CYTOGENETIC AND MOLECULAR STUDIES IN CHRONIC MYELOID LEUKAEMIA

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DEDICATIONS

I would like to dedicate this thesis to my parents

Gao Jin Hui

He Yan Ru

my family:

Gao Zheng

Chen Xiao Gang

Gao Hong

Gao Hui

Trieu Tu Kien

Trieu Verna

for whose love and hard work made my trip to England possible.

I also dedicate my thesis to my love Grace Tang Ching Yuet who brings the rainbows into the rainy days of my life as a PhD student.

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ABSTRACT

In this thesis I describe my study of the Philadelphia chromosome in various forms of leukaemia and its relationship with the clinical and haematological features of chronic myeloid leukaemia (CML).

I studied the cytogenetics of CML in chronic phase and the further chromosomal changes in the blast phase. Most of the Ph-negative CML patients I studied had strictly normal karyotypes; one patient however had a complex translocation with a masked Ph chromosome. Clinically some of these patients were indistinguishable from the Ph-positive CML while others had variable features.

I studied BCR/ABL rearrangements in 52 CML patients; 33 were Ph-positive and 19 Ph-negative. Thirty-two Ph-positive patients showed rearrangement of M-BCR; one had a chromosome 22 breakpoint located 3' outside the BCR gene but a classical BCR/ABL chimeric mRNA was present. This could be due to an alternative splicing mechanism. Of the 19 Ph-negative CML patients 9 had M-BCR rearrangement and 10 showed no evidence for BCR gene involvement. The absence of BCR gene involvement was associated with the atypical clinical features in about half the Ph-negative patients.

I constructed a long range map of the BCR and ABL genes with rare cutting restriction enzymes BssHII, MluI and NarI. This map has been used to classify the BCR gene involvement in 6 patients with M-BCR non-rearranged CML.

I studied engraftment and relapse after bone marrow transplantation for CML by cytogenetic and DNA analysis. Engraftment was demonstrated by showing donor DNA patterns; relapse was detected by the recurrence of Ph-positive metaphases or the DNA patterns of recipient leukaemic cells. Cytogenetic relapse can have different outcomes. Patients who received T-cell depleted marrow transplants had higher relapse rates than those who received non T-depleted marrow. I found no evidence for relapse in cells of donor origin.

The studies contribute to our understanding of the relationship of molecular and cytogenetic changes to the natural history of chronic myeloid leukaemia and to the results of bone marrow transplantation, currently the only treatment with curative potential.

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ABBREVIATIONS

ABL	Human proto-oncogene homologous to v-abl
A-MuLV	Abelson murine leukaemia virus
v-abl	Transforming sequence of Abelson mutant of M-MuLV
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
Ara-C	Cytosine arabinoside
BC	Blast crisis
BCR	Breakpoint cluster region
M-BCR	Major breakpoint cluster region
m-BCR	Minor breakpoint cluster region
BMT	Bone marrow transplantation
BT	Blast transformation
CFU-GM	Colony forming unit-granulocyte / macrophage
CML	Chronic myeloid leukaemia
aCML	Atypical chronic myeloid leukaemia
CMML	Chronic myelomonocytic leukaemia
°C	Degree centigrade
CP	Chronic phase
DNA	Deoxyribonucleic acid
cDNA	Complementary DNA
DNase	Deoxyribonuclease
dCTP	Deoxycytidine 5'-triphosphate
dNTP	Deoxynucleoside 5'-triphosphate

EDTA	Ethylenediaminetetraacetic acid (disodium salt)
EGF	Epidermal growth factor
GGT	Gamma glutamyl transpeptidase
G-6-PD	Glucose-6-phosphate dehydrogenase
GVHD	Graft versus host disease
GVL	Graft versus leukaemia
HLA	Human leucocyte antigen
IGLC	Immunoglobulin light chain gene
IFN	Interferon
kb	Kilo-base
kD	Kilo-Dalton
l	Litre
ml	Millilitre
ul	Microlitre
LTMC	Long term marrow culture
M	Molar
mM	Millimolar
M-MuLV	Moloney murine leukaemia virus
min.	Minute
NAP	Neutrophil alkaline phosphatase
OD600	Optical density at 600 nm
OD160	Optical density at 160 nm
^{32}P	^{32}Phosphorus
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor

