The rearrangement of the immunoglobulin and T-cell receptor genes in chronic myelogenous leukemia

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The Rearrangement of the Immunoglobulin and T-Cell Receptor Genes in Chronic Myelogenous Leukemia

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1987
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16 April 1987
(Date)
The Rearrangement of the Immunoglobulin and T-Cell Receptor Genes in Chronic Myelogenous Leukemia

A Thesis submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Tarik Mustafa Ramahi

1987
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ABSTRACT

THE REARRANGEMENT OF THE IMMUNOGLOBULIN AND T-CELL RECEPTOR GENES

IN CHRONIC MYELOGENOUS LEUKEMIA

Tarik Mustafa Ramahi

1987

Although the initial oncogenic events in chronic myelogenous leukemia (CML) take place in a pluripotent hematopoietic stem cell, with leukemic markers subsequently identifiable in granulocytic, megakaryocytic, erythrocytic, monocytic, and B lymphocytic lines, it has been extremely difficult to identify involvement of T lymphocytes. In this study T-cell receptor beta chain (TCRB) cDNA and immunoglobulin mu constant region (Cmu) cDNA were used to probe for TCRB gene and Ig heavy chain gene rearrangements in 33 patients in the chronic phase of CML, 4 patients in myeloid blast crisis, and 4 patients in lymphoid blast crisis. The goals of the study were: (i) To look for TCRB gene rearrangements in CML blast crisis in an attempt to identify T-cell blast crisis on the basis of T-cell surface antigens and rearrangement of the TCRB genes. (ii) To see if impending lymphoid blast crisis can be identified during the chronic phase of CML on the basis of clonal Ig and/or TCR gene rearrangements. (iii) To look for clinical-molecular correlations in subgroups of CML patients in lymphoid blast crisis. None of the patients in the chronic phase or myeloid blast crisis had detectable clonal rearrangements with either probe. One of the patients in lymphoid blast crisis demonstrated a clonal rearrangement of the TCRB genes in leukemic blasts, T-cell phenotype by surface marker analysis, and involvement of the breakpoint cluster region (bcr) on chromosome 22. It is concluded that this patient represents the second documented case of bona fide T-cell lymphoid blast crisis. A second patient in lymphoid blast crisis demonstrated rearrangements of both Ig heavy chain and TCRB genes, in addition to involvement of the bcr of chromosome 22, in leukemic blasts. This patient’s cells lacked B- and T-cell surface markers. The presence of dual rearrangements in this patient’s leukemic DNA reinforced the diagnosis of a blastic transformation involving an immature lymphoid progenitor. The two other patients in lymphoid blast crisis did not demonstrate rearrangements of either Ig heavy chain or TCRB genes. These results clearly establish the involvement of the T-cell line in the neoplastic proliferation of CML and demonstrate the existence of T-cell lymphoid blast crisis. They also indicate that assays for Ig and TCR gene rearrangements, as presently performed, cannot detect the occurrence of blastic transformation prior to onset of clinical manifestations of blast crisis.
INTRODUCTION

I. Chronic Myelogenous Leukemia, a Clonal Myeloproliferative Disease.

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder arising from a neoplastic transformation of a hematopoietic pluripotent stem cell [1-3]. Clinically it is characterized by excessive proliferation and accumulation of myeloid cells. Two lines of evidence support the theory that CML is a clonal disease arising in a stem cell: the presence of the Philadelphia (Ph') chromosome and the presence of monoclonal patterns of glucose-6-phosphate dehydrogenase (G6PD) isoenzymes in hematopoietic cells of diverse lineage (Figure 1).

The Ph' chromosome (q22~) is an abnormal chromosome 22 usually produced by a reciprocal translocation of the long arm of chromosome 9 to the long arm of chromosome 22 [4-9]. This characteristic cytogenetic abnormality, present in 90 to 95% of cases of typical CML, has been demonstrated in neutrophils, eosinophils, basophils, monocytes, erythrocytes, megakaryocytes, and B lymphocytes from patients with this disease [10-19]. Although it has not been conclusively demonstrated, its presence is strongly suspected in T lymphocytes derived from the neoplastic clone [20]. A possible explanation for the difficulty demonstrating the Ph' chromosome in T cells is the fact that the majority of peripheral blood T lymphocytes are long-lived cells which have matured prior to the occurrence of the neoplastic transformation. Any clonal leukemic T lymphocytes are therefore young and constitute only a small portion of the total
Figure 1. Clonal origin of human hematopoietic cells in chronic myelogenous leukemia. This schematic representation is based on the results of cytogenetic and G6PD isoenzyme studies. A plus sign indicates that the cell has been definitively shown to be involved in the leukemic clone, a question sign indicates that it is suspected of being involved, and a minus sign indicates that it has not been found to be involved in the leukemic clone based on these studies. These results indicate that the leukemic transformation most likely occurs at the level of the pluripotent hematopoietic stem cell. BFU-E denotes erythrocyte burst-forming unit, CFU-E erythrocyte colony-forming unit, CFU-C myeloid-monocytic colony-forming unit, CFU-EO eosinophil colony-forming unit, and CFU-MEGA megakaryocyte colony-forming unit. [From Koeffler and Golde, ref. 3]
T-lymphocyte pool. Hence they are largely undetectable by present methods. The Ph' chromosome is not present in somatic cells or bone marrow fibroblasts [21]. Since leukemogenesis is believed to occur as a sequence of oncogenic events in a single cell [22], the presence of this abnormal chromosome in most, if not all, hematopoietic cell lines strongly supports the hypothesis that the transforming event in CML occurs at the pluripotent stem cell level.

The Ph' chromosome arises from translocation of the long arms of chromosomes 9 and 22 in approximately 90% to 95% of patients with Ph'-positive CML. Studies carried out over the past few years on the 9;22 Ph' translocation have shown that the proto-oncogene c-abl lies on chromosome 9 close to the translocation breakpoint and is brought to chromosome 22 during translocation [23-25]. The breakpoint on chromosome 22 usually occurs within a specific region designated the breakpoint cluster region, bcr [26,27]. The standard translocation is described as t(9;22) (q34.1;q11.21) and results in the creation of a hybrid gene containing bcr sequences at its 5' end and c-abl sequences at its 3' end [23,24,26,28]. In approximately 5% to 10% of Ph'-positive CML patients different translocations have been identified, e.g. t(12;22), t(15;22), and t(17;22) [29]; all, however, produce a hybrid bcr-c-abl gene, indicating a complex cryptic translocation somehow involving both chromosomes 9 and 22. The product of this chimeric gene, a protein with strong tyrosine kinase activity, is thought to play a central role in the pathogenesis of CML [30-34]. Evidence for this role comes from the strong association
between CML and chromosomal translocations involving the bcr of chromosome 22, presumed to produce a bcr-c-abl gene. Further evidence comes from the observation that the viral homologue (v-abl) of the c-abl protooncogene is responsible for lymphoid leukemias induced by the Abelson murine leukemia virus and also produces a protein with strong tyrosine kinase activity [35-36].

In addition to the cytogenetic evidence, the clonal nature of CML is strongly suggested by G6PD isoenzyme analyses of hematopoietic cells from female CML patients heterozygous at the X-linked G6PD gene locus. A monoclonal pattern of G6PD isoenzyme expression has been identified in granulocytes, monocytes/macrophages, erythrocytes, megakaryocytes, and B lymphocytes obtained from individual patients [10,11,17,18]. A monoclonal pattern has been difficult to show in mature T lymphocytes, the explanation again being the low turnover rate of these cells. Nevertheless, the presence of a single G6PD isoenzyme in all proliferating hematopoietic cell lines, except for T cells, in each of the heterozygous female patients suggests a common origin for these cells and hence supports the clonal stem cell theory of the disease.

II. Clinical Course of CML

The course of CML is divided into two phases, an initial indolent chronic phase lasting an average of three years, followed by an aggressive terminal acute leukemia, termed blast crisis. The chronic phase is characterized by excessive proliferation of granulocytes (neutrophils, eosinophils, and basophils) in all patients, megakaryocytes in most patients, and erythro-
cytes in some patients [1,2]. Myeloid proliferation may be extreme, accounting for 85% to essentially 100% of total marrow cells, with total occupancy of all marrow space and myeloid cell counts in excess of 1,000,000 per uL in the peripheral blood. The underlying defect accounting for this unchecked expansion is presumed to be failure of transformed pluripotent hematopoietic stem cells to respond normally to feedback regulation [37-39]. They continue to proliferate and differentiate into lineage-committed stem cells (e.g. myeloid, megakaryocytic, and erythroid colony-forming cells) long after exceeding the cell density limit at which normal stem cells cease to proliferate. With loss of control at the stem cell level, leukemic cells have a growth advantage over normal stem cells and their progeny. Leukemic cells in the chronic phase retain their capacity to differentiate into mature end cells with essentially normal function. An important consequence of this normal differentiation during the chronic phase is that patients usually do not suffer from neutropenia, thrombocytopenia, or severe anemia and their respective consequences, and hence chronic CML may pass undetected.

