CYTOGENETIC AND MOLECULAR ANALYSIS OF CHRONIC AND ACUTE LEUKEMIA

CYTOGENETISCH EN MOLECULAIR ONDERZOEK VAN CHRONISCHE EN ACUTE LEUKEMIE

PROEFSCHRIFT

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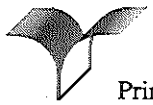

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Aan mijn ouders

Aan Arend

CONTENTS

ABBREVIATIONS

aCML	atypical chronic myelogenous leukemia
ALL	acute lymphoblastic leukemia
AML	acute myeloblastic leukemia
ANLL	acute non-lymphoblastic leukemia
APL	acute promyelocytic leukemia
BC	blast crisis
BCR	5.8 Kb breakpoint cluster region = M-bcr-1
bcr	*bcr* gene
BM	bone marrow
BMT	bone marrow transplantation
bp	base pairs
BV173	cell line derived from a CML patient in blast crisis; expresses b2a2 mRNA
c-abl	cellular *abl* oncogene
cDNA	complementary desoxyribonucleic acid
C terminus	carboxy terminus
CLL	chronic lymphocytic leukemia
CML	chronic myelogenous leukemia
CMML	chronic myelomonocytic leukemia
del	deletion
DNA	deoxyribonucleic acid
e1	first exon of the *bcr* gene
FAB	French-American-British Cooperative Group
GM-CSF	granulocyte macrophage colony stimulating factor
GVHD	graft versus host disease
GVL	graft versus leukemia effect
HL60	human acute promyelocytic leukemia cell line
HLA	human leucocyte antigen
i	iso chromosome
IFN	interferon
IgH	immunoglobulin heavy chain
IL	interleukin
IU	international units
J	joining region of immunoglobulin gene family
K562	cell line derived from a patient with CML in blast crisis; Expresses b3a2 mRNA.
Kb	kilo bases
kD	kilo Dalton
LAP	leucocyte alkaline phosphatase
LPS	lipopolysaccharide
Mar	marker

m-bcr-1	minor breakpoint cluster region=ALL breakpoint cluster region
M-bcr-1	major breakpoint cluster region=CML breakpoint cluster region=BCR
M-CSF	macrophage colony stimulating factor
MDS	myelodysplastic syndrome
MPD	myeloproliferative disease
mRNA	messenger ribonucleic acid
N terminus	amino terminus
p190	190 kD bcr-abl protein
p210	210 kD bcr-abl protein
PB	peripheral blood
PCR	polymerase chain reaction
PDGF	platelet derived growth factor
PHA	phytohemagglutinin
PWM	pokeweed mitogen
R	receptor
RA	refractory anemia
RAEB	refractory anemia with excess of blasts
RAEBt	refractory anemia with excess of blasts in transformation
RAR	retinoic acid receptor
RARS	refractory anemia with ring sideroblasts
RBC	red blood cell count
RNA	ribonucleic acid
SH	src homology
t	translocation
Tom-1	cell line derived from Ph positive ALL patient; expresses e1a2 mRNA
TPA	12-O-tetradecanoylphorbol-13 acetate
v-abl	viral *abl* oncogene
WBC	white blood cell count

CHAPTER 1

GENERAL INTRODUCTION

1.1 THE PHILADELPHIA CHROMOSOME

In 1960 Nowell and Hungerford reported the first chromosomal rearrangement associated with cancer. They discovered that a small chromosome, which they called Philadelphia (Ph) chromosome, was consistently present in the leukemic cells of patients with CML. In 1973 Rowley found that this Ph chromosome was chromosome 22q-, that resulted from a reciprocal translocation between chromosome 9 and 22. This translocation was called the Philadelphia (Ph) translocation or t(9;22)(q34;q11). It took until 1982 before de Klein et al discovered, that as a result of this translocation the *abl* oncogene was translocated from its normal position on chromosome 9 to the 22 q- chromosome. These findings formed the basis for the cytogenetic and molecular investigations in leukemia, that are described in this thesis.

1.2 NORMAL AND ABNORMAL HEMATOPOIESIS

Blood cell formation

Blood cell formation or hematopoiesis takes place primarily in the bone marrow. All elements of blood and lymph are derived from the pluripotent stem cell. Pluripotent stem cells are present in low numbers in the bone marrow. Each of them has the potential to proliferate and differentiate, giving rise to all lymphoid and myeloid blood white blood cells as well as to erythrocytes and platelets. However, under normal conditions the majority of the pluripotent stem cells are in a quiescent state, and only a few are active in blood cell formation. The processes of selfrenewal, differentiation to a more restricted phenotype, and cell death are regulated very strictly. These regulation processes concern also the more mature offspring of the stem cell e.g. the committed progenitor cell, which is a cell type whose developmental lineage is already restricted to one lineage, but is still capable of self renewal.
The regulation mechanisms are not yet fully known, but are thought to be essential for understanding normal hemopoiesis as well as the etiology of leukemia (reviewed by Sawyers, 1991). The last few years several soluble factors have been identified, that are involved in this regulation process e.g. growth factors, and small peptides that are produced by blood cells or by bone marrow stromal cells. GM-, G-, M-CSF and several interleukins such as Il-1, 3 and 6 have been identified as positive regulators (i.e. stimulators) of bone marrow stem cells and committed precursor cells. Examples of negative regulators (inhibitors) of these cells are transforming growth factor ß (TGF ß), which is produced by marrow stromal cells, and small peptides such as stem cell inhibitor (SCI), which is produced by macrophages (Graham et al, 1990, Zebo et al,1990, Williams et al, 1990, Huang et al 1990, Dexter et al, 1977, Clark et al, 1987).

In addition to these factors cell-cell contact and specific effects of the extracellular matrix play a role in regulation of stem cell function, the latter possibly by binding growth factors (Roberts et al, 1988). Disturbances in these regulation mechanisms can result in uncontrolled cell proliferation and/or failure of the progenitor cells to differentiate to mature cells.

Blood cancer

Blood cancer or leukemia is a heterogeneous group of diseases resulting from neoplastic transformation of hematopoietic cells. The main characteristics are uncontrolled proliferation of hematopoietic cells, that do (in most cases) not retain the capacity to normally differentiate to mature blood cells. This differentiation arrest can occur in every maturation stage and in every cell lineage of blood cell differentiation, resulting in distinct forms of leukemia.
Throughout the years several attempts have been made to devise a classification system of the different types of leukemia.
Based on length of survival of the patients and degree of maturation of the cells the leukemias are classified as acute or chronic. A further subclassification is made according to the predominant cell lineage involved, e.g. lymphoid, myelogenous, or monocytic and to morphological and immunological characteristics of the leukemic cells.

- Acute leukemia

The origin of acute leukemia is a single transformed progenitor cell. This might be a pluripotent stem cell or a committed precursor.
In acute leukemia the number of circulating blood cells is increased and the maturation is arrested at early stage of differentiation. The disease is characterized by the presence of large numbers of immature lymphoid or myeloid precursors in the bone marrow and the peripheral blood. These precursors subsequently replace normal bone marrow. Thereafter they often invade other tissues such as central nervous system, the eye, the skin and the testis. Displacement of normal bone marrow by large numbers of undifferentiated or immature leukemic cells results in insufficient production of mature blood cells such as granulocytes, neutrophils, eosinophils, basophils, erythrocytes, and thrombocytes, causing anemia, and susceptibility to infections and hemorrhage. Without treatment acute leukemia patients die within weeks to months after diagnosis. Acute leukemia occurs at all ages.
As a consequence of the fact that the primary cause of acute leukemia might be in the stem cell or a committed precursor and that differentiation arrest can occur in every stage of blood cell differentiation, a great variability is found with regard to the clinical, morphologic, cytochemical, cytogenetical, and immunological features.

Classification of acute leukemia:
A group of French, American, and British hematologists developed a classification system based on conventional morphological and cytochemical methods for analysis of peripheral blood and bone marrow films. This classification was called the FAB classification (Bennett et al, 1976 and 1985).
According to the predominant cell type involved, the acute leukemias are divided into two major types: Acute Lymphoblastic Leukemia (ALL) and Acute Nonlymphoblastic Leukemia (ANLL). The latter is also called Acute Myeloid Leukemia (AML). Using the FAB classification ALL is divided in three subgroups L1, L2, and L3 and AML is subdivided into seven subgroups M1-M7.
Alternatively, classification of acute leukemias can be performed according to immunological characteristics of the leukemic cells (van Dongen et al, 1988).
Which classification system is most suitable depends on the subtype of leukemia e.g. in ALL the morphological differences between the L1 and L2 subtype are very subtle, making this classification system not very useful in contrast to the immunological classification of ALL.

- Chronic leukemia

Chronic leukemias are hematologic malignancies in which the uncontrolled proliferating leukemic cells are capable of full maturation. Two subtypes of chronic leukemia are distinguished: Chronic Lymphocytic Leukemia (CLL) and Chronic Myelogenous Leukemia (CML).
CLL is the most common form of leukemia. CLL is characterized by a slow accumulation of neoplastic B lymphocytes. The peripheral blood and bone marrow of these patients show persistent absolute increase in morphological mature lymphocytes. CLL is more frequent in elderly patients. The median age of the patients is 60 years. Median survival varies from 24 months in CLL patients with anemia and thrombocytopenia as complicating features to 10 years in patients with only lymphocytosis and lymphadenopathy.
CML is a clonal myeloproliferative disorder arising from neoplastic transformation of the pluripotent stem cell. At diagnosis standard findings are leucocytosis, increased granulopoiesis, the presence of immature myeloid progenitor cells in the peripheral blood, basophilia, and hepatosplenomegaly. The course of the disease is biphasic.
The initial chronic phase lasts for 1-4 years, is characterized by leucocytosis with full maturation, and can usually be controlled by chemotherapy. Invariably a blast crisis follows, in which differentiation is blocked and usually the cells are therapy resistant.
CML accounts for 20-30% of the cases of leukemia. The median age of CML patients is 50-60 years. Median survival without treatment is 30-40 months.
Ninety five percent of the CML patients have approximately the same clinical morphological, immunological and cytogenetic characteristics. A very practical classification system for CML and diseases that sometimes might be confused with CML is the one used for the MRC trials reported by Shepherd et al (1987). Based on the peripheral blood count at presentation and morphological analysis of May-

Grünwald Giemsa stained blood films CML should be discriminated from atypical CML (aCML) and a subtype of MDS called Chronic Myelomonocytic Leukemia (CMML). The main discriminative features are the absence of dyshematopoiesis, although exceptions are possible and the lower monocyte count in CML as compared to aCML and CMML.

Some non leukemic human blood disorders such as myeloproliferative diseases (MPD) and myelodysplastic syndromes (MDS) show either uncontrolled myeloproliferation or disturbances in maturation, but both syndromes can progress to acute leukemia. The percentage of MDS patients that progresses to ANLL varies between the different studies. Geddes et al (1990) report a percentage of 9.6%, and Mufti et al (1986) 38%. Patients with normal karyotype show a lower tendency to progress into acute leukemia than patients with an abnormal karyotype (Nowell et al, 1986, Geddes et al, 1990). A variety of techniques have shown that MDS is also a clonal stem cell disease, in which the abnormal clone can give rise to granulocytes, monocytes, B and T lymphoid cells (Janssen et al, 1989, Tefferi et al, 1990). In MDS the failure to differentiate is clinically characterized by low numbers of circulating blood cells, while the bone marrow shows a normal or slightly elevated number of hematopoietic precursor cells, that fail to mature normally. Morphologically precursor cells show asynchronous maturation of the nucleus and cytoplasm, as if their normal development has been arrested.
Dysmyelopoietic syndromes are also considered by the FAB classification.
The FAB classification of MDS discriminates five groups (Bennett et al, 1982): Refractory Anaemia (RA), RA with ring sideroblasts (RARS), RA with excess of blasts (RAEB), Chronic Myelomonocytic Leukemia (CMML), and RAEB in transformation (RAEB-t).

1.3 CHROMOSOME ABERRATIONS IN CANCER

The principle that genetic changes are the basis of most human cancers, was first postulated by Boveri (1914). Soon it became evident that some of these cytogenetic changes leading to cancer, were inherited, while other were caused by environmental influences such as chemical agents. Furthermore, a number of human inherited diseases were discovered, that combine cancer proneness with an abnormal response to DNA damaging agents, such as ataxia telangiectasia (Jaspers, 1982). Cells from ataxia patients show chromosomal instability resulting in enhanced frequencies of chromosomal damage and hypersensitivity for ionizing radiation. Ataxia patients are prone to develop many different types of cancer, especially cancers of the lymphoreticular system.
Yunis et al (1983) proposed that fragile sites may act as predisposing factors for certain specific chromosomal rearrangements. In several human cancers non random cytogenetic abnormalities have been identified of which the chromosomal breakpoint location coincides with the location of fragile sites. These nonrandom chromosomal abnormalities are usually associated with special types of cancer.

The first nonrandom chromosomal aberration associated with cancer was reported in 1960 by Nowell and Hungerford. They discovered that a small chromosome was consistently present in the leukemic cells of CML patients. In 1973 Rowley found that this small chromosome was chromosome 22q-, which resulted from t(9;22)(q34;q11). Soon specific chromosome aberrations were also identified in other tumors: e.g. t(8;14)(q24;q32) was consistently found in Burkitt lymphoma. The chromosomal breakpoints indicated the localization of genes relevant in human tumorigenesis.

Study of familial cancers such as retinoblastoma and Wilms tumor, led to the identification of so called tumor suppressor genes. In 1971 Knudson proposed a theory explaining how the autosomal dominant form of inheritance of familial cancers could be accounted for by mutations that were recessive loss of function mutations involving tumor suppressor genes.
In retinoblastoma 40% of the cases are familial, the remaining 60% are sporadic cases. The essential features are that in the familial form the affected individuals inherit a mutant allele from the affected parent and then a somatic event inactivates the normal allele inherited from the other parent. The frequency of somatic mutational event is sufficiently high so that most of the individuals that inherit a mutant allele will develop one or more tumors. In contrast, the sporadic form of the tumor requires two somatic mutational events. This combination is rare and tumors therefore appear late and are generally unilateral. In 1986 Friend et al identified a homozygous deletion on chromosome 13 band q14 in two retinoblastomas. They used the sequence of this deletion to clone the retinoblastoma gene, making it possible to analyze tumors for the presence of point mutations in the retinoblastoma locus.

1.4 GENES INVOLVED IN LEUKEMIA

Blood cancer cells were easier to culture and to obtain cytogenetics from than solid tumors. As a consequence the past decade efforts have been made to identify mechanisms responsible for blood cancer.
Carcinogenesis is a multistep process in which various genetic alterations result in quantitative or qualitative modifications of various effector proteins regulating growth and differentiation of cells. Although the exact place of these changes is not always clear in the sequence of events leading to leukemia, two sets of genes have been identified playing a role in the neoplastic transformation:

1.Oncogenes

About ten years ago analysis of transforming RNA tumor viruses led to the identification of unique transforming genes, called viral oncogenes, within the retroviral genome (Bishop, 1981)). These viral oncogenes appeared to be derived from homologous cellular genes present in normal DNA of the host cell. These sequences have been incorporated into the retroviral genome during transit of the retrovirus through the host cell. The eukaryotic homologs have been termed proto-oncogenes or

cellular oncogenes (Bishop,1983). They play a role in regulation of growth and differentiation of normal cells.
However, when these proto-oncogenes are altered by mutation, translocation or amplification, their normal function is disturbed, leading to transition of proto-oncogenes into their oncogenic counterparts (oncogenes).
Further evidence for an important role of oncogenes in cancer was provided by transfection studies (Cooper, 1982, Varmus, 1984), the fact that viral oncogenes cause cancer in animals and mapping studies showing the presence of oncogenes near translocation breakpoints on chromosomes in cancer cells (Rowley, 1983).
One of the best studied example of oncogene activation by chromosomal translocation in cancer is the activation of the *c-abl* gene by the Ph translocation in CML. As an example of oncogene activation by chromosomal translocation and because of its importance for the work described in this thesis we will discuss the molecular biology of CML and Ph positive AML and ALL in more detail in chapter 3.

Altered expression of a growth factor gene
The growth factor IL-3 has been implicated in the development of a subtype of acute pre-B cell leukemia with translocation t(5;14) and eosinophilia (Meeker et al, 1990). As a result of this translocation the immunoglobulin heavy chain gene from chromosome 14 is joined to the promoter region of IL-3 upstream of the coding region and induces overexpression of a normal IL-3 protein. The coding region of the *IL-3* gene is not rearranged, but aberrant expression of the gene is responsible for the leukemia.
MDS and AML patients frequently show deletions of the long arm of chromosome 5 (5q-). On the long arm of chromosome 5 genes are localized that encode the hematopoietic growth factors IL-3, IL-4, IL-5, IL-9, M-CSF, GM-CSF and the growth factor receptors M-CSF-R and PDGF-R (Groopman et al, 1989). This suggests that truncation or loss of these genes might play a role in the etiology of this disease.

Alterations of genes involved in cellular differentiation
In addition to growth regulating genes, the cloning of translocation breakpoints and retroviral integration sites from human and animal leukemias has identified genes with high homology to known transcription factors. Some of these genes are likely to play a role in hematopoietic cell differentiation.
Recently the cloning of t(1;19) in a subset of childhood pre-B leukemias showed that part of the *E2A* gene, which codes for immunoglobulin enhancer binding proteins, is fused to a homeobox containing gene named *prl* or *PBX* on chromosome 1 (Kamps et al, 1990, Nourse et al, 1990).
Another important gene for leukemogenesis was identified in the leukemic cells of a subset of AML patients, i.e. acute promyelocytic leukemia (APL), characterized by t(15;17). This translocation creates a fusion gene between the C terminus of Retinoic Acid Receptor α (RAR α) and the aminoterminal region of a new locus called *myl* (myelocytic leukemia) (Borrow et al, 1990, de Thé et al, 1990) The region important for trans activation and target gene specificity is located in the part of RAR, that is

missing in these patients (Evans, 1988). The Myl-RAR fusion protein seems to be incapable of responding to normal levels of retinoic acid. The RAR target genes which normally induce differentiation are blocked. Interestingly complete remission of this type of leukemia can be achieved using high dose all trans retinoic acid treatment, presumably by inducing differentiation of the leukemic cells (Meng-er et al, 1988).

2.Tumor suppressor genes

Besides the important role played by tumor suppressor genes in retinoblastoma and Wilms tumor, ample evidence exists that tumor suppressor genes are also involved in leukemogenesis. Deletions of the long arm of chromosome 5 (5q-), frequently observed in MDS and AML, and deletions of the short arm of chromosome 9 (9p-) indicate that these chromosomes possibly harbour tumor suppressor loci.
Another candidate is the tumor suppressor gene *p53*, located on the short arm of chromosome 17 (McBride et al, 1986). The *p53* gene plays a role in many cancers, but its involvement in leukemia seems of minor importance. In some of the blast crisis CML patients with the chromosomal aberration i(17q) mutations or deletions of the *p53* gene have been reported, but do not seem to be a general phenomenon in leukemogenesis (Ahuja et al, 1989, Marshal et al, 1990).

1.5 INTRODUCTION TO THE EXPERIMENTAL WORK

The presence of consistent chromosomal and molecular aberrations in the leukemic cells of CML and Ph positive AML and ALL patients provides the opportunity to screen patients at diagnosis for the presence of these abnormalities and to monitor the disease during treatment.

The aim of the work described in this thesis was to determine the clinical significance of cytogenetic and molecular analysis of the Ph translocation at diagnosis and during follow up in patients with CML or Ph positive AML and ALL.

Chapter 2 describes the chromosomal aberrations that are specific for the different subtypes of chronic and acute leukemia. In CLL, AML and ALL many different non random chromosomal aberrations have been identified. Each of them is specific for a special subtype of leukemia. The clinical relevance of cytogenetic analysis in acute leukemia and the prognostic significance of cytogenetic analysis in childhood ALL is discussed.
Chapter 3 describes the molecular biology of the Ph chromosome in CML, AML and ALL. In CML and some of the Ph positive AML and ALL patients the breakpoints on chromosome 22 are clustered in a small region, called the BCR region, in the middle of the *bcr* gene. Furthermore we report the identification of a new breakpoint cluster region in the *bcr* gene in ALL patients and a new protein assay for ALL.
Chapter 4 describes the results of molecular analysis in Ph positive CML patients. We investigated the presence of a breakpoint in the BCR region and expression of *bcr-*

abl mRNA in these patients. Several unusual molecular findings were detected: breakpoint outside the BCR region, unusual localization of the breakpoint in the *abl* gene, and simultaneous expression of two *bcr-abl* mRNAs. These topics are discussed.
Chapter 5 contains the results of cytogenetic and molecular analysis in Ph positive CML patients in whom the Ph positive clone was eliminated, i.e. Ph conversion was achieved, by chemotherapy, bone marrow transplantation, interferon therapy or spontaneously.
In less than 5% of the CML patients no Ph chromosome is detected using cytogenetic techniques. These cases are called Ph negative. In **chapter 6** the results are shown of cytogenetic and molecular analysis in Ph negative CML. In some of these patients we and others detected the same *bcr-abl* rearrangement as in Ph positive CML. This finding is of clinical importance, because Ph negative CML patients without this rearrangement often have a shorter survival than rearrangement positive patients. We performed molecular analysis in all Ph negative CML patients that were referred to us. Moreover a multicentre trial was performed in order to answer the following questions: Is Ph negative CML another disease than Ph positive CML or is it a subgroup of CMML? What is the clinical significance of molecular analysis in Ph negative CML?
Chapter 7 contains the results of molecular analysis in Ph positive ALL and AML patients. Furthermore a new translocation t(12;22)(p13;q11) was identified in two AML patients. The 22q- chromosome looked much the same as a Ph chromosome. In one of these patients blood and bone marrow was available for molecular analysis. We investigated if this patient had a variant Ph translocation or that the *abl* and/or *bcr* gene were not involved.
Chapter 8 describes the results of molecular analysis in a patient with Refractory Anemia with Excess of Blasts (RAEB), a subtype of MDS, in whom t(9;22) (q34;q11) was detected by cytogenetic analysis.

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CHAPTER 2

CYTOGENETICS OF LEUKEMIA

2.1 CYTOGENETICS OF CHRONIC LEUKEMIA (CLL, CML) AND MDS

Cytogenetics of CLL

Although CLL patients have a high white blood cell count (WBC) the leukemic B cells have a low spontaneous mitotic rate and it is only recently that cytogenetic analysis of CLL patients became more successful thanks to the availability of B cell specific mitogens such as E. Coli lipopolysaccharide (LPS), and 12-O-tetradecanoylphorbol-13 acetate (TPA). Also protein A and pokeweed mitogen (PWM) stimulate mitosis in CLL. Using these mitogens in short term bone marrow and blood cultures approximately 50% of the CLL patients show karyotypic abnormalities (reviewed by Crossen, 1989). In contrast to the situation in CML, no specific translocation is found that is characteristic for the majority of the CLL patients. However, trisomy 12 is found in 30%, 14q+ in 25% of the CLL patients and 6q-, and 11q- are also associated with CLL.
Although no oncogene has been implicated in CLL, cloning of translocation breakpoints of individual patients revealed that:
-In one patient with t(11;14)(q13;q32) the *bcl-1* gene is translocated from chromosome 11 into the joining (J) region of the immunoglobulin heavy chain locus (IgH) on chromosome 14 (Meeker et al, 1989).
-In nine CLL patients with t(14;19)(q32;q13.1) the *bcl-3* gene was translocated from chromosome 19 into the cα2 region of the IgH gene on chromosome 14. This type of CLL often progresses in lymphoma (van Krieken et al, 1990).
-In one patient with t(2;14)(p13;q32) the breakpoint on chromosome 14 is in the IgH locus and on chromosome 2 outside the k-light chain locus (Fell et al, 1986).
The prognostic value of many of the chromosomal abnormalities remains to be established. Trisomy 12 and additional chromosomal abnormalities correlate with more advanced disease, and a 14q+ chromosome is often seen in patients that progress to prolymphocytic leukemia or Richter syndrome, and is also associated with poor survival (Juliusson et al, 1987, Gahrton et al,1987, Crossen et al, 1989).

Cytogenetics of CML

Cytogenetically the leukemic cells of more than 90% of CML patients are characterized by t(9;22)(q34;q11), the Philadelphia (Ph) translocation (Rowley, 1980). 5% of the CML patients show a variant Ph translocation involving chromosome 9, 22 and one or more other chromosomes (Sandberg, 1980, Heim et al, 1985).In CML blast crisis often additional chromosomal abnormalities are detected even before clinical deterioration becomes apparent. The most frequently observed chromosomal

abnormalities are +8, i(17q), and +22q- (Sandberg, 1980). An example of such a blast crisis karyotype is presented in figure 1.
The remaining 5% of the patients with CML lack a Ph chromosome and are therefore called Ph negative. Both Ph positive and Ph negative CML are discussed extensively in chapter 4 and chapter 6 of this thesis.

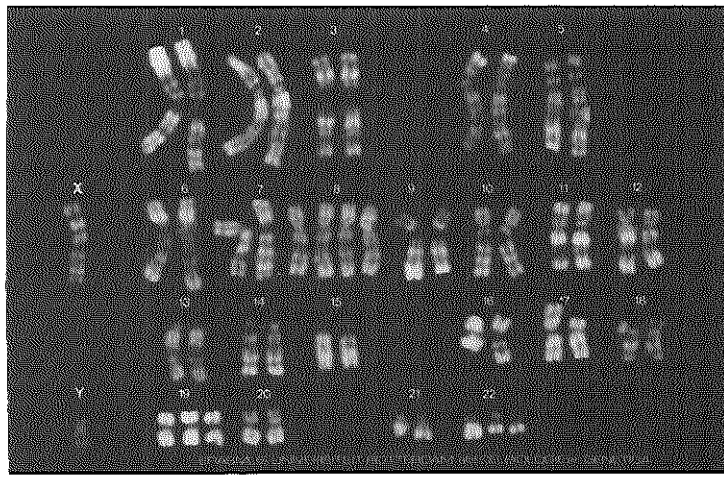

Figure 1: Karyotype of a patient with CML in blast crisis. Besides t(9;22)(q34;q11), that was also present during the chronic phase of CML, additional chromosomal aberrations were present in blast crisis: +8,+8,i(17q),+19,+22q-.

Cytogenetics of MDS

The majority of patients with de novo MDS show loss of chromosomal material rather than a reciprocal translocation or inversion, as commonly found in acute leukemia. In MDS the most frequently observed chromosomal abnormality is loss of the long arm of chromosomes 5 or 7, trisomy 8, and lesions of chromosome 20 (Jacobs et al, 1986, Yunis et al, 1986, Geddes et al (1990).
On the long arm of chromosome 5 genes are localized that encode the hemopoietic growth factors IL-3, IL-4, IL-5, M-CSF, GM-CSF, IL-9 and for the growth factor receptors M-CSF receptor and PDGF receptor (Groopman et al, 1989). This suggests that truncation or loss of one of these genes might play a role in the etiology of this disease. Another possibility is that the missing parts of chromosome 5 or 7 contain an as yet unidentified tumor suppressor gene.
Patients with normal karyotype or 5q- as single chromosomal abnormality tend to have a stable clinical course and long survival (median survival 49 months). Patients with MDS and a single chromosomal defect involving deletion 7q or monosomy 7 have a median survival of 12 months and a higher tendency to evolve into acute leukemia than the first group, whereas patients with complex chromosomal defects often in

combination with 5q-, 7q- or 20q- chromosome have poor survival i.e. median survival 4 months and the highest tendency to transform into acute leukemia (van den Berghe, 1985, Yunis et al, 1986). Patients, who show a karyotype with cytogenetic progression, also belong to this last category (Geddes et al, 1990). It should be notified that although the first appearance of new clones may reflect true karyotypic evolution associated with disease progression it is possible that in some cases they merely represent expansion of a previously existing but undetectable clone. Therefore in considering clinical significance it is clearly important to make the distinction between true karyotypic evolution or clonal expansion and technical variations due to small sample size and variability of the specimens.

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2.2: CLINICAL RELEVANCE OF CYTOGENETICS IN ACUTE LEUKEMIA

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Clinical Relevance of Cytogenetics in Acute Leukemia

A. Hagemeijer and D. C. van der Plas

Introduction

Cytogenetic analysis of leukemic cells has shown the presence of acquired chromosome abnormalities in more than 50% of cases. Since 1970, banding techniques have been applied progressively and a preliminary analysis of the data has been performed at the First, Second, and Fourth International Workshops on Chromosomes in Leukemia (IWCL) (1978, 1979, 1982) for Acute Non-Lymphocytic Leukemia (ANLL) and at the Third IWCL (1980) for Acute Lymphoblastic Leukemia (ALL) [1–4]. These analyses revealed

1. the nonrandom occurrence of some cytogenetic changes in acute leukemias and
2. the association of specific chromosomal abnormalities with specific morphological subtypes of leukemia; and
3. they emphasized the diagnostic and prognostic value of the karyotype of the leukemic cells independent of other clinical and hematological features.

Cytogenetic abnormalities also constitute a unique marker of the leukemic cells that is used in longitudinal studies to ascertain remissions, relapses, and residual diseases [5].

More recently, development of molecular genetic techniques has stimulated investigation into molecular changes characteristic for specific leukemic chromosomal translocations. With the exception of the Philadelphia translocation, most progress along this line of research has been slower than originally expected. How gene rearrangement and/or activation of oncogenes cause leukemia has not yet been demonstrated but, in the meantime, the discovery of specific molecular changes has generated new tools for diagnosis and follow-up of leukemic patients [6].

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Chromosome Abnormalities in ANLL

Occurrence and Type of Clonal Aberration

A relatively large number of reports have been published describing consecutive or selected series of ANLL patients and correlating the karyotype of the leukemic cells with morphological type of leukemia and outcome [for review see 7, 9–13]. In ANLL clonal abnormalities are found in 60%–90% of cases depending on the laboratory and the selected group of patients: in de novo adult ANLL the percentage of normal bone marrow karyotype is more important than in childhood ANLL or in secondary ANLL [7]. The clonal changes can be classified into three major categories:

1. Specific translocation associated with a particular morphological subtype of leukemia according to the French-American-British (FAB) classification (Table 1). These specific changes are more frequent in children and younger patients. The overall frequency is about one-third that of the ANLL cases.
2. Nonrandom numerical or structural changes, particularly +8, −7 or 7q−, −5

Table 1. Recognized specific chromosomal changes in de novo ANLL

Chromosome abnormalities[a]	FAB morphological subtype	Additional characteristics
t(15;17) (q22;q11 or q12)	M3 and M3 variant	In more than 90% of M3
t(8;21) (q22;q22)	M2	Auer rods
t(6;9) (p23;q34)	M2 or M4	Basophilia in M2 cases, sometimes history of MDS
t(9;22) (q34;q11)	M1 (M2, M4)	Faint peroxidase stain
inv(16), del(16), t(16;16) (p13;q22)	M4-"eo"	Abnormal eosinophils in bone marrow
t(9;11) (p21–22;q23), t11q23	M5a or M4	
t(8;16) (p11;p13)	M5b	Erythrophagocytosis

[a] For specific reports on these translocations see ref. 7, 16, and 17

or 5q–, but also trisomy 21 and 22, and deletion of part of 17p, 20q, 18q, 9q, and 13q. These changes are recurrent, found as isolated abnormality or in association. They are found in about one-third of de novo ANLLs, they are the most frequent changes in secondary leukemia, but they are also observed in myelodysplastic syndrome (MDS). They are not associated with a specific FAB morphological type and are the major component of the complex karyotypic abnormalities that characterize leukemia with very poor prognosis.

3. The remaining cases (about 25%) show numerical (like +4) or structural changes that are random and/or specific, but every single one occurs at such low frequency ($\leq 1\%$ of ANLL cases) that their diagnostic significance has not yet been established. A recent example of such miscellaneous structural change is the t(8;16) that is now recognized as a specific translocation associated with FAB-M5 acute leukemia with prominent phagocytosis [14, 15].

Clinical Significance

For obvious reasons, determination of the clonal chromosomal aberrations in ANLL is of diagnostic value: it confirms malignancy and for the specific changes, because of their specific clinical and morphological associations, it makes possible a chromosomal subclassification of leukemia [17]. Recent large studies correlating response to therapy and outcome of ANLL patients classified according to their leukemic karyotype indicated the prognostic value of the leukemic karyotype independent of other clinical parameters. At the Sixth IWCL [13] held in London in 1987 the follow-up data of the Fourth IWCL were analyzed and the results were in line with the study reported by Keating et al. [12] as well as other studies (Table 2). These studies demonstrated not only a difference in morphological diagnosis, therapeutic response, and survival for the various type of cytogenetic abnormalities but also that unique biological consequences follow these abnormalities that influence the (natural) course of the disease and that could be amenable or require specific therapeutic strategy. For example, the t(15;17) is found in more than 90% of cases with FAB-M3 and with the microgranular variant form of acute promyelocytic leukemia (APL): 20%–30% of these patients died early of hemorrhagic complications (DIC, disseminated intravascular coagulation); among the treated patients, the remission rate was high and the duration of first complete remission was relatively long, but relapses usually appeared to be therapy resistant. In contrast, patients with a t(8;21) showed a high remission rate, but also tendency to successive relapses followed by successive remissions, explaining the overall survival time, which is only slightly better than the cases with a normal diploid karyotype, matched for age and receiving the same type of treatment.

Table 2. Prognostic value of leukemic cell karyotype in ANLL median survival/CR duration in months

Karyotype	Sixth IWCL (1987)[a] 355 intensively treated		Yunis et al. (1988)[a] 185 adults		Keating et al. (1988)[a] 384 patients	
	N	Median survival	*N*	Median survival	*N* (CR)	Median CR duration
Normal	148	14	19	22+	178 (122)	14
Abn. 16	6	27	14	26+	21 (19)	22
t(15;17)	16	20	8	20	21 (11)	27
t(8;21)	25	14	9	24+	27 (25)	17
Abn.11q	15	6	16	9	5 (3)	–
−5,5q− −7,7q−	28	3	11	9	41 (15)	7
t(9;22)	–	–	5	3	12 (4)	–
Complex	52	12	39	3		
Very complex	21	3				

[a] Refs. 13, 12, and 11 respectively

The cases with inv(16), del(16), or t(16;16) showed a complete remission rate of 95% or more. But, after cessation of therapy, relapses as solid leptomeningeal tumors [18] or even peritoneal tumor have been reported. Later on, changes in therapeutic regimen seemed to have obviated these late complications [12].

Other specific translocations, i.e., t(9;22), t(6;9), or translocation involving 11q23 are correlated with very poor prognosis and short survival, as are also the recurrent abnormalities with losses of chromosomes 5 or 7. The very complex karyotypes are particularly refractory to treatment.

For other specific changes, the frequency is too low to enable any sensible analysis of survival data to be made. In Yunis' recent report of 185 consecutive AMLs [11], the cases with single miscellaneous defect (20% of total) did as well as the patients with a normal diploid karyotypes (10% of total in this series), with a median survival close to 2 years. In other series [e.g., 9, 10, 12, 13], where the proportion of normal diploid karyotype (NN) is much larger, the median survival time of this NN category is significantly shorter than in the cases with single recurrent effect, indicating the intrinsic heterogeneity of the "normal leukemic karyotype" group.

Chromosome Abnormalities in ALL

Incidence and Classification

In recent consecutive series of chromosome studies in ALL, including our own, 20%–40% of the cases show only normal metaphases, less than 10% are unsuccessful, and the remaining 50%–70% are abnormal. In childhood ALL, the abnormal karyotypes are distributed in various categories, based on changes in ploidy: high hyperdiploid karyotypes with more than 50 chromosomes (±30% in childhood ALL), hyperdiploid karyotypes with 47–50 chromosomes (±10%), pseudodiploid karyotypes (25%–30% of cases), and rare hypodiploid cases (Table 3) [19, 21–24]. In adults, the percentage of normal karyotypes and of hyperdiploid karyotypes (≥50 chromosomes) is decreased in favor of the pseudodiploid cases [20, and personal unpublished data]. The latter category is characterized by the presence of structural abnormalities and translocations that have been shown to correspond to the specific immunological phenotype with distinct prognostic significance.

Table 3. Distribution of the leukemic karyotypes in ALL in children and adults

	Normal	Hyperdiploid		Pseudodiploid	Hypodiploid
		>50 chromosomes	47–50 chromosomes		
Children	20%–40%	±30%	±10%	25%–30%	<5%
Adults	15%–20%	10%–20%	±10%	35%–50%	±5%

Technical Considerations

Cytogenetic analysis in ALL are more difficult than in ANLL. In ALL, despite high cellularity and a high percentage of leukemic cells in bone marrow (BM) and peripheral blood (PB), only a low yield of leukemic metaphases is obtained, with chromosomes very fuzzy and of poor morphology. Use of cultures and synchronization techniques increases the number of well-banded metaphases, but these show mostly a normal karyotype and are probably representative of the normal BM stem cells. This is particularly true in children and for the group of ALLs with hyperdiploid karyotype. Adult ALLs are more amenable to culture and in many cases a 24-h culture will be the method of choice. Despite these difficulties in childhood ALL, by using a direct method and careful handling of BM cells in an optimal setting, Williams (1985) demonstrated the presence of an abnormal karyotype in over 90% of cases [25]. To the best of our knowledge, these results have not been equaled in other institutions. DNA flow cytometry of leukemic cells can (should) be used in parallel with cytogenetics: indeed DNA cytometry will reliably identify two categories of patients: those with a high hyperdiploid karyotype (>50 chromosomes) and those with low hypodiploidy (≤43 chromosomes) corresponding to the groups with the best and worse prognosis, respectively [26].

Prognostic Significance of Cytogenetic Findings in ALL

In childhood ALL, the group of patients with high hyperdiploidy shows the highest response rate and a probability of cure of about 85% with modern chemotherapy [3, 17, 19, 21–24]. Translocations, particularly the Philadelphia translocation, the t(4;11), and translocations involving 8q24 in association with B-cell ALL have been shown to identify patients with the shortest survival. Intermediate response rate and survival time is found for the group of patients with normal karyotype and with slight aneuploidy (45–49 chromosomes) provided that the poor-risk translocations are absent. Translocation (1;19), 6q–, 9p–, and rearrangement of 12p are recurrent structural changes seen in these leukemic karyotypes (Table 4).

In adult ALL, discrimination between better- and worse-risk ALL is much less clear, as survival longer than 3 years is still exceptional. But, also in adults, the high hyperdiploid karyotype (>50 chromosomes) is doing better than the other karyotype [3, 20, 27, personal unpublished data].

In ALL the *high-hyperdiploid karyotype* shows recurrent, nonrandom numerical abnormalities with mainly trisomy of chromosomes X, 4, 6, 10, 14, 17, 18, and often tetrasomy 21. Structural changes and translocations are unusual and are found in about 10% of cases. Classically, the leukemic karyotype correlates with FAB-L1 (80%) or FAB-L2 (20%) morphological classification and with the common ALL (sometimes pre-B) immunophenotype. Incidentally, 0-ALL and T-ALL are found, but in these cases there is usually a specific translocation corresponding to the particular phenotype, in addition to the numerical changes. Two categories of high-hyperdiploid karyotypes do not belong to this rather homogeneous group and are best diagnosed by using DNA flow cytometry in parallel with cytogenetics, i.e., (a) the near-

Table 4. Prognostic value of karyotype in ALL

Outcome	Karyotype	Immunophenotype
Favorable	Hyperdiploid: >50 chromosomes	CALLA (80%)
Intermediate	Normal	
Poor	Translocations	
	t(1;19)	Pre-B
	t(8;14) (q24;q11)	T-ALL
	t(11;14) (p13;q11)	T-ALL
	inv(14)	
Very poor	t(4;11)	0-ALL
	t(9;22)	CALLA/Pre-B
	t(8;14) (q24;q32)	B-ALL
	near haploid	
Unclear	6q−	No specific
	del/t 9p	phenotype
	del/t 12p	

CALLA, common acute lymphoblastic leukemia antigen; T-ALL, T-cell acute lymphoblastic leukemia; 0-ALL, Null-acute lymphoblastic leukemia; B-ALL, B-cell acute lymphoblastic leukemia

diploid karyotype (26–35 chromosomes), which by duplication may present with a majority of hyperdiploid metaphases, and (b) the hypotetraploid karyotype, both types with less favorable outcome.

A normal karyotype is found in various percentages in different studies and is by nature heterogeneous since it comprises ALL cells with changes undetectable by cytogenetics as well as undetected abnormalities due to technical shortcomings. All immunophenotypes are found, but more than half of adult T-ALLs and about 30% of childhood T-ALLs have a normal karyotype. Prognosis is also intermediate, with a probability of cure of 65%–70% for children, and a median disease-free survival of 12 months in adults.

In ALL, the *specific translocations* identified correspond to specific immunophenotypes and are indicative of unfavorable outcome, thus requiring adapted treatment strategy. Other nonrandom *structural changes* are deletions (6q−, 9p−, 11q−) and unbalanced translocations involving particularly 1q, 9p, 12p, as well as 2 isochromosomes i(7q) and i(9q). Most of these recurrent abnormalities are probably secondary. They are found as a single defect or in association with other aberrations and their prognostic significance is still unclear [28].

ALL with t(4;11)

We studied 12 cases of ALL with t(4;11), 6 children including 4 infants aged 2–13 months, and 6 adults aged 22–67 years [29 and unpublished data]. All presented with high-risk clinical features and an 0-ALL phenotype (Tdt+, CD19+, CD10−, HLADR+), rearrangement of IgH in the investigated cases, and paradoxical expression of some mature myeloid or monocytic membrane antigens (CD15+). Morphologically, it is the prototype of biphenotypic leukemia. Usually the majority of blasts show a FAB-L2 morphology but monoblastic features can be expressed when studied by electron microscopy, with monoclonal antibodies or after induction with phorbol esters. In our series, all adults died within 1 year of diagnosis, four of the children survived 30–51 months, and two of them are still alive 30 months and 45 months after diagnosis.

Philadelphia-Chromosome-Positive ALL

We studied ten ALL patients with Philadelphia translocation in leukemic cell karyotype, one child and nine adults (12.5% of adult ALL in our series). The standard t(9;22) was found in six cases, variant

translocations in four instances, and in four cases the karyotype showed rather complex additional abnormalities (unpublished data). In four cases, molecular studies showed a breakpoint on chromosome 22 in the 5.8-kb *BCR* region corresponding to the breakpoint cluster region in chronic myelocytic leukemia (CML) [30]. Six cases were BCR(−) and in four of them rearrangement of the first intron of the *bcr* gene could be demonstrated; the other two cases were not investigated [6]. The median survival time was 12 months and the longest 36 months. This was an exceptional patient who relapsed after 28 months of complete remission. This series is too small to show and to allow correlation between the site of molecular breakpoint in the *BCR* gene (either first intron or *BCR* region) and outcome.

Cytogenetic Marker of Leukemic Cells

Cytogenetic abnormalities constitute a unique marker of leukemic cells. In longitudinal studies, they are used to assess the quality of hematological remission and of bone marrow autograft and to demonstrate impending relapse. Their usefulness in detecting minimal disease is limited by the requirement of a large number of metaphases, the majority being from normal progenitors. For this reason, combined techniques are used, aiming at selection or enrichment of the residual leukemic cell population in the sample karyotyped, for instance, immunolabeling of the metaphases [31], flow sorting of cells labeled with specific monoclonal antibodies [32], flow karyotyping [33], interphase cytogenetics using chromosome-specific alphoid probes to detect a known aneuploidy [34]. Cytogenetics and cell culture techniques can also be combined, e.g. using specific growth factors for recruitment of leukemic cells [35]. By far the most powerful tool for detecting minimal diseases is the new molecular technology that allows amplification of DNA or RNA sequences specifically altered in some leukemia (e.g., Ph^1+ leukemia), using the polymerase chain reaction (PCR) [6]. Unfortunately, so far this technique has only been applicable in the cases where molecular analyses of the specific chromosomal translocation have demonstrated consistent molecular rearrangements.

Molecular Studies of Specific Cytogenetic Rearrangements

In acute leukemia, the recurrent chromosomal translocations are indicative of a consistent molecular rearrangement of the genes located in the chromosomal bands involved in the translocation. Presumably, molecular analyses of these specific translocations will unravel the mechanisms causing the development of this particular morphological type of leukemia. So far the studies have been successful in demonstrating constitutive activation of the c-*myc* oncogene in Burkitt's lymphoma t(8;14) and variant translocations. Also, other leukemic T- and B-cell rerrangements of breakpoints have been characterized [36]. In the Philadelphia translocation the c-*abl* oncogene is modified and activated as a result of its translocation within the *BCR* gene on chromosome 22 [37]. These results are providing us with new tools for diagnosis of Ph+ leukemia as well as for detection of residual disease (see above). In the past few years, many oncogenes, growth factors, and growth factor receptor genes have been mapped around chromosomal regions specifically involved in leukemia [38]. In the very near future it is to be expected that the number of specific translocations molecularly characterized will increase rapidly and that new breakpoint-specific regions will be discovered.

Summary and Conclusions

The diagnostic and prognostic value of specific cytogenetic abnormalities has been established for most recurrent translocations. For less frequent changes, we still need to collect more cases for determination of their clinical significance. Optimal treatment of leukemia with modern therapeutic strategies requires knowledge of the prognostic factors, and leukemic karyotype should be one of the variable features systematically evaluated in all trials. The molecular analysis of the specific translocation will considerably increase our under-

standing of the mechanism of leukemogenesis and provide us with new tools for diagnosis. Systematic collection and conservation of acute leukemic cells, cytogenetically and immunologically characterized, would greatly facilitate and accelerate these fundamental studies.

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2.3 PROGNOSTIC SIGNIFICANCE OF KARYOTYPE AT DIAGNOSIS IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

submitted for publication.

2.3 THE PROGNOSTIC SIGNIFICANCE OF KARYOTYPE AT DIAGNOSIS IN CHILDHOOD ALL.

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ABSTRACT

Acute Lymphoblastic Leukemia (ALL) is the most frequent childhood cancer. The disease is heterogeneous. The leukemic cells in childhood ALL patients show various karyotypic abnormalities, express diverse phenotypes and respond variably to treatment. Identification of prognostic factors will make it possible to predict treatment outcome and to identify the patients that require different therapeutic approaches. The important prognostic implications of chromosome number and of the presence of several well defined structural chromosomal aberrations have been established by several groups.
We analyzed 145 children with ALL at diagnosis for cytogenetic features. In 135 cases cytogenetic analysis was successful. One hundred and one showed abnormal karyotype (70%). Structural chromosomal rearrangements were detected in 71 out of 135 cases investigated (53%), i.e. 77% of the cytogenetically abnormal cases. These cytogenetic findings were correlated with clinical outcome. In our series the ploidy of the karyotype appeared to be of prognostic importance. Our findings concerning karyotype, phenotype, and treatment outcome of this subgroup were in most cases in agreement with earlier reports from other investigators. We observed an increased risk for central nervous system relapse in childhood ALL patients with abnormalities involving the short arm of chromosome 12 in combination with pre-B or common ALL phenotype.

INTRODUCTION

Optimal sampling procedures, a direct technique for preparing metaphase spreads as well as short term cultures and improved banding techniques resulted in the identification of clonal chromosomal abnormalities in 55-90% of childhood Acute Lymphoblastic Leukemia (ALL) cases [1-5]. In 1978 Secker Walker et al [6] for the first time provided evidence that chromosome number of the leukemic cells at diagnosis had prognostic significance in childhood ALL. Subsequent studies indicated that several specific translocations such as t(4;11), t(8;14), and t(9;22) in childhood ALL predicted short survival [7-10]. Moreover the identification of such leukemia specific chromosomal aberrations stimulated molecular studies, designed to elucidate the mechanisms of lymphoid cell transformation and aberrant cell growth of the leukemic cells in ALL. The past few years the chromosomal breakpoints have been cloned and the protein products have been characterised of several translocations, that

are frequently found in ALL: such as t(1;19), t(8;14), t(10;14), t(11;14), and t(9;22). These discoveries provided new tools for diagnosis and follow up [11-17]. Furthermore these molecular studies showed that several mechanisms were responsible for the disease ALL: The chromosomal breakpoints in these specific translocations occurred in genes with a diversity of functions, such as homeobox genes, genes encoding for transcription factors, immunoglobulin and T cell receptor genes, or oncogenes.
Because treatment of ALL has improved and several new chromosomal abnormalities were identified during the past ten years, we decided to reexamine all photographs made of the metaphases from the diagnostic phase of childhood ALL patients investigated in our laboratory and to determine retrospectively the value of these cytogenetic investigations at diagnosis for predicting treatment outcome in childhood ALL. In this paper we report cytogenetic, immunologic and clinical findings in 135 children below the age of 15 years in whom the diagnosis ALL was made during the past 11 years at one single centre. The follow up period varied between 6 months and 11 years.

PATIENTS AND METHODS

Patients

Our series consisted of 135 newly diagnosed ALL patients under the age of 15 years, in whom cytogenetic analysis was successfully performed at diagnosis. In all children ALL was diagnosed between the 1st of Januari 1980 and the 1st of November 1990. They were consecutive cases of ALL, that were diagnosed and treated in the Sophia Childrens Hospital in Rotterdam. Minimum follow up was 6 months, maximum 11 years.
Children with B-ALL of Burkitt lymphoma type or with T cell lymphoma at presentation, as well as children with central nervous system leukemia at diagnosis were treated differently, because of very high risk features. For this reason they could not be validly analyzed together with the other ALL cases, and were excluded from our study.