PFGE	Pulsed field gel electrophoresis
Ph	Philadelphia
PMSF	Phenylmethylsulphonyl fluoride
PTK	Protein tyrosine kinase
p	Chromosome short arm
q	Chromosome long arm
RAS	Transforming sequence of rat sarcoma virus
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
SAA	Severe aplastic anaemia
SIS	Simian sarcoma virus
SSC	Sodium chloride + sodium citrate
SDS	Sodium dodecyl sulphate
t	Chromosome translocation
TAE	Tris (hydroxymethyl) methylamine + Acetic acid + EDTA
TBE	Tris (hydroxymethyl) methylamine + Boric acid + EDTA
TE	Tris (hydroxymethyl) methylamine + EDTA
Tris HCl	Tris (hydroxymethyl) methylamine
TY medium	Bacto-tryptone + yeast extract
UV	Ultraviolet
V	Volt

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CHAPTER ONE CLINICAL AND HAEMATOLOGICAL FEATURES OF CHRONIC MYELOID LEUKAEMIA (CML)

1.1 INTRODUCTION

1.1.1 DEFINITION

Chronic myeloid leukaemia (CML) is a haematologic malignancy of clonal origin involving a pluripotent stem cell and characterized by excessive proliferation of all myeloid cell lines (Fialkow et al, 1977; Yoffe et al, 1987a; Eaves et al, 1987; Strife et al, 1988). CML usually begins in a so-called chronic phase which is of variable duration but sooner or later it transforms into a blastic phase. About 90% of cases have a Philadelphia (Ph) chromosome (Fig. 1.1).

1.1.2 HISTORY

The earliest description of leukaemia in general can be found in 1827 (Velpeau, 1827). The increase of white cells in blood was described in 1844 (Donne, 1842; 1844). A CML-like syndrome was reported in 1845 and subsequently designated 'splenic leukaemia' in 1856 by Virchow (Virchow, 1845; 1856). However, CML is at least initially a comparatively indolent malignancy and only became the object of intense study when in 1960

Nowell and Hungerford working in Philadelphia discovered in the leukaemic cells the first consistent chromosomal change associated with human malignancy, the Ph chromosome (Nowell & Hungerford, 1960; 1961). In the present decade the role of the Ph chromosome in the pathogenesis of CML was further defined when the involvement and activation of the ABL proto-oncogene in the Ph translocation was characterized (de Klein et al, 1982; Canaani et al, 1984; Konopka et al, 1984). Much effort has since been devoted to study of the molecular mechanisms in the pathogenesis of CML; the BCR/ABL rearrangement has been analyzed at the level of gene structure, its regulation and transcription, the level of hybrid mRNA and protein structure and functions (reviewed by Groffen et al, 1987). Recent research has focused on study of BCR/ABL expression and the transforming ability of the BCR/ABL hybrid genes in animal systems.

In contrast to the other leukaemias, patients with CML have obtained little or no benefit from recent advances in the field of chemotherapy. Thus curative treatment only became a possibility in late 1970s when bone marrow transplantation (BMT) was introduced in the treatment of CML in Seattle (Fefer et al, 1979; 1982). The usefulness of BMT in the treatment of CML was soon confirmed by various workers (Goldman et al, 1981; 1986; Speck et al, 1984; Armitage et al, 1984; McGlave et al, 1987a). The use of alpha-interferon seems encouraging

for reducing the Ph-positive cell populations but its ability to prolong life is not yet clearly established (Talpaz et al, 1983; 1986).

1.2 EPIDEMIOLOGY AND PATHOGENESIS OF CML

1.2.1 INCIDENCE

CML comprises about 20% of all cases of leukaemia in the western world. A similar proportion of CML among leukaemias were also observed in China (Chu 1978; Institute of Hematology, Beijing, 1978; Boggs et al, 1987). The annual incidence of about 1 per 100,000 of the population is rather constant worldwide (Dameshek and Gunz, 1964; Gunz, 1977; Li, 1985).

1.2.2 AETIOLOGY

The aetiology of CML is still far from clear. The direct evidence that ionizing radiation is associated with CML comes from studies of CML occurring in Japanese atomic bomb survivors (Lange et al, 1954; Heyssel et al, 1960). Court Brown and Doll (1965) studied the mortality rates from leukaemia in 14,554 patients with ankylosing spondylitis treated by X-rays; they found an increased incidence of leukaemia but CML was not considered separately in this analysis. However, in the great majority of CML patients there

is no traceable history of radiation exposure.