Another characteristic of leukemic cells in the chronic phase is a tendency to undergo further neoplastic transformation with at least partial loss of the ability to differentiate [40]. As the chronic phase of CML progresses, a small percentage of patients enters an accelerated stage of the chronic phase, characterized by an increased rate of cell division, partial blockage of differentiation, and increasing resistance to therapy [41]. But in the majority of patients, after an average period of three to three and a half years, the disease directly evolves
into blast crisis. This phase is characterized by proliferation and accumulation in both bone marrow and peripheral blood of immature blast cells which fail to differentiate normally. These blast crisis cells represent an immature subclone within the Ph'-positive leukemic clone [42]. This new population of blasts often demonstrates additional chromosomal abnormalities, such as a second Ph' chromosome, trisomy 8, 19, or 21, and isochromosome 17; they may also lack the Ph' chromosome [43-45]. These blasts have a growth advantage over both normal cells and chronic phase Ph'-positive clones; this advantage is due to their failure to mature into functional end cells and be removed from the proliferating pool. Hence they accumulate in the bone marrow, saturate the peripheral blood, and may spill over into extramedullary sites. Blast crisis typically behaves like a de novo acute leukemia; cells are extremely resistant to therapy, and patients usually die within few months.

Up until the beginning of the last decade it was believed that CML blast crisis always involved transformation in the myeloid cell line. It is now believed that blastic transformation can occur in all hematopoietic cell lines. In each individual patient, however, only one cell line is usually involved. On the basis of morphologic studies it is now clear that the majority of blastic transformations, 60-75%, are myeloblastic while up to 30% are lymphoblastic [46-50]. Blast transformations in the remaining cell lines -- megakaryocytic, monocytic, erythrocytic, eosinophilic and basophilic -- make up less than 5% of the total [51-54].
The first suggestion that certain patients in blast crisis have a lymphoblastic transformation came in 1971 when it was noted that about a third of patients in blast crisis responded to treatment with vincristine and prednisone, agents often effective in acute lymphoblastic leukemia (ALL) but not acute myeloblastic leukemia (AML) [55,56]. In 1974 it was observed that approximately one third of patients in blast crisis have blast cells with lymphoid morphologic characteristics including a high nuclear to cytoplasmic ratio, one or two nucleoli, a "smudged" nuclear chromatin pattern, prominent nuclear folding, and scant basophilic cytoplasm without granulation [46-49]. Subsequently, it was found that several heterologous antisera developed against common type ALL cells reacted with blast cells in about a third of cases of blast crisis [57-61]. These antisera did not react with mature T or B lymphocytes, myelocyte progenitors, or leukemic cells in the majority of patients with AML. Moreover, the majority of blast crisis cells with lymphoid morphology and common ALL antigen (CALLA) positivity were also found to contain high levels of terminal deoxynucleotidyl transferase, TdT, an enzyme that catalyzes the polymerization of deoxynucleoside triphosphates and is found predominantly in cortical thymocytes and normal and neoplastic immature lymphoid cells of both T and B cell lineage [62-66]. By the late 1970s, this combination of findings provided convincing evidence that blast crisis involved cells of lymphoid lineage in about one-third of CML patients in blast crisis.

Over the past ten years, three lines of evidence have convincingly demonstrated that the majority of lymphoid blast crises
involve cells of B lineage. First, with the development of monoclonal antibodies, it was shown that most crisis cells with lymphoid morphology present cell surface antigens specific for B lymphocytes or their progenitors \[67,68\]. In addition, cytoplasmic immunoglobulin M (IgM) heavy chain has been demonstrated in a number of cases of blast crisis in which the blast cells were also CALLA-positive, contained high levels of TdT, and had lymphoid morphology \[69,70\]. Finally, and most convincingly, studies carried out over the last few years have demonstrated that in the majority of cases of blast cells with lymphoid morphology there is in leukemic cells rearrangement of the gene complex coding for the Ig heavy chain, a process mandatory in the early development of B lymphocytes \[71,72\].

The involvement of T-lymphocyte precursors in the blastic transformation of CML has been more difficult to establish \[70\]. Although recent immunophenotyping studies in blast crisis have yielded few cases of possible T-cell lineage, the question has remained unsettled because of the lack of universally accepted specific cell surface markers of T-cell lineage \[34,73-77\]. Recently, the structure of the gene loci encoding the alpha, beta, and gamma chains of the T-cell antigen-specific receptor (TCR) has been elucidated \[78\], and molecular probes for these genes have become available \[79,80\]. Moreover, it has become clear that rearrangement of these genes is mandatory for the development of mature T lymphocytes \[81\]. Hence, it should now be possible to assess clonality of T cells and resolve the question of T-cell involvement in CML blast crisis using a combination of
TCR gene rearrangement and cell surface antigen analyses.

III. Molecular Genetic Approach to Clonality in CML

The molecular approach to the determination of clonality in lymphoproliferative diseases is based on the fact that Ig and TCR genes in their germ-line forms are composed of discontinuous genetic elements separated by non-coding segments of DNA [78]. As noted above, rearrangement of Ig genes is mandatory in B-cell maturation, and rearrangement of TCR genes is mandatory in T-cell maturation [81-83]. These unique rearrangements are the genetic basis for the diversity and specificity of both B- and T-cell mediated immunologic processes. In molecular terms, these rearrangements come about as a result of deletions of non-coding segments of DNA and covalent linkage of discontinuous genetic elements. With these rearrangements come alterations in the locations of restriction endonuclease sites. Thus, Southern hybridization analysis can be used to distinguish the rearranged from the germ-line forms of these genes. The germ-line form of Ig and TCR genes is present in nonlymphoid cells and very early lymphoid precursors and may be present in one allele of the genes in lymphocytes. In a polyclonal B-cell proliferation, each cell undergoes a unique Ig heavy chain gene rearrangement but the total cell population contains many different rearrangements. Each specific rearrangement gives rise to a restriction fragment of unique length, but since each such fragment is present in only a single copy, none of these gene fragments can be detected as a new band on Southern blots because the method lacks the necessary sensitivity. In contrast, all cells in a monoclonal proliferation
have the same rearrangement, giving rise to a single new fragment in a concentration adequate for identification as a new band on Southern blots. Sometimes two new bands appear because of different rearrangements of both alleles of the gene. This molecular genetic method for assessing the clonality of lymphoproliferative disorders is sufficiently sensitive to detect a clonal proliferation representing 1-5% of a mixed population of cells [84-86].

The family of genes coding for the immunoglobulin polypeptides is composed of genes located on three chromosomes: genes coding for the two light chains, kappa and lambda, on chromosomes 2 and 22 respectively, and the heavy chain gene locus on chromosome 14. The heavy chain gene complex in its germ-line form is composed of discontinuous gene subsegments consisting of multiple variable regions, $V_H$, diversity regions, $D_H$, joining regions, $J_H$, and constant regions, $C$ (Figure 2) [87]. Each one of these regions is a coding segment separated from other segments by non-coding DNA. The constant regions consist of mu, delta, gamma, alpha, and epsilon regions corresponding to the various classes of Ig heavy chain — IgM, IgD, IgG, IgA, and IgE respectively [88]. The germ-line organization of the loci coding for the kappa and lambda light chains is similar to that of the heavy chain, except for the absence of D regions and presence of a single constant region [89,90]. During the development of a mature B lymphocyte there is a hierarchy of Ig gene rearrangements whereby the heavy chain gene rearrangement preceeds that of the light chain, and the kappa light chain gene complex rearranges before the lambda gene complex [91-93]. Rearrangement of the heavy chain genes begins with the juxtaposition of a single $D_H$ region with a
Figure 2. A schematic representation of the germ-line arrangement and subsequent assembly of the immunoglobulin (Ig) heavy chain gene complex. It is composed of multiple VH regions each with its own leader (L) sequence, numerous DH regions, six JH regions, and only one Cmu region per allele. During the development of a mature B lymphocyte VH, DH, and JH regions are covalently joined together by deletion of intervening DNA to form a single V-D-J segment. The resultant gene is transcribed, and the remaining intervening sequence (IVS) and JH regions are removed at the RNA level. The mu-mRNA is then translated into the cytoplasmic form of mu heavy chain polypeptide. [From Korsmeyer et al, ref. 93]
single $J_H$ and then a $V_H$ region with the new D-J segment. This process randomly assembles the gene (V-D-J) coding for the variable segment of the heavy chain polypeptide and uniquely determines its specificity (Figure 2) [82,94-98]. The first class of Ig polypeptides produced is IgM, with the constant region coded for by the $C_{mu}$ region. Because this heavy chain gene rearrangement does not involve the DNA segment between the cluster of $J_H$ regions and the $C_{mu}$ region, it can be detected with a cDNA probe to either $J_H$ or $C_{mu}$ (Figure 3). Rearrangement of light chain genes occurs by a process similar to that of the heavy chain genes [89,90]. Since every maturing B lymphocyte undergoes a unique rearrangement of the heavy and light chain genes, detection of a specific rearrangement in a population of lymphoid cells not only suggests B-cell lineage but also proves the clonality of these cells, as discussed previously [84]. Furthermore, the hierarchy of heavy and light chain gene rearrangements permits the determination of the stage of maturation of a B-lymphoid clone by analysis of the arrangement of heavy and light chain genes [91,93].

