequence. Therefore, the region shown to be positive by PCR-SSCP must be sequenced to identify if the encoded protein is functionally mutant. In cases of conservative amino acid substitution, the activity of p53 is not altered [90,91]. In addition, alternative splicing of p53 RNA in both normal and cancer cells has been observed [92]. The PCR-SSCP technique is sensitive and can still detect a mutation at a frequency of 10% [2]. The analysis of large numbers of samples for p53 mutations has been facilitated by the increasing availability and low running cost of automated DNA sequencers coupled with the specificity and sensitivity of PCR-SCCP. Comparison of the efficiency of p53 mutation detection by DNA analysis by PCR SSCP and direct sequencing and by immunocytochemistry in hematological malignancies revealed that the two methods gave concordant results in 90% of samples [93]. Rare cases may be negative by immunohistochemistry due to a nonsense mutation giving rise to a truncated p53 protein. Thus immunocytochemistry appears to be an equally sensitive method for screening for p53 mutations as PCR-SSCP in the majority of cases. It is important to point out that the majority of studies using PCR-SSCP [1,2,29,94] only screen for mutations within the identified hot-spots of the p53 gene; thus data are biased for mutations within these regions, and a number of false negative results may exist [95]. Another method used to detect point mutations in the p53 gene is denaturing gel electrophoresis [96]. This method has the advantage over PCR-SSCP in that it analyzes the whole gene, enabling the identification of polymorphisms within introns and exons throughout the gene.

5.2. The significance of p53 point mutations

Some 68% of mutations in the p53 gene are found within four of the highly conserved regions, namely, II, III, IV, and V, between codons 117 and 286 [31]. These conserved regions lie within exons 4, 5, 7, and 8 and form the core domain of the protein. The majority of mutations are missense, resulting in the incorporation of an alternative amino acid at the mutated codon and the production of an inactive p53 protein [1]. Comprehensive analysis of muta-

tions has revealed six hotspots at codons 175, 245, 248, 249, 273, and 282 ([97]; see [98] for review) within the DNA binding domain of p53 that account for 30% of all p53 mutations found in tumors. The disruption in ability of the resultant mutant p53 proteins to bind to DNA and activate transcription is dependent on the particular point mutation involved [99–101]. The 248 and 281 mutants could no longer bind to the p53 consensus sequence, but the 175 and 273 mutants maintained DNA binding activity, and the 273 mutant also maintained an intact transcriptional activation activity [99] and wild-type conformation [101]. However, the 248, 281, and 175 mutants have been found to either increase or decrease the transcriptional activity of wild-type p53 depending on the p53 DNA binding site present [101,102]. Also, the 248 mutant had a wild-type conformation as defined by antibody recognition [101]. The clustering of tumor-derived mutations within hot spots in the highly conserved regions of p53 underlines the importance of this region for p53 function, and insights into the features critical for p53 function have been gained by the elucidation of the protein structure (comprehensively reviewed in [98]).

6. Incidence of p53 mutations in nonhematological malignancies

Mutations in p53 are now the most common lesion found in human cancer [1]. The incidence of a mutated p53 is 70% in colorectal cancer, 50% in lung cancer, and 30% in breast cancer [2]. The majority of these mutations are found within the conserved DNA binding domain of p53. It has been observed that the frequency distribution of mutations found within this region is tumor tissue specific (see [31] for a review). In liver cancer, the predominant mutation is at 249, whereas in breast cancer the most common mutations are found at 174 and 273. This difference may reflect both tissue-specific lesions and environmental mutagen specificity. In some cases, specific p53 mutations have been linked to environmental carcinogens. A G to T transversion at codon 249 has been linked to aflatoxin B1 exposure during peanut cultivation in patients with liver cancer in the Qidong provence of China [103]. The incidence of this transversion is reduced in areas where aflatoxin B1 has been irradicated. Aflatoxin B1 is a potent chemical carcinogen known to cause G to T transversions in vitro. In lung cancer, 31% ($n = 52$) of uranium miners studied had the same transversion of G to T at codon 249 [104]. This mutation is rare and had previously been reported at a frequency of 0.4% in lung cancers. This specific mutation was linked to high levels of exposure to radon gas during the miners' careers. Radon is a source of alpha particles, which are known to cause mutations in DNA. In lung cancers in general, there is a predominance of transversion mutations, particularly at 157, which may be a result of the specific carcinogens present in cigarettes. However, in colon cancers the majority of lesions are transition mutations. These data indicate that mutations in

p53, the most common cancer lesion, can be caused by specific environmental carcinogens that exhibit both a tissue and mutagen specificity.

6.1. Role of p53 mutations in tumor progression

Mutations in p53 have been linked to tumor progression in several tumor types, including breast carcinomas [23,105,106], oral malignancies [107], and gastric carcinomas [108]. In gastric and colorectal carcinoma, the proportion of p53 abnormalities as detected by immunohistochemical staining was found to correlate with both disease progression and poor prognosis [108], indicating that the role of p53 in disease progression is an important prognostic indicator. However, in contrast, an extensive immunohistochemical study of lung cancer involving 125 primary tumors revealed that there was no difference in survival between p53-positive and p53-negative groups [109]. Thus the importance of the role of p53 in malignancy may be tumor tissue specific.

6.2. Association of p53 mutations with 17p abnormalities

Research into mutations in colorectal cancers resulted in early evidence for the proposal that p53 was a tumor suppressor gene [110]. The deletion of one p53 allele was accompanied by mutation of the other allele in colorectal cancers, indicating that p53 was a tumor suppressor rather than an oncogene as had originally been proposed. Analysis of breast, lung, brain, and colon tumors with deletions of 17p revealed that allelic deletion of p53 accompanied by mutation of the remaining p53 allele was a common occurence in malignancy [94]. Studies on squamous cell carcinomas revealed concurrent results [111]. In addition, it was found that point mutations occured in p53 without deletion of the other allele, indicating that 17p deletion was not necessarily the primary p53 lesion [94].

6.3. Temperature-sensitive mutations of p53

Several of the mutations identified in p53 are temperature sensitive. At 32°C the encoded mutant protein behaves as wild-type p53 but at 37°C it functions as a mutant. Thus at 32°C the p53 protein is reactive with pAb1620 and is located in the nucleus, whereas at 37°C it is reactive with pAb240 and is located mainly in the cytoplasm [112]. These mutants have been used extensively to probe the function of p53 and the role of the mutant and wild-type conformational states. A recently discovered temperature-sensitive p53 mutation is at codon 143Ala [76]. The mutant protein cannot bind DNA or activate transcription of downstream genes at the higher temperature. At the lower temperature, the protein is recognized by the monoclonal antibody PAb1620, which recognizes the wild-type p53 conformation; however, this specificity is lost at the higher temperature. Thus the temperature-sensitive mutant p53

protein alters conformation and function from wild-type to mutant upon an increase in temperature from 32°C to 37°C.

7. P53 mutations in hematological malignancies

The role of p53 and the incidence and clinical correlations of p53 mutations has been widely studied in hematological malignancies and is discussed in the following sections.

7.1. Myeloid malignancies

7.1.1. Acute myeloblastic leukemia (AML). Frequent p53 abnormalites have been reported in cell lines derived from AML patients. The HL-60 cell line exhibits a gross attenuation in the p53 gene, and 9 out of 10 myeloid leukemia cell lines studied by Sugimoto et al. [113] also exhibited p53 mutations as analyzed by RT-PCR SSCP, suggesting that the inactivation of the p53 gene may play an important role in the establishment of these cell lines. Although frequent in AML cell lines, the mutational frequency of p53 in AML cells from patients is much lower. Using RT-PCR and SSCP analysis, Sugimoto et al. [114] found no p53 mutations in six AML samples studied. Also, Fenaux et al. [115] found only one patient out of 36 who had a mutation of the p53 gene in exons 5 to 8. However, in the latter study, 4 out of 10 patients with 17p monosomy showed a point mutation or single-nucleotide deletion or insertion in exons 7 or 8. Overall, these and other studies [23,116,117] indicate a frequency of p53 mutations in AML of 5%–10%, which is much lower than that reported for solid tumors. However, these findings do not necessarily mean that mutation of p53 or abnormalities of p53 function may not be important in AML. Indeed, evidence to the contrary has recently come from a number of sources. Firstly, AML associated with p53 mutations has a very poor prognosis [16], with a median survival of 2.5 months compared to 15 months for nonmutated cases. In this study, AML patients with a p53 mutation tended to be older and to have a higher incidence of complex and unfavorable cytogenetic abnormalities; in addition, there was an absence of cases with favorable cytogenetics (t(15;17), t(8;21) inv(16)). The demonstration that murine hematopoetic cells deficient in wild-type p53 are relatively resistant to the induction of apoptosis following exposure to irradiation [19] and the topoisomerase inhibitor etoposide [42], and that p53-deficient mouse embryonic fibroblasts were dramatically resistant to a number of cytotoxic drugs, including etoposide and adriamycin [45], provides an explanation for these findings. Thus the loss of wild-type p53 function by mutation would be permissive for leukemic cell survival as the result of increased resistance to apoptosis induced by cytotoxic drugs. Also, as has been recently established in T-ALL [118], leukemia relapse in AML might occur as the consequence of p53 mutant clones that are present at diagnosis but that are below the sensitivity of

detection by SSCP and survive remission induction and consolidation chemotherapy. Evidence for this possibility comes from the studies of Wada et al. [119], who found p53 mutations in six cell lines derived from relapsed leukemia samples; however, in only three cases was the mutation present in the presenting samples by SSCP. Following sequencing of the p53 mutations and the generation of allele-specific oligonucleotide primers for PCR, the presence of mutant p53 cells in the presentation samples could be detected in a clone of less than 1% of cells. Also in another study, p53 mutations that were not detectable at presentation have been detected in patients at relapse [120]. Further studies are needed to define the possibility that a minority subclone of p53 mutant cells may be an important cause of leukemic recurrence in AML.

7.1.2. Chronic myeloid leukemia (CML). CML in the chronic phase is rarely if at all associated with p53 mutations [121]. In contrast, between 20% and 30% of cells from CML in accelerated phase or blast crisis have p53 mutations [121,122]. A variety of abnormalities have been reported, including major DNA rearrangements as well as a variety of point mutations including missense or nonsense mutations, frameshift, and splicing mutations. Blast crisis associated with p53 mutations is almost always myeloid and rarely lymphoid. Mutations may be homozygous or hemizygous and may be accompanied by loss of the short arm of chromosome 17, typically as the result of the formation of an isochromosome 17q. The frequency of p53 mutations in these cases rises to about 40%. Thus loss of one allele and mutation in the remaining allele is common in advanced CML and suggests that the complete loss of normal p53 function may be an important mechanism responsible for the transition from the chronic phase to the accelerated phase or the blast crisis. The role of the wild-type p53 protein in chronic-phase CML is intriguing, particularly since CML cells are relatively resistant to apoptosis compared to normal bone marrow cells [123].

7.1.3. Myelodysplasia (MDS). The p53 gene is mutated in about 5%–10% of MDS cases [124], generally missense mutations affecting exons 4 through to 8. In MDS the frequency of mutations is highest in patients with advanced disease (RAEB, RAEBt) [125], and as is the case for CML and AML, the frequency of mutations is highest for patients with abnormalities of chromosome 17. As in AML, the presence of a p53 mutation in MDS is associated with complex cytogenetic abnormalities [125] and with a poor response rate to intensive chemotherapy and a significantly reduced overall survival [16]. A role for p53 in the regulation of mdr-1 gene expression has been suggested following cotransfection experiments showing that mutant but not wild-type p53 could specifically activate the mdr-1 promoter [126]. In a recent study, however, no correlation was found between mdr-1 expression and p53 muta-

tion in myelodysplasia [127], suggesting that poor response to therapy associated with p53 mutations was not due to over-expression of mdr-1. There has also been no firm evidence to support a role for p53 mutations in the transformation of MDS to acute leukemia [128].

7.2. Lymphoid malignancies

7.2.1. Acute lymphoblastic leukemia (ALL). An early study revealed 80% (4 of 5) patients with ALL had immunoprecipitatable p53 [129]. However, analysis of patients with ALL by PCR-SSCP revealed a mutation frequency of 10% (2 of 21) [93], and none of the 21 ALL patients analyzed by denaturing gradient gel electrophoresis (DGGE) were shown to have a mutation [96]. It is possible that these differences are due to the samples included in the analysis. In a recent analysis of a large series of primary ALL, the frequencies of mutation were found to be 3% for common-ALL, but much higher (50%) for L3-type (Burkitts) ALL [118]. Studies in T-ALL have shown a very low incidence of mutations at presentation but a much higher incidence (30%) upon relapse [17]. Patients with p53 mutation at relapse were associated with a shortened duration of first remission, despite the mutation being undetectable at this stage. Also, the presence of p53 mutations at relapse was associated with a poor response to reinduction therapy [18]. In this study, analysis of presentation samples revealed an absence of p53 mutations in all but one patient, with a p53 mutation at relapse suggesting that either the p53 mutation was acquired after initial therapy or that a subclone with the mutation was present at diagnosis but remained undetected by PCR-SSCP. It is reasonable to suggest that the presence of the p53 mutation must be important in the ability of these cells to selectively survive chemotherapy and to mediate disease recurrence.

7.2.2. Lymphoma. PCR-SSCP analysis of high-grade B-cell non-Hodgkins lymphomas (NHL) has revealed an approximate 20% incidence of p53 mutations [130], with a very high incidence of mutations in diffuse large cell lymphoma cell lines [131], which may represent particularly aggressive tumors. In addition, mutations in p53 accompanied by the loss of the wild-type allele have been linked to disease progression in vitro in NHL [132]. In a detailed study, Kocialkowski et al. [95] examined the presence of p53 mutations by both PCR and by immunostaining in 22 high-grade NHL samples. Unlike the procedures in other studies, here the authors sequenced DNA from the entire open reading frame of the gene rather than only the 'hot spots' in exons 5–8. Mutations were found in 10 of 22 cases (45%), including three outside exons 5–8. This study shows that the incidence of p53 mutations previously reported in high-grade NHL may be an underestimate, and it is possible that these findings may have relevance to other hematological malig-

nancies. In most other studies, the analysis has been confined to the exons containing the 'hot-spots' for point mutation.

In contrast to high-grade NHL, p53 mutations are rare in follicular lymphoma [131,133]. However, cell lines bearing the t(14;18) translocation not infrequently exhibit p53 mutations [131]. This suggests that, as in other hematological malignancies, p53 mutations are associated with aggressive disease and disease progression. Further support for this conclusion comes from studies demonstrating that in about a third of cases, histological transformation of follicular lymphoma to a high-grade lymphoma was associated with the acquisition of a p53 mutation [20]. In a second study, 4 out of 5 cases that on biopsy exhibited histological evidence of transformation to high-grade lymphoma expressed mutant p53 [134]. Patients who exhibited clinical features suggestive of progression but who did not have histological evidence for this were negative for p53 mutations. In the study by Sander et al. [20], overexpression of p53 could be detected by immunocytochemistry in all cases that were positive for mutations by SSCP. Indeed, p53 positive cells by immunocytochemistry were present before evidence of histological transformation, suggesting that the routine sequential screening of biopsy material by immunocytochemistry for p53 may help identify patients at risk of transformation who may therefore be candidates for aggressive therapy.

Immunohistochemical studies in Hodgkins disease have revealed a high incidence of positive staining for p53 in Reed–Sternberg cells but not in surrounding lymphocytes [135]. Also, p53 mutations have been detected by PCR in Hodgkins cell lines [136] and in individually selected Reed–Sternberg cells from lymph nodes [137]. The clinical significance of these findings is as yet unknown, and it is also not known whether the positive staining for p53 in Reed–Sternberg cells always represents a mutation.

7.2.3. Multiple myeloma. P53 mutations are common in myeloma cell lines [138]. However, analysis of p53 mutations in 52 patients by PCR-SSCP revealed point mutations in only seven patients (13%) [21]. The presence of mutant p53 was specifically associated with advanced or aggressive disease and plasma cell leukemia. No mutations were observed in patients with indolent or chronic stable myeloma. Interestingly, three patients were observed who had developed p53 mutations associated with disease progression but who were negative when tested during the initial indolent phase of the disease. Similar results were obtained by Corradini et al. [139], who detected p53 mutations in 22% of patients with plasma cell leukemia. These findings in myeloma suggest that alterations in the p53 gene are associated with disease progression rather than initiation and help define a group of patients with a poor prognosis and a poor response to chemotherapy [21].

7.2.4. Chronic lymphatic leukemia (CLL). Analysis of patients with CLL by PCR-SSCP revealed a frequency of about 10%, mostly in patients with advanced disease (stage B, C) [16,93]. p53 mutations also appear more frequent

in Richter's syndrome, which represents the evolution of CLL to a more advanced and clinically aggressive form with a poor prognosis [16,140]. As in other hematological malignancies, CLL patients with a p53 mutation were found to have a poor response to chemotherapy [16,22].

8. Conclusions

From this chapter, it is clear that the studies of p53 undertaken in diverse hematological malignancies share several recurrent themes. Firstly, mutation of the p53 gene is usually accompanied by loss of the remaining wild-type allele [16,115,133,141,142] and hence loss of wild-type p53 function and is relatively rare in hematopoetic tumours during the chronic phase of the disease. This is best illustrated by chronic phase CML and by follicular lymphoma, where p53 mutations are very rare and if found are quite probably the harbingers of transformation [20]. Also, the presence of a p53 mutation in other hematological malignancies, including multiple myeloma, myelodysplasia, and CLL, is associated with transformation to a clinically more aggressive disease [21,125,140]. Indeed, in several well-documented cases in myeloma and in follicular lymphoma, disease progression to a histologically and clinically aggressive phase has been associated with the acquisition of a p53 mutation that was not detectable at presentation [20,21]. Thus in hematological malignancies associated with a biphasic course, as best illustrated by CML, p53 mutations are classically associated with disease progression and are frequently accompanied by the presence of other variable and frequently complex cytogenetic abnormalities [16]. The critical event occurring in tumors with p53 mutations is now seen not as resulting from the acquisition of a mutant protein but rather from the loss of wild-type p53 function. Wild-type p53, induced by DNA damage, acts to induce G1 cell-cycle arrest mediated by WAF1 expression and thus either allows DNA repair or, if DNA damage is severe, can induce apoptotic cell death. The loss of wild-type p53 function by mutation disrupts this process, preventing cell-cycle arrest and allowing the survival and replication of cells with damaged DNA. Thus there is an accumulation of chromosomal abnormalities characteristic of disease progression. Why p53 itself is the target of mutation at such a high frequency, resulting in the transformation of the chronic phase into high-grade aggressive disease, is not fully explained. One possible explanation is related to the fact that the wild-type p53 gene, which is itself induced by many DNA-damaging agents including alkylating agents, etoposide, anthracyclines, and irradiation, may be mutated as a consequence of the cytotoxic drug therapy used to treat the chronic phase of the disease. The recent demonstration that in CML, the transformation to blast cell crisis is delayed in patients treated with hydroxyurea rather than the alkylating agent bisulphan during the chronic phase may support this argument [143]. Also, perhaps mutations affecting other genes allied to p53 in function, such as WAF-1 (which is rarely

mutated in hematological malignancies [144]), are incompatible with cell survival.

Although p53 mutations are not as common in hematological malignancies as they are in many solid tumors, the presence of p53 mutations appear to have major effects in the response to treatment and the risk of disease recurrence if a response is obtained. Although the frequency of p53 mutations in AML is 5%–10%, at least 30% of patients are curable with current treatment protocols — and since this figure is unlikely to include patients with p53 mutations, the overall contribution of p53 mutations to chemoresistant AML may be significantly higher. Also, there is evidence both for AML [119] and ALL [18] that a subclone of leukemic cells with a p53 mutation that were not detectable by PCR-SSCP at presentation may mediate disease relapse following the achievement of a complete remission. In patients where p53 mutations are present at diagnosis, the prognosis appear dismal, whatever the underlying disease. Several studies in AML, ALL, myelodysplasia, CLL, follicular lymphoma, and myeloma have all demonstrated that patients with p53 mutations have advanced aggressive disease with a poor response to conventional chemotherapy [16–18,20–22]. The loss of wild-type p53 function can reduce the chemosensitivity of the malignant cells by diverse mechanisms, including the loss of signals for cell-cycle arrest and apoptosis following DNA damage [41], the loss of suppression of the bcl-2 proto-oncogene [47,48,50], and the loss of suppressor and gain of promoter effect in the mdr-1 gene [126]. Whether patients with p53 mutations are curable by high-dose chemotherapy and bone marrow or blood cell transplantation is as yet uncertain. An alternative and as yet experimental approach to these tumors is to restore wild-type p53 suppressor activity by drugs that might mimic its function or by introduction of wild-type p53 that has been demonstrated to abrogate the growth of leukemic cells in vitro and in vivo [145].

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7. Primitive hematopoietic stem and progenitor cells in human umbilical cord blood: an alternative source of transplantable cells

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1. Concepts of hematopoietic stem and myeloid progenitor cells

All the circulating blood cell elements are produced by a set of primitive cells termed hematopoietic stem and progenitor cells. Each category is considered to be hierarchical in nature, with cells from the most immature to mature being produced in a catenated fashion. Conceptually, the earliest subset of cells would be the long-term marrow-repopulating stem cell (LTMRSC). This cell would be considered to have the greatest degree of self-renewal capacity and would be the cell type that one would like to make sure was in the tissue source of transplantable cells for long-term repopulation of the blood system of the recipient. It is also this cell that would be the one investigators would like to use in a gene therapy setting for long-term expression of newly integrated genetic material by gene transfer. While the LTMRSC is inferred from animal studies, particularly those studies in mice in which serial transplantation of marrow cells from mouse to mouse to repopulate the blood system is assessed, it is not clear yet whether there is a quantitative assay for this cell in the human system [1]. The LTMRSC gives rise to more mature cells within the stem cell compartment, which can be defined by more limited cell renewal capacity. Assay of these more mature stem cells can be quantified [2] by expansion capacity of cells in suspension from a long-term culture initiating cell (LTC-IC), and by colony assays from high-proliferative-potential colony-forming cells (HPP-CFC), stem (or blast) cells (S-cell), and by a cell previously considered to be within the progenitor cell lineage, a multipotential cell (CFU-GEMM) [3]. HPP-CFC, S-cells, and CFU-GEMM all form colonies that can be replated into at least secondary culture plates with resultant secondary colony formation [2,3]. This capacity for colony replating is considered to estimate some measure of self-renewal capacity. Progenitor cells are believed to be more committed cells with a high degree of proliferative capacity and the link between stem cells and the first morphologically recognizable precursor cell for a particular blood cell lineage (e.g., myeloblast, promonocyte, proerythroblast, megakaryocyte). Cells within the progenitor category that can be quantified include subsets of CFU-GEMM and also erythroid (BFU-E), granulocyte-macrophage (CFU-GM), granulocyte (CFU-G), macrophage

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(CFU-M), and megakaryocyte (CFU-MK) progenitor cells [2]. What we know about the dynamics of human hematopoiesis under steady state or perturbed conditions, such as transplantation, comes from assessment of LTC-IC, HPP-CFC, S-cells, CFU-GEMM, BFU-E, CFU-GM, CFU-G, CFU-M, and CFU-MK. Information of the LTMRSC is at best inferred from assays of these more mature cell populations. Future efforts to develop an assay from LTMRSC currently focus on the use of mice with severe combined immunodeficiency, with or without addition to these mice of fetal tissue sources for feeder effects on human stem and progenitor cells, and on the addition to the mice of human active cytokines [1]. Additionally, in utero transplantation of sheep has been used to set up an environment in animals conducive to the growth of human cells [1,4]. While human blood cell production has been documented in mice and sheep, using these recipients as quantitative assay systems is still problematic. High-level cell engraftment of these animals is still inconsistent, and more information on transfer of human cells into secondary animals, a measure of self-renewal, is still required. While efforts continue in order to develop quantitative assays for human LTMRSC, much has already been learned regarding tissue sources of stem and progenitor cells and their clinical use for transplantation.

2. Alternative sources of transplantable hematopoietic stem and progenitor cells

The first and still most widely used tissue source for stem and progenitor cell transplantation is the adult bone marrow [5,6]. Another widely used tissue source includes adult peripheral blood, especially in the context of those cells mobilized from the marrow to blood by growth factor treatment with granulocyte-macrophage (GM) colony stimulating factor (CSF), granulocyte (G)-CSF, or interleukin (IL)-3. Until recently, the use of growth-factor-mobilized peripheral blood for transplantation was confined mainly to autologous transplants [7]. More recently, encouraging results have been noted for these cells in an allogeneic setting [8–10]. Less rarely, fetal liver cells have been used for transplantation.

Umbilical cord blood is a rich source of primitive hematopoietic stem and progenitor cells [11,12] that can now be added to the list of alternative sources of transplantable cells. This review focuses briefly on the biological and clinical aspects of cord blood stem and progenitor cells that make them a likely candidate tissue source of cells for increased applicability in transplantation in the near future.

3. Umbilical cord and placental blood transplantation

Based on a laboratory study that assessed numbers of hematopoietic progenitor cells in single collections of cord blood and the capacity to cryopreserve

and retrieve these cells in viable form, it was suggested that cord and placental blood at the birth of child should be able to serve as a source of transplantable cells [11]. This study [11] led to the first successful use of cord blood in a clinical setting [13]. The high frequency of these progenitors in cord blood [11,12], which decreased drastically and rapidly after birth [12], and later information on the extensive proliferative and replating capacity of these cells [3,14–18] further encouraged investigators to consider the clinical use of these usually discarded cells. The first transplant [13] was an outstanding success based on an extensive multi-institutional international collaboration. A male child with Fanconi anemia was transplanted with a collection of HLA-matched cord blood cells from his sister, with an extensive array of laboratory studies used to document engraftment. The recipient of the first transplant was completely cured of the hematological disorders associated with Fanconi anemia and is still healthy and completely repopulated with his sister's cells more than seven years after the transplant, which took place in October 1988 in Paris, France. This transplant was soon followed by two other transplants for Fanconi anemia [19,20] and by the first transplant for leukemia [21], a male child with juvenile chronic myelogenous leukemia who received HLA-matched cord blood from his sister. The first five — and seven of the first ten — cord blood transplants were done with cells tested and stored in cryopreserved form in the author's laboratory before transport to the sites of the transplantations, which were in Paris; Baltimore, Maryland; Cincinnati, Ohio; and Minneapolis, Minnesota. There have now been over 90 allogeneic cord blood transplants done in a sibling setting (mainly complete HLA matched, but 1-, 2-, and 3-HLA-antigen disparate cells were also used) and over 110 done in a unrelated setting (HLA-matched or 1-, 2- or 3-antigen mismatched cells used) throughout the world by a number of different centers for malignant and nonmalignant disorders. Most of the transplants have been done in children, and a feature of these sibling and unrelated transplants is the relatively low incidence of graft vs. host disease (GVHD) [22]. The low GVHD may in part be a manifestation of the lowered immunological reactivity of cord blood cells that have been reported in the literature [23–27]. Thus far the largest recipient of engrafted cord blood has been an 80-kg adult and the oldest recipient greater than 40 years old. The establishment of cord blood banks [28–33] and the use of these HLA-typed and stored cells will no doubt be able to help establish in the near future the applicability of a single collection of cord blood. In the meantime, an international cord blood transplant registry was established to coordinate reporting the results of the transplants performed [22,28]. The results of sibling transplants have been encouraging, with an approximately 70% survival rate and 55% disease-free (mainly leukemia-free) survival rate noted for the first 40-odd patients receiving complete HLA-matched or 1-antigen disparate cord blood transplants [22]. Results of related transplants have been published [13,19–22,34–44], but results of the unrelated transplants have not yet been published. Except for a report of three patients receiving fresh gene-transduced autologous $CD34^+$-column

separated cord blood cell transplants [45], all transplants have been done with cryopreserved cells that were either not manipulated at all to separate the cells or were minimally isolated by simple density cut or gelatin separation to retrieve the total nucleated or mononuclear cells prior to freezing. The rapidity with which cord blood transplants have been used since the initial cord blood transplant report, the establishment of cord blood banks, and the encouraging clinical and laboratory data suggest that in the future cord blood may be a major source of stem/progenitor cells utilized for transplantation. There are still a number of questions to be answered regarding the cellular components of cord blood and in what broad and specific contexts these cells can be used [46]. Some of what we know regarding these cells follows.

4. Characteristics of cord blood stem and progenitor cells

Cord blood stem and progenitors that can be measured are in a slow- or non-cycling state [47–49]. Yet these cells respond rapidly to stimulation by cytokines [11,12,14–18], especially to that by combinations of cytokines, and these cells can be greatly expanded through ex vivo culture [12,15–17,49–57]. Maximum cloning efficiency of these primitive cells is apparent in the presence of cytokines such as GM-CSF, IL-3 PIXY321 (a GM-CSF/IL-3 fusion protein), steel factor (SLF, also called stem cell factor, mast cell growth factor, and c-kit ligand), and the flt3/flk-2 ligand (L) [58]. These effects are noted also at the level of a single isolated stem/progenitor cell [16]. The earliest stem/progenitors in cord blood are found in the $CD34^+$ population of cells expressing the highest density of CD34 antigens (e.g., $CD34^{+++}$) on the cell surface [16]. Other phenotypic characteristics of these primitive cells are that they are $CD38^-$, $thyl^+$, $HLA\text{-}DR^+$, $CD45RA^{lo}$, and $CD71^{lo}$ expressing cells [17,18,48,59]. On a frequency basis, there are more of these phenotypically defined early cells in cord blood than in bone marrow [59]. A single immature cell can proliferate under the right culture conditions to produce tens of thousands to hundreds of thousands of cells, including cells with the same phenotypic and functional characteristics as the initial cell placed in culture [16]. It is these characteristics that make these cells useful for ex vivo expansion and gene transduction studies.

5. Ex vivo expansion and gene transduction studies

A number of laboratories have shown that cord blood cells can be greatly expanded ex vivo [12,14,15,17,18,49–57]. In some studies, this cell expansion capacity was shown to be greater than that from other tissue sources such as adult bone marrow. What is clear is that the more mature progenitors are expanded to a greater degree than the stem and immature progenitors [50].

What is not clear is whether the LTMRSC is being expanded, maintained, or lost during these procedures. Various combinations of growth factors, including those of well-characterized cytokines as well as unknown factor of factors in crude preparations including cord blood plasma [50,60], are being used with or without feeder layers of cells in usual culture plates or in bioreactors [52]. Investigators envision the time when small amounts of cells can be expanded for use in single or multiple recipients. Human cord blood is capable of highly engrafting the marrows of sublethally irradiated mice with SCID [61,62a], but not all mice engraft as well, and some do not engraft at all. Because it is hoped that such animal models will be useful for developing assays for human LTMRSC, various genetically altered new SCID mouse models are being developed in the hope that the engrafting capacity of human cells will be higher and more consistent [62a]. This includes the use of transgenic SCID mice expressing endogenous levels of human cytokines such as GM-CSF, IL-3, and SLF, non-obese-diabetic SCID mice, SCID mice expressing the membrane-bound forms of certain cytokines such as SLF, and SCID mice with inherited characteristics of these combination of genetic changes.

The ability to put new genetic material into stem and progenitor cells offers the opportunity not only to correct genetic disorders but also to change the proliferative and differentiation capacities of these cells [62b,c]. The latter possibility entails with it the capacity to enhance the ex vivo and in vivo repopulating ability of cells. Because of their extensive proliferative and replating capacity, stem/progenitor cells from cord blood would seem to be ideal targets for gene transfer. High-efficiency gene transduction has been accomplished recently using either recombinant retroviral [63–68] or adeno-associated viral (AAV) vectors [69,70]. High-efficiency transduction has been seen in HPP-CFC, CFU-GEMM, BFU-E, and CFU-GM, and this has been documented not only at the population level for highly enriched populations of stem/progenitors in $CD34^{+++}$ cord blood but also at the level of a single sorted and isolated $CD34^{+++}$ cell [64]. One potential advantage of using AAV vectors is that preincubation of cord blood cells with growth factors does not appear to be necessary for optimal transduction efficiency with this type of vector [69], in contrast to the use of retroviral vectors [66]. This finding is of importance, since preincubation of cells with growth factors could potentially cause their differentiation. If this happened to an LTMRSC, then we might not be putting the genes into these cells, but rather the more mature cells in the stem cell compartment would be transduced. At present, without an assay to define the human LTMRSC, we do not know what the transduction of efficiency of this cell is with either retroviral or AAV vectors.

With the increased use of cord blood banks for storage of cryopreserved cells, it becomes important to know whether transduced cord blood cells can be cryopreserved and retrieved in viable form with functional expression of the inserted gene, and whether frozen cells can be thawed, purified, transduced, and expanded. Efforts in this endeavor have been encouraging. Cord

blood stem/progenitors can be transduced, frozen, and recovered, and frozen cells can be recovered, purified, transduced, and expanded ex vivo with stable integration and expression of the transduced gene [71,72].

6. Concluding remarks

The use of cord blood stem and progenitor cells has come far in the relatively short time since the first cord blood transplant. This advance is due to a combination of the easy accessibility of these cells, which not too long ago were considered discarded material, and the coordinating efforts of clinicians and laboratory scientists working together and utilizing the information obtained to generate new testable ideas and experiments. Hot areas of research for the near future will involve the ex vivo expansion of cord blood cells and the capacity to transduce these cells with new genes. As mentioned above, these areas of research are not necessarily mutually exclusive. The author envisions a time when transfer of cytokine genes and the genes for receptors to these cytokines will allow expansion of selected sets of either more primitive cells or more lineage-committed cells. As with any newly emerging field, future information should shed light on how best to use cord blood for transplantation purposes.

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8. Detection of minimal residual disease in all: biology, methods, and applications

W. Mark Roberts, Zeev Estrov, Geoffrey R. Kitchingman, and Theodore F. Zipf

1. Introduction

Progress in the treatment of childhood acute lymphoblastic leukemia (ALL) has been dramatic during the last three decades [1]. Unfortunately, although the disease is initially responsive to combination chemotherapy in the vast majority of cases — i.e., complete remissions are attained — contemporary therapies result in a cure in only about 60%–70% of children [2]. The complete remission rate for adults (above the age of 18) is slightly lower, but the outcome is worse due to a higher relapse rate, often occurring early during treatment. One criterion for complete remission is that the bone marrow contain fewer than 5% blasts. However, a patient in remission may harbor up to 10^{10} malignant cells in the bone marrow that must be eradicated or controlled by therapy to achieve the desired outcome of cure [3,4]. The inability to assess this potentially large amount of leukemia in a patient who is in 'remission' has necessitated empiric strategies for deciding the duration and intensity of therapy necessary to control the disease. Thus, strategies to identify the tumor burden during remission, often termed minimal residual disease or MRD, have been applied in an effort to improve our understanding of the nature of the disease and its response to therapy during this period.

In this chapter, we examine the question: Does the detection of residual disease in ALL provide information of unique value to the clinical investigator? To answer this, we first discuss results that imply that the available technique with the most sensitivity and specificity is the polymerase chain reaction (PCR). At the time of its inception, PCR was expected to provide unique prognostic information by offering a means to determine the kinetics of residual disease over time in patients undergoing therapy and early detection of those who would eventually relapse. The discrepancies among published studies, however, have led to confusion about the technique's actual capabilities and to a widespread sense that the technique has failed to meet expectations. However, upon review of the published PCR-based clinical studies, it becomes apparent that the confusion and unrealized expectations arise from fundamentally inconsistent approaches, which themselves often arise from failure to consider limits of detection and failure to account for the fact

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that bone marrow involvement rises and falls on a continuum from treatment to relapse or cure. To see if useful patterns emerge in spite of these limitations, we have combined the data from the various studies to determine the kinetics of disease disappearance for the patients who remained in complete clinical remission. When the same process was completed for the patients who relapsed, a consistent pattern of disease persistence and reappearance also became apparent. Thus, these results suggest that PCR studies of residual disease in ALL have already shed light upon the biology of treatment response and, with appropriate design, future studies may lead to new therapeutic approaches for the 30%–40% of children with ALL who now die from this disease.

2. Methodological background

The ideal assay for detecting residual malignant lymphoid disease would be (1) applicable to the vast majority of cases, (2) specific for leukemia cells, (3) highly sensitive, and (4) quantitative. Conventional cytogenetic techniques, while specific, are only slightly more sensitive than standard morphologic assessment of bone marrow aspirate smears; furthermore, evaluating metaphases is labor intensive [5–8]. Fluorescence in situ hybridization (FISH) techniques allow us to detect genetic abnormalities in interphase cells, but the sensitivity of this method is limited, approaching only 1 malignant cell in 100 normal marrow mononuclear cells [9]. FISH, in analogous fashion to conventional cytogenetics, requires a karyotypic abnormality in chromosome number or structure for its application, a condition that is often lacking in pediatric and adult ALL cases. Immunologic methods, performed using an apparatus such as a flow cytometer (FACS), lack the ability to absolutely differentiate leukemia cells from normal marrow progenitors. Although there is no single leukemia-specific marker, combinations of monoclonal antibodies appear to identify populations of neoplastic cells, and their quantitation is precise [10,11]. However, since differentiation, characterized by changes in surface antigen expression, occurs in some ALL cases, the antibody combinations may detect a subpopulation of the total residual leukemia cells and thereby understimate the extent of disease [12]. This would be most critical in cases where the leukemia progenitor cells, the small population of cells that propagates and thereby maintains the entire population of more differentiated leukemia cells, lack one or more of the antigens identified by a panel of antibodies. While state-of-the-art techniques have pushed the potential sensitivity of immunologic techniques into the range of 1 leukemia cell in 10^4 normal marrow cells, direct comparisons to PCR suggest that immunologic techniques fail to identify residual leukemia in the majority of samples where PCR is positive [13].

The ability to grow leukemia cells in culture is the basis for direct assay of the proliferative potential of any residual progenitor cells. Recent experience

Table 1. Residual disease detection in the M.D. Anderson prospective study of childhood ALL

PCR positive	Time from diagnosis (months)	BCA Positive
22/22	1	18/20
9/11	3	7/10
16/18	6	15/18
15/18	9	15/16
12/13	12	11/13
12/18	15	10/17
11/15	18	9/13
4/10	21	5/10
4/9	24 (end of treatment)	1/7
3/6	27	1/5
2/4	30	0/2

Abbreviations: PCR, polymerase chain reaction; BCA, blast colony assay.

with cell culture using the blast colony assay (BCA) indicates that the assay is highly sensitive for detecting residual leukemia and is specific when leukemic colonies are verified by sequence analysis of a portion of the mu-heavy chain gene (IgH) [14]. Leukemic colonies have been grown for 25 of 27 cases in a prospective series in Houston (see table 1) and persist in the majority of these cases for long periods of time (i.e., $\geq$18 months). However, the assay does not readily permit quantitative estimates of residual leukemia [14].