There is considerable evidence in animal studies that viruses can induce leukaemia but little evidence that viruses cause CML in man (Gallo & Gallagher, 1974; Goldman & Jarrett, 1984; Richman et al, 1984; Jarrett, 1987). Genetic predisposition to CML appears improbable (Gunz, 1977).

Benzene is the only environmental (non-therapeutic) chemical agent known to induce leukaemia (Vighani & Saita, 1964). A retrospective European study of environmental exposure and acute leukaemia found that 5% of the patients with occupational exposure to potential carcinogens had the t(9;22) translocation characteristic of CML (Mitelman et al, 1981). No epidemiologic studies are available that relate directly to environmental carcinogens and CML (reviewed by Richman et al, 1984). The observation that leukaemic cells in patients who relapse after bone marrow transplantation may be of donor origin (Marmont et al, 1984; Smith et al, 1985) might suggest that the haemopoietic environment plays an important role in leukaemogenesis, though other interpretations, such as fusion of a normal donor cell with a leukaemic cell followed by 'diploidisation' or transfer of an aetiological agent such as a virus or other genetic material from host to donor cells are possible (Fialkow et al, 1971).

1.2.3 CLONALITY

Studies of the presence of the Ph chromosome in conjunction with expression of polymorphic genetic loci and analysis of X-chromosome linked restriction fragment length polymorphisms indicate that the initial 'neoplastic' event in CML probably occurs in a pluripotent haemopoietic stem cell. This cell is a progenitor of myeloid cells giving rise to granulocytes, macrophages, red blood cells and megakaryocytes (Fialkow et al, 1977; Yoffe et al, 1987a). Some B-lymphocytes also contain a Ph chromosome suggesting malignant transformation of a stem cell with B-lymphoid as well as myeloid potential (Martin et al, 1980; 1982). There are conflicting reports regarding T-cell involvement. The majority of peripheral blood T cells stimulated by mitogen do not have the Ph chromosome and are polyclonal by G6PD analysis (Fialkow et al, 1978; Kearney et al, 1982). Based on Southern analysis for BCR/ABL rearrangement and for T cell receptor sequences (an unique clonality marker) Bartram et al (1987a) showed that the T cell fraction from all his 12 CML patients did not have BCR rearrangement and were polyclonal. However, reports based on study of T cells in blast transformation indicated that the T cell lineage in some CML patients may be involved (Griffin et al, 1983; Allouche et al, 1985; Chan et al, 1986). There has also been a report of T-cell involvement in the chronic phase of CML (Fauser et al, 1985). In their study Fauser et al showed that T-cell progenitors gave rise to Ph-positive

mature T-cells. This may reflect the differing levels of stem cell commitment at which transformation occurs. While the majority of T cells in the peripheral blood are long-lived and antedate the development of CML or arose from progenitors not involved by CML, some T cells in some patients may be derived from a transformed haemopoietic stem cell with T-lymphoid potential.

To answer the question whether the level of pluripotent stem cell affected by CML influences the course of the disease, the underlying molecular changes (BCR/ABL chimeric gene, see Chapter 2) in different levels of stem cells from leukaemic patients with different phenotypes have been analyzed. The results indicate that although in certain patients the BCR/ABL rearrangement was detected at different levels of stem cell, the phenotype of the disease does not seem to reflect the site at which malignant transformation occurs (Gale, 1987).

1.3 GENERAL FEATURES OF CML

1.3.1 CLINICAL AND HAEMATOLOGICAL FEATURES

The majority of patients present with symptoms related to anaemia, splenomegaly or bleeding, but increasingly the diagnosis is made before the onset of symptoms - leucocytosis is recognized as a result of a routine blood test performed

for other reasons (Goldman, 1986).

Haematological examination of a patient with CML shows leucocytosis of variable degree with characteristically increased number of neutrophils, metamyelocytes, myelocytes, promyelocytes and blast cell (Spiers, 1977). Thrombocytosis and mild to moderate anaemia are present in the majority of cases (Canellos, 1976). The neutrophil alkaline phosphatase (NAP) score is almost always very low or zero. An aspirated specimen of bone marrow is usually intensely cellular, and 80 to 90 per cent of the cells are in the granulocyte series. The distribution of granulocytes shows only a slight shift toward immaturity. Marrow trephine biopsies show some degree of fibrosis; there is increased reticulin in about 25 per cent of cases. This fibrosis tends to become progressively more severe (Huguley, 1979).