Cytogenetics

Cytogenetic analysis was performed in all patients at diagnosis of ALL at the Erasmus University in Rotterdam. In this paper all cases were included from whom sufficient cytogenetic data from the diagnostic phase of ALL were available (table 1). Cytogenetic analysis was performed on bone marrow aspirates and most often also on peripheral blood, using adapted standard techniques [18]. Combinations of the following culture times were used for cytogenetic analysis: direct, 24 hours,and sometimes also 48 hours. The adaptations of the standard technique were the following:
- For the direct method bone marrow cells were transferred immediately after aspiration into tubes containing colcemid, heparine and RPMI 1640 culture medium without serum. Upon arrival in the laboratory, the cells were washed with the same

medium. Hypotonic treatment with KCL 0.075 M was applied with one change of hypotonic KCL and careful prefixation of cells with the hypotonic solution. This was followed by three times fixation in 3:1 methanol/acetic acid.
- Short term cultures were performed following standard techniques [18]. The only difference was that methotrexate synchronisation was not used in most cases. The cell cultures were treated with colcemid for 10 minutes, and harvested as described above. Air dried chromosomal preparations were made and stained using R, Q, and/or G banding techniques. Chromosomal aberrations were identified according to ISCN 1985 [19]. Always more than 10, usually 20-40 metaphases were analyzed for each patient.

Immunology
Leukemias were also classified by standard immunological methods and the results were used to group patients as null-ALL, common ALL (cALL), pre-B ALL, B-ALL, and T-ALL [20].

Classification according to clinical risk categories
As usual in the Netherlands, the patients were clinically classified as high risk when presenting with mediastinal enlargement and/or WBC $\geq 50.10^9$/l or standard risk. All patients were treated as is described in the local protocol HR 76 (=protocol C in ref.21), ALL V, VI or VII (identical to BFM 86) protocols [21-23].
Ten patients diagnosed as Burkitt or T-cell lymphoma or initial CNS leukemia were treated differently and therefore excluded from this study (table 1).

Statistical analysis
Event free survival (EFS) curves were constructed by the Kaplan Meier procedure [25] and differences were analyzed by the log rank test [25].
EFS was defined as the interval between diagnosis and relapse or death due to any cause. Patients who did not enter complete remission were assigned a failure time of zero.
Because in our series EFS did not change after 5 years, we will discuss the 5 year EFS when comparing the prognosis of the subgroups of ALL.

RESULTS

Between January 1st 1980 and November 1st 1990 cytogenetic analysis was performed in 155 children under the age of 15 years. An analyzable result was obtained in 145 cases (94%) and a cytogenetically abnormal clone was identified in 69% of the 135 analyzed patients. Follow up varied from 6 months to 11 years.

Classification by ploidy
Table 1 shows classification of the patients according to clinical risk group and ploidy of the bone marrow karyotype at diagnosis. The event free survival (EFS) was calculated for each patient. EFS curves were constructed for the high hyperdiploid (>50 chromosomes), low hyperdiploid (47-50 chromosomes), pseudodiploid and

hypodiploid (<46 chromosomes) subgroup as well as for the group of patients with normal karyotype (figure 1 and 2). The high hyperdiploid group was tentatively divided in 2 subgroups based on the presence of only numerical changes (50N) or numerical and structural aberrations (50S).
For the standard risk group Kaplan Meier plots are shown in figure 1.
For patients with a hypodiploid karyotype the most favourable outcome was found (5 years EFS 100%), followed by patients with more than 50 chromosomes and only numerical aberrations (5 years EFS 92%), then the cases with 47-50 chromosomes (5 years EFS 88%). Less favourable was the outcome for the groups of patients with more than 50 chromosomes with numerical and structural aberrations, 50S, (5 years EFS 73%), normal karyotype (5 years EFS 70%) and pseudodiploid karyotype (5 years EFS 64%). The differences observed between the Kaplan Meier curves were not statistically significant.

Table 1: Distribution according to cytogenetic findings and clinical risk categories of 155 childhood ALL cases diagnosed between 1st of January 1980 and 1st of November 1990 in the Sophia Childrens Hospital.

Karyotype	No of cases n	(%)	Standard risk	High risk[a]	Very high risk[b]
Normal diploid (N)	44	(30%)	27	15	2
Pseudodiploid (PS)	40	(28%)	20	14	6
Low hyperdiploid, (47-50)	15	(10%)	9	6	0
High hyperdiploid, (>50)	35	(24%)	28	6	1
Hypodiploid (<46)	11	(8%)	6	4	1
Total	145	(100%)	90	45	[10]
Cytogenetic failure	10	(6%)	7	3	

[a] WBC $>50x10^9$/l and/or mediastinal enlargement
[b] Cases with B-ALL(FAB L3) of Burkitt lymphoma type (n=3) and ALL with CNS leukemia at diagnosis
[ ] excluded because of different treatment

Figure 2 shows the Kaplan Meier plots for the high risk group. The 5 year EFS was 66% for the pseudodiploid group, 50% for patients with 47-50 chromosomes, 38% for the group with normal karyotype, 33% for patients with > 50 chromosomes with only numerical abnormalities, and 0% for the cases with hypodiploid karyotype.
The EFS for all categories was shorter in the high risk group as compared to the standard risk group. Except for the pseudodiploid group the clinical subdivision in high risk and standard risk is of major importance for the outcome.
Hyperdiploidy is frequent in standard risk and is a favourable prognostic feature. Only 6 patients in the high risk group presented with more than 50 chromosomes in our study, and only 2 of them survived longer than 3 years (EFS was 33%).

In both high and standard risk group a normal diploid karyotype was a rather unfavourable feature.
A major difference in outcome between the high and standard risk group was observed in patients with hypodiploid karyotype. In the standard risk group all six patients with hypodiploid karyotype are still in complete remission after a follow up period of 55-120 months. In the high risk group all 4 patients with hypodiploid karyotype died within 2 years after diagnosis. The numbers of cases in these categories are very small, and the differences between the survival curves of the subgroups do not reach statistical significance.

Patients in whom cytogenetic analysis failed.
In 10 out of 145 childhood ALL cases analyzed, no metaphases were obtained or the metaphases were of very bad quality. We did not find any specific clinical or immunological characteristic that these patients had in common. Fletcher et al [3] also addressed this question and came to the same conclusion as we did: No prognostic importance could be attributed to this category of patients.

Impact of the technique used for cytogenetic analysis on the result.
Because we wanted to know if the percentage of cells with abnormal karyotype, detected with the direct method, 24 or 48 hours cultures varied according to the ploidy status, we systematically compared for all ploidy subgroups the cytogenetic data obtained with the direct technique, the 24 and 48 hours cultures (Table 2).
When both direct and 24 hours cultures have been performed the percentage of abnormal cells was not significantly different in direct preparations compared to 24 hours cultures in most patients with pseudodiploid, or hypodiploid karyotype. In most of the patients with hyperdiploid karyotype we detected a higher percentage of abnormal cells in the direct preparations than in the 24 hours cultures. In all categories exceptions to this rule have been observed. As a consequence from each ALL patient analysis of metaphases obtained by both the direct method and 24 hour cultures are required to minimalize the risk of missing the cytogenetically abnormal clone(s).

Immunological phenotype of the clinically normal risk and high risk group.
Among the 133 immunologically tested cases analyzed here (table 3) were T-ALL:21%, c-ALL:54%, pre-B:21%, null ALL:4% and only one case of B-ALL. The other cases of B-ALL were excluded because of very high risk features requiring more intensive therapy (see table 1).
The immunophenotype of the standard and high risk group were different (table 3). In the standard risk group 86 out of 90 cases (96%) were cALL or pre-B, and only 2 cases were T-ALL. There was no patient with null ALL phenotype in this group. In contrast, in the high risk group, among 45 cases tested 25 (56%) were T-ALL and 5 (11%) were null ALL.
Kaplan Meier plots for EFS of T and non-T phenotype could only be constructed for the high risk group, because only in this group the number of patients in each subgroup were sufficient (see figure 3). From this plot we conclude that the 5 years

EFS for T-ALL phenotype is 21% while the 5 year EFS for non-T phenotype is 62%. Thus T-ALL phenotype correlates with a shorter EFS than non-T ALL phenotype, although the differences did not reach statistical significance (p=0.080).

Table 2: Impact of the technique used on the percentage of clonal abnormal metaphases in 93 cases of childhood ALL (January 1st 1980-November 1st 1990).

Karyotype	No of cases	Results of the various techniques				
		DM+24 hrs			DM only	24+48 hrs
		No difference DM≈24 hrs	DM>24hrs	24hrs>DM		
PS	34	12	7 (3)	1	11 (35-100%)	3(6-100%)
>50	34	6	14 (4)	1	8 (30-95%)	5(7-100%)
47-50	15	4	5 (2)	1	4 (31-100%)	1(20%)
<46	10	4	1	0	1 (88%)	4(12-50%)

DM: direct method
(%): % of abnormal metaphases
( ): number of cases in which only the direct method revealed abnormal metaphases.
DM>24 hours: % of abnormal metaphases higher in direct than in 24 hour cultured cells.

Table 3: Number of standard and high risk childhood ALL cases classified according to ploidy of karyotype and immunophenotype of the ALL cells.

	Standard risk (n=90)						High risk (n=45)				
Immunology	n	c	pre-B[a]	B	T	ND[b]	null	c	pre-B[a]	T	ND[b]
Karyotype											
Normal	42	11	14	0	2	0	0	4	1	9	1
PS	34	13	6	1	0	0	2	3	1	8	0
47-50	15	7	2	0	0	0	1	0	2	3	0
>50	34	26	2	0	0	0	1	3	0	2	0
<46	10	4	1	0	0	1	1	0	0	3	0
Total	135	61	25	1	2	1	5	10	4	25	1

n: Number of cases
[a] Some patients were not tested for cytoplasmic Ig and as a consequence the c-ALL category is slightly overrepresented and the pre-B underrepresented.
[b] ND: not done

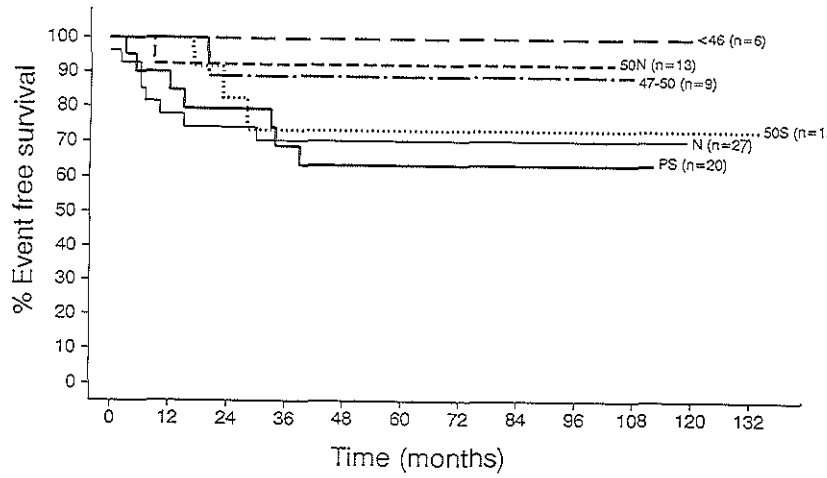

Figure 1: Event free survival of standard risk childhood ALL patients according to karyotype of the leukemic cells.

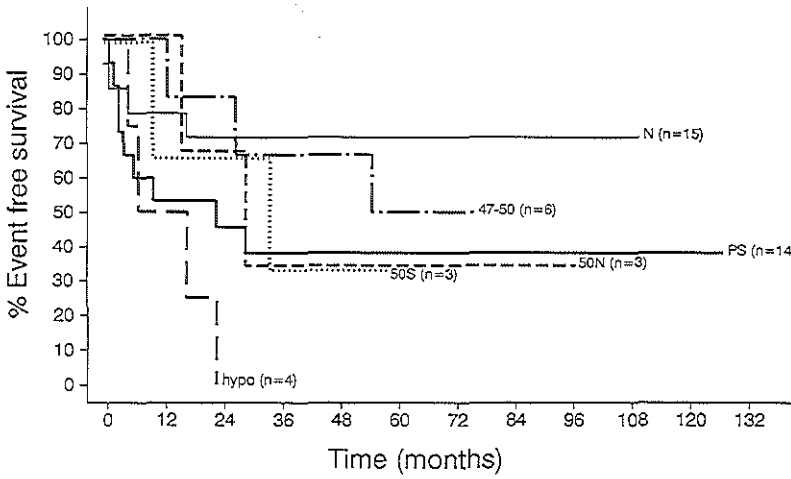

Figure 2: Event free survival of high risk childhood ALL patients according to karyotype of the leukemic cells.

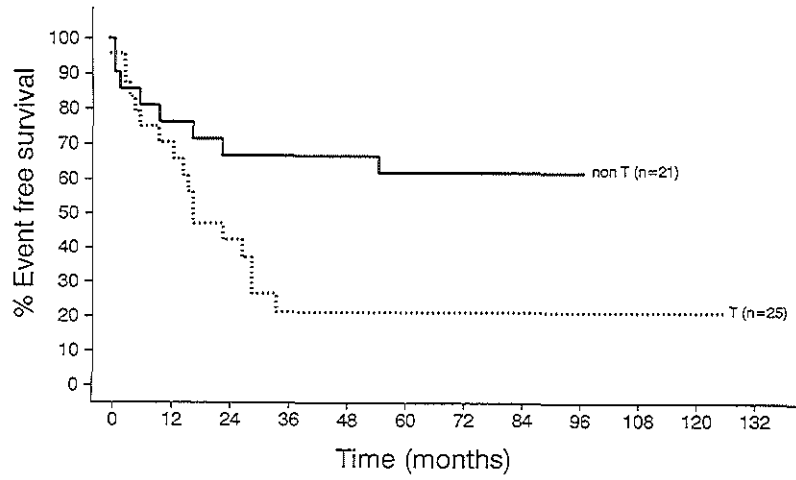

Figure 3: Event free survival of childhood ALL patients according to the immunological phenotype of the leukemic cells

Classification by structural chromosomal aberration

Structural chromosomal aberrations were detected in 71 out of 93 cases with abnormal karyotype. Most of cases showed complex changes with more than one structural abnormality.

Among 27 T-ALL, translocations involving the T cell receptor α/δ loci such as t(10;14), t(11;14) and inv(14) were found in 4 patients, del 1p32 and del 10q24 possibly involving the TALL-1 and TCL-3 loci were detected in one case each.

Other translocations such as t(1;19) or t(4;11) were strongly associated with pre-B and null ALL respectively, while other recurrent changes like 6q-, 9p- and 12p abnormalities did not seem to be associated with a specific phenotype.

The clinical, hematological and immunological data are summarised for patients with the most frequently occurring structural chromosomal aberrations in table 4. These results are described in detail according to the main structural chromosomal aberration:

t(1;19)(q23;p13)

In 3 patients we identified the balanced translocation t(1;19) and in 2 patients the unbalanced translocation derivative: der(19) t(1;19). Four out of these five patients had pseudodiploid karyotype and the remaining child had 47-50 chromosomes. Two patients showed an EFS of 5 resp. 15 months , while the other three are still in complete remission for more than 6 years. The remaining features were remarkably uniform: 4 out of 5 patients were about 2 years old (1.9-2.8 years), only the fifth patient was older(13.4 years), all patients had immunologically pre-B ALL phenotype and belonged morphologically to the FAB L1 subtype. Three out of five patients clinically belonged to the standard risk group, and the other 2 patients were of the high risk category. The latter two did not show shorter survival than the standard risk

patients. These high risk patients are in complete remission for 76+ and 97+ months respectively.

Chromosomal aberrations involving chromosome 11 band q23 were observed in four patients. Three out of four patients had t(4;11) and immunologically null ALL phenotype, in the remaining child t(8;11)(q21;q23 or q24) and cALL phenotype were found. In only one of the t(4;11) patients a short EFS of 5 months was observed. The remaining patients are still in complete remission 9, 56 and 59 months after diagnosis. Two out of the four children were less than 1 year old.

9p-
Various chromosomal aberrations result in (partial) loss of the short arm of chromosome 9, i.e. del(9p), -9, i(9q) and also 9p+, when this indicates a der(9) from an unbalanced translocation with another chromosome. In our series 16 patients had one of these chromosomal rearrangements, in nearly all cases in combination with other structural chromosomal rearrangements. The 9p- patients formed a heterogeneous group with respect to immunological phenotype, clinical risk group and duration of EFS.

The Philadelphia translocation t(9;22)(q34;q11) was observed only once, and had a poor prognosis as expected. A second patient with t(9;22) had CNS leukemia at diagnosis and was therefore not evaluable in this study.

i(17q) was observed in two patients, both had hyperdiploid karyotype. i(17q) was never found as single chromosomal aberration in ALL. One patient, who clinically belonged to the standard risk group, had numerical chromosomal aberrations in combination with i(17q). The EFS was 45+ months. The second patient was clinically high risk and showed t(4;11) in addition to i(17q). Nevertheless this patient is still in complete remission after 59+ months.

Chromosome 6q- caused by deletions or translocations was observed in 11 patients. Aberrations of chromosome 6q were never found as single chromosomal aberration, but always were part of complex chromosomal aberrations. In our series 2 patients with 6q- in combination with T-ALL phenotype had a short survival, while in those with 6q- in combination with c ALL or pre-B phenotype no relapses occurred.

12p:
Interestingly, in patients with abnormalities of the short arm of chromosome 12 and pre-B or cALL phenotype we observed an increased risk for a first relapse in the central nervous system (CNS). In 3 out of 6 patients with precursor B phenotype and 12p abnormalities a CNS relapse was observed. Two out of the three patients with 12p aberrations and CNS relapse belonged to the standard risk group and one to the high risk group. All three patients received different treatments, (HR76, VA, and VI respectively). The increased risk for CNS relapse was not associated with the clinical

risk categories, nor with the treatment protocols. When only the patients without chromosome 12p aberrations are considered, the frequency of CNS relapse is as follows: In 5 out of 86 patients in the standard risk group CNS relapse was diagnosed, while in the high risk group a CNS relapse was observed in 6 out of 41 patients. Comparison of the different treatment protocols revealed, that in the patients with pre-B or cALL without 12p abnormalities a first relapse in the CNS was detected in 2 out of 30 patients treated with protocol HR 76, in 5 out of 39 patients treated with protocol ALL V , and in 1 out of 46 patients treated with protocol VI. Because the number of patients with 12p aberrations is too low to prove that the differences in frequency of CNS relapses are statistically significant, more patients have to be investigated to confirm this finding.

Normal karyotype was identified in 27 patients belonging to the standard risk (30%) and 15 patients belonging to the high risk group (36%). In both subgroups patients with normal karyotype had a relatively poor EFS as compared to the other subgroups (see figure 1 and 2).
Almost certainly in this normal subgroup patients are included, whose leukemic cells exhibit subtle chromosomal aberrations, that were not detectable by current chromosomal analysis. In our opinion in the normal group also patients are included whose leukemic cells are not in metaphase at the moment of cytogenetic analysis. In all patients reported in this paper the total number of metaphases analyzed per patient was sufficient, but because the optimal culture time is different for the various subtypes of leukemia, we can not exclude that we have missed in some of the patients the abnormal clones. For example: in our more recent cases the optimal culture time for T-ALL was 24-48 hours, in contrast with pre-B and cALL where the direct preparations and often also the 24 hours cultures contained most of the leukemic cell populations.
Analysis of the immunologic phenotype of the bone marrow cells nor comparison of the EFS times between the patients with normal karyotype and the other cytogenetic subgroups gave an indication which cytogenetic subgroup it is, that we possibly describe incorrectly as having a normal karyotype.

Table 4: Clinical and hematological data of ALL patients with recurrent cytogenetic changes

Recurrent change	Ploidy	Age (yr)	Sex M/F	WBC x10^9/l	%Blasts Blood	Immuno Phenotype	Risk Group	EFS (months)
t(1;19)(q23;p13)	PS	1.9	M	28.5	89	pre-B	SR	106+
t(1;19)(q23;p13)	PS	2.2	M	16.7	59	pre-B	SR	5
t(1;19)(q23;p13)	PS	2.2	M	24.2	84	pre-B	SR	15*
der(19)t(1;19)	PS	2.8	F[1)]	62.0	73	pre-B	HR	97+
der(19)t(1;19)	47-50	13.4	F[2)]	85.6	93	pre-B	HR	76+
t(4;11)(q21;q23)	>50	8.0	M[3)]	95.5	83	null	HR	59+
t(4;11)(q21;q23)	PS	0.1	M	202.2	100	null	HR	5
t(4;11)(q21;q23)	PS	0.8	F	455.0	99	null	HR	9+
t(8;11)(q21;q23)	PS	6.6	M	46.6	87	cALL	SR	56+
del(6)(q14q23)	PS	4.8	F	36.5	46	cALL	SR	87+
del(6)(q16)	PS	3.3	M[4)]	5.3	6	pre-B	SR	70+
+6q-	>50	13.4	F	85.6	93	pre-B	HR	76
6q-	>50	2.7	F	5.2	36	cALL	SR	86+
t(6;12)(q16;p12)	PS	4.4	M[5)]	3.0	7	pre-B	SR	112+
+6q-	>50	4.5	M	7.9	43	cALL	SR	134+
+6q-	>50	10.0	F	36.8	89	cALL	SR	60+
del(6)(q12q16)	<46	3.9	F	2.4	17	cALL	SR	67+
6q-	PS	6.6	M	52.4	13	T	HR	36+
t(6;12)(q22;p12)	<46	4.0	M[6)]	199.0	97	T	HR	7
6q-	47-50	4.0	F	171.0	93	sup T	HR	13
t(9;22)(q34;q11)	PS	15.1	M[7)]	3.1	60	cALL	HR	17
9p-	PS	3.3	M[4)]	5.3	6	pre-B	SR	70+
9p-	PS	7.3	M	793.0	80	T	HR	0
9p-	<46	13.3	F	18.4	76	pre-B	SR	79+
del(9)(p21)	47-50	4.7	F	1.5	27	pre-B	SR	20*
9p-	PS	2.9	M	4.3	7	cALL	SR	105+
del(9)(p12)	PS	3.3	M	585.0	60	T	HR	23+
9p-/p+	>50	2.8	M	4.5	8	cALL	SR	82+
-9	<46	2.2	M	19.4	92	cALL	SR	54+
-9,i(9q)	PS	9.9	F[8)]	98.4	76	T	HR	110+
-9,i(9q)	47-50	13.4	F[2)]	85.6	93	pre-B	HR	76+
-9,i(9q)	PS	15.1	M[7)]	3.1	60	cALL	HR	17
9p+	PS	2.8	F[1)]	62.1	73	pre-B	HR	97+
9p+	<46	2.2	F	9.3	36	cALL	SR	59+
9p+	<46	1.3	M	198.7	95	null	HR	23*
9p+	<46	6.6	F	186.3	92	T	HR	5*
9p+/q-	PS	5.3	M	8.8	27	cALL	SR	39+
i(17q)	>50	8.3	M	5.7	60	cALL	SR	45+
i(17q)	>50	8.0	M[3)]	95.5	83	null	HR	59+
t(6;12)(q16;p12)	PS	4.4	M[5)]	3.0	7	pre-B	SR	112+
-12,+Mar12p+	PS	2.5	M	34.9	62	cALL	SR	12*
-12,+Mar(del12p)	PS	2.9	M	4.3	7	cALL	SR	105+
12p+	PS	3.2	F	6.2	10	cALL	SR	33*
12p+	47-50	0.8	F	120.0	91	pre-B	HR	55*
del(12)(p12)	PS	4.3	F	94.8	90	cALL	HR	21+
t(6;12)(q22;p12)	<46	4.0	M[6)]	199.0	97	T	HR	7
del(12)(p12)	PS	9.9	F[8)]	98.4	76	T	HR	110+

*CNS relapse
1)-8) identify 8 patients who are mentioned more than once in this table, because of multiple karyotypic aberrations.

DISCUSSION

In this study we determined the prognostic value of successful cytogenetic analysis at diagnosis in 135 childhood ALL cases, that were investigated in our laboratory during the past 11 years. Our observations confirm the observations of other investigators, that the ploidy of the karyotype at diagnosis has prognostical significance in childhood ALL [26,27].
Except for the pseudodiploid subgroup, the survival curves for each ploidy subgroup were different for patients belonging to the clinically high, respectively standard risk group. T-ALL phenotype was observed more frequently in the high risk group than in the standard risk group.
Furthermore many specific chromosomal aberrations were identified that were also reported by other investigators and were of special importance for treatment outcome [28].

t(1;19) or der(19) was identified in 5 patients in our series (table 4).
Shikano et al reported in 1986 [29] that this translocation predicted a bad prognosis. This was not confirmed by our data. Our series confirmed the reports of others [10,30], that the leukemic cells with t(1;19) nearly always had pre-B or cALL phenotype. In a recent paper Secker Walker et al [30] reported on 44 children and 22 adults with t(1;19). They found that patients with der(19) t(1;19) resulting from an unbalanced translocation had a significantly better survival than patients with the balanced t(1;19).
They also reported that neither age nor WBC appeared to have a significant impact on prognosis. In our series of childhood ALL the 2 patient with der(19) t(1;19) are alive and well with an EFS of 76+ and 97+ months, and the three patients with a balanced t(1;19) showed EFS of 5, 15 and 106+ months respectively. Most patients with t(1;19) reported thusfar were of pre-B ALL phenotype, as was the case with all 5 patients from our series. Recently Yamada et al [31] reported that a minority of patients with t(1;19) have cALL, null ALL and even ANLL M5 phenotype in one case.

t(4;11)(q21;q23) was detected in 3 cases of our series. One patient showed another translocation involving chromosome 11 band q23, i.e. t(8;11)(q21;q23). In agreement with earlier reports [7,32] the t(4;11) patients were young, 2/3 were less than 1 year old. Previous reports described a very short survival in these patients. The number of cases with this translocation is too low to determine the prognostic value of t(4;11) in our series. Hagemeijer et al [33] and several others reported that these patients often have a very characteristic immunological phenotype showing expression of lymphoid as well as myeloid antigens.
In our 3 patients with t(4;11) no coexpression of myeloid and lymphoid antigens was observed. Only the patient with t(8;11) expressed both myeloid and lymphoid antigens on his leukemic cells.

Infant ALL:
Several investigators [34-36] reported that ALL in children less than 1 year old , infant ALL, is a distinct entity characterised by unique clinical features (high WBC and poor response to treatment), by characteristic immunological features (coexpression of lymphoid and myeloid antigens) and in more than 50% of the cases by chromosomal translocations involving chromosome 11 band q23. In our series 2 out of 4 infant ALL patients had t(4;11)(q21;q23), one had only numerical chromosomal abnormalities and in one patient several different chromosomal aberrations were identified. We also found heterogeneity in immunological phenotype: 1 cALL, 2 null ALL and 1 pre-B ALL.
We did not observe coexpression of lymphoid and myeloid markers in our infant ALL series.
Survival duration showed a heterogeneous pattern: 2 patients relapsed and 2 patients are still in complete remission for 9+ and 104+ months respectively.

Chromosomal aberrations resulting in **(partial) loss of the short arm of chromosome 9** were detected in 16 patients. These patients formed a heterogeneous group with respect to the other karyotypic abnormalities, the immunological characteristics and the duration of EFS. Our findings are in agreement with previous reports e.g.the report from Pollak et al [38] reporting on 8 childhood ALL cases with 9p- among many other 9p-cases.
Previously suggested association of 9p- with T ALL and "lymphomatous features" [37] was not confirmed in our series, nor by the series of Pollak et al [38].
The 9p- chromosome itself is often the result of deletions of band p13 or p21p22, but can also result from other deletions or unbalanced translocations, i(9q) or monosomy 9. The leukemia in 9p- patients is thought to result from the loss of a yet unknown tumor suppressor gene, that is located on the short arm of chromosome 9 (Chilcote et al [37]). Using Southern blot and dot blot analysis Diaz et al [39] have shown the presence of homozygous and hemizygous deletions of interferon-α and -ß1 genes in 29% of the 9p-childhood and adult ALL patients in their series. They also found deletions that were not visible by routine cytogenetic analysis.

t(9;22) was observed in only 1 patient who responded poorly to treatment. This is in agreement with previous reports by Bloomfield et al [9] and others.

Translocations involving the loci for the T cell receptor (TCR) were identified in 4 patients in our series: 2 patients had t(11;14)(p13;q11), 1 patient had t(10;14)(q24;q11) and 1 patient showed inv14(q11q32). All these translocations were in the region were TCR α and TCR δ were located. In our series both patients with t(11;14) had a low EFS (0 and 5 months respectively), the other two patients are still in complete remission after 23+ and 48+ months. No survival data were presented in the papers from other authors dealing with these translocations.

Our finding that 3 out of 6 patients with 12p abnormalities and cALL or pre-B phenotype showed an increased risk for a first relapse in the CNS was not reported by others. Secker Walker et al [26] and Raimondi et al [40] reported that patients with 12p aberrations always had less than 50 chromosomes and showed a high relapse rate. This is confirmed by our findings: 4 of our 8 children with 12p abnormalities relapsed (3 of them in the CNS). However, these two authors did not identify the site of relapse.
Although the number of childhood ALL cases with 12 p aberrations reported in the literature is very limited, the prognostic significance of 12p aberrations seems to be relatively poor. Whether this is due to an increased risk for a first relapse in the CNS, as suggested by our findings, remains to be established.

Our survey has confirmed previous reports, that chromosomal aberrations are of prognostic significance in childhood ALL. We have shown that the choice of technique has impact on the percentage of abnormal metaphases detected. This is most clearly demonstrated in patients with hyperdiploid karyotype where the percentage of abnormal metaphases is higher in the direct than in the 24 hours cultures. However the quality of banding was better in the cultures than in the direct harvested cells. In our opinion the chance to miss the cytogenetically abnormal clone can be minimalized by performing direct harvesting and a 24 hours culture of bone marrow in all childhood ALL patients. These findings are in contrast to the findings in adult ALL. In adult ALL the 24 hour culture is usually the technique of choice (A. Hagemeijer, personal communication).

It is important to realize that the prognosis of the different subtypes of ALL may be improved by alternative approaches to therapy. An example is the recently improved prognosis for B-ALL using multiple agent chemotherapy. More aggressive treatment regimens for other subgroups of ALL with a poor prognosis have recently been reported to improve outcome [41]. It is thus possible that in the future the influence of cytogenetic abnormalities on prognosis will be decreased or overridden by such improvements of therapy.
In conclusion: Karyotype of the leukemic cells at diagnosis has prognostic value in predicting treatment outcome. Except for the pseudodiploid subgroup the clinical subdivision in high and standard risk is a second factor that is of major importance for predicting treatment outcome.
Specific translocations and deletions have shown to be important for prognosis as well as for cloning of the genes involved in several of the subtypes of ALL.
Combined evaluation of the clinical, cytogenetic, molecular and immunological features of childhood ALL will almost certainly contribute to a better understanding of the mechanisms that play a role in this disease.

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CHAPTER 3

MOLECULAR BIOLOGY OF THE PH CHROMOSOME

3.1 THE PH CHROMOSOME IN CML

The molecular rearrangement underlying the Ph translocation consists of translocation of the *c-abl* gene from chromosome 9 band q34 to the Ph chromosome (Fig. 1) (de Klein, 1982).
On chromosome 22 the breakpoint is located in band q11 in the *bcr* gene. The normal function of the *bcr* gene is unknown, but it is considered as a housekeeping gene.
In 1984 Groffen et al reported that in CML all breakpoints in the *bcr* gene were clustered in a region of 5.8 Kb, the breakpoint cluster region (BCR) (Groffen et al, 1984). The BCR region harbours three exons (b1-b3), and nearly all breakpoints are located either between exons b2 and b3 or between exons b3 and b4. A map of the bcr gene and the BCR region are presented in figure 2 and 3.
On chromosome 9 the breakpoints occur in band q34 in the abl gene. In the *abl* gene breakpoints are scattered over a stretch of 200 Kb (Bernards et al, 1987) i.e. always 5' of *abl* exon 2 (Fig. 4).
As a result of the Ph translocation a new chimeric *bcr-abl* gene on the Ph chromosome is generated (Heisterkamp et al, 1983), which is transcribed into a chimeric *bcr-abl* mRNA (Shtivelman et al, 1985, Grosveld et al, 1986), encoding a 210 kD bcr-abl protein (p210) (Fig. 5) (Konopka et al, 1984). This p210 has enhanced tyrosine kinase activity and is thought to play a key role in leukemogenesis (Shtivelman et al, 1987, Konopka et al, 1985).
Depending on the localisation of the breakpoint in the *bcr* gene, BCR exon b3 sequences can either be absent or present in the chimeric *bcr-abl* mRNA (see Fig. 5). In its absence BCR exon b2 is joined to *abl* exon a2 (termed b2a2 mRNA). Otherwise BCR exon b3 is joined to *abl* exon a2 (termed b3a2 mRNA).
Each of these mRNAs is translated into protein. The two corresponding 210 kD bcr-abl proteins will differ in 25 amino acids encoded by exon b3 (Heisterkamp et al, 1985).
Interestingly the disruption of the abl protein, leading to the activation of its tyrosine kinase activity in CML is comparable with the formation of the gag-abl fusion protein found in Abelson Murine Leukemia virus. This virus causes lymphoma in mice (Goff et al, 1980) and its activity is directly dependent on the presence of the v-abl protein in which the N terminal part of the abl polypeptide is substituted by a gag viral moiety, resulting in a gag-abl fusion protein with enhanced tyrosine kinase activity and tumorigenicity (reviewed in Whitlock et al, 1985, Konopka et al, 1985, Prywes et al, 1985).
An obvious question is: Does p210 cause CML?
Several attempts to express p210 in primary bone marrow cultures have yielded only lymphoid transformants in vitro, even under conditions favouring myeloid cell proliferation (McLaughin et al, 1987, Young et al, 1988). Transgenic mice carrying

a *bcr-v abl* fusion gene driven by an immunoglobulin enhancer or retroviral promoter developed lymphoid malignancies only (Hariharan et al, 1989). Final proof that bcr-abl protein induces CML came from experiments by Daley et al (1990). They transplanted irradiated mice with bone marrow that has been infected with a retrovirus encoding p210. The recipient mice developed a myeloproliferative syndrome closely resembling CML.

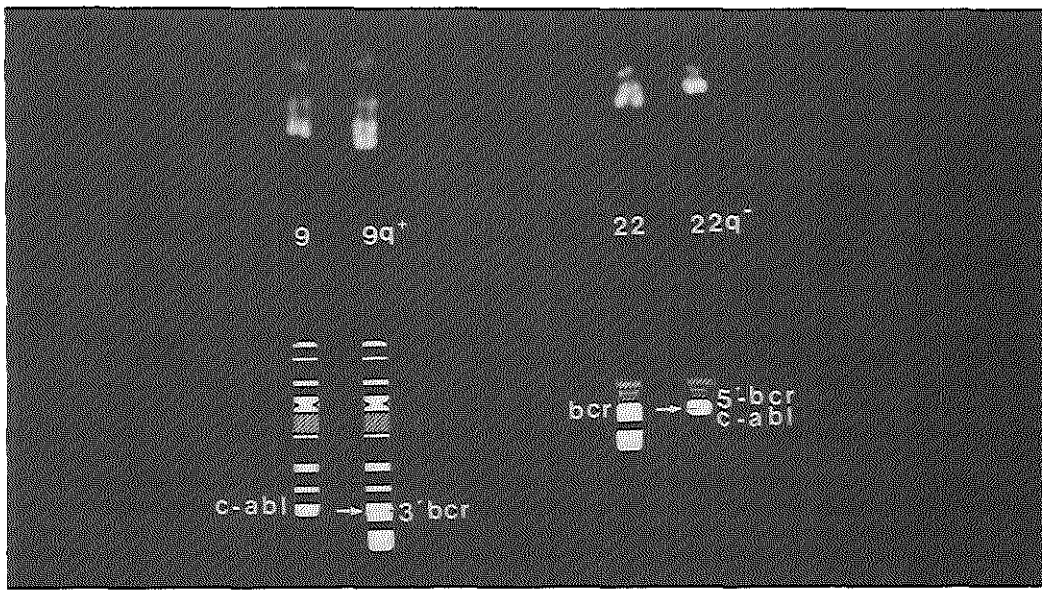

Figure 1:
Top:
Partial karyotype of standard Ph translocation t(9;22)(q34;q11) using R banding technique.

Bottom:
Scheme of molecular alterations caused by t(9;22)in CML. The regional localization of *5'-bcr*, *3'-bcr*, and *c-abl* genes are indicated.

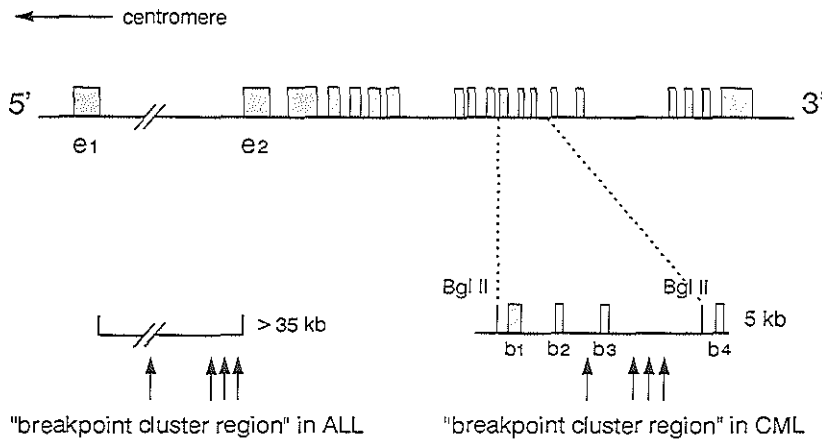

Figure 2:
Schematic representation of the *bcr* gene. In CML the breakpoint in the *bcr* gene usually occurs in the 5.8 Kb breakpoint cluster region (BCR), most frequently between exons b2 and b3 or between b3 and b4 (indicated by arrows). In acute leukemia the localization of the breakpoint in *bcr* is the same as in CML or is more 5' in the first intron of the *bcr* gene, here depicted with "breakpoint cluster region in ALL".

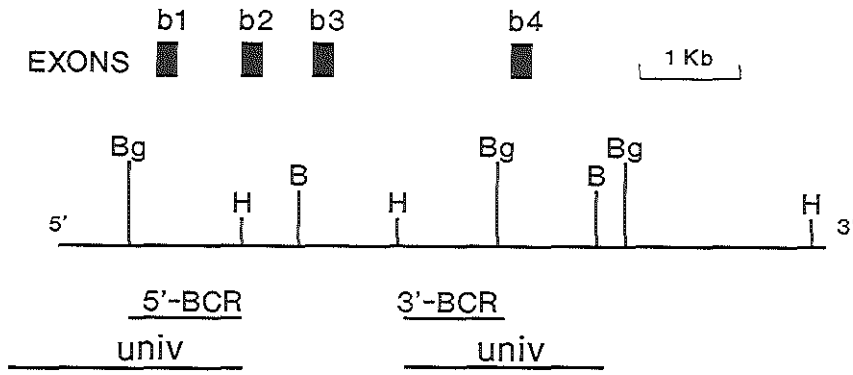

Figure 3:
Simplified restriction map of the BCR region of the *bcr* gene. Bg=BglII, B=BamHI and H=HindIII. The boxes represent exons.
5'-BCR is a 2.0 Kb BglII-HindIII fragment, 3'-BCR is a 1.2 Kb HindIII-BglII fragment and univ is the universal BCR probe, an approximately 5 Kb SalI-BamHI fragment from which a 1.2 Kb HindIII fragment is deleted, because it contains repetitive sequences. These fragments are used as probes in Southern blot analysis of CML.

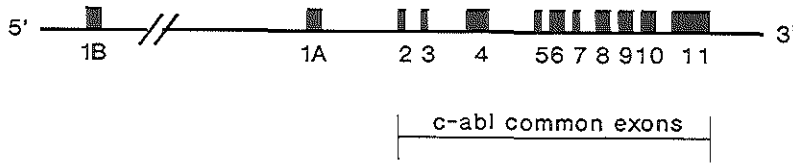

Figure 4:
Scheme of the normal *abl* gene on chromosome 9 band q34. Exons are depicted as black boxes numbered 1B-11. In patients with t(9;22)(q34;q11) the breakpoints in the *abl* gene are scattered over a region of 200 Kb, but are always 5' of *abl* exon 2.

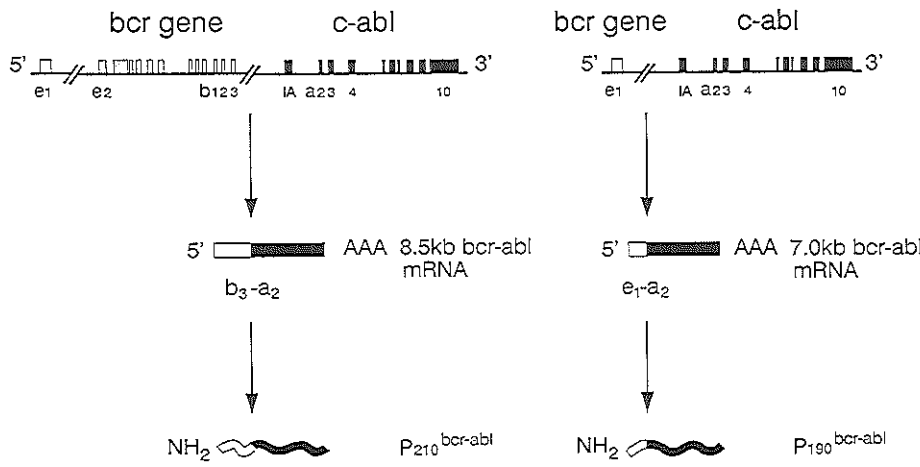

Figure 5:
On the left the *bcr-abl* recombination is depicted as occurs in CML and in part of the Ph positive ALL and AML patients. The breakpoint in the *bcr* gene occurs between exon b3 and b4, resulting in b3a2 mRNA expression. This mRNA is translated into a 210 kD bcr-abl protein.(Another possibility, that is not drawn in this figure, is that the break is between exon b2 and b3, resulting in 8.5 Kb b2a2 mRNA expression, leading to a 210 kD bcr-abl protein).
On the right the *bcr-abl* recombination is depicted in the way it occurs in the remaining Ph positive ALL and AML patients. The breakpoint in the *bcr* gene occurs in the first intron of the *bcr* gene, resulting in e1a2 mRNA expression. This mRNA is translated in a 190 kD bcr-abl protein.

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3.2 UNIQUE FUSION OF BCR AND C-ABL GENES IN PHILADELPHIA CHROMOSOME POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

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Unique Fusion of *bcr* and c-*abl* Genes in Philadelphia Chromosome Positive Acute Lymphoblastic Leukemia

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Summary

The Philadelphia (Ph) chromosome, the product of t(9:22), is the cytogenetic hallmark of chronic myelogenous leukemia. The c-*abl* oncogene on chromosome 9 is translocated to the Ph chromosome and linked to a breakpoint cluster region (bcr), which is part of a large *bcr* gene. This results in the formation of a *bcr*–c-*abl* fusion gene, which is transcribed into an 8.5 kb chimeric mRNA encoding a 210 kd *bcr*–c-*abl* fusion protein. The Ph chromosome is also found in acute lymphoblastic leukemia (Ph$^+$ ALL). Although the c-*abl* is translocated and a new 190 kd c-*abl* protein has been identified, no breakpoints are observed in the bcr (Ph$^+$bcr$^-$ ALL). Here we show that in Ph$^+$bcr$^-$ ALL, breakpoints in chromosome 22 occur within the same *bcr* gene, but more 5′ of the bcr. Cloning of a chimeric *bcr*–c-*abl* cDNA demonstrates that the fusion gene is transcribed into a 7 kb mRNA, encoding a novel fusion protein.

Introduction

The first tumor-specific chromosomal aberration, a shortened chromosome 22 (22q-) or Philadelphia (Ph) chromosome, was observed in chronic myeloid leukemia (CML) (Nowell and Hungerford, 1960). CML is characterized by a huge excess of Ph chromosome positive (Ph$^+$) mature granulocytic cells and their precursors and is clinically divided in two phases. An initial chronic phase lasts on average 3–4 years, and usually progresses to a blast crisis characterized by nonregulated outgrowth of immature myeloid or lymphoid blast cells and a loss of differentiation. CML is a clonal disorder of a pluripotent hematopoietic stem cell. The Ph chromosome is an acquired abnormality and can be found in the hematopoietic cells of all lineages and their committed progenitors (for reviews see: Champlin and Golde, 1985; Koeffler and Golde, 1981; Greaves, 1982).

The Ph chromosome is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22 t(9;22)(q34;q11) (Rowley 1973; de Klein et al., 1982). The breakpoints on chromosome 22 band q11 occur in a small region of 5.8 kb, the breakpoint cluster region or bcr (Groffen et al., 1984). Subsequent cloning experiments and DNA sequence analysis revealed that the bcr is part of a large gene, which has been called the *bcr* gene (Heisterkamp et al., 1985). The bcr contains four exons (b1–b4), and chromosomal breaks occur in two introns between exons b2, b3, and b4. Breakpoints on chromosome 9 are scattered over a distance of at least 100 kb, but are all located 5′ of the tyrosine kinase domain of the c-*abl* proto-oncogene (Heisterkamp et al., 1983; Leibowitz et al., 1985; Grosveld et al., 1986; de Klein et al., 1986a). As a result of the Ph translocation, the c-*abl* gene is transferred from its normal position on chromosome 9 band q34 to the Ph chromosome. This event creates a head to tail *bcr–abl* juxtaposition on the Ph chromosome, with the *bcr* gene closer to the centromere (Heisterkamp et al., 1983). The fusion gene is transcribed into an 8.5 kb chimeric *bcr–abl* mRNA consisting at its 5′ side of 3.2 kb *bcr* sequences linked to 5.3 kb of c-*abl* sequences, lacking the first exon of c-*abl* (Shtivelman et al., 1985, 1986; Stam et al., 1985; Grosveld et al., 1986). The hybrid RNA is translated into a hybrid protein product of 210 kd ($p210^{bcr-abl}$) and exhibits an in vitro tyrosine kinase activity that can be distinguished from the enzymatic activity of the wild-type 145 kd c-*abl* protein ($p145^{abl}$) (Ben-Neriah et al., 1986; Konopka et al., 1984, 1985; Konopka and Witte, 1985).

The presence of the Ph chromosome was thought to be pathognomonic for CML. However, in 1970 Propp and Lizzi described a patient with acute lymphoblastic leukemia (ALL) with a high percentage of Ph$^+$ marrow cells (Propp and Lizzi, 1970). To date, it is reported that 25%–30% of adult and 2%–10% of childhood ALL are Ph$^+$ (Sandberg et al., 1980; Priest et al., 1980). It is surprising that Ph$^+$ ALL and CML share the same marker chromosome, while they are clinically different diseases. Either the two diseases are different expressions of one underlying malignant process, or the marker chromosome is not the same at the molecular level.

A factor obscuring the difference between Ph$^+$ ALL and CML is the fact that following the chronic phase, 30% of the CML patients eventually develop a lymphoid blast crisis that closely resembles de novo Ph$^+$ ALL (Catovsky, 1979). In fact, cases have been reported that presented as a Ph$^+$ ALL (de novo Ph$^+$ ALL), but in which the DNA of the leukemic cells contained breaks within the bcr (bcr$^+$) and expressed the 8.5 kb *bcr–abl* mRNA (de Klein et al., 1986b). A plausible explanation for this phenomenon is that these patients have a lymphoid blast crisis following a silent or very short chronic phase of CML. In contrast to this, in three separate studies a total of eight cases of Ph$^+$ ALL that lack rearrangements in the bcr (bcr$^-$) and fail to express an 8.5 kb *bcr–abl* mRNA were described. No $p210^{bcr-abl}$ was found in the leukemic cells of these patients, but a novel c-*abl* protein of 190 kd was detected

Table 1. The Ph Chromosome in Ph+ ALL and CML

	Patient	Age	Diagnosis	Ph Chromosome	DNA Rearrangement bcr	DNA Rearrangement First Intron of *bcr* Gene	RNA	Protein
(A)	S. D.	2	cALL	+	−	+	6 + 7 kb	ND
	A. E.	14	ALL	+	−	−	6 + 7 kb	$p190^{abl}$
	F. Y.	35	cALL	+	−	−	ND	$p190^{abl}$
	P. N.	46	cALL	+	−	+	ND	$p190^{abl}$
	Tal	36	cALL	−	−	+	ND	ND
(B)			CML	+	+	−	6 + 7 + 8.5 kb	$p210^{bcr-abl}$
			ALL	+	−	+	6 + 7 kb	$p190^{bcr-abl}$

(A) Summary of data of five patients with Ph+ ALL (patients A. E., F. Y., and P. N., also see Chan et al., 1987). The Ph chromosome was observed by cytogenetic analysis in each case. Patient A. E. showed a complex variant translocation involving chromosomes 9, 17, and 22. Immunophenotyping of blast cells at presentation revealed a common ALL phenotype in all patients. The immunophenotype of patient A. E. after initialization of treatment showed coexpression of lymphoid and myeloid antigens. RNA analysis involved Northern blotting using c-*abl* kinase domain probes. Protein data are according to Chan et al. (1987). ND, not determined.
(B) Hypothetical classification of CML versus Ph+ ALL.

instead (Chan et al., 1987; Clark et al., 1987; Kurzrock et al., 1987). Others showed that in Ph+bcr− ALL c-*abl* is consistently translocated to the Ph chromosome, and 3′ *bcr* sequences are sometimes deleted (de Klein et al., 1986b; Erikson et al., 1986).