2.1. PCR evaluation of residual disease in ALL

The deficiencies enumerated for these first five methods can be overcome by PCR. When ALL cases have reciprocal chromosomal translocations between genes of known nucleotide composition, residual leukemia can be detected by PCR amplification of the leukemia-specific rearrangement from either genomic DNA or, more typically, from spliced RNA transcripts using reverse-transcription PCR (RT-PCR). Many ALL cases lack such genetic rearrangements, making an alternative PCR strategy necessary [15]. One way is to take advantage of the unique nucleotide sequence that is generated during joining of the IgH or T-cell receptor (TCR) genes during the rearrangement that occurs in lymphoid cell maturation (figure 1). Template-independent nucleotides (N) are introduced in varying number between variable (V) and diversity (D) elements and between D and joining (J) elements during this process, and nucleotides are lost from V, D, and J elements through the action of an exonuclease. Thus, the rearranged genes are unique for each leukemia cell clone and may be used as a marker for residual leukemia studies [16]. Strategies that exploit this property have been developed by many investigators and currently allow the study of greater than 90% of childhood leukemia cases [17]. Because the PCR primers for the antigen-receptor genes amplify rearrangements from normal lymphocytes as well as leukemia cells, a second

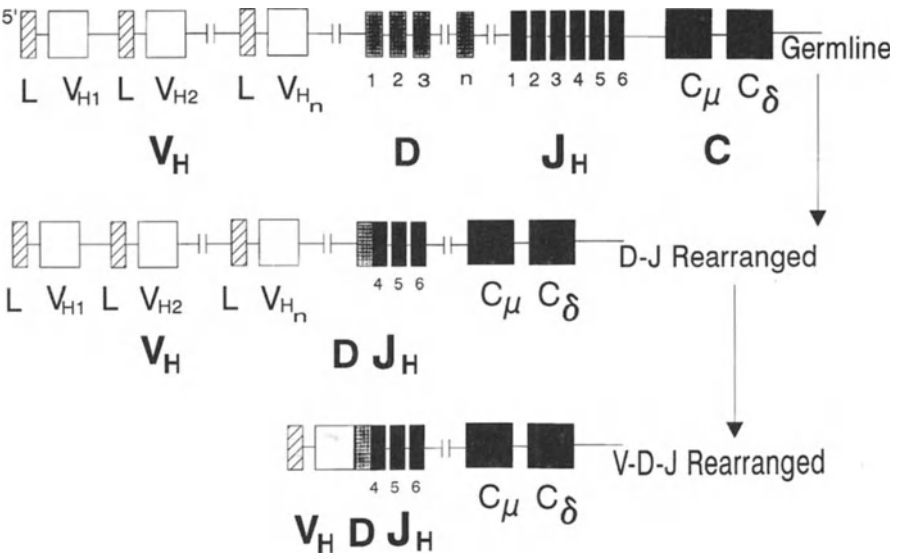

Figure 1. Immunoglobulin heavy chain rearrangement. Approximately 200 V_H segments, 40 D_H segments, and 6 functional J_H segments are recombined with a single constant C_H segment per allele.

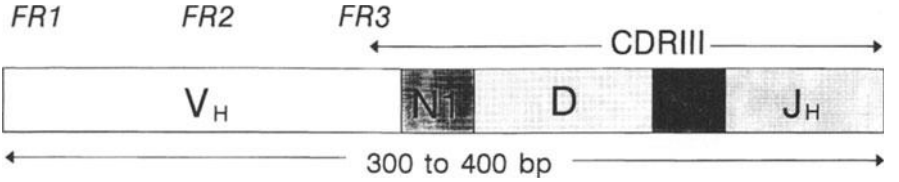

Figure 2. Structure of the V_HDJ_H rearrangement for the IgH gene. The V_H region contains three framework regions (FR1, FR2, FR3) that are highly homologous with each of seven V_H families. The N joining regions are synthesized by TdT in a template-independent fashion, leading to a large amount of junctional variability in these sequences. Consensus primers from the V_H region can be paired with a consensus J_H primer to amplify unique patient-specific CDRIII sequences by PCR.

step is necessary to specifically identify the malignant component. One such strategy involves hybridization of oligonucleotides or DNA segments isolated from the hypervariable sequence of the TCR genes or the third complementary determining region (CDRIII) of the functional IgH gene(s), termed clone-specific probes, that are unique for each patient (figure 2) [18–22]. A second strategy is to design leukemia-specific primers for a second (nested) PCR amplification. The small percentage of cases where PCR fails typically does not result from a lack of antigen-receptor gene rearrangement, but rather occurs when the unique nucleotides (N region) are too small in number either to specifically bind primers or to hybridize as a probe to leukemia-specific sequences.

A potential drawback for monitoring IgH and TCR rearrangements is that

additional rearrangements or deletions of the target sequences can occur during the course of the disease [17,23–28]. However, these effects can be minimized by selecting primers for PCR from regions in the distal part of the IgH gene, since the vast majority of disruptions occur in the proximal V–N–D sequences. By using this strategy, Steward et al. observed that false-negative testing because of clonal evolution would have occurred in only 8% of IgH-positive patients in contrast to 21% of Vδ2-Dδ3-TCR-positive patients [17]. Thus, the initial observation that suggested PCR monitoring of TCR gene sequences might e preferable due to a high clonal evolution rate (about 50%) for the IgH gene has now been reversed. Combining the two systems could reduce the rate of false-negative PCR monitoring of residual leukemia to less than 5%. Furthermore, an alternative strategy of retrieving the newly rearranged IgH gene sequence from leukemic clones grown in the blast colony assay, as shown by Ouspenskaia et al., has successfully rescued monitoring for all three cases where clonal evolution was observed in a prospective study [29].

Taking all these considerations into account, the PCR assay is broadly applicable to ALL and, given the rigorous precautions typical for laboratories using the method, is specific for leukemia cells. The greatest advantage to PCR assays is the gain in potential sensitivity. Several laboratories have detected as few as 1 leukemia cell in 10^6 normal nucleated marrow cells in control experiments. While variations in the methods applied to ensure specificity may reduce the actual sensitivity, a limit of 1 in 10^5 cells remains practical. This level of sensitivity exceeds that of any other technique currently available for detection of residual leukemia.

2.2. Sensitivity of PCR and quantitation of residual disease

PCR sensitivity arises from its exponential amplification of target sequences. However, accurate quantitation using the method can be problematic. Billadeau et al. note that a sigmoid relationship exists between the amount of PCR product formed during a reaction and the IgH gene target sequence concentration [30]. At low target concentration, no PCR product is formed, and at high target concentration the reaction saturates. The curve can be shifted by reducing the number of PCR cycles so that the upper plateau does not occur, but the nonlinear relationship still exists regardless of the target sequence and cannot be altered by changing the PCR conditions. Reliable estimates of PCR target number can only be obtained in a narrow range of about one order of magnitude under conditions associated with a relatively high target concentration. When the target concentration is very low, which is common in residual leukemia, the random factors that determine primer binding to the correct target sequence during the early amplification cycles cannot be controlled, and so any result for a single PCR reaction must be interpreted qualitatively, not quantiatively. This distinction is critical because the vast majority of PCR studies of residual childhood ALL analyze the PCR

product generated from either a single reaction or single reactions using serial dilutions. Thus, methods that attempt to accurately define the amount of PCR product formed in a single reaction, such as densitiometry or scintillation counting after clonospecific probe hybridization [18–21] or recombinant phage plaque counting [22], still result in errors as large as or larger than one order of magnitude.

An alternative strategy for estimating residual leukemia in childhood ALL was recently reported by Cavé et al. [31]. They introduced 100 copies of an internal standard that contained the same primer sequences as the leukemia target and was also of similar molecular weight. After amplification, they quantified (estimated) the tumor burden by comparing the ratio of the signals obtained with a leukemia-specific probe to that of the standard-specific probe [31]. The authors suggest that quantitation using this type of comparison assay is possible only within a log-linear range with a lower limit of detecting 1 leukemia cell in a population of 10^4 normal cells. However, in addition to the difficulty of precisely adding such a small amount of standard DNA, the authors did not consider differences in PCR efficiency between the competing sequences that occur when the sequences differ substantially in number. These differences may lead to large errors when the estimate of residual leukemia burden is based on the calculated ratio of PCR products. The distortion reaches a maximum when saturation of either reaction occurs. This artifact of 'competitive' PCR applies to reactions where coamplification of leukemia cells is performed either with normal B cells, such as the above assay, clonospecific probe methods [18–21], and the phage plaque-counting technique [22], or during coamplification of leukemic and normal marrow cells using a separate 'control' gene of different nucleotide composition as described below.

Because tube-to-tube fluctuations in product formation are unavoidable with PCR, Sykes et al. [32] have developed a strategy using limiting dilution and replication reactions at each dilution with PCR conditions optimized to generate all-or-none results. For clinical samples, five reactions were performed at each dilution, and both N-*ras*, a control gene, and the leukemia-specific IgH gene were simultaneously analyzed. The authors estimated the amount of residual leukemia by Poisson statistics using the method of Taswell applied to results taken at the limit of dilution. Although coamplification of N-*ras* and IgH sequences decreases PCR sensitivity, no account was given of the error generated by the great excess in the control gene targets over leukemic IgH targets. Furthermore, the statistical analysis of the limiting dilution assay used by these investigators requires that a linear relationship exist between the logarithm of the fraction of positive results and the target concentration and that this relationship extend over several orders of magnitude in the target concentration [33,34]. Sykes et al. present no data to indicate that this condition is satisfied.

Ouspenskaia et al. have tested a limiting dilution PCR method in a prospective study of B-precursor childhood ALL and have demonstrated that a linear

relationship does not exist between the fraction of positive reactions and leukemia target concentration [35] as supposed by Sykes et al. Instead, a sigmoid curve, reminiscent in shape of that for PCR product formation from a single PCR reaction, exists for the positive fraction of 10 replicate samples analyzed by the limiting dilution assay employed. The narrowness of the log-linear region of this curve prevents the direct application of the method of Taswell [33]; however, the investigators developed a single best-fit equation for the data from 15 ALL patients. By diluting marrow samples until the results fell on the log-linear region of the curve, they generated reliable estimates of residual leukemia with small interspecimen variation over a range of five orders of magnitude. The capability to obtain inverse confidence, or fiducial, limits was developed for these results by an algorithm comparable with Fieller's theorem as applied by Taswell in his log-linear model [35]. The assay has a standard deviation of one fourth of an order of magnitude for estimates equal to or greater than 10^{-5}.

In summary, PCR appears to most closely approximate the ideals previously outlined as desirable for methods to detect residual leukemia, since PCR can be used to study the vast majority of ALL cases, it can be readily adapted to be specific for leukemia cells, and its sensitivity substantially exceeds that of other currently available techniques. However, the deceptive simplicity of PCR becomes far more complicated if quantitative results are desired. This difficulty and other technical considerations make application to clinical residual leukemia studies far more challenging than generally appreciated.

3. Factors affecting PCR-based residual disease studies

What is the study design required to yield the maximum information about the clinical relevance of residual leukemia? Clinical experience has shown that two years or more of chemotherapy is required to cure the majority of patients [36]. Eradication of the disease is apparently a slow process, and it is reasonable to speculate that sensitive assays such as PCR may detect residual disease for many months. Thus, serial PCR analyses during therapy appear necessary to address the kinetics of disease *disappearance* for patients who remain in extended remission or disease *resurgence* for those who relapse. This strategy also reduces any errors of interpretation that result from minor fluctuations of residual disease that may occur during therapy.

After selecting the times for sample acquisition, adequate sample size is the most important factor that affects the ability to screen for residual leukemia. For example, if the sample contains only 1,000 cells for analysis, it is of little value that the assay for detecting occult leukemia has a potential sensitivity of detecting 1 malignant cell in 10^6 normal cells. In PCR, the current practical limit in a single reaction is 1 to 2 μg of DNA, corresponding to 1.6 to 3.2×10^5 cells. It is imperative that the DNA be of high quality and neither degraded

nor otherwise chemically altered, a requirement largely fulfilled when viable mononuclear hematopoietic cells are separated by density centrifugation prior to DNA extraction.

The second most important factor that determines the efficiency of antigen-receptor gene PCR for detecting residual leukemia is the strategy used to distinguish the clonal leukemia-specific product from polyclonal normal lymphocytes with rearranged IgH or TCR sequences. Most have designed clonospecific probes or oligonucleotides containing patient-specific leukemia sequences that are hybridized under stringent conditions to PCR products on Southern blots [21,31,37,38]. While the investigators have clearly indicated that the stringency can be optimized to limit the binding of the labeled probe to normal cellular DNA and that such probes are specific for rearrangements from individual patients, this specificity step can significantly diminish the sensitivity of the PCR reaction. Ouspenskaia et al. have preserved the sensitivity of optimized all-or-none PCR amplifications of IgH sequences by using a patient-specific primer, located in sequences amplified in the first PCR reaction, in a second (nested) PCR reaction and have sequenced the final PCR product directly to verify absolutely that the products represent leukemia cells [35].

To interpret the data from any single study of residual disease in ALL and to compare the results among independent investigators, it is imperative to consider the realistic sensitivity of PCR for each laboratory based on the actual number of cells that were amplified and the conditions applied to ensure leukemia specificity. Thus, studies that use DNA isolated from bone marrow slides or studies where the sensitivity of PCR is limited for other technical reasons are likely to detect fewer samples positive for residual leukemia. Interpreting the significance of positive samples is complicated by the difficulties encountered when quantitative estimates for the amount of residual leukemia are assigned. As noted above, quantitative estimates from single PCR reactions, constituting the vast majority of study designs, are likely to be associated with errors greater than one order of magnitude, making interpretations of the clinical relevance of such values exceptionally difficult. However, despite all these difficulties, we believe that the data generated from the PCR-based antigen-receptor residual disease studies to date can be interpreted and compared. Furthermore, apparently different results from these studies are not necessarily contradictory when the appropriate factors are taken into consideration.

4. Clinical studies of residual disease detected by PCR

The vast majority of studies of residual disease in ALL have been performed retrospectively on samples collected at different times during treatment and stored either as cryopreserved cells or as bone marrow smears on slides (table 2; [39]). The data for adults with ALL are included in some studies, but are not

Table 2. PCR studies of residual disease in ALL

Investigator	Study design	Patients	Sample timing
Brisco [40,42]	Retrospective	152/88	End of induction therapy
Wasserman [41]	Retrospective	44	End of induction therapy
Nizet [21,38]	Retrospective	16/25	Serial
Yamada [22]	Retrospective	8	Sporadic
Cavé [31]	Prospective	20	Serial
Bartram [37]	Prospective	71	Serial
Biondi [43]	Retrospective	17	Sporadic
Kitchingman [44]	Retrospective	11	Sporadic
Cole-Sinclair [45]	Retrospective	28	Sporadic
Yokota [46]	Retrospective	27	Sporadic
Deane [47]	Retrospective	11	Sporadic
Potter [48]	Retrospective	14	Sporadic
Estrov, Ouspenskaia [14,35,49]	Prospective	27	Serial
Neale [51]	Retrospective	17	Sporadic
Ito [50]	Retrospective	24	End of chemotherapy
Neale [52]	Retrospective	11	Extramedullary relapse
Goulden [53]	Retrospective	13	Extramedullary relapse

always separable. Therefore, the data for all patients have been grouped together, although the vast majority of the cases studied (ca. 90%) were children. While it is possible that the results for residual disease detection for adults will be significantly different, consistent with their worse outcome and potential for early disease recurrence, the data are not yet sufficient for evaluation.

4.1. End of induction therapy

Two centers have performed large retrospective studies on bone marrow samples obtained from patients who completed one month of induction therapy. The investigators, by necessity, had to extract DNA from bone marrow smears that were stained, fixed, and stored, since cryopreserved samples were not available. When the efficiency for PCR amplification of a control gene was analyzed for DNA obtained by these methods, an average number of total cells effectively studied was about 40,000, severely limiting the sensitivity of the assay [40]. Despite this, Brisco observed residual disease in 35 of 152 patients using a qualitative PCR technique [42] and in 38 of 88 patients from the same patient population who could be studied by the quantitative limiting-dilution PCR strategy described by Sykes [32,40]. The investigators suggested that the presence of any amount of residual disease at the end of induction was associated with higher rates of relapse. However, as we will show, the percentage of detectable leukemia (43%) in these cases was very small when compared to studies performed on viable, cellular bone marrow samples. Furthermore, the technical constraint of their quantitative PCR method limited its applicability to only about one half of the population of children treated in the two clinical trials evaluated by this technique.

A similar study using DNA isolated from bone marrow smears was performed by Wasserman, who identified residual disease after one month of treatment in a higher percentage of cases (33 of 44 or 75%). The authors analyzed the group of patients with residual disease further and suggested that high levels of residual disease at the end of induction were an independent predictor of relapse. The authors did not design a prospective study to test these results, perhaps because the phage clonogenic assay method for estimating residual disease is cumbersome and, as discussed earlier in section 2, may lack the necessary accuracy required for the success of such a study.

Taken together, these studies suggest that, in samples adequate for the detection of residual disease, the degree of cytoreduction for patients who attain morphologic remission from early therapy may vary over a range of about three orders of magnitude. These intriguing results suggest that prospective studies with adequate marrow samples and accurate PCR quantitation will be able to discern patients with poor treatment response to induction therapy who may be at very high risk for relapse.

4.2. Residual disease during continuation therapy

The preceding studies suggest that only 43%–75% of patients have detectable levels of residual leukemia at the end of one month of induction treatment [40–42]. The results are substantially different for studies that analyzed bone marrow samples that were either cryopreserved and studied retrospectively or processed directly on fresh bone marrow specimens. For example, the data from the studies of Cavé [31], Bartram [37], Biondi [43], Kitchingman [44], and Cole-Sinclair [45] detect residual leukemia at one month of therapy in the vast majority of cases, and the disease remains detectable in over half the patients studied during the first six months of therapy. Furthermore, the studies of Nizet [21,38], Yamada [22], Yokota [46], Deane [47], Potter [48], and Estrov and Ouspenskaia [14,35,49] extended these observations; residual leukemia was found in almost every sample analyzed during the first six months of therapy. The most recent data from a prospective study of children with B-precursor ALL treated at the University of Texas M.D. Anderson Cancer Center indicated that 26 of 27 patients have detectable residual disease during the first six months of treatment and that detection of residual disease persisted for 19 of 22 of these patients who have completed one year of treatment [49]. The median level for residual disease at the end of induction, as determined by the previously described quantitative PCR method of Ouspenskaia, was 1.7×10^{-4} and did not significantly decline on average by the end of 12 months of therapy (median 1.0×10^{-4}). A comparison of the PCR results with those from the clonogenic blast colony assay (BCA) technique performed in parallel during this study are indicated in table 1. The strong correlation between the two independent assays ($p < 0.01$) suggests that PCR is identifying leukemia cells capable of self-renewal *in vitro* and, thus, the molecular results are likely relevant to viable, malignant disease in the patient. Thus,

given PCR assays with adequate sensitivity (i.e., $\geq 10^{-5}$), and adequate bone marrow samples, it appears reasonable to conclude that almost all children will have residual leukemia detected at the end of induction therapy and, therefore, that the disease persists during early intensification and consolidation therapies.

The studies of Nizet [21,38], Bartram [37], and Estrov and Ouspenskaia [14,35,49] suggest that residual disease becomes undetectable after the first 12 months of treatment and prior to the end of chemotherapy in the majority of patients who remain in complete remission during this period. Intriguingly, however, these studies indicate that a significant number of patients still have detectable residual disease at the end of the second year of chemotherapy, a period when chemotherapy is electively terminated in many treatment protocols. The combined results from these three studies indicate that 10 of 30 patients have detectable residual disease from 19–24 months of treatment. Of the four patients with detectable residual disease by PCR at 24 months in the M.D. Anderson study (see table 1), only one had detectable leukemic colonies by the BCA clonogenic assay. Since PCR and the BCA were highly correlated during the first 21 months of treatment, one hypothesis is that PCR is a slightly more sensitive assay and is identifying low levels of residual disease missed by cell culture. Conversely, however, it could be argued that the BCA is identifying residual disease that is capable of repopulating the malignancy and that PCR may be 'too sensitive' and is detecting cells that are either crippled or dying. The answer requires longer observation for this group of patients, but the significance of residual disease persistence at the end of therapy will be discussed further in section 5.

4.3. Residual disease at the end of treatment

Three other studies have attempted to identify patients at increased risk for bone marrow relapse at the completion of chemotherapy. The study of Ito apparently lacked the sensitivity needed to adequately detect residual leukemia in a population of patients who were selected because they relapsed soon after the completion of treatment [50]. Bartram identified only 1 patient of 37 unselected patients who had detectable residual disease; notably, this is the only patient who subsequently relapsed [37]. Potter detected residual leukemia in four patients selected because they subsequently relapsed, whereas none of five patients who remained in remission had detectable disease [48]. Thus, the potential for identifying patients who might benefit from continued therapy exists, but the present data addressing this question are rather limited.

4.4. Prediction of relapse

While prognostic factors, including the determination of residual disease levels at the end of induction therapy, may be successfully utilized to design

therapy in ALL in a risk-directed fashion, the ability to accurately predict outcome for any individual patient would be of potentially greater value. The retrospective studies of Yamada [22], Biondi [43], Kitchingman [44], Cole-Sinclair [45], Potter [48], and Neale [51] and the prospective studies of Bartram [37], Nizet [21,38], Cavé [31], and Estrov and Ouspenskaia [14,35,49] all indicate, in the preponderance of cases, that bone marrow relapse is preceded by a gross increase in the amount of residual leukemia detected. The molecular prediction of relapse precedes clinical signs typically by at least three months and may be present several months prior to the morphologic diagnosis of relapse. Furthermore, the persistence of high levels of residual leukemia for long periods (i.e., >18–24 months) may also herald bone marrow relapse in patients that lack evidence for a rapid rise in leukemia cell number [38]. Of the three children in the prospective study at M.D. Anderson who have relapsed to date, each relapse occurred in the bone marrow. The quantitative estimates for residual disease in these cases revealed a net rise of leukemia cell concentration of one or more orders of magnitude occurring 3, 6, and 6 months (respectively for the three cases) prior to clinical relapse. In each case, the amount of residual disease detected after the rise increased to greater than the 10^{-3} level (0.001, figure 3). Furthermore, each case had persistent detection of residual disease by both PCR and the BCA throughout

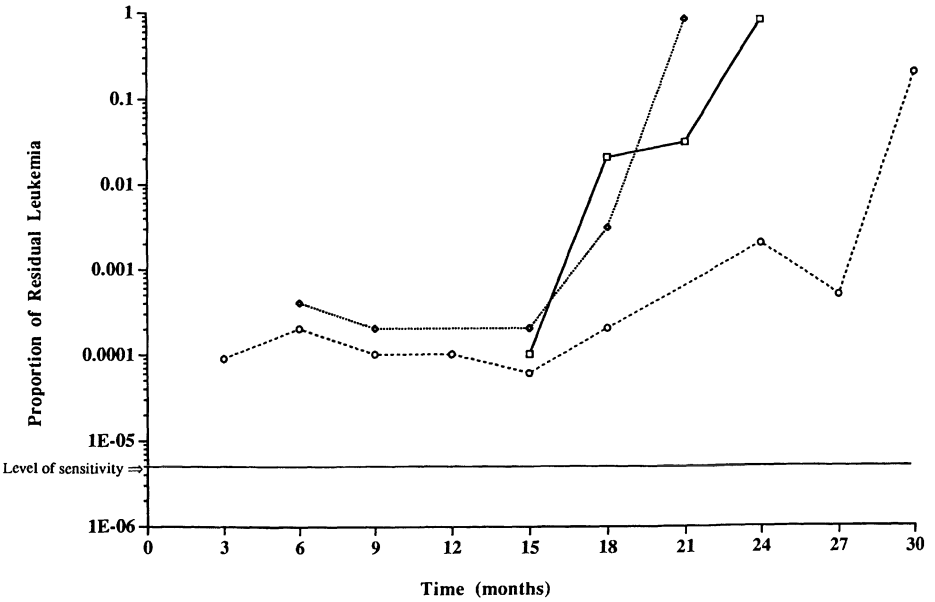

Figure 3. Chart indicating residual disease levels as determined by quantitative PCR for the three cases with relapse in the M.D. Anderson prospective study. All bone marrow samples tested for each case were positive; the axes indicate time from diagnosis and the amount of residual leukemia. The last sample in each case was obtained at the time of clinical relapse as determined by standard morphologic evaluation of bone marrow smears.

treatment and thus also fit the second criteria for predicting clinical relapse. The small number of relapses in the M.D. Anderson study precludes, at present, a meaningful statistical analysis of the pattern of residual disease reappearance in relapsing patients as compared to the pattern of residual disease disappearance for children who remain in extended remission. Given the size of the study population and the expected outcome for this group of patients, an additional 5 to 6 relapses are predicted to occur in the next 2- to 3-year period of observation; the results will be published at that time unless significant results are observed at an earlier date.

4.5. 'Isolated' extramedullary relapse

The data reported by Neale [52] and Goulden [53] clearly indicate that the bone marrow had an increased amount of residual disease detected at the time of either 'isolated' central nervous system or testicular relapse. These studies provide data consistent with the clinical impression that relapse of ALL is invariably a systemic phenomenon, although it is difficult to distinguish whether the bone marrow is 'reseeded' during extramedullary relapse or whether the failure to eradicate leukemia is similar throughout the body but is more readily detected in these so-called sanctuary sites.

5. Biologic implications

The trend in clinical treatment trials for patients with ALL is to decrease the number of routine-surveillance bone marrow aspirations, since the yield from standard morphologic assessment is low [54–56]. However, the encouraging data generated in the PCR studies to date suggest that we have already learned a considerable amount from the detection of residual disease from such samples. While the disease has been termed an acute leukemia due to the relatively rapid onset of life-threatening symptoms in the newly diagnosed patient, the persistence of residual disease suggests that the treatment response for typical patients is actually quite slow, more consistent with the 'chronic' nature of this disease. Although PCR does not directly assess viability, growth characteristics, or drug sensitivity of leukemia cells, serial studies with accurate estimates of residual disease levels have the capability to indirectly address these key issues based on whether the disease is increasing, declining, or stable. Thus, to generate data with the greatest predictive value for PCR residual disease studies, it is necessary to have frequent bone marrow samples. Unfortunately, the data from the studies of Cavé [31] and Yokota [46] definitively prove that peripheral blood is an unacceptable substitute for monitoring residual disease in ALL patients. Therefore, bone marrow specimens remain the tissue of choice for PCR analyses.

We have concentrated in this chapter on the results for residual disease detection by PCR because this assay appears to be the most sensitive tech-

nique available at present. However, it is imperative to consider that up to 10^5 leukemia cells may remain in the body of a patient who has a negative PCR result, even when the sensitivity of the assay is optimal. Thus, if it is necessary to eradicate *all* leukemia progenitor cells to achieve cure in a patient with ALL, then it would be reasonable to postulate that PCR detection of residual disease would not be possible in patients that remain in extended remission. The PCR results, therefore, could be considered surprising in that one third of cases from three studies have detectable residual disease after 24 months of treatment at a time when the cure rate is likely greater than 75%.

This leads to an interesting question: Since residual disease appears to be sustained in the marrow of some patients for prolonged periods, why don't these patients relapse? The possibilities include the following: (1) given enough time, the patients will all eventually relapse from the original leukemic clone; or (2) some patients will not relapse because mechanisms in the patient's body to control small amounts of residual disease exist, such as immunologic surveillance, or an alteration of the leukemic clone or bone marrow microenvironment has occurred during treatment, rendering the disease quiescent such that repopulation of the malignancy is no longer possible. The occurrence of very late (>10 years) relapses in ALL, although a rare phenomenon, suggests the possibility that the delicate balance between leukemic regrowth and disease quiescence may be a permanent concern for a subset of these patients. A simple interpretation of the detection of residual disease, regardless of the assay employed, requires an answer to this type of question. Conclusions related to ALL biology, however, may be different than those for other hematologic neoplasms. Thus, one of the most interesting times for studying residual disease in ALL is at the end of treatment and in the months after therapy is completed. Unfortunately, however, the prevailing clinical practice is to follow patients who have completed therapy with blood counts alone, making bone marrow sample acquisition difficult for such research studies. It is intriguing to consider that one of the early hopes for monitoring residual disease was that the duration of therapy could be shortened for patients who rapidly became PCR negative. The data, however, seem to suggest that this is *not* a practical alternative; in fact, the presence of persistent residual disease near the end of chemotherapy may hint that extending the treatment for a longer duration (e.g., >3–4 years) may improve the outcome for the subset of patients who are 'slow responders.'

6. Summary

The PCR technique appears to be the most sensitive method for detecting residual disease in ALL and can be applied to a high percentage of cases by amplifying sequences of the antigen-receptor genes. The PCR studies to date suggest that this sensitive technique can detect residual disease in virtually all patients during the first year of treatment. The residual disease becomes

undetectable in the majority of patients by the end of treatment; however, a subset of patients remain PCR positive at a time when therapy is electively discontinued. The development of a highly accurate quantitative PCR technique may allow the possibility of distinguishing the patterns of residual disease for patients who will be cured by treatment from those who relapse. If such a pattern can be discerned, then an immediate benefit for PCR monitoring will be that clinicians will have the opportunity to test whether treating patients at the time of 'molecular relapse' will help to improve the cure rate for this disease.

The PCR studies of remission marrows at the end of treatment raise a number of questions about the biology of disease persistence in patients who remain in extended 'remission.' A commitment to obtaining and analyzing bone marrow specimens in patients who have completed therapy is necessary to discern whether novel strategies, such as immunomodulatory manipulations, are needed to control or eradicated residual disease in patients who have completed planned chemotherapy. Thus, the long-term benefit of residual disease monitoring by PCR may be a better understanding of the biology and definition of 'cure' in ALL.

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9. Interleukin-6: biologic properties and role in lymphoproliferative disorders

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1. Introduction

Interleukin-6 (IL-6) is a cytokine that exemplifies the multifunctional nature common to all interleukins [1]. Due to its broad range of actions in many systems, IL-6 has been and remains the focus of intense investigation in a number of diverse research fields. Indeed, prior to its complete characterization, the molecule was variously referred to as interferon (IFN) — β_2 [2], B-cell stimulatory factor 2 [3], human hybridomaplasmacytoma growth factor [4], or hepatocyte-stimulatory factor [5]. Although such functional designations clearly and succintly conveyed a single specific action and were historically very useful, they initially led to some confusion among researchers, and it was not until the determination of the amino-acid sequence of each of these 'factors' that their common identity was recognized and the uniform use of the designation IL-6 was adopted (reviewed in [6]). With the more rapid cloning and production of recombinant molecules, such confusion with terminology rarely exists with cytokines and growth factors discovered in more recent years.

The capacity of IL-6 to modulate the growth, differentiation, and activation state of both normal and transformed human B- and T-lymphocytes suggested a possible role in the malignant transformation of these cells. Multiple myeloma was the disease in which the role of IL-6 was first intensively investigated [7–10]. Although the large amount of data relating to the role of IL-6 in myeloma will not be extensively reviewed, the model of the actions of IL-6 in myeloma provides a paradigm for exploring its activity in lymphoproliferative disorders. The major focus of this chapter will be to examine the evidence supporting a role for IL-6 in the development, proliferation, and systemic manifestations of malignant lymphoproliferative disorders.

2. Role in normal lymphoid proliferation and activation (table 1)

IL-6 is a potent and essential factor for the normal development and function of both B- and T-lymphocytes. Monocytes and T-lymphocytes are major

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Table 1. Major biologic activities of IL-6[a]

Lymphoid cells
terminal maturation of B-cells
enhancement of immunoglobulin secretion
T-cell proliferation and differentiation
MDR-1 gene expression
Hematopoiesis
enhancement of IL-3, GM-CSF, and M-CSF activities
thrombopoiesis
dilutional anemia
Hepatic
acute-phase protein synthesis
inhibition of albumin secretion
inhibition of drug metabolism via cytochrome P450
Systemic
fever
cachexia
osteoclastic bone resorption
Endocrine
enhancement of endogenous corticosteriod secretion
anterior pituitary hormone secretion (growth hormone, prolactin, LH, FSH)

[a] See text for references.

sources of IL-6 (see below), and these cells are pivotal in the modulation of B-lymphocyte maturation. IL-6 is the major determinant of terminal maturation of B-cells into immunoglobulin-producing cells [11] and is essential for the survival and proliferation of hybridoma cell lines, making the cytokine of significant research interest in monoclonal antibody formation and production (reviewed in [6]). The marked sensitivity and concentration-dependent proliferation of these hybridoma cell lines in response to exogenous IL-6 also provided the basis for early bioassays for the detection and quantitation of the cytokine.

In cell culture systems, IL-6 is a potent enhancer of IgM, IgG, and IgA responses to mitogens such as PWM [11]. IL-6 is essential for such antibody production, since IL-6 antisera effectively abrogate immunoglobulin production in response to PWM; however, cell proliferation is not diminished [12]. The ability of IL-6 to augment antibody production has been confirmed in vivo in sheep [13], and also in IL-6-deficient mice, which have greatly reduced titres of both IgG and IgM neutralizing antibody in response to soluble antigen challenge [14]. Further, IL-6 appears to be an essential component of mucosal immunity, since IL-6-deficient mice also have grossly defective IgA responses to mucosal antigen challenge [15].

IL-6 acts predominantly on activated B-cells, since resting B-cells do not express the IL-6 receptor (IL-6R) [16,17]. IL-6 may also mediate some of the actions of IL-2 on B-lymphocytes, since IL-6 antiserum greatly diminishes the level of immunoglobulin secretion in response to exogenous IL-2 [18]. Not

only are B-cells responsive to IL-6 but also there is evidence they can produce the cytokine. Freeman et al. [19] found negligible levels of IL-6 mRNA in purified resting splenic B-lymphocytes. However, IL-6 mRNA levels were greatly and rapidly increased following anti-immunoglobulin treatment of these cells, suggesting a role in cellular activation or B-cell recruitment by T-cells.

Together with IL-2, IL-6 is the major competence factor for T-lymphocyte function. IL-6 can act on the majority of T-cells, since the IL-6R is widely expressed on all stages of T-cell maturation [16,17]. IL-6 has many actions, including the upregulation of expression of the IL-2R, potentiation of PHA-induced T-cell proliferation, and the differentiation and proliferation of cytotoxic T-cells [20]. In contrast to its role in B-cell stimulation, where IL-6 in part mediates the actions of IL-2, in T-cells the proliferation in response to IL-6 is partly inhibited by antibodies to IL-2 [6,21].

3. Hematopoietic activities

In addition to the above actions specific to lymphoid proliferation and activation, IL-6 has broad actions on the hematopoietic system, predominantly as a factor synergistically enhancing the activity of early-acting thrombopoietic and granulopoietic factors. The first recognized activity was the potentiation of IL-3-induced multilineage colony formation, at least in part due to recruitment of quiescent pluripotent progenitors from G_0 into active proliferation [22–24]. Similarly, although having only weak granulopoietic activity in isolation, IL-6 potentiates the actions of both granulocyte-macrophage colony-stimulating factor (GM-CSF) [25,26] and macrophage-CSF (M-CSF) [27]. Preliminary evidence supports a role for IL-6 in the optimal in vitro expansion of peripheral blood progenitor cells [28], possibly due to its ability to synergistically enhance the activity of these other early-acting hematopoietic growth factors.

There was much early excitement over the thrombopoietic actions of IL-6 [29], although this activity is now recognized to be relatively weak compared to that of the recently identified c-*mpl* ligand [30,31]. These activities may underlie the observation of leukocytosis and thrombocytosis in animals overexpressing the IL-6 gene [32–34] and in humans receiving recombinant IL-6 [35–37].

These animal models and human therapeutic trials have also noted the rapid development of a moderate normochromic normocytic anemia. There has been no evidence of diminished erythropoiesis or hemolysis; rather, the anemia appears to be dilutional, related to an increased plasma volume [38].

Recent studies of the distribution of the IL-6R suggest that some of these hematopoietic actions may be indirect, since neutrophil and erythroid precursors express the IL-6R very weakly [16,17].

Recent therapeutic trials of the administration of anti-IL-6 antibodies have confirmed that IL-6 has a role in the steady-state hematopoiesis in humans in

vivo. Following administration of the antibody, there have been statistically significant declines in both platelet and neutrophil counts observed [39,40].

4. Other actions

4.1. Acute-phase response

IL-6 is the central regulator of the production of the 'acute-phase proteins' by the liver. The systemic response of humans to a variety of inflammatory stimuli is characterized by a distinctive coordinated temporal pattern of enhanced production of certain proteins and a reciprocal diminished production of others.

In humans, the plasma-level C-reactive protein and serum amyloid A protein are most dramatically increased during the 'acute-phase' response. Increases in serum concentrations of up to 100-fold in these proteins are commonly observed. The concentrations of other proteins, including α_1-antitrypsin, fibrinogen, haptoglobin, and ferritin may be increased up to tenfold. Conversely, transferrin, prealbumin, and albumin are reduced. It was recognized in the 1950s that the liver was the major source of all these proteins.

Early investigations focused on the activity of cell-free supernatants from activated neutrophils and monocytes on the production of these proteins by hepatocytes. A purified substance capable of regulating the full range of the involved proteins was identified and named 'hepatocyte-stimulating factor' [5]. This factor was later recognized to be IL-6. In vitro work with cultures of hepatocyte-derived cell lines has demonstrated the ability of IL-6 to appropriately regulate the mRNA expression of all the involved proteins [41,42].

Other cytokines, including IL-1 and tumor necrosis factor-α (TNF-α), can influence the production of some of the acute-phase proteins, but only IL-6 has been shown to be able to regulate the synthesis of all involved proteins (reviewed in [43]), and indeed many of the actions of IL-1 are mediated through induction of IL-6 [44].

The primary role of IL-6 in the induction of the acute-phase response was subsequently confirmed in two ways. Firstly, administration of recombinant IL-6 to both experimental animals [43,45], and to humans [35–37] reproduces the typical syndrome. Secondly, IL-6-deficient mice do not develop the biochemical features of the acute-phase response following sterile tissue damage by turpentine injection [14,46].

4.2. Fever and cachexia

Fever is a prominent component of the acute-phase response that is also due to the actions of IL-6. In rabbits, administration of IL-6 induces a dose-dependent fever that peaks 30 to 60 minutes following administration [47].

Human studies have also shown a close temporal relationship between fever and serum level of IL-6 [48]. The cause and effect relationship of this association in humans has recently been clarified by the early phase-I studies reported. IL-6 is clearly the cause, rather than the result, of the fever [35–37].

In both animal models and human disease states associated with prolonged inflammatory processes, cachexia and loss of muscle mass are common findings. In human disease states, this progressive catabolism can be extremely debilitating. IL-6 appears to be the central mediator of these processes.

In mice bearing colon carcinoma implants, tumor size and degree of weight loss correlate with serum IL-6 levels [49], and monoclonal antibodies to IL-6 (but not TNF-α) are able to prevent weight loss [50]. In such tumor xenograft models, many cytokines are likely to be released. However, the specific activity of IL-6 was confirmed by reconstituting mouse bone marrow with hematopoietic cells constitutively overexpressing IL-6 [51]. In this defined single-cytokine model, animals still displayed the same features of wasting and reduced fat stores as in the tumor-xenograft models [51]. The mechanism of these observed metabolic changes is not yet clear, although IL-6 can directly cause skeletal-muscle protein breakdown [52]. As discussed below, IL-6 may mediate loss of adipose tissue through enhanced endogenous corticosteroid secretion. No data are yet available on any direct effect of IL-6 on adipocytes or lipid metabolism.