The TCR has recently been shown to be a heterodimer consisting of alpha (TCRA) and beta (TCRB) subunits [99]. Similar to the Ig family of genes, the genes encoding these subunits undergo somatic rearrangement in the course of T-lymphocyte development [86]. The TCRB gene in its germ-line form is also composed of noncontiguous gene segments consisting of a large number of variable regions, $V_{beta}$, and duplicate sets of diversity, $D_{beta}$, and joining, $J_{beta}$, gene segments separated by the first of two
Figure 3. A. Schematic diagram of the germ-line arrangement of a portion of the Ig heavy chain gene complex depicting the locations of Eco RI (E) and Bam HI (B) restriction sites in the vicinity of the Cmu region. Also shown are cDNA probes for JH and Cmu. It is clear that hybridization with either one of these probes would detect a single Bam HI fragment of approximately 18 kb. As shown, the JH region consists of six exons (dark segments) and numerous pseudogenes (psi), and the Cmu region consists of four exons. The Cmu cDNA probe used in this study contains the first three exons of Cmu [97]. [From Korsmeyer et al, ref. 93]

B. Schematic diagram of the germ-line arrangement of the TCRB gene complex showing the locations of Eco RI (E), Bam HI (B), Sac I (S), and Hind III (H) restriction sites. In its germ-line configuration, the TCRB gene complex consists of multiple variable (Vbeta) regions each with its own leader sequence (L), and duplicate sets of diversity (Dbeta) and joining (Jbeta) regions separating two constant regions (Cbeta-1, and Cbeta-2). This map demonstrates that the two Cbeta regions would be present on a single 23 kb Bam HI fragment, on two Eco RI fragments of 4 and 11 kb, and on three Hind III fragments of 3.5, 6.5, and 8.0 kb. As shown, the TCRB cDNA probe used in this study contains V, D, J, and C regions [79]. [From Waldmann et al, ref. 84]
constant regions, $C_{\text{beta-1}}$ and $C_{\text{beta-2}}$ (Figure 3) [100-102]. Analogous to that of the Ig heavy chain genes, the process of rearrangement of the TCRB genes produces a V-D-J segment separated from one of the constant regions by noncoding DNA [103]. The organization of the TCRA genes is similar to that of the Ig light chain genes with one constant region and no diversity regions [104]. In addition to the TCRA and TCRB genes, a third gene complex, TCRG, has been identified in T cells [105]. This gene complex, whose polypeptide product is closely associated with but not a part of the TCR heterodimer, has many properties in common with TCRA and TCRB genes, including assembly from gene segments resembling V, J, and C regions, rearrangement, and expression in T lymphocytes [106]. Also similar to the Ig family of genes, there is a hierarchy of TCR gene rearrangements. TCRG genes rearrange first followed by TCRB and then TCRA genes [107]. Therefore, analysis of the arrangement of these genes allows the determination of lineage, clonality, and stage of maturation of suspected T-lymphoid proliferations [108-111].

IV. Aims of the Study

This thesis is an application of these recent developments in the molecular genetics of Ig and TCR genes to the study of lymphocyte lineage and clonality in the chronic and blast crisis phases of CML. Specifically, its purposes are three-fold:

1. To look for TCRB gene rearrangements in CML blast crisis and to see if T-cell blast crisis can be identified on the basis of T-cell surface markers and rearrangement of the TCRB genes.
2. To see if impending lymphoid blast crisis can be identified during the chronic phase of CML by the detection of clonal Ig and/or TCR gene rearrangements.
3. To look for clinical-molecular correlations in subgroups of CML patients in lymphoid blast crisis.

V. Significance

The detection of T-lymphocyte involvement in the blast crisis of CML is important for three reasons:
1. It would confirm the pluripotent stem cell theory of CML;
2. It would support the bone marrow stem cell theory of the origin of T lymphocytes; and
3. It would allow for the identification of specific clinical features in this subgroup of patients.

At the time this work was undertaken, there had been no reports of the application of TCR gene rearrangement analysis to the question of T-cell involvement in the blast phase of CML.

The second aim of this study is of extreme practical importance. It is generally agreed that the only means presently available for curing CML is bone marrow transplantation from a histocompatible donor [112]. Moreover, present data suggest that patients transplanted before the onset of blast crisis have a better chance of being cured than patients transplanted in crisis or in remission from crisis. However, in a practical sense, the question of when to perform a bone marrow transplantation is not easy to answer. Specifically, it is quite easy to reduce leukemic burden during chronic phase. Once tumor load is controlled with chemotherapy, chronic CML patients are usually asymptomatic, and
this state of well being can be maintained for about three years in the average patient and substantially longer in some patients. Furthermore, bone marrow transplantation is not a benign procedure. Patient undergoing transplantation may die during or immediately post-transplantation or may suffer from long-term complications of transplantation, e.g. graft vs. host disease and opportunistic infections. Ideally, clinicians try to maintain patients in chronic phase for as long as possible with chemotherapy and recommend transplantation when cells begin to show increasing resistance to therapy. Therefore, an objective marker of incipient blast crisis is needed to guide in choosing the most opportune time for transplantation. The identification of a clone of lymphoblasts with rearrangement of Ig or TCR genes during the chronic phase of disease, making up as few as 1% of leukemic cells and not recognizable on morphologic examination, would signal the incipient onset of blast crisis and argue strongly and objectively for early bone marrow transplantation. At the time this study was initiated, only few patients had been tested in chronic phase for Ig gene rearrangement [73,74], all with negative results, and none had been studied for rearrangement of TCR genes.

The issue of possible molecular-clinical correlations was suggested by the previously mentioned observation that most patients with lymphoid blast crisis have an initial response to vincristine and prednisone. In contrast, non-responding patients with lymphoid blast crisis have a stormy course with no or little response to other forms of therapy. It thus seemed reasonable to
inquire if there is an underlying genetic basis explaining this variability in the course of lymphoid blast crisis.
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METHODS AND MATERIALS

I. Patients and Leukemic Cells

Leukemic cells were obtained from forty CML patients. All but one had Ph'-positive CML, and they all had peripheral blood white cell counts in excess of 25,000 per μL at the time cells were obtained. Thirty-three samples were from patients in the chronic phase of disease, including four in the accelerated stage of the chronic phase. Eight samples were from patients in blast crisis, four in myeloid and four in lymphoid crisis. The diagnosis of blast crisis was based on finding of greater than 30% blasts in the bone marrow. Type of crisis was based on morphologic, histochemical and immunophenotyping studies performed on either peripheral blood or bone marrow leukocytes. Most chronic phase samples were obtained at diagnosis, but some were obtained later in the course of the disease. In few cases sequential samples were obtained.

Virtually all samples used in the study were buffy coat preparations obtained from peripheral blood. These were obtained by spinning heparinized blood at 2,000 rpm for 15 minutes and carefully removing the layer of white cells with a Pasteur pipette. The number of cells collected was estimated from the amount of spun blood and the peripheral white cell count, assuming 75% recovery. Certain cell samples were obtained by leukopheresis of peripheral blood. One sample was from spleen removed at surgery.

Clinical information on patients was obtained from hospital records and physicians caring for patients.
II. Special Laboratory Studies

All special laboratory studies were performed in clinical laboratories at the Yale-New Haven Medical Center and the University of Alabama Medical Center, Birmingham. Cell surface marker studies were performed using a panel of monoclonal antibodies directed against CALLA, surface immunoglobulins and other B-cell antigens, T-cell antigens, and myelocyte/monocyte antigens. TdT enzyme assays were performed on peripheral blood or marrow smears by immunofluorescence. Cytogenetic studies were performed by standard procedures.

III. Isolation of DNA

Genomic DNA was extracted from control and leukemic cells by modification of the procedure of Gross and Bellard et al [113]. DNA for negative controls was prepared from human placentas and normal skin fibroblasts. DNA for positive controls was prepared from Molt-3 (a T-cell leukemic cell line) and from lymphomatous tissue obtained from two patients with suspected T-cell lymphomas.

Cell pellets frozen in liquid nitrogen were ground to a fine powder in a mortar and pestle. The powder was then suspended in TNE-A buffer (10mM Tris Cl/10mM NaCl/10mM EDTA, pH 8.0; 2 ml for every $1 \times 10^7$ cells). Proteinase K and Na dodecyl sulfate (SDS) were then added sequentially to final concentrations of 50 ug/ml and 0.5% respectively. Each suspension was incubated 3-6 hours at 37 degrees C with gentle shaking. The viscous suspension was then extracted twice with equal volumes of phenol saturated with TNE-B.
(500 mM Tris Cl/10 mM NaCl/10 mM EDTA, pH 8.0), and the aqueous phases, containing DNA, were removed with a wide bore Pasteur pipette. DNA was then precipitated with 2.5 volumes of chilled ethanol and removed from suspension by spooling it onto a glass rod. Excess ethanol was removed by rubbing the DNA against the inside of an inverted Eppendorf tube. After complete drying, the DNA was dissolved in TNE-C (50 mM Tris Cl/10 mM NaCl/10 mM EDTA, pH 8.0; 2 ml for every 1 X 10^7 cells) by gentle overnight shaking at 37 degrees C. DNA was then digested with pancreatic RNAse (40 ug/ml) at 37 degrees for one hour with constant shaking. After complete digestion, the solution was extracted twice with equal volumes of phenol/TNE-B and then dialyzed against 100 volumes of sterile TNE-D (10 mM Tris Cl/10 mM NaCl/0.5 mM EDTA, pH 8.0) using 5-6 changes of buffer with a minimum of three hours per change. After dialysis, the concentration of DNA was determined by absorbance at 260 nanometers. It was then stored at 4 degrees C.