We report here the underlying genetic mechanism in Ph+bcr− ALL. Chromosomal breaks still occur within the *bcr* gene, but they take place in the putative first intron of the *bcr* gene, which is 5′ of the bcr involved in CML. In this way an alternative *bcr*–c-*abl* fusion gene is created, which is transcribed into a chimeric 7 kb *bcr*–*abl* mRNA. cDNA cloning and sequencing of the chimeric part of the 7 kb RNA of a Ph+bcr− ALL patient show that this was the product of an in-frame fusion of the *bcr* and c-*abl* open reading frames. Most likely the RNA encodes a Ph+ ALL-specific $p190^{bcr-abl}$ hybrid protein since this product was shown to be present in another Ph+bcr− ALL patient with a chromosomal breakpoint in the first *bcr* intron. These findings establish the existence of two molecularly distinct Ph chromosomes in Ph+ ALL versus CML.

Results

Breakpoint Analysis of the *bcr* Gene in Ph+bcr− ALL

This investigation was started with Southern blot analysis of the DNA from five Ph+ ALL patients S. D., A. E., F. Y., P. N., and Tal (Table 1A). The DNAs were digested with BamHI, BglII, HindIII, and EcoRI and hybridized with *bcr* cDNA probes covering the 5′ two-thirds of the gene. No rearranged fragments could be detected, indicating that breakpoints map either farther 5′, outside the *bcr* gene, or within large introns, escaping detection by the combination of probes and restriction enzymes used. To check for the presence of breakpoints in the chromosomal region encompassing the *bcr* gene, the DNA of patient S. D. was digested with the restriction enzymes MluI and SalI. The fragments were separated by field inversion gel electrophoresis (Carle et al., 1986). After blotting and hybridization, both digests showed rearranged fragments of 800 and 300 kb, respectively. The hybridization of rearranged fragments with both 5′ *bcr* gene and c-*abl* cDNA probes (data not shown) indicates the presence of chromosomal breakpoints in the vicinity of the *bcr* and c-*abl* genes of patient S. D.

Cloning of a Novel Chimeric cDNA from a Ph+bcr− ALL Patient

Northern blot analysis was performed with a c-*abl* cDNA probe (comprising the c-*abl* tyrosine kinase domain) of the RNA from peripheral blood cells of Ph+bcr− ALL patient S. D. A relatively strong 7 kb mRNA and a weak 6 kb mRNA were detected (Figure 1, lane 2). It is clear from the comparison of the RNA to K562 RNA that the CML-specific 8.5 kb mRNA is not present in the leukemic cells of patient S. D. The same observation has been reported for other Ph+bcr ALL patients, although a novel $p190^{c-abl}$ protein was expressed (Kurzrock et al., 1987; Chan et al., 1987; Clark et al., 1987). If a new species of c-*abl* mRNA were present, encoding $p190^{c-abl}$, then the RNA comigrates on Northern blots with either the normal 7 kb or the 6 kb (types B and A, respectively) c-*abl* mRNA (Shtivelman et al., 1986).

To analyze the structure of the c-*abl* mRNAs present in the leukemic cells of patient S. D. a λgt10 cDNA library was constructed from the same RNA preparation used for the Northern blot analysis. The first strand cDNA synthesis was primed both with oligo(dT) and with a 17-mer oligonucleotide complementary to a sequence in the third c-*abl* exon. After synthesis of the second strand and EcoRI linker addition, a cDNA library of 2×10^6 pfu was generated. The library was screened with a 300 bp SauI–HindII c-*abl* cDNA fragment (comprising c-*abl* exon 2 and the 5′ half of exon 3), mapping at the 5′ side of the 17-mer primer. Nine hybridizing plaques were found. Southern blot analysis of the EcoRI-digested DNA of these phages showed that the 1.6 kb insert of clone D11 hybridized not only to the c-*abl* SauI–HindII probe, but also to a 600 bp *bcr* cDNA fragment (EcoRI–BamHI) representing the 5′ end of a full-length *bcr* cDNA clone. In Figure 2 the results of extensive restriction enzyme analysis and Southern blotting of clone D11 DNA are summarized: the first 5′ 1200 bp of clone D11 are colinear with the 5′ side of clone KW3 (a full-length

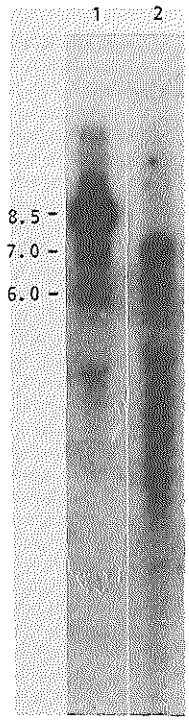

Figure 1. c-*abl* mRNA Analysis of a Ph+ ALL Patient

Northern blot of K562 (lane 1) and RNA of patient S. D. (lane 2). K562 is a CML cell line. Sizes are indicated in kb. Total RNA (20 μg per lane) was electrophoresed on a formaldehyde, 1% agarose gel, blotted onto nitrocellulose, and hybridized to a c-*abl* probe containing the tyrosine kinase domain. After washing, a Kodak XAR5 film was exposed to the filter for 5 days.

bcr–c-*abl* cDNA from K562), diverging just 3′ of the BglII site, from which point on it is colinear with the restriction map of c-*abl* exons 2 and 3. Sequence analysis showed that the clone ends 15 bp downstream of the KpnI site (Figure 2), with the 17-mer primer used for the first strand cDNA synthesis. Thus, clone D11 represents the result of a unique *bcr–abl* fusion.

Sequence Analysis of the Chimeric Portion of Clone D11

Sequencing of the fusion point in clone D11 reveals an in-frame joining of *bcr* to c-*abl* sequences (Figure 3, line 1; see Figure 4B for sequencing strategy). This indicates that the chimeric RNA in patient S. D. potentially encodes a *bcr–abl* fusion protein. Since the last 2 nucleotides of the linking codon are derived from c-*abl* exon 2 (Figure 3, lines 1, 4, 5, and 6; Shtivelman et al., 1985; Grosveld et al., 1986), the first nucleotide should come from *bcr*. This predicts that the *bcr* exon involved contains a splice donor sequence at this position. To verify this prediction, we investigated the exon–intron border of the *bcr* exon corresponding to the most 3′ *bcr* part of the chimeric clone D11. From an EMBL-3 genomic library, a phage clone that hybridized to the same 600 bp EcoRI–BamHI *bcr* cDNA probe (derived from the 5′ end of the *bcr* gene) as clone D11 was isolated. The hybridization of the DNA in the phage appeared to be confined to a 7 kb EcoRI fragment, which was analyzed in detail (Figure 4A). Sequencing of the presumptive 3′ border of the exon revealed a perfect splice donor site. The first nucleotide of the linking codon, a G-residue, is directly followed by the GT dinucleotide defining the 5′ border of the intron (Figure 3, line 3). Thus, linking of the *bcr* exon and c-*abl* exon 2 (Figure 3, line 4) in the chimeric RNA of patient S. D. occurred via the use of normal RNA splicing signals (GT–AG rule; Breathnach and Chambon, 1981).

Restriction enzyme and hybridization analysis showed the genomic *bcr* sequences to be colinear with *bcr* sequences contained in the cDNA clones D11 and KW3. KW3 is a full-length 8.5 kb *bcr–abl* cDNA clone derived from the CML cell line K562 (Lozzio and Lozzio, 1975). S1 mapping analysis has not been performed, so the presence of small introns in this *bcr* exon may have escaped our attention. We anticipate that this 1.7 kb of DNA is the first exon of the *bcr* gene. The restriction map of the 5′ end of the exon was found to be colinear with the published *bcr* cDNA maps up to an NaeI site, representing the possible 5′ end of the gene (Mes-Masson et al., 1986; Hariharan and Adams, 1987). To simplify terminology, we will refer to

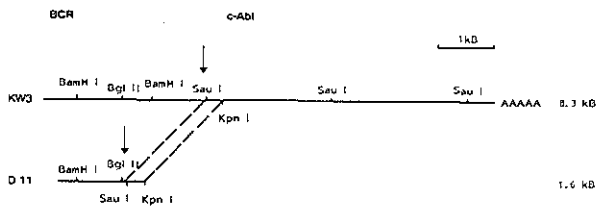

Figure 2. Hybrid *bcr–abl* cDNA from a Ph+ ALL Patient

Restriction enzyme maps of two hybrid *bcr–abl* cDNAs, KW3 and D11. (A) KW3 is a full-length 8.3 kb *bcr–abl* cDNA derived from a λgt Wes DNA library from the CML cell line K562. (B) D11 is a 1.6 kb *bcr–abl* cDNA cloned from a λgt10 cDNA library of Ph+ ALL patient S. D. The first strand was primed with the phosphorylated 17-mer, 5′-pATACTCAGCGGCATTGC -3′ complementary to a sequence in c-*abl* exon a3.

Details of the cDNA cloning procedures are described in Experimental Procedures. The restriction sites in D11 for PstI, BamHI, XhoI, EcoRI, BglII, HindII, SalI, and PvuII are located at the same position as in the 5′ part of KW3, while those for SauI, HindII, and KpnI sites are colinear with the most 5′ c-*abl* sequences in KW3. Arrows indicate the location of *bcr–abl* fusion points.

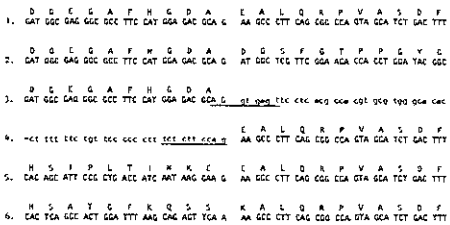

Figure 3. Sequences of Three Different *bcr–abl* Fusions

Nucleotide sequences and predicted amino acid sequences of the open reading frames. Intron sequences are in small type; splice acceptor and donor sequences are underlined. (Sequences 4, 5, and 6 are according to Grosveld et al., 1986.) Line 1, D11 *bcr–abl* fusion, first *bcr* gene exon, second *c-abl* exon (Ph+ ALL); line 2, *bcr* gene cDNA, 3′ first exon, 5′ second exon; line 3, *bcr* gene genomic, 3′ first exon, 5′ first intron; line 4, *abl* gene genomic, 3′ first intron, 5′ second exon; line 5, *bcr–abl* fusion b3–a2, third exon of *bcr*, second *c-abl* exon (CML); line 6, *bcr–abl* fusion b2–a2, second exon of *bcr*, second *c-abl* exon (CML).

this exon and the following intron as the first exon and intron of the *bcr* gene.

The fine mapping data of clone D11 reveal that D11 has terminated 120 bp before the supposed *bcr* AUG start codon (Figure 4). Therefore, we sequenced the first 170 bp of the open reading frame of the genomic exon (as indicated in Figure 4A). Comparison of this genomic open reading frame sequence to the *bcr* cDNA sequence of this region (Mes-Masson et al., 1986; Hariharan and Adams, 1987; our unpublished results) shows exact colinearity. Thus, it is likely that translation of the chimeric RNA of patient S. D. initiates at the putative *bcr* start codon.

In Ph+bcr− ALL, Chromosomal Breakpoints Occur in the First Intron of the *bcr* Gene

Cloning and mapping of the genomic *bcr* gene reveals a minimum size of 90 kb for this gene (Heisterkamp et al., 1985; N. H., unpublished results). This establishes the *bcr* gene to be large. The EcoRI restriction map is depicted in Figure 5A. The precise assessment of its size awaits cloning of a gap in the first intron. This first intron covers at minimum 30 kb of DNA.

The structure of cDNA clone D11 predicts that the chromosome 22 breakpoint in Ph+ ALL patient S. D. occurs in this first intron. At the 3′ end of the intron (Figure 5B), a cluster of recognition sites is found for the most commonly used restriction enzymes in our Southern blot analysis (EcoRI, BamHI, BglII, and HindIII). The cluster prevents the detection of breakpoints mapping to the 5′ side of it with cDNA probes. Using a 0.59 kb PstI–KpnI intron probe located at the 5′ side of this cluster (Figure 5B), we could detect rearranged fragments in two patients (P. N. and Tai, Figure 6). The Southern blot in Figure 6 was hybridized twice. The first time, a 660 bp PstI–BamHI *bcr* cDNA probe mapping just 3′ of the first *bcr* intron failed to detect extra bands (Figure 6, bands without size indication or arrows). The second hybridization was performed both with this 660 bp *bcr* cDNA probe and with the 590 bp PstI–KpnI intron probe. This time rearranged fragments appeared, which are indicated by arrows in Figure 6 (the sizes of the normal genomic fragments detected by the 0.59 kb PstI–KpnI intron probe are indicated).

Patient P. N. is a Ph+bcr− ALL patient expressing $p190^{c\text{-}abl}$ (Chan et al., 1987) and patient Tai was initially diagnosed as a Ph+ CML who relapsed after treatment and remission, with a bcr− ALL lacking the Ph chromosome. The Southern blot is from this Ph− period and shows that while cytogenetically no Ph chromosome is observed, there is a breakpoint in the first intron of the *bcr* gene. In patient S. D. we have been unable to detect chromosomal breakpoints with the 0.59 kb probe. Considering the structure of the cDNA cloned from this patient, the breakpoint should map elsewhere in this large intron, at a position that is not covered by the present analysis. We speculate that the same is true for two other patients, in whom we fail to detect a chromosomal breakpoint (Table 1A). Confirmation awaits Southern blot analysis with new probes that scan the entire intron for the presence of breakpoints.

Discussion

The results presented here provide direct evidence for a unique *bcr-abl* fusion in de novo Ph+ ALL. Surprisingly, the *bcr* gene is still involved in this Ph translocation, although Ph+bcr− ALL is clinically distinct from CML. The *bcr* gene on chromosome 22 appears to be large, covering at minimum 90 kb of genomic DNA. Chromosomal breaks in Ph+bcr− ALL occur in the putative first *bcr* intron (spanning at least 30 kb), which is 5′ of the bcr involved in CML.

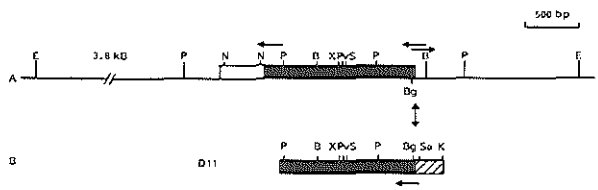

Figure 4. Restriction Enzyme Comparison between the First Exon of the *bcr* Gene and cDNA Clone D11 and Sequence Strategy

(A) 7 kb human genomic EcoRI fragment including the putative first exon of the *bcr* gene (also see Figure 5A). The black box indicates the *bcr* open reading frame from the AUG start codon on. The hatched open box indicates the most 5′ sequences that are colinear with the 5′ end of full-length *bcr* cDNA clones. The slashes indicate that the figure is not drawn to scale.

(B) cDNA clone D11. *bcr* coding fragments are indicated as a black box; *abl* coding fragments, as a striped box. Sequenced fragments are indicated by arrows. E, EcoRI; P, PstI; B,BamHI; X, XhoI; Pv, PvuII; S, SalI; Bg, BglII; Sa, SauI; K, KpnI; N, NaeI.

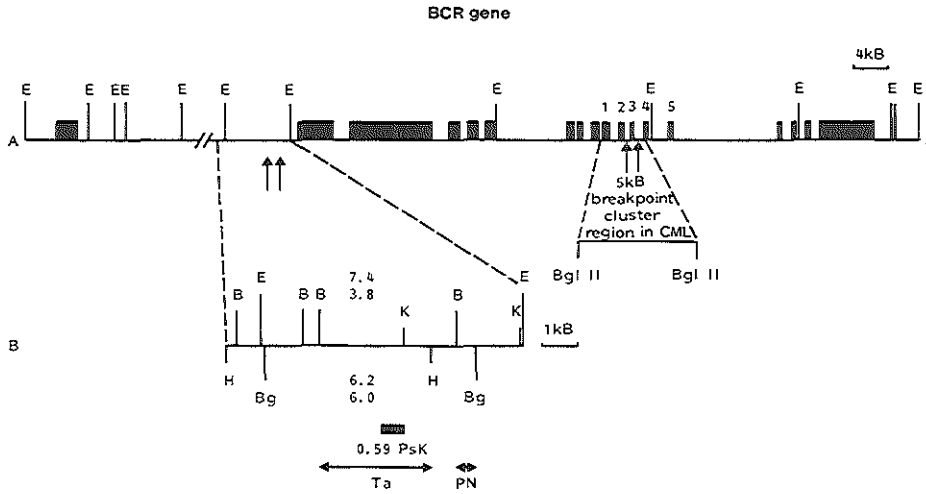

Figure 5. Genomic Organization of the *bcr* Gene and Breakpoints in Ph+ Leukemias

(A) Black boxes indicate restriction enzyme fragments hybridizing to cDNAs of the *bcr* gene. The exons numbered 1–5 of the bcr are mapped precisely (Heisterkamp et al., 1985). A detailed map of the most 5' EcoRI fragment of 7 kb containing the first exon of the *bcr* gene is presented in Figure 4. The slashes indicate a gap in the cloned sequences. Arrows indicate breakpoints.

(B) The 590 bp PstI–KpnI probe that detects the breakpoint fragments is indicated below the map. Numbers indicate size of the fragments detected by the probe in kb. The region in which a break occurs in the DNA patients Tal and P. N. is indicated by a horizontal arrow. E, EcoRI; B, BamHI; Bg, BglI; H, HindIII; K, KpnI. Note on terminology: *bcr* gene refers to the 90 kb gene on chromosome 22; bcr is the breakpoint cluster region localized in the middle of this *bcr* gene. The bcr consists of four small exons, historically named b1–b4. The first exon of the *bcr* gene is found more than 50 kb upstream of the bcr involved in CML.

Our data indicate that chromosomal breakpoints in different patients can be scattered over the intron; for example, in patient S. D. it was not possible to detect the breakpoint with the available probes; however, from the structure of the chimeric cDNA clone D11 isolated from this patient, it is clear that there should be a breakpoint present in this large intron. It will be useful to generate a set of single copy probes that monitor the entire large first intron for the presence of chromosomal breakpoints in Ph+bcr− ALL patients. It may provide a second means to define these

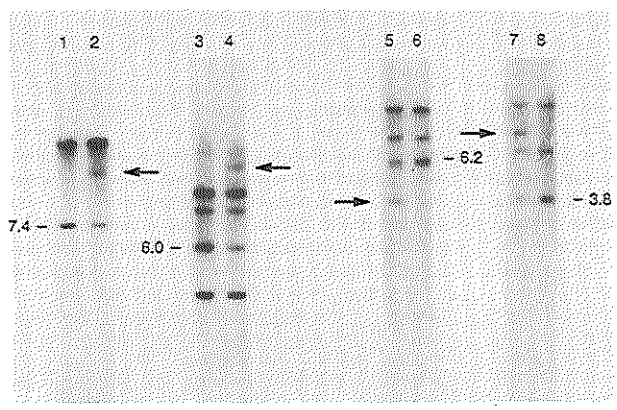

Figure 6. Southern Blot of Two Leukemia Patients with a Break in the First Intron of the *bcr* Gene

DNA of leukemic cells was digested with EcoRI (lanes 1 and 2), BglII (lanes 3 and 4), HindIII (lanes 5 and 6), and BamHI (lanes 7 and 8), separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to the 590 bp first intron PstI–KpnI probe described in Figure 5B. Lanes 1 and 3, NALM 6 (cell line derived from a Ph− ALL; Hurwitz et al., 1979). Lanes 2 and 4, patient P. N.; lanes 5 and 7, patient Tal; lanes 6 and 8, patient G (Ph− CML). Arrows indicate abnormal bands. Sizes of unrearranged bands detected by the 590 bp PstI–KpnI probe are in kb.

patients on a molecular basis, particularly in cases in which in vitro phosphoprotein analysis for the presence of $p190^{c\text{-}abl}$ cannot be performed.

The first *bcr* intron breakpoint analysis could help in diagnosis to discriminate between a lymphoid blast crisis of CML and de novo Ph^+bcr^- ALL. This discrimination is difficult because clinically both leukemic states are similar: they have an extremely poor prognosis and the leukemic cells have a similar blast morphology. Moreover, the cells express the same composite immunophenotype of B cell precursors (Janossy et al., 1977; Greaves, 1982), with clonal rearrangements of Ig-H genes (Ford et al., 1983). The criteria used to date are not always conclusive; in Ph^+ ALL some Ph^- cells can be present in the bone marrow, Ph^+ cells can be eliminated from the marrow by therapy (as judged by cytogenetic analysis), and nonrandom chromosomal abnormalities in addition to the Ph chromosome are seen only rarely compared with the blast crisis of CML, in which they are common (Sandberg et al., 1980). In one of the ALL patients (Tai) no Ph chromosome was detected, while the Southern blot showed a breakpoint in the first *bcr* intron. This underlines a role for molecular diagnosis in the clinical management of ALL, as the presence of a Ph chromosome drastically worsens the prognosis (Bloomfield et al., 1986; Priest et al., 1980). Although we favor the idea that all chromosome 22 breakpoints in Ph^+bcr^- ALL patients will be found in the first *bcr* intron, the data do not exclude that they can also occur 5′ of the *bcr* gene, in another as yet unidentified gene. This question will be resolved by Southern blot analysis of a larger number of Ph^+bcr^- ALL patients using the appropriate probes.

A new chimeric *bcr–abl* cDNA was cloned using RNA from the leukemic cells of a 2 year old Ph^+bcr^- ALL patient. The cDNA shows an in-frame joining of the first exon of the *bcr* gene and the second *c-abl* exon. Joining of the two exons occurs via normal splicing of the precursor mRNA using the splice donor site of the *bcr* first exon and the splice acceptor site of the *c-abl* second exon. The supposition that the *bcr* exon indeed represents the first exon of the *bcr* gene is based on circumstantial evidence: all 5′ *bcr* cDNAs cloned to date have their 5′ termini in a stretch of 300 bp (Hariharan and Adams, 1987; Mes-Masson et al., 1986; our unpublished results). These sequences, together with the *bcr* moiety of cDNA clone D11, are contained in one probably continuous exon of 1.7 kb. The total size of the chimeric mRNA in patient S. D. is calculated to be 7 kb (1.7 kb of *bcr* and 5.3 kb of *c-abl* sequences). Thus on Northern blots this RNA will comigrate with the normal 7 kb type B *c-abl* and the 7 kb *bcr* mRNAs and will be difficult to identify by this analysis. In two reports on Ph^+bcr^- ALL (Clark et al., 1987; Kurzrock et al., 1987) aberrant *c-abl* transcripts of 6.5 and 7.4 kb were found, respectively. This could be the result of sizing problems on the Northern blots. Alternatively, since Clark et al. did not detect hybridization of the 6.5 kb transcript to a probe derived from the 5′ part of the *bcr* gene, it may be that as yet unidentified genes are fused to the *c-abl* gene in these cases. Further analysis will clarify these discrepancies.

The chimeric RNA encodes a *bcr–c-abl* fusion protein of 1530 amino acids (426 *bcr* amino acids [Hariharan and Adams, 1987] and 1104 *c-abl* amino acids [Shtivelman et al., 1986]), which is 476 or 501 amino acids residues shorter than the $p210^{bcr\text{-}abl}$ fusion protein in CML (depending on the presence or absence of *bcr* exon b3; Heisterkamp et al., 1985). We propose that the new fusion protein represents the $p190^{c\text{-}abl}$ immunoprecipitated with *abl* antibodies and specifically found in Ph^+ ALL (Chan et al., 1987; Kurzrock et al., 1987; Clark et al., 1987). The 500 amino acid difference between $p210^{bcr\text{-}abl}$ and $p190^{c\text{-}abl}$ is not reflected in their mobility on SDS–PAGE gels, i.e., $p190^{c\text{-}abl}$ runs too slowly. This may be due to the *c-abl* moiety of the protein, since the molecular weight of the normal *c-abl* proteins measured by this method is 145 kd, while these two proteins consist of only 1130 and 1148 amino acids, respectively. Besides the cloning of the cDNA from patient S. D., support for this supposition comes from patient P. N., who contains a breakpoint in the first *bcr* intron and was shown to express $p190^{c\text{-}abl}$ (Chan et al., 1987). The second patient (Tai) shows rearranged *bcr* gene fragments with the 0.59 kb PstI–KpnI first intron probe that comigrate with rearranged bands in BamHI and BglII digests hybridizing with a 0.52 EcoRI probe (Chan et al., 1987) located 17 kb upstream of the *c-abl* second exon (L. M. W., unpublished results). This strongly indicates the presence of a chimeric *bcr* first exon–*c-abl* gene in patient Tai.

Final proof for the existence of a $p190^{bcr\text{-}abl}$ awaits the development of antibodies directed against peptides encoded by the first *bcr* exon, studies now in progress. In concordance with the proposed structure for $p190^{bcr\text{-}abl}$ in Ph^+ ALL is the observation that the available antisera against the *bcr* gene do not precipitate $p190^{bcr\text{-}abl}$, as they are not directed against peptides derived from the first exon of the *bcr* gene (Clark et al., 1987).

The data suggest the existence of two molecularly distinct Ph chromosomes in CML and Ph^+ ALL, the first containing chromosome 22 breakpoints in the 5.8 kb bcr and the second containing breakpoints in the first intron of the *bcr* gene (Table 1B), giving rise to different *bcr–abl* fusion proteins. At present, it is unclear whether there is a strict correlation between the two types of Ph chromosomes and the two different leukemias. There are cases initially scored as Ph^+ ALL that show breakpoints in bcr and express the 8.5 kb chimeric mRNA. Most likely those cases represent lymphoid blast crises of CML, in which the chronic phase of the disease escaped detection (de Klein et al., 1986b). On the other hand, there are indications that fusion of the first exon of the *bcr* gene to *c-abl* may not be restricted to Ph^+ ALL. In two cases of chronic phase of typical CML no chromosomal breakpoints were detected with either probes covering the 5.8 kb bcr or full-length *bcr* cDNA probes. Moreover no 8.5 kb *bcr–abl* mRNA was found (L. Selleri, personal communication). In addition, patient Tai was initially diagnosed as having CML, but relapsed with ALL after treatment. The breakpoint detected in the first *bcr* intron in the ALL phase may have been present in the CML phase as well.

Although there may be exceptions, we expect that the presence of $p190^{c\text{-}abl}$ will be largely restricted to Ph^+ ALL

(Table 1B). Therefore, the presence of this molecule is associated with an acute leukemia in which there is autonomous growth of immature invasive lymphoid blast cells, while $p210^{bcr-abl}$ in CML is present in an initially nonagressive tumor with an excess of apparently normal myeloid cells of all differentiation stages. The acquisition of the Ph chromosome in CML is strongly correlated with the chronic phase of the disease. However, no consistent quantitative changes of the 8.5 kb *bcr–abl* mRNA are detected in the chronic phase versus the blast crisis in CML (Shtivelman et al., 1987). Thus it is thought that the onset of the blast crisis originates from a second event unrelated to a *bcr–abl* joining. In concordance with this is the fact that a blast crisis of CML is usually accompanied by additional nonrandom chromosomal abnormalities such as trisomy 8 or 19 or isochromosome 17q (Champlin and Golde, 1985).

De novo Ph^+bcr^- ALL also contains a *bcr–abl* fusion product, but lacks a chronic phase. Either a second hit escapes undetected, or the $p190^{bcr-abl}$ has a transforming capacity that is considerably stronger than that of $p210^{bcr-abl}$. Deletion of 476 or 501 *bcr* amino acids in $p190^{bcr-abl}$ compared with $p210^{bcr-abl}$ removes a part of the *bcr* protein that may be involved in controlling the action of the c-*abl* moiety of the protein. This hypothesis has to be tested in vivo, as there is no direct correlation between tyrosine phosphorylation activity in vitro of the different *abl* proteins and their transforming capacity in the target cell. Although the tyrosine kinase activity of $p210^{bcr-abl}$ is very similar to that of $p160^{v-abl}$ (Konopka and Witte, 1985; Witte, 1986), expression under several promoters (SV40, M-MuLV LTR, and bcr promoter) of the 8.5 kb full-length *bcr–abl* cDNA fails to transform NIH 3T3 fibroblasts, while the M-MuLV-LTR-driven v-*abl* gene does induce transformation (our unpublished results).

Introduction of full-length 7 kb and 8.5 kb *bcr–c-abl* cDNAs under control of the *bcr* gene promoter into the germ line of mice may offer an in vivo system to test for the difference in transforming properties between $p210^{bcr-abl}$ and $p190^{bcr-abl}$.

Experimental Procedures

General Methods

Nucleic acid probes were labeled by the method of Feinberg and Vogelstein (1983). Sequence analysis was done by the chain termination method (Sanger et al., 1980), using single-stranded DNA derived from pTZ18/19 vectors according to the suppliers protocol (Pharmacia), or the chemical degradation method (Maxam and Gilbert, 1980).

Genomic libraries were generated in EMBL-3 or cosmid vectors as described by Grosveld et al. (1986) and de Klein et al. (1986). RNA extraction was done as described by Chirgwin et al. (1979) or Auffray and Rougeon (1980). Oligo(dT) column chromatography was performed according to Aviv and Leder (1972), and a 17-mer oligonucleotide was prepared on an Applied Biosystems 361A DNA synthesizer.

Field inversion gel electrophoresis was performed according to Carle et al. (1986). The programmable power inverter was purchased from MJ Research Inc.

cDNA Cloning

Total RNA (50 μg) extracted from peripheral blood cells of patient S. D. was treated with RNAase-free DNAase (Promega). The RNA was denatured in 10 mM methylmercuric hydroxide. Priming was performed with oligo(dT) (12–18), and a c-*abl* third exon-specific phosphorylated 17-mer, 5′-pATACTCAGCGGCATTGC-3′. First strand synthesis was done with cloned M-MuLV reverse transcriptase (BRL); second strand synthesis was according to the RNAase H method (Gubler and Hoffman, 1983). Blunt ends on the cDNA were generated with T4 polymerase, followed by internal EcoRI site methylation with EcoRI methylase (Sigma). In between reactions, the cDNA was purified on Elutip-D columns (Schleicher and Schuell). EcoRI linkers were ligated onto the cDNA with T4 ligase and RNA ligase (Maniatis et al., 1982), and after EcoRI digestion, the cDNA was size-selected on a Sephacryl S-1000 column. cDNA larger than 1 kb was ligated into the EcoRI site of λgt10 (Huynh et al., 1984). Phage DNA was packaged using packaging extracts (VCS).

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3.3 IMMUNOLOGICAL CHARACTERIZATION OF THE TUMOR SPECIFIC BCR-ABL JUNCTION IN PHILADELPHIA CHROMOSOME POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA

Blood 1990, 76:136-141

Immunologic Characterization of the Tumor-Specific *bcr-abl* Junction in Philadelphia Chromosome-Positive Acute Lymphoblastic Leukemia

By Janneke van Denderen, Dorien van der Plas, Toon Meeuwsen, Netty Zegers, Wim Boersma, Gerard Grosveld, and Willem van Ewijk

Philadelphia (Ph')-positive acute lymphoblastic leukemia (ALL) is highly associated with two forms of chimeric *bcr-abl* proteins: P190$^{bcr-abl}$ and P210$^{bcr-abl}$. Whereas P210$^{bcr-abl}$ also occurs in chronic myeloid leukemia, P190$^{bcr-abl}$ is uniquely expressed in Ph'-positive ALL. As a consequence, P190$^{bcr-abl}$ is preeminently a tumor-specific marker in leukemic cells of ALL patients. Because P190$^{bcr-abl}$ is composed of the normal *bcr* and *abl* proteins, the major part of the P190$^{bcr-abl}$ molecule comprises nontumor-specific determinants. The joining region between *bcr* and *abl*, newly generated during the Ph' translocation, is exclusively a tumor-specific epitope on the P190$^{bcr-abl}$ molecule. Therefore, only antibodies against the *bcr-abl* joining region will detect the tumor-specificity of P190$^{bcr-abl}$. In this study a polyclonal antiserum, termed BP-ALL, was raised against a synthetic peptide corresponding to the *bcr-abl* junction in P190$^{bcr-abl}$. The reactivity of BP-ALL with native P190$^{bcr-abl}$ derived from a Ph'-positive ALL cell line (TOM-1) was tested using immunoprecipitation analysis. BP-ALL reacted highly specifically with P190$^{bcr-abl}$ but not with P210$^{bcr-abl}$ isolated from chronic myeloid leukemia cell lines. Peptide inhibition studies further confirmed the fine specificity of BP-ALL. Our data indicate that the tumor-specific *bcr-abl* junction domain is exposed in an antigenic fashion on the P190$^{bcr-abl}$ molecule.

THE PHILADELPHIA (Ph') chromosome, a minute chromosome 22, is found in 90% of all patients with chronic myeloid leukemia (CML).[1] Therefore, this chromosome is regarded as the cytogenetic hallmark of CML. However, the Ph' chromosome has also been reported to occur in some patients with other types of leukemia. Thus, in the leukemic cells of 2% to 3% of all patients with acute myeloid leukemia, the Ph' chromosome can be detected.[2] Moreover, in childhood acute lymphoblastic leukemia (ALL), a 2% to 10% incidence is reported, whereas in adult ALL the Ph' chromosome is found to be the most frequent chromosomal aberration, with an incidence of 20% to 30%.[3,4]

Although the Ph' chromosomes in CML and acute leukemias are cytogenetically indistinguishable, molecular analyses have shown important differences.[5,6] In all cases the Ph' chromosome is the result of a reciprocal translocation between chromosomes 9 and 22.[7-9] Here, the *abl* oncogene has moved from chromosome 9 into the *bcr* gene on chromosome 22. On the *bcr* gene two regions are defined in which breakpoints occur. These regions are termed major breakpoint cluster region 1 (M-*bcr*-1) and minor breakpoint cluster region 1 (m-*bcr*-1).[10] In all Ph'-positive CML patients the *abl* oncogene has moved from chromosome 9 into M-*bcr*-1, localized in the middle of the *bcr* gene on chromosome 22. The result of this particular translocation is a chimeric *bcr-abl* gene, encoding a 8.5-kilobase (kb) messenger RNA (mRNA) that is translated into a 210-Kd protein, P210$^{bcr-abl}$.[11-13]

With respect to the Ph' translocation process, Ph'-positive ALL patients are heterogeneous[14]: 50% of adults and 10% of children with Ph'-positive ALL show the same *bcr-abl* rearrangement as found in CML. Accordingly, these patients express P210$^{bcr-abl}$.[14] In contrast, a P190$^{bcr-abl}$ protein is demonstrated in the other group of ALL patients, including almost all cases of childhood ALL.[15-17] We have previously shown a different *bcr-abl* rearrangement in such patients.[5,6] Here, breakpoints were noticed in the fist intron of the *bcr* gene, termed m-*bcr*-1. The first *bcr* exon (e1) is then spliced to exon 2 of the *abl* gene (termed a2), resulting in a 7.0-kb *bcr-abl* mRNA. Therefore, in the chimeric P190$^{bcr-abl}$ protein the e1-a2 *bcr-abl* junction is present.

Because P210$^{bcr-abl}$ as well as P190$^{bcr-abl}$ are expressed exclusively in malignant cells in CML and ALL, these proteins are by definition tumor-specific. However, P210$^{bcr-abl}$ and P190$^{bcr-abl}$ are composed of parts of *bcr* and *abl* molecules, which are, as such, normal cellular proteins. The only tumor-specific determinant on the chimeric proteins is formed just in the joining region between *bcr* and *abl*. Recently we have shown that the *bcr-abl* junction in P210$^{bcr-abl}$ is antigenically exposed on this protein.[18]

In this study we investigate the expression of the e1-a2 *bcr-abl* junction in P190$^{bcr-abl}$ in cell lines and in leukemic cells of a Ph'-positive ALL patient. Our data also indicate that the *bcr-abl* junction in P190$^{bcr-abl}$ can be recognized by antibodies. These experiments indicate that the tumor-specific joining regions in the *bcr-abl* fusion proteins are new immunologic markers for CML and ALL diagnosis.

MATERIALS AND METHODS

Cell Lines

TOM-1 is a cell line derived from a Ph'-positive ALL patient.[19] The cell line was kindly provided by Dr M. Okabe (Sapparo, Japan). K562 and BV173 are cell lines derived from patients during blast crisis of CML.[20,21] Cells were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 μg/mL penicillin, and 60 μg/mL streptomycin.

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Patients

Patient L. is a 52-year-old and patient K. is a 44-year-old male ALL patient. Both patients exhibited a standard Philadelphia translocation, t(9,22)(q34;q11). In both patients the diagnosis precursor B-ALL was based on immunologic and hematologic criteria obtained at presentation. Blast cells were isolated from peripheral blood by Ficoll-Hypaque centrifugation. After cryopreservation using a controlled freezing apparatus (Planer Biomed, Sunbury-on-Thames, UK), the cells were stored under liquid nitrogen.

Peptide Synthesis, Purification, and Conjugation

Peptides were synthesized using solid-phase synthesis as described previously.[18] Purification of the peptides was performed by gel filtration and reverse phase high performance liquid chromatography. A cysteine residue was added to the amino terminus during synthesis. Peptides were crosslinked through free sulfhydryl groups to the carrier protein chicken γ globulin (CGG) using m-maleimidobenzoyl-sulfosuccinimide-ester (MBS).[22]

Immunizations, Antisera, and Purification of Immunoglobulins (Igs)

Antiserum BP-ALL. Flemish rabbits (MBL-TNO, Rijswijk, The Netherlands) were primed intracutaneously with 250 μg protein complex consisting of peptide e1-a2 coupled via MBS to CGG, emulsified in an equal volume of complete freund adjuvans (CFA). Rabbits were boosted twice after intervals of 4 weeks; the first time with 250 μg of protein in CFA, the second time with the same dose in incomplete freund adjuvans. Fourteen days after the last boost, rabbits were bled and sera were collected.

*Anti-*bcr *antiserum.* Anti-*bcr* antiserum is a polyclonal rabbit antiserum directed against the amino terminal part of the *bcr* protein. This antiserum was prepared as described previously.[18]

The Ig fraction of both antisera was purified by precipitation with 16% Na_2SO_4 (final concentration). The precipitates were dissolved in phosphate-buffered saline and desalted using Sephadex G-25M columns (Pharmacia, Uppsala, Sweden).

Immunoprecipitation and Protein Kinase Reaction

Immunoprecipitation and protein kinase reaction were performed as described previously,[18] except that, in the present study, the tyrosine kinase reaction was performed for 10 minutes at 30°C instead of 10 minutes at 37°C.

Amplification of cDNA by the Polymerase Chain Reaction (PCR) Method

Total RNA was extracted according to the LiCl method,[23] followed by cDNA synthesis by reverse transcriptase. The cDNA amplification was performed using the PCR as described previously.[5] For the amplification, *bcr* primers from exon e1 and b2 and an *abl* primer from exon a3 were used. In Fig 1 the primers on the respective cDNA molecules are shown. Twenty-four cycles of the PCR were performed using *Taq* polymerase. The samples were electrophorized, and transferred to nylon filter (zeta probe). Filters were hybridized to ^{32}P end-labeled oligonucleotides, spanning the e1-a2, and b2-a2 b3-a2 junctions.[6] After hybridization the blots were washed and exposed to Fuji XR films (Tokyo, Japan).

RESULTS

Peptides Corresponding to the bcr-abl *Junction in P190$^{bcr\text{-}abl}$*

Based on the previously published nucleotide sequence,[5] a peptide was synthesized corresponding to the *bcr-abl* junction in P190$^{bcr\text{-}abl}$. The peptide sequence was chosen in such a way that a short peptide, probably one antigenic determinant, with optimal hydrophilicity pattern was selected. This peptide, termed SP e1-a2, consists of the following amino acids:

cys-ala-phe-his-gly-asp-ala-glu-ala-leu-gln

e1 a2

As a control peptide b3-a2 (SP b3-a2) was constructed corresponding to the b3-a2 joining region in the CML-specific protein P210$^{bcr\text{-}abl}$. SP b3-a2 has the following amino acid sequence:

cys-lys-gln-ser-ser-lys-ala-leu-gln-arg-pro

b3 a2

Amino-terminal cysteine residues were added to both peptides to provide a coupling site for carrier molecules.

Because fusion of the *bcr* and *abl* genes in both P190$^{bcr\text{-}abl}$ and P210$^{bcr\text{-}abl}$ occurs in a coding triplet, the glu in SP e1-a2 and the second lys in SP b3-a2 are newly generated by the translocation process.[5,12] Figure 1 schematically shows the respective chimeric *bcr-abl* mRNAs and proteins as occurring in ALL and CML. All fusion proteins share the *abl* carboxy terminal amino acids, but differ at the amino terminal *bcr* sequence. As a consequence the synthetic peptides also differ at their amino terminus and have the same *abl* terminal amino acids.

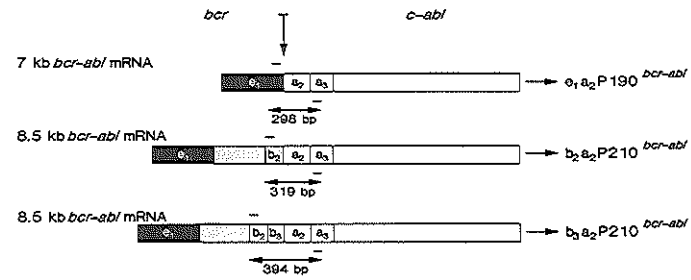

Fig 1. Schematic representation of the chimeric *bcr-abl* mRNAs and proteins. The small horizontal bars indicate the localization of the primers used in the PCR. The vertical arrow indicates the *bcr-abl* fusion point.

cDNA Analysis of the bcr-abl *Joining in TOM-1 Cells*

TOM-1 cells were used as a source of $P190^{bcr\text{-}abl}$ protein. First, presence of $P190^{bcr\text{-}abl}$ mRNA comprising the e1-a2 *bcr-abl* junction in our TOM-1 cells had to be confirmed. To this purpose the highly sensitive PCR technique was performed. The *bcr-abl* cDNA was amplified and hybridized to oligonucleotides specific for the e1-a2, b2-a2, and the b3-a2 joining in the chimeric cDNA.

As shown in Fig 2, a 298-base pair (bp) fragment, specific for the e1-a2 joining, was amplified in the TOM-1 cells (lane 3, e1-a2 probe). As expected, this fragment was not amplified in both CML cell lines K562 and BV173. However, a 394-bp fragment specific for the b3-a2 joining was found to be present after amplification in K562 cells (Fig 2, lane 1, b3-a2 probe), while a 319-bp fragment, specific for a b2-a2 joining, was detected in BV173 cells (Fig 2, lane 2, b2-a2 probe).

Antibody Binding to bcr-abl *Chimeric Proteins*

Antiserum BP-ALL was raised against SP e1-a2. Reactivity of BP-ALL with native *bcr-abl* chimeric proteins $P190^{bcr\text{-}abl}$ and $P210^{bcr\text{-}abl}$ in different cell lines was tested with an immunoprecipitation assay followed by an autophosphorylation reaction. As shown in Fig 2, TOM-1 cells contain mRNA comprising the e1-a2 *bcr-abl* junction. However, to confirm that this mRNA was indeed translated into protein, we searched for the presence of $P190^{bcr\text{-}abl}$ in TOM-1 cells. Fig 3 shows that $P190^{bcr\text{-}abl}$ can be precipitated from these cells using an antiserum directed against the amino terminal side of the *bcr* protein (lane 2). Two CML cell lines, K562 and BV173, showed presence of $P210^{bcr\text{-}abl}$ after immunoprecipitation with the same anti-*bcr* antiserum (Fig 3, lanes 4 and 6).

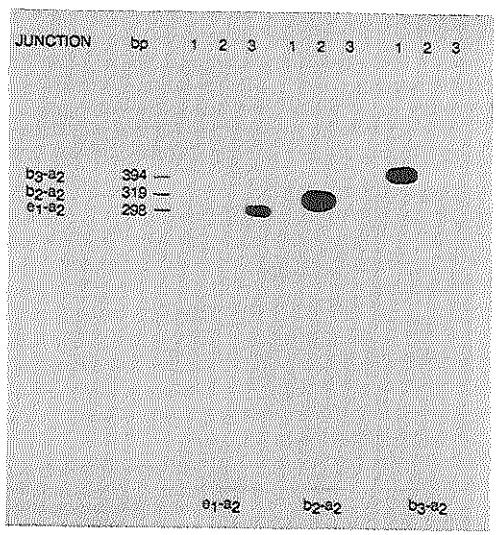

Fig 2. Amplification of the *bcr-abl* junction from K562 (lanes 1), BV173 (lanes 2), and TOM-1 (lanes 3) cDNA. Hybridization was performed to oligonucleotides spanning the e1-a2, b2-a2, and b3-a2 *bcr-abl* junction.

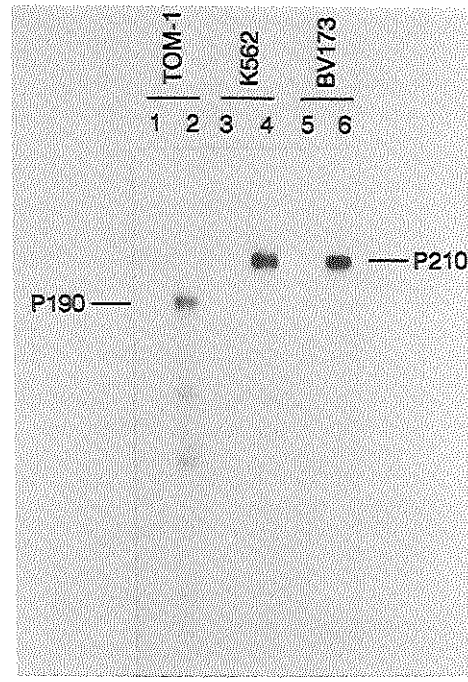

Fig 3. Immunoprecipitation analysis of *bcr-abl* chimeric proteins from TOM-1, K562, and BV173 cells with a polyclonal antiserum directed against the amino-terminus of the *bcr* protein. The immunoprecipitation was followed by autophosphorylation of the proteins. The cells were lysed and proteins were precipitated with 25 μL Na_2SO_4-precipitated normal rabbit serum (lanes 1, 3, and 5), and with 25 μL Na_2SO_4-precipitated anti-*bcr* antiserum (lanes 2, 4, and 6).

Next, we tested the reactivity of antiserum BP-ALL with $P190^{bcr\text{-}abl}$. Incubation of BP-ALL with a lysate of TOM-1 cells resulted in a clear precipitation of $P190^{bcr\text{-}abl}$ (Fig 4, lane 1). In contrast, BP-ALL did not precipitate $P210^{bcr\text{-}abl}$ from either K562 or BV173 (Fig 4, lanes 2 and 3). These results imply that antiserum BP-ALL specifically recognizes the e1-a2 junction domain in $P190^{bcr\text{-}abl}$.

We performed peptide blocking studies to confirm this notion. We first incubated BP-ALL with cognate peptide SP e1-a2 or, as a control, with a peptide corresponding to the b3-a2 *bcr-abl* junction. Next, TOM-1 lysate was added. As shown in Fig 5, preincubation of BP-ALL with cognate peptide SP e1-a2 prevented precipitation of $P190^{bcr\text{-}abl}$ completely (lane 2). However, precipitation of background bands was not inhibited. Preincubation of BP-ALL with SP b3-a2, absorbing antibodies directed against a2, had no effect on the immunoprecipitation (lane 4). This observation strengthens the notion that $P190^{bcr\text{-}abl}$ is precipitated by antibodies in

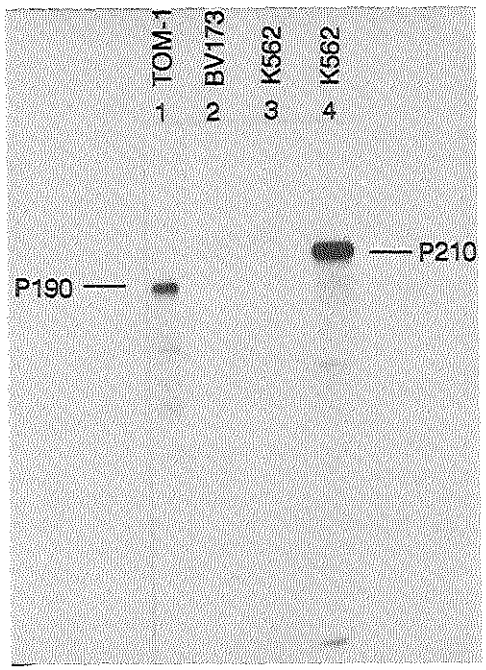

Fig 4. Immunoprecipitation analysis of the specificity of antiserum BP-ALL. TOM-1, BV173, and K562 cells were lysed and proteins were precipitated with 25 μL Na_2SO_4 precipitated serum BP-ALL (lanes 1, 2, and 3). In lane 4 proteins from a lysate of K562 cells were precipitated with 25 μL Na_2SO_4-precipitated anti-*bcr* antiserum. The immunoprecipitation was followed by autophosphorylation of the proteins.

serum BP-ALL that are specifically directed against the e1-a2 junction.