4.3. Bone resorption

Bone is also a major site of activity of IL-6. Fibroblasts, osteoclasts and osteoblasts in the local bone environment produce IL-6, which is a potent inducer of osteoclastic bone resorption [53]. It has recently been demonstrated that IL-6-mediated bone resorption is a major factor in post-menopausal osteoporosis [54,55]. Also, in some experimental systems, IL-6 can mediate humoral hypercalcemia through its potent induction of bone resorption [56,57]. There are less data to support such a role in clinical hypercalcemia complicating solid tumors or lymphoma [58,59].

4.4. Endocrine effects

IL-6 has a number of complex and interrelated effects on the endocrine system that are under ongoing investigation (reviewed in [60]). The major site of activity is in the hypothalamic–pituitary axis.

At the level of the hypothalamus, IL-6 enhances endogenous corticosteroid secretion through induction of corticotropin releasing factor, and consequently ACTH [61]. In addition to the capacity of IL-6 to induce CRF secretion, it also directly enhances ACTH secretion from the anterior pituitary [62]. The adrenal secretion of corticosteroids is also directly activated by IL-6 [63]. Thus IL-6 potently induces endogenous corticosteroid secretion by acting at all levels of the hypothalamic–pituitary–adrenal axis. The enhanced corticos-

teroid secretion may transiently serve to potentiate the influence of IL-6 on acute-phase proteins, since corticosteroids increase the expression of the IL-6R by hepatocytes [64] and the presence of corticosteroids is necessary for maximal hepatic IL-6 responsiveness [65]. However, corticosteroids also ultimately inhibit production of IL-6, at least from monocytes and macrophages, but probably other sources also [66].

Many other anterior pituitary hormones are secreted in response to IL-6. These include growth hormone, prolactin, LH, and FSH [60].

Other endocrine organs that secrete, or respond to, IL-6 include the thyroid, the pancreas, and both male and female reproductive organs [60]. The physiological role of the cytokine in these systems is less well characterized.

5. Structure and properties of IL-6 (table 2)

Human IL-6 has been cloned, sequence and expressed in *E. coli*. Recombinant human IL-6 has entered therapeutic trials [35–37,67].

The human molecule consists of a single chain of 184 amino acids incorporating four cysteine residues and two potential N-glycosylation sites [3]. This mature protein is the product of a 212-amino-acid precursor protein following the cleavage of a 28-amino-acid signal peptide. A variety of N- and O-glycosylation and phosphorylation patterns are seen depending upon the tissue of origin, although these do not appear to alter biologic activity (reviewed in [43]).

Table 2. Major features of the human IL-6 gene, protein, and receptor[a]

Protein
212-amino-acid precursor
184-amino-acid mature protein
source-specific glycosylation and phosphorylation pattern
four conserved cystein residues necessary for function
Gene
located at chromosome 7p 15–21
approximately 5kB in size
five exons and four introns
regulated by cAMP, AP-1, NF-κB, glucocorticoids, IL-1, NF-IL6
Receptor
80-kDa, 449-amino-acid ligand-binding component (IL-6R)
membrane bound and soluble forms
member of cytokine-receptor family
shared motif of 'WSXWS box'
130kDa signal-transducing component (gp 130)
predominantly membrane bound
shared signaling component for many cytokine receptors

[a] See text for references.

The number and position of the cysteine residues is conserved between the murine and human molecules despite only 42% homology of the proteins overall [68], suggesting that these residues are critical in determining the tertiary structure of the protein and biologic activity. An identical pattern of cysteine residues is also seen in the G-CSF protein, and the structure of the G-CSF gene also shows homology to the IL-6 gene, suggesting a possible common evolutionary link (reviewed in [12]). The structure–function relationships of the IL-6 molecule are under active investigation. At this stage, it is clear that the amino-terminal portion of the molecule is not required for biologic function [69], and the protein is predicted to assume a tertiary structure of four antiparallel-α-helices [70].

6. IL-6 receptor (table 2)

An enormous amount of recent experimental work has clarified the identity, structure, and function of the IL-6 receptor system (reviewed in [71–73]). The cell-surface receptor complex for IL-6 consists of two distinct components; an 80-kDa 449-amino-acid molecule that associates directly with IL-6 (IL-6R) [74] and a 130-kDa signal-transduction molecule (gp130) that associates with the IL-6/IL-6R complex [75].

The IL-6R itself exists in both a membrane-bound and a soluble form (sIL-6R) [72]. IL-6R usually exists as a monomeric protein and has significant homology to a large family of cytokine receptors, including IL-2R, IL-3R, IL-4R, IL-5R, IL-7R, erythropoietin receptor, G-CSFR, GM-CSFR, leukemia-inhibitory factor (LIF)-R, and ciliary neurotrophic factor (CNTF)-R. IL-6R has a single transmembrane domain and a short intracytoplasmic domain without signal-transduction capacity. The region of shared homology of these receptors is a sequence of approximately 200 amino acids that consists of a single immunoglobulin-like domain of approximately 90 amino acids at the amino-terminal end of the extracellular region of the receptor and four common cysteine residues necessary for intrastrand cross-linking. All receptors in the IL-6R family also have a common amino-acid sequence motif in the proximal extracellular domain. This motif, which consists of the sequence tryptophan–serine–one amino acid–tryptophan–serine and has been called the 'WSXWS box,' is essential for optimal ligand binding [76].

Although the involvement of either the surface-bound IL-6R or the sIL-6R is necessary for the activity of IL-6, neither alone is sufficient to allow signal transduction. This function is performed by the gp130, which, when expressed with either IL-6R or sIL-6R, forms a high-affinity receptor complex. Given that gp130 directly interacts weakly, if at all, with IL-6 [77], most investigators propose that the interaction between the complex of IL-6 bound to IL-6R and gp130 alters the configuration of IL-6R, enhancing the affinity of its binding to IL-6. The use of accessory signal-transduction molecules is a theme common to all receptors in the IL-6R family, and the gp130 signaling

molecule is also used for this purpose by LIF, CNTF, and oncostatin-M (OSM).

The sIL-6R maintains the same functional capacity of membrane-bound IL-6R and binds IL-6 with the same affinity, demonstrating that the transmembrane and short intracellular portions of the IL-6R are not essential for function, having only an anchoring role. Similarly, once associated with gp130, IL-6 that is complexed with either sIL-6R or IL-6R induces identical signal-transduction events, resulting in identical cellular responses. This clearly demonstrates that the functional domain of the IL-6R lies within the extracellular portion of the molecule.

In addition to the sIL-6R, there is evidence that a soluble cleavage form of the gp130 molecule (sgp130) may also be present in human serum [78]. In vitro data has demonstrated the potential for sgp130 to associate with the complex of sIL-6R and IL-6, moderately reducing the capacity of such a complex to bind to and activate membrane-bound gp130. It has been postulated, but is yet to be demonstrated, that sgp130 has such a capacity in vivo and may therefore act as a negative regulator of the activity of IL-6.

7. Signal transduction

The understanding of the signal-transduction events involved in the actions of IL-6 is evolving rapidly, and the details are not yet entirely clear. The current level of understanding has been reviewed by some of the senior investigators in the field [79–83]. In spite of this state of flux in the understanding of the process, some of the major early events have been clearly established.

Although gp130 has no basal kinase activity, IL-6/IL-6R binding induces tyrosine-specific protein phosphorylation. It appears that the proximal intracytoplasmic portion of gp130, specifically a conserved 'proline-x-proline' motif, is essential for this function [84]. Initiation of the signaling cascade also requires homodimerization of gp130 molecules following binding to IL-6R linked to IL-6 [85]. Site-directed mutagenesis has established that the dimerization and kinase activation sites are distinct [85].

Following binding of IL-6, the high-affinity receptor complex may, at least in some circumstances, assume the form of a hexameric complex consisting of two molecules each of IL-6, IL-6R, and gp130 [86]. The gp130 homodimers then bind and activate cytoplasmic tyrosine protein-kinases [85]. Recent data have clearly shown that these include members of the Jak/Tyk family of tyrosine kinases [87–90]. The gp130 molecules forming the homodimer then undergo tyrosine-specific phosphorylation [85]. The cellular events more distal to this remain unclear, but in diverse cell types are known to involve the phosphorylation and activation of a variety of signaling molecules involved in at least two distinct signaling cascades, including members of the ERK family of serine/threonine protein MAP kinases [89,91,92], *ras* [91,93], *raf* [89], phospholipase Cγ-1, phosphatidylinositol 3′-kinase [82], and members of the STAT

family of tyrosine kinases, Stat3 and Stat1 [83,94]. Many of these intracellular events are common to the signaling cascade of numerous cytokines, and the mechanism allowing specificity of cellular response to such signals is an area of great research and clinical interest.

It appears that the final intranuclear mediator of IL-6 activity is a transcription factor called NF-IL6 that binds to specific IL-6 responsive elements within the DNA of many genes [95]. Site-specific serine (amino acid 231) and threonine (amino acid 235) phosphorylation of NF-IL6 by MAP kinases are essential for DNA binding [91]. NF-IL6 has a 'basic leucine zipper' type motif, homologous to other DNA-transcription factors such as C/EBP.

8. Sources and regulators of IL-6 (table 3)

The biologic activity ultimately found to be due to IL-6 was first reported in the supernatant of fibroblast cultures [2], and fibroblasts are a rich and widespread source of the cytokine in vivo. It is likely, however, that the major cellular sources of IL-6 in most circumstances are peripheral blood monocytes and tissue macrophages [96,97]. Other sources of substantial quantities of IL-6 are vascular endothelial cells [98]. A very large number of other cell types have also been demonstrated to produce IL-6, although the biologic significance of such sources in the normal physiology is less clear (reviewed in [43]). These cell types include, but are not limited to, keratinocytes, synovial lining cells, amnion cells, endometrial stromal cells, astrocytes and microglial cells.

Table 3. Major sources and regulators of IL-6[a]

Cellular sources
fibroblasts
monocytes/macrophages
B- and T-lymphocytes
vascular endothelial cells
keratinocytes
synovial lining cells
amnion
endometrial stromal cells
astrocytes and micoglial cells
Inducers
IL-1
lipopolysaccharide
phytohemaglutinin
IFN-γ
GM-CSF
TNF
platelet-derived growth factor
Inhibitors
glucocorticoids

[a] See text for references.

As described above, both normal B- and T-lymphocytes are also able to synthesize and secrete functional IL-6.

Many well-recognized inflammatory cytokines can induce the expression and secretion of IL-6. Overall, the most potent of these is IL-1 [47,99], but the relative potency and importance of the regulatory molecules vary depending upon the cellular source of IL-6 (reviewed in [43]). Using peripheral blood monocytes, the bacterial products LPS and PHA are significantly more potent inducers of IL-6 than is IL-1 [100]. Other moderately active stimuli for monocyte and macrophage IL-6 production include interferon-γ and GM-CSF [43]. The major inhibitor of IL-6 production from monocytes is corticosteroid.

Conversely, IL-1 is the most potent stimuli for IL-6 production from both fibroblasts and endothelial cells, whereas LPS and TNF-α are only weak to moderate inducers in these systems. Platelet-derived growth factor is also an active inducer of IL-6 secretion from fibroblasts [99,101]. There are only scanty data available on the soluble factors important in the regulation of IL-6 production by normal B- and T-lymphocytes.

Such variability in the soluble factors controlling production of IL-6 from various sources makes it difficult to predict the overall influence of circulating levels of the respective factors on the balance of IL-6 production in the whole organism in vivo. Preliminary data in mice injected with increasing doses of various cytokines provide some information [102]. Using circulating concentrations of IL-6 as the endpoint, TNF-α appears to be the most potent inducer of IL-6 secretion. In this model, IL-1, IL-2, and interferons-α and -γ also had significant activity. Comparable studies in humans receiving such cytokines in therapeutic settings have not yet been performed.

9. IL-6 gene structure and regulation (table 2)

The human IL-6 gene was cloned even before it was recognized that its protein product was actually IL-6 [103]. It is located on chromosome 7p15-21 [104], spans approximately 5 kb, and consists of five exons and four introns. Overall, there is only moderate homology with the mouse gene [68]. However, specific regions show greater than 90% sequence conservation. The areas of greatest homology are those involved in regulation of expression, particularly the 3′ untranslated region and the proximal 5′ flanking region.

The IL-6 gene has a number of transcriptional enhancer elements identified that are very important in controlling gene expression. These include recognition sites for cAMP, activating protein-1 (AP-1) [68], NFκ-B [105], the glucocorticoid receptor, and IL-1 response elements [106]. There is an additional 14 base-pair palindromic site that specifically binds NF-IL6 [95]. NF-IL6 and NF-κB appear to be the most potent regulators of IL-6 gene expression [107], and they act synergistically to enhance transcription [108].

There is evidence emerging that important ubiquitous cell-cycle regulatory proteins may also influence the regulation of the IL-6 gene. Santhanam et al.

[109] have demonstrated that the tumor suppressor gene products, wild-type p53 and the retinoblastoma protein, can inhibit the level of expression of the IL-6 gene. Not only do tumor-associated mutant forms of p53 lack this repressor function, but some actually enhance IL-6 gene expression [110]. The mechanism of regulation by the retinoblastoma protein is unknown; however, wild-type p53 acts, at least in part, through modulation of the activity of NF-IL6 [110].

10. Laboratory issues and measurement

Recognition of the important physiological and pathologic role of cytokines such as IL-6 provided an urgent need for simple, reliable, accurate, and reproducible means of measuring their presence and activity in both laboratory settings and patient samples. This was initially hampered by the very low concentration of IL-6 in most physiologic situations, making detection in normal persons, and hence determination of an appropriate normal range, problematic. This problem has been overcome by the development of more sensitive assays. Currently available assays are able to reproducibly detect as little as 0.094 pg/ml of IL-6, whereas the lower limit of sensitivity in earlier systems was as high as 100 to 200 pg/ml. This greatly enhanced sensitivity now means that serum IL-6 can be detected and quantified in almost all normal individuals, allowing reference ranges to be very accurately determined, rather than relying upon detectability alone to indicate elevated serum levels of IL-6. The sensitivity of the assay system used is an important feature of early studies that must be borne in mind when interpreting data.

Prior to full characterization of IL-6, such measurements relied upon detection of biologic activities postulated to be unique to IL-6, such as proliferation of certain factor-dependent cell lines. Those used included the B9, MH60, and 7TD1 murine hybridoma cell lines and the T1165 murine plasmacytoma cell line [111]. The major difficulty with such assays is that the biologic activity quantified is rarely unique to the molecule purportedly measured.

More recently, increasingly sensitive immunoassays for IL-6 have become available for use. Although such assays are truly specific for IL-6, they create other potential problems in interpretation that have yet to be clarified. The major issue relates to determining what the particular assay system is actually measuring. This point has been highlighted and reviewed thoroughly by May et al. [112]. These investigators have demonstrated the presence of IL-6 bound to numerous carrier proteins in human serum. The major proteins characterized were complement factors 3b and 4b, C-reactive protein, and albumin. Earlier reports also suggested that α2-macroglobulin may also function as a carrier protein for IL-6 [113].

May et al. [112] further investigated the ability of a variety of assay systems to measure such 'complexed' IL-6 with variable results. They found that the B9 murine hybridoma bioassay system did not detect such 'complexed' IL-6,

whereas the immunoassays tested did recognize bound IL-6. It is necessary to clarify such issues before a uniform and biologically relevant system for measurement of IL-6 in patient sera can be broadly applied for clinical use. A similar analysis of the available assays for sIL-6R is required [114].

One group has developed a novel assay that specifically detects IL-6 bound to sIL-6R [115]. The assay is an ELISA utilizing a monoclonal antibody to IL-6 to capture the protein, and a distinct monoclonal antibody conjugated to alkaline phosphatase directed against sIL-6R for detection. This overcomes some of the above uncertainties regarding biologic activity, as it is established that the complex of IL-6/sIL-6R retains full functional capacity. However, the kinetics of dissociation of the pair in serum is not known.

Further difficulties in the interpretation of single determinations of serum IL-6 levels are introduced by recent data demonstrating that there may be diurnal variations over as much as a threefold range in patients with inflammatory arthritis [116]. Fluctuations of a similar amplitude have also been reported to correlate with the sleep–wake cycle in healthy volunteers [117].

There are also a number of important issues in sample collection and processing that can very significantly alter assay results. There are convincing data that indwelling peripheral venous cannulae induce local IL-6 production from vascular endothelial cells, making fresh venipuncture the preferred method of sample acquisition [118]. The blood should be collected into EDTA and the serum separated immediately, or rapidly cooled to 4°C, to minimize ex vivo IL-6 production by monocytes activated by the sampling process. Specific protease inhibitors such as aprotonin are not required [119]. Lithium heparin is unsuitable as an anticoagulant [120]. Further, repeated freezing and thawing of stored samples can significantly diminish the detection of IL-6 [119]. Although such issues may appear mundane and unimportant, failure to adhere to optimal technique renders interpretation of results difficult and may obscure the correlates and associations of elevated cytokine levels.

11. Evidence for a role of IL-6 in the systemic effects of lymphoma

Early in the history of the clinical investigation of lymphoproliferative disorders, it was recognized that patients frequently developed a clinically distinct constellation of systemic symptoms, collectively referred to as 'B-symptoms' [121–123], and biochemical abnormalities resembling the 'acute-phase' response [124]. By convention, B-symptoms are defined by the presence of any one or more of '*(a) unexplained weight loss of more than 10% of the body weight during the six months before initial staging investigation; (b) unexplained, persistent, or recurrent fever with temperatures above 38°C during the previous month; and (c) recurrent drenching night sweats during the previous month*' [123]. Although not all studies have been unanimous in their conclusions, most data support the judgment that the presence of such symptoms

independently confers a poor prognosis in Hodgkin's disease [125]. The situation is less clear in the more heterogenous studies of patients with aggressive non-Hodgkin's lymphomas, where the presence of B-symptoms is certainly a powerful prognostic indicator in univariate analysis, but loses much of its significance in multivariate analysis [126,127].

12. IL-6 as a mediator of B-symptoms

12.1. Non-Hodgkin's lymphoma (table 4)

As discussed above, the potent capacity of exogenous IL-6 to induce fever, sweats, and weight loss/cachexia certainly suggest it as one of the possible mediators of B-symptoms in non-Hodgkin's lymphomas. Data are rapidly accumulating that support this hypothesis.

The earliest studies proposing IL-6 as the mediator of B-symptoms were reported independently by Stasi et al. [128] and Kurzrock et al. [129]. Stasi et al. [128] found that the mean plasma IL-6 level was 18 pg/ml among 16 patients with any B-symptom, compared to 5 pg/ml among those 15 patients with no symptoms ($p = 0.011$). These authors also measured IL-1, IL-2, IL-3, IL-4, IL-7, IL-8, TNF-α, and IFN-γ without noting any correlation between these inflammatory cytokines and the presence of B-symptoms. They have confirmed these findings in two separate group of patients [130,131].

In a concurrent and independent study, Kurzrock et al. [129] found, using

Table 4. Clinical correlates of IL-6 in non-Hodgkin's lymphomas[a]

presence of B-symptoms in newly diagnosed and relapsed patients
direct linear correlations with:
erythrocyte sedimentation rate
C-reactive protein
white blood cell count
platelet count
inversely related to serum albumin
elevated serum levels associated with:
higher serum level of β_2-microglobulin
poorer performance status
higher International Index prognostic score
macrophage IL-6 expression associated with higher proliferative rate
serum levels predictive of development of lymphoma in:
posttransplantation setting
human immunodeficiency virus infection
serum levels predictive of poorer failure-free and overall survival

[a] See text for references.

an ELISA assay with a lower limit of sensitivity of 22 pg/ml, that those patients with B-symptoms among a group of 32 patients with non-Hodgkin's lymphoma had significantly higher levels of IL-6 than those without such symptoms ($p < 0.005$). In this study, the level of other potentially pathogenic cytokines, IFN-γ, TNF-α, and IL-1 α, were not different between these groups.

Preceding studies using relatively sensitive bioassays had noted elevated serum levels of IL-6 in patients with non-Hodgkin's lymphoma, but had failed to detect any association with B-symptoms [132,133]. The interpretation of these studies was complicated by the inclusion of patients with a variety of histologic types of lymphoma.

We have recently reported [134] further supporting data from a group of 58 patients with newly diagnosed diffuse large cell lymphoma. In this study, serum levels of IL-6 were significantly higher in those patients with B-symptoms (median 8.8 pg/ml compared to 3.1 pg/ml, $p = 0.012$).

These studies clearly associate IL-6 levels with the presence of B-symptoms in patients with non-Hodgkin's lymphoma, both at the time of diagnosis and relapse. However, they do not establish the causality of such an association. The pathogenic role of IL-6 was recently proven by the elegant studies of Emilie et al. [39]. These investigators developed a murine monoclonal antibody that prevents the binding of human IL-6 to its receptor. Administration of this antibody to four patients with lymphoma and B-symptoms resulted in resolution of fevers and sweats within hours. The symptoms did not recur during therapy, despite progression of the underlying lymphoma in most cases. An example of such a response is shown in figure 1. Further, treated patients reversed the previous trend of progressive weight loss and gained an average of 1.4 kg over the three-week treatment period [39].

Although the above data clearly demonstrate a pathogenic role for IL-6 in the systemic symptoms of patients with non-Hodgkin's lymphoma, some uncertainty remains. Not all patients with elevated serum levels of IL-6 report B-symptoms, and not all patients with B-symptoms have elevated serum levels of IL-6. One contributing factor may be that other inflammatory cytokines may mediate B-symptoms in some cases. Further, different patients will certainly have varying levels of awareness of the same severity of symptoms. It is also likely that the difficulties discussed above in actually determining what level of biologically active IL-6 is present contribute to the lack of a clear 'cut-point.' The duration and consistency of elevated serum IL-6 levels are also likely to explain much of the observed heterogeneity. Recent work clearly demonstrates cyclic variation in serum levels of IL-6 [116,117], and a single serum sample, although clearly useful, cannot provide a precise measure of the 'area under the curve' of the time course of the serum IL-6 concentration in an individual. Repetitive sampling studies should clarify the presence and nature of any cyclic variation in serum concentrations of IL-6 in various disease states and improve the ability to detect the relationship between serum levels and symptoms.

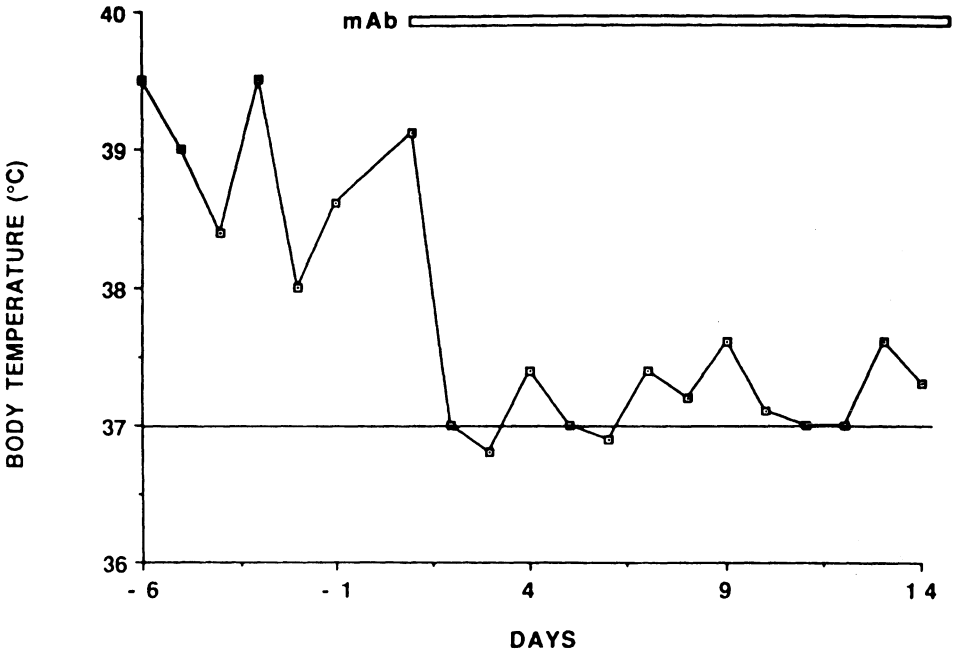

Figure 1. Effect of anti-IL-6 monoclonal antibody (m Ab) on lymphoma-associated fever. Body temperature curve for one representative patient. (From [39], with permission.)

12.2. Hodgkin's disease

Gause et al. [135–137] were the investigators who first examined the relationship between serum levels of IL-6 and B-symptoms in patients with newly diagnosed Hodgkin's disease. In a cohort of 56 previously untreated patients, they documented elevated serum levels of IL-6 in 57%, but did not find any correlation with the presence of B-symptoms. The assay used was an ELISA with a sensitivity of 10 pg/ml. A further small study by Blay et al. [138] of 24 untreated patients also failed to note any correlation between serum levels of IL-6 and B-symptoms, but utilized a very insensitive assay (lower limit of sensitivity 70 pg/ml).

In contrast, Kurzrock et al. [129] noted a striking correlation between the presence of B-symptoms and serum levels of IL-6 in a group of 28 patients with relapsed Hodgkin's disease ($p < 0.005$).

We have recently reported the results of a larger study of 65 newly diagnosed patients using a very sensitive ELISA method (lower limit of sensitivity 0.35 pg/ml) [139]. Similar to the earlier studies, elevated serum IL-6 levels were found in the majority of patients (47 of 65, or 72%). Although those patients with B-symptoms had higher serum levels, this difference did not reach statistical significance (median 5.6 pg/ml compared to 2.5 pg/ml).

Other investigators have found correlations between the presence of B-

symptoms and the serum levels of other soluble mediators, such as the soluble receptor for IL-2 [140,141] and the soluble CD30 antigen [142–144]. There are few studies of the administration of antibodies to these putative mediators in patients with Hodgkin's disease to clarify the causal relationship of these cytokines. There are small numbers of patients treated with antibodies to soluble CD30 reported [145,146], and these cases do not support a pathogenic role for soluble CD30 as a mediator of systemic symptoms.

In conclusion, the major mediator of B-symptoms in patients with newly diagnosed Hodgkin's disease remains to be elucidated, but a strong correlation between serum IL-6 levels and these symptoms appears to exist in patients with relapsed disease.

13. The role of IL-6 in the biochemical/hematologic abnormalities of lymphoma

As discussed above, IL-6 has a large number of diverse actions influencing the endocrine, immunologic, hematopoietic, and hepatic systems. Patients with lymphoproliferative disorders frequently manifest abnormalities reflecting stimulation or dysregulation of these systems. Could IL-6 be responsible for some of these abnormalities?

13.1. Non-Hodgkin's lymphoma (table 4)

The early studies of Stasi et al. [128,130] reported significant correlations ($r \approx 0.7$) between the erythrocyte sedimentation rate (ESR) and serum level of IL-6 in patients with non-Hodgkin's lymphoma. They have recently confirmed these findings in a separate group of patients [131]. The major determinant of the ESR is the plasma fibrinogen concentration [147]. The known capacity of IL-6 to greatly enhance hepatic fibrinogen synthesis [43,148] in concert with the other acute-phase proteins suggests that this relationship is causal. Our recent analysis [134] in 20 patients with diffuse large cell lymphoma confirms the correlation between the ESR and serum IL-6 level ($r = 0.56$, $p = 0.01$) (figure 2).

Given the other activities of IL-6, we explored the relationship between serum levels of IL-6 and serum albumin, white cell count, and platelet count. As predicted by the actions of IL-6 in experimental systems, there were direct linear correlations between the serum level of IL-6 and white cell count ($r = 0.32$, $p = 0.012$) and platelet count ($r = 0.30$, $p = 0.019$), and an inverse relationship with serum albumin ($r = -0.62$, $p < 0.0001$) (figure 3). We found no correlation between serum IL-6 concentration and the level of LDH, consistent with the observations that administration of IL-6 to patients does not result in an increase in LDH [37] and that IL-6 transgenic mice have a normal level of serum LDH [149].

Elevated serum levels of IL-6 are also associated with a number of addi-

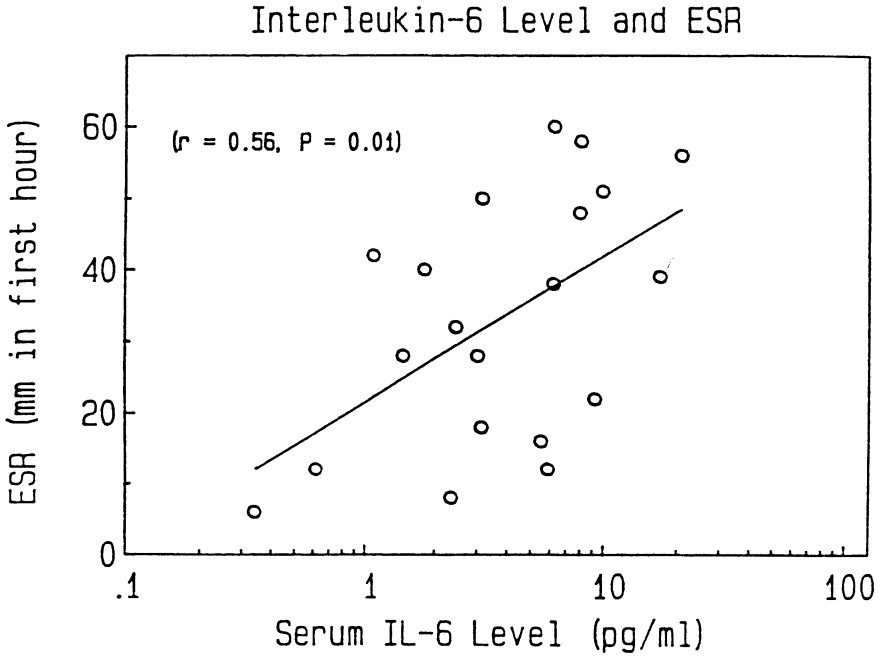

Figure 2. Correlation between serum IL-6 level and erythrocyte sedimentation rate in 20 newly diagnosed patients with diffuse large-cell non-Hodgkin's lymphoma ($p = 0.01$ by Spearman Rank Sum correlation coefficient). __ represents linear regression line. (From [134], with permission.)

tional adverse prognostic indicators, including higher serum levels of β_2-microglobulin, lower performance status, and a higher International Index score [134].

IL-6 probably has a similar role in the systemic manifestations of the lymphoproliferative disorder Castleman's disease [150,151]. There is a recent report of a single patient treated with a prolonged course of anti-IL-6 antibody [152]. Similar to the results obtained in patients with non-Hodgkin's lymphomas, treatment was accompanied by prompt resolution of the fever, anemia, thrombocytosis, and hypoalbuminemia. In this instance there was no objective regression of the tumor mass, and symptoms and signs recurred promptly on withdrawal of the antibody. This case clearly illustrates the protean systemic manifestations caused by excessive production of IL-6 by a localized disease process.

13.2. Hodgkin's disease (table 5)

Unlike the consistent findings of correlation between the ESR and serum level of IL-6 in non-Hodgkin's lymphoma, there are contradictory reports in patients with Hodgkin's disease. The early studies of Gause et al. claimed no relationship between serum IL-6 level and ESR, albumin, white cell count, or

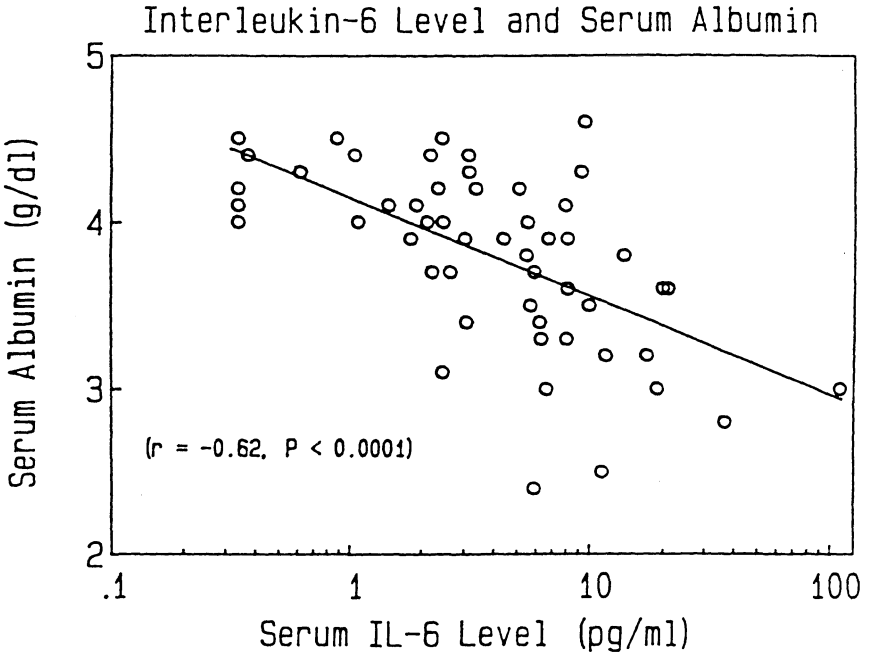

Figure 3. Correlation between serum IL-6 level and serum albumin level in 53 newly diagnosed patients with diffuse large-cell non-Hodgkin's lymphoma ($p < 0.0001$ by Spearman Rank Sum correlation coefficient). __ represents linear regression line. (From [134], with permission.)

Table 5. Clinical correlates of IL-6 in Hodgkin's disease[a]

Serum levels elevated in most newly diagnosed patients
no clear relationship with B-symptoms
inconsistent reports, but likely correlated with:
erythrocyte sedimentation rate
white blood cell count
platelet count
β_2-microglobulin
inversely with serum albumin
inadequate data to evaluate prognostic role in newly diagnosed patients
In relapsed patients
associated with B-symptoms
associated with a poorer survival

[a] See text for references.

platelet count in 56 patients with newly diagnosed Hodgkin's disease [135–137]. Possibly due to the insensitivity of the assay used (lower limit of detection 70pg/ml), Blay et al. [138] also reported no relationship between the serum level of IL-6 and ESR, platelet count, white cell count, serum albumin, or fibrinogen among 24 patients with Hodgkin's disease.

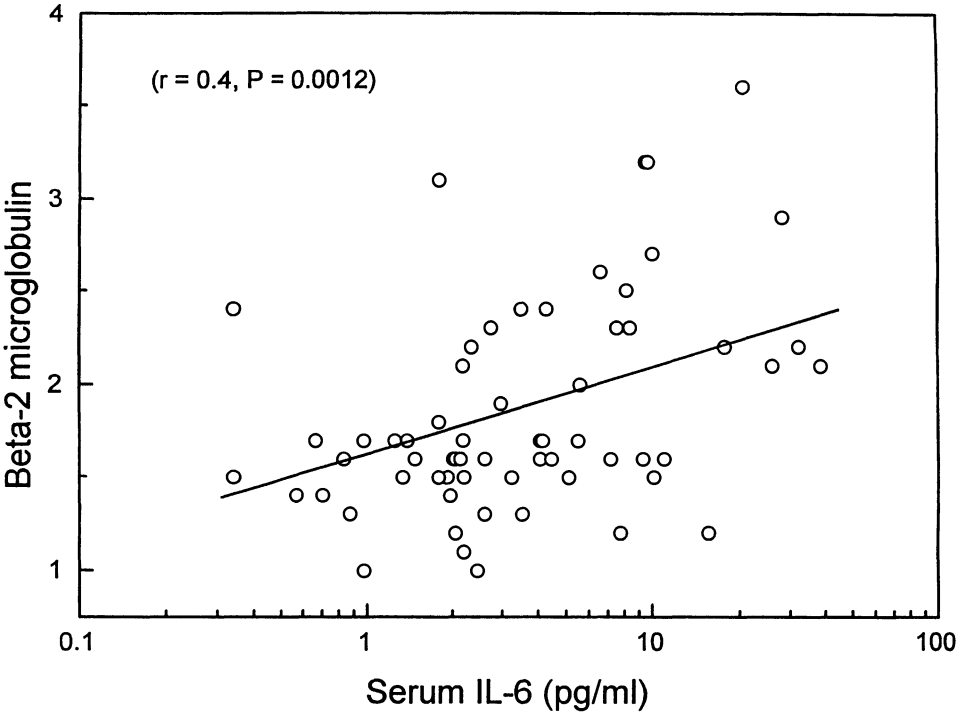

Figure 4. Correlation between serum IL-6 level and serum β_2-microglobulin level in 63 newly diagnosed patients with Hodgkin's disease (p = 0.0012 by Spearman Rank Sum correlation coefficient). __ represents linear regression line.

In contrast to these reports, and consistent with our studies in patients with non-Hodgkin's lymphoma, we found strong correlations between the serum level of IL-6 and ESR (r = 0.64, p = 0.0007), white cell count (r = 0.36, p = 0.003), and platelet count (r = 0.53, $p < 0.0001$). We also demonstrated an inverse relationship of IL-6 with serum albumin ($r = -0.43$, p = 0.0003) [139].

In addition, we also reported a linear correlation between the serum levels of IL-6 and β_2-microglobulin (r = 0.40, p = 0.0012) (figure 4). As with ESR, there are preclinical and clinical data suggesting that IL-6 may be pathogenic in this relationship. IL-6 can increase the expression of the major histocompatibility complex (MHC) class-1 genes in human fibroblasts [153] and of the MHC class-1 protein in colon cancer cells [154]. Since β_2-microglobulin is the light chain component of the MHC class-1 protein, IL-6 may thus increase the amount of β_2-microglobulin shed from the tumor or nonmalignant tissue, increasing serum levels. This activity has previously been documented for other cytokines, notably IFN-γ [155].

Although other cytokines, particularly IL-1, sIL-2R [140,141], and sCD30 [142–144] have been investigated in patients with Hodgkin's disease, none of these has been demonstrated to have any relationship with the biochemical and hematologic features discussed.

14. The influence of IL-6 on the toxicity of chemotherapy

Intriguing recent work suggests that IL-6 may also have a role in the toxicity and patient tolerance of chemotherapy for lymphoma. It has long been recognized that acute illnesses can result in increased serum levels of certain drugs normally cleared by the liver (reviewed in [156]). Since IL-6 is the major factor controlling hepatic acute-phase protein production in such circumstances, its role in hepatic drug metabolism has come under scrutiny.

Both rat and human hepatocyte culture studies have shown that IL-6 reduces the intracellular levels and activities of various cytochrome P-450 enzymes [157–159]. This effect is seen at concentrations of IL-6 similar to those observed in patients with Hodgkin's disease or non-Hodgkin's lymphoma [139,160]. These reductions are mediated via reduced gene transcription, most likely through NF-IL6 consensus sequences in the promoters of the involved genes [159].

Such observations are relevant to drug disposition in patients in clinical situations. Chen et al. [156] prospectively followed and correlated the serum levels of IL-6 and cyclosporine in patients undergoing bone marrow transplantation. Cyclosporine is known to be metabolized by a cytochrome P450 isoenzyme influenced by IL-6 in vitro [161]. They observed up to threefold increases in the serum level of cyclosporine metabolites temporally related to increases in serum concentrations of IL-6 [156].

Although the precise enzymes involved are yet to be determined, many of the most potent cytotoxics used in the treatment of lymphomas, particularly the anthracyclines and the vinca alkaloids, are catabolized by the liver [162], possibly involving cytochrome P450. Many historical data correlate impaired hepatic function with increased toxicity from these drugs. This suggests that, at clinically encountered serum concentrations, IL-6 may inhibit hepatic enzyme activity sufficiently to diminish the clearance, and hence increase the toxicity, of commonly used chemotherapeutic agents.