IV. Southern Hybridization

Genomic DNA samples were digested with restriction endonucleases Eco RI, Bam HI, Hind III and Sac I (obtained from New England Biolabs, Beverly, MA, and International Biotechnologies Inc., New Haven, CT) under conditions specified by the suppliers. For each sample, approximately 15 ug of DNA was digested with 45-75 units of enzyme, the excess of enzyme added to insure complete digestion. Reaction mixtures were incubated at 37 degrees C for 16-20 hours after which digested DNA was precipitated by addition of 1/10th volume of 3.0 M Na acetate, pH 7.0, and two volumes of chilled ethanol. To insure complete precipitation, the suspension
was cooled to -20 degrees C for 2 hours. DNA was then centrifuged down in a table-top Eppendorf centrifuge and the liquid phase was discarded. After complete drying, DNA was dissolved in 50 ul 0.1x TE buffer (TE: 10mM Tris-HCl/1mM EDTA, pH 7.4) by vigorous vortexing and then allowed to stand for one hour to insure complete solution. Five uL of a dye solution containing 0.2% bromphenol blue and 20% glycerol in 0.1x TE buffer was then added to each DNA sample, and the DNA was size-fractionated by electrophoresis on an 0.8% agarose gel made in TAE buffer (40mM Tris acetate/1mM EDTA, pH 8.0). A Hind III digest of lambda phage DNA (Boehringer Mannheim Biochemicals, Indianapolis, IN) was run on each gel to provide size markers. Electrophoresis was carried out at 35-50 volts for 14-20 hours to obtain 15 cm migration of the bromophenol blue dye front. After electrophoresis was completed, the gel was stained in a solution of ethidium bromide, 0.5 ug/mL, for 10 minutes, and then photographed with a UV lamp to ascertain complete digestion of DNA samples and migration of size markers. Following an additional 10 minutes of UV exposure, the gel was immersed in 300 ml HCl solution (21.5 mL concentrated HCl in 1 L solution) for 10 minutes twice with constant shaking. After washing three times with distilled water, the gel was immersed twice in 300 mL of denaturing solution (0.5 M NaOH/1.5 M NaCl) for 25 minutes each time with constant shaking. Again after washing three times with distilled water, the gel was immersed in 300 mL of neutralizing solution (1M Tris Cl/1.5M NaCl, pH 8.0) for 25 minutes each time, also with constant shaking. Denatured DNA was then transferred to nitrocellulose filter paper (Type BA, 45 micrometer porosity, Schleicher and Schuell) previously pre-
wetted for 5 minutes with distilled water, using the method of Southern [114]. Transfer of DNA was carried out for approximately 20 hours using 10x SSC (20x SSC: 3.0M NaCl/0.3M Na citrate) as the transfer solution. After transfer, the nitrocellulose paper was rinsed with 6x SSC and allowed to dry in room air. It was then baked in a vacuum oven for 2 hours at 80 degrees C. After cooling the nitrocellulose paper was soaked for 6 hours at room temperature with constant shaking in a solution consisting of 5x Denhardt's solution (10x stock solution containing 5 gm Ficoll, 5 gm polyvinylpyrrolidone, 5 gm bovine serum albumin-Pentax fraction V, and water up to 500 mL) and 6x SSC. The nitrocellulose paper was then prepared for hybridization by incubation at 37 degrees C for 1 1/2 hours in 1x Denhardt's solution, 50% deionized formamide, and 4x SSC. It was then hybridized with the appropriate denatured radioactive probe in a mixture of 1x Denhardt's solution, 50% formamide, 4x SSC, 0.20% SDS, and 10 ug/mL denatured salmon sperm DNA (50 uL of hybridization fluid per squared cm of nitrocellulose paper). The probe and salmon sperm DNA were denatured immediately before addition to the hybridization mixture by immersion in a boiling water bath for 5-10 minutes and then quenching in a dry ice-ethanol mixture. Hybridization was carried out for 36 hours at 37 degrees C with constant shaking. Maximal contact between the hybridization solution and the nitrocellulose paper was facilitated by placing the paper in a plastic bag in which the solution has been placed and meticulously excluding all air bubbles. The bag was then sealed and placed in another plastic bag containing a large
volume of water and air. After hybridization, the filter paper was washed 4 times, 15 minutes each time with 2x SSC/0.1% SDS solution at room temperature, followed by 2 washings with 0.25x SSC/0.1% SDS at 64 degrees C, the first washing for 2 1/2 hours and the second for a half hour. The filter paper was allowed to dry and then autoradiographed for 1-7 days at -70 degrees C using Kodak XAR-5 X ray film and a Dupont Chronex intensifying screen.

V. Probes

Three human DNA probes were used in this study: an Ig heavy chain probe, a TCRB probe, and a bcr probe. The Ig probe was a 1.3 kb Eco RI cDNA fragment containing the first three exons of the mu constant region (C\text{\textsubscript{mu}}) of the heavy chain gene complex (Figure 3) [106]. This probe was kindly supplied by Dr. P. J. Buckley of the Department of Pathology. It was prepared from a plasmid originally provided by Dr. P. Leder, Harvard Medical School, Boston.

The TCRB probe was a 770 bp cDNA segment obtained from mRNA of the Jurkat-2 cell line, a T-cell leukemic line [103]. It contains V, D, J, and C regions of the TCRB gene (Figure 3). The complete sequence of this probe has been determined [79]. The plasmid was originally provided to Dr. Paul Lebowitz by Dr. Tak Mak of the Ontario Cancer Institute, Toronto. The probe was inserted at the unique Pst I site in pBR322.

A large scale preparation of plasmid pBR322 containing the TCRB probe was provided by Dr. Lebowitz. The plasmid (420 ug of DNA) was digested with the restriction enzyme Pst I (New England Biolabs) according to the supplier's recommendations. The diges-
tion mixture was size-fractionated by electrophoresis on a 1% agarose gel in 1x TAE buffer. Following ethidium bromide staining, two distinct bands were seen under UV light, the one at approximately 800 bp corresponding to the cDNA insert. This band was excised from the gel and placed in a segment of dialysis tubing with 1x TAE buffer. The DNA was then electroeluted in an electrophoresis apparatus using 100 volts for 3 hours. Polarity was reversed for 5 minutes at the end of the electroelution in order to elute DNA from the dialysis bag. Solid NaCl was then added to the DNA solution to a final concentration of 0.2 M. The DNA was then chromatographed on an Elutip-DNA column (Schleicher and Schuell) using salt elution according to the supplier's instructions. After completion of chromatography, the DNA was precipitated by adding 2 volumes of ethanol. DNA was then recovered by centrifugation, allowed to dry, and dissolved in 50 uL of water. The concentration of DNA was estimated by small scale agarose-ethidium bromide electrophoresis with a cDNA probe of similar size and known concentration, followed by comparison of fluorescence under UV light.

The probe for the bcr of chromosome 22 was a 1200 bp Bgl II/Hind III fragment purchased from Oncogene Science Inc. (Mineola, NY).

All three probes were radiolabeled by incorporation of radioactive dCTP and dGTP into the double stranded DNA by the method of nick translation. Radioactive alpha-P\(^{32}\)-dCTP and -dGTP (both with specific activities of 3000 Ci/mmol) were obtained from Amersham Inc. (Arlington, IL). Nick translation reactions contained, in a total volume of 22 uL, 1x nick translation buffer
[113], 1 nmole each of dATP and dTTP, 70 uCi of $^{32}\text{-dCTP}$ and $^{32}\text{-dGTP}$, 1 ug probe, and 3 uL of premixed E. coli DNA polymerase and pancreatic DNAse (Boehringer Mannheim). The reaction mixture was incubated for 2 hours at 15-16 degrees C after which the reaction was terminated by addition of 4 ul of 0.2M EDTA, pH 8.0. The radioactive probe was isolated by chromatography through a Sephadex G-100 column, using 1x TE buffer for elution. Total counts of radioactivity were determined by scintillation counting. Probes were labeled to specific activities of 1-2 X $10^8$ cpm/ug DNA. For hybridization, 1-5 X $10^6$ cpm of probe were used for every mL of hybridization fluid.
RESULTS

I. Chronic Phase CML

Thirty-three patients were studied in the chronic phase of CML, including four in the accelerated stage. None of these patients showed rearrangement of either the Ig heavy chain or the TCRB genes.

Figure 3 schematically depicts the structure of a portion of the Ig heavy chain gene complex. As shown, there are two Bam HI restriction sites in the vicinity of the C mu region, one 5' to the JH region and the other 3' to the C mu region. Digestion of germ-line DNA with Bam HI should yield an approximately 18 kb restriction fragment that includes both C mu and JH regions. A rearrangement bringing VH and DH segments 5' to a JH segment removes the existing 5' Bam HI site and brings a new Bam HI site 5' to the new V-D-J segment, consequently resulting in a new restriction fragment length. This fragment is recognized by both C mu and JH probes since there is no Bam HI site between the C mu and JH regions. Therefore, all rearrangements 5' to the C mu region would be detected by a C mu probe in Bam HI digests.