Precipitation of P190$^{bcr-abl}$ From Leukemic Cells of a Ph'-Positive ALL Patient

Finally, we investigated whether antiserum BP-ALL precipitated P190$^{bcr-abl}$ from leukemic cells from a Ph'-positive ALL patient. Peripheral blood leukocytes of patient L. were enriched by Ficoll-Hypaque centrifugation. Next, blast cells were lysed and incubated with BP-ALL. The immunoprecipitation was followed by the autophosphorylation assay. Figure 6 shows a clear precipitation of P190$^{bcr-abl}$ from patient L (lane 2). Moreover, analogous to the chimeric proteins in the cell lines, the P210$^{bcr-abl}$ protein from patient K was not recognized by BP-ALL (Fig 7, lane 3). This indicates that the e1-a2 joining region also in leukemic cells of a Ph'-positive patient antigenically is exposed on the P190$^{bcr-abl}$ protein.

DISCUSSION

Chromosomal abnormalities in ALL are frequently observed and have prognostic significance.[24] Particularly, the presence of the Philadelphia chromosome in ALL is of

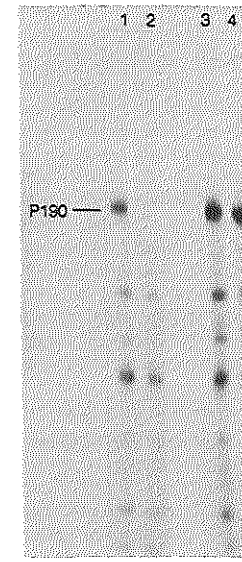

Fig 5. Immunoprecipitation analysis of TOM-1 cells with 25 μL Na_2SO_4-precipitated antiserum BP-ALL (lanes 1 and 3), after preincubation of BP-ALL with 0.25 mmol/L SP e1-a2 (lane 2) and after preincubation with 0.25 mmol/L SP b3-a2 (lane 4).

clinical importance. Ph'-positive ALL is associated with a lower remission rate and an overall worse prognosis than Ph'-negative ALL.[4,25] Especially in childhood ALL the Ph' chromosome implicates a bad prognosis.[25] It is obvious that for clinical diagnosis antibodies directed against the tumor-specific *bcr-abl* joining will be useful tools.

Recently we described the development of a polyclonal antiserum directed against the b2-a2 *bcr-abl* junction in P210$^{bcr-abl}$.[18] Now we report on the production of an antiserum, BP-ALL, specifically recognizing the e1-a2 *bcr-abl* joining region in P190$^{bcr-abl}$. The specificity of the polyclonal antiserum, raised against a synthetic peptide corresponding to the e1-a2 junction, was tested in an immunoprecipitation assay, followed by an autophosphorylation. TOM-1 cells

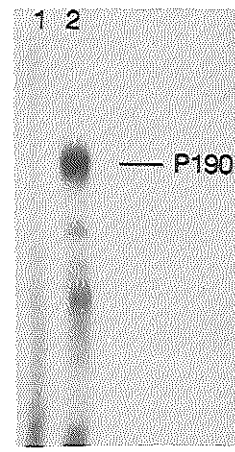

Fig 6. Immunoprecipitation analysis of Ficoll-Hypaque–enriched peripheral blood cells of ALL patient L. Cells were lysed and proteins were precipitated with 25 μL Na_2SO_4-precipitated normal rabbit serum (lane 1), and with 25 μL Na_2SO_4-precipitated serum BP-ALL (lane 2).

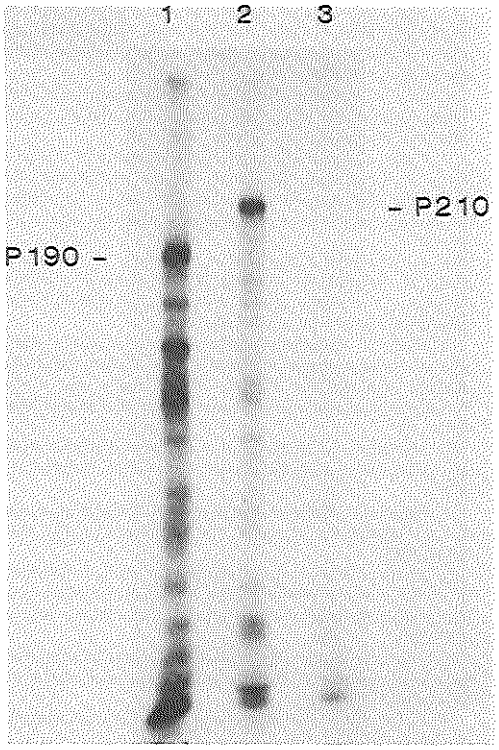

Fig 7. Immunoprecipitation analysis of Ficoll-Hypaque–enriched peripheral blood cells of ALL patient K. (lanes 2 and 3). Cells were lysed and proteins were precipitated with 25 μL Na_2SO_4-precipitated anti-*bcr* antiserum (lane 2), and with 25 μL Na_2SO_4-precipitated serum BP-ALL (lane 3). As a control, TOM-1 cells in lane 1 were precipitated with 25 μL Na_2SO_4-precipitated BP-ALL.

were used as a source of Ph'-positive cells. These cells are derived from a Ph'-positive patient by Okabe et al.[19] Since the exact *bcr-abl* rearrangement in TOM-1 was not described, we performed the highly sensitive PCR technique to verify which *bcr-abl* junction occurred in TOM-1 cells. In these experiments we amplified cDNA from TOM-1 cells comprising the e1-a2 *bcr-abl* junction, indicating a breakpoint in the m-*bcr*-1. Therefore, presence of $P190^{bcr\text{-}abl}$ in these cells could be expected. We confirmed this notion by immunoprecipitation of $P190^{bcr\text{-}abl}$ from TOM-1 cells using an antiserum directed against the amino-terminus of the *bcr* gene.

We subsequently showed that antiserum BP-ALL, raised against a peptide corresponding to the e1-a2 junction, reacted in a highly specific way with the native protein $P190^{bcr\text{-}abl}$ in TOM-1 cells. We argue that $P190^{bcr\text{-}abl}$ is precipitated by antibodies in BP-ALL that specifically recognize the e1-a2 junction. Antibodies reacting with e1 and/or a2 sequences only do not play a role, since no other *bcr-abl* chimeric proteins, b2-a2 and b3-a2 $P210^{bcr\text{-}abl}$, which also contain e1 and a2 sequences, were immunoprecipitated by BP-ALL. Moreover, the reaction of BP-ALL with $P190^{bcr\text{-}abl}$ was specifically inhibited by blocking with cognate peptide. Thus, precipitation of $P190^{bcr\text{-}abl}$ was completely abrogated after preincubation of BP-ALL with peptide e1-a2. Preincubation with a related peptide SP b3-a2, which eliminates antibodies from BP-ALL directed against a2 sequences, had no effect on the immunoprecipitation of $P190^{bcr\text{-}abl}$. These observations provide strong evidence that the tumor-specific *bcr-abl* junction e1-a2 is expressed in an antigenic way on the $P190^{bcr\text{-}abl}$ molecule.

The e1-a2 joining region in $P190^{bcr\text{-}abl}$ in leukemic cells of a Ph'-positive ALL patient is similarly exposed as in the TOM-1 cell line. This observation confirms the idea that the e1-a2 epitope can be used as an immunologic marker for Ph'-positive ALL.

The data presented in this report have implications for ALL diagnosis and classification. Here we have demonstrated the antigenic expression of the tumor-specific e1-a2 *bcr-abl* junction in $P190^{bcr\text{-}abl}$. In general, antibodies directed against this determinant will be valuable tools in ALL diagnosis and detection of minimal residual disease after clinical treatment, such as chemotherapy and bone marrow transplantation. However, some Ph'-positive ALL patients express $P210^{bcr\text{-}abl}$, just as in CML. It has been suggested that these patients are, de facto, CML patients in lymphoid blast crisis without or with a very short chronic phase.[9] Ph'-positive ALL patients expressing $P190^{bcr\text{-}abl}$ would then be de novo ALL patients. Without necessarily subscribing to this hypothesis, it is of interest to investigate whether Ph'-positive ALL with $P190^{bcr\text{-}abl}$ differ from the cases of Ph'-positive ALL expressing $P210^{bcr\text{-}abl}$ in terms of clinical features like prognosis, response to therapy, and survival. To this purpose a large group of ALL patients has to be studied. The development of well-defined reagents for immunocytochemistry, such as monoclonal antibodies directed against the tumor-specific *bcr-abl* junction in $P190^{bcr\text{-}abl}$ and $P210^{bcr\text{-}abl}$, is therefore invaluable for accurate diagnosis of ALL and CML.

ACKNOWLEDGMENT

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CHAPTER 4

MOLECULAR DIAGNOSIS OF PH POSITIVE CML

4.1 MOLECULAR INVESTIGATIONS OF PH POSITIVE CML

The breakpoints in chromosome 22, caused by the Ph translocation, are clustered in a region of 5.8 Kb, the breakpoint cluster region (BCR) (Groffen et al, 1984). Detection of BCR breakpoints can be performed by Southern blotting followed by hybridization with BCR specific probes.
As alternative for DNA analysis, *bcr-abl* mRNA expression can be analyzed by PCR analysis followed by hybridization to oligonucleotides that specifically recognize the various *bcr-abl* junction regions.
Molecular analysis is not routinely performed in Ph positive CML patients, because the Ph chromosome itself can be used as tumor specific marker in these patients.
During the past four years we performed Southern blot analysis and if necessary also PCR and / or PFGE in a selected group of Ph positive CML patients.
These cases were molecularly analyzed for the following reasons: Unusual cytogenetic findings, clinical evaluation of a new BCR probe (paper included in the appendix), determination of the BCR breakpoint localisation or of the type of *bcr-abl* mRNA expressed prior to interferon treatment. The results of these molecular investigations are reported and discussed in this chapter.

MATERIAL AND METHODS

Samples:
Bone marrow aspirates and blood samples were sterile and heparinized. All sampling was part of diagnostic and clinical follow up procedures and obtained only after informed consent of the patients. Some patients were referred to us from other laboratories in the Netherlands or from abroad.

Chromosome analysis:
Following established procedures blood and bone marrow cells are cultured without stimulation (Hagemeijer et al, 1979), and harvested. Chromosomes are identified using R and or G banding techniques and classified according to ISCN (1985). The constitutional karyotype is determined using PHA stimulated blood cultures.

Southern blot analysis
This technique is applied to demonstrate a breakpoint in the BCR region of the *bcr* gene (Groffen et al,1984; Manniatis et al, 1982). DNA is extracted from blood and bone marrow cells, digested with restriction enzymes, electrophoresed through an agarose gel, and blotted onto nylon membrane, followed by hybridisation to ^{32}P labelled BCR specific probes.
The following BCR probes are used (fig.1):
5'-BCR probe: 2 Kb BglII-HindIII fragment.
3'-BCR probe: 1.2 Kb HindIII-BglII fragment.
universal BCR probe: approximately 4 Kb SalI-BamHI fragment covering the whole BCR region, except for a HindIII-HindIII fragment, that is deleted from the probe, because it contains repetitive sequences (Blennerhasset et al, 1988, see appendix).
Autoradiograms are made. Detection of aberrant sized restriction fragments mark the presence of a breakpoint in the BCR region. In t(9;22) the 5'-BCR probe recognizes the Ph chromosome, while the 3'-BCR probe recognizes the 9q+ chromosome.

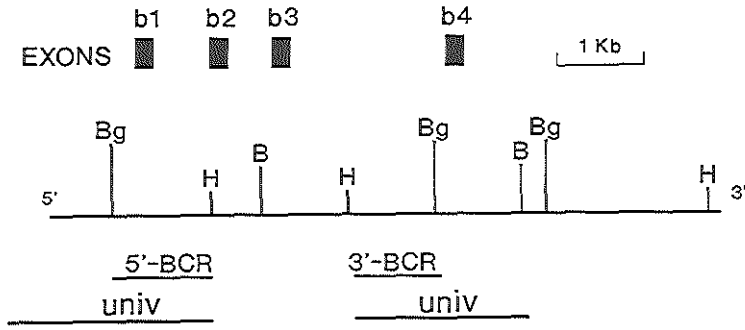

Figure 1. Simplified restriction map of the BCR region of the *bcr* gene. Bg=BglII, B=BamHI and H=HindIII. The boxes represent exons.
5'-BCR is a 2.0 Kb BglII-HindIII fragment, 3'-BCR is a 1.2 Kb HindIII-BglII fragment and univ is an approximately 5 Kb SalI- BamHI fragment from which a 1.2 Kb HindIII fragment is deleted, because it contained repetitive sequences. These fragments are used as probes in Southern blot analysis in CML.

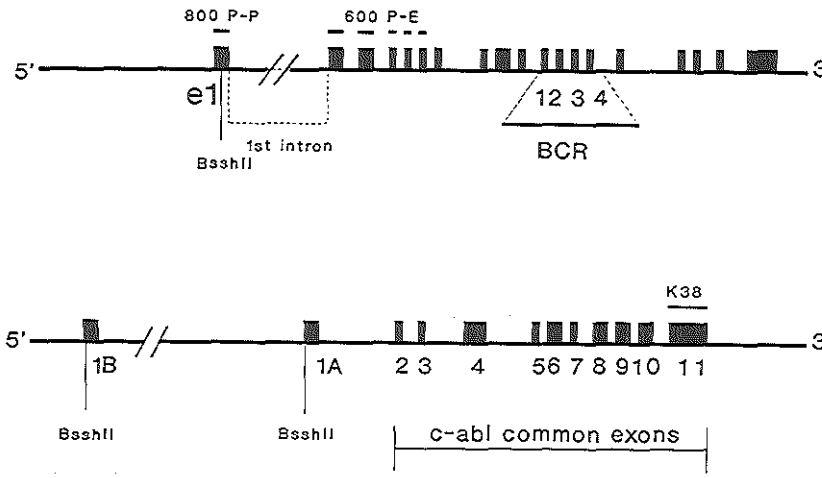

Figure 2. Simplified map of the *bcr* (top) and *abl* (bottom) genes. The localization of the *bcr* probes that are used in pulsed field gel electrophoresis (PFGE) are represented by 800 P-P and 600 P-E. The restriction sites of BsshII, the restriction enzyme most frequently used by us in PFGE, are indicated.

Pulsed field gel electrophoresis (PFGE):
PFGE is performed when the breakpoints are expected to be scattered over a large region e.g. to search for breakpoints in the *bcr* or *abl* gene. Blood or bone marrow cells are mixed with low melting point agarose, poured in a mould in order to form plugs. Thereafter the cells in the plugs are lysed, treated with proteinase K, and high molecular weight unsheared DNA is obtained. After digestion with rare cutting restriction enzymes, the plugs are placed in the slots of a 1% agarose gel, and pulsed field gel electrophoresis is carried out. After blotting onto nylon membrane and hybridization to specific probes autoradiograms are made. The following probes were used:
800 bp PstI-PstI cDNA probe covering the first exon of the *bcr* gene (e1).
600 bp PstI-EcoRI cDNA probe corresponding to coding sequences of the second exon (e2) and the adjacent four small exons of the *bcr* gene, that are all located 5' of the BCR region.
1.7 Kb *abl* cDNA probe (K38) corresponding to the 3' end of the human *c-abl* cDNA.
For maps of the *bcr* and *abl* gene see fig. 2. Detection of aberrantly sized restriction fragments mark the presence of a breakpoint in the gene investigated. Using this technique fragments varying in size from 4-1200 Kb can be detected (Chu et al, 1986).

Polymerase Chain Reaction (PCR):
The PCR is performed to detect *bcr-abl* mRNA.
Total RNA is extracted from blood or bone marrow cells (Auffray et al, 1980).
cDNA preparation and amplification with the PCR are performed as described by Hermans et al (Hermans et al, 1988). The primers used in the PCR reaction are schematically drawn in Fig.3. In short: using a sense primer containing b1b2 sequences (primer2) and an antisense primer containing a3 sequences (primer 1) a fragment is amplified covering the *bcr-abl* junction region. Usually we also add an antisense primer containing e1 sequences. This has the advantage that in case in a CML patient an e1a2 mRNA is expressed, this can be detected in the same PCR analysis. As an internal positive control, half of the cDNA product is used to amplify an *abl* fragment, that should always be present irrespective of the Ph translocation. To amplify this *abl* fragment a sense primer containing a2 sequences (primer 4) and an antisense primer containing a3 sequences (primer 1) are used. The amplified fragments are electrophoresed through a 2% agarose gel, blotted onto nylon membrane and hybridised to two ^{32}P labelled oligonucleotides, that specifically recognize the bcr-abl fusion region b2a2, b3a2 or e1a2 in the amplified fragment.
With the PCR technique as few as 1 Ph positive cell in 100,000 normal cells can be detected.

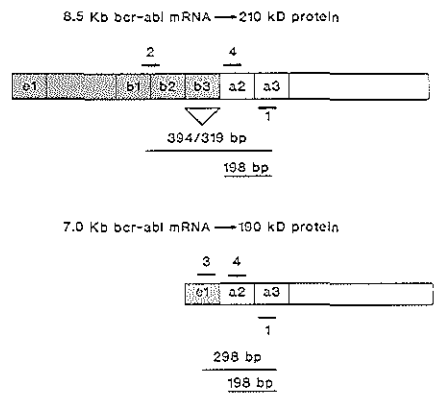

Figure 3. Schematic representation of the 8.5 Kb *bcr-abl* mRNA. The localisation of the *bcr* and *abl*primers is shown. Using primer 1 and 2 a fragment of 394 or 319 bp is amplified, depending on the presence or absence of exon b3 in the mRNA. These fragments have a b3a2 and a b2a2 junction respectively. Using primer 1 and 3 a fragment of 298 bp is amplified with an e1a2 junction. Primers 1 and 4 are used as an internal control to amplify an *abl* fragment of 198 bp.

RESULTS AND DISCUSSION

Screening Ph positive CML patients for BCR breakpoints

In addition to the Ph positive CML patients, that are included in the multicentre trial reported by Blennerhasset et al (see appendix), another 46 were analyzed using Southern blotting. The results are summarised in table 1.
We expected to find a BCR breakpoint in all Ph positive CML patients. However, in one patient (table 2 patient 8), with a standard Ph translocation and an isodicentric Ph chromosome, no BCR breakpoint was detected. In another patient (table 2 patient 9, indicated by BCR?), an aberrant restriction fragment was present in one out of three restriction enzyme digests, which can be the result of a restriction enzyme polymorphism or a BCR breakpoint. In these 2 patients additional analysis was required using the PCR technique or Pulsed Field Gel Electrophoresis. Furthermore another 10 patients were selected for additional PCR analysis out of the initial 46 Ph positive patients (table 2).
In all cases the type of *bcr-abl* junction identified by PCR analysis corresponded with the localization of the BCR breakpoint as determined by Southern blotting. Moreover the PCR experiments elucidated several unexpected findings.

Table 1: Results of Southern blot analysis in 46 Ph positive CML patients.

Number of cases	*Karyotype*	*Southern blot*
36	standard Ph	BCR+
1	standard Ph, i(Ph)	BCR-
1	standard Ph	BCR?
8	variant Ph	BCR+

Table 2: Results of molecular analysis in 12 Ph positive CML patients.

Patient	*Karyotype*	*Southern blot*	*PCR*	*PFGE*
1	t(9;22)	BCR+	b2a2	
2	t(9;22)	BCR+	b2a2	
3	t(9;22)	BCR+	b2a2	
4	t(9;22)	BCR+	b2a2	
5	t(9;22)	BCR+	b2a2	
6	t(9;22)	BCR+	b2a2	
7	t(9;22)	nv	b3a2	
8	t(9;22), i(Ph)	BCR-	e1a2	abl+,bcr?
9	t(9;22)	BCR?	b2a3	abl+,bcr+
10	t(9;22)	BCR+	b2a2+b3a2	
11	t(9;22)	BCR+	b2a2+b3a2	
12	t(9;22)	BCR+	b2a2+b3a2	

1. Breakpoint on chromosome 22 outside BCR

In nearly all CML patients the breakpoints on chromosome 22 are found in the 5.8 Kb BCR region of the *bcr* gene. However, Southern blot analysis failed to detect a breakpoint in the BCR region in 1 out of 46 Ph positive CML patients. In this patient whose karyotype shows t(9;22) in a minority of cells, and an iso Ph chromosome in 1-4 copies per cell (fig.4), the breakpoint on chromosome 22 must be located outside the BCR region (Table 2, patient 8). Using PFGE we searched for a breakpoint in the *bcr* and *abl* genes. An aberrant restriction fragment was present in the DNA digested with the enzyme BsshII after hybridization with *abl* probe (K38), indicating a possible breakpoint in the *abl* gene (data not shown). Unfortunately we could not prove the chimeric *bcr-abl* origin of this fragment, because the size of the aberrant fragment detected by K38 *abl* probe was the same as the germ line band detected after hybridization with *bcr* probes. Therefore the PFGE was not conclusive in this patient. To solve this problem PCR analysis of bone marrow of this patient (Table 2, patient 8) was performed, showing expression of *bcr-abl* mRNA with an e1a2 junction i.e. the first exon of the *bcr* gene (e1) is joined to the second exon (a2) of the *abl* gene (Fig 5). This type of mRNA is very unusual in CML, but is frequently found in Ph positive AML and ALL (see Hermans et al, 1987 and chapter 3 and 7 of this thesis). As the karyotype of this patient showed both a Ph chromosome and an iso Ph chromosome in varying numbers, we do not know if the e1a2 *bcr-abl* mRNA originates from the Ph or the iso Ph. An attempt to answer this question by in situ hybridization was not successful.

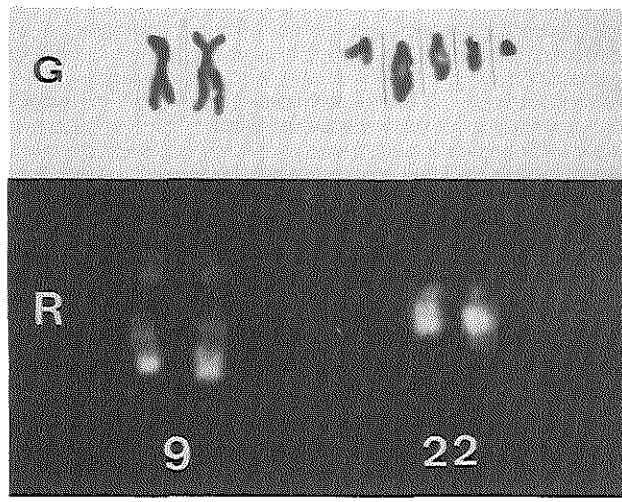

Figure 4. Partial karyotype of patient 8 (table 2).
Top: partial karyotype using G banding. From left to right are visible: normal chromosome 9, 9q+, normal 22, i(Ph), i(Ph), i(Ph), and 22q-.
Bottom: partial karyotype of another metaphase using R banding. From left to right: normal chromosome 9, 9q+, normal 22, i(Ph).

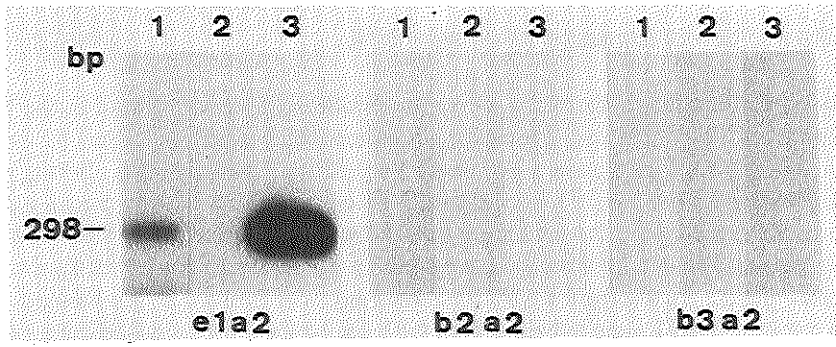

Figure 5. Autoradiogram of PCR analysis after hybridization to oligonucleotides that specifically recognize the *bcr-abl* junctions. Lane 1: patient 8 (table 2), lane 2: normal leucocytes, and lane 3: bone marrow of a Ph positive ALL patient as positive control. Hybridization with breakpoint specific oligonucleotides demonstrates expression of *bcr-abl* mRNA with an e1a2 junction in lane 1 and 3.

Table 3: Summary of reported cases of Ph positive CML with chromosome 22 breakpoint outside BCR region. The breakpoint localization in *bcr* gene and corresponding mRNA are indicated.

No of pat	*Southern/PFGE*	*PCR/Northern blot*	*Reference*
1	1st intron and BCR region.	8 Kb mRNA	Bartram et al, 1987
1	BCR-	no bcr-abl mRNA	Selleri et al, 1987
2	9-12 Kb 5' of BCR		Saglio et al, 1988
4	BCR-	1 normal abl mRNA, others not tested.	Saglio et al, 1988
2	20 Kb 3' of BCR	c_3a_2 bcr-abl mRNA, 9 Kb bcr-abl mRNA	Saglio et al, 1990
1	320 Kb 3'of central bcr sequences	b_2a_2 bcr-abl mRNA	Min et al, 1990
1	1st intron bcr	e_1a_2 bcr-abl mRNA	Selleri et al, 1990

A limited number of CML patients with a breakpoint outside BCR has been reported by other investigators and is summarized in table 3.

From the available data it can be concluded that:
1. Breakpoints outside the BCR region are very rare in CML.
2. These breakpoints can be located either 3' or 5' of the BCR region.
3. The generation of a *bcr-abl* fusion gene, consisting of *5'-bcr* and *3'-abl* sequences was observed in all CML patients, and therefore is likely to play an important role in

leukemogenesis of CML.
4. A special case is reported by Min et al (Min et al, 1990): the breakpoint on chromosome 22 is located 320 Kb downstream of the BCR region, and an unusual splicing mechanism results in expression of b2a2 mRNA.
5. The reported CML patients with chromosome 22 breakpoints outside BCR were clinically indistinguishable from other Ph positive CML patients, but more patients have to be investigated to determine the clinical and prognostic significance of this finding.

2. Simultaneous expression of two different *bcr-abl* mRNAs

PCR analysis revealed simultaneous expression of *bcr-abl* mRNA with b2a2 and b3a2 junctions in 3 out of 12 Ph positive CML patients investigated (Table 2, patient 10,11,12). The autoradiogram of the PCR analysis of two of them is presented in figure 6. In lane 1 and 2 of the left panel a faint additional fragment of 394 bp is visible, that hybridizes to the b2a2 oligonucleotide. This fragment is almost certainly a heteroduplex molecule consisting of one strand of DNA with a b2a2 and another one with a b3a2 junction between *bcr* and *abl*. This was confirmed by additional control experiments in which K562 and BV173 RNA or cDNA were mixed respectively before and after the PCR reaction. Only in the samples that were mixed before PCR amplification we detected the 394 bp fragment which hybridized to the b2a2 specific oligonucleotide).
In one of the three patients the BCR breakpoint was mapped between exon b3 and b4 using Southern blotting, and in the same fragment (between b3 and b4) or 150 bp more 5'(between b2 and b3) in the two other patients (data not shown).
Two explanations can account for these results:
1. The BCR breakpoint is located between exon b3 and b4, and alternative splicing results in expression of both b2a2 and b3a2 mRNA.
2. A mixed population of Ph chromosomes is present in these patients: The main population, that is also detected by Southern blotting, has a breakpoint between exon b3 and b4 and results in expression of b3a2 mRNA, whereas a small population, that is below the detection level of Southern blotting, has a breakpoint between exon b2 and b3, resulting in expression of b2a2 mRNA.
With the techniques available in our lab we can not prove which of the two explanations is correct. A staining technique with antibodies, that specifically recognise b2a2 and b3a2 junctions in the bcr-abl proteins on single cell level would be very useful to solve this problem, but is not yet available.
Simultaneous expression of two *bcr-abl* mRNAs detected by PCR analysis has also been reported by other investigators (e.g. Dobrovic et al, 1988; Marcella et al, 1989; Lee et al, 1989). The clinical significance of this finding is as yet unclear. The few cases reported did not differ clinically from other Ph positive CML cases.
In conclusion:
The PCR technique has proven to be a very useful and reliable technique for screening CML patients for the presence of *bcr-abl* mRNA expression. Besides the PCR

technique S1 protection assays (Dreazen et al, 1988) and RNAse assays in combination with antisense RNA probes (Shtivelman, 1986, 1987) have previously been applied for this purpose. The pitfalls of the latter two techniques have been demonstrated by Hermans et al (Hermans et al, 1988). The alternative splicing in the CML blast crisis cell line K562 reported by Shtivelman et al (1986, 1987) was due to an artefact in the RNAse protection assay. Using PCR analysis only b3a2 mRNA expression was detected in the cell line K562.

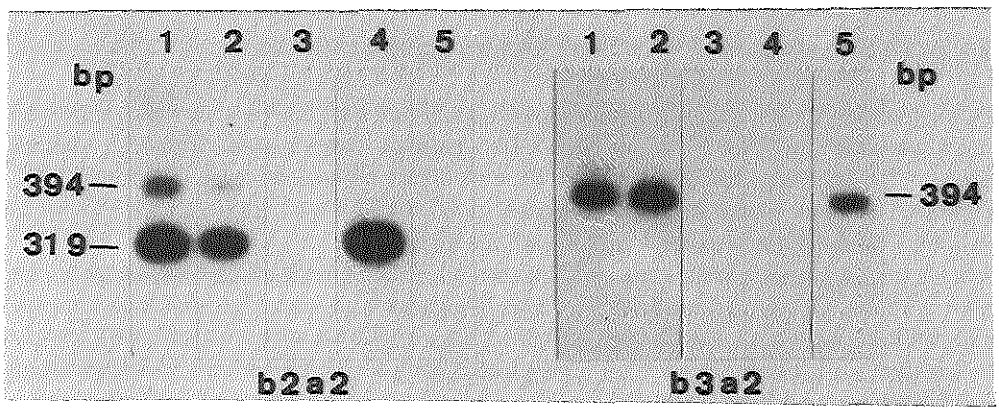

Figure 6. Autoradiogram of the PCR experiment of patient 10 and 11 from table 2 and positive and negative control samples. Left panel: hybridization with b2a2 specific oligonucleotides. Right panel: hybridization with b3a2 specific oligonucleotides.
Lane 1: patient 10, lane 2: patient 11, lane 3: normal leucocytes, lane 4: cell line BV173, and lane 5: cell line K562.
The patients in lane 1 and 2 show expression of b2a2 and b3a2 mRNA. For explanation of the double hybridization signal in lane 1 and 2 of the left panel see text.

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4.2 BCR-ABL mRNA LACKING ABL EXON A2 DETECTED BY POLYMERASE CHAIN REACTION IN A CML PATIENT

Leukemia 1991, 5:457-461

bcr-abl mRNA Lacking *abl* Exon A2 Detected by Polymerase Chain Reaction in a Chronic Myelogeneous Leukemia Patient

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Using the polymerase chain reaction and Southern blot analysis the expression was detected of a *bcr-abl* mRNA lacking *abl* exon a2. This was due to a corresponding unusual localization of the breakpoint in the *c-abl* gene and was seen in a patient with Philadelphia (Ph) chromosome positive chronic myelogeneous leukemia in chronic phase. This type of mRNA has been described only once before in two Ph-positive acute lymphoblastic leukemia patients, by Soekarman *et al.* (1). The *abl* exon a2 sequences, which are missing in the three reported patients, code for a part of the SH3 region of the abl protein, which is supposed to be involved in negative regulation of the kinase domain. The clinical significance of this finding is discussed.

INTRODUCTION

Chronic myelogenous leukemia (CML) is cytogenetically characterized by the presence of the Philadelphia (Ph) chromosome, which originates from the Ph translocation t(9;22) (q34;q11) (2).

On chromosome 22 the breakpoint is located in band q11 in the *bcr* gene. In CML the breakpoints in the *bcr* gene are clustered in a region of 5.8 kb, the breakpoint cluster region (BCR) (3). The BCR region is part of a gene, the *bcr* gene, and contains four exons (b1–b4) (4). In nearly all CML patients the chromosome 22 breakpoint is located between exons b2 and b3, or between b3 and b4.

On chromosome 9 the breakpoints are scattered over a region of more than 200 kb in the c–*abl* gene, either between exons 1B and 1A, or between 1A and the second exon of c-*abl*, which is referred to as *abl* exon a2 in this paper (Figure 1) (5). However, the breakpoint is always located 5′ of c-*abl* exon a2. The *bcr-abl* fusion gene on the Ph chromosome is transcribed into a *bcr-abl* mRNA, in which either BCR exon b2 or b3 is fused to *abl* exon a2 (6,7). These chimeric mRNAs encode bcr-abl fusion proteins of 210 kD (p210), which have enhanced *in vitro* tyrosine kinase activity as compared to the normal abl protein (8) and have transforming activity. In Abelson murine leukemia virus (AMLV) the transforming potential of v–abl is dependent on the tyrosine kinase activity (9). Therefore regulation of the tyrosine kinase activity of p210 bcr-abl is thought to play an important role in the leukemogenesis of CML.

The Ph translocation is also observed in acute lymphoblastic leukemia (ALL) and acute myeloblastic leukemia (AML), although less frequently than in CML. The breakpoint in the *abl* gene always occurs at the same position as in CML, i.e. 5′ of *abl* exon a2. In 50% of the Ph-positive acute leukemias the breakpoint in *bcr* is also identical to that observed in CML, resulting in the same *bcr-abl* products as are observed in CML. In the remaining 50% the breakpoint in the *bcr* gene is located more 5′, i.e. in the first intron. In the latter cases an mRNA is transcribed, in which the first exon of the *bcr* gene (e1) is spliced to *abl* exon a2, resulting in an e1a2 junction (10). This mRNA is translated into a bcr-abl fusion protein of 190 kD (p190), that also shows enhanced tyrosine kinase activity and transforming potential (8).

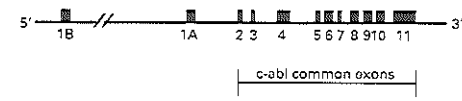

Figure 1. Schematic representation of the normal *abl* gene. The exons are depicted as black boxes and numbered according to Groffen *et al.* (3).

Recently Soekarman *et al.* (1) reported an aberrant localization of the chromosome 9 breakpoint in the *abl* gene in two Ph-positive ALL patients. This new breakpoint was located in the intron between *abl* exons a2 and a3, resulting in a *bcr-abl* mRNA lacking *abl* exon a2. In their first patient, *bcr-abl* fusion in the mRNA occurred between exons b2 and a3, resulting in a 210 kD fusion protein, whereas in the second patient *bcr* exon e1 appeared to be fused to *abl* exon a3, resulting in a fusion protein of 190 kD. Both proteins showed enhanced tyrosine kinase activity in autophosphorylation assays as compared to the normal abl protein, but in the same range as observed in other ALL patients.

In the novel bcr-abl fusion protein 58 amino acids are missing, which are encoded by *abl* exon a2. Interestingly, the last 17 amino acids encoded by a2 are part of a stretch of 50 amino acids that form the src-homology 3 region (SH3) of the abl protein (11). This is one of the regions that is highly homologous in abl and scr. The SH3 region is thought to have a negative regulatory effect on the kinase domain, termed SH1 (12,13). Therefore the finding of partial deletion of the SH3 region in the two ALL patients indicates that this regulatory region is inactive in the bcr-abl fusion proteins.

In this paper Southern blot and polymerase chain reaction (PCR) analysis are reported of bone marrow cells of a Ph-positive CML patient in chronic phase, with a b2a3 chimeric mRNA and a chromosome 9 breakpoint between *abl* exons a2 and a3, much the same as in the two ALL patients reported by Soekarman *et al.* (1). The significance of this finding is discussed. A PCR and

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LEUKEMIA

Southern blot strategy is proposed, which can be used for screening patients for the presence of this new *bcr-abl* rearrangement.

MATERIALS AND METHODS

Patient

A 59-year-old man was first seen in December 1988 with complaints of tiredness. Physical examination showed splenomegaly. Laboratory investigations revealed Hb 6.0 mmol/l, leucocyte count 254×10^9/l and thrombocyte count 180×10^9/l. The peripheral blood smear contained 42% myelocytes, 10% promyelocytes, 5% metamyelocytes, 3% normoblasts, 14% bands, 24% segmented neutrophils, and 2% eosinophils. Blood urea, creatinine, and electrolytes were within the normal range. Plasma lactate dehydrogenase was elevated (1153 U/l). The bone marrow smear showed increased myelopoiesis. Based on these findings, a diagnosis of CML was made.

The patient was treated with hydroxyurea. During the next 2 years he remained in chronic phase CML. In December 1990 acceleration of the disease occurred. The bone marrow biopsy showed myelofibrosis. To control the serious anemia the patient frequently received blood transfusions.

Samples

Sterile bone marrow aspirates and blood samples were taken and heparinized. Sampling was part of diagnostic and clinical follow-up procedures and was obtained only after informed consent of the patient. After Dextran separation, leucocytes were frozen and stored in liquid nitrogen until DNA and RNA analysis was performed.

Cytogenetics

The karyotype of blood and bone marrow cells was investigated at diagnosis and during the next 2 years at 6 month intervals using standard cytogenetic procedures (14). Chromosomes were identified using the R-banding technique, and classified according to ISCN (1985) (15). The constitutional karyotype was determined and found to be normal using PHA stimulated blood cultures.

DNA Probes

The following DNA probes were used: 5'-BCR, 2 kb BglII–HindIII fragment; 3'-BCR, 1.2 kb HindIII–BglII fragment; abl, 0.9 kb SauI–KpnI fragment, spanning the 0.6 kb intron between exons a2 and a3.

Southern Blotting

This was performed using standard techniques (16).

Reversed PCR Analysis

cDNA synthesis and amplification using the PCR technique were performed as described by Hermans *et al.* (17). The localization of the primers used in the PCR reaction is shown schematically in Figure 2. In brief: oligonucleotides 1, 2 and 3 were used as primers to amplify the fragment covering the *bcr-abl* region. As an internal positive control in the PCR reaction, an *abl* fragment was amplified, using oligonucleotides 1 and 4. This *abl* fragment is always present, irrespective of the Ph translocation. Four oligonucleotides specific for the breakpoint junctions e1a2, b2a2, b3a2, b2a3, and two oligonucleotides specific for b2 and a2 sequences were used as probes. The first three oligonucleotides mentioned are the same as described by Hermans *et al.* (17). The sequences of the remaining oligonucleotides are: b2a3, 5' GCTGACCATCAATAAGGAAGGTGAAAAGCTCCGGGTCTTA 3'; b2, 5' GTGAAACTCCAGACTGTCCACAGCA 3'; and a2, 5' TCCACTGGCCACAAAATCATACAGT 3'.

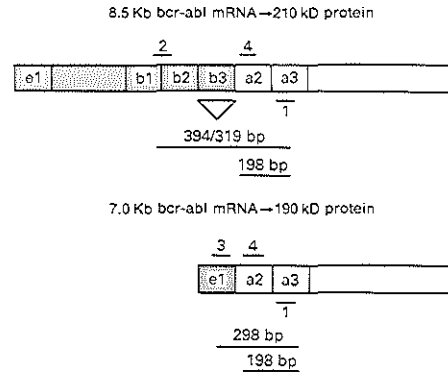

Figure 2 Schematic representation of the 8.5 and 7.0 kb *bcr-abl* mRNA. The localization of *bcr* and *abl* primers is shown. Using primer 1 and 2, a fragment of 394 or 319 bp is amplified, depending on the exon b3 in the mRNA. These fragments have a b3a2 and a b2a2 junction, respectively. Using primer 1 and 3 a fragment of 298 bp is amplified with an e1a2 junction. Primers 1 and 4 are used as an internal control to amplify an *abl* fragment of 198 bp, which should always be present irrespective of the presence of the Ph translocation.

RESULTS AND DISCUSSION

At diagnosis the karyotype of all 32 metaphases analysed from blood and bone marrow was 46,XY,t(9;22)(q34;q11), showing a standard Ph translocation. Treatment was started with hydroxyurea. For the next 2 years, cytogenetic analysis was performed every 6 months and showed persistence of the Ph translocation in all metaphases examined, without additional cytogenetic aberrations. Both clinically and cytogenetically the patient was in chronic phase of CML for these 2 years.

Molecular analysis was performed on the bone marrow sample obtained at diagnosis. Using Southern blot analysis the breakpoint in the BCR region was determined (Figure 3). A rearranged band was detected in the BglII, but not in the BamHI digested DNA after hybridization with the 5'-BCR probe. Hybridization with the 3'-BCR probe showed no rearranged bands. This can be explained by: (1) a breakpoint in the 5' part of BCR in combination with a deletion in the 3' part of BCR; (2) a breakpoint in the 5' part of BCR, resulting in the BamH1 digested DNA in an aberrant BamH1 *bcr-abl* fragment of the same size as the germline BamH1 *bcr* fragment; or (3) by a BglII polymorphism without a BCR breakpoint.

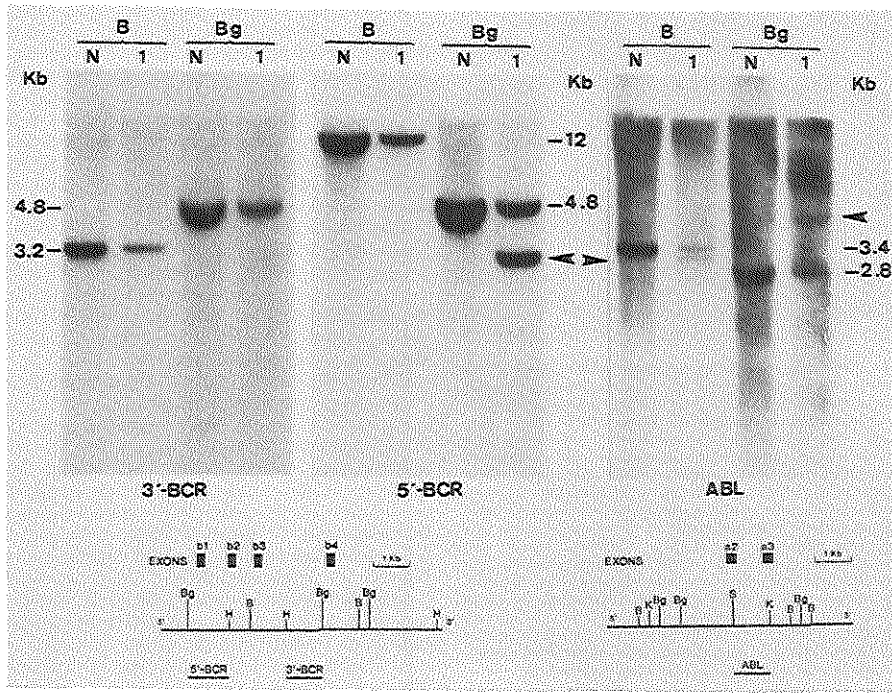

Figure 3. Upper panel: Southern blot analysis of the CML patient (1) and normal thymus DNA (N). DNA was digested with the restriction enzymes Bg1II (Bg) and BamH1 (B). The following probes were used: 1.2 kb HindIII–Bg1II BCR fragment (3'-BCR), 2.0 kb Bg1II–HindIII BCR fragment (5'-BCR), and a 0.9 kb SauI–KpnI fragment spanning the intron between *abl* exon a2 and a3. The sizes of the germline fragments are given in kilobases (kb). Additional restriction fragments are indicated by arrowheads. Lower panel: A simplified restriction map of the normal BCR region and the part of the *abl* gene surrounding *abl* exons a2 and a3 is depicted. (Bg) Bg1II; (B) BamH1; (K) KpnI; (S) SauI. Exons are represented by black boxes. The locations of the 3'-BCR, 5'-BCR, and ABL fragments used as probes in the Southern blot analysis are shown.

To check which of the explanations was correct, it was investigated if *bcr-abl* mRNA was expressed in the leukemic cells of this patient. PCR analysis was performed on cDNA preparations obtained from total RNA of the bone marrow cells of the patient. A scheme indicating the localization of the PCR primers and the expected size of the amplified fragments is shown in Figure 2. No fragment of the expected size, i.e. 298, 319, or 394 bp, corresponding to mRNA with an e1a2, b2a2, or b3a2 junction, was visible on gel, nor was such a fragment detected after hybridization with oligonucleotides which specifically recognize these *bcr-abl* fusion regions in the amplified fragment (data not shown). However, a fragment of 145 bp was amplified instead (Figure 4), using primers 1 and 2. The fragment failed to hybridize under stringent conditions to e1a2, b2a2, or b3a2 specific oligonucleotide probes. It also failed to hybridize to an oligonucleotide containing exon a2 sequences, but hybridized readily to an oligonucleotide containing exon b2 sequences. The most likely explanation for these hybridization data and the size of the amplified fragment (319 − 174 = 145 bp, i.e. b2a2 − a2 = b2a3) is that the leukemic cells of this CML patient express a *bcr-abl* mRNA with a b2a3 junction.

This was checked by determining the breakpoint in the *abl* gene by Southern blotting (Figure 3). The Southern blot containing the Bg1II and BamHI digested DNA was hybridized to a 0.9 kb SauI–KpnI genomic fragment spanning the *abl* intron between exon a2 and a3. Indeed, both in Bg1II and BamHI digested DNA an aberrant restriction fragment was detected, indicating a breakpoint in this region.

Final proof for the presence of a b2a3 junction in the *bcr-abl* mRNA was provided by hybridization of the PCR product to an oligonucleotide specific for the b2a3 fusion region. This oligonucleotide readily hybridized to the 145 bp PCR fragment of the CML patient, whereas it failed to hybridize to the PCR product of a Ph-negative BCR unrearranged CML patient (Figure 4), or to the 319 bp PCR product of a CML patient with a b2a2 junction (data not shown), which served as negative controls on the PCR reaction and the hybridization, respectively.

In conclusion, the leukemic cells of this CML patient

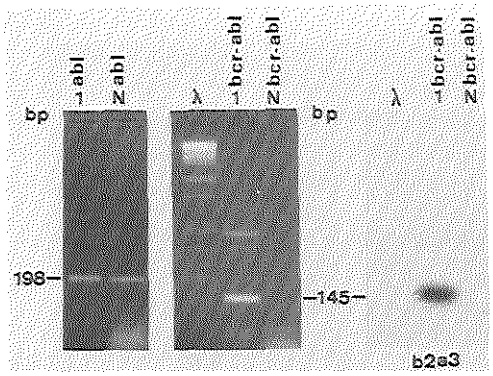

Figure 4. The two panels on the left show the UV picture of the ethidium bromide stained agarose gel containing the PCR products of the blood cells of the CML patient (1), and of a Ph-negative CML patient without *bcr-abl* rearrangement (N), serving as control. Bacteriophage λ DNA digested with the restriction enzyme PstI is used as molecular weight marker. Abl indicates a lane in which primers 1 and 4 (see Figure 2) are used to amplify a 198 bp *abl* fragment, which should always be present irrespective of the presence of the Ph translocation. Bcr-abl on top of a lane indicates that primers 1 and 2 are used to amplify a *bcr-abl* fragment. A 145 bp fragment was detected in the patient (1). As expected, no *bcr-abl* fragment was amplified in the negative control patient (N). The panel on the right shows the autoradiogram of the same agarose gel after blotting onto nylon membrane and hybridization to an oligonucleotide which specifically recognizes the junction between BCR exon b2 and *abl* exon a3 (b2a3). The 145 bp PCR product of the patient (1) readily hybridized to the b2a3 specific oligonucleotide, whereas no hybridization signal is detected in the negative control patient (N).

express *bcr-abl* mRNA, in which BCR exon b2 is fused to *abl* exon a3. In the BCR region the breakpoint is located between exon b2 and b3, in the *abl* gene the breakpoint occurs at an unusual location, i.e. in the intron between exon a2 and a3.

To the best of our knowledge the CML patient described in this paper and the two ALL patients described by Soekarman *et al.* (1) are the only three cases reported with a breakpoint in the *abl* gene 3′ of exon a2. Theoretically the predicted frequency of occurrence of *bcr-abl* mRNA lacking *abl* exon a2 is 0.3% of the *bcr-abl* rearranged patients (0.6 kb:200 kb = 0.003), assuming that the breakpoints in *abl* are randomly distributed (1). However, in the leukemia patients, in whom the breakpoint location in the *abl* gene has been determined, it was always found 5′ of *abl* exon a2 (5,7,18–20). In this respect a paper by Hooberman *et al.* (21) is very interesting. These authors reported that in one out of six Ph-positive and in one out of five Ph-negative ALL or blast crises CML patients breakpoints in the BCR gene were found, but no *bcr-abl* mRNA expression could be detected by PCR analysis. These patients possibly express the *bcr-abl* mRNA lacking *abl* exon a2, which was not detected in the PCR because of the use of a 3′-primer corresponding to *abl* exon a2 sequences. To our opinion it would be worth checking a selected group of patients for the expression of mRNA with a fusion between one of the *bcr* exons and *abl* exon a3. This especially concerns all Ph-positive ALL or AML patients and all CML patients, in whom a breakpoint in the BCR region is detected using Southern blotting, but none of the known *bcr-abl* mRNAs has been found using PCR analysis. The additional analysis, which is required in these cases is either PCR analysis using an *abl* primer containing *abl* exon a3 instead of a2 sequences, or hybridization of the Southern blot to the SauI–KpnI *abl* probe. It is expected that using this strategy more patients will be identified with this unusual breakpoint location in the *abl* gene.

Concerning the disease caused by this new *bcr-abl* rearrangement, the following data reported in this paper are important. Firstly, the expression of *bcr-abl* mRNA with a b2a3 junction is not restricted to acute leukemia or blast crisis of CML, but also occurs in the chronic phase of CML. Secondly, the CML patient reported in this paper and the two ALL patients reported by Soekarman *et al.* (1) were clinically indistinguishable from other Ph-positive CML or ALL patients. In the ALL patients the tyrosine kinase activity of the p210 proteins in the autophosphorylation assay did not differ from other Ph-positive ALL patients. In the CML patient this assay could not be performed because the percentage of blasts was too low for this assay. In the bcr-abl fusion protein of the patients, 17 out of 50 amino acids, forming the SH3 region, are missing. The SH3 domain is a negative regulator of the kinase domain of the abl protein (11–13).