15. The role of IL-6 in drug resistance

Preclinical experimental data suggest that IL-6 may influence the resistance of lymphoma cells to many cytotoxics through enhanced expression of the multidrug resistance (MDR) -1 gene [163]. The MDR-1 gene encodes a transmembrane glycoprotein that mediates cellular resistance to many naturally occurring cytotoxics by functioning as an efflux pump [164,165]. Resistance to many of the active drug classes in lymphoma, including the vinca alkaloids and anthracyclines, can be mediated through overexpression of MDR-1 (reviewed in [166,167]). Although there are many technical difficulties in definitively ascertaining expression of functional MDR-1 protein, such a phenotype probably predicts for a poor response to therapy in lymphoma [168], myeloma [169] and acute myeloid leukemia [170,171].

Based upon the recognition of the conservation of an NF-IL6-like consensus sequence in the promoter region of human, mouse, and hamster MDR-1 genes, Combates et al. [163] have recently established that NF-IL6 binding, but not binding of closely related transcription factors such as C/EBP, is able to greatly enhance expression of the MDR-1 gene. As described above, NF-IL6 is the major intracellular mediator of the actions of IL-6 following receptor binding, and many lymphoma cell lines [172–174] and patient samples express receptors for [175] and are able to respond to IL-6. The links between the actions of IL-6, MDR-1 expression, drug resistance, and poor clinical efficacy of therapy in the clinic remain speculative, but illustrate a potentially beneficial area of further investigation. Currently, there are no data exploring the possible association between IL-6 levels, IL-6R expression, and the MDR-1 phenotype in lymphoma. If IL-6 is confirmed to have a role in the expression of the MDR-1 phenotype, the inhibition of its binding would be a novel mechanism for restoring drug sensitivity.

16. Evidence for a pathogenetic/promotional role in lymphoma

The available data suggest that although IL-6 is a potent growth stimulatory cytokine for B-lymphocytes, in isolation it is insufficient to induce malignant transformation, but probably has a promotional role in such transformation.

Kishimoto's group was the first to develop an IL-6 transgenic mouse [34]. They inserted the human IL-6 gene fused to the human immunoglobulin heavy chain gene enhancer to generate a strain of mice with very high serum concentrations of human IL-6. These mice developed massive polyclonal plasmacytosis with organ infiltration and polyclonal hypergammaglobulinemia. Although some mice died as a result of the progressive plasmacytosis, these cells were not transplantable into syngeneic mice, failing to demonstrate the properties of malignant transformation. Similar results have been reported in animals with isolated hepatic expression of the IL-6 transgene [149]. When followed for more than 18 months, however, some of these IL-6 transgenic mice develop true lymphomas [176].

Further work from Kishimoto's group suggests that additional genetic alterations are required before IL-6 transgenic mice manifest malignant lymphoid transformation. These investigators back-crossed their C57BL/6 IL-6 transgenic mouse strain with BALB/c mice, which have an inherent genetic tendency to develop plasmacytomas with prolonged immune stimulation, and found that the resultant mice did develop transplantable monoclonal plasmacytomas with defined cytogenetic abnormalities resulting in *myc* oncogene rearrangement [177].

Further data supporting a 'multi-step' model for IL-6 tumorigenicity have come from many studies of EBV-immortalized lymphoblastoid cell lines. In these models, cells inherently capable of IL-6 secretion were found to be more tumorigenic [178], and similarly, cells transfected with the IL-6 gene were

more tumorigenic than the untransfected parent strains [179,180]. The potential role for virus-induced IL-6 secretion and lymphomagenesis is discussed below.

17. Autocrine/intracrine

Multiple myeloma is the IL-6 responsive tumor that has been most widely studied. There is evidence supporting both autocrine and paracrine sources of the IL-6 stimulation ([181,182]; reviewed in [8,9,40]) with most data in favor of a predominantly paracrine model (reviewed in [10]). The investigation of the growth-stimulatory role of IL-6 in lymphoma is still in its infancy, and a similar controversy exists. Some data potentially support both autocrine and paracrine roles.

The first area of investigation that supports an autocrine role for IL-6 in lymphoma comes from cell line studies. A number of cell lines proliferate in response to the addition of IL-6 [172,173] and also constitutively secrete IL-6 [172,174]. IL-6 also functions as an autocrine growth factor for EBV-immortalized B-cells [183]. In various systems, antibodies to IL-6 have been reported to have significant [172,184] or little [174] inhibitory effect on growth. These data are consistent with an autocrine growth-stimulatory role for IL-6 in at least some instances of lymphoma. The failure of inhibition by exogenous anti-IL-6 antibodies [174] does not necessarily disprove an autocrine role for IL-6, since some cells may respond to intracellular IL-6 without the need for extracellular secretion or binding of the cytokine. This private autocrine loop is also known as an 'intracrine' loop. Such activity has recently been shown for human myeloma cells [185] and hairy cell leukemia cells in response to exogenous TNF [186]. The possible intracrine actions of IL-6 in other lymphoproliferative disorders have not yet been explored.

A number of viruses have been implicated in the development of non-Hodgkin's lymphomas, predominantly Epstein–Barr virus (EBV) in the post-transplantation setting [187,188] and human T-cell lymphotrophic virus type-1 (HTLV-1) [189]. Some recent data suggest that the induction of constitutive expression of IL-6 by the *tax* gene product in HTLV-1 infected cells may be one of the mechanisms by which HTLV-1 induces tumor formation [190,191].

There are less conclusive clinical data supporting an autocrine role for IL-6 in non-Hodgkin's lymphomas, but it certainly is implicated. Freeman et al. [19] very clearly demonstrated the expression of the IL-6 gene by Northern analysis in approximately 50% of B-cell lymphomas studied. Other studies have demonstrated relatively low levels of IL-6 in malignant cells by immunohistochemical methods [192–194a], although malignant lymphoma cells certainly expressed IL-6R in most cases [175,193].

Perhaps there is more evidence consistent with an autocrine role for IL-6 in Hodgkin's disease. Both the IL-6R and IL-6 gene expression have been dem-

onstrated in Hodgkin's disease-derived cell lines [194–196]. Hodgkin's cells from tissue samples of patients have also shown expression of IL-6 in 50% to 80% of cases [194,196–199]. There are fewer data available on the expression of the IL-6R in primary tissue samples, with the only reported study finding expression in 8 of 16 cases [194,196]. These receptors appear to be able to mediate cell growth, since exogenous IL-6 was able to greatly enhance the proliferation of Hodgkin's-disease-derived cell lines expressing the receptor [196]. However, anti-IL-6 antibodies did not inhibit the basal proliferation of such cell lines [195]. As discussed above, this finding does not necessarily disprove an autocrine role for IL-6 in this setting.

18. Paracrine

There is some histopathologic evidence that is more supportive of a paracrine role for IL-6 in non-Hodgkin's lymphomas. As discussed above, monocytes and macrophages are the major sources of IL-6, and it appears that the total amount of IL-6 present in the local lymph node environment may be predominantly due to macrophage production. Four groups have independently shown significantly greater reactive cell IL-6 expression than in malignant lymphocytes themselves [192–194,200]. Müller et al. [200] have shown a correlation between the proliferative rate of the malignant cells and the degree of IL-6 expression by reactive macrophages in a group of 121 node biopsies from patients with non-Hodgkin's lymphoma. This would be consistent with an in vivo paracrine proliferative action of IL-6.

The only studies of proliferation of lymphoma cells from direct tissue samples from patient biopsies failed to demonstrate any stimulatory effect of IL-6 on the low-grade MALT lymphoma cells [201]. However, serum levels of IL-6 are rarely elevated in patients with low-grade lymphomas, and data in intermediate- and high-grade disease are required.

There are some provocative prospective data on the predictive value of serum IL-6 levels and the development of lymphomas both in the post-transplant setting [202] and in patients infected with the human immunodeficiency virus (HIV) [203]. The long lead time prior to the clinical detection of the lymphomas in both reports suggest that the link with serum IL-6 level is not merely an early manifestation of a preexisting disorder, but truly predicts for the subsequent development of malignancy. The B-cell stimulatory properties outlined above would be consistent with IL-6 playing a promotional role in the development of such tumors.

The primary initiating event in both situations may be a viral infection, with most available data supporting a role for EBV [187,188,204]. EBV infection induces IL-6 secretion from monocytes [205], and recent data clearly demonstrate that cyclosporine also enhances IL-6 secretion by T-lymphocytes and potentiates EBV replication, possibly also enhancing tumorigenesis as shown for EBV-immortalized cell lines.

19. Prognostic significance of IL-6

The foregoing discussion provides compelling preclinical and clinical grounds to suspect that the serum level of IL-6 may provide an easily determined and accurate assessment of the likely outcome of patients with aggressive lymphoproliferative disorders. The available preliminary data support this suggestion.

The first data supporting the prognostic potential of IL-6 levels came from the study of Kurzrock et al. [129] of patients with relapsed Hodgkin's disease. Among the 26 patients studied, the median survival of those with increased serum IL-6 (≥22 pg/ml) was only 10 months, whereas that of the group with undetectable serum IL-6 had yet to be reached with a median follow-up time of 37.5 months ($p = 0.0012$). Further studies in patients with uniform treatment are needed to confirm this finding.

The very high complete remission rate of patients with newly diagnosed Hodgkin's disease, coupled with the long natural history, makes it difficult to rapidly obtain accurate prospective information on the prognostic significance of new parameters. This is applicable to the study of IL-6, where no studies with adequate follow-up to accurately assess outcome data have yet been reported. The studies of Gause et al. [135,136] have not yet noted any survival difference, although the median follow-up of patients was less than two years at the time of reporting. Longer follow-up of this cohort is clearly required.

In contrast, the lower complete remission rate and shorter median remission duration in patients with aggressive non-Hodgkin's lymphoma make prognostic information more rapidly attainable. We used the stored sera of a cohort of uniformly staged and treated patients with diffuse large-cell lymphoma to explore the prognostic significance of serum levels of IL-6 [134]. Fifty-seven patients were studied and treated with an intensive alternating anthracycline-based therapy [206]. Seventy-eight percent of the lymphoma patients had serum IL-6 levels above the normal range (0–1.9 pg/ml). There was no significant difference observed in the complete remission rate according to the IL-6 level (77% for those with a normal level, compared to 61% for those with elevated levels). With a median follow-up of 25 months (range 7 to 37 months), those patients with an elevated baseline serum level of IL-6 had an inferior relapse-free and overall survival rate ($p = 0.042$ and $p = 0.05$, respectively) compared to those patients with normal serum levels of IL-6 (figures 5 and 6).

20. Potential therapeutic implications

Based upon early cell line studies demonstrating inhibition of cell growth [207] and supportive data in some animal models [208,209], there was some initial enthusiasm for the therapeutic potential of the administration of IL-6. There have been no objective responses noted in the phase-I trials reported to date

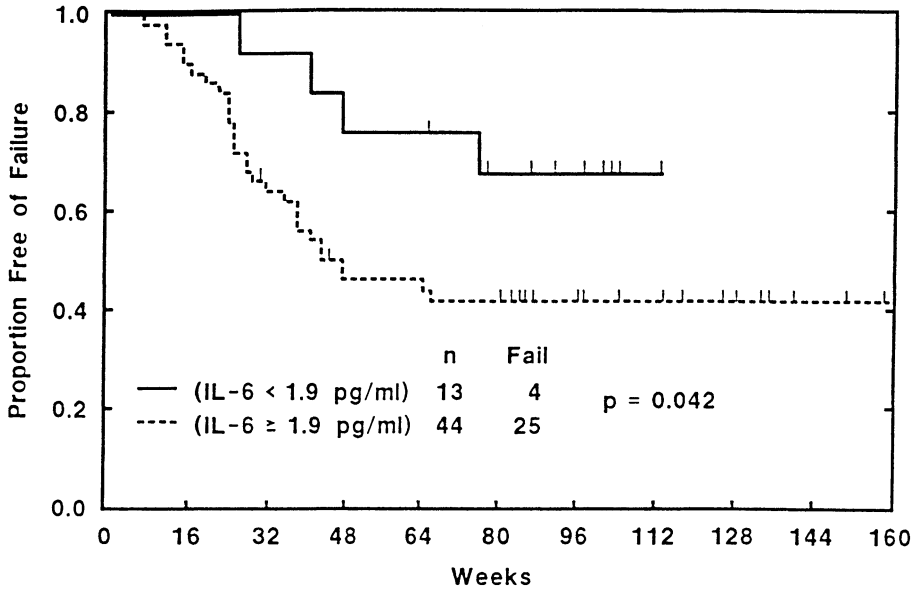

Figure 5. Freedom from treatment failure in 57 patients with newly diagnosed and uniformly treated diffuse large-cell lymphoma according to their pretreatment serum level of IL-6 (curve plotted by Kaplan-Meier method; $p = 0.042$ by Gehan–Breslow test). (From [134], with permission.)

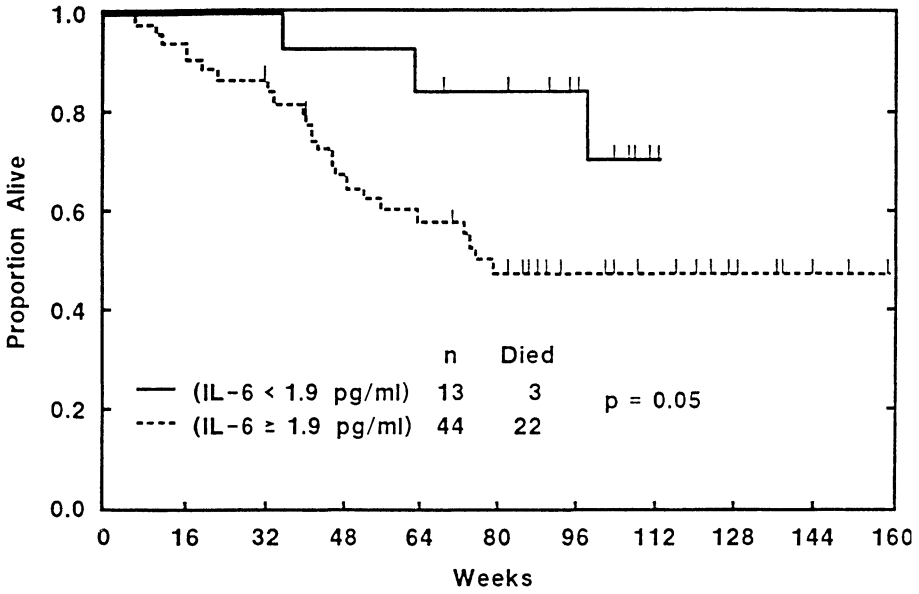

Figure 6. Overall survival of 57 patients with newly diagnosed and uniformly treated diffuse large-cell lymphoma according to their pretreatment serum level of IL-6 (curve plotted by Kaplan–Meier method; $p = 0.05$ by Gehan-Breslow test). (From [134], with permission.)

[35–37,67]. Conversely, it now appears that the inhibition of action of IL-6 may have beneficial consequences in many tumor types. For example, high serum levels of IL-6 are associated with a poor response to therapy and survival in patients with multiple myeloma [210,211], renal cell carcinoma [212], ovarian cancer [213], and melanoma [214]. Indeed, there has been concern expressed regarding the tumor-promoting potential of IL-6, since a number of patients have developed rapid unexpected disease progression during administration of IL-6 [215].

Our increasing understanding of the role of IL-6 in lymphoproliferative disorders provides opportunities in two broad areas to improve therapy. Firstly, it may allow us to target certain high-risk populations, and secondly IL-6 may provide a novel therapeutic target in itself.

The ability to tailor the intensity of therapy to the specific requirements of individual patients has become increasingly important. The financial costs, and the acute and chronic toxicities of dose-intensive therapies for both Hodgkin's disease and non-Hodgkin's lymphoma, demand that these be applied in an appropriate manner. It is intuitively obvious that it should be an aim of treatment to obtain the best possible outcome for an individual patient at the lowest cost and with the least morbidity. In patients with aggressive lymphoproliferative disorders, this means aiming to obtain a cure. One of the major advances in this field of clinical research is the recognition of specific groups of patients where this goal can readily be obtained with standard, relatively nontoxic therapies [125–127].

Over many years, a number of investigators have repeatedly demonstrated the prognostic value of markers such as the ESR, serum β_2-microglobulin, serum albumin, and the presence of B-symptoms [125–127,216–219]. These relationships suggest that it is possible that many currently recognized prognostic factors may be surrogate measures of the activity of IL-6. Although measuring the phenotypic expression of the activity of IL-6 (e.g., hypoalbuminemia) may provide as accurate prognostic information, the exploration of the underlying cause will allow meaningful exploration of the biologic relationship between the measured variable and the outcome for the patient. Thus, investigation of the role of IL-6 in lymphoproliferative disorders will both enhance our ability to select the appropriate therapy for individual patients and expand our understanding of the underlying pathobiology of the disease.

Expanding their earlier work using anti-IL-6 antibodies in the therapy of myeloma [40], French investigators have recently demonstrated the clinical utility of disrupting IL-6 in patients with HIV-associated lymphomas [39]. Eleven patients were treated with a murine monoclonal antibody to human IL-6 with negligible toxicity. Despite rapidly progressive disease at the institution of therapy in most patients, stabilization was achieved in five patients, and an objective partial remission in one additional patient. Immunization against the murine protein was only observed in two of these highly immunocompromised patients. More impressive even than the antitumor activity was

the amelioration of weight loss and fevers (discussed above). These preliminary data clearly demonstrate the activity of IL-6 in the growth and systemic effects of IL-6 in human lymphomas and provide an impetus for improving the potency of binding inhibitors of IL-6 [220] and the efficacy of other interventions to inhibit the activity of IL-6 in vivo.

In human myeloma cells, exposure to IFN-α or -γ downregulates expression of the IL-6R, disrupting autocrine growth stimulation [221,222]. There are no data available to determine whether such a mechanism has a role in the antitumor activity of IFN-α in either low-grade [223,224] or aggressive non-Hodgkin's lymphomas [225]. Alternative methods of IL-6-directed therapy potentially also include the admministration of antibodies capable of blocking the IL-6R [226–228], radio- or immunotoxins targeting the IL-6 receptor [229], specifically designed ribozymes selectively able to cleave IL-6 mRNA [230], or the use of antisense oligonucleotides to inhibit IL-6 gene expression [185,231]. This field is rapidly expanding, and new laboratory developments are certain to increase the number of therapeutic modalities capable of disrupting the actions of IL-6 in vivo available to the clinician.

21. Unanswered questions

The investigation of the role of IL-6 in lymphoproliferative disorders is progressing very rapidly on a number of fronts. At the basic research level, the major area of interest is to develop a more accurate and refined understanding of the signal-transduction machinery used by the IL-6 receptor system. Of particular importance is understanding how such a complex cascade can share many components with other cytokine signaling systems and yet still maintain the high degree of specificity necessary for the coordinated function of the intact organism.

The biological understanding of the actions of IL-6 remain further advanced than the knowledge of the clinical relevance of such actions. There are at least three major questions to be addressed. Firstly, how is IL-6 related to the development of lymphoproliferative disorders? Is its excessive secretion and activity directly causative, or does it play a promotional or accessory role? Secondly, if IL-6 is pathogenic in the development and proliferation of these tumors, is this primarily due to autocrine or paracrine sources? What data are available suggest that the mechanism of autocrine secretion will not be simply one of gene translocation — for example, as seen with c-*myc* in Burkitt's lyphoma. The IL-6 gene locus is very rarely involved in the translocation events documented in patients with lymphomas. The mechanism of constitutive autocrine secretion may be due to the disruption of the complex regulatory factors normally responsible for the control of gene expression.

The third, and most directly clinically applicable question, is what benefits effective abrogation of the actions of IL-6 will have both on systemic disease manifestations and on disease growth and progression in patients with malig-

nant lymphoproliferative disorders. It is unclear what proportion of malignant lymphomas (Hodgkin's and non-Hodgkin's) are dependent upon IL-6 for viability and proliferation, as opposed to simply possessing the capacity to proliferate in response to IL-6, in addition to other growth-stimulatory molecules. In the former case, intervention aimed at disruption of IL-6 may be potently cytoreductive. Conversely, in the latter case, even the most effective blocking of IL-6 activity will not result in meaningful tumor regression.

Regardless of the ultimate effectiveness of IL-6-directed therapy in lymphoproliferative disorders, a more complete understanding of the involvement of this pleotropic cytokine in both normal and malignant lymphoid cell biology will enhance our knowledge of the pathogenesis of malignant transformation and also improve our understanding of the causes for the clinical manifestations of these diseases, the great heterogeneity in responsiveness to cytotoxic-based therapy, tretament-related toxicity, and ultimately outcome.

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10. Autologous bone marrow transplantation for leukemia and lymphoma

Koen van Besien and Sergio Giralt

1. Introduction

The hematologic malignancies, namely, acute and chronic leukemias and lymphomas, are among the most chemosensitive malignancies. Responses and complete remissions occur in the majority of patients with lymphoma and leukemia. Unfortunately, recurrence usually follows, indicating the persistence of cancer cells with a higher degree of resistance. For many chemotherapeutic agents, however, there is a steep dose–response curve. This is particularly true for alkylating agents, for which any level of in vitro resistance can be overcome by dose escalation [1–3]. The concept of dose intensity has been applied in clinical medicine by dose escalation of selected agents to maximally tolerated levels. The agents utilized have myelosuppression as their dose-limiting toxicity. The dose is increased to a level that is considered myeloablative, and patients are rescued by infusion of previously collected blood and marrow stem cells. A large number of pilot studies in the 1980s established the feasibility of this approach in selected patients with hematologic malignancies. In recent years, improvements in supportive care and in stem cell collection and purging techniques have increased the applicability of high-dose chemotherapy. In addition, recent results of prospective studies, some of them randomized, have better defined the efficacy of this procedure for various malignancies.

This chapter will provide an overview of the current clinical experience with high-dose chemotherapy and autologous bone marrow transplantation in hematologic malignancies, particularly with reference to

1. acute myelogenous leukemia,
2. acute lymphoblastic leukemia,
3. chronic myelogenous leukemia,
4. aggressive lymphoma,
5. low-grade lymphoma,
6. chronic lymphocytic leukemia, and
7. Hodgkin's disease.

The chapter ends with some conclusions and suggestions for future directions.

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2. Autologous bone marrow transplantation in acute myelogenous leukemia (AML)

Allogeneic bone marrow transplantation (BMT) was long considered the optimal treatment for many patients with AML [4]. However, the lack of available donors and the considerable problems associated with this procedure make it available to only a minority of patients [5]. Autologous bone marrow transplantation has therefore been investigated as an alternative treatment and has emerged as a promising strategy.

2.1. Autologous bone marrow transplantation for refractory AML and AML in relapse

Many patients with recurrent leukemia fail to respond to salvage chemotherapy. Such patients can be cured in approximately 20% of cases by allogeneic bone marrow transplantation, including some who never achieved remission [6,7]. With similar conditioning regimens and the infusion of syngeneic marrow, long-term disease-free survival was achieved in 8 of 34 patients with refractory leukemia [8].

For those who lack an HLA-identical or syngeneic donor, high-dose chemotherapy and reinfusion of marrow obtained in first remission has been attempted. This led in early studies to a high rate complete remissions [9–14]. With few exceptions [12–14], those remissions were not durable. It is conceivable that in the future, different conditioning and marrow purging techniques could result in a better outcome for patients with recurrent AML and make this approach more generally available.

One example of an innovative approach was reported by Meloni et al. [14]. They treated three patients with high-dose chemotherapy and bone marrow transplant as induction treatments at the time of first recurrence. These treatments were then successfully consolidated with high-dose cyclophosphamide and TBI, followed by infusion of marrow that was collected in second remission.

2.2. Autologous BMT for AML in second or subsequent remission

Patients with recurrent leukemia can occasionally achieve a second remission, but when treated with chemotherapy alone, they usually relapse, although a small subset may achieve prolonged remissions. Allogeneic BMT may improve the outcome, but is available to only a minority of patients. Consolidation of second remission with autologous BMT has been attempted in a number of small and several large prospective studies (for an overview of published results, see table 1). We will present in some detail the results of the largest and most recent prospective studies.

Chopra et al. [12] present data on 34 patients who underwent autologous bone marrow transplantation for recurrent AML between 1985 and 1989.

Table 1. Autologous bone marrow transplantation in AML: results of phase II studies

Author, year, reference	N pat	Age		Status at BMT			Purging[a]	Conditioning[a] (N Pat)	% BMT mortality	Relapse rate		Disease-free survival		Comments
		median	range	CR1	Rel1/ CR2	>CR2/ REFR				CR1	All	CR1	ALL	
Meloni et al. 1985 [10]	13	28	6–41		13		none	COATA (4) or BVAC (9)	30%		38%		30%	
Meloni et al. 1990 [15]	21	24	1–47		21		none	BVAC	5%		45%		52%	
Meloni et al. 1994 [16]	199	23	1–60	139	60		none	BVAC (140) or BuCy (69)	7.5%			SR[‡]: 42% HR[‡]: 0%	CR2: 41%	[‡]SR, Standard risk; HR, high risk
Chopra et al. 1991 [12]	34	40	21–62		34		none	BuCy	12%		Rel1: 70% CR2: 43%		Rel1: 52% CR2: 33%	
Spinolo et al. 1990 [11]	15	32	21–55		12	3	none	CBV	13%		73%		14%	
De Fabritiis et al. 1989 [35]	7	19	5–37	6	1		S4-7 + C[b]	BAVC	14%	33%	42%	50%	42%	
Michel et al. 1988 [177]	11	10	1–16	11			in vivo	MEL → MEL	0	36%		64%		Double autograft
Meloni et al. 1989 [14]	3	28	3–36		3		in vivo	BAVC → CyTBI	33%		0%		66%	Double autograft for untreated relapse
McMillan et al. 1990 [39]	82	40	16–57	82			none (56), in vivo (26)	BACCT 26 double autografts	6%	48%		48%		Possible benefit to double autograft DFS 67% vs. 40% ($p = 0.052$)
Cahn et al. 1985 [178]	18	24	3–44	18			none (2) mafosfamide (16)	TACC (17), Cy-TBI (1)	28%	22%		44%		
Sanz et al. 1993 [43]	24	40	14–62	24			PBSC	BuCy	12%	60%		35%		
Lenarsky et al. 1990 [179]	13	5	1–21		13		4HC	BuCy	0		39%		61%	
Robertson et al. 1992 [36]	12	41	25–49	1	10	1	CD33 + C[b]	AraTBI (4), CyTBI (4), BuCy (4)	0	1/1	66%	0/1	33%	
Ball et al. 1990 [180]	30	42	11–57	6	18	6	PM-81, AML-2-23	CyTBI (28), BuCy (1), BuVP (1)	22%	33%	40%	66%	40%	
Beelen et al. 1989 [181]	20	40	16–53	20			none	BuCy	12%	38%		55%		
Rosenfeld et al. 1989 [13]	24	33	10–61		11	13	4HC	BuCy (15), ByCyAra (3), CyTBI (6)	25%		58%		19%	
Yeager et al. 1986 [182]	25	31	4–53		20	5	4HC	BuCy (23), CyTBI (2)	20%		46%		43%	
Stewart et al. 1985 [183]	13	16	13–38	13			none	CyTBI	14%		67%		22%	

Table 1. (continued)

Author, year, reference	N pat	Age median	Age range	Status at BMT CR1	Status at BMT Rel1/ CR2	Status at BMT >CR2/ REFR	Purging[a]	Conditioning[a] (N Pat)	% BMT mortality	Relapse rate CR1	Relapse rate All	Disease-free survival CR1	Disease-free survival ALL	Comments
Chang et al. 1986 [184]	1	16			1		long-term culture	CyTBI	0		0/1		1/1	Cells were obtained in relapse and reinfused after long-term culture
Gorin et al. 1990 [40]	234	30	1–65	234			none (194), mafosfamide (69)	TBI (131), chemo (132)		55%		39%		European survey on role of purging Purging benefits standard-risk AML
Koeppler et al. 1990 [185]	7	43	25–62	7			none	CyVPAra	0%	14%		86%		Limited follow-up
Chao et al. 1993 [18]	50			34	16		none (20), 4HC (28), 4HC + VP (2)	BuVP	12%	P: 21 U: 67	P: 28% U: 62%	P: 63% U: 27%	P57% U32%	P vs. U $P = 0.06$[c]
Linker et al. 1993 [17]	58	38	17–59	32	19	7	4HC	BuVP	17%	22%	24%	76%		DFS for advanced patients 56%. 9/10 toxic deaths in advanced patients
Laporte et al. 1994 [19]	84	36	16–53	64	20		Mafosfamide	CyTBI	NS	25%		58%	52%	20 of 125 (84 AML, 41 ALL) died from treatment-related causes
Korbling et al. 1989 [20]	52	35	17–53	22	23	7	Mafosfamide	CyTBI	5.8%	36%	53%	61%	45%	
Korbling et al. 1991 [42]	23	41	5–48	20			PBSC	CyTBI	0%	65%		35%		
Solh et al. 1989 [186]	14	9	0–17	9	5		none	BuCy	0%	0%	20%	100	92%	
Löwenberg et al. 1984 [187]	8	32	15–48	8			none	CyTBI	0%	37%		63%		
Shaw et al. 1994 [188]	23	6	0–14	21	2		none	BuCy	9%	33%	35%	62%	57%	

[a] For details on purging and conditioning, see references.
[b] Monoclonal antibody purging.
[c] fP vs. U: Purged vs unpurged.
Abbreviations: CR1, first complete remission; Rel1, first relapse; CR2, second complete remission; REFR, refractory disease; DFS, disease-free survival; PBSC, peripheral blood stem cells.

Twenty-five were in second remission and nine were in untreated first relapse. The data were obtained in a retrospective fashion from 12 centers in Great Britain. The conditioning regimen consisted of busulfan 16 mg/kg and cyclophosphamide 200 mg/kg in 19 patients and of busulfan 16 mg/kg and cyclophosphamide 120 mg/kg in 15 patients. All patients received unpurged marrow. Twelve of 25 patients grafted in second remission remained free of disease with a median follow-up of 26 months (range 8–62 months). For 10 patients, the duration of second remission exceeded that of first remission.

Meloni et al. [15] reported 24 patients who underwent autologous BMT as consolidation of second remission between 1984 and 1988. The chemotherapy schedules that were used to induce first and second remission varied. All patients received the BVAC conditioning regimen prior to autologous BMT, consisting of carmustine 800 mg/m^2, amsacrine 150 mg/m^2, etoposide 450 mg/m^2, and cytarabine 900 mg/m^2. All patients received unpurged marrow. There was one treatment-related death. Eleven of 21 patients remained alive and free of disease, with a median follow-up of 40 months (range 24 to 63). For those 11 patients, the duration of second remission exceeded that of first remission. Their experience was recently updated [16], 60 patients have been autografted in second remission since 1984. Event-free survival projected at 10 years is 41%.

Linker et al. [17] reported 26 patients who underwent autologous BMT as consolidation of second or third remission between 1986 and 1992. The chemotherapy schedules that were used to induce first and second remission varied, but two thirds of the patients had relapsed after previous exposure to high-dose cytarabine. All patients received busulfan 16 mg/kg and etoposide 60 mg/kg as a conditioning regimen prior to autologous BMT. Marrow was purged with 4-Hydroperoxycyclophosphamide (4HC). In all patients, marrow was collected during the remission immediately preceding BMT. There were nine treatment-related deaths. Thirteen patients continue in remission, with a median follow-up of 22 months (range 3 to 56 months).

Chao et al. [18] reported 12 patients who received busulfan and etoposide as conditioning for autologous bone marrow transplantation in second remission. Three received unpurged marrow, and nine received marrow purged with 4HC. Ten patients continue in remission, with a median follow-up of 31 months.

Laporte et al. [19] reported 20 patients who underwent autologous BMT as consolidation of second or third remission between 1983 and 1993. All patients received cyclophosphamide 60 mg/kg and TBI as conditioning regimen prior to autologous BMT. Marrow was purged with mafosfamide. With a median follow-up of 64 months, leukemia-free survival was 34% + 11%.

Korbling et al. [20] reported 30 patients who underwent autologous BMT as consolidation of second (22), third (5), or fourth (2) remission. All patients received hyperfractionated TBI and cyclophosphamide 200 mg/kg as a conditioning regimen prior to autologous BMT. Marrow was purged with mafosfamide. There were two treatmentrelated deaths. Actuarial disease-free

survival is 34% with a median follow-up of 19 months. In 11 of 30 patients, the remission duration after BMT exceeded that of their first remission.

These reports are representative of the majority of publications in this field. Most reports concern small groups of highly selected patients, accrued over a prolonged period of time. It is unlikely that such patient populations are representative of leukemia patients in general. Although it is undoubtedly true that prolonged disease-free survival can be obtained in some patients, it is far from clear which patients benefit most and whether autologous bone marrow transplantation is truly superior to salvage chemotherapy. Most reports provide information on the delay between remission and transplant, which accounts for bias due to time censoring [21]. Information on other important prognostic features, especially cytogenetics, is usually lacking. The exact impact of autologous BMT in the treatment of recurrent leukemia therefore remains difficult to assess.

High-dose chemotherapy and autologous bone marrow transplantation in second remission is not a trivial procedure. Hematopoietic recovery is slow, with delays in platelet recovery and neutrophil recovery commonly occurring [19]. The delayed hematopoietic recovery is probably caused by a disease-specific defect in the hematopoietic precursors, but is accentuated by the use of purging techniques [19]. It predisposes to infectious and toxic complications and leads to treatment-related mortality ranging from 5% to 33%.

2.3. Autologous BMT for AML in first remission

With modern chemotherapy, a substantial fraction of younger patients with AML can be cured [22]. The cure rates depend on prognostic features such as cytogenetic abnormalities associated with the disease, white blood cell counts at diagnosis, and response to initial chemotherapy.

There are a number of reports on autologous BMT in first-remission AML, with disease-free survival rates ranging from 33% to 66%. Although apparently superior to results of chemotherapy, these results are heavily influenced by time censoring and patient selection.

Linker et al. [17] report a disease-free survival of 100% for 15 patients with M3 or M4EO consolidated in first remission. For 17 patients with other FAB subtypes, actuarial relapse rate at three years was 48%. Meloni et al. [16] report a disease-free survival of 0 among 32 patients with high-risk disease (post-MDS, secondary leukemia, or two cycles to CR) as opposed to 42% among 127 patients with standard-risk AML. In other reports, details on prognostic features are often lacking.

The issue of patient selection has been addressed in several prospective studies confirming various consolidation modalities.

In the Dutch prospective study [23], (table 2), all patients received induction therapy consisting of an anthracycline and cytarabine ± thioguanine, followed by one consolidation treatment with the same agents. Patients under age 46 who had an HLA-matched sibling donor then underwent allogeneic

Table 2. Studies evaluating allogeneic vs. autologous BMT in AML in first remission. Patients with an HLA identical sibling undergo allogeneic BMT

Author, year, reference	N pat	Age	Conditioning	CR (%)	Allo-BMT (%)	Auto-BMT (%)	No BMT (%)[a]	Disease-free survival			Survival		
								Allo-BMT	Auto-BMT	no BMT	Allo-BMT	Auto-BMT	no BMT
Löwenberg et al. 1990 [23]	117	15–60	CyTBI	90 (77%)	21 (23%)	32 (36%)	32 (36%)	51%[b]	34%[b]	<9%[b]	66%[b]	35%[b]	
Ferrant et al. 1991 [24]	107	<56	AraCy TBI	96 (89%)	20 (21%)	33 (35%)	43 (45%)	64%[c]	28%[c]	5%[c]	71%[d]	31%[d]	9%

[a] Not transplanted for various reasons. For details, see text.

[b] $p = 0.05$.

[c] $p = 0.0098$.

[d] $p = 0.028$.

Abbreviations: CR: number of patients who achieved complete remission.

BMT; the others underwent bone marrow harvest, one additional cycle of consolidation treatment, and autologous BMT with unpurged marrow. Of 117 patients entered into the protocol, 90 achieved complete remission. Twenty-three (23%) underwent allogeneic bone marrow transplantation. Thirty-two (36%) underwent an autologous bone marrow transplantation, and 37 (41%) were not transplanted for reasons such as early relapse ($n = 18$), insufficient graft ($n = 3$), refusal ($n = 6$), and other reasons ($n = 10$). The actuarial three-year relapse-free survival was 51% for those undergoing allogeneic BMT and 35% for those undergoing autologous BMT ($p = 0.12$). For those patients who did not undergo BMT, disease-free survival was extremely poor.

Ferrant et al. [24] reported a single-institution study with a similar design. Of 107 patients entered, 96 achieved complete remission. Twenty-four patients had an HLA identical sibling, and 20 underwent an allogeneic BMT in first remission (the upper age limit for allogeneic BMT was 56 in this study). Thirty-three patients (44% of the first-remission patients) underwent autologous BMT in first remission. Forty-three patients in first remission (48% of the first-remission patients) were not transplanted, for reasons such as early relapse ($n = 9$), refusal ($n = 11$), or medical problems ($n = 23$). The four-year leukemia-free survival was 71% for allogeneic BMT recipients and 31% for recipients of autologous BMT ($p = 0.028$). An 'intention to treat' analysis shows a leukemia-free survival of 53% for those who had a donor and of 16% for those who lacked a donor ($p = 0.003$).

Both studies showed a benefit for allogeneic bone marrow transplantation over autologous BMT. They also illustrated the selection process, limiting the availability of autologous BMT to a minority of patients with acute leukemia.

Four randomized studies have compared autologous BMT with intensive consolidation chemotherapy for patients who lack an HLA identical donor (table 3). In a French study [25], patients in first remission received one consolidation treatment and were then randomized to double autologous bone marrow transplantation or to four cycles of intensive chemotherapy. Three of 15 patients randomized to undergo autologous BMT were not transplanted. In an 'intention to treat' analysis, disease-free survival was significantly better for allogeneic BMT (61%) than for autologous BMT (41%) or for intensive chemotherapy (16%). The difference in disease-free survival between autologous BMT and intensive chemotherapy was marginally significant ($p = 0.06$).

In the GOELAM study [26], patients in first remission who had an HLA identical sibling underwent allogeneic BMT. All others received one consolidation treatment with high-dose cytarabine and were then randomized to high-dose chemotherapy and autologous BMT or to a second intensive consolidation. The four-year disease-free survival was 48% for allogeneic BMT, 59% for intensive chemotherapy, and 52% for autologous BMT. None of the three treatment arms was significantly superior.

In the MRC trial [27], all patients received two inductions followed by two

Table 3. Studies evaluating autologous BMT vs. intensive chemotherapy vs. allogeneic BMT in AML in first remission. Patients with an HLA-identical sibling undergo allogeneic BMT; others are randomized to autologous BMT vs. intensive chemotherapy

							DFS			Survival			
Author, year, reference	N pat	CR (%)	Conditioning	Allo	Auto	Chemo	Allo	Auto	Chemo	Allo	Auto	Chemo	Comments
Reiffers et al. 1989 [25]	85	68 (72%)	allo:CyTBI auto:MEL×2	20	15[a]	20	66%	41%	16%				CR patients received one consolidation before BMT. Three patients assigned to ABMT were not transplanted. DFS is superior for allo BMT ($p < 0.09$).
Burnett et al. 1994 [27]	1499	81%	NS							58%[c]	54%[c]	52%[c]	Results are provided on intention to treat basis. Allo was given after four rounds of chemo. Patients without a donor were randomized to ABMT or Stop.
Zittoun et al. 1995 [189]	990	623 (66%)	CyTBI or BuCy	ass[a]:168 com[b]:144	ass:128 com:95	ass:126 com:104	55%	48%	30%	59%	56%	46%	All patients received 1–2 induction treatments and one intensive consolidation. Those without donor were randomized to ABMT or second intensive chemotherapy. DFS: ABMT > chemo ($p = 0.05$); Survival: no significant difference ABMT vs. chemo.
Harousseau et al. 1994 [26]	470	326 (77%)	allo:CyTBI auto:BuCy	57	58	57	48%	52%	56%	56%	56%	59%	CR patients received allo-BMT or one consolidation followed by autologous BMT vs. a second consolidation. DFS and survival are not significantly different between the treatment arms.