Figure 4 shows representative Southern hybridization results of peripheral leukocyte DNA from chronic phase CML patients digested with Bam HI and probed with the C mu region probe. All patients studied demonstrated a single 18 kb Bam HI fragment representing the germ-line pattern.

The structure of the TCRB gene complex is also depicted in Figure 3. As shown, there are two constant (C beta) regions within this gene locus. As expected from the location of restriction
Figure 4. Southern hybridization analysis of peripheral blood leukocyte DNA from seven patients with chronic phase CML (lanes 1-7) and nonleukemic control DNA (lanes 8-10) digested with Bam HI and probed with Ig Cmu probe. A single approximately 18 kb band appears for both patients and controls.
sites, digestion with Eco RI should produce two fragments containing C region sequences, an approximately 11 kb fragment containing C$_{\text{beta-1}}$ and an approximately 4 kb fragment containing C$_{\text{beta-2}}$ (Figure 5). Occasionally, however, a third fragment of approximately 8.5 kb is observed in germ-line DNA. This band was originally thought to represent a rare polymorphism within the 11 kb fragment containing the C$_{\text{beta-1}}$ region. However, this explanation is no longer tenable for three reasons: (i) the 8.5 kb fragment has not been seen in the absence of the 11 kb fragment, which would be expected in a homozygote for the 8.5 kb polymorphic fragment; (ii) in one normal individual, the 8.5 kb fragment was seen in digests of lymphocyte, but not granulocyte DNA [115]; and (iii) in the case of one patient from whom serial leukemic DNA samples were examined (patient M.P., Figure 10), this fragment was present in some samples (samples 2,3) but absent in other samples (samples 1,2). All these findings suggest that the 8.5 kb DNA fragment arises from technical factors associated with DNA extraction or Southern blotting.

Figure 3 also indicates that a C$_{\text{beta}}$ probe also recognizes a 23 kb Bam HI fragment containing both C$_{\text{beta}}$ regions (Figure 5); two Sac I fragments, a 6.0 kb fragment containing C$_{\text{beta-1}}$ and a 5.6 kb fragment containing C$_{\text{beta-2}}$; and three Hind III fragments, the smallest at 3.5 kb containing C$_{\text{beta-1}}$, and the other two at 8.0 kb and 6.5 kb containing portions of C$_{\text{beta-2}}$.

Although the TCRB probe used in this study contained V, D, J, as well as C regions [Dr. Tak Mak, written communication], and although in previously published studies it detected bands corresponding to V and C regions [109], it recognized only the C
Figure 5. Control Southern hybridization results showing the constant region germ-line pattern of the TCRB gene complex. Hybridization with a TCRB probe reveals a single 23 kb Bam HI fragment (odd lanes) containing both Cbeta regions, and two Eco RI fragments (even lanes), an 11 kb fragment containing Cbeta-1 and a 4.2 kb fragment containing Cbeta-2. The 8.5 kb fragment appearing in Eco RI digests is thought to contain a portion of Cbeta-1. Its unpredictable presence is thought to be an artifact of DNA extraction. Control DNA was obtained from human placentas and skin fibroblasts.
regions in the studies reported here. The most likely explanation for this behavior relates to the stringency of the washing conditions employed in Southern hybridization. Both V and J regions in assembled V-D-J coding segments involve a large degree of variability from one cell to another, variability that is the genetic basis of immunologic diversity and specificity. A particular probe with single V and J segments may thus fail to hybridize to V and J regions containing highly variable sequences. Regardless of what the true explanation may be, the results of this study should not be affected. Any rearrangement of the beta chain gene complex will assemble a V-D-J coding segment 5' to one of the C\textsubscript{beta} regions and thereby effect an alteration in the location of restriction sites 5' to the J region. Since some of these endonucleases (notably Eco RI and Bam HI) lack sites in the sequence between the J and C regions, the resultant new restriction fragments should therefore be detectable by probes to the C\textsubscript{beta} regions.

Figures 6-9 show representative Southern analyses of TCRB gene structure in chronic phase CML patients. Studies were carried out with four restriction endonucleases: Eco RI, Bam HI, Hind III, and Sac I. Germ-line patterns were obtained in all cases.

One of the patients in the accelerated stage of the chronic phase, M.P., who later entered a lymphoid blast crisis, had an unusual pattern for the TCRB gene on digestion with Eco RI. Samples of genomic DNA from one point in the accelerated stage of the chronic phase lacked the fragment containing the C\textsubscript{beta}-2 region (Figure 10, sample 2). This is felt to be artifactual for
Figure 6. TCRB Southern hybridization results of peripheral blood leukocyte DNA from six representative chronic phase CML patients (lanes 1-12) digested with Eco RI (odd lanes) and Bam HI (even lanes). Lanes 13-16 show control results. All chronic phase CML patients demonstrated the germ-line pattern.
Figure 7. TCRB Southern hybridization results of peripheral blood leukocyte DNA from five representative chronic phase CML patients, digested with Bam HI. Lanes 1-5 show patient results, and lane 6 shows control results. All chronic phase CML patients had the 23 kb germ-line fragment. The two extra bands in lane 5 were not present on repeat digestion, and hence are not significant.
Figure 2: A novel framework for decision support systems in personalized medicine.
Figure 8. TCRB Southern hybridization results of peripheral blood leukocyte DNA from seven representative chronic phase CML patients, digested with Hind III. Lanes 1-7 show patients' results and lanes 8-10 show control results. The germ-line pattern, shown by the controls, consists of a 3.5 kb fragment containing Cbeta-1 and two fragments, 6.5 kb and 8.0 kb, containing portions of Cbeta-2. The germ-line pattern appeared in genomic DNA samples obtained from all CML patients in this study.
Figure 9. TCRB Southern hybridization results of peripheral blood leukocyte DNA from eleven representative chronic phase CML patients, digested with Sac I. Lanes 1-11 show patients' results, and lanes 12-14 show control results. The germ line pattern, consisting of a 6.0 kb fragment containing Cbeta-1 and a 5.4 kb fragment containing Cbeta-2, was seen in peripheral blood leukocyte DNA from all chronic phase CML patients studied.
two reasons. The first is that fragments containing $C_{\beta-2}$ appeared in Hind III and Sac I digests of the same DNA, and there was no change in the size of the Bam HI fragment which contains both $C_{\beta-1}$ and $C_{\beta-2}$ (data not shown). Secondly, the $C_{\beta-2}$ fragment appeared in DNA samples obtained from this patient later in the course of the disease, both in accelerated chronic phase and blast crisis. Furthermore, complete absence of this fragment in the presence of a normal $C_{\beta-1}$ fragment can only be explained by complete deletion of $C_{\beta-2}$ and presence of normal $C_{\beta-1}$. This is unlikely, not only because such a deletion should be present in all enzymatic digests, but also because it would be a nonphysiologic deletion that cannot be explained by a functional rearrangement.

II. Myeloid Blast Crisis

Four patients were studied in myeloid blast crisis. None of these patients demonstrated rearrangement of the Ig heavy chain genes using Bam HI digestion or the TCRB genes using Eco RI, Bam HI, Hind III, and Sac I digestions (results not shown).

III. Lymphoid Blast Crisis

Four patients were studied in lymphoid blast crisis. Of these patients one, B.S., had rearrangement of the TCRB gene alone; a second, E.B., had rearrangements of both Ig heavy chain and TCRB genes; while the other two, A.K. and the aforementioned M.P., had no detectable rearrangements.
Figure 10. TCRB gene analysis of peripheral blood leukocyte DNA from patient M.P.. Lanes 1-5 and 7 show Eco RI digests, and lanes 6 and 8 show Bam HI digests. Four serial samples of DNA were obtained in the course of accelerated chronic phase. Sample 1 (lane 1) obtained early in the accelerated stage, sample 2 (lanes 2 and 5,6) obtained two months later, sample 3 (lane 3) obtained ten months after sample 1, and sample 4 (lane 4) obtained at onset of blast crisis, approximately two years after sample 1. Lanes 7 and 8 are controls. It is noted that sample 2 DNA lacked the band corresponding to Cbeta-2 (arrows). Bands containing Cbeta-2 were, however, present in Hind III and Sac I digests of sample 2 DNA (results not shown). It is also noted that an 8.5 kb Eco RI band appeared in DNA of samples 2 and 3 but was absent in DNA of samples 1 and 4.
A. Patient B.S.

Patient B.S., a female, presented at age 29 with a classic clinical and laboratory picture of the chronic phase of CML. Cytogenetic studies revealed a t(9;22) translocation, and Southern hybridization with a bcr probe revealed new bands indicating involvement of the bcr in the translocation with presumed formation of a hybrid bcr-c-abl gene (Figure 11). The patient’s leukocyte count was initially controlled on daily busulfan, but five months after diagnosis she entered blast crisis, and nine and a half months after diagnosis she died.