Important for understanding the mechanism of activation of the *abl* oncogene is to compare these patient data to the data reported by Jackson *et al.* (22) and van Etten *et al.* (23), who investigated the effect of deletions in the mouse abl type IV protein. Jackson *et al.* (22) and van Etten *et al.* (23) reported that in mice, the deletion of 53 amino acids in the SH3 domain of the abl type IV protein, which is homologous to the human SH3 domain, results in full activation of the transforming potential, both with respect to fibroblast and B-lymphoid cell transformation *in vitro* and leukemogenic activity *in vivo*. The same is found in v-abl, in which the SH3 domain is absent. Activation of the kinase domain of the abl protein can be reached either by replacement of 5′-abl by bcr sequences or viral gag sequences or by deletion of the SH3 domain. As argued by Soekarman *et al.* (1) partial deletion of the SH3 domain in the three patients has no extra effect on kinase activity of the fusion proteins due to the fact that the inhibiting function of the SH3 domain is most probably already overruled by the presence of 5′-*bcr* sequences in the bcr-abl proteins.
possible clinical and prognostic implications of this finding. It is expected that molecular analysis in patients such as these will contribute to a better understanding of the mechanisms of regulation of the kinase activity of the abl protein, and thus of leukemogenesis.

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CHAPTER 5

PH CONVERSION IN CML: DISAPPEARANCE OF THE PH CHROMOSOME

INTRODUCTION

CML is a stem cell disorder involving the granulocytic, erythrocytic, and megakaryocytic series. Although cytogenetic analysis of blood and bone marrow usually demonstrates 100% Ph positive metaphases, 2-4 weeks in vitro culture of bone marrow from Ph positive CML patients has produced both Ph positive and Ph negative colonies. This finding demonstrates the existence of cytogenetically normal stem-cells in at least some patients (Chervenick et al, 1971, Benjamin et al, 1979, Coulombel et al, 1983, and Dubé et al, 1984).

In CML the chronic phase, which has a duration of 3-4 years, is followed by a blast crisis, which is nearly always fatal.

Chemotherapy with busulphan or hydroxyurea controls the clinical symptoms well during the chronic phase, but is neither capable of delaying the development of a blast crisis, nor of eliminating the Ph positive clone. Thus chemotherapy of CML has so far been considered palliative, but not curative.

Allogeneic bone marrow transplantation is an alternative for some of the patients, but is applicable only to a restricted number of cases, because of the older age of most of the CML patients, and because a HLA matched donor is often not available.

During the last fifteen years new therapies have been devised, e.g. intensive chemotherapy, and more recently α-interferon treatment. These are aimed to eliminate the leukemic clone(s) and to give the functional, but suppressed normal stem-cells the opportunity to repopulate the bone marrow, in order to cure the patient. The disappearance of the Ph chromosome from the bone marrow of the patient is called "Ph conversion". As a consequence of elimination of the Ph positive clone the corresponding molecular markers, i.e. the BCR breakpoint, the *bcr-abl* mRNA, and 210 kD bcr-abl protein also disappear from the blood and bone marrow of the patient. This process can be observed by follow up studies of blood and bone marrow using chromosome analysis, Southern blotting, the very sensitive PCR technique or protein analysis.

In this chapter we will discuss how reduction of the percentage of Ph positive cells and finally Ph conversion can be achieved. This will be illustrated by cases reported in literature and by patients investigated by us.

RESULTS AND DISCUSSION

Ph conversion after busulphan therapy

Conventional chemotherapy prolongs the duration of the chronic phase by several months and diminishes the clinical symptoms caused by splenomegaly, leucocytosis, and thrombocytosis. However, Ph conversion is rare.
Long term remissions have accidentally been observed in CML patients after prolonged busulphan or other chemotherapy treatment (Finney et al, 1972, Benjamin et al, 1979, Hagemeijer et al, 1979, Fegan, 1989). In these patients Ph conversion is usually preceded by bone marrow aplasia due to myelosuppression. Reduction of the percentage of Ph positive metaphases was documented by follow up cytogenetic studies of bone marrow. In the case reported by Fegan also PCR analysis was performed and no *bcr-abl* mRNA expression was detected.
Patients with mixed Ph positive and Ph negative colonies (Ph mosaicism) in their bone marrow survive longer than those with only Ph positive colonies. These observations succeeded in clinical trials in which the effects of new therapies, designed to acquire Ph conversion i.e. intensive chemotherapy, bone marrow transplantation or α interferon, were compared to hydroxyurea treatment of CML patients.

Ph conversion after intensive chemotherapy

Very few CML patients are reported, who have lost the Ph chromosome temporarily or permanently after intensive chemotherapy. Cunningham et al (1979) report 12 out of 37 patients showing temporarily more than 60% reduction of the percentage of Ph positive metaphases and another 7 out of these 37 patients in whom the Ph chromosome completely disappeared using the L5 protocol. Another series is reported by Kantarjian (1985) who observed a temporary fall in the percentage of Ph positive cells to less than 30% in 18 out of 34 CML patients using the ROAP 10 protocol. In both reports the reduction of the percentage Ph positive cells was often temporarily, and the survival was hardly better than in patients treated with hydroxyurea or busulphan, while considerable myelosuppressive toxicity was observed.

Spontaneous Ph conversion

Sporadically CML patients have been reported, who developed Ph mosaicism during conventional chemotherapy followed by complete elimination of the Ph positive clone after cessation of chemotherapy (Singer et al, 1984, Wodzinsky et al, 1989). This process has been termed "spontaneous Ph conversion". In the two patients reported by these two authors the first cytogenetic analysis has been performed several years after diagnosis of CML, showing Ph mosaicism. Cytogenetic investigations have not been done at diagnosis. Cytogenetic follow up studies demonstrate Ph mosaicism under hydroxyurea respectively busulphan treatment. Repeated analysis when the patients were no longer receiving chemotherapy showed a decreasing percentage of Ph positive

cells finally resulting in complete loss of the Ph chromosome. This finding is confirmed by Southern blot and PCR analysis in the first patient and Southern blot analysis and protein analysis in the second case. Interestingly both patients had a remarkable long survival i.e. 25 years and more than 15 years respectively.

Spontaneous Ph conversion was observed in one patient investigated by us. Patient H was a 44 year old man in whom CML was diagnosed in 1982. Cytogenetic analysis at diagnosis showed a standard Ph translocation in all metaphases. During 1.5 year he was treated with busulphan, resulting in Ph mosaicism and clinical and hematologic remission. No episode of bone marrow aplasia or severe thrombopenia was documented. In 1984 chemotherapy could be discontinued and the patient remained in good health and in remission. In 1986 cytogenetic analysis of bone marrow revealed only normal metaphases. In 1989 repeated chromosome analysis showed that the Ph chromosome had disappeared from the blood and bone marrow. A bone marrow sample was sent to our lab for molecular analysis. Blood stem cells from the diagnostic phase (1982) had been stored in liquid nitrogen and thus were available for molecular investigation.
The results of molecular analysis are presented in figure 1 and 2.
In the blood stem cells from 1982 a BCR breakpoint was detected using Southern blotting (fig.1). PCR analysis showed expression of b2a2 *bcr-abl* mRNA (fig.2).
In the bone marrow sample of 1989, in which no Ph chromosome was found, no *bcr-abl* mRNA expression was detected using PCR analysis (fig.2).
In conclusion: In this CML patient spontaneous Ph conversion was observed i.e. Ph mosaicism occurred after busulphan treatment, followed by disappearance of the Ph positive clone after cessation of chemotherapy. At the time of molecular investigations the number of Ph positive metaphases was below the detection level of the most sensitive technique, the PCR, which means that less than 1 in 10^6 cells was Ph positive.
The question remains if this patient is definitively cured. When a recidive occurs, will it be recurrence of the same CML with the same molecular characteristics or is the patient at risk for a secundary leukemia? The answers to these questions are of extreme interest and can only be obtained by clinical, hematological, cytogenetic and molecular follow up studies.
Although very rare, cases of Ph conversion have been reported (see first part of this chapter). Usually a narrow correlation exists between disappearance of the Ph and hematological and clinical remission. In contrast some very rare cases of active CML disease with late appearance of the Ph or early disappearance without cure (Hagemeijer, 1979) have been reported. Unfortunately none of these cases haS been analyzed molecularly.
Another case of particular interest with respect to Ph conversion was the patient mentioned in chapter 4 table 2 as patient 5. In this 27 year old patient, who has accidentally been exposed to benzene, the Ph chromosome was found in 100% of the in bone marrow cells. In addition to the Ph translocation, 50% of the bone marrow cells also showed trisomy 8. Without treatment the patient came in clinical remission

and the cytogenetic aberrations regressed. Seven years after diagnosis this patient had only 30% Ph positive metaphases in the bone marrow, and no additional chromosome 8 was found (Smadja et al, 1986). Thirteen years after diagnosis the patient relapsed and clinically had CML disease with 100% Ph positive metaphases in bone marrow [Smadja et al, 1986]. Only this last bone marrow was available for molecular analysis. We performed Southern blot analysis and detected a breakpoint in the BCR region. PCR analysis showed expression of *bcr-abl* mRNA with a b2a2 junction. From that time on the patient was treated with hydroxyurea and interferon. Because the patient did not respond to this treatment, BMT will be performed.
We are very much interested in the results of follow up studies in this patient.

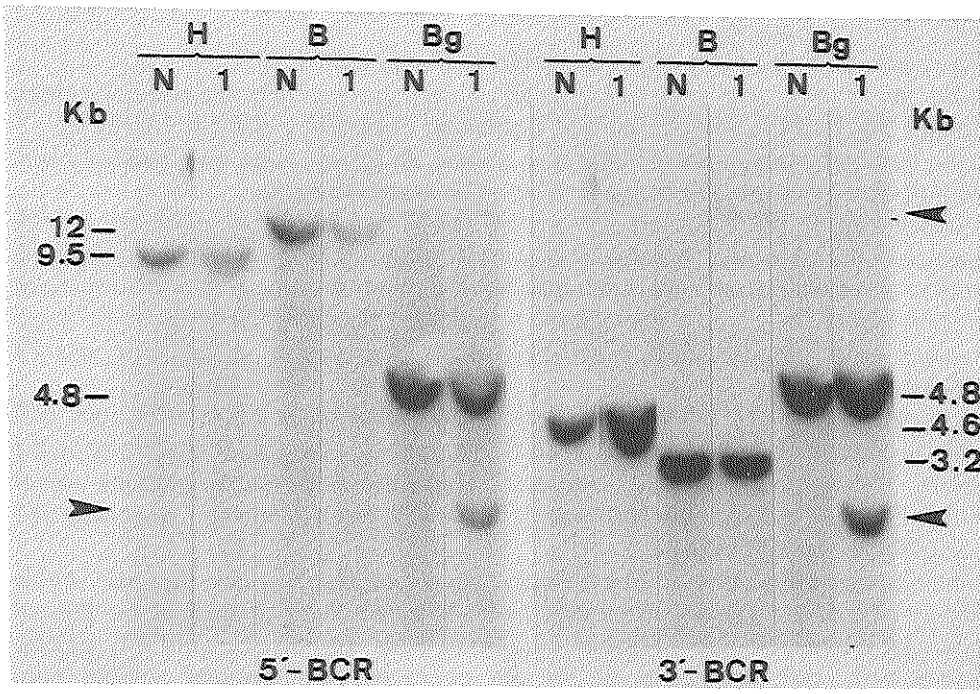

Figure 1. Autoradiogram of Southern blot analysis of peripheral blood stem cells from patient H at the diagnostic phase of Ph positive CML. Lane 1: patient H, lane N: normal human thymus. Restriction enzymes: H: HindIII, B: BamHI, Bg:BglII. Hybridization with 5'-BCR probe is shown in the left panel, and hybridization with 3'-BCR probe the right panel. The aberrant bands in the BglII as well as in the BamHI digest point to a breakpoint in the BCR region localised in the BamHI-HindII fragment (see also BCR restriction map included in chapter 4).

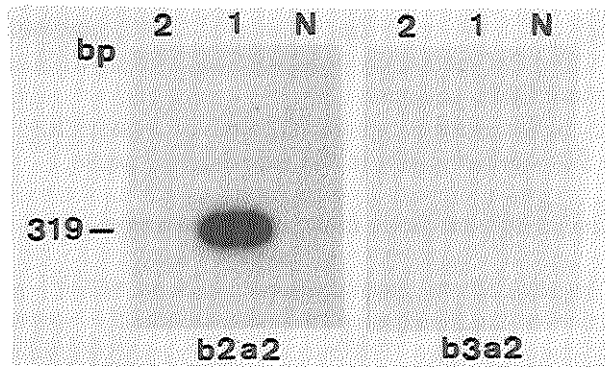

Figure 2. Autoradiogram of the PCR experiment performed with sequential samples of patient H. Lane 1: RNA derived from the blood stem cells at diagnosis of CML in 1982. Lane 2: RNA derived from bone marrow obtained in 1989 in remission. N: RNA derived from normal leucocytes as negative control. In lane 1 a 319 bp amplified fragment is seen that hybridised with an oligonucleotide specific for the b2a2 junction of the *bcr-abl* mRNA (left pannel). No *bcr-abl* fragment was detected in the sample from 1989, (lane 2), nor in the normal control.

Ph conversion after autologous bone marrow transplantation

In 1979 the first results were reported of chemotherapy followed by autologous BMT in CML (Goldman, 1979)). The strategy followed by Goldman and later also by others was: Blood or bone marrow stem cells were collected at diagnosis or after a course of chemotherapy and stored in liquid nitrogen. When accelerated phase or blast crisis developed, autologous blood stem cell transplantation was performed preceded by high dose chemotherapy with or without total body irradiation. The aim was to obtain a second chronic phase of CML. This second chronic phase lasted for 8-40 weeks, as was reported by the different investigators (Goldman, 1979, and 1981, Korbling, 1981, Reiffers, 1983, Gorin, 1982, Thomas, 1984, Phillips and Herzig, 1984)).

Sporadically patients were reported who lost the Ph chromosome after auto grafting (Brito-Babapulle, 1981). In one patient the Ph conversion lasted for 24 months.
In order to try to cure CML patients, strategies were developed aimed to eliminate the Ph positive cells from the autograft in vitro before transplantation. Elimination of the Ph positive cells from the autograft in vitro was performed using one of the following techniques:
1. Dexter type long term cultures of bone marrow result in the presence of predominantly Ph negative progenitor cells in some, but not in all CML patients, as was demonstrated by Coulombel et al (1983) and Dube et al (1984). Turhan et al (1990) demonstrated complete disappearance of the Ph positive BCR positive cells concurrent with the appearance of new clones of normal cells in 1 out of 3 CML patients investigated.
2. In vitro lysis of leukemic cells can be obtained using lymphokine activated killer (LAK) cells. Combined application of IL-2 or interferon and LAK cells leads to

increased cytotoxicity of LAK cells. Teichman et al (1990) reported successful lysis of leukemic cells using this method in 4 out of 6 blast crisis CML patients and in 2 out of 5 chronic phase CML patients.
Results of clinical trials using purging followed by autografting can not yet be reported, because this method is still in the experimental phase.

Ph conversion after allogeneic bone marrow transplantation

The results of allogeneic bone marrow transplantation (BMT) are encouraging. Complete suppression of the Ph chromosome was frequently obtained by allogeneic BMT preceded by chemotherapy. The success rate varied according to the patient selection. BMT performed during chronic phase of CML in patients younger than 30 years, resulted in the highest percentage long term remissions i.e. 50-60% . BMT performed during accelerated phase or blast crisis resulted in 15-30% long term remission (Speck, 1984, Thomas, 1986, Talpaz, 1990). Most of the failures were due to graft versus host disease (GVHD) and other complications of the BMT procedure. Attempts to reduce the failure rate by preventing GVHD using T cell depleted transplants, resulted in lower incidence and severity of GVHD, but in an increased risk of graft failure and a markedly increased relapse rate (Clift, 1987, Hale, 1988, and reviewed by Appelbaum, 1990).
The actuarial relapse rate was 9% in CML patients who received untreated transplants during the chronic phase of the disease, while it was 48% in those receiving T cell depleted transplants (Goldman, 1988). When BMT was performed during the accelerated phase using T cell depleted donor marrow, the relapse rate was nearly 100% (Clift, 1987).
Depleting the donor marrow of T cells helps in preventing GVHD, but also abrogates Graft Versus Leukemia (GVL) effect. It is assumed that T lymphocytes in the donor marrow are capable of mediating GVHD on one hand and GVL effect on the other (Gale and Reisner, 1986). Although the exact mechanism is unknown,evidence is found in animal models that at least some of the cells responsible for GVHD are clearly distinct from those causing GVL effect (Bortin, 1979, Truitt, 1986). This creates the possibility to separate the lymphocyte subpopulations, that mediate GVHD from those mediating GVL and to give back the latter subpopulation after BMT.
Alternative strategies to lower the relapse rate using T cell depleted donor cells for BMT are:
1. Partial T cell depletion.
2. Transfusion of graded numbers of donor T lymphocytes post BMT.
3. Administration of α interferon or IL-2 post BMT.

When relapse of CML occurs it is nearly always caused by failure to eliminate the original leukemic clone, and seldom by leukemia in donor cells. Evidence for this was provided by follow up studies in sex mismatched donors and recipients, and by molecular investigations showing an identical BCR breakpoint localization before BMT and in the relapse (Ganesan, 1987, Gao, 1988). If relapse occurs, it nearly always takes place during the first two years after BMT. Occasionally relapses have been

reported up to 4 years after BMT (Goldman, 1988).
A limitating factor of allogeneic BMT in CML is, that most CML patients are ineligible for BMT. Firstly, because CML usually occurs in older patients (mean age 50-60 years), whereas the best results of BMT are reported in the younger age group. Secondly, because in only 20-25% of the CML patients a HLA matched donor, most frequently a relative, is available. In an attempt to find suitable donors for these patients partially matched family donors and (partially) matched unrelated donors were tested.
Partially matched family donors differing in two or more HLA A,B, or DR antigens from the acceptor, were unsuitable. Better results were obtained when the difference was in only one of these antigens. Allogeneic BMT using partially matched unrelated donors resulted in severe acute GVHD in 47% of the patients and in a high rate of fatal infections (Gingrich, 1988). However the preliminary data of allogeneic BMT using matched unrelated donors are thusfar equally good as the results obtained in HLA matched siblings. These results are hopeful and might be very helpful in increasing the availability of suitable donors for BMT in CML.

Ph conversion after interferon therapy

From the first part of this chapter it can be concluded, that new therapeutics are needed to treat CML patients.
Since 1981 promising results were reported using interferons for treatment of CML. In general, interferons have antiproliferative, antiviral, immune-modulating and differentiation inducing activity (Borden, 1981). Based on their antigenic and physiochemic properties the interferons (IFN) are divided in three classes: IFN α, produced by leucocytes, IFN ß produced by fibroblasts, and IFN γ produced by T lymphocytes. IFN α has significant anticellular effects on normal myeloid cells as well as on CML progenitor cells in vitro (Verma, 1978, Oladipupo-Williams, 1981, Neuman, 1982). IFN α also has profound effects on the marrow microenvironment as has been demonstrated in long term bone marrow cultures. Especially the reticulo-fibroblasts are inhibited by IFN α (Galvani et al, 1990).
IFN γ has a similar suppressive effect on myeloid colony formation as IFN α, and induces differentiation of leukemic cells (Ball, 1984). In vitro synergistic antiproliferative activity exists between IFN γ and IFN α as well as between IFN γ and other lymphokines such as tumor necrosis factor and several interleukines.

Since 1981 clinical trials were started to test IFN α and later also IFN γ and combinations of the two as therapeutics for treatment of CML.

Clinical trials with α IFN

The first clinical trials were performed using IFN α produced either by virus stimulated leucocytes or by recombinant DNA technology. No difference in clinical effect was observed between these two types of α interferons. The first clinical trial

using IFN α was reported by Talpaz (Talpaz, 1986). Between 1981-1984, 51 Ph positive CML patients were treated with 3-9 million IU of partially purified α interferon per day. Complete hematologic remission was achieved in 71% of the patients, while in 41% reduction of the percentage of Ph positive metaphases was demonstrated. In 1990 Talpaz reported the results of 7 years follow up of these patients: in 7 out of 51 patients complete suppression of the Ph chromosome was demonstrated in at least one test and an additional 3 patients showed suppression of the percentage of Ph positive metaphases to less than 35% (Talpaz, 1990). The best results were obtained in newly diagnosed untreated CML cases. The median survival of the patients enroled in 1981 was 64 months. The mechanism of Ph suppression by IFN is still unknown.

A second study was performed by Talpaz et al between 1984 and 1988 (Talpaz, 1987 and 1990). Forty five newly diagnosed CML patients were treated with 5 million IU of recombinant IFN α per day. The response rate again was approximately 75% . In 23% complete cytogenetic response was achieved in at least one follow up test, and in an additional 4 patients partial suppression of the Ph chromosome was observed, resulting in less than 35% Ph positive metaphases. This was confirmed by Southern blot analysis, which has the same sensitivity as chromosome analysis.

In 1987 a clinical trial was started in the Netherlands comparing the effect of IFN α (Intron A, Schering corporation) versus hydroxyurea treatment in newly diagnosed CML patients in the age group of 18-60 year. All patients included in this trial should be Ph positive or show *bcr-abl* rearrangement as detected by Southern blot or PCR analysis. Although this study is not finished yet and follow up of most patients is short, we will present the cytogenetic and molecular data obtained thusfar. Clinical and hematologic data are not yet free for publication.
Seven patients were excluded from entering the study, because they lacked the Ph chromosome as well as molecular evidence for *bcr-abl* recombination. Thusfar 43 patients entered the study. Initially all patients were treated with hydroxyurea until WBC was between 5-10 10^9/l. Thereafter twenty patients were randomized on interferon (3 million IU/day, 5 out of 7 days) and twenty three on hydroxyurea(dose adjusted to keep WBC at 5-10 10^9/l).
We performed cytogenetic analysis in eighteen of the twenty patients of the interferon group and on twenty of the twenty three patients of the hydroxyurea group. (The remaining patients were investigated in other laboratories). Cytogenetic follow up was done every 6 months.
No reduction of the percentage of Ph positive metaphases was observed in the patients treated with hydroxyurea and three patients developed a blast crisis during hydroxyurea treatment.
In the patients treated with IFN α cytogenetic improvement was observed in three patients, while additional chromosomal abnormalities occurred during IFN treatment in three patients (see Table 1).
Only one patient developed a myeloid blast crisis during IFN α treatment.

Besides the Ph negative patients several Ph positive CML patients were investigated using molecular analysis prior to the trial. The results of Southern blotting and PCR analysis are included in chapter 1. The most important findings were:
The leukemic cells of two patients showed expression of both b2a2 and b3a2 mRNA before or during treatment with interferon resp. hydroxyurea. During the two years follow up the karyotype of blood and bone marrow of both patients revealed persistence of 100% Ph positive metaphases.
In blood and bone marrow of another patient expression of an unusual *bcr-abl* mRNA with an b2a3 junction was detected. This was caused by an unusual localization of the breakpoint in the *abl* gene.
Longer follow up will be necessary to collect data about the percentage of cytogenetic remissions and more importantly, about the temporary or persistent character of the remissions.

Table 1: Karyotypic evolution during IFN α treatment as was observed in 6 out of 20 patients treated with IFN α. A summary is presented of the percentage of Ph positive metaphases and additional chromosomal aberrations detected during IFN α treatment of CML patients, who were investigated in Rotterdam.

Patient	Months of IFN treatment				
	0	6	12	18	24
1	100%	30%	20%	37%	6%
2	20%Ph 80%Ph,+8	29%Ph 17%Ph,+8	19%Ph 3%Ph,+8		
3	100%	50%	100%	15%	3%
4	100%	35%	57%	67%	
5	100%	100%	100%	52%Ph 48%Ph,+8q-	
6	100%	97%	100%	94%Ph 3%Ph,+22q-	

Clinical trial with IFN γ

In 1987 Kurzrock et al reported preliminary results of a clinical trial testing the effectiveness of IFN γ treatment of newly diagnosed CML patients (Kurzrock et al, 1987). Using 0.25-0.5 mg IFN γ/m^2/day cytogenetic improvement was observed in approximately 40% of the patients that achieved complete hematological remission. The follow up period is too short to draw conclusions as yet. Maximal cytogenetic response is expected 2 years after starting this treatment. The side effects of IFN γ were comparable to that of IFN α treatment in this study.

Clinical trial with IFN α + IFN γ

Preliminary data of a large study of the CALGB (Ozer et al, unpublished data, 1990, reviewed by Silver et al, 1990) testing the effect of alternating courses of IFN α and IFN γ compared to IFN α alone, suggested no therapeutic gain of the alternating courses over IFN α alone. IFN γ caused more severe toxicity than IFN α in this study. Interestingly it was found that patients who were resistant to IFN α treatment did react to IFN γ and viceversa.

A summary of the conclusions from these and other IFN trials (Alimena, 1989, Anger, 1989) is:
1. Thusfar the highest percentage of Ph reduction and Ph conversion and the lowest toxicity was achieved using long term treatment with IFN α.
2. Both duration of treatment and dose are important, as leucopenia often coincides with disappearance of the Ph chromosome.
3. Best results were obtained in newly diagnosed CML patients.
Response to treatment has also been observed in patients treated with IFN α, because of relapse after BMT (Higano et al, 1989).
4. In some patients severe muscle and bone pain occurred during IFN α treatment, but was never observed during IFN γ therapy. These patients always became resistant to IFN α. Therefore these symptoms should always be a reason to stop treatment with IFN α.
5. Development of nonneutralizing and neutralizing antibodies has been observed in patients treated with interferon, but in the majority of patients this observation is of little clinical significance.
6. Even in patients with complete hematologic and cytogenetic response *bcr-abl* rearrangement was often detected using the PCR technique, indicating minimal residual disease (Dhingra et al, 1989).
In other patients, in whom clinical and hematological complete remission was obtained by treatment with IFN α or γ, no *bcr-abl* mRNA expression was detected using PCR analysis. The latter results indicate that IFN α and γ are able to suppress the Ph positive cells to a level below the detection level of the PCR analysis and possibly to eliminate them completely. Longer follow up studies are necessary to determine in what percentage of the former group of patients long term clinical remissions will be

obtained. This knowledge is essential to determine the prognostic value of a positive result of PCR analysis in such patients.

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CHAPTER 6

CYTOGENETIC AND MOLECULAR DIAGNOSIS OF PH NEGATIVE CML

6.1 CYTOGENETIC AND MOLECULAR ANALYSIS OF PH NEGATIVE CML

Blood 1989, 73: 1038-1044

Cytogenetic and Molecular Analysis in Philadelphia Negative CML

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We studied the clinical, hematologic, cytogenetic and molecular biologic features in four patients with Philadelphia (Ph) negative chronic myeloid leukemia (CML). In all four cases the clinical and hematologic characteristics were indistinguishable from Ph positive CML. Cytogenetic analysis showed a normal karyotype in two patients and chromosomal translocations apparently not affecting chromosome 22 in the other two cases. Southern blot analysis using probes of the *bcr* region, demonstrated a *bcr* breakpoint in all four patients. In situ hybridization with *bcr*, c-*abl*, and c-*sis* probes showed unusual hybridization sites for 5'-*bcr* and c-*abl* indicating complex chromosomal rearrangements affecting three different chromosomes in the four patients investigated. Using polymerase chain reaction (PCR) followed by hybridization to oligonucleotide probes specific for the *bcr-abl* fusion region, the expression of a chimeric *bcr-abl* mRNA was detected. In these patients we demonstrated that (*a*) CML with a breakpoint in the *bcr* region without cytogenetically detectable Ph chromosome is characterized by the same genomic recombination of 5'-*bcr* and c-*abl* as CML with standard Ph translocation and (*b*) unusual localization of 5'-*bcr* and c-*abl* sequences caused by complex Ph translocation does not interfere with transcription of the *bcr-abl* fusion gene.

THE PHILADELPHIA CHROMOSOME (Ph) is found in 94% of chronic myeloid leukemia (CML) patients. In the majority of cases it originates from the standard Ph translocation t(9;22)(q34;q11).[1] As a result of this translocation the c-*abl* oncogene is translocated to a specific site in a gene on chromosome 22 band q11 called breakpoint cluster region (*bcr* region).[2] The part of chromosome 22 distal to the breakpoint is translocated to chromosome 9 band q34.

The 5'*bcr-abl* DNA on the Ph chromosome is transcribed into an 8.5 kilobase (kb) chimeric *bcr-abl* mRNA. Depending on the position of the breakpoint in *bcr* either *bcr* exon b2 or b3 are spliced to *abl* exon a2, resulting in either b2a2 or b3a2 chimeric mRNA.[3-10] This 8.5 kb *bcr-abl* mRNA encodes an *abl* protein of 210 kilodalton (kD), which has in vitro enhanced tyrosine kinase activity compared with the normal *abl* protein.[9-11] Approximately 5% of the Ph-positive CML patients have cytogenetic variants of the Ph translocation.[12,13] Southern blot and in situ hybridization studies have shown that in variant Ph translocation molecular recombination of 5'-*bcr* and c-*abl* takes place in exactly the same way as in standard Ph-translocation.[14-20] The remaining 6% of CML patients show no Ph chromosome and are classified as Ph-negative. Ph-negative CML patients have different clinical and hematologic features, ie, older median age (>65 years), monocytosis, thrombopenia, poor response to chemotherapy, rapid transformation to acute leukemia, and shorter survival than Ph-positive CML.[12,15,21,22] Nevertheless a few Ph-negative CML patients present with clinical and hematologic features that are indistinguishable from Ph-positive CML, ie, median age <60 years, higher WBC with basophilia but without monocytosis, no dysplastic changes in bone marrow cells and same survival as in Ph-positive CML.[23]

We report in this article detailed cytogenetic and molecular analysis in four patients with a disease indistinguishable from Ph-positive CML, with either a normal karyotype (two cases) or chromosomal translocations apparently not affecting chromosome 22 (two cases). Southern blot analysis and in situ hybridization studies demonstrated genomic recombination of 5'-*bcr* and c-*abl* sequences caused by complex Ph translocations. cDNA analysis using the polymerase chain reaction (PCR) showed expression of *bcr-abl* mRNA. These findings are identical to the findings in Ph-positive CML.

CASE REPORTS

Patients were diagnosed and treated in three different European centers: patient no. 1 in Rome, patient no. 2 and 3 in Paris, and patient no. 4 in Amsterdam. A summary of clinical and hematologic data at diagnosis is given in Table 1.

Patient no. 1. In September 1984 CML was diagnosed that responded to hydroxyurea (2.5 g/d): splenomegaly regressed and hematologic findings returned to normal values. Hematologic remission was maintained until January 1986 when thrombocytosis appeared increasing to 1,000 × 10^9/L, but responded well to busulfan (2 mg/d). In December 1987, the patient developed a nonlymphoid blast crisis and died in June 1988 due to septic complications.

Patient no. 2. CML was diagnosed in February 1983. Clinical and hematologic remission were obtained with hydroxyurea and lasted 3 years. In April 1986 accelerated phase occurred with 20% myeloblasts in bone marrow (BM). 6-Mercaptopurine was added to hydroxyurea, and again hematologic stabilization was obtained. In October 1986 a splenectomy was performed. The spleen showed important myeloid metaplasia. In April 1987 engraftment with allogenic BM was realized with success.

Patient no. 3. In May 1977 CML diagnosis was made and successfully treated with busulfan. Chronic phase lasted 8½ years. In January 1986 the first signs of accelerated phase appeared, characterized by bone pains, asthenia, blast cells in BM (10%) and

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Table 1. Clinical and Hematologic Data

	Patient No. 1	Patient No. 2	Patient No. 3	Patient No. 4
Sex/age at diagnosis	M, 40	F, 33	M, 55	F, 64
Organomegaly	Spleno- and hepatomegaly	Splenomegaly	Hematomegaly	None
Hb (mmol/L)	9.0	8.0	8.0	6.8
WBC (10^9/L)	128	80.6	20	121
Myeloblasts	1%	5%	Normal differential count	—
Promyelocytes	2%	4%		5%
Myelocytes	20%	6%		10%
Metamyelocytes	14%	—		5%
Neutrophils	53%	71%		65%
Eosinophils	1%	2%		1.5%
Basophils	1%	2%		2.5%
Lymphocytes	8%	9%		8%
Monocytes	—	1%		3%
Platelets (10^9/L)	470	510	450	218
Bone marrow	Hypercellular granulocytic + megakaryocytic hyperplasia	Hypercellular typical CML no myelofibrosis	Hypercellular myeloid hyperplasia normal maturation	Hypercellular myeloid + megakaryocytic hyperplasia
LAP	4	4	Unknown	9
(normal value)	(20–100)	(20–80)		(25–100)
Duration of chronic phase (yr)	3.25+	3	8.5	7
Survival (yr)	3.75+	5+	9.3	7.3

peripheral blood (10%) and thrombopenia (72×10^9/L). In September 1986, blastic transformation occurred with 55% of undifferentiated blast cells in bone marrow aspirate. The patient was treated with vincristine, daunorubicine, novantrone, and aracytidine. No remission was obtained and the patient died in October 1986 of aplasia, cardiac and renal failure, and hemorrhage.

Patient No. 4. CML was diagnosed in the beginning of 1980, following incidental discovery of granulocytosis without other symptoms. The patient reacted favorably to repeated courses of busulfan. In February 1987 a steady increase of myeloblasts in the peripheral blood was seen, followed within a month by a full blown myeloblastic crisis. Among other drugs treatment with alpha-2-interferon had no success. The patient died 2 months later.

MATERIALS AND METHODS

Samples. Bone marrow aspirates and blood samples were sent to Rotterdam for molecular investigations. The samples were sterile and heparinized and reached the laboratory within 24 hours after aspiration. All sampling was part of diagnostic and clinical follow-up procedures and obtained only after informed consent of the patients.

Cytogenetics. The karyotype of leukemic cells was investigated at diagnosis using standard cytogenetic procedures. Chromosomes were identified using G and/or R banding techniques and classified according to ISCN (1985).[24] Because of unusual findings the cytogenetic analyses were repeated several times locally and in Rotterdam at the time of the molecular investigation. The constitutional karyotype of each patient was determined and found normal using PHA stimulated blood cultures.

DNA probes. The following probes were used in Southern blot analysis and in situ hybridization[7]: c-*abl*, 0.6 kb *Bam*HI + 1.1 kb *Hin* dIII-*Eco*R1 fragment; c-*sis*, 1.7 kb *Bam*HI fragment; 5′-*bcr*, 2 kb *Bgl*II-*Hin* dIII fragment; 3′-*bcr*, 1.2 kb *Hin* dIII-*Bgl*II fragment. In standard Ph translocations the 5′-*bcr* probe recognizes the 22q- derivative and the 3′-*bcr* probe recognizes the 9q+ derivative.

Southern blotting was performed following standard techniques.[2,25] In situ hybridization was performed as reported previously.[26] The probes were ^{3}H-labeled, using the method of Feinberg and Vogelstein[27] to a specific activity of 10^8 cpm/μg DNA. After hybridization and autoradiography the labeled sites were scored on R or Q banded metaphases and assigned to a chromosomal band or region. Grain distribution in the in situ hybridization experiments was tested on its statistical significance using the Poisson distribution followed by the binomial method.

cDNA preparation and amplification with the PCR were performed as described by Hermans et al.[28] Oligonucleotides[28] 2 and 3 (Fig 1) were used as primers to amplify a fragment covering the *bcr-abl* region. As internal positive control in the PCR, an *abl* fragment was amplified, using oligonucleotides 1 and 3, that is always present, irrespective of the Ph translocation. Two 40 mer oligonucleotides specific for the breakpoint junctions b2a2 and b3a2 were used as probes.

RESULTS

Cytogenetics

Patient no. 1. At diagnosis 75 metaphases were analyzed after Giemsa staining and GTG banding technique without evidence of a recognizable Ph chromosome. However, one of

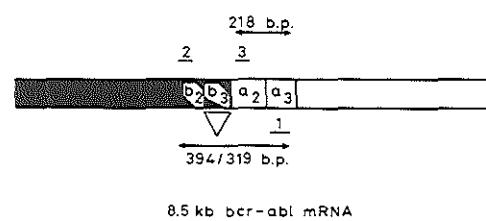

Fig 1. Schematic representation of 8.5 kb *bcr-abl* mRNA. Localization of primers used in PCR experiment is given. ▽ indicates that exon b3 may be absent or present in 8.5 kb *bcr-abl* mRNA. The size and localization of the two fragments amplified in the PCR experiments are presented.

the two chromosomes 22 appeared slightly shorter, one chromosome 19 appeared smaller and sub-metacentric and one chromosome 9 appeared identical to the 9q+ derivative in the standard t(9;22). R-banding studies confirmed these findings (Fig 2). A complex chromosomal translocation t(9;19;22) resulting in a masked Ph was retained as probable interpretation of the karyotype, but routine karyotyping showed two apparently normal chromosomes 22, which was the reason for inclusion of the case in this study.

Patient no. 2. At diagnosis 26 metaphases were analyzed and 80 metaphases in subsequent studies. All metaphases examined showed the same karyotype that was equivocal in R and G bands. There were two possible interpretations: either a simple translocation t(9;14) or a complex Ph translocation t(9;14;22) (Fig 2).

Patient no. 3. The BM karyotype of patient no. 3 was studied twice during the chronic phase of the disease. All 50 metaphases showed a 46,XY normal male karyotype (Fig 2). Molecular investigations were performed at the time of acceleration 8 years and 10 months after diagnosis. At that time the BM karyotypes were 46,XY in 90% and 46,XY, t(1;21)(p21;q22) in 10% of the metaphases. Six months later, in blast crisis 45 metaphases were analyzed, showing additional abnormal clones: 46,XY(16%)/46,XY,t(1;21)(13%)/46,XY,del (6)(p22)(13%)/49,XY,+10,+21,+22 (58%).

Patient no. 4. The BM karyotype of patient no. 4 was found to be normal: 46,XX in repeated investigations of blood and BM cells during the chronic phase of the disease (Fig 2). In January 1987 hematologic and clinical data indicated progression of CML, 20% of the metaphases were abnormal: 47,XX,+8,i(17q). Remarkably, the type of aberrations is the same as often described in progression of Ph positive CML.

Southern Blot Analysis

DNA from bone marrow and/or blood cells of the four patients was digested with *Bgl*II, *Bam*HI, and *Hin* dIII and hybridized to 5'- and 3'-*bcr* probes. All patients showed extra bands with more than one probe and more than one restriction enzyme indicating the existence of a breakpoint in the *bcr* region of chromosome 22 (Fig 3). The breakpoint was found in the *Hin* dIII-*Bgl*II fragment of the *bcr* region in patient no. 1, 2, and 4 (ie, between *bcr* region exon 3 and 4) and in the *Hin* dIII-*Bam*HI fragment in patient no. 3 (ie, between *bcr* region exon 2 and 3). These results are similar to our observations in more than 50 CML patients with standard t(9;22).

In Situ Hybridization Studies

The absence of a cytogenetically recognizable Ph chromosome in the presence of a *bcr* breakpoint prompted investigation of the chromosomal localization of the various genes of interest using in situ hybridization. To this aim metaphase spreads of the four patients were hybridized to four different probes: (1) c-*abl*, (2) 5'-*bcr* mapping proximal to the breakpoint, (3) 3'-*bcr* mapping distal to the breakpoint, and (4)

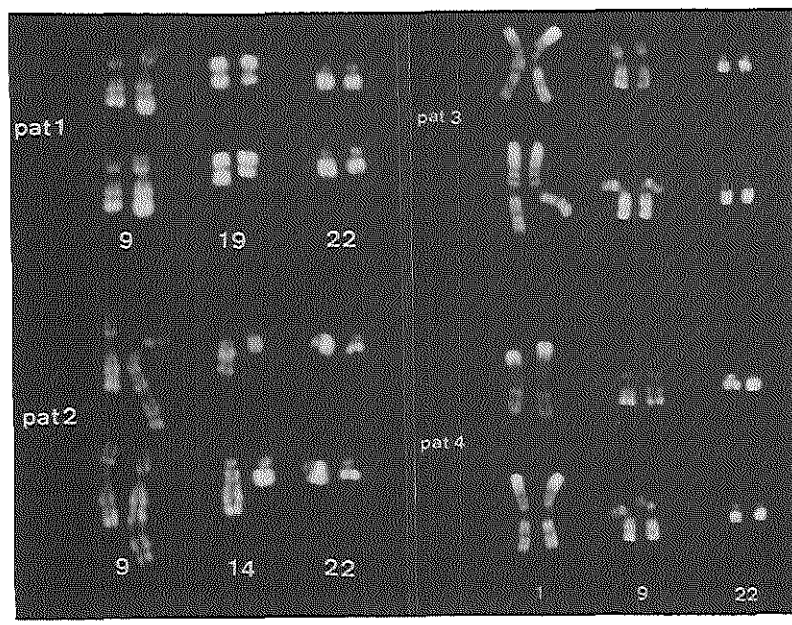

Fig 2. Partial karyotype of patients no. 1, 2, 3, and 4. R-bands with acridine orange. Patient no. 1 shows t(9;19;22), patient no. 2 t(9;14;22), and patients no. 3 and 4 have normal karyotype.

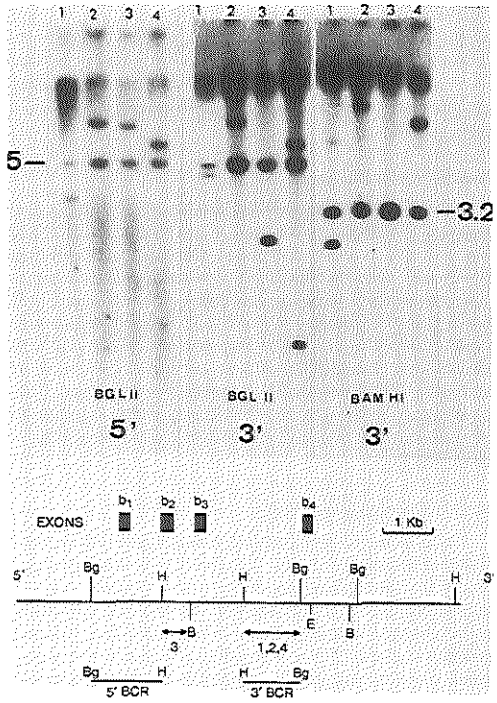

Fig 3. Southern blot showing genomic DNA from Ph-negative CML patients no. 1, 2, 3, and 4 digested with *BglII* and *BamHI*. The filter with *BglII* digests was probed with 3'- and 5'-*bcr* probe. The filter with *BamHI* digests was probed with 3'-*bcr*. Size of germline bands is marked. Extra bands are found in all lanes except in *BamHI* digest of patient no. 3 probed with 3'-*bcr*, indicating the presence of a breakpoint in the *bcr* region in all four patients. Below the Southern blot a simple restriction map of the *bcr* region is given.[2] The 5'-*bcr* and 3'-*bcr* probes are indicated. Bg, *BglII*; H, *Hin* dIII; B, *BamHI*; E, *EcoRI*; b1-b4, *bcr* region exon 1-4. Arrows marked 1, 2, 3, and 4 indicate the fragment in which the breakpoint on chromosome 22 is mapped in patients no. 1 through 4.

c-*sis* as an indicator of the distal part of chromosome 22. Results are detailed in Table 2 and summarized in Fig. 4.

Patient no. 1. In patient no. 1, with presumably a complex t(9;19;22) (Fig 2), it appeared that 5'-*bcr* and c-*abl* probes hybridized to the shortened q arm of the 19q− while 3'-*bcr* and c-*sis* hybridized to the 9q+ as in classical t(9;22). It is obscure whether the centromere of the chromosome designated as 19q− belongs to chromosome 19 or 22.

Patient no. 2. In patient no. 2 specific hybridization indicated a complex t(9;14;22). Indeed, 5'-*bcr* and c-*abl* probes strongly hybridized to the smallest chromosome therefore identifying itself as Ph or 22q−. 3'-*bcr* and c-*sis* only hybridized to chromosome 22 and to the 14q− that are cytogenetically indistinguishable in this case.

Patients no. 3 and 4. c-*abl* and 5'-*bcr* showed an abnormal localization on the distal part of 1p in patients no. 3 and 4. This part of chromosome 1 showed the same staining properties as the distal part of chromosome 22. In both cases c-*sis* only maps to chromosome 22, while 3'-*bcr* maps on chromosome 22 and in patient no. 3 also on 9q34. In patient no. 3 in the minority of cells with t(1;21) specific hybridization of c-*abl* and 5'-*bcr* occurred on the normal chromosome 1, not involved in the translocation t(1;21). These results indicated that in both patients a complex rearrangement has occurred between chromosomes 1p36, 9q34, and 22q11, resulting in the presence of hybridization sites for c-*abl* and 5'-*bcr* probes on 1p36.

Statistics Used in the In Situ Hybridization Experiments

Grain distribution in the in situ hybridization experiments was tested on its statistical significance using the Poisson distribution followed by binomial method in the case of chromosomes 9 and 9q+. In the case of chromosomes 14q−, 19q−, 22, and 22q− only the Poisson distribution was applied, since the contribution of these chromosomes to the DNA-content of a metaphase is relatively small.[29] Binomial distribution was tested in the case of chromosome 1, since this chromosome contributes to a relatively large part of the genomic DNA. The P value for both Poisson and binomial distribution was determined at 10^{-3}.

All experiments were statistically significant for the probes used with the exception of the 3'-*bcr* probe, which showed no statistical significance according to the Poisson distribution in patient no. 1 on chromosomes 9q+ and 22, and in patients no. 3 and 4 on chromosome 9. Though, in the case of patients no. 1 and 3, experiments with the 3'-*bcr* probe could be demonstrated to be statistically significant for the chromosomes 9q+ and 9, respectively, when the binomial distribution alone was tested.

Amplification of cDNA by the PCR

The results of amplification of cDNA followed by hybridization to breakpoint specific oligonucleotides are shown in Fig 5. A 319 base pairs fragment (bp) corresponding to b2a2 joining in the *bcr-abl* mRNA has been found in patient no. 3, a control CML patient with standard Ph translocation and cell line BV173. A 394 bp fragment corresponding to b3a2 joining has been found in patients no. 1 and 4 and cell line K562. As expected neither b2a2 nor b3a2 joining have been detected in leukocytes of a healthy control.

DISCUSSION

In this article we report clinical, cytogenetic and molecular data obtained in four patients with CML and absence of Ph chromosome in bone marrow metaphases. In these patients clinical features at presentation and long survival (3.75 to 9.3 years) contrasted with the atypical symptomatology and rapid transformation to blast crisis usually associated with Ph-negative CML. Reclassification of these cases as myelodysplastic (CMML) or myeloproliferative syndrome[21,30] does not apply to these four patients who had all characteristics of classic CML except for the Ph chromosome.

Southern blotting demonstrated a *bcr* breakpoint in the four cases, similar to our findings in Ph-positive CML

Table 2. In Situ Hybridization Studies

Patient and Karyotype	Probes	No. of Metaphases Analyzed	No. of Labeled Sites on Chromosomes and Specific Bands								
			Total	9	(q33-34)	9q+	(q-distal)	22, der (22)	22q−	Other Chromosomes Involved	No. of Background Grains
Patient No. 1	c-*abl*	30	96	11	(10)	3		2†		19/19q−:1/8 (8)‡	46
46, XY,	c-*sis*	192	346	10		32	(22)	24		19/19q−:6/5	103
t (9;19;22)	5′-*bcr*	30	97	2		3		10		19/19q−:1/7 (7)‡	42
	3′*bcr*	32	86	2		8	(7)	6		19/19q−:1/1	50
Patient No. 2	c-*abl*	50	165	20	(18)	8		3§	11	no. 14:3	95
46, XX	c-*sis*	50	158	9		10		24	2	no. 14:3	120
t (9;14;22)	5′-*bcr*	50	156	5		4		16	13	no. 14:3	61
	3′-*bcr*	50	157	4		6		24	1	no. 14:3	94
Patient No. 3	c-*abl*	63	230	27	(19)			4		no. 1:28 (20)‖	150
46, XY	c-*sis*	50	187	9				22		no. 1:22	97
	5′-*bcr*	65	210	10				18		no. 1:25 (15)‖	113
	3′-*bcr*	66	214	21	(14)¶			27		no. 1:19	153
Patient No. 4	c-*abl*	100	208	36	(29)			4		no. 1:26 (17)‖	106
46, XX	c-*sis*	63	182	9				22		no. 1:15	103
	5′-*bcr*	50	146	7				19		no. 1:19 (12)‖	75
	3′-*bcr*	56	174	12	(5)#			21		no. 1:17	118

*The number of labeled sites on specific parts of chromosomes or specific bands is written in parentheses.

†In patient 1 chromosome 22 and der(22) were indistinguishable in mitoses studied after in situ hybridization.

‡Number of labeled sites on the deleted arm of 19q−.

§In patient 2 chromosome 22 and 14q− are indistinguishable by cytogenetics only.

‖Number of labeled sites on chromosome 1 (p34-p36).

¶Statistically significant using the binomial distribution alone.

#Statistically not significant according to the Poisson and binomial distribution.