[a] ass = assigned.
[b] com = completed.
[c] p = N.S.

consolidation treatments. Those with an HLA-identical sibling underwent allogeneic BMT; the others were randomized between autologous BMT and no further treatment. Eighteen hundred patients were accrued to the trial. In a preliminary analysis of 766 patients in first remission, disease-free survival was not significantly different between the three treatment arms.

Zittoun et al. [189] reported a cooperative study by the EORTC and GIMEMA groups. Patients received one or two induction treatments followed by one intensive consolidation. Those with an HLA-identical donor then underwent allogeneic BMT. The others were randomized between autologous BMT and consolidation with one additional round of intensive chemotherapy. Overall survival and disease-free survival were respectively 59% and 55% for those assigned to allogeneic BMT, 56% and 48% for those assigned to autologous BMT, and 46% and 30% for those assigned to intensive chemotherapy, Overall survival was not significantly different between the treatment arms. Disease-free survival was significantly superior for those undergoing autologous BMT compared with those receiving intensive consolidation. This is the only large multicenter trial that has been reported in detail. It is characterized by a median age of 33 years and a large percentage of patients not completing the assigned treatments. In fact, only 343 of 424 (81%) patients assigned to a consolidation treatment completed the treatment as assigned.

Finally, in a retrospective review of patients reported to the International Bone Marrow Transplant Registry and to the North American Autologous Bone Marrow Transplant Registry, Keating et al. [29] found no difference in two-year leukemia-free survival between patients undergoing autologous BMT or allogeneic BMT in first remission.

The results of these trials and of the registry review by Keating et al. therefore fail to establish a role for autologous BMT in first remission. Most studies, in fact, indicate a recurrence rate that is substantially higher after autologous BMT than after allogeneic BMT. It is, however, not excluded that autologous BMT may benefit selected patient subgroups. Only careful analysis of prognostic features in large patient populations will be able to demonstrate this. It should also be kept in mind that unpurged bone marrow was used in the prospective studies and in most patients reported to the registry. As discussed below, it is likely that leukemic cells contained in the marrow contribute to recurrence, and conceivably results could be improved with adequate purging techniques.

2.4. Bone marrow purging

AML involves the marrow, and even at the time of complete remission, a small number of viable tumor cells remain in the marrow and may contaminate a bone marrow harvest. Several lines of evidence suggest that, at least in some cases, these cells contribute to recurrence. The Baltimore team first showed

that in vitro treatment of the bone marrow as assessed by the elimination of CFU-GM colonies was associated with a significant decrease in relapse [30]. Later, the same team correlated the sensitivity to 4HC of clonogenic leukemia cells grown in remission with the posttransplant outcome [31].

Experiments utilizing retroviral marking of infused unpurged bone marrow cells indicate that infused stem cells contribute to long-term hematopoiesis and that reinfused leukemic cells can contribute to recurrence [32] Elimination of these leukemic cells from the bone marrow prior to reinfusion may therefore substantially reduce the risk of recurrence and has been attempted by a number of investigators.

The most common way of purging marrow consists of incubating it with a chemotherapeutic agent. In the U.S., 4-Hydroperoxycylophosphamide is the most commonly utilized agent [31]. In Europe, mafosfamide, another cyclophosphamide derivative, is used [33]. Typically, cells obtained at bone marrow harvest are incubated with the chemotherapeutic agent for a predetermined period of time at a fixed concentration. These chemotherapeutic agents affect normal stem cells as well as leukemic cells. Typically, this procedure will lead to an elimination of all mature CFU-GM and will affect the rate of engraftment. One group has adapted this technique to allow adjustment of the mafosfamide dose to the sensitivity of the leukemic cells [33]. Such an approach, although cumbersome, may be important for determining the efficacy of purging methods [19].

Other methods of purging include the use of monoclonal antibodies directed against the leukemic cells. The immunophentypic heterogeneity of myeloid blasts makes such an approach applicable only to selected patients [34,35]. CD33 is an antigen expressed on the surface of leukemia cells of approximately 90% of patients with AML and may therefore be more widely applicable. Its use was associated with delayed but durable engraftment [36].

Newer methods, currently in phase I trials, rely on stimulation of LAK cells by in vitro incubation with interleukin-2 [37,38].

Finally, double autologous bone marrow transplantation with repeat harvest after the first conditioning has also been used [25,39]. The first intensive conditioning is meant to eradicate minimal residual disease and therefore can be considered as 'in vivo' purging. In at least one report, those patients who underwent double transplantation had a better outcome than comparable patients who did not undergo the second transplant [39].

None of the purging techniques has been tested in a randomized fashion, and convincing evidence of a contribution to improved disease-free survival is lacking. There is, however, substantial indirect evidence of their effect. An analysis from the European Bone Marrow Transplant Registry [40] indicated that purging of marrow with mafosfamide benefits subpopulations of patients, especially those transplanted early after achieving remission and slow remitters. Strikingly, among patients transplanted in first CR, late relapses (>1 years post-BMT) occurred only in those receiving unpurged marrow. Chao et

al. [18] also found that for patients treated with busulfan and etoposide, recurrence was lower among those receiving chemopurged marrow.

2.5. Stem cells

The use of peripheral blood stem cells is based on the observation that pluripotent stem cells reside in the peripheral blood and that they may cause persistent hematopoiesis [41]. The combination of growth factors and intensive chemotherapy allows the rapid collection of large numbers of hematopoietic progenitor and stem cells, which assures rapid and permanent hematopoietic recovery after high-dose chemotherapy. This significantly reduces the morbidity and mortality associated with this procedure. Early trials of its use in AML resulted in a rather high recurrence rate, possibly due to contamination of the stem cell product with leukemic cells [42,43].

Techniques of stem cell collection are rapidly evolving, allowing collection of the total number of required cells within a few collections in the majority of patients. It is foreseeable that in the near future single large-volume pheresis will be possible. Such developments will make purging of stem cell products feasible and may make peripheral blood stem cell transplantation the procedure of choice for patients with AML.

3. Autologous bone marrow transplantation in acute lymphocytic leukemia (ALL)

Autologous bone marrow transplantation for ALL was initially undertaken more than a decade ago. The results vary considerably depending on the stage of the disease and other factors such as age and type of ALL (determined by cytogenetic and immunophenotypic characteristics)

Pediatric ALL is a highly curable disease, and therefore transplant is recommended only for patients with recurrent disease and for occasional patients with high-risk features such as a high white blood cell count at diagnosis, hypodiploid cytogenetics, or the presence of t(9;22) or t(4;11) [44,45]. Adult ALL has a less favorable prognosis. In particular, patients with high white count at diagnosis and those with particular cytogenetic features such as Philadelphia chromosome (approximately 20% of adults with ALL) have a poor prognosis with conventional chemotherapy [46]. Investigational treatments are justified, and autologous BMT has been attempted for those who lack HLA identical donors. As in other malignancies, the original conditioning regimens were derived from those used for allogeneic BMT and included cyclophosphamide and TBI. Other chemotherapeutic agents such as etoposide and cytarabine have sometimes been added in an effort to increase the efficacy of the chemotherapy. Combinations of TBI with other agents such as melphalan [47] or VM26 [48] have also been tested, with encouraging early results.

3.1. Autologous bone marrow transplantation in recurrent ALL

Results of recently published trials are summarized in table 4. As can be noted, the results vary widely. For patients with recurrent disease, disease-free survival ranges between 10% and 50%. The results are affected by differences in conditioning and in the method of marrow purging, but most importantly by patient selection. The best results have been obtained in pediatric patients who relapsed after a prolonged first remission [44]. Some subgroups of patients with very long initial remissions had up to an 80% disease-free survival after autologous BMT. For patients with short initial remissions, disease-free survival was limited.

3.2. Autologous bone marrow transplantation for ALL in first remission

A number of patients with ALL have undergone bone marrow transplantation as consolidation of first remission. The results are summarized in table 4. Disease-free survival ranges from 21% to 66%. As in AML, the results are influenced mainly by patient selection. In those studies focusing on the high-risk patients, the outcomes are typically poor [49]. It therefore appears that high-risk patients do not benefit from autologous bone marrow transplantation. It is, however, possible that autologous BMT benefits other subgroups of patients. This issue has been addressed in two European prospective studies [50,51]. In one study [51] patients with ALL in first remission who had an HLA-identical sibling were consolidated with allogeneic BMT. All others underwent autologous BMT after conditioning with cyclophosphamide and TBI, followed by a randomization to maintenance interleukin-2. Of the 135 patients were registered, 126 achieved remission and 120 were HLA typed. Forty-three had an HLA identical sibling and underwent allogeneic BMT; 77 were to receive autologous BMT, of whom 30 were randomized to receive interleukin-2. Event-free survival was 68% for those undergoing allogeneic BMT, 29% for those receiving autologous BMT + interleukin-2, and 27% for those undergoing autologous BMT without interleukin-2 maintenance.

In the LALA87 trial [50], patients in first remission with a sibling donor underwent allogeneic BMT. Those without a donor were randomized to further chemotherapy vs. autologous BMT. Ninety-six patients were randomized to chemotherapy and 95 to autologous BMT. In this study, no significant difference was observed between any of the three treatment arms when the results were analyzed on an intention-to-treat basis. However, for patients with high-risk features, there was a trend for an improved outcome after allogeneic BMT. When comparing autologous BMT vs. chemotherapy, the incidence of late relapse was decreased among those undergoing autologous BMT.

At present, there is no clear indication for autologous bone marrow transplantation in first-remission ALL. While allogeneic bone marrow transplanta-

Table 4. Studies of autologous BMT in ALL

Author, year, reference	N pat	age		Status at BMT				Purging		Conditioning	% BMT mortality	Relapse rate		Disease-free survival		Comments
		median	range	CR1	CR2	>CR2	Rel	anti-body	chemo			CR1	All	CR1	ALL	
Kersey et al. 1987 [55]	45	<16		3	17	25	0	45	0	CyTBI (32), AraTBI (13)	4%		79%		20%	No influence of remission number or duration CR1 on survival. Update [44] DFS = 18%
Doney et al. 1993 [190]	89	18	2–47	10	27	25	27	48	2	CyTBI (81), VPCyTBI (8)	19%	27%	≥CR2: 69%, Rel: 90%	50%	≥CR2: 21%, Rel: 0%	
Blaise et al. 1990 [191]	22	31	7–47	22				18	4	CyTBI (3), MelTBI (18), CyMelTBI (1)	9%	52%		40%		
Davis et al. 1988 [48]	11	17	9–25	2	6	3		0	0	TBI + VM26, Ara, Pred	27%	0%	36%	0	36%	
Pico et al. 1986 [192]	24	10	3–37		14	5	5	4	17	BACT	21%		63%		17%	1/5 transplanted in relapse remains free of disease. 2/7 with CNS disease remain free of disease
Stoppa et al. 1990 [58]	12	16	4–47	6	4	1	1	12		MelTBI (7), CyMelTBI (2), CyTBI (2), BuMel (1)	0	50%	50%	50%	50%	
Soiffer et al. 1993 [193]	22	28	18–54	1	11	10		22		AraCyTBI (9), CyTBI (13), VPCyTBI (2)	17%	54%			20%	
Billett et al. 1993 [194]	66	8	3–18	1	42	23		66		CyTBI ± Ara	18%		60		32	Outcome is related to duration of CR1
Gilmore et al. 1991 [49]	27	18	11–45	27				27		CyTBI ± Ara	8%	67%		32%		

Schroeder et al. 1991 [47]	24	<16			17	7		6		MelTBI	16%	33%		50%		
Zintl et al. 1990 [195]	15	7	1–11	6	7	2		1		CyTBI	7%	54%	31%	43%		
Hervé et al. 1991 [196]	311	<16		66	177			32%	39.5%	TBI (69%), other (31%)	8%		45%		CR2: 33%	Registry survey. 66 patients >CR2 or relapse, no details reported.
Colleselli et al. 1991 [197]	56	11	4–19		36	20			32	VCR, Cy, TBI (29) other (27)	14%	45%		21%		
Spinolo et al. 1990 [11]	7	27	21–37		3	4		0	0	CBV	0		86%		14%	
Laporte et al. 1994 [19]	41	29	17–55	35	6			0	41	CyTBI	10%	37%		57%		
Cahn et al. 1986 [198]	26	12		6	16	3	1	9	13	AraMelTBI		62%		66%	28%	Two patients received marrow purged with mafosfamide and monoclonal
Cahn et al. 1991 [178]	15			4	11			4	11	CyTBI (14), BACT (1)	7%	50%	27%	50%	67%	11 children, 4 adults
Gonzalez-Chambers et al. 1991 [59]	10	33	13–52	1	6		3	0	10	CyTBI	20%	50%		30%		
Santos et al. 1989 [60]	31			12	19			0	31	CyTBI	71%	CR2 88%	25%	CR2 10%		
Fière et al. 1994 [50]	95		15–50	95				NS	NS	NS	NS			41%		DFS allo-BMT, chemo and auto-BMT is similar
Attal et al. 1994 [51]	77		<55	77				0	0	CyTBI ± IL-2				26%		DFS alloBMT > AutoBMT (see text)

Abbreviations: CR1, first complete remission; CR2, second complete remission; >CR2, beyond second complete remission; Rel, transplantation in early or over relapse.

tion seems to benefit at least a fraction of patients that are incurable with other treatments [52,53], such a patient population has not been identified for autologous BMT. Application of this procedure to all patients in first CR does not appear to improve their outcome and cannot be recommended outside an investigational protocol.

3.3. Marrow purging in ALL

In ALL, as in AML, it is likely, though unproven, that residual leukemia cells contaminate the marrow and contribute to recurrence in patients undergoing autologous BMT. For this reason, virtually all BMT studies in ALL have utilized purging of autologous marrow with either monoclonal antibodies plus complement [54,55], immunotoxins [56,57], antibody-bead immunoconjugates [58], or pharmacologic agents such as mafosfamide [19] or 4HC [59,60].

The phenotype of B-cell or pre-B-cell ALL is fairly well defined and uniform, thus allowing the application of a monoclonal antibody technique to the majority of patients. Typically, antibodies are directed against lymphoid antigens that spare myeloid hematopoietic progenitors. Such antigens include CD9, CD10, CD19, and CD24 [44]. Antibodies used for purging of T-cell leukemias have targeted antigenic determinants with broad reactivity. These include CD5 and CD7 and have been used alone or in combination with 4HC [57,61]. Randomized studies of purging agents are not available; therefore, the exact contribution of purging in preventing disease recurrance cannot be accurately assessed. It is likely that purging, by eliminating residual leukemia cells, contributes to cure in some patients. Similarly, in other patients, residual leukemia is probably responsible for recurrence, as has been suggested by studies correlating the pretransplant leukemia burden with recurrence [62].

4. Autologous bone marrow transplantation in chronic myelogenous leukemia (CML)

The natural history of CML involves an indolent chronic phase followed by a disease acceleration or a blast crisis that is rapidly fatal. Conventional chemotherapy has not resulted in prolonged survival or duration of the chronic phase. Newer approaches involving interferon chemotherapy have resulted in prolongation of the chronic phase and an improved survival for a minority of patients [63]. But allogeneic and syngeneic bone marrow transplantations remain the only curative treatment modalities for CML [64]. Cure is effected by dual mechanisms in allogeneic BMT. One mechanism is cytoreduction by high-dose chemoradiotherapy, and the other is a poorly defined graft vs. leukemia effect that contributes to eradication of minimal residual disease. In syngeneic BMT, graft vs. leukemia effects are not present, and although the cure rates are lower than with allogeneic BMT, they are still in the range of 40% [65,66].

4.1. Autologous bone marrow transplantation for CML in chronic phase

Autologous BMT for CML in chronic phase relies on the following concepts: (1) the results of syngeneic BMT could be imitated if the patients could be provided with a stem cell rescue product that contains only nonmalignant hematopoietic progenitors; and (2) if no complete eradication of the leukemic clone can be achieved, a massive reduction in tumor cell burden will reduce the number of target cells for secondary mutational events, and thus delay the onset of blast crisis.

Different methods have been used for purification of benign hematopoietic cells in CML. Some are nonspecific and based on in vitro growth advantages for normal progenitors [67] or on collection of cells during the transient phase of Philadelphia negative hematopoiesis that is sometimes achieved after intensive chemotherapy [68–70]. Other methods rely on differences in immunophenotype between Philadelphia positive cells and normal hematopoietic progenitors [71–73]. Finally, in recent experiments, antisense oligonucleotides are used directed against the BCR-ABL gene or against the MYB oncogene [74,75].

The results of autografting of CML in chronic phase are summarized in table 5. Early studies were performed with bone marrow or peripheral blood cells collected during the chronic phase of the disease. Such an approach resulted in rapid engraftment in most patients and the appearance of Philadelphia negative hematopoiesis in some. Occasionally, failure of engraftment has occurred. Cytogenetic remissions were usually partial and transient, and there is conflicting evidence on whether autologous bone marrow transplantation with unpurified marrow leads to a survival benefit for the patients.

Hoyle et al. [76] recently published an update on the British experience with 21 patients treated between 1984 and 1992 for CML in the chronic phase. Twelve of these patients survived at a median of 82 months from the time of autografting (range 9 to 105 months), six died of leukemia in transformation, and three died from other causes. Autografted patients had a significantly longer survival at five years postautograft than chemotherapy patients (56% vs. 28%). Eleven of 17 patients who had cytogenetic analyses performed in the first six months after transplantation had some Philadelphia-negative cells detected. After 12 months, only three patients had Philadelphia-negative cells.

Mc Glave et al. compiled results from seven different centers [77]. Approximately 40% of the patients transplanted in chronic phase achieved a prolonged survival.

On the other hand, Khouri et al. [78] compared patients undergoing autologous BMT for CML chronic phase with patients who were concurrently treated without transplantation. Patients were matched for prognostic features such as age, decade of therapy, and response to interferon. No significant improvement in survival could be found for those undergoing bone marrow transplantation. Therefore, at the current time, autologous BMT for CML in

Table 5. Studies of autologous BMT for CML in chronic phase

Author, year, reference	N pat	Conditioning	Cells			% Ph- neg methaph (in evaluable patients)	Median survival after BMT	Comments
			Source	Time of harvest	Manipulation			
Khouri et al. 1994 [78]	22	NS	BM	NS	none	NS	34 Mo	Survival not significantly different from matched controls
Barnett et al. 1994 [67]	16	VP, Cy, TBI (1), Bu, Cy, Mel (15)	BM	CP	10-day culture	100% (in 11/13 patients), 79%–94% (2 patients)	Not reached, survival 81%, median follow-up 2.6 years	Four graft failures. All responding patients had cytogenetic recurrence.
Hoyle et al. 1994 [76]	21	BuMel (13), CyTBI (3), Bucy (4), Bu (1)	PBSC	CP	none	some Ph-ve metaphases in 11/17 patients	5-year survival 56%	All patients had recurrence of the Ph-clone. Survival is significantly better than that of a historical control group, not treated with BMT
McGlave et al. 1994 [77]	142	various	BM or PBSC	NS	none, IFN, in vitro culture	NS	Actuarial survival 58%	Pooled results from 8 transplant groups.
De Fabritiis et al. 1990 [199]	12	Bu, Mel	BM (10), PBSC (2)	100% Ph-neg (4) 23–98% Ph-neg (8)	none	100% (2/12)10–90% (8/12)	NS	11/12 remain in chronic phase 18 months (16–20) after BMT
McGlave et al. 1994 [200]	22	CyTBI	BM		In vitro IFN	15–100%	Actuarial survival 71%	All patients had recurrence of the Ph- clone
Carella et al. 1994 [80]	11	CyTBI	PBSC	100% Ph-neg	none	100%		5/11 remain alive an 100% Ph-ve
Simonsson et al. 1994 [81]	26	NS	BM	100% Ph-(20), Ph+(6)	none	NS	23 patients are alive	

For details of conditioning regimens, see references

the chronic phase remains an investigational treatment and should not be applied outside the setting of a clinical trial.

On the other hand, there are good indications that the use of Philadelphia-negative, or partially Philadelphia-negative stem cell products will contribute to prolongation of remissions after autologous BMT. Indeed, in our experience, a direct correlation could be observed between the level of Philadelphia-negative cells in a bone marrow or stem cell product and the percentage of Philadelphia-negative cells after transplant in the patients [79]. In addition, the Italian and Swedish experience with Philadelphia-negative products obtained after intensive chemotherapy [68,80,81] and the Canadian experience with in vitro cultured marrow indicate the frequent occurrence of cytogenetic remissions and achievement of prolonged hematologic remissions in small groups of patients [67]. McGlave et al., utilizing interferon treated marrow, also obtained a high incidence of transient cytogenetic remissions. With current technologies, cytogenetic remissions are still transient, and it is unlikely that any of the patients thus treated are cured. It can be hoped that further improvements in cell manipulation techniques will ultimately result in the procurement of products containing only normal hematopoietic progenitors, which may result in restoration of permanent normal hematopoiesis.

4.2. Autologous bone marrow transplantation for CML in accelerated phase or blast crisis

Autologous BMT has frequently been attempted in transformed CML in an attempt to eradicate the transformed clone and to return the patient to a chronic phase. Since the original report by Buckner et al. [82], a number of cases have been reported of autologous bone marrow transplantation for CML in blastic phase with marrow collected during the initial chronic phase of the disease. The majority of these reports are summarized in table 6. While such approaches have usually resulted in rapid engraftment and a return to chronic phase, transformation has usually rapidly reoccurred. This result is not surprising, given the equally poor results obtained with allogeneic bone marrow transplantation in this disease [63].

Reiffers et al. [83] indicated that treatment with interferon alpha could prolong the duration of the second chronic phase after autologous BMT and prolong the median survival to approximately 19 months after transplantation. The use of Philadelphia-negative products, obtained after intensive chemotherapy [68,79,80] or by in vitro culture [67], has resulted in the achievement of more prolonged hematologic and cytogenetic remissions in some patients. Therefore, it can be hoped that, as in chronic-phase CML, further improvements in stem cell purification techniques will result in better outcome for transplantation even in this disease. Given the extreme chemotherapy resistance of transformed CML, it has to be anticipated, however, that despite infusion of benign hematopoietic progenitors, recurrence may often occur due to endogenous leukemia cells surviving the conditioning.

Table 6. Autologous bone marrow or stem cell transplantation in transformed CML

Author, year, ref	No. patients			Conditioning	Cells		% BMT mortality	Achievement of CP2		% Ph neg cells (No of pat with Ph-neg cells)	Median survival in months
	total	Acc/ CP2	BC		Source	Time of collection		% pat	duration		
Karp et al. 1985 [201]	7		7	BCNU ± Ara	PBSC	CP	14%	71%	NS	0	11 (2–16)
Buckner et al. 1977 [82]	7		7	CyTBI (4), BCNU, CyTBI (3)	BM	CP	86%	43%	NS	54% (in 1 of 4)	2 (1–4.5)
Preisler et al. 1984 [202]	16		16	Ara + Dox + VC + Pred + Ara (10); Ara (4), Ara, Bu (2)	PBSC (15), BM (1)	CP	5/16	31%	11 (1–12)	100 (IN 1 Patient)	7.5 (0.5–17)
Vogler et al. 1983 [203]	1		1	CyTBI	BM	cytogenetic remission	0	100%	>14 mo	100%	>14 mo
Thomas et al. 1984 [204]	11		11	AD (4), MadHAt (7)	BM	CP	NS	55%	5.5 (2–8.5)	NS	4 (0.5–11)
Lemonnier et al. 1986 [205]	13	4	10	TACC (10), TACC + TBI (3)	BM	CP	30%	46%	4(1.5–26)	100% (in 1 of 5)	3 (0.5–34)
Phillips and Herzig 1984 [206]	4		4	CyTBI	BM	CP		50%	6, 26	100% (in 1 patient)	7.5 (2–32)
Vellekoop et al. 1986 [69]	11	1	10	PipTBI	BM	CP (8) cytogenetic remission (2), partial cytogenetic remission (1)	27%	56%	5.5 (4–12.5)	100% (3/6), 95% (1 of 6) 5% (1/6)	5.5 (1–19)
Kantarjian et al. 1991 [207]	7	6	1	CBV	BM	CP (3), cytogenetic remission (1) partial cytogenetic remission (3)	14%	0 of 2[a]		100% (2/6), 92% (1/6), 55% (1/6), 45% (1/6), 20% (1/6)	13 (1–19)

Barnett et al. 1994 [67]	6	5	1	VPCyTBI (2), BuCyMel (4)	BM + 10 d culture	CP2 (1), CP3 (1), AP (3), BP(1)	17%	4/4[b]	NS	100% (4), 94% (1), 85% (1)	19 (1–>51)
Haines et al. 1984 [208]	51	6	45	DAT ± MEL (9); DAT ± TBI (12), Super-RATE ± MEL (30)	PBSC	CP	NS	94%	NS	14–36 (in 3 of 11)	6 (0.5–35)
Reiffers et al. 1991 [83]	47[c]	23	24	grp 1 Super RATE (4), DAT + Cy + TBI (13)	BM	CP	29%	91%	3 (1–19)	100 (1 of 29), 66–99 (3 of 29)	~3.5 (NS–21)
				grp2:Ara, Cy, TBI (13)			0		10 (1–48+)	36–65 (5 of 29)	~9.5 (1–49+)
				grp3:Bu, Mel (17)			0		18 (1–39+)	10–35 (5–29)	~19 (NS–40+)
Carella et al. 1991 [68]	5		5[d]	VP, Cy, TBI	PBSC	cytogenetic remission (5)	100%			100% (in 5 of 5)	NS (5+–15+)
McGlave et al. 1994 [77]	58	30	28[e]	various	BM or PBSC	NS					AP: 36 months (SE: 24 months) BC; 4 months (SE: 5 months)
McGlave et al. 1994 [200]	22	22		CyTBI	BM + γIFN	NS		NS			Actuarial survival 15% at 3 years

[a] 5 were treated in 2nd CP.

[b] 2 were harvested and treated in CP2 and CP3, respectively.

[c] grp1, single autograft followed by hydroxyurea; grp2, double autograft, second graft conditioned with MEL, maintenance with hydroxyurea; grp3, double autograft, maintenance with interferon.

[d] All achieved transient chronic phase after intensive systemic chemotherapy.

[e] Some were in second chronic phase (exact number not specified); pooled data from 8 centers.

For details of conditioning regimens, see references.

5. Autologous bone marrow transplantation in intermediate and high-grade non-Hodgkin lymphoma (NHL)

5.1. Outcome of high-dose chemotherapy and conditioning regimens in recurrent lymphoma

Patients with recurrent NHL have a poor prognosis with conventional dose salvage treatment. Complete remission rates of approximately 30% can be achieved, but only 25% of the complete remissions are durable [26]. When patients with chemosensitive recurrences are consolidated with high-dose chemotherapy, cure rates of 30% to 50% have been reported [75,84–95]. The mortality ranges from 5% to 20% and depends heavily on patient selection. Result of several large studies are summarized in table 7. These studies include a majority of patients with intermediate grade and immunoblastic lymphoma, but also a substantial number of patients with other disease histologies. Many different conditioning regimens have been used. Some are based on regimens used for allogeneic BMT and use combinations of cyclophosphamide and TBI [58,85,86,89,96–101]. In some regimens, cyclophosphamide has been combined with or replaced by other drugs such as etoposide [75,95,102], melphalan [58,103], or cytarabine [87,97,104]. TBI-containing regimens are effective but also potentially toxic, especially when used in patients who have previously received radiation to the lung and mediastinum [105]. Pulmonary complications seem to be even more frequent when cytarabine has been incorporated in TBI-containing regimens [87,97,104]. An increased incidence of secondary hematologic disorders has also been associated with the use of TBI-containing regimens [70].

Chemotherapy-based regimens have several advantages, including ease of administration and potentially reduced side effects. A commonly used regimens is the BEAC regimen consisting of high-dose BCNU, cytarabine, etoposide, and cyclophosphamide [92,93,106]. Although highly effective, this regimen has been associated with a high incidence of cardiac and pulmonary toxicity [92,93]. This is possibly due to the association of BCNU and cyclophopshamide with cytarabine, and was also noted with the BACT and TACC regimens [93,107–109]. BVAC is similar to BEAC, but with a dose reduction in cyclophosphamide and an increase in the doses of BCNU and cytarabine. In one report, it was associated with a high incidence of fatal sepsis in patients with NHL (9 out of 21 patients) [110]. The CBV regimen, a combination of carmustine, etoposide, and cyclophosphamide, has not been associated with excessive toxicity [94]. BEAM has been used frequently in Europe [91,111]. In it, cyclophosphamide has been replaced with high-dose melphalan. Other regimens incorporate platinum compounds: examples include cisplatinum, etoposide, and cyclophosphamide (CVP) [112–114], ifosfamide, carboplatin, and etoposide [115]; and cyclophosphamide, carboplatin, and etoposide [116]. Such regimens are active and have a low treatment-related mortality. Busulfan and cyclophosphamide have been rarely

Table 7. Autologous BMT in aggressive lympoma; selected phase II studies

Author, year, reference	N Pat	Conditioning	pbsc	bm	Age (range)	Histology				Disease status				%BMT Mortality	Disease-free survival	Relapse
						LGL	TR	IGL/ + IBL	LBL/ DSNCC	HODGKIN	CR1	SR/UR	REFR			
Gulati et al. 1988 [124]	31	CyTBI	0	31	27 (15–41)	0	2	29	0	0	14	11	6	16%	48%	35%
Nademance et al. 1992 [123]	20	CyVPTBI (17) OR CBV (3)	16	4	29 (19–54)	0	0	16	4	0	20	0	0	0%	85%	15%
Freedman et al. 1993 [122]	26	CyTBI	0	26	43 (21–59)	0	0	25	1	0	26	0	0	0%	84%	11%
Weisdorf et al. 1991 [86]	70	CyTBI (57), CyBCNUAra (13)		70	37 (2–63)	15		34	21	0	6	43	21	18%	23%	
Vose et al. 1993 [90]	158	TBI (89) Non-TBI (69)	53	105	40 (3–68)	0		158	0	0	0	90	68	NS	32%	
Rapoport et al. 1993 [92]	100	BEAC (90), OTHER 10	13	86	40	11		42		47		73	27	12%	act 40%	34%
Wheeler et al. 1993 [94]	78	CBV	2	76	43 (16–66)	0	10	58	8	0	3	58	17	8%	58% IGL/IBL	
Philip et al. 1987 [84]	100	CyTBI (39), CHEMO (61)	0	100	35 (16–61)			100			0	44	66	34%	19%	
Gribben et al. 1989 [111]	50	AraCyBCNU (8)[a], BEAC (11), BEAM (31)[b]	0	50	38 (16–57)	0		31	9	0	0	20	29	20%	24%	54%
Colombat et al. 1990 [91]	46	TACC (12) BEAM (23) CyTBI (10)	0	46	35 (16–62)	7		20	19	0	12	26	8	11%	55%	
Phillips et al. 1990 [96]	68	CYTBI	0	68	39	12		38	16			22	46	21%	16%	57%
Gulati et al. 1992 [102]	44	CY/VPTBI	0	44	37 (25–56)	9	0	35	0		0	35	9	36%	act 57%	6%

Table 7. (Continued)

Author, year, reference	N Pat	Conditioning	pbsc	bm	Age (range)	Histology				Disease status				%BMT Mortality	Disease-free survival	Relapse
						LGL	TR	IGL/ + IBL	LBL/ DSNCC	HODGKIN	CR1	SR/UR	REFR			
Takvorian et al. 1987 [85]	49	CYTBI	0	49	42 (23–63)	6	19	43			8	41		4%	63%	27%
Lazarus et al. 1992 [113]	79	BCNU, VP, PLAT	0	79	36 (16–63)	5		33	4	35		41	38	7.5%	50%	42%
Freedman et al. 1990 [99]	100	CyTBI	0	100	43 (24–64)	34		64	2			100		2%	61% act 50%	39%
Gingrich et al. 1990 [110]	41	BVAC	0	41	16–59	2		18	1	20				38%	48%	12%
Przepiorka et al. [118]	34	TBC	10	24	42 (21–60)	4		26	4		3	24	7	15%	act 35%	32%
Peetersen et al. 1990 [89]	100	various	100		33 (3–61)	5		48	26	20	3	24	73	21%	27%	52%
Horning et al. 1994 [75]	96	CyVPTBI	32	64	15–55	10		57	5	24	10 (NHL)	47 (NHL)	15 (NHL)	7.8%	60% for NHL	29% NHL
Weaver et al. 1994 [95]	53	CyVPTBI	12	41	44 (18–56)	12		13	18	10	1	31	21	17%	58%	28%
Van Besien et al. [93]	48	BEAC	47	1	41 (17–61)		5	43			5	32	11	25%	31%	43%

[a] 3 double transplants.

[b] 1 double transplant.

For details of conditioning regimens, see references.

Abbreviations: LGL, low-grade lymphoma; TR, transformed lymphoma; IGL, intermediate grade lymphoma; IBL, immunoblastic lymphoma; LBL, lymphoblastic lymphoma; CR1, first complete remission; SR, sensitive relapse; UR, untreated relapse; REFR, refractory disease; act, actuarial.

used as conditioning regimens for non-Hodgkin's lymphoma [117]. Recently, we have used the combination of busulfan, cyclophosphamide, and thiotepa in a group of patients with end-stage lymphoma [118]. Grade 3–4 toxicity occurred in 14% of the patients treated at the highest dose level. Disease-free survival at 18 months was 35%.

5.2. Prognostic factors in transplantation for recurrent lymphoma

Several prognostic features for the outcome of high-dose chemotherapy and autologous BMT for lymphoma have been recognized. Those patients with disease that does not respond to salvage chemotherapy usually do not benefit from intensification treatment [84,89,90,92–94,102,105]. Performance score at the time of transplantation is another important predictor for the outcome of BMT, but may be related to the stage and responsiveness of the disease [89,96].

Several other features have been recognized that allow a more adequate separation for good and poor prognosis patients. Vose et al. [90] constructed a prognostic model based on four parameters. Those with a tumor mass of 10 cm or more, or with two of the following characteristics: elevated LDH; chemotherapy resistance; or three or more prior chemotherapy regimens, had a three-year failure-free survival of 10% compared with a 45% three-year failure-free survival in other patients. In the good prognosis group, failure-free survival was significantly better for those receiving peripheral stem cell transplantation as opposed to bone marrow transplantation.

Appelbaum et al. [105] identified a group of patients with advanced disease who had a poor prognosis. This group included those with refractory disease and those with more than one prior recurrence.

In other studies, similar or slightly different prognostic variables have been identified [92–94,102,119]. Outcome of autologous BMT is in all studies related to tumor bulk, measured by either LDH [90,93] or tumor size [90], and to the chemosensitivity of the disease, as identified by the number of recurrences [90,93,105], the response to the last chemotherapy [90,92–94,96,102,105], or in some studies disease histology [94]. Other important predictors of outcome include the performance score [89,119] and a history of prior chest irradiation when TBI containing regimens are used [89,119].

Finally, an intriguing observation was reported by the Nebraska group, who found that in good-prognosis patients, recurrence rates were lower after peripheral stem cell transplantation compared with autologous bone marrow transplantation [90]. This finding has been attributed to a possible immune effect of lymphocytes infused during autologous stem cell transplant. In an analysis of patients with Hodgkin's disease treated at the same institution, no difference in outcome was found between stem cell and bone marrow recipients [95].

5.3. *Chemotherapy vs. autologous BMT for recurrent lymphoma*

Patients enrolled in BMT trials are selected by age and performance status and possibly other factors. The survival advantage for patients treated with high-dose chemotherapy and autologous BMT can therefore at least partially be explained by patient selection.

The Parma trial was specifically designed to compare conventional-dose salvage chemotherapy with transplantation. Patients with recurrent intermediate grade or immunoblastic lymphoma were treated with DHAP chemotherapy. After two cycles, they were randomized to consolidation with BEAC and autologous BMT vs. further treatment with DHAP. The final analysis of this study has recently been reforted and showed a significant advantage in survival (53% vs 32%, P = 0.038) and disease-free survival (46% vs 12%, P = 0.001) for patients with chemotherapy sensitive disease, who underwent BMT.

One other study compared in a retrospective fashion the outcome of conventional chemotherapy with bone marrow transplantation for patients with recurrent lymphoma after front-line treatment with a uniform regimen, the LNH84 protocol [121]. After reinduction, some patients were consolidated with autologous BMT, and others received chemotherapy alone. The outcome was significantly better for those undergoing autologous BMT, even after correction for age and response to chemotherapy.

5.4. *Autologous BMT in NHL in first remission*

Based on the encouraging results with high-dose chemotherapy for patients with recurrent lymphoma, pilot studies have addressed the use of autologous BMT in patients with high-risk lymphoma in first remission [122–124]. Unfortunately, commonly used risk factors in lymphoma predict better for achieving a remission than for maintaining it [125], and a substantial number of patients enrolled in these studies may already have been cured prior to the transplant.

The issue of whether or not high dose chemotherapy benefits patients with intermediate grade lymphoma prior to recurrence can be better addressed in randomized studies. Three such studies have been recently completed.

1. In the French study [74], patients with poor prognostic features who achieved complete remission after four induction cycles of conventional chemotherapy were randomized to intensification or to sequential chemotherapy. The intensification treatment consisted of two cycles of high-dose methotrexate followed by high-dose CBV and autologous BMT. Sequential therapy consisted of two cycles of high-dose methotrexate followed by several rounds of treatment with a combination of ifosfamide, etoposide, asparaginase, and cytarabine.

Between October 1987 and December 1991, 881 patients were registered. Of these, 790 patients were eligible for analysis, 520 (66%) of whom achieved a complete remission. Fifty-six patients were not randomized because of re-

fusal (n = 26), poor performance status (n = 20), or protocol violation (n = 10); 460 patients were randomized, 230 to BMT and 234 to sequential chemotherapy. However, only 174 of 230 patients scheduled to undergo BMT did so. The reasons for not undergoing BMT were patient refusal (n = 26) and early relapse (n = 23). Two patients died of transplant-related complications, and one patient in the sequential chemotherapy arm died from embolic complications related to asparaginase treatment.

All analyses were carried out on an intention-to-treat basis. The three-year disease-free survival rate was 52% (45%–59%) in the sequential chemotherapy arm and 59% (52%–66%) in the autologous BMT arm (p = 0.46). Three-year disease-free survival for those patients who actually underwent autologous BMT was 68%. When the international index [125] was applied to the patients studied, approximately 50% of the patients were found to be in a high-risk category (C or D). Ninety-five such patients had been randomized to chemotherapy and 108 to autologous BMT. Three-year disease-free survival was 41% for those in the chemotherapy arm and 60% for those in the autologous BMT arm (p = 0.07). Therefore, the benefit of intensification may apply only to the subgroup of patients who truly were in a high-risk category [228]. Also, 23 of the 87 recurrences in the autologous BMT arm occurred before the patients were actually transplanted, and this may explain partially the relatively poor results in the autologous BMT arm.