Laboratory data obtained on entry into blast crisis are shown in Table I. The bone marrow was packed with lymphoid-appearing blasts, the majority CALLA-positive. In the peripheral blood 76% of the white cells were Leu-1-positive, 20% were Leu-2-positive, 13% were Leu-3-positive, and 24% were OKT6-positive, all suggestive of T-cell phenotype. Only 10% were B4-positive, indicating a small percentage of B cells. Lymphoid blast crisis of probable T-cell lineage was diagnosed, and the patient was treated with vincristine and prednisone with no response. Table 2 shows laboratory data on two subsequent occasions, three and four months after onset of blast crisis. These data show that the percentage of total T cells (Leu-1-positive), helper T cells (Leu-3-positive), and thymocytes (OKT6-positive) in the periphery rose significantly during the course of blast crisis. It was also observed that the majority of cells became TdT positive, while CALLA-positive cells became weakly positive and then disappeared. The percentages of B-lymphoid cells (B1-, B4-, and HB2-positive) and myeloid-monoctytic cells (OKM1-positive) were also very small.
Figure 11. Breakpoint cluster region (bcr) analysis of leukemic blast cell DNA obtained from patient B.S. (P1) and patient E.B. (P2). C represents control results. DNA was digested with Bgl II (Bg), Hind III (H), Bam HI (B), and Eco RI (E). The appearance of new bcr fragments (arrows) in DNA from both patients indicates a translocation involving bcr of chromosome 22.
Table 1. Clinical and laboratory data of patient B.S. at onset of blast crisis.

<table>
<thead>
<tr>
<th>Patient:</th>
<th>B.S.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
<td>29</td>
</tr>
<tr>
<td>Sex:</td>
<td>Female</td>
</tr>
<tr>
<td>WBC count:</td>
<td>51,000-81,000 (range over 7 days)</td>
</tr>
<tr>
<td>Percentage blasts:</td>
<td>13-37% (peripheral blood)</td>
</tr>
<tr>
<td>Cytogenetics:</td>
<td>46XX, t(9;22) (q34;q11)</td>
</tr>
<tr>
<td>Bone marrow analysis:</td>
<td>Immature lymphoid blasts, majority CALLA-positive</td>
</tr>
</tbody>
</table>

**Peripheral blood cell surface markers:**

1. **T cells:**
   - Total T cells (Leu 1) 73%
   - T suppressor cells (Leu 2) 20%
   - T helper cells (Leu 3) 13%
   - Thymocytes (OKT6 Ag) 24%
   - SRBC rosettes (Leu 5) 24%
   - Natural killer cells 3%
   - OKT10 Ag 2%

2. **B cells:**
   - HB2 <1%
   - B4 10%
   - Surface Ig (M,D,G,A) (+)
   - Cytoplasmic Ig (M,D,G,A) <1%

3. **CALLA** 76%

4. **Myeloid/Monocytic**
   - OKM1 Ag 1%

5. **HLA-DR**
   - (-)
<table>
<thead>
<tr>
<th>Cells</th>
<th>Len 1 (%)</th>
<th>Len 2 (%)</th>
<th>Len 3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helper cells</td>
<td>25%</td>
<td>20%</td>
<td>13%</td>
</tr>
<tr>
<td>Suppressor cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Natural killer cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Serial cell surface marker analyses on patient B.S. during the course of blast crisis: I. At onset of blast crisis. II. Three months after onset of blast crisis. III. Four months after onset of blast crisis.

<table>
<thead>
<tr>
<th></th>
<th>I.</th>
<th>II.</th>
<th>III.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (peripheral blood)</td>
<td>79,000</td>
<td>187,000</td>
<td>80,000</td>
</tr>
<tr>
<td>Percentage blasts + lymphocytes</td>
<td>99%</td>
<td>93%</td>
<td>99%</td>
</tr>
<tr>
<td>TdT</td>
<td>(+)</td>
<td>(+)</td>
<td></td>
</tr>
<tr>
<td>Peripheral blood cell surface markers:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total T cells (Leu 1)</td>
<td>73%</td>
<td>91%</td>
<td>88%</td>
</tr>
<tr>
<td>T suppressor cells (Leu 2)</td>
<td>20%</td>
<td>3%</td>
<td>23%</td>
</tr>
<tr>
<td>T helper cells (Leu 3)</td>
<td>13%</td>
<td>25%</td>
<td>38%</td>
</tr>
<tr>
<td>SRBC rosettes (Leu 5)</td>
<td>24%</td>
<td>3%</td>
<td>40%</td>
</tr>
<tr>
<td>Transferrin receptor (Leu 9)</td>
<td></td>
<td>100%</td>
<td>73%</td>
</tr>
<tr>
<td>Thymocytes (OKT6)</td>
<td>24%</td>
<td>48%</td>
<td>60%</td>
</tr>
<tr>
<td>OKT10</td>
<td>2%</td>
<td>6%</td>
<td>2%</td>
</tr>
<tr>
<td>Natural killer cells</td>
<td>3%</td>
<td>&lt;.5%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>(2) B cells (B4)</td>
<td>10%</td>
<td>&lt;.5%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>(3) CALLA</td>
<td>76%</td>
<td>80%</td>
<td>&lt;1%</td>
</tr>
<tr>
<td>(4) Myelocyte/Monocyte (OKM1)</td>
<td>1%</td>
<td>1%</td>
<td>4%</td>
</tr>
<tr>
<td>(5) HLA-DR</td>
<td>(-)</td>
<td>&lt;.5%</td>
<td>1%</td>
</tr>
</tbody>
</table>
Although the gene rearrangement
processes make it difficult to be certain
that the alternative explanation holds,
seems a partial digestion product.
Peripheral blood drawn three months after the onset of blast crisis (corresponding to column II, Table 2) was used for Ig and TCR gene rearrangement studies. As shown in Figure 12, no Ig heavy chain gene rearrangements were detectable in Bam HI digests of the patient’s leukemic cell DNA.

Southern hybridization of the patient’s leukemic DNA with the TCRB probe revealed a new Eco RI fragment of approximately 5.0 kb in addition to the germ-line pattern (Figure 13). However, only the germ-line pattern was seen in digestions with Bam HI, Sac I, and Hind III (Figure 14). Although the new band on Eco RI digestion suggests a gene rearrangement, the results with the other three enzymes make it difficult to be certain of this conclusion. However, the alternative explanation, that the new Eco RI band represents a partial digestion product, seems extremely unlikely for three reasons: (i) Two repeat digestions carried out with a considerable excess of new enzyme overnight followed by a second addition of a similar amount of enzyme and digestion for 4-6 additional hours gave identical results. (ii) The same DNA sample did not give partial digestion products with Bam HI, Hind III, or Sac I, nor did it give partial bcr gene products when digested with Bam HI or Hind III. (iii) From the Eco RI cleavage sites on the TCRB gene map, it is difficult to explain the production of a 5.0 kb partial digestion product. The possibility that this 5.0 kb Eco RI fragment might represent a variable region fragment can also be dismissed since none of the sixteen other Eco RI digests on the same nitrocellulose paper subjected to the same washing conditions showed this band. On the other hand, the possibility that the 5.0 kb Eco RI band reflects a genuine rearrangement of
Figure 12. Ig heavy chain gene analysis of leukemic cell DNA from patients E.B. (lane 1) and B.S. (lane 3). DNA was digested with Bam HI and probed with Cmu cDNA. The results show a clear rearrangement in DNA from patient E.B. but no detectable rearrangement in DNA from patient B.S.. Lane 2 shows the germ-line pattern in control DNA.
Figure 13. TCRβ gene analysis of Eco RI digested genomic DNA obtained from leukemic blast cells from patient B.S. (lane 2) and from two suspected T-cell lymphomas (lanes 3 and 4). A new band of approximately 5 kb appears in digests from patient B.S. and one of the lymphomas (lane 3, bottom arrow). This band most likely represents a new fragment containing Cβ-1. DNA from the second lymphoma clearly has a rearrangement 5' to Cβ-1 as suggested by appearance of a new band containing that region (lane 4, top arrow). Lane 1 demonstrates the germ-line pattern in a control.
Figure 14. TCRB gene analysis of DNA from leukemic cells of patient B.S. (lanes 3 and 6) and the two suspected T cell lymphomas (lanes 1, 4 and 2, 5) digested with Hind III (Lane 1-3) and Sac I (lane 4-6). Although the three samples demonstrated nongerm-line Eco RI fragments, as shown in Figure 13, they all show germ-line patterns in these digests.
the genome is supported by the appearance of an identical Eco RI fragment in DNA from a suspected T-cell lymphoma (Figure 13, lane 3). In addition, examination of the TCRB gene map (Figure 3) reveals that a V-D-J rearrangement 5' to $C_{\text{beta}-1}$ will not be detected by a constant region probe in Hind III and Sac I digests, because these enzymes possess restriction sites in the DNA segment between $J_{\text{beta}}$ and $C_{\text{beta}-1}$. Such a rearrangement may also pass undetected in Bam HI digests if it does not produce an appreciable change in the length of the 23 kb germ-line fragment. Therefore it is quite conceivable that this rearrangement is detectable only by Eco RI digestion. It is also significant that in Eco RI digested DNA from a second suspected T-cell lymphoma (Figure 13, lane 4) there is a nonambiguous rearrangement involving the $C_{\text{beta}-1}$ band in the presence of germ-line pattern with the three other enzymes (figure 14). All these arguments suggest that the 5.0 kb band represents a clonal rearrangement of the TCRB gene complex in leukemic cells of patient B.S..