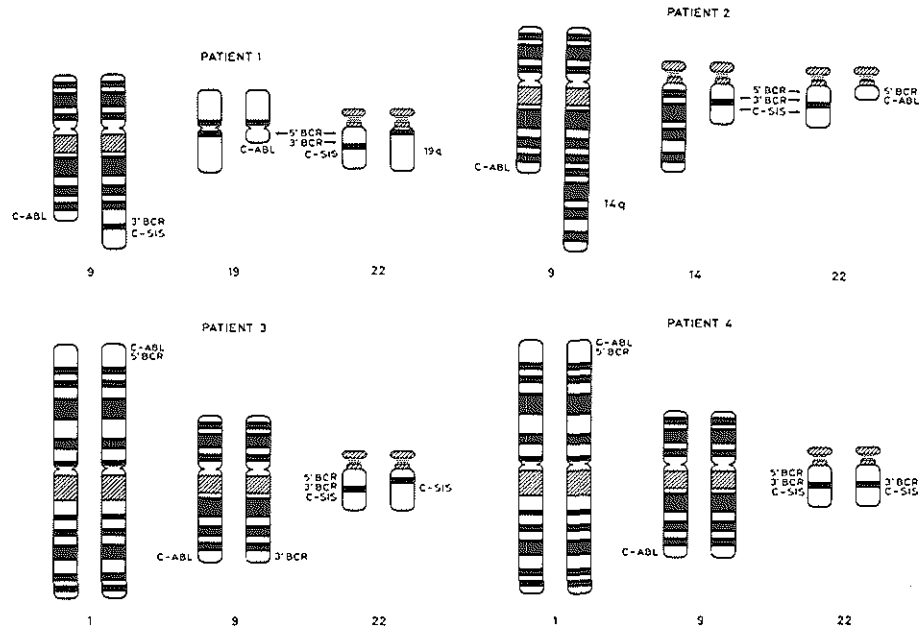

Fig 4. Summary of results of in situ hybridization with 5′-*bcr*, 3′-*bcr*, c-*sis*, and c-*abl* probes in Ph-negative CML patients no. 1, 2, 3, and 4. Normal chromosomes on the left. Localization of the different probes by in situ hybridization is presented.

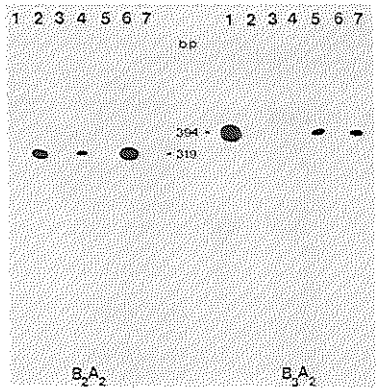

Fig 5. Autoradiogram of the PCR experiment: Using *bcr* and *abl* primers (Fig 1) a cDNA fragment containing the *bcr-abl* fusion region was amplified. After gel electrophoresis and blotting onto nylon membranes hybridization was performed to breakpoint specific oligonucleotide probes b2a2 and b3a2. A 319 bp fragment corresponding to a b2a2 joining has been found in lane 2 (BV173 cell line), lane 4 (Ph positive CML), and lane 6 (patient no. 3). A 394 bp fragment corresponding to a b3a2 joining has been found in lane 1 (cell line K562), lane 5 (patient no. 1), and lane 7 (patient no. 4). As expected no *bcr-abl* joining has been found in lane 3 (leukocytes of a healthy control).

patients with standard t(9;22). Cytogenetically patients no. 1 and 2 showed chromosomal rearrangements involving 9q34 and chromosomes 19 and 14 respectively, while involvement of chromosome 22 was dubious. In situ hybridization studies demonstrated that complex translocations t(9;19;22) and t(9;14;22) had occurred. As a result of these translocations 5'-*bcr* and c-*abl* probes hybridized to the same chromosomal region, ie, 19q− in patient no. 1 and 22q− in patient no. 2. Similar results were reported by Bartram et al[31] in a Ph-negative CML with t(9;12), and 1 year later by Kurzrock et al[32] in a Ph-negative, *bcr*-positive CML patient with t(9;11). In a CML patient with complex t(9;13;15) and two normal chromosomes 22 we also found a *bcr* breakpoint by Southern blot analysis (case referred to us by D. Riviere [Brest, France], unpublished observation, 1985). These data strongly suggest that in apparently Ph-negative CML, with chromosomal rearrangement of 9q34, molecular evidence for a *bcr* breakpoint and/or *bcr-abl* recombination will usually be found and that these cases constitute a rare type of variant Ph translocation.

A normal bone marrow karyotype was found in patients no. 3 and 4. In situ hybridization studies unexpectedly showed hybridization of c-*abl* and 5-*bcr* probes to the chromosomal region 1p35-36 in addition to 9q34 (c-*abl*) and 22q11 (5'-*bcr*). c-*Sis* hybridized to chromosome 22 as expected. In patient no. 3, 3'-*bcr* hybridized to chromosome 22 and also to 9q34, which is an indication that t(9;22) almost certainly was the first step in a two-step rearrangement in this patient. In situ hybridization studies have been reported in four cases of CML, Ph-negative, *bcr*-positive, and a normal karyotype.[19,33] In all four cases c-*abl* was found to hybridize to 22q11 and the rearrangement was interpreted as an insertion of c-*abl* in the *bcr* gene. In patients no. 3 and 4, the exact mechanism of chromosomal rearrangements is not completely elucidated, but they are to the best of our knowledge the first cases of Ph-negative CML with an apparently normal karyotype and translocations or insertions of 5'-*bcr* and c-*abl* on 1p35-36.

The PCR experiments described here are of crucial importance because they demonstrate the presence of *bcr-abl* mRNA in the three cases investigated (patients no. 1, 3, and 4). The b3a2 joining detected in patients no. 1 and 4 and the b2a2 joining in patient no. 3 corresponded to the mapping of the breakpoint on chromosome 22 by Southern blot analysis. The results of Southern blot analysis and the PCR experiment indicated that in these Ph-negative CML patients *bcr-abl* fusion has taken place analogous to Ph-positive CML patients and that transcription was not influenced by the unusual chromosomal localization of 5'-*bcr* and c-*abl* in these patients. The occurrence of *bcr-abl* rearrangements in Ph-negative CML and the clinical significance of such findings have been studied by others.[19,23,31-37]

From the 50 cases with normal karyotypes that have been investigated by Southern blotting, including our own patients, 20 showed a breakpoint in the *bcr* region.[19,22-36] In ten cases RNA or protein analysis demonstrated transcription or translation[19,36] of the chimeric *bcr-abl* gene. The clinical criteria for CML diagnosis, the frequency of molecular rearrangements, and the interpretation of data are different in each study. Nevertheless most investigators[31-37] concluded from their studies that in Ph-negative CML the finding of *bcr-abl* molecular rearrangements indicated an undetected Ph translocation and therefore classify these patients to the group of Ph-positive CML with the prognostic and therapeutic consequences attached to this diagnosis. The remaining cases (Ph negative, *bcr* negative) constitute the group of Ph-negative CML with atypical (ie, myelodysplastic) hematologic features and a relatively short survival. Dreazen et al[19] challenged this hypothesis and claimed the presence of the *bcr* rearrangement in the majority of their Ph-negative CML patients and the finding of atypical features.

Our data support the former observations and indicate that in Ph-negative CML, the finding of molecular evidence for the presence of a *bcr-abl* fusion gene is diagnostic for classical CML. Larger prospective studies are needed to clarify the clinical relevance of molecular investigations in CML.

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6.2 PHILADELPHIA NEGATIVE CML: COMPARISON WITH PH POSITIVE CHRONIC MYELOID LEUKEMIA (CML) AND CHRONIC MYELOMONOCYTIC LEUKEMIA

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Philadelphia-Negative (Ph^-) Chronic Myeloid Leukemia (CML): Comparison With Ph^+ CML and Chronic Myelomonocytic Leukemia

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To better understand the Philadelphia-negative (Ph^-) chronic myeloid leukemia (CML) and its relationships with Philadelphia-positive (Ph^+) CML and chronic myelomonocytic leukemia (CMML), a study was undertaken by the Groupe Français de Cytogénétique Hématologique. Thirty-five Ph^- CML patients were investigated and compared with 55 chronic phase Ph^+ CML and 100 CMML patients. There were 12 M-BCR positive (M-BCR^+) and 23 M-BCR negative (MBCR^-) patients. No clinical or biologic differences were found between Ph^+ and Ph^-, M-BCR^+ patients. In the Ph^- group, M-BCR^+ and M-BCR^- patients differed significantly in age (47.7 ± 6.6 *v* 67.0 ± 6.1 years, respectively; $P = .001$), leukocytosis (153.4 ± 135.1 *v* 58.5 ± 37.7 10^9/L, $P = .002$), relative monocytosis (1.8% ± 1.2% *v* 5.6% ± 1.4%, $P = .048$), absolute basophilia (8.5 ± 9.7 *v* 0.9 ± 1.5 10^9/L, $P = .001$), percentage of immature myeloid precursors (IMP) in peripheral blood (29.0% ± 9.5% *v* 15.3% ± 8.1%, $P = .001$), and percentage of erythroblasts in bone marrow (BM) (6.5% ± 3.5% *v* 14.6% ± 3.6%, $P = .001$). Karyotypic abnormalities other than the Ph chromosome occurred in 0 of 12 M-BCR^+ at diagnosis and 7 of 23 M-BCR^- Ph^- CML ($P = .033$). None of the 13 investigated BCR^- patients had detectable BCR/ABL transcripts using polymerase chain reaction (PCR) and none had an N-RAS mutation. Cytologic findings showed a marked morphologic difference between M-BCR^+ and M-BCR^- patients, especially in the monocytic lineage. Dysmyelopoietic features in CMML and M-BCR^- patients were very similar, and the differences were of quantitative order only. Using four criteria (monocytosis, percentage of IMP, basophilia, and percentage of erythroblasts in BM), patients could be divided into typical and atypical CML and this classification correlated well with molecular findings. We conclude that, while Ph^-, M-BCR^+, and Ph^+ CML are identical diseases, Ph^-, M-BCR^- CML, and CMML have many similarities and might be only different aspects of a same entity.

OVER THE LAST 10 years an increasing number of investigators[1-14] have focused on the Philadelphia chromosome-negative (Ph^-) chronic myeloid leukemia (CML). These patients do not have a Ph chromosome but a proportion of them, varying according to the selection criteria, have a rearrangement within the major breakpoint cluster region (M-BCR). Using different criteria (clinical, morphologic, or molecular), there have been many controversies about the existence of an entity called atypical chronic myeloid leukemia (aCML) that would cover the patients lacking a Ph chromosome and a rearrangement within M-BCR, and about whether this disorder should be regarded as distinct from CML and from chronic myelomonocytic leukemia (CMML).[7]

However, due to heterogeneity in the criteria used for Ph^- patients[2,4,6,10,13,14] and due to the large diversity in the clinical and biologic presentation of CMML,[12,15,16] there is room for debate on this subject.

Therefore, we undertook a study on Ph^- CML to better understand this disorder, and to try to answer the question of whether Ph^- M-BCR^- CML should be regarded as a defined entity or as a subset of CMML.

MATERIALS AND METHODS

Patients. Between December 1988 and March 1990, all patients referred to the participating centers with a tentative diagnosis of Ph^- CML were included in this study, provided that they met five inclusion criteria as the initial diagnosis material: (1) absence of the Ph chromosome on analysis of at least 25 bone marrow (BM) and peripheral blood mitoses; (2) persistent, unexplained leukocytosis greater than 20.10^9/L; (3) more than 75% of peripheral white blood cells (WBC) belonging to the granulocytic lineage; (4) a peripheral blast cell percentage equal or less than 5%; and (5) presence of immature myeloid precursors (IMP) (promyelocytes, myelocytes, and metamyelocytes) in peripheral blood.

Patients with another chronic myeloproliferative syndrome (polycythemia vera,[17] essential thrombocythemia,[18] idiopathic myelofibrosis, chronic neutrophilic leukemia, chronic myeloproliferative syndrome with hypereosinophilia) and patients whose peripheral monocytosis and marrow blast percentage make them meet the French-American-British (FAB) criteria for CMML[19] were excluded from analysis.

Seven cases studied in previous years and that met the criteria of this study were also included, provided initial material was available for investigation.

The Ph^- CML patients were compared with 55 consecutive Ph^+, M-BCR^+, CML patients in chronic phase referred to one of the institutions (UCL, Brussels, Belgium) between 1985 and 1990 and with 100 CMML patients included in another prospective study of our group.[15]

The following parameters were investigated: (1) clinical: age, sex, and organomegaly; (2) hematologic: hemoglobin, platelet count, WBC count, differential count on at least 300 cells, immature precursors in the peripheral blood, and BM cytology; (3) biologic: neutrophil alkaline phosphatase (NAP) and serum lysozyme; (4) cytogenetic: peripheral blood and BM; and (5) molecular: presence or absence of M-BCR rearrangement at the genomic level, polymerase chain reaction (PCR) studies on RNA in patients whose material was available for the detection of the BCR/ABL hybrid message and N-RAS mutations.

Survival analysis was performed using the method of Kaplan and Meier.[20]

Morphology. The pretreatment blood films and BM aspirates were first evaluated in each center, and were subsequently reviewed by a team of morphologists (G.F., Hopital St Louis, Paris, France; M.Z., CHRU Lille, France; and J.R., UCL Brussels, Belgium) without previous knowledge of the clinical, cytogenetic, and molecular findings. The presence of dysmyelopoietic features in BM cells and their severity were assessed using standard criteria.[19] In addition, iron stains were performed in all patients to

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exclude refractory anemia with ringed sideroblasts. BM biopsies, available in all but one patient at diagnosis, were reviewed to rule out idiopathic myelofibrosis.

Cytogenetics. Chromosome studies were performed on peripheral blood and BM cells cultured for 24 to 48 hours without mitogens. Metaphases were G-banded with Wright's stain or trypsin technique and R-banded with heating techniques. A minimum of 25 metaphases were analyzed and classified according to the ISCN guidelines. The karyotypes of all patients were first reviewed in subgroup meetings of the Groupe Français de Cytogénétique Hématologique (GFCH). Karyotypic abnormalities raising problems were reviewed a second time during general workshops of the same group.

DNA analysis. High molecular weight DNA was prepared and restriction digested with at least three restriction enzymes according to standard protocols.[21] After electrophoresis on a 0.8% agarose gel and transfer to nylon membrane, the DNA was hybridized to either a probe covering most of the M-BCR sequences (universal probe) or to two probes 3′ and 5′ in M-BCR. In seven M-BCR$^-$ patients, N-RAS mutations (codons 12, 13, and 61) were sought for using the polymerase chain reaction (PCR) and specific oligo-nucleotide hybridization according to the method described by Cogswell et al.[11]

RNA analysis. Total RNA was extracted from samples of 13 M-BCR nonrearranged patients, reverse transcribed, and submitted to PCR for the detection of hybrid BCR-ABL transcripts (e_1a_2, b_2a_2, and b_3a_2) as previously described by the participating centers.[22-24]

RESULTS

Patients. The records of 42 patients were addressed for inclusion. After morphologic review, two patients had to be excluded because of a peripheral blastosis greater that 5%. Four patients were reclassified as CMML. Another patient had to be excluded because cytogenetic data were not available at diagnosis.

Cytogenetics. No patient had evidence of standard or variant t(9;22). There were seven chromosomal abnormalities at diagnosis that are detailed in Table 1, part B. In three patients, the karyotype became abnormal during evolution of the disease. After classifying the patients into M-BCR$^+$ and M-BCR$^-$, the analysis showed that no M-BCR$^+$ patient had karyotypic abnormalities at diagnosis (two became abnormal in blastic crisis), whereas seven abnormal karyotypes were found at diagnosis in the M-BCR$^-$ group (another patient also became abnormal in blastic crisis) (Table 2).

DNA and RNA analysis. Twelve of 35 patients had a rearrangement within M-BCR and 23 were negative. Among the latter, 13 could be screened for the presence of a hybrid BCR/ABL transcript using PCR. PCR was performed to try to detect a rearrangement occurring within the BCR gene but downstream to M-BCR, which could lead to the formation of the typical message, as seen in a few Ph$^+$ CML.[25,26] None of them were shown to be positive. Among the seven M-BCR$^-$ patients who were tested for possible N-RAS mutations, none was positive.

Clinical and morphologic studies. Clinical, biologic, and hematologic parameters were studied after classifying the patients into two (M-BCR$^+$ and M-BCR$^-$) groups and compared using the Mann-Whitney test. M-BCR$^+$ patients were also compared with the 55 Ph$^+$ CML patients, and the M-BCR$^-$ group with the 100 CMML patients.

The details of this comparison are shown in Table 3.

The first comparison between Ph$^-$, M-BCR$^+$ and Ph$^-$, M-BCR$^-$ patients showed six parameters differing significantly: age (years): 47.7 ± 6.6 versus 67.0 ± 6.1, respectively, $P = .001$; leukocytosis: 153.4 10^9/L ± 135.1 versus 58.5 10^9/L ± 37.7, $P = .002$; relative monocytosis: 1.8% ± 1.2% versus 5.6% ± 2.8%, $P = .048$; absolute basophilia: 8.5 10^9/L ± 9.7 versus 0.9 10^9/L ± 1.5, $P = .001$; percentage of IMP in PB: 29.0% ± 9.5% versus 15.3% ± 8.1%, $P = .001$; and the percentage of erythroblasts in bone marrow: 6.5% ± 3.5% versus 14.6% ± 3.6%, $P = .001$. There were no differences with respect to spleen size, absolute monocytosis, hemoglobin level, platelet count, serum lysozyme level, and NAP score.

In Ph$^-$, M-BCR$^+$ patients, dysmyelopoiesis was mild. The most frequently observed abnormalities were a maturative defect of the granulocyte granules and nuclear abnormalities (nonlobulated nucleus) in the megakaryocytic lineage. The monocytic lineage did not show any maturative defect.

In the Ph$^-$, M-BCR$^-$ group, dysgranulopoiesis was more pronounced, nuclear abnormalities being associated with a maturative defect of the granules, and there were nuclear abnormalities in the monocytic series. Megakaryocytic lineage was less severely involved and the percentage of nuclear abnormalities was less than 15%. These observations are summarized in Table 4.

When comparing the Ph$^+$ and Ph$^-$, M-BCR$^+$ patients, no significant differences with respect to the investigated parameters could be found.

Interestingly, the comparison between the Ph$^-$, M-BCR$^-$ and the CMML patients showed significant differences in relative (5.6% ± 1.4% *v* 31.4% ± 19.2%, $P < .001$) and absolute (3.3 10^9/L ± 2.1 *v* 10.9 10^9/L ± 7.3, $P = .036$) monocytosis, absolute basophilia (0.9 10^9/L ± 1.5 *v* 0.07 10^9/L ± 0.8, $P < .001$), and percentage of IMP in PB (15.3% ± 8.1% *v* 3.1% ± 2.3%, $P = .001$). As far as age, sex ratio, spleen size, leukocytosis, platelet count, hemoglobin level, and percentage of BM erythroblasts were concerned, there were no differences between these two groups of patients. There were no qualitative differences for dysmyelopoiesis, but the fraction of dysplastic cells of the erythroid and megakaryocyte lineage was much more important in CMML, as was the number of patients involved (Table 4).

Survival. The median survival of the Ph$^-$, M-BCR$^+$ patients was not reached at 58 months. In the M-BCR$^-$ cohort, the median survival was 36 months, which was not significantly different from the median of 30 months observed for the 100 CMML patients.

DISCUSSION

Our study confirms the similarity of Ph$^+$ and Ph$^-$ M-BCR$^+$ CML, which is in good agreement with previously published reports focusing on the correlation between molecular and cytologic findings.[3,5,7,10]

Table 1. Clinical, Cytogenetic, and Molecular Characteristics of the 35 Ph⁻ CML Patients

Patient No.	Initials	Sex	Age	Cytogenetics* (no. of cells)	M-BCR	BCR/ABL mRNA	N-RAS Mutation	Evolution Survival (mo)
A. M-BCR⁺								
1	C.M.	F	56	M [33] 46, XX	+	+	ND†	10+‡
2	F.I.	M	50	M [47] 46, XY	+	ND	ND	18+
3	M.M.	M	30	M [46] 46, XY	+	ND	ND	BMT 2.88; 42+
4	M.B.	M	36	M [36] 46, XY	+	ND	ND	BMT 7.88; 8 in blastic crisis
5	L.F.	F	58	B, M [37] 46, XX	+	ND	ND	36 Nonhematologic cause
6	M.P.	M	51	B, M [29] 46, XY	+	ND	ND	131 Nonhematologic cause
7	G.A.	F	59	M [25] 46, XX Blastic crisis: M [11] 46, XX, [7] 46, XX t(9;12)(p21;p11)	+	ND	ND	17 in blastic crisis
8	G.D.	M	31	B, M [149] 46, XY	+	ND	ND	29+
9	B.M.	M	56	B, M [45] 46, XY	+	ND	ND	20+
10	A.G.	F	42	M [33] 46, XX	+	ND	ND	3+
11	K.J.	M	35	B, M [108] 46, XY	+	ND	ND	BMT 8.89; 58+
12	J.V.D.W.	M	23	B, M [44] 46, XY Blastic crisis: M [24] 47, XY +Mar 21	+	+	ND	13 In lymphatic blastic crisis
B. M-BCR⁻								
13	C.M.	F	79	B [14] 46, XX [5] 47, XX, +8 [2] 48, XX, +8, +8 [6] 46, XX, t(8;8)(q21;q24)	–	–	–	16 In accelerated phase
14	D.R.	M	63	M [25] 46, XY	–	–	–	12 In accelerated phase
15	G.E.	M	74	M, B, [33] 46, XY	–	–	–	14+
16	H.G.	F	77	M, B, [28] 46, XX	–	–	–	14+
17	L.F.	M	70	M, B, [3] 46, XY [2] 46, XY, 12p– [3] 46, XY, 13p+, –15, +der (15) t(15;?)(p11;?) [14] 46, XY, –15, +der(15) t(15;?)(p11;?)	–	–	–	32+
18	L.A.	M	67	M, B, [15] 46, XY, del (20)(q12)	–	–	–	11 In blastic crisis
19	R.C.	M	65	M, B, [50] 46, XY	–	–	–	10+
20	S.L.	F	64	M, [7] 46, XX, [1] 47, XX, +C Blastic crisis: M, B, [21] 46, X, I DIC(Xq13) [3] 47, X, I DIC(Xq13), + I DIC(Xq13)	–	ND	ND	5+
21	S.C.	M	54	M, B, [28] 46, XY [1] 46, XY, t(3;22)(p14;p11)	–	ND	ND	Splenectomy 01.89 42+
22	D.J.	M	55	M [31] 46, XY	–	ND	ND	59 In blastic crisis
23	P.M.	M	69	M, B, [26] 46, XY Blastic crisis: B [5] 46, XY [22] 46, XY, –17, +I(17q)	–	ND	ND	26 In blastic crisis
24	V.B.	M	34	M, B, [44] 46, XY, t(5;12)(q31;p12.13)	–	–	ND	14+
25	B.R.	M	68	M [25] 46, XY	–	ND	ND	19 In blastic crisis
26	P.H.	F	64	M [20] 47, XX +13	–	ND	ND	14+
27	C.R.	M	79	M [68] 46, XY	–	ND	ND	22+
28	J.M.	F	34	B, M, [45] 46, XX	–	ND	ND	BMT 05.89; 13, Cerebral abscess
29	R.S.	F	75	M [27] 46, XX	–	–	ND	7+
30	V.C.	M	48	M [80] 46, XY, t(5;17)(q31;p11)	–	–	ND	54 In accelerated phase
31	A.C.P.M.	F	78	M [37] 46, XX	–	ND	ND	28+
32	G.H.J.	M	64	B, M, [28] 46, XY	–	ND	ND	10+
33	J.S.	M	67	M [32] 46, XY	–	ND	ND	11+
34	W.Kn.	M	72	M [27] 46, XY	–	–	ND	12+
35	W.Kr.	M	71	M [29] 46, XY	–	–	ND	18+

*B, peripheral blood; M, bone marrow.

†ND, not done.

‡10+, still alive after . . . months.

Table 2. Karyotypic Abnormalities According to the M-BCR Status

	M-BCR+ N = 12	M-BCR- N = 23
Chronic phase	12 x: normal	16 x: normal
		1 x: + 13
		1 x: del(20)(q12)
		1 x: t(5;12)(q31;p12.13)
		1 x: t(5;17)(q31;p11)
		1 x: +8, +8+8, t(8;8)(q21;q24)
		1 x: 12p−, 13p+, der 15
		1x: 47 X, i DIC(Xq13), + i DIC(Xq13)
Blastic crisis	1 x: t(9;12) (p21;p11)	1 x: i(17q)
	1x: + Mar 21	

Abbreviation: N, number of patients.

The comparison between Ph-, M-BCR+ and Ph-, M-BCR- patients shows that these two disorders are different entities: they differ significantly in age, total WBC count, relative monocytosis, absolute basophilia, percentage of immature precursors in peripheral blood, and percentage of erythroblasts in BM (a finding that is equivalent to the lower myeloid:erythroid ratio already reported by Ezdinli et al[7]). NAP score was not helpful in our experience and this is in agreement with the findings of Kantarjian et al.[8]

In their study, Shepherd et al[7] found that three entities (typical CML or CGL, atypical CML, and CMML) could be defined using morphologic criteria: morphology of granulocytes, monocytosis, absolute basophil count, and the number of mature and immature granulocytes in PB. When we tried to apply these criteria to our series (Table 5), we found a good, though not perfect, correlation between the morphologic features and the molecular findings: four M-BCR- patients satisfied the CGL criteria while the aCML (19 patients) group contained only M-BCR- patients; this is in agreement with two previously published studies.[13,14] However, when we added the percentage of erythroblasts in bone marrow to these criteria (<15% for typical CML) we found a perfect agreement between the molecular findings and the cytologic features. Several groups[3,5,7,10,14] have already focused on that correlation, but with some differences. In these studies, there remained a few patients who had either typical CML features without

Table 4. Dysmyelopoietic Features in BM

Cell	Morphologic Abnormalities	% of Patients	Severity*
M-BCR+			
Granulocytes	Granules: abnormal or devoided	50	2
	Nucleus	0	—
Monocytes	Nucleus	0	—
Erythrocytes	Megaloblastosis	14	1
Megakaryocytes	Micromegakaryocytes	10	1
	Nucleus: nonlobulated	30	2
M-BCR-			
Granulocytes	Granules: abnormal or devoided	66	3
	Nucleus: abnormal segmentation	60	2
Monocytes	Nucleus: abnormal segmentation	44	2
Erythrocytes	Megaloblastosis	12	1
Megakaryocytes	Micromegakaryocytes	44	1
	Nucleus: multiple small nuclei	14	1
CMML			
Granulocytes	Granules: abnormal or devoided	81	3
	Nucleus: abnormal segmentation	62	2
Monocytes	Granules: abnormal or devoided	81	2
	Nucleus	31	3
Erythrocytes	Megaloblastosis	44	1
Megakaryocytes	Micromegakaryocytes	62	2
	Nucleus: multiple small nuclei or giant	31	1

*Severity 1, 1% to 15% abnormal cells; 2, 16% to 50% abnormal cells; 3, >50% abnormal cells.

Table 3. Clinical and Morphologic Characteristics (mean with ranges)

	Ph+ N = 55	P	Ph- M-BCR+ N = 12	P	Ph- M-BCR- N = 23	P	CMML N = 100
Age (y)	45.2 (7-76)	—	47.7 (23-59)	.001	67.0 (34-80)	—	71.8 (26-94)
Sex ratio (M/F)	30/25	—	8/4	—	15/8	—	64/36
Spleen size (cm)	5.0 (0-20)	—	3.7 (0-15)	—	4.8 (0-18)	—	2.7 (0-18)
Leukocytes (10^9/L)	113.9 (20-350)	—	153.4 (27-420)	.002	58.5 (21-287)	—	34.0 (2.5-480)
Monocytes (10^9/L)	2.6 (0-19)	—	2.3 (0-12)	—	3.3 (0-19)	.036	10.9 (1-221)
Monocytes (%)	2.3 (0-9)	—	1.8 (0-3)	.048	5.6 (0-26)	<.001	31.4 (5-76)
Basophils (10^9/L)	5.5 (0-11)	—	8.5 (0.5-22)	.001	0.9 (0-12)	<.001	0.07 (0-2.7)
IMP in PB (%)	30.4 (5-61)	—	29.0 (19-40)	.001	15.3 (4-45)	.001	3.1 (0-25)
NAP score	13.7 (0-144)	—	15.7 (0-70)	—	39.2 (0-92)	—	55.1 (0-190)
Hemoglobin (g/DL)	12.0 (6.3-15.0)	—	12.3 (9-15.6)	—	11.4 (6.9-13.5)	—	10.9 (5.8-16.3)
Platelets (10^9/L)	529 (162-1,638)	—	336 (56-676)	—	308.7 (20-765)	—	202 (12-1.667)
BM erythroblasts (%)	8.0 (1-21)	—	6.5 (1-11)	.001	14.6 (6-26)	—	16.2 (8-32)

Ranges are given between parentheses.
Abbreviation: N, number of patients.

Table 5. Classification of the 35 Ph⁻ CML Patients According to Morphology and Characteristics

	Typical CML	Atypical CML
A. Classification according to the criteria published by Shepherd et al[7]		
M-BCR⁺	12	0
M-BCR⁻	4	19
B. Using BM erythroblasts as a fourth criteria (≥ 15%)		
M-BCR⁺	12	0
M-BCR⁻	0	23

M-BCR rearrangement or atypical features with M-BCR rearrangement.[3,6] In the study by Shepherd et al,[7] dysgranulopoiesis played a major role in the classification of typical CML, atypical CML and CMML, a finding that we were unable to confirm, and they did not emphasize the role of the myeloid:erythroid ratio that we found significantly correlated with the molecular pattern. The absence of predictive value of dysgranulopoiesis in our study is worth mentioning, as is the fact that in the group of 55 Ph⁺ CML investigated in parallel, moderate dysgranulopoiesis of up to 50% of the cells or more was not unusual. Finally, this study shows that although there are a few (four) M-BCR⁻ patients who are by multiple parameters undistinguishable from typical CML, as reported by Kurzrock et al[13] and Selleri et al,[14] a careful examination of the myeloid:erythoid ratio in BM may help to reclassify them as atypical CML. Future studies of the underlying molecular abnormalities in this little subset of patients will certainly be of great interest, as these studies can give clues to mechanisms involved in the generation of CML.

Cytogenetics was also of some help in discriminating these two groups of patients: while none of the Ph⁻, M-BCR⁺ patients had karyotypic abnormalities at diagnosis, 7 of 23 M-BCR⁻ had an abnormal karyotype. This difference was statistically significant. This frequency of abnormalities also is in good agreement with most of reported data.[11,13,14] In their study of four Ph⁻, M-BCR⁺ patients, van der Plas et al[22] found two abnormalities that could be considered Ph variant translocations, while in the two remaining cases karyotype was normal at diagnosis and the abnormalities occurred later on in the evolution, as usual in Ph⁺ CML. The study by Kantarjian et al[8] does not segregate the abnormalities according to the molecular status.

The most striking finding to us was the similarity of the Ph⁻, M-BCR⁻ patients and the 100 CMML patients used for comparison. They were identical as far as age, sex ratio, spleen size, survival, hemoglobin, platelet count, and myeloid:erythroid ratio were concerned. Due to the heterogeneity of the therapies used, survival data have only an indicative value. Of course, there were significant differences (total WBC count, basophilia, high percentage of IMP in PB and monocytosis), but the study itself by its design could have produced these differences. While the 100 CMML patients were selected only on the basis of the FAB criteria for myelodysplastic syndromes (MDS),[19] the criteria of this Ph-CML study required a high percentage of granulocytes (which excludes the possibility for the monocytosis to be as high as in unselected CMML) and the presence of IMP in PB (which obviously results in a higher percentage than in the CMML group). In our study on CMML, we found a significant percentage of patients having characteristics (high number of IMP and basophils in PB) classically related to typical CML. Although not systematically investigated in all patients, it seems worth mentioning that 2 of 10 M-BCR⁻ patients had a monoclonal peak, a finding that has been reported in CMML.[15,16] The frequency and type of cytogenetic abnormalities were very similar to what is described in CMML,[12,15,16] except that no deletion of chromosome 7 (7q−/−7) was found in this study. Two patients (nos. 24 and 30) had an abnormality of the long arm of chromosome 5 (5q31), one of which, [t(5;12)(q31;p12-13)], has been reported only once in a CMML case.[28] Taking all these facts into account, we indulge on speculating that CMML and so-called Ph⁻ atypical CML might be two aspects of the same disorder, with a tendency for atypical CML to express in a more balanced way between the granulocytic and the monocytic lineage. This opinion is supported by two other studies: Cogswell et al[11] also speculated that atypical CML could be regarded as a subgroup of CMML, and Kurzrock et al[13] reported that the evolution of M-BCR⁻ patients was quite close to what could be expected in CMML.

In conclusion, our study confirms the good correlation between molecular, clinical, and morphologic findings in Ph⁻ CML, especially if the myeloid:erythroid ratio in BM is taken into account, and also raises the question of whether Ph⁻, M-BCR⁻ CML should continue to be regarded as an entity distinct from CMML.

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Appendix. Groupe Français de Cytogénétique Hématologique

Chairman: J. Tanzer, Poitiers
Secretary: P. Bernard, Bordeaux

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(Continued on following page)

Appendix. Groupe Français de Cytogénétique Hématologique (Cont'd)

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6.3 REVIEW OF CLINICAL, CYTOGENETIC AND MOLECULAR ASPECTS OF PH NEGATIVE CML

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10TH ANNIVERSARY ARTICLE

Review of Clinical, Cytogenetic, and Molecular Aspects of Ph-Negative CML

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ABSTRACT: *Between 1985 and 1989, many cases of Philadelphia (Ph) chromosome negative chronic myelogenous leukemia (CML) were reported. For this review, the following selection criteria were used: the original articles on Ph-negative cases should provide clinical, hematologic, cytogenetic as well as molecular data. In addition, eight unpublished cases of Ph-negative CML are included that were studied in our institute during the last two years. Our purpose was to correlate presence or absence of the Ph rearrangement with the clinical features in an attempt to test whether the entity "Ph-negative CML" really exists and to identify the pathologic characteristics, frequency of occurrence, prognosis for survival, and underlying molecular mechanisms. Data on Ph-negative CML patients were compared with data on Ph-positive CML, atypical CML (aCML), and chronic myelomonocytic leukemia (CMMoL), reported in the same papers as the Ph negative patients. Essential for comparison of data from the different investigators appeared to be a clear description of criteria they used to establish the diagnosis CML, or alternatively a complete presentation of data for all patients reported in the articles. In most cases, Ph-negative CML was distinguishable from CMMoL and aCML, using simple criteria, e.g., differential count of peripheral blood and absence of dysplasia in the bone marrow. Cytogenetic analysis showed normal karyotype in most cases of Ph-negative CML. Interestingly, in cases with abnormal karyotype, chromosome 9 band q34 was relatively frequently involved in translocations with other chromosomes than chromosome 22, suggesting a variant Ph translocation not visible by cytogenetic techniques. This assumption was confirmed by molecular analysis, demonstrating bcr–abl rearrangement in 9 out of 10 of the latter cases. Results of cytogenetic and molecular investigations in 136 cases of Ph-negative CML reviewed in this article clearly indicated that molecular techniques are valuable tools for identification of bcr–abl rearrangements, indicative for the Ph translocation. The different mechanisms responsible for bcr–abl rearrangement in Ph-negative CML patients are discussed. The question remains whether all Ph-negative CML patients will have bcr–abl rearrangements, or whether alternative mechanisms will be identified that are responsible for this disease.*

INTRODUCTION

Chronic myelogenous leukemia (CML) is a hematopoietic malignancy arising from neoplastic transformation of the pluripotent bone marrow stem cell. Standard findings at presentation are leukocytosis, increased granulopoiesis, sometimes increased thrombopoiesis, presence of immature granulocytic progenitors in peripheral blood, basophilia and/or eosinophilia, decreased leukocyte alkaline phosphatase (LAP), and hepatosplenomegaly. The course of the disease is biphasic. During the chronic phase, with a median duration of 1–4 years, the response to chemotherapy is usually good;

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thereafter, the disease transforms ineluctably into acute leukemia, the so-called blast crisis.

In 1960, Nowell and Hungerford [1] described the presence of a specific cytogenetic marker, the Philadelphia (Ph) chromosome, in blood and bone marrow cells of CML patients. The Ph chromosome was soon considered to be the most reliable biologic marker for diagnosis of CML. At the same time, a subset of Ph-negative CML patients was identified, i.e., CML patients in whom no Ph chromosome was found with the use of cytogenetic techniques. In early studies, these Ph-negative CML patients comprised 10%–15% of all CML cases [2–4]. Absence of the Ph chromosome correlated with early transformation to blast crisis and reduced survival, as compared to Ph-positive CML.

In subsequent years, progress has been made in nosological classification of hematopoietic disorders in the field of cytogenetics as well as in molecular biology, which increased our knowledge and understanding of these diseases. Relevant points for this review are described.

First, hematologic and morphologic characteristics of myelodysplastic syndromes (MDS) [5], myeloproliferative diseases (MPD) [6, 7], juvenile CML (jCML) [8], and ANLL [9] have been carefully defined, and the established criteria are increasingly used internationally.

Second, chromosomal rearrangement corresponding to the Ph chromosome has been demonstrated, i.e., t(9;22)(q34;q11) in more than 90% of the cases [10]. In 5% of the cases [11–15], variant translocations are identified in which additional chromosomes are involved. Usually the diminutive aspect of 22q− is conserved, although secondary rearrangements of the Ph chromosome may result in a 22q+ or 22p+ derivative, called masked Ph [14, 15].

Third, molecular rearrangement underlying the Ph translocation has been identified and was found to be quite consistent in all cases. Translocation of the c-*abl* oncogene is observed from chromosome 9 band q34 to a specific site in the *bcr* gene on chromosome 22 band q11, called the breakpoint cluster region (*BCR*) [16]. The newly formed chimeric *bcr–abl* gene on the Ph chromosome is transcribed into an 8.5-kb *bcr–abl* mRNA, which encodes a 210-kD *bcr–abl* protein. This gene product has enhanced tyrosine phosphokinase activity, as compared to the normal 145-kd *abl* protein [17–19].

Bcr–abl recombination takes place both in standard and variant Ph translocations [14, 20–24], even in cases in which chromosome 9 involvement is cytogenetically not detectable or when a masked Ph chromosome is present [14, 23, 24]. Demonstration of BCR breakpoints or detection of hybrid *bcr–abl* mRNA or protein in leukemic cells of CML patients can be used as an alternative for cytogenetics. The molecular rearrangement is that consistent, that its detection guarantees the presence of the Ph translocation. If necessary, the new or unusual chromosomal location of the hybrid *bcr–abl* gene can be investigated by in situ hybridization experiments on metaphase chromosomes, using *bcr* and *abl* probes.

In this article we review clinical, cytogenetic, and molecular data on Ph negative CML cases that have been reported during the last 4 years by different investigators. In addition, eight unpublished cases are included that were studied in our institute during the last 2 years. The purpose was to correlate presence or absence of the Ph rearrangement with clinical features in order to answer the following questions: 1) Does the entity "Ph-negative CML" exist? 2) What are the pathologic characteristics of Ph-negative CML, the frequency of occurrence, and prognosis for survival? 3) What molecular changes are responsible for the development of Ph-negative CML?

PATIENT POPULATION REVIEWED

During the last 4 years, at least 128 cases of Ph-negative CML were reported. Per definition, these cases presented with clinical features suggestive for CML and were cytogenetically characterized by absence of the Ph chromosome in the leukemic cells.

Table 1 Clinical, hematologic, cytogenetic, and molecular data of Ph-negative CML patients studied recently in our institute

Name (Sex/age [yr])	AP (F/56)	WK (M/71)	JS (M/67)	JV (M/74)	JW (M/23)	GJ (M/64)	LM (F/75)	KN (M/72)
WBC (10^9/L)	32.9	60	28.9	89	405.7	24.7	70.9	60
% Blasts	2	1	0	10	0	2	0	3
% Promyelocytes	0	1	0	9	12	2	3	2
% Myelocytes	7	9	2	9	18	3	5	6
% Metamyelocytes	2	10	5	3	18	10	4	5
% Bands	12	18	0	0	0	26	7	0
% Neutrophils	64	46	90	53	38	45	71	70
% Eosinophils	0	3	0	2	3	0	4	2
% Basophils	0	3	0	2	3	0	0	5
% Lymphocytes	11	3	3	10	0	11	5	4
% Monocytes	2	6	0	2	0	1	1	3
Thrombocytes (10^9/L)	153	505	508	504	263	315	81	268
RBC (10^{12}/L)	3.2	3.1	3.9	3	3.01	2.86	2.53	2
LAP	ND	ND	ND	ND	1 (N20–90)	ND	ND	42 (N50–100)
Hepato/splenomegaly	–	+	+	+	+	+	+	+
Karyotype	N	N	N	N	N	N	N	N
BCR breakpoint	–	–	–	–	+	–	–	–

Abbreviations: LAP, leukocyte alkaline phosphatase; N, normal; ND, not done; RBC, red blood cells; WBC, white blood cells.

All cases reviewed were also analyzed for molecular rearrangements, corresponding to the Ph translocation. Of an additional eight unpublished Ph-negative CML cases, investigated by us recently, the clinical, hematologic, cytogenetic, and molecular data are presented in Tables 1 and 3.

The possibility to apply molecular analysis is relatively recent, and as a consequence, long-term clinical follow-up and survival data, taking into account the molecular status of the patients often were not available. To the best of our knowledge, double reporting of cases was tracked down and avoided.

RESULTS AND DISCUSSION

Clinical and Hematologic Features of Ph-Negative CML

For 20 years, based on clinical, hematologic and cytogenetic data, the general opinion was that Ph-negative CML patients formed a separate group as compared to Ph-positive cases. Ph-negative CML cases are characterized by older median age (>65 years), monocytosis, thrombopenia, poor response to chemotherapy, rapid transformation to acute leukemia and shorter survival [2, 3, 21, 25]. In 1985 and 1986, respectively, Pugh et al. [26] and Travis et al. [27] initiated an interesting discussion. They extensively reviewed their cases previously classified as Ph-negative CML (25 and 22 cases, respectively), using strictly defined morphologic criteria for MDS [5] and MPD [6, 7]. Their conclusion was that all but one case had to be reclassified as MDS or MPD, rather than CML. This statement was exaggerated, since it was not taken into account, that clinical heterogeneity is also seen in Ph-positive CML cases. Indeed, in the presence of the Ph chromosome, diagnosis of CML is rarely questioned, and atypical features are easily disregarded. It was the merit of Spiers et al. [28] and Galton and colleagues [29–31] to establish hematologic and morphologic criteria for

CML, atypical CML (aCML), and CMMoL. Not all hematologists are in agreement with these rather strict proposals, but they reflect on it and describe their own discriminating features for establishing the diagnosis of CML. The criteria for CML followed by the different investigators are summarized in Table 2.

Generally, there is consensus on the most essential features, i.e., leucocytosis, basophilia, hepatosplenomegaly, absence of absolute monocytosis, and absence of MDS or ANLL characteristics. As a consequence, the percentage of cases of Ph negative CML has been reduced from 10%–15% [2–4] to less than 5%, mainly by elimination of cases fulfilling the presently established criteria for CMMoL, jCML, MDS, and ANLL. Discrimination between Ph-positive and Ph-negative CML is not possible using clinical or hematologic characteristics only. The remaining group of patients with Ph-negative CML still appears heterogeneous and comprises cases that are clinically and hematologically indistinguishable from Ph-positive CML, including long survival and good therapeutic response. Other cases are atypical but resemble CML more than other well defined hematologic disorders. These are designed as aCML [30, 31].

Cytogenetic Findings in Ph Negative CML

In 127 patients, cytogenetic studies were performed at diagnosis or during the chronic phase of CML. In 9 other patients analysis was performed after blastic transformation. During chronic phase, the karyotype was found to be normal in 48 patients (Table 3); abnormal in 15 cases (Table 4); and Ph-negative, not specifying other chromosomal abnormalities, in 64 cases (Table 5). Among the 15 abnormal karyotypes, 10 showed a translocation involving chromosome 9 band q34, which is the chromosomal site involved in the Ph translocation (Table 4A). This is highly suggestive for a variant Ph translocation, in which the microscopic aspect of chromosome 22 is not visibly altered. Molecular studies confirmed this assumption, as discussed later. The rest of this group of chronic-phase CML patients with cytogenetically abnormal karyotype showed random clonal abnormalities (Table 4B). In a few patients, other translocations are detected, usually associated with subtypes of ANLL such as t(8;21), described by Wiedemann et al. [31], in a patient with atypical CML (Table 4D).

Three out of nine cases in blast crisis showed cytogenetic abnormalities (Table 4C). Remarkably, trisomy 8 and i(17q) were found in the latter cases [32]. These abnormalities are identical to the ones associated with blastic transformation of Ph-positive CML. The resemblance between Ph-negative and Ph-positive CML is also expressed in the clonal and multipotent stem cell origin of both Ph-positive and Ph-negative CML [33] and in the occurrence of lymphoid, myeloid, mixed and undifferentiated blast crisis of Ph-negative and Ph-positive CML [34, 35].

Molecular Investigations in Ph-Negative CML

The purpose of molecular investigations in Ph-negative CML is to identify the patients with bcr–*abl* rearrangement on DNA, RNA, or protein level. Comparison of cytogenetic, molecular, and clinical data between Ph-negative CML patients with or without *bcr* rearrangement and Ph-positive CML patients is important to determine the functional meaning of the Ph chromosome itself. Therefore, the strategy followed by all investigators was to screen Ph-negative CML cases for:

1. The presence of *BCR* breakpoint using Southern blot analysis.
2. Localization of c-*abl*, *bcr*, and c-*sis* oncogenes on the chromosomes applying in situ hybridization techniques.
3. Expression of bcr–*abl* mRNA using Northern blot, RNAse protection assay, or polymerase chain reaction (PCR) techniques. Both the RNAse protection assay and PCR technique give the opportunity to identify which *BCR* exon is fused to *abl*. In CML patients with t(9;22), usually *BCR* exon 2 (b2) or *BCR* exon 3 (b3) is fused to *abl* exon 2 (a2), resulting in b2a2 or b3a2 bcr–*abl* fusion region [36].

Table 2 Clinical criteria used to establish CML diagnosis and number of patients reviewed

Reference	Kurzrock et al. [44]	Bartram et al. [32, 47, 58]	Fitzgerald et al. [59]/ Morris et al. [46]	Dreazen et al. [41]/ Ganesan et al. [42]	Kantarjian et al. [60]	Ohyashiki et al. [61]	Shepherd et al. [30]/ Wiedemann et al. [31]	Others[a] [34, 37, 42, 43, 45, 55, 62–67]
Splenomegaly		+		+				
WBC (10^9/L)	>20	>30[b]		>100	>20	>20		
Absolute basophilia in PB		+	+	+			+	
Lack of absolute monocytosis (i.e., <1000/mm^3)	+		+			+		
Decreased LAP		+						
BM hyperplasia without dysplasia	+	+	+		+	+	+	
Absence of ANLL features, <20% myeloblasts + promyelocytes in BM at presentation	+	+				+		
Absence of MDS features	+	+			+	+		
Other criteria		[c]		[d,e]			[f]	
No. of patients with CML	4	41	2	4	23	1	25[g]	19
No. of patients with CML BC		3				1		5
No. of patients with aCML				5		3	10	7

[a]No detailed criteria for CML diagnosis were presented in these case reports and papers, but often clinical and hematologic data of the individual patients were mentioned.

[b]WBC > 100 × 10^9 L in Ref. [58].

[c]All stages of neutrophilic series present in differential count and good response to hydroxyurea or busulphan.

[d]Platelets more than 300 × 10^9 L.

[e]Survival more than 1 year from diagnosis.

[f]Peaks of neutrophils and myelocytes plus metamyelocytes in differential count, more than 15% immature granulocytes in peripheral blood.

[g]CML patients in which the diagnosis was not verified are included [31].