In a Dutch study [126], patients with stage II–IV lymphoma who had a partial remission after three induction cycles of CHOP chemotherapy, but no bone marrow involvement, were randomized to either three additional cycles of CHOP or to autologous BMT after conditioning with cyclophosphamide and TBI. Of the 294 patients registered, 234 were evaluable after three courses of CHOP. Complete remission was observed after three cycles in 94 of 234 (40%), a partial remission without marrow infiltration in 89 of 234 (38%), a partial remission with marrow infiltration in 21 of 234 (9%), and no response, progressive in 30 of 234 (13%). Sixty-one patients were randomized — 30 to autologous BMT and 31 to CHOP chemotherapy. In the autologous BMT arm, 7 patients never received autologous BMT, 8 relapsed, 2 died from toxicity, 12 are in complete remission and 1 is too early. In the CHOP arm, 13 showed a relapse, 17 are in CR, and 1 is too early. The conclusions of this study, which was reported in abstract form, are that autologous BMT does not improve the outcome of slow responders and that slow response does not predict for outcome in general.

Finally, Gianni et al. [127] reported a comparison between MACOP-B versus an innovative high-dose sequential chemotherapy program supported by growth factors and autografting. Preliminary findings suggest a better tumor response in patients receiving high-dose chemotherapy, but survival reportedly is not different, perhaps due to lack of follow-up and due to a high treatment-related mortality in the transplant group [127].

While these reports provide interesting information and indicate a potential role for autologous BMT in high-risk patients, the results are preliminary and

further studies are needed. Possibly such studies should specifically target very high-risk populations, including those with a high tumor burden who fail to achieve remission with conventional treatments [128].

5.5. *Burkitt's and lymphoblastic lymphoma*

High-grade lymphomas account for a large proportion of childhood lymphomas but represent only a small proportion of adults with non-Hodgkin's lymphoma and are therefore usually included in large series of patients with aggressive lymphoma, without specifying their results.

In Burkitt's lymphoma, high-dose chemotherapy and autologous bone marrow transplantation have been attempted in patients with recurrent disease, but also as consolidation of first remission and as intensification treatment for those with partial responses.

The results of autologous BMT for patients with recurrent disease are summarized in table 8. The majority of patients received chemotherapy-based

Table 8. Result of autologous BMT in recurrent and refractory DSNCC lymphoma (Burkitt's and non-Burkitt's); individually reported cases

				Outcome		
Author, year, reference	Conditioning	Age	N pat	DFS	Progression	Treatment-related death
Petersen et al. 1989 [87]	AraTBI	24	1		1	
Philip et al. 1986 [129]	BACT	3–16	12	4	7	1
	BEAM	3–24	6	3	1	2
Philip et al. 1992 [209]	BEAM	17, 22	2	2		
Gaspard et al. 1988 [210]	BEAM	8	1	1		
Stoppa et al. 1990 [58]	Bu-mel	15	1		1	
Appelbaum et al. 1978 [211]	BACT	3–33	14	3	7	4
Vaughan et al. 1987 [212]	Ara-Cy-TBI	26	1		1	
Ostronoff et al. 1992 [213]	BACT	18–39	4		4	
	BEAM	38	1	1		
	Bu-Cy	17	1		1	
Hartmann et al. 1984 [214]	BACT	3–13	10	4	4	2
Avet-Loiseau et al. 1991 [215]	Bu-Cy	7–18	5	1	4	
	Bu-Mel	813	2	2		
	Bu-Cy-Mel	3–10	6	1	5	
Total			67	22 (33%)	36 (54%)	9 (13%)

For details of conditioning regimens, see references.

Table 9. Result of autologous BMT in DSNCC lymphoma (Burkitt's and non-Burkitt's) in first remission or partial response

Author	Type BMT	Conditioning	Age	N pat	Outcome
Transplantation in CR 1					
Vaughan et al. 1987 [212]	auto	BACT	4	1	Progression day 35
Strauss et al. 1991 [216]	auto	TBI-CY-VP	?	3	DFS 3
Philip et al. 1986 [129]	auto	BACT	4–14	4	DFS 1 (657+ days) progression 3 (day 30, 46, 60)
		BEAM	6–10	4	DFS 1 (277+ days) progression 2 (day 35, day 37), sepsis 1
		Cy	10	1	CR 657+
Stoppa et al. 1990 [58]	auto	Mel-TBI	41, 43	2	DFS 15+ months, 11+ months
Ostronoff et al. 1993 [213]	auto	Cy-TBI	29, 26	2	+toxicity 2
Total				17	DFS 8
Transplantation in PR 1					
Petersen et al. 1989 [87]	auto	Ara-TBI	19	1	Progression + day 27
Vaughan et al. 1987 [212]	auto	BACT	29	1	Progression + day 41
		Cy-TBI	26	1	Progression + day 35
Philip et al. 1988 [130]	auto	BACT	4–8	8	DFS 5 (46, 47, 49, 52, 70 months) progression 2 (day 60 and day 70), toxic death 1
		BU-Cy	15	1	DFS 27+ months
		BEAM	7	1	DFS 26+ months
Avet-Loiseau et al. 1991 [215]	auto	Bu-Cy	16	1	DFS 276+ weeks
		Bu-Mel	5	1	DFS 116+ weeks
		Bu-Cy-Mel	3	1	DFS 88+ weeks
Kaizer et al. 1979 [217]	auto	Adr-Cy-TBI	6	1	DFS 25+ months
Total				17	DFS 11

For details of conditioning regimens see references.

regimens, although the use of TBI-containing regimens has been reported as well. Overall, approximately 30% of the reported patients achieved long-term disease-free survival. Patients with refractory disease had a poor outcome [129]. The inclusion of many patients with poorly responsive disease probably accounts for the very high failure rates in adult patients.

The results of autologous BMT in first complete or partial remission are summarized in table 9. Philip et al. [130] used high-dose intensification in 10 children with Burkitt's disease in partial response after induction therapy. The presence of residual disease was demonstrated by biopsy in seven patients and by the presence of persistent neurologic symptoms in three. One patient died from infectious complications, two relapsed, and seven achieved durable

Table 10. Results of autologous BMT in recurrent or refractory lymphoblastic lymphoma; individually published cases

Author	Conditioning	Age	N pat	Outcome		
				Disease-free survival	Progression	Treatment-related death
Petersen et al. 1989 [87]	Ara-TBI	34	1		1	
Avet-Loiseau et al. 1991 [215]	Bu-CY (2), Bu-Mel (2), Bu-Cy-Mel (4)	7–17	8	1	7	
Hartmann et al. 1984 [214]	BACT	5–14	6	1	4	1
Sweetenham et al. 1994 [218]	various	16–57	109	CR2:29%, REFR < 15%		25

[a] One patient died from sepsis six years after BMT.
For details of conditioning regimens, see references.

Table 11. Results of autologous BMT for lymphoblastic lymphoma in first remission

Author, year, reference	Conditioning	Age	N pat	Outcome		
				Disease-free survival	Progression	Treatment-related death
Sweetenham et al. 1994 [218]	various	16–53	63%			6 (5%)
Santini et al. 1989 [219]	CyTBI	15–43	18	14	4	0
Milpied et al. 1989 [220]	CyTBI (9), MelTBI (4)	16–39	13	9	4	0
Baro et al. 1992 [221]	various	14–52	14	11	2	1
Verdonck et al. 1992 [222]	CyTBI	16–31	9	6	2	1 (secondary AML)
Morel et al. 1992 [136]	CyTBI		5	3	2	0

For details of conditioning regimens, see references.

remissions, indicating the efficacy of this approach in this poor prognosis setting.

High-dose chemotherapy and autologous bone marrow transplantation as consolidation of first remission in high-risk patients has only rarely been attempted [129,131], and it is unclear whether cure rates are improved by

intensive consolidation in patients who have already achieved a complete remission.

The results with autologous BMT for lymphoblastic lymphoma have recently been reviewed by the European Bone Marrow Transplant Registry. The Registry analyzed 214 patients, including 105 who underwent BMT in first remission. The six-year actuarial survival was 63% for patients transplanted in first complete remission, 31% for those transplanted in second complete remission, and 15% for those with resistant disease. The results in patients with recurrent disease are consistent with those reported by others, as summarized in table 10. These results indicate that a fraction of patients with recurrent disease are cured by autologous BMT. As in ALL, allogeneic BMT has better results and should be considered the treatment of choice in patients with a sibling donor [132].

The results in first complete remission are summarized in table 11. They are encouraging but can at least be partially explained by patient selection and time censoring and are not necessarily better than those obtained with intensive chemotherapy approaches [133–136].

6. Autologous BMT in low-grade lymphoma

Low-grade lymphomas are indolent disorders with a median survival of 7 to 9 years. Several features allow the identification of patients with more aggressive disease. These features include tumor bulk (stage and size of lymph nodes, LDH), response to chemotherapy, and, for patients with follicular lymphomas, male sex. At the time of first recurrence, the prognosis is usually limited [137–138]. Conventional salvage regimens, while effective, rarely lead to prolonged remissions, and the use of more aggressive regimens appears appropriate.

Several groups have used autologous BMT as treatment for recurrent low-grade lymphoma [139,140]. The patients usually had chemosensitive recurrences and underwent autologous BMT after achieving a second complete remission or a good partial remission. Because of the frequent involvement of the bone marrow with lymphoma, purging procedures are usually applied.

Two large series have been reported. Freedman et al. [139] reported 69 patients with low-grade B-cell non-Hodgkin's lymphoma with chemosensitive relapse or partial remission who underwent autologous BMT. Of these patients, 51 had low-grade lymphoma at the time of transplantation, and 18 had transformed histology. The bone marrow was treated with a cocktail of monoclonal antibodies combined with complement. Conditioning consisted of high-dose cyclophosphamide and TBI. Actuarial disease-free survival was estimated at 53% for those with low-grade histologies. Those in complete remission at the time of bone marrow transplant had a significantly better

outcome than those in partial remission. With prolonged follow-up, however, the difference in outcome between those transplanted in CR versus those transplanted in PR is no longer significant [141].

Rohatiner et al. [140] reported 64 patients with recurrent follicular lymphoma who underwent autologous BMT as consolidation of second or subsequent remission. The marrow mononuclear cell component was treated in vitro with three cycles of the monoclonal anti-CD20 and complement. Conditioning consisted of high-dose cyclophosphamide and TBI. With a median follow-up of three years, 35 patients continue in remission. The actuarial disease-free survival at three years is 50%. Disease-free survival for patients treated in this fashion was superior to that of a historical control group treated at the same institution before 1985.

Both studies have a median follow-up of several years. The early treatment-related mortality of autologous BMT is less than 5%. With prolonged follow-up, however, there has been a continued pattern of relapse after autologous BMT. In addition, it must be kept in mind that patients undergoing this procedure were highly selected. They had recurrent disease that was highly responsive to salvage therapy and had achieved a second CR or a very good PR. It is not entirely certain whether the survival of such patients is better than that of patients with similar characteristics who do not undergo autologous BMT [138,142]. When we have extended autologous BMT to patients with more extensive disease, the results have been poor [143]. Also, when autologous BMT has been used as consolidation of first remission in poor-prognosis low-grade lymphoma, the results have been disappointing [143]. At the current time, we recommend allogeneic bone marrow transplantation for patients with recurrent low-grade lymphoma who have an HLA-identical sibling. This procedure has resulted in durable remissions in the majority of patients treated [144].

The Dana Farber group has stressed the importance of purging in autologous BMT for bcl-2-positive low-grade lymphoma. They used a PCR technique to detect the presence of lymphoma in bone marrow before and after purging [145] and in the peripheral blood before and after bone marrow infusion [146]. They found that bone marrow involvement was always present before purging. After purging with a cocktail of three monoclonals, PCR negativity was obtained in approximately 50% of the cases. The achievement of PCR negativity correlated with excellent disease-free survival after BMT. On the other hand, those whose bone marrow remained involved after purging had a high recurrence rate. In addition, in many of the latter patients, circulating bcl-2 positive cells could be detected within several hours after marrow infusion, suggesting that the infused lymphoma cells were viable and potentially clonogenic.

These findings contrast with the clinical observation of frequent reoccurrence of lymphoma at sites of previous bulk disease, suggesting the persistence of clonogenic cells at those sites. In addition, Johnson et al. [147] were unable to confirm the data obtained by the Dana Farber group. Utilizing different

purging methods and slightly different PCR techniques, they obtained PCR negativity in only a small fraction of patients, and PER negativity did not correlate with disease-free survival in this study. More definitive answers concerning the contribution of reinfused lymphoma cells to tumor recurrence will probably be obtained only with gene-marking studies of reinfused bone marrow.

7. Autologous BMT in chronic lymphocytic leukemia (CLL)

CLL has many features in common with low-grade lymphomas, including an indolent course, frequent reoccurrences, and the presence of extensive bone marrow involvement. Up to 40% of patients with B-CLL are younger than 60 years of age at the time of diagnosis, making them eligible for studies of high-dose chemotherapy [148].

Khouri et al. [149] reported data on 11 patients with CLL who underwent autologous BMT after conditioning with high-dose cyclophosphamide and TBi. Bone marrow had been collected in a prior, fludarabine-induced remission and was purged with anti-CD19. Six patients achieved a complete remission, four a nodular partial remission, and one a partial remission. With limited follow-up, 6 of 11 patients continue in remission.

Rabinowe et al. [150] reported autologous BMT in 12 patients with poor-prognosis CLL. At the time of BMT, 2 patients were in complete remission and 10 had minimal disease (<2 cm lymph nodes and <20% bone marrow involvement). Conditioning consisted of high-dose cyclophosphamide and TBI, and marrow was purged with three B-cell antibodies (CD20, CD10, and B5) and complement. One patient died from diffuse alveolar hemorrhage, one had persistent disease, and 10 achieved a complete remission.

Both studies, while preliminary because of a limited follow-up, indicate the efficacy of high-dose chemotherapy to induce remissions in CLL. Further studies with more prolonged follow-up are required to assess the impact of high-dose chemotherapy and autologous BMT on survival of these patients.

8. Autologous BMT in Hodgkin's disease

Hodgkin's disease affects mainly younger patients and is highly curable with conventional chemotherapy, radiation therapy, or combinations of the two.

High-dose chemotherapy has been used for those patients who recur after adequate front-line therapy. A number of different conditioning regimens have been used. The majority of these include high-dose cyclophosphamide (6–7.2 gr/m^2), BCNU (300–600 mg/m^2), and VP16 (900–1200 mg/m^2) (CBV)[151–154]. At lower doses of BCNU, this regimen is well tolerated.

At the higher dose levels, there is a considerable incidence of pulmonary toxicity [151]. Reece et al. [151] recently reported the use of CBVP, in which platinum was added to CBV. Other commonly used regimens include BEAM, which has been widely used in Europe and is well tolerated [155], and BEAC [92], a variant of CBV with added cytarabine. TBI [156] or TLI [157] including regimens are attractive because of the efficacy of radiation therapy in Hodgkin's disease. Unfortunately most patients will have received extensive radiation by the time they are referred to BMT, making further radiation therapy unpractical. Finally, the use of busulfan- and cyclophosphamide-containing regimens has been reported to be effective [156,158]. In a univariate analysis, however event-free survival was inferior for patients receiving busulfan and cyclophosphamide, compared to those receiving TBI-containing regimens [156].

Excellent results have been reported with high-dose chemotherapy and autologous BMT as treatment for first recurrence of Hodgkins disease. Reece et al. [151] reported 58 patients with Hodgkin's disease in first relapse after primary chemotherapy treated with high-dose chemotherapy (CBV or CBV +P) and autologous BMT. All but six of these patients were given a median of two cycles of conventional chemotherapy ± involved field radiation therapy prior to admission for BMT. There were three treatment-related deaths, and 13 patients have relapsed a median of 0.7 years after BMT. At a median follow-up of 2.3 years, the actuarial progression-free survival was 64%. Forty-three patients in this series were originally from British Columbia, where cancer care is centralized. An additional 16 patients had been diagnosed with recurrent Hodgkin's disease in British Columbia during this time interval, but had not undergone BMT. Alternative treatments included radiation treatment with curative intent ($n = 8$), allogeneic BMT ($n = 1$), and conventional chemotherapy ($n = 4$), as well as refusal of therapy ($n = 3$). These findings indicate that in this disease, high-dose chemotherapy is applicable to the large majority of patients.

Prognostic factors identified in a multivariate analysis included the presence of B-symptoms at relapse, extranodal disease at relapse, and short duration of first remission. For those patients with no or only one risk factor, progression-free survival was 100% and 81%, respectively. Similar low mortality rates have been reported for autologous BMT in first recurrence of Hodgkin's disease by other groups [159].

The results of a number of other relevant reports are summarized in table 12. In general, the results of autologous bone marrow transplantation have been superior to those achieved with conventional-dose chemotherapy at the time of first relapse. But fairly good results have also been reported with conventional salvage chemotherapy, especially among patients with prolonged initial remissions. The best results have been reported by the group from Milan [160]. They reported a 51% freedom from progression with ABVD treatment for patients who relapsed after initial MOPP induced complete remissions that were longer than one year. For failure of MOPP/ABVD treat-

Table 12. Results of autolgous BMT in recurrent Hodgkin's disease

Author, year, reference	N pat	Disease status						Conditioning regimen	Stem cell source	CR/PR	Mortality	Alive, disease free	Event-free survival	Adverse prognositic factors
		PIF	CR	UR	SR	RR	NSR							
Philip et al. 1986 [223]	17	11			3	3		Various, usually BACT (+TBI in 4)	marrow	9/0	9	3	NS	
Carella et al. 1987 [224]	50	18		7	13	12		CBV	marrow	24/16	2	12	>40% at 3 years	
Jagannath et al. 1989 [154]	61			17	12	32		CBV(+LTR in selected patients)	marrow	29/18	4	23	38% at 2 years	>2 Prior regimens, po status
Phillips et al. 1986 [225]	26	5		8		13		Cy + TBI (+LRT in selected patients)	marrow	18/3	6	7	38% at 4.5 years Overall survival	Lower performance st longer diagnosis to BM
Hurd et al. 1990 [226]	33	NS	NS	NS	NS	NS	NS	CBV (+LRT in selected patients)	marrow: 23 blood: 8 both: 2	25/NS	0	12	32% at 28 months	Marrow involvement at blood cell harvest
Jones et al. 1990 [158]	28				20	8		Cy + TBI or BuCy (4)	purged marrow	NS	6	10	Approximately 50% at 20 months	Resistant relapse stat
Kessinger et al. 199 [153]	56	3	NS	NS	NS	NS	NS	CBV (+LRT in selected patients)	blood	29/18	4	28	37% at 3 years	Marrow involvement at
Gianni et al. 1991 [161]	25	16		9				Cy → VCR + MTX/LV → CDDP or VP16 → MelTBI ± LRT	blood	18/NS	0	12	49% at 3 years	Failure to achieve remi primary therapy
Reece et al. 1991 [227]	56	12		32	1	6		CBV ± LRT	marrow	44/2	12	30	47% at 5 year	Low performancestatu
Rapoport et al. 1993 [92]	47	12					35	BEAC (43) TBI/EAC (2), BuCy (2)	marrow (44), blood (3)	NS	11	24	49% at 3 years	Disease status
Horning et al. 1994 [75]	24	2			22			CyVPTBI	blood (23), marrow (1)		1	NS	55% at 3 years	
Chopra et al. 1993 [155]	155	46+ 7 in PR		44	26	32		BEAM (+LRT in selected patients)	marrow	43/72	26	NS	50% at 5 years	Tumor mass, relapse treatment before BMT, f
Reece et al. 1994 [151]	58	0	0	6	NS	NS	52	CBV ± cisplatin (+LRT in selected patients)	marrow (57) blood (1)	NS/NS	3	42	64% at 2.3 years	B symptoms at relapse year, extranodal diseas
Linch et al. 1993 [164]	20[a]	10		6	2	2		BEAM (+LRT in selected patients)	marrow	8/6	2	NS	53% at 3 years	NS

[a] One withdrew consent and was not transplanted.

Abbreviations: PIF, primary induction failure; CR, complete remission; UR, untreated telapse; SR, sensitive relapse; RR, refractory relapse; NSR, relapse, not specified; LRT, local radiotherapy.

For details of conditioning regimens, see references.

ment, the freedom from progression is 46% in this series. A fraction of the patients with second recurrence can still be salvaged with autologous BMT, and overall outcome may be similar.

In many centers, including our own, autologous BMT is offered preferentially to patients with specific high-risk factors, such as (1) failure to achieve a remission, (2) short (less than one year) initial remission, and (3) two or more treatment failures [154,155,157,158,161]. While these high-risk features also predict for an inferior outcome with high-dose chemotherapy, it is generally believed that a fraction of such patients can be salvaged by high-dose chemotherapy. This approach is also supported by an analysis of cost-effectiveness [162,163]. In a recent report on 155 patients selected in this fashion and treated with BEAM and autologous BMT [155], failure-free survival was 55% for the entire study population and 33% for those with primary refractory disease. These results are comparable to those of other reports, as indicated in table 12. In addition, in patients selected according to these criteria, a randomized prospective trial has indicated an improved disease-free survival (53% vs. 10%) for those receiving BEAM versus those receiving several cycles of melphalan, etoposide, carmustine, and cytarabine at conventional doses (miniBEAM)[164].

While there is little disagreement on transplantation for patients with refractory disease, it has been more difficult to identify those patients with Hodgkin's disease in first remission who are destined to relapse. Carella et al. reported 15 patients who underwent autologous BMT in first complete remission because of the presence of several high-risk features [165]. At the time of the report, 13 of the 15 patients were alive and in remission, one had died from treatment-related causes, and one had relapsed. Further follow-up and accrual of more patients will be necessary to validate such an approach.

9. Conclusion

Autologous BMT has become one of the most promising areas of clinical and basic cancer research. The data obtained over the past decade are encouraging and document the validity of the dose-intensity concept. On the other hand, recurrence rates and morbidity associated with the procedure have been high, making it applicable only to very selected patients.

In recent years, there has been a rapid evolution in the use of cytokines and peripheral blood stem cell apheresis. This has resulted in a significant reduction in duration of pancytopenia and a reduction in complications and treatment-related deaths. Recent trials in patients with good performance status have typically had mortalities in the range of 2% [166,167], which is comparable to mortality of conventional chemotherapy trials. Many centers are now treating patients with high-dose chemotherapy as outpatients, thus reducing expense. It is to be expected that the improvements in supportive care will continue. Methods of in vitro stem cell expansion are under develop-

ment [168,169]. If successful, such methods may lead to a complete abolition of pancytopenia.

Further improvements can also be expected from posttransplant methodologies for eradicating minimal residual disease. Such treatments rely on targeting residual tumor with monoclonal or polyclonal antibodies linked to radioemitters [170,171] or cellular toxins [172]. Nonspecific stimulation of the immune system with interleukin-2 [37,38] or cyclosporin withdrawal [173] with the purpose of inducing autologous GVHD has also been attempted. Finally, anti-idiotypic vaccinations are sometimes used in situations of minimal residual disease.

Hematopoietic stem cells are an ideal target for genetic manipulation. Trials are underway in metastatic ovarian cancer to establish chemotherapy resistance of normal hematopoietic stem cells by introducing the MDR gene, thereby hopefully allowing the administration of larger doses of chemotherapy. We are likely to witness an explosion in the applications of molecular techniques in the field of BMT for a variety of tumors. Finally, thanks to improvements in supportive care, high-dose chemotherapy can be reexamined in diseases where it was formerly thought to be ineffective due to unacceptable toxicity or lack of prolonged remissions, such as in small cell lung cancer or osteosarcoma.

Unfortunately, for any progress, there is a price to pay [174]. Until recently, the late side effects of autologous BMT were thought to be minimal; however, there have been three reports in the last year documenting the frequent occurrence of secondary myelodysplastic disorders after autologous BMT for lymphoid malignancies [70,175,176]. The cumulative incidence of secondary myelodysplastic disorders is over 10% at five years in each of these studies. A better understanding of the pathogenesis, incidence, and severity of this syndrome will probably emerge in coming years. Currently identified risk factors include increased exposure to chemotherapy, older age, and the use of radiation therapy. Modification in conditioning regimens and avoidance of potentially leukemogenic agents may be necessary as more patients are treated.

Current debate often centers on transplant versus so-called conventional chemotherapy in the treatment of malignant hematologic disorders. Such issues will soon be something of the past. High-dose chemotherapy and the use of cellular support strategies, as well as the manipulation of stem cells by either genetic manipulation or other methodologies, will become an integral part of cancer treatment in the same way that blood transfusions and later platelet transfusions became an essential part of supportive care.

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11. Retinoblastoma gene in malignancy

Lance C. Pagliaro

1. Introduction

The central importance of genetic damage in the development of tumors was suspected prior to the advent of molecular biology because chemical carcinogens were noted to be potent mutagens as well [1]. Hereditary cancer syndromes provided further evidence of a genetic basis for carcinogenesis [2]. The identities of the genes involved remained unknown until the 1970s, when the study of animal RNA tumor viruses led to the discovery of several vertebrate genes that could acquire transforming properties through mutation [3,4]. The consequences of these genetic alterations are now beginning to be understood. Knudson proposed that at least two separate mutational events are required, based on his observations of hereditary and nonhereditary retinoblastoma tumors in children [5]. According to his two-hit hypothesis, sporadic tumor formation requires the independent occurrence of both mutations within the same cell, whereas in individuals who inherit one mutation in the germline, only the second mutation is required. Using the estimated frequency of spontaneous mutation, Knudson showed that the hypothesis correctly predicts multiple bilateral tumors in patients with the inherited retinoblastoma susceptibility trait, and solitary unilateral tumors in nonhereditary cases.

The retinoblastoma gene (*RB1*) takes its name from a rare tumor, but actually plays an important role in cell-cycle regulation of normal cells in most vertebrate species and is disrupted in a wide variety of human neoplasms [6]. It is the prototype of a class of genes that suppress cancer cell growth and is increasingly important to our understanding of the molecular pathology of cancer [7]. This discussion will review the evidence that structural alteration of the *RB1* gene constitutes the primary etiology of retinoblastoma and contributes to the development of other solid tumors and hematopoietic neoplasms. The structure and function of the *RB1* gene product will be reviewed, and the significance of low or absent levels of the protein in human tumors will be discussed. The results of *RB1* restoration will be examined with respect to the implications for development of novel therapeutic strategies.

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2. Genetic basis of retinoblastoma

2.1. Paradigm of tumor suppressor gene

Identification of the genetic loci involved in retinoblastoma began with the karyotyping of patients with hereditary retinoblastoma. Visible deletions in the long arm of chromosome 13 were frequently observed [8]. Subsequently, comparison of chromosome 13 DNA polymorphisms in tumor cells and somatic cells from the same patients revealed loss of heterozygosity (LOH) in tumor cells at the same region of chromosome 13 [9]. These observations suggested that, in the case of retinoblastoma, the first and second mutational events consist of loss or mutation of genetic material from the same locus on homologous chromosomes, and point to complete inactivation of a single gene as the most likely cause of this particular tumor. The *RB1* gene has been cloned and, with confirmation of its suspected role in the development of tumors, is the prototype of a class of genes called tumor suppressor genes [10].

Pedigree analysis of patients with retinoblastoma reveals an autosomal dominant pattern of inherited susceptibility [2,5]. The germline transmission of a mutant *RB1* allele from a heterozygous parent results in heterozygosity for the gene in 50% of offspring. When heterozygosity is demonstrated through restriction fragment length polymorphism (RFLP) analysis of *RB1*, then looked for in retinoblastoma tumor tissue, the tumor shows LOH, signifying deletion of the wild-type allele [11]. In heterozygous children, multifocal retinal tumors arise as a result of LOH occurring independently in different cells. This response to LOH is specific to retinal progenitors and is not shared by most other cell types or by mature retinal epithelial cells [12]. The paradigm of LOH as a fundamental process in carcinogenesis has been found to apply to other tumor suppressor genes, and the view that carcinogenesis is usually, if not always, a multistep process is now widely accepted [4,7,13].

2.2. Regulation of RB1 expression

The *RB1* promoter has a high G+C content and lacks CCAAT or TATA motifs; these are characteristics identified with 'housekeeping' genes, or genes expressed at a relatively constant level [14]. In normal mammalian tissues, expression of *RB1* is ubiquitous [15]. Increased levels of *RB1* transcripts and of the gene product, $p110^{RB}$, are seen following mitogenic stimulation of resting B- and T-lymphocytes. In reactive lymphoid tissue, $p110^{RB}$ is expressed mainly in germinal centers [16,17]. Levels of $p110^{RB}$ also increase following induction of differentiation in embryonal carcinoma P19 cells [18] and K562 myeloid leukemia cells [19]. The *RB1* promoter contains an E2 element that is repressed by $p110^{RB}$ and a *cis*-acting element susceptible to regulation by p53.

There is evidence that both $p110^{RB}$ and the product of the p53 gene repress *RB1* transcription [20,21].

3. *RB1* mutations in malignancy

3.1. Nonocular tumors in retinoblastoma survivors

Somatic mutation of *RB1* and resulting LOH can occur not only in retinal progenitor cells of children, but in any dividing cell at any time in the life of the individual. Since treatment of retinoblastoma is largely successful, many patients survive into adulthood and have been studied for the incidence of other cancers. Certain solid tumors, particularly bone and soft tissue sarcomas, occur more often than expected and demonstrate LOH [22]. Most neoplastic diseases, however, do not affect retinoblastoma survivors more often than the general population. It is apparent that *RB1* is not required to prevent tumor formation in all cell types [12,16,23].

3.2. Solid tumors

Sporadic cases of retinoblastoma and of RB-negative sarcomas can occur without prior germline mutation [23]. In fact, absent *RB1* expression has been found in a wide variety of solid tumors and hematopoietic neoplasms, including those that do not occur more frequently in retinoblastoma survivors (table 1) [24–34]. LOH at the *RB1* locus occurs in the majority of small-cell lung cancer tumors [26]. In the case of bladder carcinoma, absence of *RB1* expres-

Table 1. Structural abnormalities and aberrant expression of the *RB1* gene in primary human solid tumors

Reference	Disease	No. pts.	LOH	Homozygous deletions	Absence of $p110^{RB}$ protein
Wadayama et al.	Osteosarcoma	63	39	9	30/56
Ishikawa et al.	Bladder ca.	27	5		0/5
	Renal cell ca.	30	2		1/2
Hensel et al.	SCLC	26	6/6		12/14
Xu et al.	NSCLC	36			10
T'ang et al.	Breast ca.	41		3	
Kim et al.	Ovarian ca.				
	Low grade	15	0		0
	High grade	29	15		2/26
Murakami et al.	Hepatocellular ca.	7	6		
Cryns et al.	Parathyroid ca.	5	5		
Ruggeri et al.	Pancreatic ca.	10			2
Cance et al.	Sarcomas	44			13
Hamel et al.	High-grade glioma	10			3

sion is associated with worse prognosis [35]. In ovarian cancer, LOH is associated with high-grade histology, although preservation of immunoreactive $p110^{RB}$ is commonly observed [29]. Whereas *RB1* inactivation is a tumor-initiating step in retinoblastoma and in a limited group of solid tumors, it apparently occurs as a late step in a much greater number of tumor types, probably contributing to tumor progression in these cases.

3.3. Hematologic malignancy

Normal hematopoietic tissue has a high rate of proliferation. Assuming normal mutation rates, patients with germline *RB1* mutations should produce hundreds of hematopoietic cells with LOH every day. Yet, the incidence of leukemias and lymphomas in retinoblastoma patients is no higher than in the general population [36]. The lack of an association between germline *RB1* mutations and development of hematopoietic malignancies is a strong argument against a role for *RB1* in the transformation of normal to leukemic cells, even through *RB1* is normally expressed in hematopoietic cells. There are several possible reasons for differences in the consequences for cells when they lose *RB1* expression. For example, normal hematopoietic cells may possess other means of growth control that are absent in retinal progenitor cells. Alternatively, normal hematopoietic cells may die when *RB1* expression is lost due to a unique requirement for its expression, whereas retinal progenitor cells survive and proliferate. Nevertheless, DNA studies of cells obtained from patients with hematopoietic neoplasms have shown that *RB1* deletions and mutations do occur, most likely as a late event contributing to progression of disease.

Cytogenetic abnormalities in acute myelogenous leukemia (AML) do not frequently involve chromosome 13, although deletion at 13q14 (the site of *RB1*) is occasionally observed [37]. Southern blotting has the potential to detect structural deletions that may be missed with cytogenetics alone. In a series of 69 patients with AML, however, Chen and coworkers found only one case of apparent *RB1* deletion by Southern blotting [38]. Ahuja and coworkers found 5 of 54 AML samples, 1 of 17 chronic myelogenous leukemia (CML) chronic phase, 6 of 42 myeloid blast crisis, and 1 of 9 lymphoid blast crisis with abnormal *RB1* gene structure [39]. The absence of detectable structural abnormalities of the *RB1* gene in the majority of AML and CML cases does not exclude the possibility of additional cases with subtle gene alterations that are undetected, or of aberrancy involving transcriptional or posttranscriptional regulation. Blast cells from patients with AML show increased colony formation in vitro following incubation with an RB-antisense oligonucleotide, providing further evidence that decreased expression of *RB1* contributes to the neoplastic phenotype [40].

The study by Chen et al. showed 1 of 52 acute lymphoblastic leukemia (ALL) cases with structural rearrangement (homozygous deletions) of the *RB1* gene detected by Southern blotting [38]. Ginsberg and coworkers re-

ported *RB1* deletions detectable by Southern blotting in 1 of 26 cases of ALL and in the T-cell ALL cell line HSB-2. The patient sample with *RB1* deletion also demonstrated undetectable *RB1* transcript, whereas HSB-2 has multiple transcripts of abnormal size [41]. Ahuja and coworkers found abnormal *RB1* gene structure in 2 of 17 T-cell ALL cases, 5 of 22 CALLA-positive pre-B-cell ALL cases, and 3 of 11 Philadelphia chromosome-positive ALL cases [39]. *RB1* deletions have also been reported in childhood ALL, with frequency ranging from 5% to 32% [42,43].

Evidence for involvement of *RB1* is relatively common in chronic lymphocytic leukemia (CLL). Stilgenbauer et al. reported that CLL cells from 31% of the patients in their study had monoallelic deletions of the *RB1* gene according to the results of fluorescence in situ hybridization (FISH) using probes to the *RB1* gene [44]. Monoallelic deletion is analogous to LOH in that it may provide a mechanism whereby a recessive loss-of-function mutation is expressed. The high frequency of LOH in CLL has not been confirmed, nor is it known how LOH affects the function of the gene product, $p110^{RB}$. Aberrations affecting the long arm of chromosome 13 are the most common structural cytogenetic abnormalities in CLL, and most of these involve the site of the *RB1* gene. In a large European study, 35 of 391 patient samples with evaluable peripheral blood lymphocyte cultures showed deletions or translocations affecting band 13q14 [45].

Normal resting B- and T-lymphocytes have low levels of *RB1* expression that increase upon mitogenic stimulation [16]. In parallel fashion, low-grade non-Hodgkin's lymphomas (NHLs) show weak expression of $p110^{RB}$ by immunohistochemistry, whereas high-grade NHLs show increased expression [17]. There is a subset of intermediate- and high-grade lymphomas that do not express $p110^{RB}$ (discussed below), but structural rearrangement of the *RB1* gene is rarely found [46]. The frequency of *RB1* alterations is variable among other types of lymphoid neoplasms. Corradini et al. reported 1 of 29 cases of multiple myeloma with a DNA rearrangement consistent with deletion of part of the *RB1* gene, and no rearrangements among 15 with monoclonal gammopathy of undetermined significance (MGUS) or 16 with plasma cell leukemia [47]. In a study from the University of Arkansas involving 23 patients with multiple myeloma, Dao and coworkers found an abnormal chromosome 13 by routine cytogenetics in 17% and monoallelic deletion of *RB1* detected by FISH in 48% [48].

In summary, genetic abnormalities at the *RB1* locus are uncommon in most hematologic malignancies. The exceptions are CLL and multiple myeloma, where monoallelic deletions of *RB1* are commonly detected by FISH or cytogenetics. It remains possible that cytogenetic abnormalities are significant due to loss of other genes on chromosome 13, although results of FISH with *RB1*-specific probes yield the highest estimates of monoallelic loss. The clinical significance of *RB1* disruption in these indolent lymphoid neoplasms has yet to be determined. Measurement of the *RB1* gene product in leukemia and lymphoma cells, discussed in the next section, suggests that involvement in

NHL and myeloid leukemias may be more frequent than is apparent from studies of gene structure.

4. RB1 gene product

4.1. Protein structure

The *RB1* gene product, $p110^{RB}$, is a 110-kilodalton-molecular-weight nuclear protein [49]. It has A and B domains forming the A/B pocket that binds SV40 T-antigen, and a C-terminal domain that binds the nuclear tyrosine kinase c-abl [50]. The C-terminal portion of the protein also contains a basic region with sequence similarity to a known bipartite nuclear localization signal (NLS), which is both necessary and sufficient to direct the protein into the nucleus [51]. $p110^{RB}$ is the prototype of the RB family of proteins, which also includes p107, p130, and p300 [16]. $p110^{RB}$ also contains sequence homology with intermediate filaments of the cytoskeleton and has been observed to form filaments in solution [52]. $p110^{RB}$ contains 11 phosphotryptic serine and threonine residues and may exist in various states of hypo- or hyperphosphorylation [53]. Phosphorylation appears to be an important means of post-translational control, mediated by the D and E cyclins and cyclin-dependent kinases (cdk's), primarily cdk4 [54–56]. Quiescent cells in tissue culture contain predominantly hypophosphorylated $p110^{RB}$, whereas actively proliferating cells contain hyperphosphorylated $p110^{RB}$ [6].

4.2. Tumor suppressor function

The oncoproteins of certain tumor viruses, namely, simian virus 40 (SV40), adenovirus, and human papilloma virus, are known to bind hypophosphorylated $p110^{RB}$ [57–59]. Viral point mutations that interfere with binding to $p110^{RB}$ also abolish transforming capacity [60]. There is now a wealth of evidence to support the model that hypophosphorylated $p110^{RB}$ possesses tumor suppressor activity whereas the phosphorylated form is inactive, and that viral oncoproteins function by binding and blocking the A/B pocket of $p110^{RB}$, preventing the binding of its normal protein partners [50,56].

Proteins that bind the A/B pocket domain of $p110^{RB}$ include the transcription factor E2F [60] and D cyclins [54,55]. $p110^{RB}$ also binds DNA, suggesting that it participates in a protein complex with gene regulatory function [49]. The binding of $p110^{RB}$ to double-stranded DNA most likely occurs on the basis of charge–charge interactions. The $p110^{RB}$ amino acid sequence does not contain any of the recognizable DNA binding motifs, nor has a unique DNA binding motif been described such as in the case of p53. Binding of hypophosphorylated $p110^{RB}$ to E2F prevents transcription from genes controlled by the E2 promotor. E2F normally regulates cell-cycle genes such as c-

myc, c-*myb*, DNA polymerase-α, ribonucleotide reductase, and thymidylate synthase [60]. Phosphorylation of $p110^{RB}$ allows E2F-driven transcription to proceed. In mammalian cells, phosphorylation of $p110^{RB}$ is linked to the cell cycle and occurs at a point late in G1. Microinjection of an RB-related peptide effectively blocks synchronized cells from entering S phase [61]. $p110^{RB}$ also binds c-*myc* in vitro, but a significant functional interaction in vivo has not been found. There is, however, evidence for an in vivo interaction between the related protein p107 and c-*myc* [62].