It is interesting that the pattern obtained for patient B.S. is identical to that obtained for one of the two suspected T-cell lymphomas. This suggests either the presence of a limited repertoire of possible rearrangements involving the TCRB genes or an association between the development of neoplasia and specific patterns of rearrangement [86].

B. Patient E.B.

Patient E.B., age 70, also a female, presented at another hospital with a picture of acute leukemia. Her leukemic cells
were not typed, and she was treated with aggressive chemotherapy (daunamycin, cytosine arabinoside and thioguanine) with a transient remission. During the period of remission both her peripheral blood and bone marrow were typical of CML. Moreover, cytogenetic studies performed at this time revealed a t(9;22) translocation and Southern analysis carried out later using a bcr probe clearly showed new fragments suggesting bcr-c-abl junction formation (Figure 11). In view of the typical CML picture in the peripheral blood and bone marrow during remission, the 9;22 translocation, and bcr junction fragments, this patient's most likely diagnosis was CML presenting in blast crisis. After her brief remission, the patient relapsed. At that time 70-80% of bone marrow blasts were TdT-positive, suggesting lymphoid blast crisis, but lymphocyte marker studies with Leu 4 and 14 antibodies (pan-T and B cell antibodies) were both negative. Southern analyses were undertaken on DNA from leukemic cells obtained during this relapse.

As shown in Figure 15, hybridization with the TCRB probe revealed new Bam HI and Hind III bands and deletion of the 11 kb Eco RI, 6.5 kb Sac I, and 3.5 kb Hind III germline bands. These results clearly indicate rearrangement of the TCRB gene complex resulting in a deletion of the $C_{\beta-1}$ region. Shortening of the germ-line Bam HI fragment is also consistent with this interpretation. The new Hind III band, however, is not predicted by this interpretation and is most likely the product of a partial digestion. The appearance of normal germline bands corresponding to fragments containing $C_{\beta-2}$ suggests that the genomic rearrangement that resulted in deletion of $C_{\beta-1}$ was a considerable
Figure 15. TCRB gene analysis of leukemic cell DNA obtained from patient E.B. (P) and digested with Eco RI (E), Bam HI (B), Sac I (S), and Hind III (H). C represents results for a normal control. Arrows pointing to the right demonstrate absence of the germ-line Eco RI, Sac I, and Hind III fragments (11 kb, 6.5 kb, and 3.5 kb, respectively) containing Cbeta-1. Arrow pointing left shows a new Bam HI fragment of approximately 14 kb containing both Cbeta-1 and Cbeta-2. The new Eco RI (in control DNA) and Hind III (right-most arrow) bands were not seen on repeat digestions and therefore most likely represent partial digestion products.
distance 5' to $C_{\text{beta-2}}$ and therefore did not alter the locations of restriction sites immediately 5' to $C_{\text{beta-2}}$. It is interesting that the pattern of rearrangement of the TCRB gene in patient E.B.'s leukemic cells is identical to that obtained for DNA from the Molt-3 T-cell leukemia cell line (data not shown). This also supports the hypothesis of a limited number of possible rearrangements, or the presence of an association between certain rearrangements and the development of leukemia.

Hybridization studies of patient E.B.'s genomic DNA with the Ig $C_{\text{mu}}$ probe also showed a clear rearrangement (Figure 12). On Bam HI digestion, a single band of approximately 16 kb appeared instead of the germ-line 18 kb band. This pattern was also similar to that obtained for the Molt-3 cell line.

In both TCR and Ig hybridization studies there was complete absence of some germ-line bands. This suggests not only that peripheral leukocytes were essentially all clonal blasts but also that in these blasts there was rearrangement of both alleles.

Patient E.B. survived only five months after initial presentation.

C. Patients M.P. and A.K.

The two remaining patients studied in lymphoid blast crisis, M.P. and A.K., were also female. Hybridization studies of genomic DNA from these patients showed no rearrangement of either Ig $C_{\text{mu}}$ or TCRB genes (Figures 10 and 16).

It is noteworthy that patient M.P. remained in chronic phase for nine years before converting to blast crisis while patient A.K. converted after approximately two and a half years. Crisis
Figure 16. Southern hybridization results of blast crisis leukemic DNA from patients M.P. and A.K.. A. TCRB gene analysis of patient M.P. DNA (lanes 1 and 3), patient A.K. DNA (lanes 2 and 4), and control DNA (lanes 5 and 6) digested with Eco RI (lanes 1 and 2) and Sac I (lanes 3-6). Both patients demonstrated the germ-line patterns. B. Ig heavy chain gene analysis with Cmu probe. Lane 1 shows patient M.P. DNA and lane 2 shows patient A.K. DNA. Lane 3 shows the germ-line pattern in a control. There are no detectable rearrangements.
in M.P. was fatal within one week while A.K. underwent bone marrow transplantation and succumbed to recurrent leukemia about one year later.
DISCUSSION

Much has been learned about the course and nature of CML since its initial description by Minot [116]. A neoplastic transformation occurs at the level of the bone marrow pluripotent hematopoietic stem cell with subsequent involvement of granulocytic, erythrocytic, megakaryocytic, monocytic, and lymphocytic cell lines. In the chronic phase there is excessive proliferation of myeloid cells with retention of their capacity to differentiate. Both cytogenetic and isoenzyme studies provided strong evidence of the monoclonal nature of the disorder. At some point in the course of the chronic phase, a second oncogenic event occurs in an immature lineage-committed cell within the proliferating clone. As a result of this second transformation, an immature "blastic" subclone emerges with development of the blast crisis phase of disease. Approximately one-third of blastic clones are of lymphoid lineage, the majority B cells. At the time this study was initiated, T-lymphoid blast crisis had not been convincingly demonstrated.

In this study, analysis of Ig heavy chain and TCRB gene rearrangements was applied to the study of CML in both chronic and blast crisis phases of the disease. Prior studies had looked into Ig gene rearrangements in patients with lymphoid blast crisis and few patients in the chronic phase of the disease [71,72]. Almost all patients (17/18) in lymphoid crisis were shown to have an Ig heavy chain gene rearrangement in DNA from blastic cells. In contrast, none in chronic phase had demonstrated Ig gene rearrangements. Prior to this study, there were
no reported analyses of TCR gene rearrangements in CML.

None of the 33 randomly selected chronic phase CML patients examined in the present study demonstrated rearrangement of either the Ig heavy chain gene or the TCRB gene in peripheral blood cells. These results are thus in agreement with those of previously reported small scale studies addressing the issue of Ig gene rearrangement [71,72]. We may thus conclude that with the level of sensitivity used in this study one cannot detect transformed lymphoid clones in the peripheral blood of chronic phase CML patients. Hence, one cannot yet use analysis of Ig and TCR gene rearrangements to identify early transformation to lymphoid crisis and to predict the onset of clinically evident blast crisis.

The present study does not exhaust approaches for identifying the early conversion to lymphoid blast crisis by detection of Ig and/or TCR gene rearrangements. Improvements in the methods can be made by increasing the sensitivity of detection of rearrangements and by employing bone marrow for assay.

The sensitivity of the methods employed in this study allows for the detection of a clone constituting no less than 1% of the total number of leukocytes [86]. Increasing the sensitivity of the methods is therefore an obvious way to improve on this study. Recently it has become possible to prepare radioactive cDNA probes with 5-10 times the specific activity used in this study. This method, developed by Feinberg and Vogelstein [117], uses binding of random primers followed by primer extension to produce radioactively labeled cDNA with specific activity as high as $1 \times 10^9$ cpm/ug DNA.
It is also noteworthy that this study made use of peripheral blood samples. Since blast crisis begins with an oncogenic event in an immature bone marrow cell, and since the transformed clone proliferates in the marrow before spilling over into the peripheral blood, it would seem more likely that early conversion to lymphoid crisis could be picked up in the marrow rather than peripheral blood. Therefore, future attempts to identify Ig and TCR gene rearrangements during the chronic phase of CML should be directed to the bone marrow. Although it is conceivable that marrow studies may suffer from sampling error, they still seem more likely to detect the presence of blastic clones than peripheral blood studies.

Finally, approximately half of the chronic phase samples examined in the present study were obtained from patients at or shortly after diagnosis. Only four were from patients in the accelerated stage of chronic phase and only one of these patients went on to develop lymphoid blast crisis. Future attempts to demonstrate Ig and TCR gene rearrangements should thus concentrate on patients who are well into chronic phase or are beginning to show early signs of progression, for example a requirement for higher doses of suppressant drugs.

None of the four patients studied in myeloid blast crisis demonstrated rearrangement of Ig heavy chain or TCRB chain genes. Although this result was expected [71,72], analyzing myeloid crisis cells was of potential interest for two reasons: (i) Myeloid leukemia cells occasionally demonstrate lymphoid features, e.g. lymphoid surface markers or TdT positivity. This is an example of
so-called lineage infidelity. (ii) Occasional patients demonstrate two distinct leukemic cell lines, e.g., one myeloid and one lymphoid. The presence of Ig or TCR gene rearrangements in the peripheral blood samples of patients with myeloid blast crisis would have suggested one of these two possibilities, more likely the latter.