Table 3 Molecular data on CML patients with normal karyotype of leukemic cells

No. of cases	BCR breakpoint	bcr-abl mRNA	P210 bcr-abl	In situ hybridization	Reference
A. Patients with CML (n = 48)					
1	ND			c-*sis* on 22	Bartram et al. (1984) [62]
1	+	−		c-*abl* on 9q34	Bartram et al. (1985) [47]
1	−		−		Kurzrock et al. (1986) [44]
2	−		ND		Kurzrock et al. (1986) [44]
1	+				Bartram et al. (1986) [58]
6	−				Bartram et al. (1986) [58]
1	+			c-*abl* on 9q/22q c-*sis*, 3′*bcr* on 22	Morris et al. (1986) [46] and Fitzgerald et al. (1987) [59]
1	+			ND	Morris et al. (1986) [46] and Fitzgerald et al. (1987) [59]
1	+[a]	ND		ND	Ganesan et al. (1986) [42] and Dreazen et al. (1987) [41]
1	+	+, (b_2a_2 + b_3a_2)		c-*abl* on 9/22 *bcr* on 22q11 c-*sis* on 22	Ganesan et al. (1986) [42] and Dreazen et al. (1987) [41]
1	+	+, (b_3a_2)		c-*abl*, *bcr* on 22q11	Dreazen et al. (1987) [41]
1	+	ND		c-*abl*, *bcr* on 22q11	Dreazen et al. (1987) [41]
1	+				Ohyashiki et al. (1988) [61]
2	+				Weinstein et al. (1988) [63]
1	+				Eisenberg et al. (1988) [64]
7[c]	+		+(4/4)[b]		Wiedemann et al. (1988) [31]
4[c]	−		−		Wiedemann et al. (1988) [31]
4	+				Bartram et al. (1988) [32]
1	+				LoCoco et al. (1989) [65]
1	+	+, (b_2a_2)		5′*bcr*, c-*abl* on 1p 3′*bcr* on 9 c-*sis* on 22	Van der Plas et al. (1989) [45]
1	+	+, (b_3a_2)		5′*bcr*, c-*abl* on 1p 3′*bcr*, c-*sis* on 22	Van der Plas et al. (1989) [45]
1	+	+, (b_2a_2)			Van der Plas et al. (this report)

Table 3 (*Continued*)

No. of cases	BCR breakpoint	bcr-abl mRNA	P210 bcr-abl	In situ hybridization	Reference
7	–	– (5/5)[b]			Van der Plas et al. (this report)
B. Patients with CML BC (n = 6)					
1	–				Bartram et al. (1986) [58]
4			–		Maxwell et al. (1987) [34]
1	+				Ohyashiki et al. (1988) [61]
C. Patients with atypical CML (n = 16)					
1	+[a]				Ganesan et al. (1986) [42]
1	+				Ganesan et al. (1986) [42]
2	+	+ (b_2a_2)			Dreazen et al. (1987) [41]
2	–				Ohyashiki et al. (1988) [61]
4	–		–, (2/4)[b]		Wiedemann et al. (1988) [31]
6	–				Cogswell et al. (1989) [55]

Abbreviation: ND, not done.

[a]Extra bands in one restriction enzyme digest.

[b]Number of cases observed/number of cases investigated.

[c]CML diagnosis could not be verified.

4. Detection of 210 kD *bcr–abl* protein (P210), e.g., by means of autophosphorylation assays.

The results of these molecular investigations are presented in detail in Tables 3–6 together with the corresponding cytogenetic data. (An overview of these data for CML patients is provided in Table 7.)

In summary, we can make the following points:

1) Fifty-eight out of 136 Ph-negative CML patients (including the cases in which CML diagnosis could not be verified [31]) showed evidence of *BCR* rearrangement, *bcr–abl* mRNA expression, or the presence of a 210 kD *bcr–abl* protein.

2) Southern blot analysis detected a BCR breakpoint in 9 out of 10 Ph-negative CML patients with cytogenetic abnormalities involving chromosome 9 band q34, indicative for variant Ph translocation. Only one patient showed involvement of chromosome 9 band q34 without *BCR* rearrangement, although clinical and hematologic data were in favor of *CML* diagnosis [37]. However, it should be noticed that molecular data are scarce in this article: No details are mentioned about number of restriction enzyme digestions or probes used. Therefore, it cannot be ruled out that this patient also has a *BCR* rearrangement that was not detected in this study. When really no *BCR* breakpoint can be found using Southern blot analysis, it is worthwhile to search for a breakpoint more 5′ in the BCR gene, e.g., using PCR technique on cDNA or pulse field gel electrophoresis (PFGE) on DNA. This case is possibly comparable with Ph-positive, *BCR*-negative cases that are described by Selleri et al. [38, 39] and had a breakpoint in the first intron of the *BCR* gene or with the Ph-positive *BCR*-

Table 4 Cytogenetic and molecular data on patients with abnormal karyotype of leukemic cells

No. of cases	Karyotype	*BCR* breakpoint	*bcr–abl* mRNA	P210 *bcr–abl*	Reference
A. CML patients with translocations involving 9q34 (n = 10)					
1	t(9;12)(q34;q21)[a]	+			Bartram et al. (1985) [43]
1	t(9;11)(q34;q13)	+	+	+	Kurzrock et al. (1986) [44]
1	t(8;9)(?;q34)	+			Bartram et al. (1988) [32]
1	t(9;18)(q34;?)	+			Bartram et al. (1988) [32]
1	t(9;12)(q34;q13)	+			Weinstein et al. (1988) [63] and Eisenberg et al. (1988) [64]
1	t(9;11)(q34;q11)	+			Weinstein et al. (1988) [63] and Eisenberg et al. (1988) [64]
1	t(8;9)(q22;q34)	+			Weinstein et al. (1988) [63]
1	t(2;9)(?;q34)	+			Wiedemann et al. (1988) [31]
1	t(3;7)(q21;q32), t(4;9)(q21;q34),del(8)(q22)	–[b]			Wang et al. (1988) [37]
1	t(9;9)(p13;q34)	+			Sessarego et al. (1989) [67]
B. CML patients with translocations not involving 9q34 (n = 5)					
1	t(20;21)(q11;q22)	+			Weinstein et al. (1988) [63]
1d	N/t(5;6)	+		ND	Wiedemann et al. (1988) [31]
1d	t(3;5)	–		ND	Wiedemann et al. (1988) [31]
1d	t(9;15)(q22;q22),t(11;20)	–		–	Wiedemann et al. (1988) [31]
1	t(11;22)(q23;q13),del 7q, del(13)	–		–	Wiedemann et al. (1988) [31]
C. CML patients in BC with abnormal karyotype, 9q34 not involved (n = 3)					
1	46,XY/47,XY,+8	–			Bartram et al. (1986) [58]
1	46,XY/46,XY,i(17q)	–			Bartram et al. (1986) [58]
1	46,XX,t(7p–q+,13q+,13q–),	ND	+		Andrews et al. (1987) [66]
D. Atypical CML patients with abnormal karyotype (n = 4)					
1	del(16)(q22)	+[c]			Ganesan et al. (1986) [42] and Dreazen et al. (1987) [41]
1	7q–	–			Ohyashiki et al. (1988) [61]
1	t(8;21)	–			Wiedemann et al. (1988) [31]
1	47,XY,+8	–			Cogswell et al. (1989) [55]

Abbreviation: ND, not done.

[a]Results in situ hybridization studies: c-*abl* on 12q–, 5′-*bcr* on 12q–, 3′-*bcr* on 9q+, c-*sis* on 22.

[b]No detailed molecular data presented in this article.

[c]Extra bands in one restriction enzyme digest only.

[d]CML patient in which diagnosis could not be verified.

? Localization of breakpoint not mentioned.

Table 5 Molecular data on patients with no abnormalities of chromosome 22 in leukemic cells (karyotype not further specified)

No. of cases	*BCR* breakpoint	*bcr–abl* mRNA	P210 *bcr–abl*	Reference
A. CML patients (n = 64)				
6	+			Shepherd et al. (1987) [30]
2	–			Shepherd et al. (1987) [30]
4	–			Eisenberg et al. (1988) [64]
11	+	5/5 +		Kantarjian et al. (1988) [60]
12	–			Kantarjian et al. (1988) [60]
1[a]	–		–	Wiedemann et al. (1988) [31]
27	–			Bartram et al. (1988) [32]
1	+			LoCoco et al. (1989) [65]
B. Atypical CML patients (n = 5)				
4	–			Shepherd et al. (1987) [30]
1	–			Wiedemann et al. (1988) [31]

[a]CML patient in which diagnosis could not be verified [31].

negative CML patient described by Bartram et al. [40], which had a breakpoint in the *bcr* gene located 5′ of the *BCR* region but 3′ of the region described by Selleri et al. [38, 39]

3) In 20 out of 25 cases of aCML, no *BCR* breakpoint was detected. The five exceptions with a *BCR* breakpoint were all reported by the same research group [41, 42]. It would be interesting to reexamine the differential count and other clinical data to check if these patients really belong to the group of aCML or resemble more CML. To the best of our knowledge, no CMMoL or juvenile CML cases are published in which a *BCR* rearrangement was identified. In conclusion, *bcr–abl* rearrangement is strongly associated with the morphologic features of CML, although few exceptions still exist.

4) The percentage of Ph-negative CML patients with *BCR* rearrangement versus no *BCR* rearrangement varied between the different authors, e.g., Bartram et al. [32, 58] reported 3 out of 12 cases *BCR*-positive; Ganesan et al. [42] and Dreazen et al. [41], 5 out of 5; Fitzgerald and Morris [46, 59], 2 out of 2; Wiedemann et al. [31], 8 out of 8 (5 out of 9 among the cases that were not morphologically reexamined); Kantarjian et al. [60], 11 out of 23; and our group, 5 out of 12 [45, this report]. In our opinion, there are two main reasons responsible for these differences. First, the different authors used clinical, hematologic and morphologic criteria that are not exactly the same, resulting in differences in diagnosis. Second, some authors [41, 42] diagnosed *BCR* breakpoints on extra bands in only one out of several different restriction enzyme

Table 6 Molecular data on CMMoL patients

No. of cases	*BCR* breakpoint	*bcr–abl* mRNA	P210 *bcr–abl*	In situ hybridization	Reference
A. Normal karyotype (n = 3)					
2	–				Morris et al. (1986) [46]
1	ND			c-*abl* on 9q	Fitzgerald et al. (1987) [59]
B. Ph negative, karyotype not further specified (n = 18)					
1	–				Shepherd et al. (1987) [30]
17	–				Kantarjian et al. (1988) [60]

Abbreviation: ND, not done.

digests. In such cases, the occurrence of a restriction enzyme polymorphism is a more likely cause for the aberrant fragment than the presence of a *BCR* breakpoint. In such cases, additional analysis, e.g., at the protein or RNA level, is required to prove *bcr–abl* rearrangement.

5) Molecular data presented in Tables 3–5 indicate that several mechanisms can play a role in Ph-negative CML. A summary follows.

Bcr–abl recombination takes place in the same way as in Ph-positive CML but is cytogenetically not visible. Examples of complex Ph translocations in Ph-negative CML are provided by Bartram et al. [43], Kurzrock et al. [44] and our data [45]. In situ hybridization studies of Ph-negative CML patients reported by Bartram et al. [43] and our own group [45] provided evidence that 5'-*bcr* and c-*abl* were localized on the same chromosomal segment. However, in these special cases, the hybrid *bcr–abl* gene was present on a third chromosome instead of on the Ph chromosome. In these cases, the localization of the hybrid *bcr–abl* gene indicated that complex Ph translocations had occurred, although the aspect of chromosome 22 was visibly unaltered.

Insertion of part of the *abl* gene in the *bcr* gene without reciprocal translocation to chromosome 9 has been described by Morris et al. [46] and Dreazen et al. [41].

Based on investigations in a Ph-negative CML patient in which BCR was rearranged without juxtaposition of c-*abl*, Bartram [47] proposed the hypothesis that *bcr* or *abl* can work in combination with yet another oncogene. Thus far, there is no further evidence for this hypothesis.

Several other possibilities remain open for discussion in Ph-negative, BCR-negative cases indistinguishable from Ph-positive CML on clinical and hematologic as well as morphologic criteria. Three hypothetic mechanisms could explain these phenomena:

1) The breakpoint might be located outside the BCR, but within the BCR gene as described by Selleri et al. [38, 39] and Bartram et al. [40] in Ph-positive CML cases. Both authors reported breakpoint localizations more 5' in the *BCR* gene.

2) Abl possibly cooperates with an as yet unknown oncogene.

3) Neither *bcr* nor *abl* are responsible for the disease in exceptional cases, but other oncogenes might be. Thus far, the few data available on this subject do not identify candidate genes for this latter hypothesis [48–54]. Recently, Cogswell et al. [55] reported that using the polymerase chain reaction very few *ras* mutations were detectable in CML, i.e., in 1 out of 18 Ph-positive CML patients in blast crisis and in 0 out of 39 Ph-positive CML cases in chronic phase. However, in Ph-negative, BCR-negative atypical CML (aCML), they [55] demonstrated the presence of *ras* mutations in 54% (i.e., 7/13) of the cases. This high frequency of *ras* mutations is comparable with results obtained by Padua et al. [56] in CMMoL patients. CMMoL and aCML also share several clinical and hematologic features. The authors therefore conclude that aCML is a subgroup of CMMoL and that both diseases belong to MDS rather than CML.

CONCLUSION

Correct diagnosis of CML is essential when efforts are made to correlate clinical features with molecular changes in Ph-negative CML. The data reviewed in this article do not identify any clinical or hematologic characteristic that is unique for Ph-negative CML. We expected that in nearly all Ph-negative CML patients, indistinguishable from Ph-positive CML on clinical and hematologic grounds, *bcr–abl* rearrangement will be detected using molecular analysis. The data on Ph-negative CML reviewed in this article show the presence of *bcr–abl* rearrangement in 43% of the cases (Table 7). Although no evidence was found for *bcr–abl* rearrangement in the remaining 57%, in many cases no definitive proof was provided to rule out this possibility. On the other hand, it can not be denied that several CML patients are reported with classical CML disease without the presence of *bcr–abl* rearrangement. Very recently, this was confirmed by Kurzrock et al. [57], who reported on 11 Ph-negative, BCR-negative CML

Table 7 Distribution of Ph-negative CML patients in chronic phase or blast crisis according to results of cytogenetic and molecular studies

No. of cases	Karyotype	Molecular evidence for BCR rearrangement	
		Yes	No
54	Normal	28	26
10	Abnormal, 9q34 involved	9	1
8	Abnormal, q34 not involved	3	5
64	Ph-negative, karyotype not specified	18	46

cases investigated in the MD Anderson Cancer Center using Southern and Northern blot analysis. They represented about 3% of the CML cases studied in the same period in that institute. In addition to our findings, Kurzrock et al. reported that, although the early stage of BCR-negative and BCR-positive CML shows striking resemblance, disease progression manifests distinctly.

In the Ph-negative patients (the aCML patients) that do not fulfill all criteria for CML, a more heterogeneous picture can be expected, showing activation of other oncogenes than *bcr* and *abl* e.g., *ras*, in some cases.

A controlled multicenter study of Ph-negative CML patients who are clinically, hematologically, and cytogenetically well characterized should form the basis for future molecular investigations necessary to elucidate the mechanisms responsible for Ph negative CML and to apply this knowledge to determine choice of therapy and prognosis.

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CHAPTER 7

CYTOGENETIC AND MOLECULAR DIAGNOSIS OF PH POSITIVE ACUTE LEUKEMIA

7.1 BCR-ABL REARRANGEMENT IN ACUTE LEUKEMIA

The Ph chromosome is quite rare in acute leukemia. It is observed in 2-3% of the patients with AML, in 2-10% of the childhood ALL cases, and in 20-30% of the adult ALL cases.
The molecular biology of the Ph translocation is reported in detail in the paper by Hermans et al, that is included in chapter 3.
In this chapter we present the results of molecular investigations performed in acute leukemia patients in order to detect *bcr-abl* rearrangement.

MATERIALS AND METHODS

see chapter 4.1.

RESULTS

We studied 6 Ph positive ALL cases and 8 acute leukemia patients with cytogenetic aberrations involving either chromosome 9 or 22.
A summary of the results of molecular analysis in acute leukemia patients is presented in table 1.
In 3 out of 6 Ph positive ALL patients a breakpoint in the BCR region was detected using Southern blot analysis. In the remaining three patients PCR analysis showed expression of *bcr-abl* mRNA with an e1a2 junction, indicating that the breakpoint in the *bcr* gene had occured in the first intron.

Furthermore, we investigated by PFGE 4 ALL and 1 AML patient, who were suspected to have variant Ph translocations. All 5 patients showed chromosomal aberrations involving the q arm of chromosome 9, but had normal chromosomes 22. No rearrangements in the *bcr* gene were observed in these patients. However, in 1 ALL and one AML patient an aberrant restriction fragment was observed in the BsshII digest after hybridization with the K38 abl probe (i.e. *3' abl* sequences).
Additional investigations using PCR analysis showed that *bcr-abl* mRNA was not expressed in both patients.
In conclusion: Possibly the *abl* gene is rearranged in the absence of *bcr* rearrangement. Another possibility is that the additional *abl* fragment is caused by CpG methylation of a BsshII site instead of by a breakpoint. Additional PFGE using another restriction enzyme could provide further evidence for the presence of a

breakpoint in *abl*, and in situ hybridisation using *abl* probe will demonstrate if the *abl* gene is translocated to another chromosome.

We analyzed 3 AML patients who were also suspected to have variant Ph translocations, because their karyotypes showed translocations involving chromosome 22 band q11, but no involvement of chromosomes 9. Molecular analysis failed to provide evidence for the presence of *bcr-abl* recombination in these 3 patients.
In conclusion: in none of the acute leukemia patients having chromosome 9q or chromosome 22q11 abnormalities we were able to prove a variant Ph translocation by detection of *bcr-abl* rearrangement. However in all ALL patients with t(9;22) we detected *bcr-abl* rearrangement. The latter is important, because it can be used as tumor specific marker for follow up during treatment, especially for detection of minimal residual disease. Moreover the presence of the Ph translocation in Ph positive acute leukemia is now recognized as a high risk feature by itself, independent of hematological parameters and thus important for the choice of therapy.

Table 1: Molecular analysis in acute leukemia

Patient	*Diagnosis*	*Karyotype*	*Southern*	*PFGE*	*PCR*
1	Ph+ALL	t(9;22),*	BCR+		b3a2
2	Ph+ALL	t(X;5;9;22),*	BCR+		
3	Ph+ALL	t(9;22),*	BCR+		
4	Ph+ALL	t(9;22),*	BCR-		e1a2
5	Ph+ALL	t(9;22)	BCR-		e1a2
6	Ph+ALL	t(9;22)	BCR-		e1a2
7	ALL	9q+,*		-	
8	ALL	9q on 7p,*		-	
9	ALL	9q+,*		-	
10	ALL	del(9q21q34),*		abl+,bcr-	no bcr-abl mRNA
11	AML	t(6;Mar9),*		abl+,bcr-	no bcr-abl mRNA
12	AML	t(21;22),*	BCR-	-	no bcr-abl mRNA
13[1)]	AML	t(12;22)	BCR-	-[2)]	no bcr-abl mRNA
14	AML	t(1;22),*		-	

* : also other chromosomal abnormalities present.
\- : no breakpoint found in bcr or abl gene using PFGE.
1) : this patient is reported in more detail in chapter 7.2.
2) : possibly breakpoint 5' of *bcr* gene.

7.2 MOLECULAR ANALYSIS OF A NEW CHROMOSOMAL REARRANGEMENT IN ACUTE NONLYMPHOCYTIC LEUKEMIA

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ABSTRACT

In the bone marrow cells of two patients with acute nonlymphocytic leukemia (ANLL) we demonstrated t(12;22)(p13;q11). Because the breakpoint on chromosome 22 is cytogenetically the same as in the Ph translocation and the appearance of the 22q- chromosome in t(12;22) is cytogenetically indistinguishable from the Ph chromosome we thought that this translocation might be a variant Ph translocation, in which the involvement of chromosome 9 was not detectable using chromosomal analysis. Therefore we investigated one of these patients for the presence of *bcr-abl* rearrangement was. Using Southern blotting, pulsed field gel electrophoresis, PCR analysis, and in situ hybridization we found that no *bcr-abl* rearrangement was detectable in bone marrow cells of this ANLL patient with t(12;22). Additional experiments are required to determine the exact breakpoint localization on chromosome 12 and 22.

INTRODUCTION

In acute nonlymphocytic leukemia (ANLL) many chromosomal aberrations are identified that are associated with specific hematological subgroups of the French-American-British (FAB) classification e.g. t(8;21)(q22;q22q) in FAB M2 and inv 16(p13-q22) in FAB M4.
In the bone marrow cells of two ANLL patients we identified t(12;22)(p13;q11). This translocation was recently reported by Callen et al (1991) as a new chromosomal rearrangement in ANLL. Because the breakpoint on chromosome 22 is cytogenetically the same as in the Ph translocation and the appearance of the 22q- chromosome in t(12;22) is cytogenetically indistinguishable from the Ph chromosome we thought that this translocation might be a variant Ph translocation, in which the involvement of chromosome 9 was not detectable using chromosomal analysis. In one of these patients, a 19 year old woman with ANLL M4, blood and bone marrow cells were available to perform molecular analysis with the aim to find out if *bcr-abl* rearrangement was detectable in this ANLL patient with t(12;22).

METHODS

See chapter 4.

RESULTS AND DISCUSSION

Chromosome analysis of bone marrow cells of ANLL patient 1 and 2 showed the karyotype 46,XY,t(12;22)(p13;q11), and 47,XX,+8,t(12;22)(p13;q11) respectively. Partial karyotype of patient 2 is shown in figure 1.

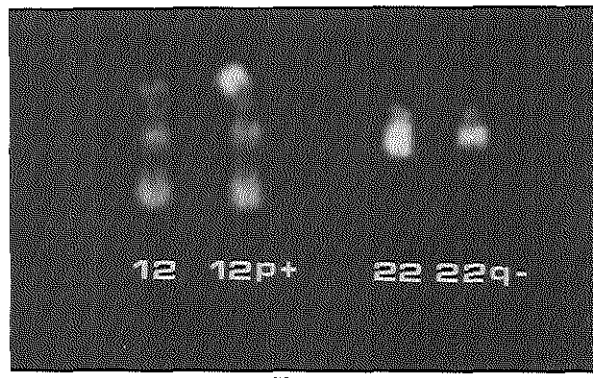

Figure 1: R banding: Partial karyotype of patient 2, an AML patient with t(12;22)(p13;q11).

Molecular analysis was performed on bone marrow cells of patient 2, showing the following results:

Southern blot analysis of DNA digested with BglII, BamHI, EcoRI, and PstI and hybridized to 5'-BCR, 3'-BCR and universal BCR probe showed no aberrant restriction fragments indicating the absence of a breakpoint in the BCR region (results not shown).

In situ hybridization on metaphases showed that:
5'-BCR, 3'-BCR and *c-sis* probes all were localised on chromosome 12p+, and on the normal chromosome 22.
3'-abl probe was localised on chromosome 9.
Conclusion: the *abl* gene is not translocated to chromosome 22 q- as is the case in the Ph translocation. The breakpoint on chromosome 22 in t(12;22) is located 5' of the BCR region of the *bcr* gene.

Pulsed field gel electrophoresis (PFGE) was performed using DNA digested with the rare cutting restriction enzymes BsshII and MluI in order to investigate the presence of breakpoints in the *bcr* and *abl* gene. A restriction map of the *abl* and *bcr* gene is presented in chapter 4.1 figure 2. No breakpoint was detected in the BsshII digest using the following probes: an 800 bp Pst 1 *bcr* cDNA probe (*bcr* exon e1), a 600 bp PstI- EcoR1 *bcr* cDNA probe (*bcr* exon 2, 3 and several small exons) and *3'-abl* cDNA sequences (K38). These findings exclude the presence of a breakpoint in the *bcr* or *abl* gene.

In the Mlu1 digest besides the normal 1000 Kb bcr fragment, an aberrant restriction fragment was detected after hybridization with the 800 bp Pst1 *bcr* cDNA probe. Hybridization with *3'-abl* probe (K38) revealed no aberrant bands.
We conclude that the aberrant restriction fragment can be explained by either a breakpoint in this Mlu1 fragment (that reaches 800 Kb more 5' than the BsshII fragment) or by CpG methylation of a Mlu 1 site. Additional restriction enzyme digestions and hybridization to probes located 5' to the *bcr* gene are necessary to find out which of the two explanations is correct.

Polymerase chain reaction (PCR).
Using the PCR technique to amplify the *bcr-abl* junction no *bcr-abl* mRNA expression was detected. Coamplification of a normal *abl* fragment, that serves as internal control on the quality of the RNA, the cDNA synthesis and the PCR reaction worked nicely.

From the molecular analysis it can be concluded that the *bcr* and *abl* genes are not involved in t(12;22) in the ANLL patient 2.
We did not yet systematically analyze the breakpoint on chromosome 12, but hybridization of the Southern blots to probes containing sequences of the Ki-ras oncogene, that map on 12 p13, did not show aberrant restriction fragments.

To the best of our knowledge, this is the first patient with t(12;22) that has been investigated at the molecular level. t(12;22) as sole structural chromosomal abnormality is very rare in ANLL patients as can be seen in table 1. t(12;22) does not seem to be associated with one special FAB subtype of ANLL. The breakpoint on chromosome 22 is reported to occur in band q11 or q12 by the different authors. We think that all authors described the same translocation and that differences between the two chromosomal breakpoints is often hardly visible.
This translocation has also been reported in a few patients with CML (Engel et al, 1977; Verma et al, 1979). In CML t(12;22) often appears to be a variant Ph translocation i.e. t(9;12;22) (Bartram et al, 1985).
In MDS translocations involving chromosome 12p13 have been reported to be strongly associated with eosinophilia (Keene et al, 1987).

In conclusion t(12;22)(p13;q11 or q12) has been reported as the sole structural aberration in 8 ANLL patients (including the 2 patients reported in this paper).
In this paper we demonstrated that in one ANLL patient t(12;22)(p13;q11) does not appear to be a variant Ph translocation, although the 22q- chromosome in t(12;22) is cytogenetically indistinguishable from the Ph chromosome.

Table 1: Reported ANLL cases with t(12;22) as sole structural chromosomal abnormality

Patient	*FAB*	*Karyotype*	*Reference*
1	M1	46,XY,t(12;22)(p12;q11)	Hagemeijer et al, 1981
2	M2	47,XX,+8,t(12;22)(p13;q12)	Mittelman, 1988
3	M2	47,XX,+8,t(12;22)(p13;q12)	Mittelman, 1988
4	M2	47,XX,+8,t(12;22)(p13;q12),-21,+r	Mittelman, 1988
5	M4	47,XX,+8,t(12;22)(p13,q12)	Callen et al, 1991
6	M4	47,XX,+8,t(12;22)(p13;q12)	Callen et al, 1991
7	?	46,XY,t(12;22)(p13;q11)	this report
8	M4	47,XX,+8,t(12;22)(p13;q11)	this report

?: FAB classification unknown

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CHAPTER 8

CYTOGENETIC AND MOLECULAR DIAGNOSIS IN PH POSITIVE MYELODYSPLASTIC SYNDROME

8.1 CYTOGENETIC AND MOLECULAR STUDIES OF THE PHILADELPHIA TRANSLOCATION T(9;22) OBSERVED IN A PATIENT WITH MYELODYSPLASTIC SYNDROME

Leukemia 1989, 3: 236-238

BRIEF COMMUNICATION

Cytogenetic and Molecular Studies of the Philadelphia Translocation t(9;22) Observed in a Patient with Myelodysplastic Syndrome

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We report here the case of a patient with refractory anemia with excess of blasts (RAEB) which evolved into RAEB in transformation. The standard Philadelphia (Ph) chromosome was found by cytogenetic study at diagnosis and during evolution. Southern blot analysis showed breakpoint cluster region (*bcr*) rearrangement as observed in chronic myelogenous leukemia (CML).

INTRODUCTION

PHILADELPHIA (Ph) chromosome is a very unusual feature outside chronic myelogenous leukemia (CML) and some acute leukemias. We report here the case of a patient with refractory anemia with excess of blasts (RAEB) whose bone marrow cells showed a standard Ph chromosome identified both by cytogenetic and molecular studies.

CASE REPORT

An 85-year-old male patient was referred to our hospital in July 1985 for evaluation of an aregenerative anemia revealed by an asthenia. He had no pathological records. Physical examination was normal except for pallor. There was no splenomegaly. Peripheral blood showed RBC count of 2.12×10^{12}/liter; hemoglobin level was 6.1 g/100 ml; mean cell volume (MCV) was 100 fl and reticulocytes were 10×10^9/liter; WBC count was 3×10^9/liter consisting of 20% (600/mm^3) polymorphonuclears, 2% (60/mm^3) eosinophils, 0% basophils, 52% (1560/mm^3) lymphocytes, 20% (600/mm^3) monocytes, 4% (120/mm^3) atypical monocytes, 1.5% (45/mm^3) blast cells, and 0.5% (15/mm^3) myelocytes. Platelets were 286×10^9/liter; some of them were giant platelets. Serum iron was 39 μM ($N < 27$) with iron binding capacity at 42.6 μM ($N = 48.6$–67.2). Lactate dehydrogenase (LDH) was 350 IU/liter ($N < 330$). Ferritin was 305 ng/ml ($N > 10 < 200$). A2 hemoglobin was 3.50% and F hemoglobin was 0.90%. The serum vitamin B_{12} level was 996 μg/ml ($N < 750$). Serum lysozyme was normal; leucocyte alkaline phosphatases were not scored because of reduced numbers of polymorphonuclears. Bone marrow aspiration was hypercellular with 71% of myeloid cells including 16% myeloblasts, 23% promyelocytes, 22% myelocytes, 4% metamyelocytes, and 6% polymorphonuclears; erythroblasts were 16% and showed dyserythropoiesis. Abnormal megakaryocytes were observed. Diagnosis of RAEB was made, according to the FAB classification (1).

He was only treated with blood cell transfusions. His hematological condition remained stable for 18 months until February 1987. Blood transfusion frequency was increased; WBC count decreased to 2.0×10^9/liter consisting of 2% (40/mm^3) polymorphonuclears, 0% eosinophils, 1% (20/mm^3) basophils, 48% (960/mm^3) lymphocytes, 3% (60/mm^3) monocytes, 38% (760/mm^3) atypical monocytes, 1% (20/mm^3) myelocytes, and 7% (140/mm^3) blast cells. In March 1987, bone marrow aspiration showed a rise of myeloblasts which were at 23%. He was then considered in RAEB in transformation (19 months after diagnosis). His physical condition worsened, and he died in May 1987 (21 months after diagnosis of RAEB and 2 months after occurrence of RAEB-t).

CYTOGENETICS

Cytogenetic studies were carried out on bone marrow cells using short-term culture (24 and 48 hr). Chromosomes were stained using RHG techniques. At diagnosis (August 1985), all 22 mitoses examined showed the standard Ph chromosome: t(9;22)(q34;q11) (Fig. 1). In March 1987, second cytogenetic studies were done in Rotterdam and Paris. All 39 metaphases examined showed the standard Ph translocation. No other changes were seen, and cells with a normal male karyotype were not detected as well at diagnosis as during evolution.

MOLECULAR STUDIES

Southern blot analysis was performed: 15 μg DNA was digested with *Bgl*II, *Bam*HI, and *Hind*III, separated by electrophoresis on a 0.7% agarose gel, blotted onto nylon membranes, and hybridized to the bcr probe, which was oligolabeled according to Feinberg and Vogelstein (2). The breakpoint cluster region (bcr) probe designated *phl/bcr* 3 (3) encompassed the entire breakpoint cluster region with additional 5′ and 3′ flanking segments. A 1.5-kb *Hind*III fragment containing repetitive sequences was deleted in this probe. Southern blot analysis showed hybridization of the bcr probe to the expected germ line bands and to one additional band in *Bgl*II and *Bam*HI DNA digests and to two additional bands in *Hind*III digests (Fig. 2 lane 3). These findings indicate a breakpoint in the part of the *bcr* gene recognized by this probe (bcr region). These results are in accordance with our observations in Ph(+) CML (Fig. 2, lane 2). The Southern blot of a Ph-negative patient showed only the germ-line bands (Fig. 2, lane 1).

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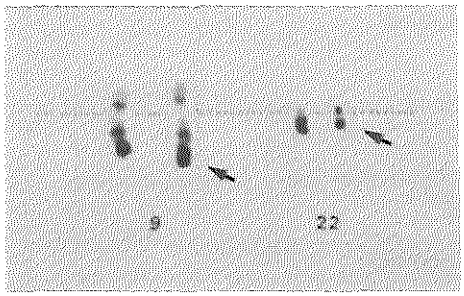

Figure 1. Partial karyotype of bone marrow cells of RAEB patient showing the t(9;22) (q34;q11) R-bands.

DISCUSSION

The presence of the standard Philadelphia chromosome in the bone marrow of this patient with myelodysplasia is a rather unusual finding.

The Ph chromosome is characteristic for CML. In this genetic rearrangement, the *c-abl* oncogene is translocated from chromosome 9 to the *bcr* gene on chromosome 22. The *bcr* breakpoints appear to be clustered in a 5.8-kb DNA segment (4). The *bcr-abl* genomic recombination is transcribed in a novel 8.5 kb mRNA, that encodes a *bcr-abl* chimeric protein of 210 kD with in vitro tyrosine kinase activity (5, 6).

Heterogeneity regarding Ph positive diseases has been found at the clinical, cytogenetic, and molecular level and will be briefly reviewed at the hand of this unusual case. CML patients with variant forms of Ph and some cases cytogenetically Ph negative have been shown to have a breakpoint in the 5.8-kb *bcr* region (7). In addition, RNA studies and/or in situ hybridization experiments demonstrated or were highly suggestive for a bcr-abl recombination (8, 9). In probably less than 1% of

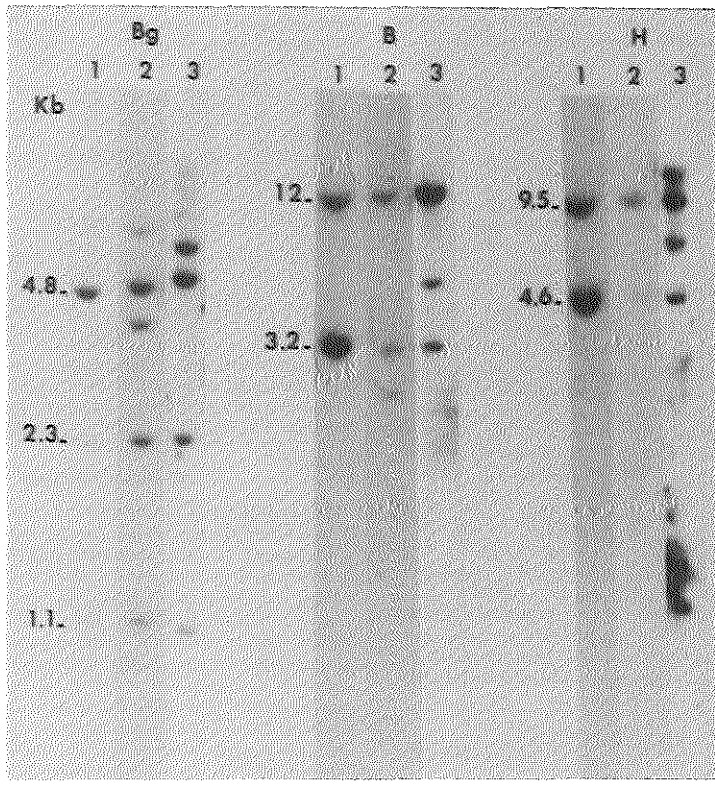

Figure 2. Southern blot of DNA from bone marrow cells digested with *Bgl*II (Bg), *Bam*HI (B), and *Hind*III (H) restriction enzymes and hybridized to the Ph1/bcr-3 probe. The size of the germ line band is indicated. The DNAs blotted were from a Ph^1-negative myeloid leukemia with t(5;12) (lane 1), a Ph^1-positive CML (lane 2), and the Ph^1-positive RAEB described here (lane 3).

Ph[1]-positive CML, a breakpint on chromosome 22 has been found outside the classical *bcr* region (10), without apparent modification of the clinical symptoms of CML. This is in contrast to the CML cases, Ph negative and bcr negative, who present with atypical hematological features such as dysplastic features of myelopoiesis, normal basophils count, moderate monocytosis and relative thrombocytopenia, and shorter survival (8). In Ph positive ALL (and AML), heterogeneous location of *bcr* breakpoints is observed: about 40% of the cases show a breakpoint in the *bcr* region (Ph positive, *bcr* positive cases), while the others do not (Ph-positive, *bcr*-negative cases) (11). In the latter cases, a breakpoint in the first intron of *bcr* gene has recently been found that corresponds to another *bcr-abl* recombination with its own chimeric mRNA and chimeric *bcr-abl* protein of 190 kDa (12). The clinical and prognostic significance of these various types of bcr-abl activation are not yet clear and await prospective studies of large series of patients.

Ph chromosome in RAEB is a rather exceptional observation. To the best of our knowledge only four other cases of Ph positive myelodysplasia have been reported. The first one (13) had Ph chromosome in three of eight examined cells at diagnosis. The patient developed fatal acute leukemia after 3 years with one of two examined cells with Ph chromosome and no additional abnormalities. Two other cases (14, 15) had a therapy-related preleukemia occurring after treatment of Hodgkin's disease and prolonged use of phenylbutazone, respectively. Cytogenetic studies showed Ph chromosome in all examined cells. The first patient died 9 months after onset of preleukemia of septicemia and disseminated intravascular coagulation, the second case died of acute leukemia, and the last case (16) had an idiopathic acquired sideroblastic anemia; cytogenetic study showed Ph chromosome in 1/3 of bone marrow cells. No leukemic transformation was observed during the next 45 months.

In our observation, a standard Ph translocation was found in all 61 metaphases examined, which suggests a stem cell origin. Indeed in myelodysplastic syndrome (MDS), cytogenetic investigations usually reveals a mosaic normal/abnormal clonal karyotype. In our case, the normal stem cell compartment was either completely regressed or replaced by Ph-positive progenitors. Precursors of red cells are not cytogenetically evaluable in bone marrow cultured for 1 or 2 days, but they were clearly pathological by cytology. Southern blot indicated a bcr breakpoint conforming to the CML model. Unfortunately, RNA studies could not be performed. But because of cytogenetic and DNA conformity to the CML model, it seems safe to assume that a chimeric mRNA is transcribed coding to a p210 bcr-abl protein. We have no explanation why in this patient RAEB is observed rather than CML. It is another example of the diversity of clinical expression of Ph[1]-positive stem cell disorder that could be related to additional factors such as the age of the patient and/or other (e.g., molecular) events superimposed to the *bcr-abl* activation and inductive of RAEB phenotype rather than CML.

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CHAPTER 9

GENERAL DISCUSSION

During the last few years an increasing number of translocation breakpoints have been cloned and characterised molecularly e.g. t(9;22), and more recently t(6;9), t(15;17) and t(1;19) (Hermans et al, 1987, von Lindern et al, 1990, Kamps et al, 1990). This knowledge was applied to develop new diagnostic tools, that could be used for diagnostic purposes and for follow up during treatment. Soon DNA, RNA and protein assays became available for patients whose leukemic cells carry one of these well characterised translocations.
In this thesis we focussed on t(9;22) in CML, ALL and AML.

CYTOGENETIC AND MOLECULAR ANALYSIS IN CML

The leukemic cells in ninety percent of the CML patients show t(9;22)(q34;q11). Five percent is characterized by variant Ph translocation, i.e. translocations involving chromosome 9, 22 and one or more other chromosomes. In less than five percent of the CML patients no Ph chromosome is detected. The latter are called Ph negative. Our results and the data of other investigators show that CML with variant or standard Ph translocation as well as Ph negative CML with a breakpoint in the *bcr* gene, all represent of the same disease. The only exception is formed by Ph negative CML without detectable *bcr* breakpoint. The latter is probably not the same disease, but resembles more MDS.
The fact that Ph negative CML patients with *bcr-abl* rearrangement have a prognosis that differs from the prognosis of Ph negative CML patients without *bcr-abl* rearrangement indicates that in addition to the cytogenetic classification system a molecular classification of CML is required.
Interestingly transition from chronic phase CML to blastic phase is often preceded by the presence of additional chromosomal aberrations such as +8, i(17q), +22q-, and sometimes t(3;21). Therefore chromosomal analysis in CML is used to detect the Ph chromosome as well as additional chromosomal aberrations. However trisomy 8 is sometimes also seen in chronic phase CML, but in these cases it is usually present in a low percentage of the metaphases. In our laboratory chromosome analysis was performed at diagnosis and during follow up of all CML patients. In our opinion additional molecular analysis is required in CML in the following circumstances:
- When a CML patient turns out to be Ph negative.
- In patients with possible variant Ph translocations involving chromosome 9 or 22.
- Prior to therapies that can result in Ph conversion e.g. bone marrow transplantation or interferon treatment. Determination of the *bcr* breakpoint or the type of mRNA expressed in the diagnostic phase facilitates follow up when the patient has minimal residual disease or has became Ph negative. This is especially important for the detection of patients with unusual positions of the breakpoint in the *bcr* or *abl* genes. The latter finding was more commonly observed than we expected.

CYTOGENETIC AND MOLECULAR ANALYSIS IN ALL AND AML.

In ALL and AML many structural chromosomal abnormalities are found that are of clonal origin and specific for certain subgroups of ALL and AML (as is demonstrated in chapter 2.2 and 2.3). Identification of these specific chromosomal abnormalities is of clinical importance for the prognosis. In acute leukemia specific chromosomal aberrations can be used as an independent prognostic factor. Furthermore, in addition to morphologic and immunologic features, they can serve to characterize the leukemic cells in more detail.

An important step forward in molecular analysis in acute leukemia was the cloning of a new cluster of breakpoints in the first intron of the *bcr* gene by Hermans et al in 1987 (chapter 3.2). A breakpoint in the first intron of the *bcr* gene turned out to be present in nearly all children, and in 50% of the adult patients with Ph positive ALL and Ph positive AML (Heisterkamp et al, 1990 and our own unpublished data). In the remaining Ph positive acute leukemia patients a breakpoint in the BCR region of the *bcr* gene was found, i.e. at the same position as observed in CML.

Sometimes it was very difficult to distinguish Ph positive ALL from blast crisis of CML in the diagnostic phase. In most cases clinical and cytogenetic follow up studies provided the answer. It should be noted that detection of a breakpoint in the first intron of the *bcr* gene, e1a2 mRNA expression or production of p190 bcr-abl protein in most cases is an argument in favour of the diagnosis acute leukemia, but this is not absolute. Sporadically CML cases are reported that have a breakpoint in the first intron of the *bcr* gene (Selleri et al, 1987, and the CML patient reported in chapter 4.1 of this thesis).

Ph positive AML is a very rare disease, and not many molecular data have been published. The breakpoint in the *bcr* gene is either in the first intron of the *bcr* gene or in the BCR region.

We experienced that molecular analysis, aimed to detect *bcr-abl* rearrangement in acute leukemia patients, turned out to be very useful in patients in whom chromosome analysis was of inferior quality or when the chromosomal aberrations were very complex. Furthermore molecular techniques were very helpful for detection of minimal residual disease in patients, who are in clinical hematologic and cytogenetic remission after therapy. In the latter cases especially the very sensitive PCR technique was useful.

Molecular techniques are also required to determine if a variant Ph translocation has taken place in patients showing chromosomal aberrations involving either chromosome 9 or 22. One such a patient, whose karyotype was t(12;22)(p13;q11), would have been described as Ph positive based on chromosome analysis, but turned out to be Ph negative at the molecular level (chapter 7.2). The leukemic cells of this patient showed no rearrangement in the *bcr* or in the *abl* gene using Southern blotting and PFGE, and consequently no expression of *bcr-abl* mRNA using PCR analysis. In situ hybridization showed no translocation of the *abl* gene (chapter 7.2). A review of literature showed that thusfar 8 AML patients with t(12;22) have been reported (including three patients investigated in our laboratory). In none of the other patients

molecular analysis has been performed. We conclude that t(12;22) (p13;q11) is a new specific translocation in AML.

REMAINING QUESTIONS

Although progress has been made in understanding and treating ALL, AML and CML the mechanism by which *bcr-abl* rearrangement is caused is still unknown. Malignant transformation of a cell is a complex multistep process, and it should be kept in mind that oncogenic activation such as activation of the *abl* oncogene by t(9;22) is only one of these steps. We still haven not characterized the other steps, that must play an important role in leukemogenesis. Several other interesting questions and controversial issues remain that I would like to discuss briefly:

In which cell does transformation occur?
In CML transformation occurs in the pluripotent bone marrow stem cell (Fialkow, 1977, Nowell and Hungerford, 1960, Yoffe, 1987). In most CML patients all precursor and mature blood cells except for T lymphocytes carry t(9;22). Rare cases of CML have been reported in which also part of the T lymphocytes had t(9;22) (Griffin et al, 1983, Chan et al, 1986). The reason for this difference is unknown.

ALL is considered to represent clonal expansion of lymphoid precursor cells which have undergone malignant transformation and are arrested at distinct stages of differentiation. Most cases of ALL involve B lymphocytes and their progenitors. T lymphocytes are less often involved. Even rarer is ALL involving both B and T cells or lymphoid as well as myeloid cells (hybrid leukemia). These data suggest that the typical site of transformation in ALL is the B cell progenitor. However recent data suggest that sometimes pluripotent stem cells are the site of transformation in ALL and that the leukemic phenotype can be modulated by hematopoietic growth factors (e.g. Griessinger et al reported in 1990 that GM-CSF and IL-3 stimulate myeloid differentiation of CD3 positive T ALL with T cell receptor ß, γ, and δ rearrangement, while IL-2 produces cytotoxic T cell phenotype). Furthermore, hybrid leukemias such as ALL with t(9;22) or t(4;11) are almost certainly caused by malignant transformation of a pluripotent stem cell or an uncommitted lymphoid progenitor (Tachibana, 1987, Turhan et al, 1988, and Kalousek et al, 1985).

AML originates from a single transformed progenitor. In certain cases transformation occurs at the pluripotent stem cell level, whereas in other cases a committed precursor is the target (Fialkow, 1981, Fialkow, 1987, and Keinänen, 1988). As a consequence a great variability is observed between different subtypes of AML.

Does a preleukemic phase precede CML, ALL, and AML?
In studies of allelic biochemical markers (e.g. G6PD in females) in Epstein Barr Virus transformed B cells derived from a chronic phase CML patient Fialkow et al (1981) and Martin et al (1982) showed, that most clones expressed the same markers as the

leukemic clone and had a high frequency of cytogenetic abnormalities. However, they did not always show t(9;22). These data suggest that CML is a multistep process in which t(9;22) might not always be the first step. The initial step may be development of clonal hematopoiesis unrelated to the Ph chromosome. This clone seems to have increased susceptibility to chromosomal aberrations, in particular t(9;22).
This multistep model of leukemogenesis in CML is supported by observations of Lisker(1982) reporting a patient with late appearance of the Ph chromosome, and its disappearance with progression. However, as long as we are not able to recognize this clonal Ph negative expansion, we can not detect a preleukemic phase in CML.
Last year we had the opportunity to perform DNA analysis using Southern blotting on blood from a patient one year before she was diagnosed to have CML. No BCR breakpoint was detected in this blood sample, while the blood sample of the diagnostic phase clearly showed a BCR breakpoint. Regretfully, no material was available that was suitable for cell culture to search for other leukemic characteristics.

Most data suggest that leukemia is a multistep process. ALL is almost certainly preceded by a preleukemic phase. The initial step may be physiologic e.g. immune response to infection, antigen or immunization which causes reversible expansion of the lymphoid compartment. However in rare instances there may be further clonal progression to a pre leukemia. When this is followed by imbalance between self renewal and differentiation in B lymphoid stem cells, this might result in ALL. This last step almost certainly is caused by additional genetic alterations.
This model of leukemogenesis is supported by the observation that some ALL patients in clinical and hematological remission have persistence of cells from the leukemic clone detected by PCR analysis without leukemia recurrence. Possible explanations for these findings are either re-establishment of a pre leukemic phase or eradication of the clone containing extra genetic aberrations or effective immuno surveillance.

The multistep model of leukemogenesis also is observed in AML. In some AML patients preleukemic phase, called myelodysplastic syndrome (MDS), precedes the AML phase, but other AML patients do not clearly show such a preleukemic phase.

Why is in children the cure rate in ALL higher than in most other leukemias and is it necessary to eliminate all ALL, AML, or CML cells to cure the patient?
In children cure rates (i.e. long term remissions) in ALL are higher than in AML and CML.
Possible explanations are:
Firstly, in the normal individual lymphocytes die for more than 99% in thymus and bone marrow. If this happens also to ALL cells, it is reasonable to suggest that it may not be necessary to eradicate all ALL cells to achieve cure of ALL. Reduction of the clone size so as to prevent death of the patient may permit normal physiologic mechanisms, such as cell death in the thymus, to eradicate small numbers of residual leukemic cells.
Except for lymphocytes most of the blood cells survive until they reach the end of

their normal life span. Therefore reducing the clone size without complete eradication will eventuate in relapse more easily in AML and CML than in ALL.
Secondly, most ALL cells are sensitive to drugs that have a more favourable therapeutic margin than drugs that are cytotoxic to AML cells. Especially for adult patients toxicity of drugs often prevents administration of the doses needed to kill the leukemic cells. Hopefully new therapies such as growth factors or immune therapy will overcome this problem.
In CML chemotherapy does not eliminate the transformed stem cell, as was discussed in chapter 5 of this thesis. The results of interferon or allogeneic BMT look more promising.
Thirdly, the occurrence of relapse is directly related to the selfrenewal capacity of the residual leukemic cells. In many of the ALL patients malignant transformation occurs at the level of the (pre) B cell i.e. a stem cell committed to B lymphopoiesis, and not at the level of the pluripotent stem cell. The selfrenewal capacity of the former is much lower than of the pluripotent stem cell. An exception is formed by ALL with t(4;11) or t(9;22) which are caused by transformation of a pluripotent stem cell. In AML transformation can occur at the level of the pluripotent stem cell or of the committed stem cell.
In CML transformation is at the level of the pluripotent stem cell.
For reasons explained above elimination of the leukemic clone is necessary in AML and CML. In ALL reduction of the clone size might be sufficient.