Binding of the nuclear tyrosine kinase c-abl to a C-terminal domain of $p110^{RB}$ inhibits tyrosine kinase activity. Interaction with c-able is prevented by phosphorylation of $p110^{RB}$, but not by viral oncoprotein binding. The presence of two separate binding pockets suggests that one of the biological functions of $p110^{RB}$ is to bring specific proteins together, for example, c-abl and a substrate for c-abl [50].

A high percentage of RB-positive tumor cell lines contain mutations or deletions of the *MTS1* gene on chromosome 9. The gene product of *MTS1* is p16, which binds and inhibits cdk4. It is likely that absence of p16 promotes cell-cycle progression by allowing phosphorylation of $p110^{RB}$. In this manner, *MTS1* functions as a tumor suppressor gene by altering the posttranslational modification of $p110^{RB}$ [63].

4.3. Cytokine effects

Regulation of $p110^{RB}$ activity occurs primarily at the posttranslational level, i.e., through changes in phosphorylation, rather than at the level of the gene. There is evidence that extracellular signaling from soluble cytokine proteins regulates the phosphorylation of $p110^{RB}$ as a mechanism for control of cell growth and differentiation [64]. These cytokines include interferons [65,66], transforming growth factor β (TGFβ) [54,67], and interleukin-6 (IL-6) [65]. Conversion of $p110^{RB}$ to the unphosphorylated form precedes G0/G1 arrest induced by IL-6 or interferons (α and β) [65], and interferon-gamma-mediated responses that are absent in RB-negative breast cancer cells are restored by infection with the wild-type *RB1* gene [66]. The inhibitory effect of TGFβ on proliferation and differentiation of hematopoietic cells is partially reversed by RB antisense oligonucleotides, indicating a requirement for $p110^{RB}$ function [67]. TGFβ downregulates cdk4 expression, allowing $p110^{RB}$ to remain in the unphosphorylated form [54].

4.4. Animal model

There is a high degree of homology between mouse and human *RB1* [16]. Mice with germline *RB1* mutations ('knockout mice') have been described. Homozygous mutants die before the 16th day of gestation, with abnormal maturation of the central nervous system and hematopoietic tissues. Nevertheless, a striking degree of tissue and organ formation is possible in the complete

absence of p110^{RB}. Heterozygous mice survive to adulthood and do not form retinal tumors, but do have an increased incidence of pituitary tumors associated with LOH [68,69]. In adult mice, loss of p110^{RB} activity results in slowly growing tumors, with increased prolifeation of cells partially offset by widespread apoptosis. Inactivation of p53 results in disappearance of apoptosis and rapid tumor growth [70].

5. Levels of p110^{RB} in normal and neoplastic tissues

Monoclonal antibodies that bind p110^{RB} have been used to study levels in various tissues and tumor types. In vertebrate species, p110^{RB} is detected in virtually all proliferating tissues. In mouse embryos, the *RB1* transcript reaches maximum levels at gestational days 12.5–14.5 and is present in all tested tissues [15,16]. Malignant cells from human tumors often have readily detectable levels of p110^{RB}. Expression may be heterogeneous, with varying fractions of RB-negative cells present within different tumors (table 1). Using immunohistochemistry, solid tumors that undergo loss or inactivation of the *RB1* gene, such as transitional cell carcinoma of the bladder, may be distinguished from normal stroma by complete absence of nuclear staining [71]. RB-negative human tumor cell lines have been derived from retinoblastoma, osteosarcoma, synovial sarcoma, prostate, bladder, renal cell, breast, cervical, small-cell lung, and non-small cell lung cancers [56], ALL [72], and NHL [41].

Cells from hematopoietic malignancies are easily accessible and amenable to study of p110^{RB} expression. Several laboratories have reported the successful use of flow cytometry to measure p110^{RB}, but few clinical studies to date have made use of this technique [40,71,73]. Early reports based on Western blotting suggested that absence of p110^{RB} occurs in up to 25% of AML cases and 45% of CML blast crisis [39]. Subsequent studies have found instead that less than 10% of myeloid leukemias completely lack p110^{RB}, although absence of p110^{RB} is still detected more commonly than gene rearrangements, and there is heterogeneity in the level of expression among RB-positive cases, with lower p110^{RB} levels associated with shorter survival [74,75]. The downward trend in estimates of RB negativity may be due to the use of anti-p110^{RB} antibodies with higher affinity in later studies [71]. There have also been cases of RB-negative AML that were RB-positive at relapse, raising doubt as to the significance of the original finding [76]. Towatari et al. studied 22 cases of CML blast crisis using Western blotting and found 5 with absent p110^{RB}, all of which had the megakaryoblastic phenotype, whereas the RB-positive cases had lymphoid or other myeloid phenotypes suggesting a phenotypic correlation with *RB1* inactivation, the strength of which has not been confirmed [77]. Absence of p110^{RB} is more common in CML blast crisis than in the chronic phase, suggesting that inactivation of *RB1* contributes to disease progression [39,77].

Levels of p110^{RB} in ALL have been reported to be undetectable by Western

blotting in cases with structural gene rearrangements [39,41,43]. Furukawa et al. have reported undetectable $p110^{RB}$ expression in 1 of 12 ALL cases studied by Western blotting alone [78].

Levels of $p110^{RB}$ are heterogeneous in CLL, with low or absent levels found on Western blotting in approximately 30% of untreated patients [79]. It is unclear how the loss of $p110^{RB}$ expression affects the biology of this slowly proliferating neoplasm, and no correlations of $p110^{RB}$ level with clinical characteristics have yet been reported. Absence of $p110^{RB}$ detection by immunohistochemistry has been observed in 5 of 14 multiple myeloma cases and 3 of 9 plasma cell dyscrasias [47].

Levels of $p110^{RB}$ in NHL reflect its normal association with activated or proliferating lymphocytes. Low-grade lymphomas, which have a low growth fraction, show weak or absent expression, whereas intermediate- and high-grade lymphomas tend to have higher expression. There is a significant correlation between intensity of immunohistochemical staining for $p110^{RB}$ and staining with Ki-67, which is a monoclonal antibody specific for a nuclear antigen detected in all phases of the cell cycle except G0 [17]. There is a subset of high-grade NHLs that has high Ki-67 but absent $p110^{RB}$, in which the inactivation of $p110^{RB}$ might have pathological significance. The frequency of absent $p110^{RB}$ expression by immunohistochemical analysis in high-grade NHL has been reported at 16% to 58% [17,46].

In summary, heterogeneity of $p110^{RB}$ expression is readily demonstrated in AML and CML blast crisis. The frequency of low or undetectable expression is greater than that of detected structural gene abnormalities. Decreased expression may be highly correlated with megakaryoblastic phenotype in CML blast crisis, and lower levels of expression in AML correlated with decreased survival. Absence of $p110^{RB}$ expression is common despite absence of detectable *RB1* gene alterations in high-grade NHL, whereas both gene and protein are affected with low frequency in ALL, and with higher frequency but uncertain significance in CLL and plasma cell dyscrasias.

6. Effect of $p110^{RB}$ restoration on tumor growth

The role of *RB1* inactivation in tumor cells has been investigated through genetic reconstitution of *RB1* expression in RB-negative cancer cell lines. The retinoblastoma cell line WERI-Rb27 and osteosarcoma cell line Saos-2 have partial deletions of *RB1* and express no functional $p110^{RB}$. Infection with an *RB1*-expressing viral construct resulted in decreased growth of bulk cultures, although *RB1*-infected clones that express $p110^{RB}$ and still grow well in culture were eventually isolated. When injected subcutaneously into nude mice, *RB1*-infected WERI-Rb27 showed complete suppression of tumorigenicity compared to control vector-infected cells in the opposite flank [80]. Similar experiments with other solid tumor cell lines have yielded variable results. Decreased growth and clonogenicity in vitro and decreased tumorigenicity in

vivo have been demonstrated following genetic reconstitution of *RB1* expression in RB-negative prostate cancer [81], transitional cell carcinoma [82], and non-small cell lung cancer [83] cell lines. The majority of RB-negative cell lines, however, show little effect of *RB1* restoration in vitro [16]. The data show that reintroduction of the *RB1* gene into an RB-negative background can reverse some aspects of the neoplastic phenotype, but that these effects are variable and probably dependent on factors other than prior inactivation of endogenous *RB1*. This observation is reminiscent of the variable effects of *RB1* inactivation in normal cells from different tissues.

The effect of increased *RB1* expression was also studied in normal RB-positive mice. Transgenic mice bearing multiple copies of a human *RB1* transgene, in addition to two copies of murine wild-type *RB1*, have been characterized. Organ structure was apparently normal, but most transgenic mice were smaller than their nontrasgenic littermates, with size inversely proportional to *RB1* gene copy number and level of expression [84]. These data provide evidence for an *RB1* gene-dose effect, and that differences in intracellular $p110^{RB}$ concentration may have functional significance.

Using recombinant human $p110^{RB}$ from *E. coli*, it is possible to test the protein at concentrations up to 500 nM for its effect on cells in culture. We asked whether direct exposure to $p110^{RB}$ could regulate cancer cell growth. There are several precedents for extracellular proteins affecting gene expression through cellular uptake and nuclear translocation, including interleukin-1 (IL-1) [85] and basic fibroblast growth factor (bFGF) [86]. The bladder cancer cell line 5637 (ATCC HTB-9) expresses no functional $p110^{RB}$ and has been previously reported to exhibit decreased growth in vitro, decreased colony formation in soft agar, and reduced tumorigenicity in vivo following transfection with the wild-type *RB1* gene [82]. We asked whether exposure of the 5637 parent cell line to recombinant $p110^{RB}$ would result in growth inhibition. Incorporation of tritiated thymidine by 5637 cells was inhibited by direct exposure to $p110^{RB}$ concentrations above 100 nM. Next, to further investigate the effect of recombinant $p110^{RB}$ on self-renewal in a neoplastic cell population, we looked for an effect on clonogenicity of blast cells from patients with AML. Growth inhibition was observed in blast cultures from 8 of 20 patients following exposure to recombinant $p110^{RB}$ and was dose responsive. In six cases self-renewal of blast stem cells, measured as clonogenic cell recovery, was decreased when cells were cultured for two days with $p110^{RB}$ at a concentration of 200 nM. Our data also show that $p110^{RB}$ is internalized by cells in vitro and translocated to the nucleus. There were no differences in immunohistochemically detectable $p110^{RB}$ expression between samples that were growth inhibited and those that were not, suggesting that heterogeneity of response is governed by other factors, such as differences in the degree to which exogenous protein is phosphorylated by specific kinases [75].

The mechanism for internalization of $p110^{RB}$ is not known. One possibility is that recombinant $p110^{RB}$ reaches the interior of cells through endocytosis

when there is a sufficient concentration gradient across the plasma membrane. Other growth-regulating macromolecules have been shown to cross the plasma membrane through receptor-mediated or non-receptor-mediated endocytosis. For example, the HIV tat protein is found in the extracellular matrix and is internalized by cells; attachment to the cell surface involves interaction of basic domains on tat with $\alpha_v\beta_5$ integrin [87]. Insulin enters cells by receptor-mediated endocytosis at low concentrations and by fluid-phase endocytosis at high concentrations (>100 ng/ml) [88]. Both tat and insulin reach the nucleus and affect gene expression once internalized. A 60-kDa peptide derived from the *Drosophila* homeobox gene *antennapedia* is internalized by rat embryonic nerve cells, enters the nucleus, and regulates morphologic differentiation; internalization is blocked by an antibody that binds the α-2,8-polysialic acid (PSA) chains of the neuronal cell adhesion molecule [89]. It has been proposed that structural similarity between PSA and double-stranded DNA may be the basis for binding of the homeobox peptide to the cell surface and subsequent internalization. Cultured endothelial cells exposed to bFGF are found to have bFGF present in the cytoplasm and nucleus [86], and a similar requirement for receptor-mediated internalization and nuclear translocation is seen for epidermal growth factor, platelet-derived growth factor [90], and interleukin-1 [85]. Endocytosis of bFGF is mediated by binding to high-affinity receptors as well as lower-affinity binding to heparan-sulfate proteoglycans [86]. While there is no evidence for a high-affinity $p110^{RB}$ receptor, binding to proteoglycans on the cell surface or other nonspecific binding might occur. Localization to the nucleus is normally mediated by an NLS at the C-terminal portion of $p110^{RB}$, and such signaling may also mediate protein trafficking from endocytic vesicles.

An alternative, though less likely, mechanism for cellular uptake of $p110^{RB}$ is by diffusion directly through the plasma membrane. An intact plasma membrane does not normally permit diffusion of macromolecules as large as $p110^{RB}$, but membranes may be altered to increase permeability. Cellular uptake of $p110^{RB}$ has been reported to occur following permealization with detergent [73].

7. Conclusions

7.1. Role of RB1 in hematopoietic neoplasia

Abnormalities of the *RB1* gene are found in all types of leukemia. The frequency of decreased protein levels parallels gene status in ALL, but is more common than detectable gene abnormalities in AML, CML blast crisis, and NHL. Decreased protein levels may signify subtle structural gene alterations, such as point mutation, which escape detection during Southern or RFLP analysis, or may be due to deregulation of *RB1* expression that does not involve a structural gene abnormality. There may be additional means of

inactivating $p110^{RB}$ without a decrease in the level of the protein. Indeed, this appears to be the case when the tumor suppressor gene *MTS1* is inactivated, resulting in increased phosphorylation of $p110^{RB}$.

The importance of *RB1* expression in the regulation of AML blast cell proliferation is demonstrated by the growth-inhibitory effect of recombinant $p110^{RB}$ and the growth-stimulatory effect of RB-antisense oligonucleotide. Loss of *RB1* expression may contribute to the progression from chronic phase to accelerated phase or blast crisis in CML. The role of *RB1* inactivation in CLL and other indolent lymphoid neoplasms has yet to be determined and may involve regulation of processes independent of cell-cycle progression, such as differentiation and senescence.

7.2. Prospects for tumor suppressor therapeutics

The attraction of tumor suppressor genes as targets for therapy lies in the potential for reversal of the neoplastic phenotype through restoration of the gene or gene product. In the case of *RB1*, cancer cell lines lacking expression of the gene show decreased proliferation following reconstitution of *RB1* expression through gene insertion, and following introduction of exogenous $p110^{RB}$ through microinjection or direct exposure to extracellular protein. Both normal and neoplastic cells are sensitive to the level of $p110^{RB}$ within the cell. The apparent autoregulatory function of $p110^{RB}$, whereby the gene product downregulates its own transcription, suggests that maintenance of a specific protein concentration is critical to the cell. Increased *RB1* expression in mice transgenic for multiple copies of the gene results in dwarf mice. AML blasts show increased clonogenicity when *RB1* expression is blocked with an antisense oligonucleotide and decreased clonogenicity when exposed to exogenous $p110^{RB}$. Yet, in the case of RB-negative cell lines as well as primary AML blasts, not all cell types are growth inhibited by $p110^{RB}$.

The specificity with which $p110^{RB}$ affects a restricted subset of neoplasms is poorly understood. Patients who carry a mutant *RB1* gene in the germline have a 90% incidence of retinoblastoma and increased incidence of osteosarcoma and soft tissue sarcomas, but normal incidence of most other neoplasms. The occurrence of *RB1* mutations in a wider variety of solid tumors and hematopoietic neoplasms probably does not reflect a role in tumor initiation, but rather, a role in tumor progression. The effect of *RB1* inactivation is consistent with its normal function as a repressor of mitosis; but, for reasons that have yet to be determined, it only affects a limited group of human malignancies. Also unexplained is the apparent species specificity of LOH in the setting of heterozygous *RB1* germline mutation: retinal tumors predominate in humans and pituitary tumors in mice.

Restoration of tumor suppressor genes through viral infection is currently under investigation as a potential therapeutic modality [91,92]. Genetic reconstitution of *RB1* has been studied only in RB-negative cell lines, where decreased tumorigenicity is observed in a restricted subset of tumor types. One

of the those cell lines, the transitional cell carcinoma cell line 5637, is not only growth inhibited by introduction of the *RB1* gene, but also by direct exposure to extracellular $p110^{RB}$. This appears to be due to uptake of exogenous $p110^{RB}$ by the cell and translocation to the nucleus. Proteins that function extracellularly as regulators of growth and differentiation, such as interferons, IL-6, and TGFβ, may act in part through their effects on the phosphorylation status of $p110^{RB}$, so that delivery of exogenous $p110^{RB}$ to the nucleus in sufficient amounts mimics the effect of the signaling peptide. Cytokines alone may have no effect on cancer cells that bear mutations of critical mediators such as $p110^{RB}$, but this escape from regulation is reversed by delivery of the tumor suppressor protein itself. In vivo studies with recombinant $p110^{RB}$ may reveal more efficient means of achieving internalization and nuclear translocation of $p110^{RB}$, offering the possibility of a new approach to cancer treatment.

Notes

Since this manuscript was prepared, *in vivo* gene transfer of *RB1* using a replication-defective recombinant adenovirus vector has been reported (Chang MW, Barr E, Seltzer J, Jiang YQ, Nabel GJ, Nagel EG, Parmacek MS, Leiden JM (1995) *Science* 267:518–522). In this experiment, adenovirus was administered intra-arterially to pigs after the femoral artery intima had been injured with a balloon catheter. The gene, a mutant RB that resists phosphorylation, was expressed in arterial smooth muscle and inhibited the hyperproliferative response to injury seen in controls. The animals exhibited no apparent adverse effects from the gene transfer. Adenovirus vectors, unlike retrovirus vectors, can infect cells in any stage of the cell cycle, resulting in episomal gene expression which lasts 2 to 3 weeks. Adenovirus-mediated *RB1* gene transfer may be clinically useful in certain diseases, for example bladder cancer where infection of tumor cells with adenovirus can be accomplished through intravesical administration. These approaches will be investigated in future clinical trials.

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12. Treatment of relapse after allogeneic bone marrow transplantation

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1. Introduction

Disease relapse after allogeneic bone marrow transplantation (BMT) for hematologic malignancies remains the single most important cause of treatment failure [1–4]. Improvements in supportive care, prophylaxis of graft versus host disease (GVHD), and treatment of infectious complications have resulted in a decrease in transplant-related mortality during the years. However, modifications in the conditioning regimen and other strategies to prevent relapse have failed, and the relapse rate after allogeneic transplant has remained constant throughout the years [5,6].

Since allogeneic BMT is being used more frequently and in earlier stages of disease, many centers are now being confronted with a growing population of patients whose first relapse occurs after their allograft. These patients will demand and require further therapy for their disease.

We review the major treatment modalities for patients relapsing after allogeneic BMT, with emphasis on some of the more novel treatment strategies.

2. Natural history of leukemia relapse after allogeneic BMT

The survival of patients who relapse after an allogeneic BMT depends on the specific leukemia diagnosis, stage of the disease, interval from transplant to recurrence, the patient's performance status, and treatment. Patients with acute leukemia who relapsed after allogeneic BMT and receive no further treatment have a median survival of 3 to 4 months, with no long-term survivors [7,8].

Patients achieving a complete remission with conventional chemotherapy have median survival durations of 8 months for acute myelogenous leukemia (AML) and 14 months for acute lymphoblastic leukemia (ALL). Patients not achieving a complete remission have survivals comparable to patients receiving no treatment at all. The most important adverse prognostic factor for achieving a complete remission is a short remission duration after transplant.

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Patients with remissions of less than one year have less than a 10% chance of achieving a complete remission with conventional chemotherapy [7,8].

Arcese recently reviewed the natural history of patients with chronic myelogenous leukemia (CML) relapsing after an allogeneic BMT. Patients with isolated cytogenetic relapses have a median survival of greater than six years compared to three years for patients relapsing into a clinical chronic phase [9]. Spontaneous cytogenetic remissions have been reported in recipients of unmanipulated allografts with cytogenetic relapses, but have not been described in patients receiving T-cell-depleted allografts [10,11].

Patients with CML who relapse into blast crisis or accelerated phase have a median survival duration of less than six months, with poor response to interferon or conventional chemotherapy [9].

3. Approach to treatment in patients relapsing after allogeneic BMT

Patients relapsing after an allogeneic BMT will usually be in a chimeric state in which the recurrent leukemia is of recipient origin, but the residual normal hematopoietic and immune systems remain donor in origin. Strategies to induce remission should therefore not only consider conventional antineoplastic chemotherapy but also novel therapeutic approaches that exploit this unique chimeric situation (see table 1). Such strategies may employ selective stimulation of donor-derived hematopoiesis or augmentation of the graft-versus-leukemia (GVL) reaction either through cytokines or cellular therapy.

Table 1. Therapeutic approaches for patients relapsing after allogeneic transplants

Antineoplastic Therapy	
Conventional chemotherapy	Anthracyclines
	Vinca alkaloids
	Ara-C
	Steroids
	Alkylators
	Topo II inhibitors
Investigational chemotherapy	Topo I inhibitors
	Nucleoside analogues
	Phase I agents
Second transplants	
Enhancement of Graft vs. Leukemia	
Discontinue immunesuppression	
Cytokines	Interferon
	Interleukin-2
	G-CSF
	Others
Cellular therapy	Donor lymphocyte infusions

3.1. Cyclosporine withdrawal

Abrupt cyclosporine withdrawal has been reported to induce hematologic and cytogenetic remissions in patients with CML, AML, and ALL. All patients reported have developed GVHD [12–14]. Recently, a retrospective analysis from the Seattle group suggests that patients with CML and two consecutive abnormal cytogenetic tests benefited from cyclosporine discontinuation without exacerbation of GVHD [15]. Although further evaluation of this phenomenon is needed, it is reasonable to discontinue immunosuppressive therapy in patients who relapse after allogeneic BMT as an initial effort to induce remissions.

3.2. Conventional chemotherapy and radiotherapy

For patients ineligible or unwilling to receive investigational therapy, conventional chemotherapy can prolong survival and improve quality of life in patients obtaining a complete remission.

Patients with AML can be succesfully treated with a combination of cytosine arabinoside and anthracyclines, while patients with ALL have responded to combination therapy with vinca alkaloids, steroids, and an anthracycline [7,8,16–18].

Extramedullary relapses after allogeneic BMT occur in 20% of patients who undergo BMT for AML and 6% to 30% of patients with ALL [7,8,19]. Most occur in the presence of marrow relapse. Patients with central nervous system (CNS), testicular, or symptomatic extramedullary disease may receive localized radiation therapy. Isolated extramedullary relapses will precede overt marrow relapses, and further therapy is warranted for long-term disease control after radiation therapy [20,21].

3.3. Second allogeneic BMT

Second transplants have been succesfully performed in selected patients who relapse after allogeneic BMT. Treatment-related mortality is between 30% and 40%, with approximately 20% of the patients becoming long-term survivors [22–31]. Patients with early relapse, or those who experienced serious toxic effects or GVHD during the first transplant, are poor candidates for repeat high-dose chemotherapy and should be offered alternative approaches.

Most second transplants have involved the same donor. Since patients undergoing a second transplant generally remain chimeric and retain donor-derived immunity, the preparative regimen is not required to be immunosuppressive [30]. For patients with more than one possible donor, there are no data that support the use of an alternative HLA-compatible donor as a means to achieve a greater GVL effect. For patients relapsing after a syngeneic BMT, an allogeneic transplant could be considered if a histocompatible donor is available.

Allogeneic peripheral blood progenitor cells are being actively explored as a source of stem cells for transplantation. Potential advantages could include more rapid recovery and, because of the large lymphocyte doses infused, a greater antileukemic effect. This approach is feasible, but its efficacy is unknown [32–34].

3.4. Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) can induce cytogenetic and hematologic remissions in patients relapsing after allogeneic BMT. Fluorescence in situ hybridization of the bone marrow cells did not detect differentiation of the leukemic clone and was most consistent with preferential stimulation of donor cell populations [35]. The experience so far suggests that patients with circulating blasts or extramedullary relapses are unlikely to respond too G-CSF and that some of these patients may have had acceleration of their disease during treatment. Seven of the eight responding patients have subsequently relapsed between 1 and 20 months after remission was reinduced [36]. This novel approach to therapy requires further evaluation to determine the mechanism of response. Assessment of the in vitro sensitivity of both normal marrow and leukemic cells could be useful in predicting response and requires evaluation [37].

3.5. Interferon

IFN-α has direct activity against leukemic cells and may enhance a GVL effect by upregulating MHC antigen expression and stimulating T-lymphocyte and NK-cell activity [38,39].

Treatment with IFN-α has been effective in selected patients with recurrent CML after allogeneic BMT [11,40,41]. For patients with CML relapsing after an allogeneic BMT, the actuarial probability of six-year survival was 36%. Survival was associated with four independent variables: CML phase, time from transplant to relapse, IFN-α therapy, and female sex [1]. For patients with a hematologic relapse after BMT, the cytogenetic response rate was 22% for those receiving IFN-α therapy versus 2% for those not receiving IFN-α. IFN-α delayed disease progression, improving the two-year probability of survival for patients with isolated cytogenetic relapse or hematologic relapse, but did not seem to effect long-term survival [9].

3.6. Interleukin-2

Interleukin-2 (IL-2) is a potentially active agent for treatment of leukemia relapse after allogeneic BMT. It has modest but definite activity as a single agent, and it can enhance the antileukemic and cytotoxic activity of T-lymphocytes and NK cells [42–45].

In a pilot trial, we have explored the combination of IFN-α and low-dose

IL-2 for patients with acute or chronic leukemia relapsing after allogeneic BMT. One of five patients with acute leukemia had clearing of her bone marrow and peripheral blood with restitution of donor hematopoiesis but died from a fungal pneumonia. However, three patients developed exacerbation of acute GVHD, and four patients required dose reduction [36]. Further study of this combination may be warranted in conjunction with other therapeutic modalities such as donor lymphocyte infusions.

3.7. Donor lymphocyte infusion

The antileukemic effects of unmanipulated donor peripheral blood lymphocytes in patients who had relapsed after allogeneic BMT was described by Kolb in 1990 [46]. These initial obsevations have been confirmed by other investigators (table 2) [47–56]. Results from the largest reported series suggest that patients with CML recurring as isolated cytogenetic relapse or chronic phase respond better to this treatment than patients with more advanced disease or acute leukemia. Thirty-one of 39 patients with isolated cytogenetic relapse or chronic phase achieved cytogenetic remissions compared with 5 of 12 patients with more advanced CML and 8 of 23 patients with acute leukemia [47].

Neither the effector cells or the mechanism responsible for the antileukemic effect of donor lymphocytes is understood. Donor-derived CD4+ and CD8+ T-cell clones have been reported to have antileukemic activity [57–59]. Natural killer cells have also been implicated in this process [60–62]. Likewise, donor cells reactive against recipient nonleukemic cells have been shown to inhibit colony formation in vitro [62].

Recent data suggest that a dose of at least 1×10^7 CD3+ cells/kg is needed

Table 2. Donor leukocyte infusions for CML relapse after allogeneic BMT

Ref	*N*	10^8 cells infused per/Kg	Aplasia	GVHD	Median time to documented response	Complete responses
46	3	4.4–7.4	NS	2/3	16 weeks	3/3
47	7	NS	3/9	4/9	NS	2/7
48	11	0.9–7.9	7/11	9/11	8 weeks	6/11
49	7	2.6–3.6	NS	2/7	12 weeks	5/7
51	3	3.8–12.3	1/3	3/3	12 weeks	2/3
52	14	1.8–10.1	2/14	9/14	16 weeks	8/14
53	6	0.34–5.2	2/6	5/6	12 weeks	4/6
54	8	3–5.5	6/8	5/6	16 weeks	5/8
63	18	0.001–5	3/18	1/18	NS	11/18
64	7	0.8–1.2	3/7	0/7	15 weeks	3/7
55	51	NS	NS	NS	NS	36/51

Ref, reference; N, number; Dx, diagnosis; TCD, T-cell depletion; GVHD, graft-versus-host disease; CML, chronic myelogenous leukemia; CP, chronic phase; NS, not stated; AP, accelerated phase; BC, blast crisis; CG, isolated cytogenetic relapse.

to achieve a response and that CD8+ lymphocytes may not be essential for the antileukemia effect, at least in CML [63,64].

GVHD and pancytopenia secondary to marrow aplasia have been important causes of treatment failure in most published series. These complications account for most of the 20% treatment-related mortality that has been associated with donor lymphocyte infusions. In patients with isolated cytogenetic relapses, the incidence and severity of the aplasia postlymphocyte infusion seems to be less than when patients receive donor lymphocyte infusion with more advanced disease. GVHD, which has been reported to occur in up to 80% of patients receiving donor lymphocytes, may be related to the number and type of cells infused. Some investigators have suggested that by infusing less lymphocytes, responses can still be achieved, but with a lower incidence of GHVD. We recently have explored the use of CD8-depleted donor lymphocyte infusions. This strategy has been effective in inducing remissions in patients with CML relapsing after an allogeneic BMT with a low incidence of GVHD and a high response rate. Our experience supports the hypothesis that CD8+ lymphocytes are not essential for a graft-versus-leukemia effect to occur, but contribute significantly to the pathogenesis of GVHD.

Patients with acute leukemia relapsing after an allogeneic BMT have also responded to donor lymphocyte infusions, but at a much lower level than CML (table 3). The response rate to donor lymphocyte infusions seems to be similar in patients receiving concomitant chemotherapy rather than in those receiving donor lymphocytes alone (3 of 12 vs. 2 of 9) [55]. This therapeutic strategy warrants further study, especially in patients relapsing early after allogeneic transplantation. It is also possible that infusion of CD34+ cells in addition to donor lymphocytes might facilitate recovery after salvage chemotherapy for patients relapsing after an allogeneic BMT.

4. Summary and therapeutic recommendations

Relapse after allogeneic BMT remains an important cause of treatment failure and requires systematic study to develop adequate treatment strategies.

Table 3. Donor leukocyte infusions for acute leukemia relapse after allogeneic BMT

Ref	*N*	10^8 cells infused per/Kg	Concomitant chemotherapy	Aplasia	GVHD	Complete responses
50	4	3.3–9.2	Amsacrine/Ara-C:3 None:1	NS	3/4	1/4
55	23	NS	NS	NS	NS	8/23
65	13	1.18–16.4	NS	NS	3/3	3/13
66	5	4.9–6.7	Mitoxantrone/VP16:3 Retinoic Acid:1 None:1	NS	4/5	4/5

Ref, reference; N, number; GVHD, graft-versus-host disease; NS, not stated.

The chimeric state following allogeneic BMT allows for innovative approaches.

Our approach is summarized in figure 1. For CML patients relapsing with an isolated cytogenetic relapse or into chronic phase, an initial trial of IFN-α or donor lymphocyte infusions is our recommended approach. High-dose chemotherapy and second BMT are reserved for patients who fail to respond, or who have relapsed into blast crisis.

Patients with acute leukemia relapsing at least one year after initial transplant, who have a good performance status, and who had no major complications during their first transplant should be considered for a second marrow or blood stem cell transplant. This could possibly be followed by some form of investigational therapy to prevent relapse. Patients whose remission lasted less than one year from initial transplantation are more difficult to treat because of their resistance to therapy and generally debilitated condition. These patients have occasionally responded to novel approaches, such as G-CSF, cytokines, or donor lymphocyte infusions. Further study of these treatments are required to determine their true value in the setting of relapse postallogeneic transplantation.

Relapse prevention after the initial transplant is now feasible and is being actively pursued [67–69]. More effective preparative regimens may be possible using novel chemotherapy or targeted radiotherapy approaches [70]. Mainte-

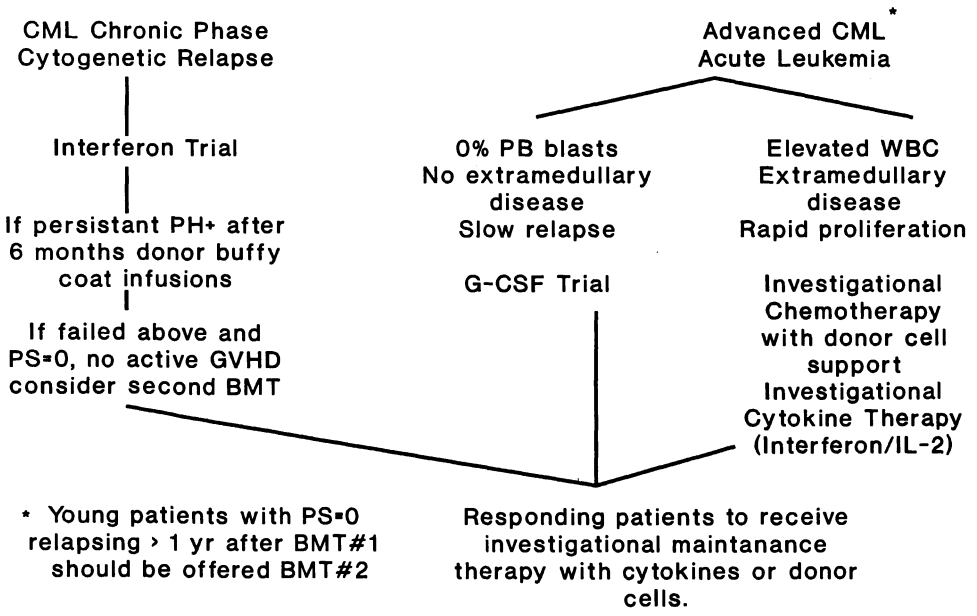

Figure 1. Suggested approach for treatment of relapse after allogeneic BMT.

nance chemotherapy or immunotherapy post-BMT is also being actively explored, but no definite conclusions as to the efficacy and utility of either can be made at this time [67–69].

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13. Acute lymphocytic leukemia: a comprehensive review with emphasis on biology and therapy

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1. Introduction

Acute lymphocytic leukemia (ALL) is a malignant hematologic disorder resulting from the clonal proliferation of lymphoid precursors with arrested maturation [1]. The disease can originate in lymphoid cells of different lineages, thus giving rise to B-cell or T-cell leukemias, or sometimes mixed lineage leukemia (see table 1). There are 3,000 to 5,000 new cases of ALL diagnosed every year in the United States [2,3]. In adults it represents 20% of all leukemias [4] and 1% to 2% of all cancers [5]. The disease has historic relevance, since it was one of the first malignancies reported to respond to chemotherapy [6] and was later among the first malignancies that could be cured in a majority of children [7]. Since then, much progress has been made, not only in terms of treatment for ALL but also, importantly, in understanding the heterogeneity of the disease. As information has accumulated about molecular aberrations, immunophenotyping, chromosomal abnormalities, and prognostic factors, more rational therapies have been designed. Since most cases are diagnosed in children [4], our current knowledge has originated from studies in the pediatric population. As differences between childhood and adult ALL became apparent, more research and progress has been made in adults.

2. Etiology

The etiology of ALL is not known. However, some associations have to be considered [8]:

2.1. Genetic factors

Some reports have identified families with multiple members affected by leukemia [9]. Several genetic syndromes have also been associated with leukemia, best characterized by Down's syndrome, and others such as Bloom syndrome, ataxia-telangiectasia, Wiskott–Aldrich syndrome, and Fanconi anemia [10–12].

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Table 1. Immunophenotype in ALL: expression of differentiation markers

B-lineage	HLA-DR	CD19	CD24	CD10	CD20	CD21	CD22	CD23	cIg	sIg
Early pre-B	+	+	+	+	±	−	Cyt	−	−	−
Pre-B	+	+	+	+	+	+	+	−	+	−
Transitional B	+	+	+	±	+	+	+	−	+	+μ[a]
Mature B	+	+	+	−	+	+	+	+	−	+

[a] μ heavy chains but no light chains.

T-lineage	CD7	CD2	CD5	CD1	CD4	CD8	CD3
Early	+	+	+	−	−	−	−
Intermediate	+	+	+	+	±	±	−
Mature	+	+	+	−	+	+	+

2.2. *Parental and socioeconomic factors*

Children of mothers older than 35 years of age may have an increased risk of leukemia [13]. A history of prior fetal loss, especially if there have been multiple miscarriages, has also been identified as a risk factor for the offspring [14]. The association of increased weight at birth and childhood ALL has been reported consistently [13,15].

2.3. *Environmental factors*

Exposure to radiation is associated with a definite risk for ALL [16–18]. Exposure to different chemicals, particularly benzene, has also been associated with an increased risk of leukemia [19]. The exposure to electromagnetic fields has been repeatedly linked to an increased risk for ALL [20–22], but the evidence is inconclusive. Several studies have suggested clustering of cases of childhood leukemia. These usually represent a group of cases occurring within a population, whose incidence is higher than that expected for the general population [23]. This clustering of cases has been attributed to the close proximity of environmental hazards, such as nuclear plants. However, evidence of this exposure is lacking in most cases. This and other epidemiologic data, such as the increased incidence of common ALL (cALL) with higher socio-economic status and isolation, has led to the hypothesis of an infectious etiology for common ALL in children [24,25]. According to this hypothesis, cALL at childhood peak ages might arise following unusual patterns of exposure to common infectious agents. In more developed societies with better hygiene and less social contacts early in infancy, common infections are frequently delayed beyond the first year of life and until a higher level of social contacts is made [24,25].

3. Classification

ALL is a heterogeneous group of disorders, with several subgroups that have distinct clinical and prognostic features. Several attempts at classification of ALL have been made. The two most relevant ones are the morphologic and immunophenotypic classifications.

3.1. Morphologic classification

Morphologic classification follows the guidelines proposed by the French–American–British (FAB) Cooperative Working Group [26,27]. It identifies three subgroups of ALL (table 2): L1 is the most common variety in children, representing 85% of all cases, but only 30% of adult cases [28,29]; L2 is the predominant variety in adults, affecting 60% to 70% of all cases, but less than 15% of childhood ALL [93,94]; and L3, which represents less than 5% of all cases. The original FAB classification is not always reproducible, and a scoring system has been added to increase concordance between observers [27].

3.2. Immunophenotypic classification

Immunophenotypic classification of ALL is more clinically relevant and is based on the expression of certain antigens on the surface of leukemic cells. Normal lymphocytes express specific antigens in an orderly fashion through their different stages of differentiation [30]. According to Greaves [31], lymphoblasts represent an interruption at different steps of differentiation of normal lymphocytes. Therefore, expression of antigens on the cell surface indicates the specific step in differentiation where transformation occurred. Several classifications have been proposed for normal [32,33] and leukemic [34,35] lymphocytes. The immunophenotypic classification of ALL with the frequency of each subtype is presented in table 3.