One of the four patients studied in lymphoid blast crisis, B.S., demonstrated a germ-line configuration of the Ig heavy chain gene but appeared to have a clonal rearrangement of the TCRB gene in her leukemic blast cells. In addition, the patient’s crisis cells demonstrated TdT-positivity, and cell surface marker studies detected T-cell surface antigens (monoclonal antibodies Leu 1, 2, 3, and 5; OKT6 Ag; OKT10 Ag) and virtual absence of B-lymphoid and myeloid/monocytic surface antigens. The presence of TCRB gene rearrangement and absence of Ig heavy chain gene rearrangement taken together with the marker studies provide conclusive evidence that this patient had T-cell blast crisis. Furthermore, the presence of nongerm-line bcr fragments in crisis cells indicated that they were indeed part of the Ph'-positive clonal proliferation.

While work on patient B.S. was in progress, Chan and Greaves et al [118] reported another case of T-cell blast crisis, also on the basis of a TCRB gene rearrangement and cell surface marker studies. These two cases are the only confirmed cases of T-cell blast crisis. Their significance is clear. First, they establish a new subset of patients with lymphoid blast crisis. Such patients can now be examined for particular clinical features and response to therapy. Secondly, since CML arises from a bone
marrow stem cell, they confirm the bone marrow stem cell origin of T lymphocytes. Thirdly, they further confirm the pluripotent stem cell theory of CML. Now all hematopoietic cell lines, including T lymphocytes, have been shown to be involved in clonal CML proliferation. The difficulty in detecting Ph'-positive G6PD-monoclonal mature T lymphocytes in the peripheral blood during the chronic phase of CML has been circumvented by demonstrating the involvement of Ph'-positive T-lymphoid cells in blast crisis. The difficulty in detecting mature T lymphocyte involvement in the chronic phase has reasonably been attributed to the long survival of mature T lymphocytes. The rarity of T-lymphoid blast crisis can be explained similarly. Blast transformation occurs in Ph'-positive clonal cells. Assuming that this transformation is a random process, the frequency of its occurrence in a specific cell line should be determined by the relative rates of production of the various cell lines within the Ph'-positive clonal expansion. If it is also assumed that T cells have no growth advantage over other cell lines, the fact that they have a low rate of production accounts for the rarity of T-lymphoid blast crisis. Nevertheless, the presence in this study of one case of T-cell lymphoid blast crisis out of four lymphoid blast crisis cases suggests that it may not be as rare as previously thought. It is of course difficult to draw any statistically significant conclusions from such a small number of cases.

The case of patient E.B. was less well defined. Originally thought to have ALL, she almost certainly had CML presenting in lymphoid blast crisis. Two points support this diagnosis. First,
during her remission post-chemotherapy, she had an abnormal periph-eral blood picture typical of chronic phase CML with both elevated white blood cell count and a differential demonstrating myeloid forms of intermediate maturity. Secondly, bcr analysis in relapse DNA showed a breakpoint within that region. Whereas breakage within the bcr region is seen in virtually all cases of Ph'-positive CML, it has been noted in about half of patients diagnosed as having Ph'-positive ALL [26]. However, the idea has recently been advanced that the latter patients actually have CML and that they present with lymphoid blast crisis while patients with true Ph'-positive ALL have a breakpoint on chromosome 22 far upstream from the bcr region [119]. The diagnosis of lymphoid blast crisis was made in patient E.B. during her terminal relapse on the basis of blast cell morphology and TdT positivity. However, the only cell surface marker studies performed, with the antibodies Leu 4 and 14 (detecting pan-B and -T cell antigens, respectively), were negative. The finding of distinct rearrangements of both Ig heavy chain and TCRB genes clearly supported the diagnosis of lymphoid blast crisis.

Two plausible hypotheses may be advanced to explain the dual rearrangement of Ig and TCRB genes. The first is that this patient may have had two lymphoid clones, one with a rearrangement of the Ig heavy chain genes, and hence of B-cell lineage, the other with rearrangement of TCRB genes, implying T-cell lineage. There is currently no way to test this hypothesis since leukemic cells were not sorted prior to DNA extraction and the patient is dead. There have, however, been no previous reports of mixed B-cell T-cell lymphoid blast crisis, and this is unlikely
to be the case here, especially since cell surface antigen studies were negative for both cell lines.

The second, more plausible, explanation is that the patient had a lymphoid blast crisis involving an immature cell of either B- or T-cell lineage, or at a stage of maturation preceding the divergence of the two cell lines. This hypothesis is supported by the lack of B- and T-cell surface antigens. Dual rearrangements of Ig heavy chain and TCRB genes have been known to occur in immature blasts of some subtypes of ALL. In fact, 10% of patients with T-cell ALL (on the basis of cell surface antigens) and 30% of patients with non-B non-T cell ALL (on the basis of lack of B- and T-cell surface antigens and surface Ig) have cells with rearranged Ig heavy chain and TCRB genes [84,86,108,120]. Furthermore, the presence of such dual rearrangements has also been reported in more mature B lymphocytes, although with lower frequency than in immature cells. Specifically, 10% of B-cell CLL patients have rearranged TCRB genes in clonal lymphocytes [84,120].

Dual rearrangements of Ig and TCR genes have been found to be more frequent in leukemias of the precursor B-cell series. One of the more plausible explanations advanced for this observation is that rearrangement of both B- and T-cell genes within the same cell may be accomplished by use of the same molecular recombinational mechanisms. Moreover, immature B cells are postulated to lack the signals present in mature cells that terminate gene rearrangement [107].

Regardless of the explanation, the presence of dual rear-
rangements in the absence of cell surface antigens in crisis cells from patient E.B. suggests that the blast transformation occurred in an immature lymphoid cell. Although statistically it is more likely to be of B-cell lineage, the extent of TCRB gene rearrangement, resulting in complete deletion of $C_{\text{beta-1}}$, argues for a T-cell lineage. It may even be artificial to speak of divergent B- and T-cell lines at this stage of cell maturation. The lineage of patient E.B.'s clonal lymphoid cells may be investigated further by analysis of the arrangement of Ig light chain and TCRA genes. Mature B cells tend to have rearrangement of at least one light chain gene complex, and mature T cells tend to have rearrangement of the TCRA gene in addition to TCRG and TCRB genes [107]. Based on the apparent immaturity of this clone, however, it is likely that results of such studies would be negative.

Two patients, M.P. and A.K., with lymphoid blast crisis demonstrated no detectable rearrangement of either Ig or TCRB genes. This finding is somewhat surprising since all lymphoid cells, unless quite immature, should be committed to either B- or T-cell lines and have started the gene rearrangement process [72]. Other than cell immaturity, the only explanation for failure to detect Ig or TCR gene rearrangements is that the transformations in these patients occurred in more mature B-cell precursors (with Ig heavy chain gene rearrangement but not TCRB gene rearrangement) and that a relatively slight alteration in the size of the large Bam HI fragment used to detect Ig heavy chain rearrangement couldn't be detected. Obviously the next step for further characterization of the blasts in these two patients is
analysis for rearrangement of the kappa and lambda light chain genes.

It is of interest that both lymphoid blast crisis patients with rearrangement of the TCRB genes had particularly aggressive disease with short time to development of crisis and short survival (patient B.S. with crisis at 5 months and death at 9 1/2 months post-diagnosis; patient E.B. with presentation in crisis and death at 5 months post-diagnosis) whereas the two patients lacking rearrangement of these genes had more prolonged courses (patient A.K. transplanted prior to crisis with death in crisis 4 years post-diagnosis; patient M.P. with crisis and death 9 years post-diagnosis). Although these four patients do not allow for statistically significant conclusions, this observation is still intriguing. It raises the question of whether patients who develop lymphoid blast crisis with rearranged TCRB genes undergo blastic transformation earlier than patients without this gene rearrangement. It is unfortunate that additional blast crisis patients with TCR gene rearrangements are not yet available for analysis. However, review of the few reported cases of possible T-cell lymphoid blast crisis based on surface markers alone does not support this hypothesis [20,73-77]. In fact, it may be more likely that a T-lymphoblastic transformation would occur in a patient who had remained in chronic phase for a relatively long period of time, a period adequate to allow for penetration of the Ph'-positive clone into the T-cell compartment and thus provide a substrate for T-lymphoblastic transformation. Further investigation of blast crisis patients with and without TCR gene rear-
rangements is necessary to address these speculations.

It is well established that chromosomal translocations in certain lymphoid malignancies bring proto-oncogenic genes under control of Ig heavy or light chain promoters. For example, in Burkitt's lymphoma with 8;14, 2;8, and 8;22 translocations, the c-myc gene on chromosome 22 comes under control of an Ig gene promoter, while in follicular lymphomas with a 14;18 translocation, the bcl-1 gene comes under control of the Ig heavy chain promoter. These events are thought to play an important role in the genesis of these malignancies. It has been speculated that similar events may occur in T-cell malignancies whereby proto-oncogenes may come under control of TCR gene promoters. Therefore, leukemic blast cell DNA from patients B.S. and E.B. was examined by Southern analysis for rearrangement of the c-myc gene by Dr. C.M.I. Ahmed in the laboratory of Dr. P. Lebowitz. Rearrangement was not detected, suggesting that in these two patients a translocation of the c-myc gene into either a TCR or Ig gene locus had not occurred.
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