5. Is the presence of leukemic cells after bone marrow transplantation indicative for the occurrence of relapse?
Several investigators (Petz et al,1987, Arthur et al, 1988, Morgan et al, 1989, Pignon et al, 1990, and Delfau et al,1990) have reported that, using cytogenetics and especially using PCR analysis, small numbers of leukemic cells were detectable in the majority of the patients even after long intervals (up to one year) after allogeneic bone marrow transplantation.
The most fascinating observation of these investigators was that the presence of small numbers of leukemic cells even months after BMT did in most cases not result in progression to hematologic relapse.
In most of the cases the presence of low numbers of leukemic cells after BMT was transient: e.g. Morgan et al (1989) reported that no leukemic cells were detected by them using PCR analysis 5-7 years after allogeneic transplant for CML. Thus it seems that all leukemic cells were eradicated in these patients and that they are truly cured. The mechanism responsible for this "late" eradication of leukemic cells is still unknown.

6. What will be the role of cytogenetic and molecular analysis of ALL, AML and CML in the near future?
The last few years the breakpoints of t(1;19) in B-ALL, t(6;9) in AML, and t(15;17) in APL have been cloned, providing new tools for diagnosis and monitoring of the disease. We expect that soon more of such specific chromosomal aberrations will be

characterised molecularly. Furthermore new treatments such as interferon, or growth factor therapy require follow up studies using both immunologic and cytogenetic markers, as well as very sensitive molecular techniques, such as PCR.
How can we identify the patients with high risk? Sokal et al (1985) reported the most important prognostic factors in CML patients younger than 45 years of age: Sex, spleen size, percentage of blasts in peripheral blood and bone marrow, and the presence of a Ph chromosome. The localisation of the breakpoint in the BCR region did not show to be a significant prognostic factor (Morris et al, 1990, Tefferi et al, 1990, Selleri et al, 1989). Thusfar not enough patients are identified with breakpoints outside BCR or at an unusual position in the *abl* gene to draw conclusions about the prognostic value of these findings. Our results obtained thusfar indicate that identification of unusual breakpoints in the abl or bcr gene is of importance for us to facilitate follow up studies for minimal residual disease, but for the patient has no consequences for prognosis and choice of treatment.
The work reported in this thesis shows the results of such diagnostic and follow up studies using combinations of chromosome analysis and molecular techniques. Even in patients with standard Ph translocation the results of molecular analysis revealed several new mechanisms of oncogenic activation. In our opinion combined clinical, cytogenetic and molecular investigations such as described in this thesis will contribute to better understanding of the mechanisms that are of clinical importance for leukemogenesis.

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SUMMARY

Cytogenetic studies revealed that a considerable number of tumors, especially leukemias, is associated with consistent, specific chromosomal aberrations. The most frequently observed specific chromosomal aberration in leukemia is the Philadelphia (Ph) translocation. This is a reciprocal translocation between chromosomes 9 and 22, resulting in a shortened chromosome 22, the 22q- or Ph chromosome, and a longer chromosome 9, the 9q+ chromosome. At the translocation breakpoints of chromosome 9 and 22 the *abl* and *bcr* proto oncogenes are located. As a consequence of the Ph translocation part of the *abl* proto oncogene is translocated to the Ph chromosome, creating a new chimeric *bcr-abl* gene. This *bcr-abl* gene is transcribed in a *bcr-abl* mRNA, which is translated into a bcr-abl protein. The latter is responsible for leukemogenesis in Ph positive leukemias (chapter 3). The Ph chromosome, the chimeric *bcr-abl* gene, its RNA and protein products can be used as tumor specific markers for diagnosis and follow up of these leukemias. The results of such diagnostic and follow up studies are described in this thesis.

In 95% of the CML patients the Ph chromosome is present, and a chromosome 22 breakpoint in the BCR region of the *bcr* gene is detected in nearly all patients (chapter 4). In contrast, in Ph positive AML and ALL this breakpoint on chromosome 22 was observed in only 50% of the cases. The remaining Ph positive patients had a chromosome 22 breakpoint that was located more 5' in the *bcr* gene i.e. in a newly discovered second breakpoint cluster region (chapter 3.2, 3.3, and 7).

Sporadically we identified unusual breakpoints in the *bcr* or *abl* gene (chapter 4).

Modern treatment such as bone marrow transplantation, multiagent chemotherapy and interferon treatment are aiming to eradicate all Ph positive cells, thus resulting in disappearance of the Ph chromosome (Ph conversion). Cytogenetic analysis in combination with sensitive molecular techniques, e.g. PCR analysis, are required for follow up studies and detection of minimal residual disease in these patients. We present the results of a follow up study in patients treated with interferon α. Furthermore the case history and cytogenetic and molecular data obtained in a CML patients, in whom the Ph chromosome disappeared spontaneously are discussed.

In 5% of the CML patients no Ph chromosome is observed. They are called Ph negative. Using combinations of cytogenetic and molecular techniques we detected in several Ph negative CML patients the same *bcr-abl* recombination as in Ph positive CML (chapter 6). The latter Ph negative patients show the same survival rates as Ph positive CML. In contrast

Ph negative patients in whom no *bcr-abl* rearrangement is detected have a shorter survival and clinically resemble more CMML, a subtype of MDS, than CML.

Furthermore we report that in acute leukemias, that are characterised by a large variety of chromosomal aberrations, cytogenetic analysis at diagnosis can predict survival duration. Especially the number of chromosomes present in the leukemic cells and the presence of specific chromosomal aberrations are important for predicting treatment outcome (chapter 2.3 and 2.4).

In conclusion: in many different subtypes of leukemia cytogenetic analysis and, when

the molecular rearrangement underlying the cytogenetic aberration are known, also molecular analysis can be performed at diagnosis and during follow up. These investigations will help to elucidate the mechanisms responsible for leukemia, and are of importance for diagnosis, prognosis and choice of treatment.

SAMENVATTING

Cytogenetisch onderzoek van tumoren, in het bijzonder van leukemiëen, heeft aangetoond dat vele van deze tumoren geassocieerd zijn met consistente, specifieke chromosomale afwijkingen. Juist op de plaats, waar breukpunten optreden in de chromosomen van de tumorcellen, blijken genen te liggen, die in normale cellen een funktie vervullen bij allerlei fysiologische processen, maar wier funktie tgv de chromosomale afwijkingen verandert, waardoor normale cellen veranderen in kanker cellen.

De meest voorkomende chromosomale afwijking bij leukemie is de Philadelphia (Ph) translokatie. Dit is een reciproke translokatie tussen chromosoom 9 en 22, die resulteert in een verkort chromosoom 22, ook wel 22q- of Ph chromosoom genoemd, en een verlengd chromosoom 9, het 9q+ chromosoom. Op de translokatie breukpunten van chromosoom 9 en 22 liggen de *abl* en *bcr* proto oncogenen. Een deel van het *abl* gen verhuist tgv de Ph translokatie naar het Ph chromosoom, waardoor een nieuw chimeer *bcr-abl* gen ontstaat. Dit *bcr-abl* gen wordt overgeschreven in een *bcr-abl* mRNA, wat wordt vertaald in een bcr-abl eiwit. Dit eiwit is verantwoordelijk voor het ontstaan van leukemie in Ph positieve leukemiëen (hoofdstuk 3). Het Ph chromosoom, het chimere *bcr-abl* gen, mRNA en zijn eiwit zijn uniek voor deze leukemische cellen en kunnen worden gebruikt als tumor specifieke markers voor diagnose en vervolg studies van deze leukemiëen. De resultaten van dit soort diagnostiek en vervolg studies zijn beschreven in dit proefschrift.

In 95% van de patienten met chronisch myeloide leukemie (CML) is het Ph chromosoom aanwezig. In vrijwel al deze patienten kan een breukpunt in de breukpunt cluster regio (BCR) aangetoond worden (hoofdstuk 4).

In acuut myeloide (AML) of acuut lymfatische leukemie (ALL) daarentegen komt het Ph chromosoom maar in een deel van de patienten voor. In slechts 50% van de Ph positieve AML en ALL patienten wordt eenzelfde chromosoom 22 breukpunt gevonden als in CML. In de overige gevallen bevindt het breukpunt zich meer 5' in het *bcr* gen, en wel in een nieuw ontdekte tweede breukpunt cluster regio (hoofdstuk 3.2, 3.3 en 7).

In een klein deel van de patienten ontdekten we ongebruikelijke breukpunten in het *bcr* of in het *abl* gen. Dit is belangrijk voor de diagnostiek, maar voor zover we nu kunnen beoordelen heeft het geen consequenties voor de levensverwachting van de patient (hoofdstuk 4).

Nieuwe behandelings methoden zijn erop gericht het Ph chromosoom te laten verdwijnen (Ph conversie). Voor vervolg studies en het aantonen van "minimal residual disease" in deze patienten is cytogenetisch onderzoek in combinatie met zeer gevoelige moleculaire technieken, zoals PCR analyse, noodzakelijk. In hoofdstuk 5 worden de resultaten van diagnostiek en vervolg studie vermeld van CML patienten, die behandeld zijn met interferon α. Bovendien beschrijven we in dit hoofdstuk de ziektegeschiedenis, en de resultaten van cytogenetisch en moleculair onderzoek van een CML patient, bij wie het Ph chromosoom spontaan verdwenen is.

In 5% van de CML patienten wordt geen Ph chromosoom waargenomen. Ze worden

Ph negatief genoemd. Gebruik makend van combinaties van cytogenetische en moleculaire technieken hebben we aangetoond dat in verscheidene van deze Ph negatieve CML patienten dezelfde *bcr-abl* recombinatie plaats heeft gevonden als in Ph positieve CML (hoofdstuk 6). De Ph negatieve CML patienten, bij wie *bcr-abl* recombinatie aangetoond werd, hadden dezelfde overlevingsduur als Ph positieve CML patienten. Daarentegen hadden de Ph negatieve CML patienten, bij wie geen *bcr-abl* recombinatie aantoonbaar was, een kortere overlevingsduur en klinische en hematologische kenmerken, die meer leken op CMML, een subgroep van MDS, dan op CML.
Tevens rapporteren we, dat in acute leukemiëen, welke een grote verscheidenheid aan specifieke chromosomale afwijkingen vertonen, uit het cytogenetisch onderzoek bij diagnose de overlevings duur voorspeld kan worden. Vooral het aantal chromosomen dat in de leukemische cellen aanwezig is en de aanwezigheid van specifieke chromosale afwijkingen blijken belangrijke parameters te zijn om het resultaat van behandeling te voorspellen (hoofdstuk 2.3 en 2.4).
In conclusie: Cytogenetisch en, indien de moleculaire afwijkingen die hieraan ten grondslag liggen, ook moleculair onderzoek van de verschillende soorten leukemie spelen een steeds belangrijkere rol in de diagnostiek en bij het vervolgonderzoek van leukemie. De resultaten van deze onderzoeken verschaffen inzicht in de mechanismen die leukemie kunnen veroorzaken en zijn belangrijk voor het stellen van de diagnose, het voorspellen van de levensverwachtig en het kiezen van de behandelingsmethode.

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APPENDIX

CLINICAL EVALUATION OF A DNA PROBE ASSAY FOR THE PHILADELPHIA (PH) TRANSLOCATION IN CHRONIC MYELOGENOUS LEUKEMIA

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Clinical Evaluation of a DNA Probe Assay for the Philadelphia (Ph^1) Translocation in Chronic Myelogenous Leukemia

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We report the clinical evaluation of an improved DNA probe assay for the characteristic genetic marker of human CML, observed by cytogenetics and designated the Philadelphia chromosome (Ph^1). The Ph^1 chromosome results from the fusion of c-*abl* proto-oncogene sequences from chromosome 9 to *phl* gene sequence on chromosome 22. (The *phl* gene is often referred to as *bcr*. However, for clarity we prefer to reserve the designation "bcr" for the region within the *phl* gene in which translocation breakpoints have been found to occur. We also find it useful to distinguish between two such regions in *phl*, bcr-210 and bcr-190, named after the 210- and 190-kDa *phl/abl* fusion proteins resulting from translocations with breakpoints in the respective regions. We refer to the corresponding chromosomal translocations as Ph^1(bcr-210) and Ph^1(bcr-190).) DNA, extracted from peripheral blood (PB) or bone marrow (BM) and digested with restriction endonuclease *Bgl*II, is hybridized with a probe (*phl*/bcr-3) spanning a breakpoint cluster region within *phl*. Rearrangements are revealed by the presence of one or two novel junction fragments. Clinical specimens from leukemic patients with active disease were compared by cytogenetic and DNA probe analysis at seven centers in the United States and Europe. The probe assay identified the *phl* rearrangement in 190 of 191 cases of Ph^1-positive CML, as well as in 12 of 27 clinically diagnosed CML specimens lacking a typical Ph^1 chromosome. DNA rearrangements also were seen in two of six cases of Ph^1-positive ALL. No false positive results were obtained among 93 non-leukemic controls. Mixing experiments showed that the DNA probe assay can detect as few as 1% leukemic cells in a specimen. A preliminary study of CML patients in remission after allogeneic BM transplantation revealed a small fraction of residual Ph^1-positive leukemic cells in a significant number of such patients.

INTRODUCTION

THE ACTIVATION of cellular oncogenes plays a crucial role in neoplastic disease (1, 2). Substantial evidence shows that specific chromosomal abnormalities, often involving the sites of known proto-oncogenes, are associated with various forms of leukemia (3). Chromosomal translocations can cause oncogenic activation both by altering the control of gene expression and by causing protein structural changes which lead to the deregulation of the enzymatic activities of oncogene-encoded proteins (1, 2). These mutations may result in profound abnormalities in the control of proliferation and differentiation in a given cell lineage.

The Philadelphia (Ph^1) chromosome offers one of the most clearly documented examples of a translocation which leads to the activation of a human cellular oncogene. The characteristic cytogenetic abnormality is the $22q^-$ chromosome, usually resulting from a reciprocal translocation t(9;22)(q34;q11) (4). This marker chromosome is found in the leukemic cells of more than 95% of patients with CML (4, 5). A Ph^1 chromosome, indistinguishable by cytogenetics from that found in CML, is also observed in the leukemic cells of about 17–25% of adults with ALL (6–8). A lower incidence has been reported for childhood ALL (about 5%) (9, 10) and ANLL (less than 1%) (11, 12).

The Ph^1 chromosome can be used as a prognostic indicator. CML patients with this marker generally show increased survival compared to patients with Ph^1-negative CML (13–15). By contrast, the presence of the Ph^1 chromosome in the leukemic cells of ALL patients correlates with decreased survival (10, 16).

The critical molecular consequence of the Ph^1 translocation is a specific gene fusion (Fig. 1). A segment of the c-*abl* proto-oncogene, located at chromosome 9 band q34, becomes joined to a segment of the *phl* gene, located at chromosome 22 band q11 (17–20). In Ph^1-positive CML the translocation breakpoint almost invariably lies within a 5.8-kb "breakpoint cluster region" of *phl*, designated here as bcr-210 (21). Detailed mapping has revealed that translocation breakpoints in c-*abl* occur within intervening sequences (introns) located in the 5′ region of the gene, almost always upstream of c-*abl* exon 2. The breakpoints within the bcr-210 region of *phl* occur in any of three introns separating the four small coding exons (numbered 1–4) in this region. The Ph^1(bcr-210) translocation diagrammed in Figure 1 depicts a hybrid gene with a junction between c-*abl*, in the intron bounded by exons 1B and 1A, and *phl*, in the intron bounded by exons 3 and 4 of the bcr-210 cluster region. Transcription of such a fused *phl*/c-*abl* gene, followed by RNA splicing, gives rise to an 8.5-kb polyadenylated RNA, with *phl* sequences at the 5′ end and *abl* sequences (almost invariably beginning with exon 2) at the 3′ end (22–24). This mRNA encodes a fusion protein of approximately 210 kDa, designated P210 *phl/abl* (25, 26). Compared to the normal c-*abl* gene product, a polypeptide of 145 kDa, the hybrid P210 *phl/abl* protein exhibits elevated, constitutive protein-tyrosine kinase activity (27–29).

Molecular analysis of Ph^1-positive ALL reveals two classes of rearrangements, both involving *phl* and c-*abl*. One group

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Abbreviation: bcr, breakpoint cluster region.

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LEUKEMIA

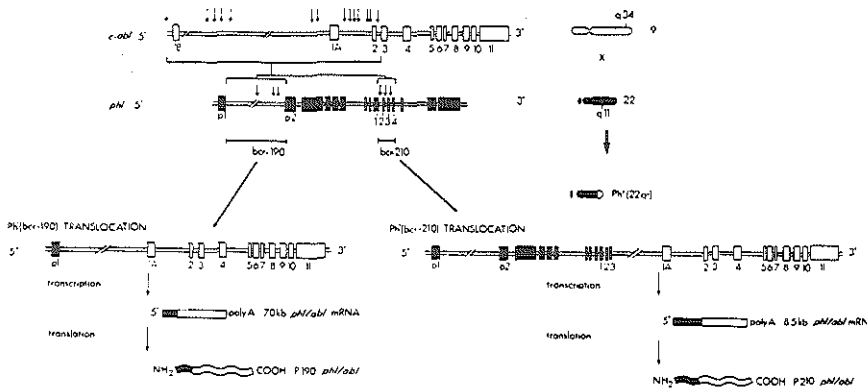

Figure 1. Molecular basis of the Ph' translocations. Generation of Ph' (22_q) chromosomes by recombination between *phl* and c-*abl*, and structures of spliced mRNA and of protein products. Chromosomal localization of genes and translocation event are depicted at right and schematic gene structures at left. Arrows indicate known positions of translocation breakpoints (individual cases for c-*abl*, representative examples for *phl*). Alternative regions in *phl* gene introns in which translocation breakpoints are found are labeled bcr-190 and bcr-210. Exons are indicated by boxes, introns by double lines. 5' and 3' refer to chemical polarity of mRNA or sense strand of DNA. Wavy boxes, protein products (amino-terminal and carboxyl-terminal ends indicated); open symbols, c-*abl* sequences; closed symbols, *phl* sequences.

exhibits Ph'(bcr-210) translocations indistinguishable from those found in CML, as shown by chromosomal breakpoints within bcr-210, and synthesis of 8.5-kb mRNAs and P210 *phl/abl* fusion proteins. The second group, here designated Ph'(bcr-190), has translocation breakpoints lying nearer to the 5' end of the *phl* gene, within the large intron located between exons p1 and p2 (Fig. 1) (30). The Ph'(bcr-190) translocations are associated with the expression of a 7-kb chimeric *phl/c-abl* mRNA (30), encoding a 190-kDa *phl/abl* fusion protein (P190) with elevated tyrosine protein kinase activity (31–34). The P190 and P210 fusion proteins differ structurally only in the amount of *phl*-derived sequence at the amino-terminal end of the polypeptide.

The precise role of the *phl/abl* fusion proteins in leukemogenesis is not known. However, the consistent and specific presence of the P210 and P190 proteins in leukemic cells argues forcefully that their expression is an important step in the pathogenesis of Ph'-positive CML and ALL.

The molecular dissection of the Ph' translocation offers novel approaches to the differential diagnosis of human leukemias. In particular, because of the relatively small size of the bcr-210 in the *phl* gene, it is possible to utilize hybridization with a single DNA probe to detect any rearrangement of genomic DNA in this region (35–37). We report here the results of the clinical evaluation of such a DNA probe assay. Specimens obtained from CML patients, patients with other leukemias, and non-leukemic controls were assessed for the presence of a Ph' translocation by both cytogenetic and molecular hybridization analysis. The results show that the DNA probe assay provides a highly specific and sensitive clinical diagnostic test for Ph'(bcr-210) translocations.

METHODS

Sample Source. BM and/or PB cell specimens obtained from leukemic individuals and non-leukemic controls were evaluated at seven independent clinical trial sites by both DNA probe and cytogenetic (karyotype) analysis. The participating trial sites and the total number of patient specimens studied per site are as follows: Erasmus University, 25 (I); Leukaemia Research Fund Centre, 53 (II); Vanderbilt University Medical School, 59 (III); M. D. Anderson Hospital and Tumor Institute, 91 (IV); Memorial Sloan-Kettering Cancer Center, 97 (V); University of Rochester, 24 (VI); Oncogene Science, Inc., 65 (VII).

The diagnosis of CML, ALL, or ANLL was based upon clinical and hematological criteria. Samples used in both karyotype and DNA probe procedures were obtained from patients on the same day. Samples were obtained from 218 CML patients with active disease. In 12 of these cases both blood and marrow specimens were obtained simultaneously from the same patient and tested by DNA probe analysis.

An additional 34 BM specimens were obtained from CML patients after allogeneic BM transplantation.

Cytogenetics. BM samples were cultured overnight, in the absence of mitogenic stimulation, in Ham's F10 medium supplemented with 10% FCS. Standard cytogenetic procedures were used. Slide preparations were pretreated with trypsin and either stained with Giemsa to yield G-banded chromosomes, or stained with quinacrine dihydrochloride to yield Q-banded chromosomes. At least 25 metaphase spreads were analyzed from each sample (38). The karyotype was reported according to conventions of the International System for Human Cytogenetic Nomenclature (39).

Purification of phl/bcr-3 DNA Probe. A 4.5-kb DNA probe, called *phl*/bcr-3, was designed to detect all Ph'(bcr-210) translocations. This probe was described previously (35). It consists of genomic sequences which span the entire 5.8-kb bcr-210 region of the *phl* gene, but lacks an internal *Hin*dIII restriction fragment of 1.6 kb containing repetitive sequences. The *phl*/bcr-3 fragment was molecularly cloned using the plasmid pSP65 (Promega), and propagated in *Escherichia coli* K12 strain HB101 by standard techniques (40, 41). For fragment purification, plasmid DNA was isolated and digested with restriction endonucleases, and the 4.5-kb insert was separated from vector sequence by electrophoresis in low melting temperature agarose (40). The fragment was excised from the gel and the agarose was melted by heating at 65°C for 30 min. DNA was extracted twice with phenol, equilibrated with 0.3 M sodium acetate (pH 5.0), then once with phenol/chloroform/isoamyl alcohol (25:24:1). The extracted DNA fragment was precipitated with ethanol (70% final, v/v) and 0.2 M sodium acetate (pH 5.6) in the presence of 20 μg/ml Dextran T-500 as carrier. The

precipitated DNA was rinsed two times with 70% ethanol and dissolved in Tris-EDTA buffer.

Isolation of Cellular DNA. High molecular weight DNA was isolated from cells of PB or BM aspirates. Nucleated cells were obtained from PB by centrifugation on Ficoll-Hypaque (Pharmacia) according to the manufacturer's recommendations. WBC were lysed with SDS (40), and DNA was obtained by extraction with phenol/chloroform (1:1) and precipitation with two volumes of ethanol. RNA and protein were removed from the DNA by digestion with RNase A (final concentration 20 μg/ml) for 30 min at 37°C followed by proteinase K (final concentration 100 μg/ml) for at least 30 min at 37° C. The DNA was extracted again with phenol/chloroform (1:1), followed by ethanol precipitation as described (41). The yield of DNA was determined by absorbance at 260 nm.

Hybridization Analysis. The purified cellular DNA was digested with *Bgl*II restriction endonuclease, and the resulting fragments were separated by electrophoresis on an agarose gel (0.7–0.8%, w/v) and transferred to a nitrocellulose or nylon filter by the method of Southern, with minor modifications (41, 42). In several cases, as specified in the text, the analysis was repeated with *Bam*H1 restriction endonuclease. The *phl*/bcr-3 DNA probe was labeled with ^{32}P to a specific activity of 2×10^8–1×10^9 cpm/μg by either nick translation (43) or the random primer method of Feinberg and Vogelstein (44, 45). Filter hybridization with at least 2.5×10^6 cpm/ml of labeled probe was performed overnight at 65°C in a solution containing 10% dextran sulfate as described elsewhere (46). Filters were subjected to several consecutive washing steps for 30 min at 65°C under increasingly stringent conditions (2.5 × SSC to 0.1 × SSC; SSC = 0.15 M NaCl, 0.015 M sodium citrate). After the final wash, filters were dried, and hybridized probe was detected by autoradiography using XAR-2 film (Kodak) at −70°C with intensifying screens (Du Pont Lightning Plus) for 4 hr or longer.

RESULTS

The phl/bcr-3 Probe Test. As a consequence of a reciprocal Ph^1 translocation, two novel junctions should be present in genomic DNA, corresponding to the 22_q^- and 9_q^- chromosomes, respectively. If the breakpoint in *phl* lies within the bcr-210 region (Fig. 1), then molecular hybridization with a DNA probe spanning this region should reveal two rearranged fragments in genomic DNA digested with an appropriate restriction endonuclease. The *phl*/bcr-3 probe (Fig. 2 and Methods) encompasses the entire bcr-210 region, with the exception of an internal 1.6-kb *Hin*dIII fragment found to contain repetitive sequences. Figure 2 indicates the human genomic DNA fragments, generated by several restriction endonucleases, that hybridize with this probe. For example, digestion with *Bgl*II normally yields three detectable fragments of 4.8, 2.3, and 1.1 kb (Fig. 2). A translocation involving bcr-210 would disrupt one of these fragments and generate two new fragments. Because only one copy of chromosome 22 is generally rearranged in Ph^1-positive leukemic cells, DNA from such cells would be expected to yield up to five DNA fragments that can hybridize with the *phl*/bcr-3 probe, i.e., the three germ line DNA fragments and two junction fragments. We have found *Bgl*II to be a suitable restriction enzyme for the identification of rearranged *phl* bcr-210 regions using the *phl*/bcr-3 probe (35), because of the excellent electrophoretic resolution of the three hybridizable fragments generated by this enzyme, and the apparent absence of polymorphism of the four relevant *Bgl*II target sites in human genomic DNA.

The results of DNA probe analysis to detect Ph^1(bcr-210) rearrangements in the K562 cell line and in cells of five representative Ph^1-positive CML patients are shown in Figure 3. The HL-60 cell line, derived from a human promyelocytic leukemia with no Ph^1 chromosome, and BM cells of a Ph^1-negative CML patient served as controls lacking bcr-210 rearrangements. Genomic DNA was digested with *Bgl*II, and Southern blot hybridization with the *phl*/bcr-3 probe was carried out as described (Methods). The three germ line fragments (4.8, 2.3, and 1.1 kb) are present in every case. No additional DNA fragments can be detected in DNA from either HL-60

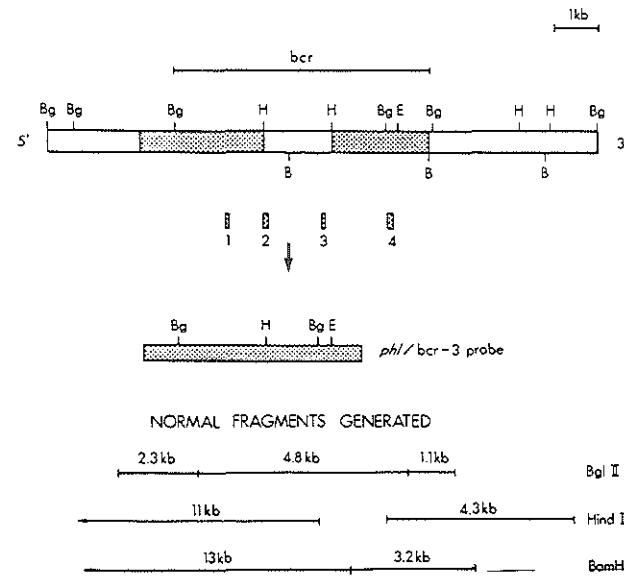

Figure 2. Restriction map of *phl* and construction of the *phl*/bcr-3 probe. The position of the bcr-210 breakpoint cluster region (labeled bcr), and regions of genomic DNA present in the *phl*/bcr-3 probe (cross-hatched boxes) are indicated. Small boxes labeled 1–4 indicate positions of coding exons in bcr-210 region of *phl* gene. Fragments in normal genomic DNA generated by digestion with several restriction endonucleases, and detectable by the probe are indicated (size in kb) at bottom. Restriction endonuclease cleavage sites: E = *Eco*RI, B = *Bam*HI, Bg = *Bgl*II, H = *Hin*dIII.

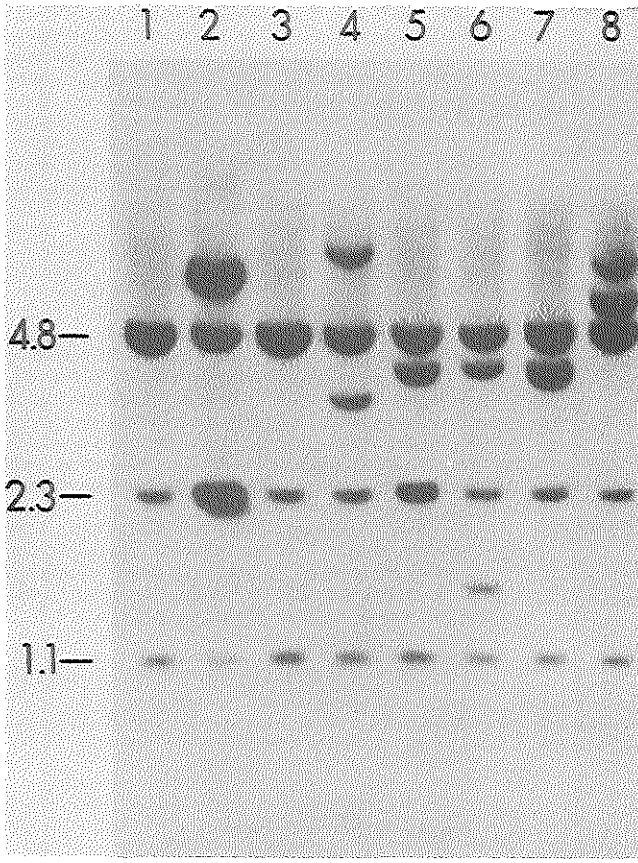

Figure 3. Assay for Ph¹(bcr-210) translocations by hybridization with *phl*/bcr-3 probe. DNA from control cell lines or patient specimens was digested with *Bgl*II and analyzed for the Ph¹(bcr-210) translocation by DNA probe assay as described (Methods). Sources of DNA: lane 1, HL-60 cell line (human acute promyelocytic leukemia); lane 2, K562 cell line (human CML); lane 3, Ph¹-negative CML patient specimen; lanes 4–8, Ph¹-positive CML patient specimens. Positions of germline bands (sizes in kb) are indicated at left.

cells (Fig. 3, lane 1) or from leukemic cells of the Ph¹-negative CML patient (Fig. 3, lane 3). In DNA from the CML cell line K562, one novel band is observed (Fig. 3, lane 2). This presumably represents a 22_q^- fragment, since cytogenetic analysis revealed no 9_q^+ chromosome in these cells (23). The intensity of the rearranged band confirms the finding that the chimeric *phl*/*c-abl* gene is amplified in K562 (23). In three of the samples from Ph¹-positive CML patients, two novel bands can be seen, corresponding to the junctions present in the 22_q^- and 9_q^+ chromosomes (Fig. 3, lanes 4, 6, and 8). Another CML patient sample (Fig. 3, lane 5) also appears to have two rearranged DNA fragments, but one co-migrates with the 2.3-kb germ line fragment, as judged by the intensity of the corresponding autoradiographic band. In the analysis of one of the Ph¹-positive leukemic DNA samples, only a single extra DNA fragment is apparent (Fig. 3, lane 7). It is possible that a small rearranged fragment ran off the gel or that a 9_q^+ fragment is not present in these cells.

Assay Threshold of Detection. In order to determine the minimal fraction of leukemic cells detectable by the probe test, we performed mixing experiments using DNA samples obtained from PB of a CML patient and from a non-leukemic individual. By cytogenetic analysis, all dividing BM cells analyzed from the CML patient were found to contain a Ph¹ chromosome. Normal and leukemic DNA were mixed in varying ratios, the DNA was digested with *Bgl*II restriction endonuclease, and a total of 15 μg DNA per slot was separated by gel electrophoresis and analyzed by Southern hybridization with ^{32}P-labeled *phl*/bcr-3 probe. The results of this experiment are shown in Figure 4. The anticipated germ line fragments (4.8, 2.3, and 1.1 kb) are present in both the normal and the leukemic DNA samples. A single novel junction fragment (labeled Ph¹) of approximately 4 kb is observed in the CML patient DNA. The intensity of the corresponding band is roughly proportional to the fraction of leukemic DNA in the input sample, a conclusion supported by densitometric scanning, and 10% Ph¹-positive DNA is readily detectable in a short autoradiographic exposure (Fig. 4A). We repeated the analysis using smaller percentages of leukemic DNA, and a longer exposure time (Fig. 4B). The results of this and comparable experiments indicate that the *phl*/bcr-3 probe assay can routinely detect less than 5% leukemic DNA, and that as little as 1% leukemic DNA can be

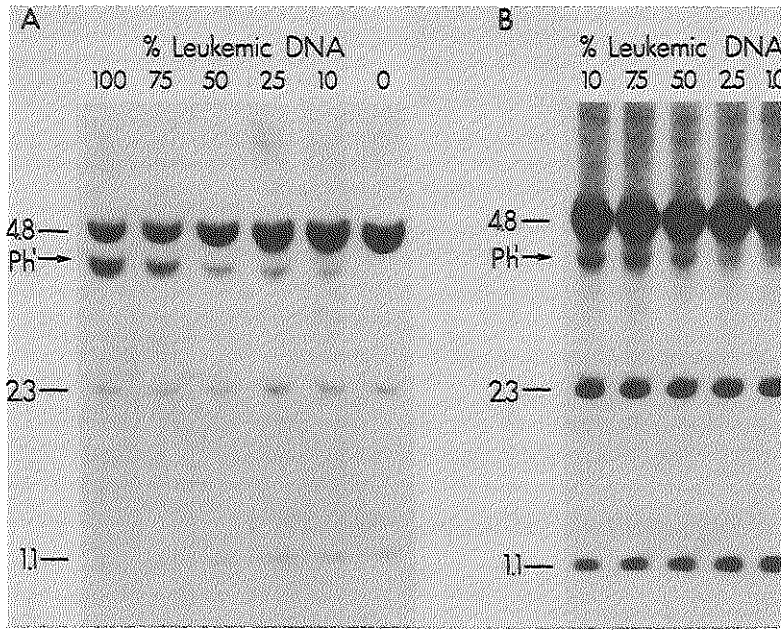

Figure 4. Threshold of detection of *phl*/bcr-3 DNA probe analysis. DNA from a CML patient and a normal donor were mixed and analyzed by digestion with *Bgl*II and blot hybridization with the *phl*/bcr-3 probe, by the method of Southern, as described in the text. A and B show independent experiments using DNA from the same individuals. Percentage of leukemic DNA is denoted above each lane. Positions of normal germline DNA fragments (sizes in kb) are indicated at left. Arrow labeled Ph¹ indicates position of the rearranged DNA fragment from the CML patient. Autoradiographic exposure times: A, 4 hr; B, 20 hr.

observed under favorable conditions. Reconstruction experiments in which leukemic and normal cells were mixed in varying ratios prior to DNA isolation gave similar results (data not shown).

The threshold of detection for rearranged DNA fragments in the probe assay would be expected to show some dependence on fragment size, since smaller fragments give a less intense hybridization signal. However, to date, all Ph¹(bcr-210) samples have shown at least one rearranged *Bgl*II restriction fragment larger than 2.3 kb. Thus, the reconstruction experiment of Figure 4 should accurately represent the situation in the vast majority of clinical specimens.

Clinical Evaluation. The DNA probe assay was compared with cytogenetic analysis in the diagnosis of Ph¹-positive leukemia at seven trial sites. Karyotyping was carried out on cells obtained from BM aspirates, while the probe analysis was carried out on DNA extracted from PB or BM. Specimens used for the two assays were obtained on the same day, usually at the time of initial presentation at a clinic. In all cases *Bgl*II was used to digest genomic DNA. In cases of apparent discrepancy between cytogenetic observation and the DNA probe assay, the probe analysis was repeated using DNA digested with at least one other restriction endonuclease (usually *Bam*HI).

The results of karyotype and DNA probe analysis of patients with active leukemia and of non-leukemic controls are summarized in Table 1. Both the karyotype and DNA hybridization assays revealed Ph¹ translocations only in samples from patients with leukemia, and not in any of 93 non-leukemic controls. This confirms the specificity of the probe test. Among 218 cases of clinically diagnosed CML with active disease, 191 were Ph¹-positive by cytogenetic analysis. Hybridization with

Table 1. Comparison of *phl*/bcr-3 DNA Probe Assay and Cytogenetic Analysis in Clinical Diagnosis of Ph¹-positive Leukemia

Diagnosis (Number)		Karyotype Ph¹	DNA Probe Assay Positive/No. Tested		Total
			Sample Source		
			PB	BM	
CML	(218)	+	121/121[a]	69/70	190/191[b]
		−	4/8	8/19	12/27
ALL	(24)	+	2/4	0/2	2/6
		−	0/6	1/12	1/18
ANLL	(33)	+	0/1		0/1
		−	1/21	0/11	1/32
Non-leukemic	(93)	−	0/70	0/23	0/93

[a]From 12 Ph¹-positive CML patients DNA was obtained from both PB and BM, with consistent probe test results. These 12 cases are included only under BM.

[b]The *phl*/bcr-3 DNA probe assay was carried out on DNA digested with *Bgl*II. In three cases of Ph¹-positive CML, novel *phl*/*abl* junction fragments were not seen after *Bgl*II digestion. However, in two of these cases the probe test was positive on DNA digested with *Bam*H1. In the one remaining discrepant case, probe analysis was carried out only on DNA digested with *Bgl*II, and *Bam*H1 digestion was not attempted.

the *phl*/bcr-3 probe of genomic DNA digested with *Bgl*II showed definitive evidence of DNA rearrangement in 188 of 191 Ph^1-positive CML cases. In two of the three remaining cases, the probe analysis was repeated using DNA digested with *Bam*H1, and novel junction fragments were observed, indicating the presence of a bcr-210 rearrangement. In the third discrepant case, insufficient DNA was available to repeat the analysis using a second restriction endonuclease, so it could not be determined whether this represented an example of a rearrangement occurring outside of the bcr-210 region.

The DNA probe assay revealed novel bcr junction fragments in an additional 12 clinically diagnosed CML cases in which no Ph^1 chromosome was observed by karyotype analysis. In eight of these cases a normal diploid karyotype was reported. In three cases a variant translocation was observed (t(9;14;22), t(9;19;22), and t(2;9), respectively), and in one case only an apparently unrelated translocation, t(1;21), was reported.

Among six cases of cytogenetically Ph^1-positive ALL, two showed bcr-210 rearrangements using the DNA probe test on genomic DNA digested with *Bgl*II. It seems very likely that in each of the four remaining cases the breakpoint on chromosome 22 was located within the bcr-190 region of *phl* (Fig. 1), and therefore could not be detected by hybridization with the *phl*/bcr-3 probe. In one additional case of ALL the probe test disclosed a Ph^1(bcr-210) rearrangement that was not revealed by karyotyping.

Only one example of a cytogenetically detectable Ph^1 chromosome was reported among 33 cases of clincally diagnosed ANLL, and this specimen was negative by DNA probe analysis. Conversely, in another case the probe test revealed a bcr-210 rearrangement in the absence of an obvious Ph^1 karyotypic abnormality.

A diagnostic test for the Ph^1(bcr-210) rearrangement has potential utility in monitoring patients during the course of therapy (74). At two test sites (III and V) preliminary studies were carried out on specimens from CML patients several months after undergoing allogeneic BM transplantation (Table 2). A total of 34 marrow specimens was analyzed from 31 such patients. With one exception, described below, the samples were obtained at a time when the patient was in apparent clinical remission. In about one-half of these cases, both cytogenetic and DNA probe analyses were negative. However, in the remaining cases either one or both assays revealed a Ph^1 translocation, indicative of residual leukemic cells. The DNA probe test was more successful than cytogenetic analysis in detecting these Ph^1-positive cells (15 vs. 10 positive specimens), but there were three cases in which hybridization with the *phl*/bcr-3 probe did not corroborate positive karyotypic results.

During this study a few CML patients were tested sequentially at intervals before and after allogeneic BM transplantation. Two cases proved of particular interest.

From one patient, at site V, an initial specimen of splenic tissue was obtained prior to treatment, and the presence of a Ph^1(bcr-210) chromosome was verified by both karyotype and DNA probe analysis. Eight months after allogeneic transplantation the patient's disease was in remission, and both cytogenetic and probe assays were negative. A specimen taken at 15 months after transplantation still showed a normal diploid karyotype, and the patient remained in clinical remission. However, DNA probe analysis carried out at this time revealed a rearranged bcr-210 DNA fragment, suggesting the presence of a population of Ph^1-positive leukemic cells. This patient is still being followed.

In the second case (studied at site III) the diagnosis of Ph^1-positive CML was confirmed at presentation by both cytogenetic and DNA probe assays. The patient was treated and received a BM transplant. Several months later the patient was clincially disease free, and the karyotype appeared normal, but the *phl*/bcr-3 probe test revealed a rearranged DNA fragment. Densitometric analysis suggested that this represented a population of no more than 5% cells with a Ph^1 translocation. Several months later the patient developed a clinical relapse resembling an acute lymphoblastic crisis. At this time cytogenetic analysis was attempted, but adequate chromosome spreads were not obtained. However, the DNA probe test showed a high level of a rearranged bcr-210 DNA fragment in BM cells. Comparison of the mobility of the junction fragments obtained by restriction endonuclease digestion with *Bgl*II showed that the same rearrangement was present in DNA obtained during the chronic phase of CML, during apparent remission after transplantation, and at the time of the clinical relapse. This implies that the lymphoblastic cells observed at relapse were derived from the same leukemic clone that was present in the patient during the chronic phase of CML prior to transplantation. The patient has subsequently died, and no additional karyotyping could be done for confirmation.

Table 2. Detection of Ph^1 Translocation in CML Patients after BM Transplantation

Test Results		Number of Samples
Karyotype	DNA Probe	
−	−	16
+	+	7
+	−	3
−	+	8

DISCUSSION

Elucidation of the molecular structure of the Ph^1 translocation resulted in the first oncogene-based assay for the diagnosis of a specific form of human cancer, CML (21). We have constructed an improved DNA probe, *phl*/bcr-3, to detect rearrangements in the bcr-210 segment of the *phl* gene which are associated with activation of the c-*abl* oncogene. The assay entails Soutern blot hybridization of the probe to genomic DNA fragments generated by digestion with *Bgl*II. The 4.5-kb *phl*/bcr-3 probe spans the entire bcr, bcr-210, of the *phl* gene. Prior studies mainly have employed a 1.2-kb probe from the 3′ portion of this region. The use of this shorter probe may lead to false negative results in approximately 10–20% of Ph^1-positive CML patients (23, 47, 48).

In the multisite clinical study described here, the *phl*/bcr-3 probe test correctly identified 99% of clinically diagnosed cases of Ph^1-positive CML. Furthermore, no false positive results were obtained among 93 samples from non-leukemic individuals. This indicates that the four *Bgl*II restriction sites critical to the assay are highly conserved and that extraneous *Bgl*II sites in this region occur rarely, if at all, in the human population. In approximately 1% (2 of 190) of cases the use of a second restriction endonuclease, *Bam*H1, instead of *Bgl*II, was required to obtain a positive DNA probe test in a Ph^1-positive CML.

The DNA probe test also revealed rearrangements of the characteristic bcr of the *phl* ge..e (bcr-210) in 12 of 27 cases (44%) of karyotypically Ph^1-negative CML, only 3 of which

showed complex chromosomal rearrangements. In agreement with this observation, others have demonstrated both a bcr-210 rearrangement and the expression of a P210 *phl/abl* tyrosine protein kinase in some CML cases lacking a marker chromosome (36, 49–52). Wiedemann et al. (36) recently reported that in Ph^1-negative CML the presence or absence of the DNA rearrangement correlates with clinical and morphological features. Among 12 such cases reviewed in their study, 5 were clinically indistinguishable from Ph^1-positive CML, and these were marked by molecular rearrangements of bcr-210 and, where tested, expression of P210 *phl/abl*. The remaining seven cases involved no bcr rearrangement detectable by the *phl*/bcr-3 probe test. Upon independent review, these leukemias were reclassified on the basis of cellular morphology and clinical features as either atypical CML (6 cases) or CMML (1 case). Thus, irrespective of the presence of a visible Ph^1 chromosome, CML involving a breakpoint in the bcr-210 region appears to define a distinct clinical entity.

Among 191 patients with Ph^1-positive CML, as judged by karyotype, only one did not appear to have a DNA rearrangement in bcr-210. This discrepant case was not confirmed by analysis of DNA using a second restriction endonuclease, and it remains possible that a breakpoint was present in the bcr-210 region but was not detected. Alternatively, although almost all translocation breakpoints in Ph^1-positive CML lie within bcr-210 (21, 35, 53, 54), a small minority may lie outside of this cluster region (55, 56) (our data would indicate a frequency of less than 1%).

A somewhat higher frequency (3 of 34) of specimens from patients in apparent remission after allogeneic BM transplantation were Ph^1-positive by cytogenetic analysis but showed no evidence for a bcr-210 rearrangement by DNA probe test with a single restriction endonuclease (*Bgl*II). A partial explanation for this observation may be that leukemic cells were infrequent in these samples, so that both cytogenetic and DNA probe assays were pushed near their thresholds of detection, leading to some discrepant results due to sampling variation. For example, in some cases Ph^1-positive cells might have grown out selectively during in vitro culture for cytogenetic analysis. It is also possible that secondary deletions or entirely new translocations are selected preferentially in this group of patients. However, in the one case in which specimens from a single patient were studied sequentially during chronic phase CML, during a post-transplant clinical remission, and at the time of a lymphoblastic relapse, the same bcr-210 rearrangement was observed at each point. Similarly, Ganesan et al. reported four CML cases in which leukemic cells present before allogeneic BM transplantation and after relapse were derived from the same clone, as judged by identity of the bcr-210 rearrangement (57).

The chromosomal rearrangements in Ph^1-positive ALL recently have come under intense scrutiny. Two classes of *phl/abl* gene fusions have been found. One group has molecular characteristics indistinguishable from those in Ph^1-positive CML, and the *phl*/bcr-3 probe therefore can be used to diagnose the presence of the Ph^1(bcr-210) translocation. The second group is distinguished by rearrangements with breakpoints in a *phl* gene intron located upstream of those typically involved in CML, defining a region we provisionally designate bcr-190. The Ph^1(bcr-190) translocations cannot be identified utilizing the assay described here, although the *phl*/bcr-3 probe would detect such rearrangements if sufficiently large DNA restriction endonuclease fragments were generated and resolved, for example by pulsed field gel electrophoresis (58, 59). In ALL the Ph^1 chromosome generally is associated with extremely poor prognosis (10, 16, 60). It will be of interest to determine the particular clinical implications of the Ph^1(bcr-210) and Ph^1(bcr-190) rearrangements, which give rise to similar but distinct *phl/abl* fusion proteins (P210 and P190, respectively) with elevated protein-tyrosine kinase activity.

The same molecular heterogeneity in the precise location of breakpoints may be involved in Ph^1-positive ANLL as in Ph^1-positive ALL (61, 62). In our study only 2 of 33 ANLL specimens showed evidence for a Ph^1 translocation. In one case cytogenetic analysis established the presence of a Ph^1 chromosome, but no rearrangement of bcr-210 could be found by DNA probe assay. Assays for activated *abl* kinase would suggest whether such cases involve rearrangements in bcr-190, or elsewhere on chromosome 22. In the second example the probe test revealed a bcr-210 rearrangement, but a Ph^1 chromosome was not apparent.

In summary, the *phl*/bcr-3 DNA probe assay compares very favorably with karyotype analysis as a specific diagnostic test for CML. The probe assay can be carried out on PB specimens as well as BM aspirates; it identifies the translocation in at least 99% of cases of cytogenetically Ph^1-positive CML, and reveals a significant number of *phl/abl* translocations not detected by karyotype analysis. Furthermore, the test offers useful information, which cannot be obtained from cytogenetics, on the differential diagnosis of atypical CML, CMML, and those cases of Ph^1-positive ALL and ANLL involving bcr-210 rearrangements. As treatment of the leukemias becomes increasingly sophisticated, it seems highly probable that the precise diagnosis of these diseases, at the molecular level, will be accompanied by the development of distinct therapeutic strategies for each identifiable class.

The DNA probe test is likely to find a second major use in monitoring CML patients, particularly to determine the response to therapy. A key consideration for this application is assay sensitivity. Reconstruction experiments indicate that Southern hybridization with the *phl*/bcr-3 probe test can reveal leukemic cells present at 1% in a PB or BM cellular population. The threshold for detection of Ph^1-positive cells by karyotype analysis depends on the number of metaphase spreads studied per sample, but is usually in the range of 10% of the cell population (63). Thus, under routine laboratory conditions the probe assay appears to be severalfold to 10-fold more sensitive than conventional cytogenetic analysis.

Hematological remission of CML can be induced by human rIFN-αA and possibly IFN-γ (64–66). In some patients the fraction of BM cells containing the Ph^1 chromosome drops significantly. This has been documented most convincingly by molecular hybridization analysis (67, 68). Similarly, the DNA probe assay should have utility in monitoring the response of CML patients to other biological response modifiers, such as G-CSF (69).

Recent advances in BM transplantation suggest that this approach will play an increasingly important role in the treatment of CML (57, 70–73). Although the systematic study of allogeneic transplant recipients was outside the main focus of the present work, we found that the *phl*/bcr-3 DNA probe assay readily revealed residual leukemic cell populations in nearly half of such patients sampled during clinical remission after transplantation (Table 2). The superior detection threshold of

the probe test and its precision in the identification of Ph[1](bcr-210) translocations clearly make it a powerful tool to supplement clinical observation and cytogenetic analysis in following the complex biology of BM transplantation.

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