Table 2. FAB classification of acute lymphoblastic leukemia

Type	Incidence		Characteristics	CR (%)	Three year survival (%)
	Adult	Children			
L1	31	85	Small, homogeneous cells; round nucleus; scanty cytoplasm.	85	40
L2	60	14	Large, heterogeneous cells; irregular nucleus, cleft, nucleolus; more cytoplasm.	75	35
L3	9	1	Large, homogeneous; regular nucleus; vacuolated, basophilic cytoplasm. Burkitt's leukemia Poor prognosis	65	10

Table 3. Immunophenotypic classification of acute lymphoblastic leukemia

Type	Markers	Incidence (%)		Observations
		Children	Adults	
Early pre-B	cIg−	65–70	50–60	Express at least one B-cell marker CALLA + or −
Pre-B	cIg+	15–20	15–25	Express at least one B-cell marker CALLA + or − Worse prognosis than early pre-B
B	sIg+	<5	<5	Extramedullary lymphomatous masses, CNS involvement, hyperuricemia, ARF Burkitt's leukemia
T	CD2 CD3 CD5 CD7 Others (CD4, CD8)	10–15	20–25	High WBC, CNS involvement, thymic mass

Although this classification is useful clinically, some cautionary notes have to be added. First, the phenotype of the lymphoblasts may not correlate with any normal phenotype, including some cases with simultaneous expression of antigens normally present at different ends of the differentiation spectrum (i.e., asyncronous antigen expression) [36], although some of these lymphoblasts may actually have a rare normal counterpart [37]. Approximately 5% to 10% of childhood ALL cases [38,39] and 30% of adult ALL cases [40–42] express myeloid markers. It is not clear whether these cases represent transformation of a pluripotent cell or an as yet unidentified progenitor coexpressing markers and features from several lineages [43,44]. No marker is absolutely lineage specific; in fact, CD19, CD2, and CD4 can be found in at least 50% of AML with t(8;21), APL, and AML with monocytic features, respectively [45–47]. Therefore, it has been suggested that at least two markers corresponding to a different lineage need to be present to diagnose a mixed-lineage leukemia [39].

There are some non-lineage-dependent markers. The most common is CD10, also known as common acute lymphocytic leukemia antigen (CALLA), which is a membrane-bound neutral endopeptidase [48]. It can be expressed in both B- and T-cell ALL [49]. CD34 is a marker of a very early pluripotential cell, including the stem cell [50], and is most frequently expressed in non-T, non-B cell ALL cases [51]. Cells coexpressing CD38 and represent lineage-committed cells [52]. This represent 20% of normal bone marrow cells, as well as activated plasma cells and T-cells, and is a common marker in both T-cell and B-cell leukemias [53]. CD71 is another marker of activation and is more common in T-cell than in B-cell leukemias [53].

The immunologic classification of ALL correlates with clinical characteristics, with certain features associated with specific subtypes:

3.2.1. Early pre-B-cell ALL. Nearly 70% of childhood cases and adult ALL are early pre-B-cell ALL [54]. The hallmark of this immunophenotype is the lack of expression of cytoplasmic or surface immunoglobulins. Patients are frequently young (1 to 9 years old) and have low WBC counts [48]. Nearly half of those younger than one year old, 10% of older children, and 10% to 40% of adults with this immunophenotype are CALLA negative [55,56]. Lack of expression of CD10 is associated with pseudodiploidy, high WBC counts, and poor prognosis. CD10-negative early pre-B ALL probably represents a more immature counterpart of CD10-positive early pre-B ALL [57].

3.2.2. Pre-B-cell ALL. This group is characterized by the expression of cytoplasmic immunoglobulin heavy chains [58,59]. It represents approximately 20% of all cases of ALL, and almost all express CD10 [54]. These patients have higher hemoglobin levels, WBC counts, and LDH levels. Cytogenetics are often pseudodiploid, with frequent association with t(1;19), and are less likely to be hyperdiploid [60–61]. Poor-prognosis characteristics and poor outcome are correlated to the t(1;19) abnormality [62]. Other studies suggest that among patients with t(1;19), pre-B immunophenotype correlates with a worse prognosis than early-pre-B immunophenotype [63]. More than three fourths of pre-B cell childhood ALL patients express CD34, a feature frequently accompanied by hyperdiploidy, low incidence of CNS involvement at presentation, and good prognosis [64,65].

3.2.3. Transitional pre-B-cell ALL. This subtype has been recently characterized. It is present in approximately 1% of all cases. The hallmark is the expression of μ heavy chains on the cell surface, but without light chains [66]. Patients with this phenotype have L1 or L2 morphology and low WBC counts and are hyperdiploid. They have a better outcome than patients with mature B-cell ALL.

3.2.4. Mature B-cell ALL. Mature B-cell ALL occurs in less than 5% of patients with ALL [54]. It frequently presents with bulky extramedullary disease, including abdominal lymphadenopathy and CNS involvement [67]. Morphologically, it often represents the L3 subtype of the FAB classification. Some cases of mature B-cell ALL do not show the L3 morphology and have lymphoma-like features with particular karytoypic abnormalities such as $6q^-$, $14q^+$, t(11;14), or t(14;18).

3.2.5. T-cell ALL. T-cell ALL is present in nearly 15% to 20% of cases of childhood and adult [68] ALL, although its incidence may decrease with age [69]. It is associated with male gender, older age, high WBC counts, CNS involvement, and mediastinal masses [70], the latter associated with mature thymocyte phenotypes [71]. Patients with T-cell ALL lacking CD10 expression have a worse prognosis [49].

3.3. *Cytogenetic classification*

Several cytogenetic abnormalities have been described in ALL. These can be numeric or structural [72,73], as shown in table 4. Ploidy is of significant prognostic value, whether determined by karyotype or by DNA content. When analyzed by DNA index (i.e., DNA content in leukemic cells versus that in normal cells), patients with an index greater than 1.16 correspond approximately to more than 50 chromosomes. These patients have the best prognosis, with a four-year EFS close to 90% [74]. Hyperdiploidy is present in 25% to 30% of children with ALL [75] but only 10% to 20% of adults. The index classification should be accompanied by regular cytogenetics, since the presence of additional structural abnormalities (as occurs in 60% of hyperdiploid cases) makes the prognosis not as good as with the numeric abnormality alone [76]. Patients with a DNA index greater than 1.16 usually present with good prognostic features (i.e., age 1 to 9 years, low WBC counts, early pre-B phenotype), but those with high-risk features should be treated as good risk if hyperdiploid [77]. Hyperdiploid patients with near tetraploid cells (approximately 1% of all cases) [75,78] and those with 47 to 50 chromosomes (i.e., a DNA index >1 but <1.16; 15% of all cases) [75] have an intermediate prognosis [79]. The near-tetraploid group represents an older age group frequently with a T-cell phenotype [80]. Patients with 47 to 50 chromosomes often have additional chromosomes 21, X, 8, and 10, and in 76% of cases also have additional structural abnormalities [81]. When trisomy 21 is the sole chromosomal abnormality, patients may have a particularly good prognosis, in part due to the association with other good prognostic features [82].

Hypodiploid cases, most frequently from loss of chromosome 20 [83], represent 6% of all cases of ALL [75]. Although commonly presenting with good

Table 4. Cytogenetic classification of acute lymphoblastic leukemia

		Incidence (%)	
	Cytogenetic abnormality	Children	Adults
Numeric abnormalities	Hyperdiploid	40–50	10–20
	47 to 50 chromosomes	15–20	5–10
	>50 chromosomes	25–30	5–10
	Diploid	10–30	25–35
	Hypodiploid	7–10	5–10
Structural abnormalities	Pseudodiploid	40–50	50–60
	t(9;22)(q34;q11)	3–5	15–25
	t(8;14), t(8;2), and t(8;22)	3–5	5–10
	t(4;11)(q21;q23) [and others involving 11q23]	5	5
	t(1;19)(q23;p13.3)	5–7	<5
	14q11 abnormalities	<5	5–10
	7q35 abnormalities	<5	<5
	Others	5–15	5–15

prognostic features these cases have an intermediate prognosis [83]. Patients with near haploid disease (<1% of all cases) [75] have a very poor prognosis [84–86]. Eight to ten percent of all patients have a normal diploid karyotype [75], but the frequency is as high as 30% in T-cell ALL [87]. The prognosis of disease with a normal karyotype is intermediate [79]. Numeric chromosomal abnormalities (hyper- and hypodiploid) are less common in adults and have much less impact in outcome than they do in children [85]. Adults with diploid ALL may have the best prognosis [85].

Pseudodiploid ALL represents a large percentage of patients [75,88]. Overall, these patients have a poor prognosis. Some of the specific abnormalities deserve special attention:

3.3.1. Translocation t(9;22)(q34;q11) or Philadelphia-chromosome (Ph). The Ph-chromosome is present in less than 5% of children with ALL [89–91], but in 15%–30% of adults [92,88,93]. When assessed by molecular techniques, Ph-related abnormalities are present in up to 30% of adults with ALL [94,95]. Ph-positive ALL is associated with older age, high WBC counts, and L2 morphology [90,93]; in adults, it is also associated with a higher frequency of expression of CD10 and CD34 [93]. Nearly half of patients with Ph-positive ALL may have additional chromosomal abnormalities, most frequently monosomy 7 [96]. At the molecular level, the Ph-chromosome in ALL may be different from the one seen in CML. The breakpoint at band 34 of the long arm of chromosome 9 splices the proto-oncogene c-abl to band 11 of the long arm of chromosome 22 in the bcr gene [97]. In 50% to 80% of ALL cases, the breakpoint in 22q11 falls between exons b1 and b2 of the major breakpoint cluster region (M-bcr) [98] as opposed to between b2 and b3 or b3 and b4 in CML [97]. This translates into a different protein product of only 190kDa ($p190^{BCR/ABL}$), compared to that of CML (210kDa, $p210^{BCR/ABL}$) [97,98]. Both proteins have increased tyrosine kinase activity [99]. $p190^{BCR/ABL}$ can induce acute leukemia in transgenic mice [100] and may have comparatively higher transforming potential than $p210^{BCR/ABL}$ [101]. Twenty percent to 50% of adults with Ph-positive ALL express p210 rather than p190 [95]; some of these patients may have a blastic phase of a previously unrecognized CML [102].

The outcome of patients with Ph-positive ALL is poor, with significantly low CR rates (75% in children, 50% to 70% in adults) [90,93] and long-term disease-free survival rates (less than 10%) [90,93,94].

3.3.2. t(8;14), t(8;2), and t(8;22). These translocations are present in most cases of mature B-cell ALL [103] and Burkitt's lymphoma [104]. The proto-oncogene c-myc present in band 24 of the long arm of chromosome 8 is juxtaposed to an immunoglobulin locus, most frequently the heavy chain (chromosome 14q32), but sometimes the light chains κ (2p12) or λ (22q11) [105]. This results in overexpression of myc [106], a transcription factor that

interacts with other proteins (MAX, MAZ) and binds to DNA [107]. In transgenic mice, overexpression of c-myc driven by immunoglobulin enhancers induces lymphoid malignancies [108].

3.3.3. t(4;11) and other abnormalities in band 23 of the long arm of chromosome 11 (11q23). Abnormalities in 11q23 are frequently associated with acute myelogenous leukemias developing in patients treated for ALL and other malignancies [109–112]. ALL with 11q23 abnormalities can present de novo and is seen in approximately 5% of childhood ALL and less often in adults [113]. Patients with 11q23 abnormalities are frequently young, black, with high WBC counts [113,114], with CD10 negative, and with early pre-B-cell disease [115]. Coexpression of myeloid markers is a common feature. It is the most common chromosomal abnormality in infant ALL [116], and when investigated at the molecular level, it affects more than 70% of cases [117]. It carries a poor prognosis and may be the major culprit of the poor prognosis in infant ALL; infants who do not have this chromosomal abnormality may have an outcome comparable to that of intermediate-risk childhood ALL, but infants with 11q23 rearrangements have a three-year event-free survival of 13% compared to 67% for those without this abnormality [118]. Chromosome 11q23 codes for a gene called MLL or ALL-1 [119,120] with unknown function. The frequent involvement of this gene in mixed lineage and myeloid leukemias suggests a role in lineage differentiation.

3.3.4. t(1;19). This is the most common translocation in childhood ALL, present in 5% of all cases [121–123], but is very uncommon in adults. It is frequently associated with a pre-B cell immunophenotype [61,124]. Patients with this abnormality frequently carry other poor prognostic factors (high WBC, high LDH) and have a poor prognosis [61]. At the molecular level, it results in the fusion of E2A, an immunoglobulin enhancer-binding protein coded for in chromosome 19p13, with PBX, a homeobox protein that binds to DNA and is probably a transcription activation factor, coded for in chromosome 1q23 [125]. This results in the constitutional expression in pre-B-cells of a gene (PBX) that is normally not expressed in these cells.

3.3.5. 14q11 and 7q35 abnormalities. These regions contain the loci for the α/δ and β T-cell receptor (TCR) gene, respectively. They are rearranged in patients with T-cell ALL [126–130]. The most frequent abnormality involving these genes is a t(11;14)(p13;q11), present in 7% of T-cell ALL [87,131], which fuses the TCR α/δ to a gene called rhombotin 2 or Ttg-2 [132]. A less common translocation, t(11;14)(p15;q11), present in only 1% of T-cell ALL [222], affects rhombotin 1 or Ttg-1 [133]. The rhombotin (Ttg) family of genes is involved in transcription regulation via a LIM-domain-mediated protein interaction, which in turn could prevent transcription activation by LIM-domain protein partners [134]. Other partner genes for TCR α/δ include HOX11 in the t(10;14), a homeobox gene [135] that binds DNA and activates gene expres-

sion; TAL1/SCL in t(1;14), a basic helix-loop-helix protein that binds DNA and can control transcription either directly or by dimerization with other DNA binding proteins [136,137]; and c-myc in a t(8;14) [138]. Translocations affecting TCR β are less common [130,139,140]. Partner genes involved in these translocations include TAL2, similar to TAL1/SCL, in a t(7;9)(q34;q32) [141]; LYL1 in t(7;19), analogous to TAL2 [136,137]; rhombotin 2/Ttg2 in t(7;11); and TAN-1 in t(7;9) [142].

3.3.6. Others. Abnormalities in the short arm of chromosome 9 occur in 5% to 10% of childhood and adult ALL [143,144]. It is associated with high WBC counts, older age, T-cell immunophenotype, a high rate of extramedullary relapse [145], and poor outcome. This abnormality affects 9p21–22, which contains the IFN-α and IFN-β genes [146]. Abnormalities in 6q occur in 6% of cases [88], and their clinical and prognostic significance is uncertain [147]. The short arm of chromosome 12 is affected in 10% of cases of childhood ALL [148], usually of B-lineage, with great heterogeneity of the specific abnormality. Patients with this abnormality may have a higher incidence of CNS relapse [149]. Mutations of N-ras have been detected in 6% of childhood ALL, clustered in codons 12 and 13, and may be a poor prognostic feature [150].

4. Prognosis

Several prognostic factors have been identified for childhood [75,151,152] and adult [92,153,154] ALL, and risk categories have been defined and used to guide therapy. Some of the better defined prognostic factors are given below.

4.1. Age

Infants under 1 year of age and children older than 10 years of age have a worse prognosis than patients 1 to 9 years old [75,151,155]. Adults have a worse prognosis than children, with the worst outcome associated with age older than 60 years [92,153,156].

4.2. WBC

The WBC count at presentation is a highly significant prognostic variable. In children, the cutoff at which good prognosis is defined varies in different centers [77], but counts greater than 50×10^9/L are clearly associated with a poor outcome [57,77]. In adults, the cutoff value is not clear but is probably lower than that in children; the cutoff for prognostic significance has ranged from 5 to 50×10^9/L [157].

4.3. Cytogenetics

Cytogenetics is probably the most important prognostic factor for ALL [158]. In children, ploidy is the most important prognostic factor [74,159,160]. Patients with hyperdiploid ALL, in particular those with more than 50 chromosomes, have the best prognosis [79,88].

4.4. Immunophenotype (table 5)

T-cell ALL represents a group with historically poor outcome, having a five-year event-free survival of 50% in children [161] and 10% to 20% in adults. Recent studies have shown a similar or better outcome to that of other immunophenotypes [92,153], probably from the inclusion of cyclophosphamide and cytarabine in the treatment of this subgroup of patients [162,163]. Within the T-cell phenotype, those with pre-T phenotype (CD7+, CD2−, CD1−, CD4−, CD8−) have a worse prognosis.

Mature B-cell ALL has historically been associated with a poor prognosis. The introduction of hyperfractionated cyclophosphamide, high-dose methotrexate, and cytarabine have significantly improved the results, both in children [67,164] and in adults [165]. The best prognosis among B-lineage ALL is associated with early pre-B-cell phenotype, in particular when associated with CD10 [58].

Wide variations in the incidence of myeloid markers from less than 20% to more than 40% have been reported, most commonly in adults. Some investigators have reported an associated poor prognosis [166], but not others [167,168], especially when adjusted for other poor prognostic factors [42].

4.5. Other prognostic factors

Expression of CD34 has been correlated with a favorable outcome in children with pre-B phenotype [169], but not in adults [167]. Expression of MDR-associated protein P-170 has been reported to confer a poor prognosis on both children and adults [170]. Patients with MDR-positive ALL at diagnosis have lower CR rates, higher relapse rates, and shorter survival than MDR-negative patients [170]. Males and blacks may have a worse prognosis [171,172]. Late response to therapy is a poor prognostic feature in all age groups [92,153,173,174].

4.6. Prognostic models

Several prognostic models have been proposed using these risk factors. In children, an NCI-sponsored workshop has used age and WBC counts to define risk [77]. Patients aged 1 to 9 years with a WBC less than 50×10^9/L represent 68% of all B-precursor ALL in children and have a four-year event-free

survival of approximately 80%. Patients older than 10 years or with WBC counts greater than 50×10^9/L have a four-year event-free survival of approximately 64% [77]. There is a strong correlation between age and WBC counts. In infants, almost 50% of patients have counts of 50×10^9/L or more, whereas less than 20% of older children have counts that high [151] and 50% have counts lower than 10×10^9/L [77,151]. In adults, 25% of all patients have counts greater than 50×10^9/L.

In adults, several prognostic models have been proposed with close similarities; these are summarized in table 5. High-risk groups represent the majority of adult ALL cases. Hoelzer et al. identified time to CR of more than four weeks, age greater than 35 years, WBC count higher than 30×10^9/L, and null-cell phenotype as poor prognostic features [153]. Patients with none of these features (27% of cases) have a five-year remission rate of 62%, compared to 28% for those with one or more of these features [153]. Ph-positive ALL is a definite poor prognostic group, while the prognosis of mature B-cell ALL is changing; favorable outcomes (CR rates of 80% to 90%; long-term disease-free survival rates of 40% to 60%) have been reported with short-term dose-intensive regimens. The WBC cutoff is dependent on whether the particular model incorporates older age as a poor prognostic factor, since an inverse correlation is noted between age and WBC counts. In the model of Hoelzer et al. [153], age of more than 35 years (more than 50% of patients) is a poor prognostic factor; thus the WBC count cutoff is high (30×10^9/L). In a model from MDACC [153], age does not enter into the model as a poor prognostic factor; consequently the WBC count cutoff is lower (5×10^9/L) (table 5).

Table 5. Prognostic models for adult ALL

Risk category	Hoelzer et al. [142]	Kantarjian et al. [161]	Gaynor et al. [143]
Standard risk			
% of all patients	27	28	40
Long-term DFS (%)	62	70	61
Intermediate risk			
% of all patients			22
Long-term DFS (%)			43
High-risk			
% of all patients	73	72	38
Long-term DFS (%)	28	27	20
High-risk features	WBC $>30 \times 10^9$/L	WBC $\geq 5 \times 10^9$/L	WBC $>20 \times 10^9$/L
	Age >35 years		Age >60 years
	Null-cell ALL	B-cell ALL	Null- or B-cell ALL
	CR after >4 weeks	CR after ≥2 induction courses	CR after >5 weeks
		Ph-positive ALL	Ph-positive
		CNS leukemia	

DFS, disease-free survival.

5. Treatment

Modern therapy has changed the outcome of patients with ALL. In children, ALL is now a highly curable disease, with cure rates ranging from 60% to 85%. Therapy of ALL in adults has followed the lead of childhood ALL. About 75% of adults with ALL (range 65% to 90%) achieve a complete remission, but despite significant progress in the last three decades, only 20% to 40% are cured [175–177]. The value of individual components of the different phases of ALL therapy is sometimes controversial.

5.1. Induction

With the combination of vincristine and prednisone, 40% to 60% of patients achieve a CR, although the median remission duration is short (3 to 7 months) [178,179]. The addition of anthracyclines to this combination improves the CR rate to 85%, compared to 47% without anthracyclines ($p = 0.003$) [180]. The combination of these three drugs is now standard for remission induction in adult ALL, with CR rates of 70% to 85%. Doxorubicin and daunorubicin are the commonly used anthracyclines and have produced similar results [180,181]. Mitoxantrone may also be effective [182]. Among the corticosteroids, prednisone and methyl-prednisolone are most frequently used; however, dexamethasone penetrates the CNS–blood barrier better and exhibits better in vitro antileukemic activity [183]. With this induction chemotherapy, the mortality associated with therapy is 10% or less.

Other chemotherapeutic agents have been incorporated into induction regimens in an attempt to improve CR rate and CR duration, including cyclophosphamide, L-asparaginase, cytarabine, and less frequently etoposide, teniposide, and m-amsacrine. The benefit from these modifications is difficult to determine, but overall results seem to be equivalent to those with vincristine, anthracyclines, and corticosteroids. In one study, half the patients treated with induction therapy with vincristine, asparaginase, daunorubicin, and corticosteroids were randomized to receive additional cyclophosphamide during induction. The CR rate was 84% for both arms, and the continuous CR rate at three years was also similar (47% versus 43%) [184]. In another study, however, the addition of cyclophosphamide may have improved outcome for patients with T-cell ALL [185]. The addition of high-dose cytarabine to the induction regimen did not improve the results and led to an increased toxicity and induction mortality [186]. Cytarabine at lower doses, together with thioguanine and daunorubicin, was added to vincristine and prednisone in another study, resulting in a CR rate of 91%, but the median remission duration was only 15 months [187]. Cytarabine during induction may also improve outcome in T-cell ALL [188]. L-asparaginase has been added to induction with no improved CR rates [188–191], although in children remission duration may be prolonged [192]. In one study, L-asparaginase was used instead of anthracyclines in the induction regimen, resulting in a similar out-

come but with the potential benefit of decreased cardiotoxicity [193]. Using methotrexate instead of anthracycline produced equivalent results [194]. More intensive regimens with growth factor support may induce rapid tumor-burden reductions and potentially better outcome [195]. Preliminary results with such an approach are encouraging [196,197]. Duration of neutropenia may be shortened [196,198], but there is the potential risk of stimulation of leukemic cells by growth factors [199].

Some subsets of patients may require a different approach for induction of remission. Patients with T-cell ALL may benefit from the addition of cytarabine and cyclophosphamide [92,153,185,188]. In mature B-cell ALL, the use of hyperfractionated cyclophosphamide alternating with high-dose methotrexate and cytarabine has resulted in cure rates of 50% to 60% in children and more recently also in adults [165,197,200–202].

5.2. Consolidation

Intensive consolidation has demonstrated its value in childhood ALL. High-dose methotrexate [203], sometimes in combination with 6-mercaptopurine (6MP) [204,205] or with teniposide and cytarabine [206], and the use of asparaginase [192] have significantly contributed to the cure rate of 70% to 80% in childhood ALL. Delayed intensification has also improved the outcome in childhood ALL [207], but its benefit in adult ALL has been dificult to prove. Some studies have failed to demonstrate that consolidation improves results in adult ALL [208], while others concluded that it improves the outcome [177]. This discordance may be due to the difficulty in assessing the specific value of individual components or phases of the overall treatment. Some of the most effective regimens reported in the literature have included some form of consolidation, but its intensity varied from asparaginase alone [191] to combinations including cyclophosphamide, cytarabine, 6MP, and methotrexate [92,153,157]. These studies have resulted in median remission durations of 20 to 24 months and three-year survival rates of 35% to 45%. In one study, 61 patients were randomized to receive three courses of consolidation at monthly intervals with doxorubicin, cytarabine, and asparaginase versus no consolidation. The three-year disease-free survival was 38% with and 0% without consolidation [209]. In contrast, the European Organization for Research and Treatment of Cancer (EORTC) randomized patients to receive a three-month consolidation schedule with methotrexate, cytarabine, and thioguanine, versus maintenance therapy after induction of CR without consolidation. No difference in disease-free survival between the two groups was observed [210]. Similarly, the Cancer and Leukemia Group B (CALGB) randomized patients after induction to receive two courses of consolidation with cytarabine and daunorubicin versus maintenance with 6MP and methotrexate. The CR duration and overall survival were similar for both arms [211]. It is difficult to obtaind definitive conclusions from these studies because of the limitations involved. These include (1) the limited time and

intensity of the consolidation used in these patients, and (2) the use of schedules that did not include some of the most effective agents in consolidation for childhood ALL, such as high-dose methotrexate and 6MP, high-dose asparaginase, and cyclophosphamide with cytarabine. The use of high-dose cytarabine may be beneficial in some patients. Rohatiner et al. found a trend for prolonged remission duration using high-dose cytarabine consolidation in patients with high blast cell counts or T-cell morphology [188]. A German multicenter trial used high-dose cytarabine with mitoxantrone for intensification in high-risk patients and achieved a continuous CR rate at four years of 43%, compared to 23% for those not receiving this therapy. However, older patients were frequently not offered the high-dose therapy, and the difference in results may be at least partially explained by the presence of higher-risk patients in the control arm [212]. The Eastern Cooperative Oncology Group (ECOG) used high-dose cytarabine consolidation without improvement in outcome. In this trial, however, some patients received very short induction regimens, and no patient received maintenance therapy [213].

In conclusion, consolidation may be beneficial when adequate drugs at adequate doses are used. Some subsets may benefit from specific agents, i.e., T-cell ALL from cytarabine and cyclophosphamide, and Ph-positive ALL and other high-risk groups from high-dose cytarabine.

5.3. Maintenance

The benefit of maintenance therapy has been established in several studies in childhood ALL. Maintenance therapy is usually given with 6MP and methotrexate and continued for two years. However, when adequate serum levels of these drugs are not achieved, the outcome may be as poor as when no maintenance is given [214,215]. Maintenance therapy including these drugs has been used in adult ALL [92,153,157,191,216] in different schedules. Some have used relatively intensive maintenance with regimens including high-dose methotrexate, daunorubicin, mercaptopurine, and prednisone (M-DOMP) [157], or vincristine, prednisone, doxorubicin, 6MP, oral methotrexate, actinomycin-D, cyclophosphamide, and carmustine [216]. Others have used less intensive regimens with oral 6MP and methotrexate reinforced by monthly doses of vincristine and prednisone and occasionally the addition of doxorubicin or carmustine and cyclophosphamide [153,191].

Some studies have used no maintenance after induction of remission. The ECOG used consolidation with high-dose cytarabine and methotrexate, asparaginase, cyclophosphamide, doxorubicin, vincristine, and prednisone, with no maintenance. The four-year disease-free survival was only 13%, with a median remission duration of 9.6 months [213]. The CALGB used four intensification courses with several agents including cytarabine, 6MP, methotrexate, and asparaginase, but no maintenance. The median remission duration for these patients was only 11.2 months [182]. One study from Italy randomized patients to receive conventional maintenance or a more intensive

schedule with 6MP and methotrexate alternating with the same drugs used for consolidation. No difference in disease-free survival was observed [217]. Conventional maintenance with 6MP and methotrexate could therefore be as effective as more intensive regimens in adult ALL.

Some subgroups are not treated with maintenance chemotherapy. Patients with mature B-cell ALL do not need maintenance with 6MP and methotrexate. These patients are usually treated with 3 to 8 months of dose-intensive therapy, which results in disease-free survival rates of 40% to 60%. Patients with Ph-positive ALL do not benefit from 6MP and methotrexate maintenance. These patients should be included in investigational approaches including the use of alpha interferon, high-dose cytarabine, immunomodulation, dose-intensive chemotherapy with autologous stem cell transplant (with or without purging), and gene-targeted therapy if allogeneic BMT is not feasible in first CR.

5.4. CNS prophylaxis and treatment

CNS disease is present at diagnosis in only 5% of children and adults [218,219]. However, without adequate prophylaxis, 50% to 75% of patients will eventually have CNS disease [220,221]. CNS prophylaxis has reduced the incidence of CNS relapses to less than 10% [118]. Different alternatives have been used for prophylaxis of CNS disease, including cranial radiation, intrathecal chemotherapy with methotrexate and cytarabine, and high-dose systemic chemotherapy [222,223]. These can result in neurologic sequelae including intellectual dysfunction, seizures, and dementia [224], as well as extraneural complications such as slow growth, particularly in children [225]. Complications may be more common in patients receiving cranial radiation [224,226], and prophylaxis without the use of cranial radiation may be as effective [227].

Adults with ALL frequently receive prophylaxis to the CNS with intrathecal (IT) chemotherapy and cranial radiation, which has resulted in a lower incidence of CNS relapse [228,229]. The complications associated with CNS prophylaxis in adults are frequently asymptomatic and are detected only by electroencephalogram or CT scans [230]. It is questionable whether the reduced incidence of CNS leukemia has resulted in improved survival. In a study randomizing patients to receive cranial radiation and IT methotrexate or no CNS prophylaxis, the three-year CNS relapse rate significantly decreased from 45% to 20% with prophylaxis. This did not translate, however, into an improved survival [228]. In our studies in adult ALL, early intervention with IT chemotherapy and high-dose systemic chemotherapy without cranial radiation has proven to be highly effective prophylaxis, particularly for patients with high-risk for CNS relapse [219]. Recent studies have emphasized intrathecal therapy plus high-dose systemic chemotherapy over cranial irradiation as CNS prophylaxis in both childhood and adult ALL.

Several reports have identified risk factors for the development of CNS

leukemia. In children, high WBC counts, T-cell or B-cell disease, young age, lymphadenopathy, thrombocytopenia, hepatomegaly, and splenomegaly are associated with a higher risk of CNS relapse [220,231]. In adults, mature B-cell phenotype, high serum LDH levels, and high proliferative index (i.e., cells in S+G2M compartments ≥14%) have been identified as risk factors for CNS disease [232]. Patients with none of these factors (40% of patients) had an incidence of CNS leukemia at one year of 5%, compared to more than 50% for those with high LDH and high proliferative index [232]. The intensity of CNS prophylaxis could then be adjusted to the risk of CNS disease according to this model [219].

Patients who develop CNS leukemia should receive more aggressive therapy. One proposed therapeutic scheme includes IT methotrexate alternating with IT cytarabine twice weekly until the CSF clears, then weekly for one month, and once monthly thereafter for two years [211]. Cranial radiation may be indicated in these patients [201,211]. For those patients with 5 or less WBC/μl CSF together with blasts, prophylaxis as for CSF blast-negative patients may be equally effective [227]. Patients with cranial nerve root involvement may benefit from selective irradiation to the base of the skull.

5.5. Allogeneic BMT

Allogeneic BMT is an effective alternative for therapy in ALL, but the timing of the transplant is controversial. Allogeneic BMT in first remission has resulted in long-term disease-free survival in 22% to 60% of patients in different series [233–235]. This wide range derives from the variability in patient selection, since factors such as age, phenotype of the disease, WBC count, sex mismatch, and the type of GVHD prophylaxis used significantly influence the outcome of BMT [236]. The International Bone Marrow Transplant Registry (IBMTR) reported a 44% five-year actuarial leukemia-free survival for patients transplanted in first remission [237]. Several other studies have reported long-term disease-free survival of 40% to 70% [238–240]. Patients receiving allogeneic BMT usually represent a highly selected population of young patients with no organ dysfunction. To clarify the value of allogeneic BMT in patients in first remission, Horowitz et al. [241] conducted a retrospective analysis of patients who received intensive consolidation and maintenance with the Berlin–Frankfurt–Munster (BFM) regimen versus those receiving an allogeneic BMT in first remission. After accounting for age and lead-time bias to BMT, the five-year leukemia-free survival was 38% with chemotherapy and 44% with transplant [241]. With chemotherapy, the five-year probability of relapse was 59% and the probability of treatment-related death 4%; for those treated with BMT, the probabilities were 26% and 39%, respectively [241]. No specific subgroups that could benefit from BMT in first remission could be identified in this study [233].

In a prospective study by Fière et al. [242], patients younger than 40 years with an HLA-identical sibling who achieved remission after induction chemo-

therapy were assigned to allogeneic BMT; if older than 50 years, patients received consolidation with chemotherapy; and all others were randomized to consolidation with chemotherapy alone or autologous BMT. The estimated three-year disease-free survival was 43% for patients receiving an allogeneic BMT, 39% for autologous BMT, and 32% for chemotherapy (non-statistically significant difference). The older patient subgroup had a significantly shorter three-year disease-free survival of only 24% [242]. In an update of this study, the five-year disease-free survival was 45% for the allogeneic BMT group and 31% for the control group (combining the autologous BMT and chemotherapy groups) ($p = 0.1$) [243] (table 6). When patients with high-risk disease (i.e., Ph-positive ALL, null or undifferentiated ALL, age >35 years, WBC count $>30 \times 10^9$/L, or time to CR >4 weeks) were analyzed separately, the five-year disease-free survival was 39% with allogeneic BMT, and 14% with other therapies ($p = 0.01$). For standard-risk patients (62.5% of the total population), the five-year disease-free survival was 48% and 43% respectively (non-significant difference). This study shows the bias inherent in selecting patients for BMT: 62.5% of patients included had standard-risk ALL, whereas in most studies (table 5) standard-risk ALL composes less than 30% of patients. Barrett et al. reported a two-year disease-free survival of 38% in patients with Ph-positive ALL transplanted in first remission [244]. Since these patients have a cure rate of less than 10% with chemotherapy alone, they are more likely to benefit from allogeneic BMT in first remission [243,244]. However, this population has to be selected carefully, since treatment-related mortality is still significant with BMT and the sequence of chemotherapy in first CR and BMT in first relapse or subsequently may yield the best cumulative cure rate.

For patients refractory to conventional therapy or in first relapse or second remission, allogeneic BMT is the treatment of choice. In patients refractory to chemotherapy, the actuarial three-year disease-free survival with BMT was 23% in one study [245], which is more than can be expected with conventional salvage chemotherapy. For patients in second remission, several studies have reported long-term disease-free survival of 18% to 45% (average 30%) [233–236].

Patients relapsing after allogeneic BMT may still respond to salvage chemotherapy. The outcome depends on the time from BMT to relapse: patients who relapse less than 100 days after BMT have a CR rate of 18%, compared to 71%

Table 6. Results with allogeneic and autologous BMT in ALL (from 243)

	Allogeneic BMT	Autologous BMT
No. patients included	116	95
No. patients acutally transplanted (%)	95 (79%)	63 (66%)
Three-year survival (%)	56	49
Three-year DFS (%)	44	39

DFS, disease-free survival.

if the relapse occured more than one year after BMT [246]. Remissions, however, are usually short in either case.

5.6. Autologous BMT

Two large studies have failed to demonstrate an advantage in disease-free survival for patients receiving autologous BMT compared to chemotherapy alone [241,242]. In the series from the French Group on Therapy for ALL, chemotherapy and autologous BMT produced comparable disease-free survival at three year (39% vs. 32%; $p = 0.8$). However, late relapses (i.e., after three years) were mainly seen in the chemotherapy arm [242]. A study from MDACC planned an intensive consolidation including autologous BMT for patients achieving a CR [157]. Of 79 patients who achieved a CR, 32 relapsed before the time of the BMT. Among the other 47 patients, 21 could not receive a BMT because of age, medical contraindications, or socioeconomic reasons. There was no difference in three-year remission rate between the 26 patients who received the BMT and the 21 patients who did not receive it (60% vs. 49%, respectively). The three-year survival was also similar (58% vs. 62%, respectively). These studies suggest that autologous BMT is not more effective than consolidation chemotherapy. The fact that a plateau in disease-free survival may be seen after three years suggests that some patients, particularly those who are noncompliant or who are not willing to receive long-term therapy, may benefit from this one-time procedure. Several studies with autologous BMT also exemplify the problems with patient selection. Only 20% to 50% of patients for whom autologous BMT is planned can actually receive the transplant [157,243].

For patients with refractory ALL or those in relapse, autologous BMT can result in long-term disease-free suvival of 20% to 30% [247–250]. The best results are achieved in patients with first CR duration of more than one year. These patients can have a disease-free survival of 40% to 50% [247]. Better methods of bone marrow purging are needed to reduce relapse rates in order to make this therapy a better alternative. 'In vivo' purging via mobilization of normal hematopoietic precursors after intensive chemotherapy is a promising approach [251].

6. Survival

The long-term prognosis is excellent in children. More than 90% achieve CR, and 60% to 70% will be cured. In adults, the results are not as good. Although initial reports from large studies suggested a long-term disease-free survival of more than 50% [252,253], these studies included mostly younger patients and excluded those with Ph-positive disease. With longer follow-up and less selective inclusion criteria, the long-term disease-free survival has dropped to 20% to 35% [153,190]. These figures represent the cure rate expected with current

therapies in most studies. Although a major improvement from older studies, these results still speak for the need for further research to improve results towards what is now observed in childhood ALL.

7. Biologic and prognostic investigations

7.1. Minimal residual disease

Flow cytometric sorting and immunophenotyping, clonogenic assays, and detection of leukemia-specific DNA or RNA sequences by Southern blot or PCR can be used to detect residual disease below the level of sensitivity of morphologic evaluation [254]. This may have prognostic significance. In one study of patients treated with autologous BMT, all 42 patients with more than 51 malignant cells per 10^6 total cells (as measured by multiparameter flow cytometry and cell-sorting with assays for leukemic progenitor cells) relapsed within 1 year, but only 41% of those with lower levels of residual disease relapsed [255].

One approach for the detection of minimal residual disease is to identify the clonal rearrangement of Ig- or TCR-genes. Amplification of this leukemia-specific marker by polymerase chain reaction (PCR) can identify 1 leukemic cell in 10^5 normal cells [256]. During induction chemotherapy, there is a 3- to 4-log reduction in the number of leukemic cells, but even after achieving CR some residual disease can be documented [257]. In some patients, an increase in leukemic cells can be demonstrated by this technique months before the increase becomes clinically evident [257]. The major limitation is that, since the specific rearrangement is unique for each clone, specific probes have to be generated for each patient.

An alternative approach is the blast-colony assay described by Estrov et al. [258]. With this assay, in vitro growth of lymphoblastic colonies during CR was observed in patients who later relapsed. However, the presence of disease as detected by this method is not always associated with relapse, and a threshold for prediction of adverse outcome has not been established.

7.2. Multidrug resistance detection

The multidrug resistance gene (MDR) encodes for a membrane glycoprotein, p170, that is thought to function as an efflux pump [259]. MDR expression confers resistance to some chemotherapeutic agents (e.g., vinca alkaloids, taxoids, anthracyclines, and epipodophylotoxins) [260]. In adults, the frequency of MDR positivity increases after relapse (10% at diagnosis, 50% after relapse) [261]. The expression of MDR is associated with lower CR rate (56% for MDR-positive vs. 93% for MDR-negative; $p = 0.05$) and a higher relapse rate (100% vs. 46%, respectively; $p = 0.05$). This results in a survival advantage for MDR-negative patients [261].

7.3. Expression of bcl-2

Bcl-2 is an oncogene involved in regulation of cell death. Overexpression of bcl-2 results in inhibition of programmed cell death [262]. B-cell precursor ALL cells overexpress bcl-2, resulting in prolonged survival of leukemic cells [263]. An association between bcl-2 overexpression and glucocorticoid resistance has been suggested [264]. Overexpression of bcl-2 was documented in all patients with ALL studied by Gala et al. [265], except those with Burkitt's phenotype. Overexpression, however, was not associated with a poor prognosis [265].

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