1.3.2 CML HAS THREE PHASES: CHRONIC PHASE, ACCELERATED PHASE AND BLAST TRANSFORMATION

Classically CML begins in a relatively non-aggressive chronic phase. In this stage a patient usually has only mild symptoms related to CML. Death in chronic phase related to leukaemia is rare. The interval from the diagnosis of chronic phase to blast transformation varies from some months to many years; the longest chronic phase reported is over 17 years (Richman et al, 1984). Once blast transformation occurs, survival is

very brief with a median of approximately 2 months (Canellos, 1976). The chronic phase of CML typically has the following features: splenomegaly occurs at presentation in 90% of patients, lymphadenopathy is uncommon and its presence suggests acceleration of the disease process; the leucocyte count at diagnosis is greater than 100 X 10^9/l in 77% of cases; 8% of patients have platelet count above 200 X 10^9/l (Richman et al, 1984). Although some degree of basophilia is characteristic of CML, a progressive rise in the percentage of basophils is generally regarded as a poor prognostic sign, suggestive of accelerating disease (Theologides, 1972). Striking eosinophilia may be associated with eosinophilic leukaemia and an aggressive clinical course despite the presence of only mature eosinophils (Bentley et al, 1961; Krauss et al, 1964; Gruenwald et al, 1965). The percentage of blasts in the peripheral blood and bone marrow is usually less than 10% in the chronic phase.

Sooner or later the easily controlled chronic phase of CML changes to a more resistant disease, typically within 2-6 years of presentation. In approximately two-third of cases, this leads to a phase designated as acceleration. In the remaining one-third of patients with previously stable CML, blast crisis develops abruptly without a preceding accelerated phase. A small proportion of CML patients present in blast crisis with no identifiable preceding chronic phase.

In most CML patients the accelerated phase lasts some months. New symptoms such as fever and bone pain may develop. Progressive painful splenomegaly, lymphadenopathy and sternal tenderness may be observed. The raised leucocyte count may become refractory to previously effective doses of chemotherapy while the platelet count is easily depressed. Other poor prognostic signs such as basophilia, marrow fibrosis, or new cytogenetic abnormalities may appear (Spiers, 1977; 1979). In spite of the clinical evidence for a change in the disease process, the blood and/or bone marrow may not show any major increase in the numbers of immature cells such that a diagnosis of 'blast crisis' can not formally be made.

The symptoms and signs of established blast crisis of CML are generally similar to those of the acute leukaemias and are primarily related to anaemia, thrombocytopenia and granulocytopenia. The three most common clinical manifestations are progressive splenomegaly, malaise and unexplained fever. The criteria for defining blast crisis are not precise. Most studies suggest that the finding of more than 30% blast cells in the peripheral blood or marrow is diagnostic of blast crisis (Karanas & Silver, 1968; Canellos, 1976). By morphological assessment CML blast crisis is a heterogenous form of acute leukaemia; about one-third of cases have lymphoblastic morphology, while the remaining two-thirds

of cases have myeloblastic morphology (Rosenthal et al, 1977). There are differences in lymphoblastic and myeloblastic crises in clinical presentation and in response to therapy. Patients with lymphoblastic transformation are more likely to have severe thrombocytopenia and absent marrow megakaryocytes, while patients with myeloblastic transformation are more frequently severely anaemic, but have less severe thrombocytopenia. Patients with lymphoblastic disease tend to respond to vincristine and prednisone, agents useful in the treatment of acute lymphoblastic leukaemia (ALL) (Rosenthal et al, 1977; Richman et al, 1984).

1.4 THE PHILADELPHIA CHROMOSOME

1.4.1 Ph CHROMOSOME IN CML

In 1960 Nowell and Hungerford discovered a minute aberrant chromosome in the metaphases of the leukaemic cells of patients with CML (Nowell & Hungerford, 1960; 1961). This minute chromosome was later called the Philadelphia (Ph) chromosome after the city in which it was discovered. Using chromosome banding techniques the Ph chromosome was later found to be the result of reciprocal translocation between the long arms of chromosomes 9 and 22, t(9;22)(q34;q11) (Rowley, 1973; Fig. 1.1a & b). The Ph chromosome is found in all bone marrow cell lineages: myelocytic, monocytic, megakaryoblastic

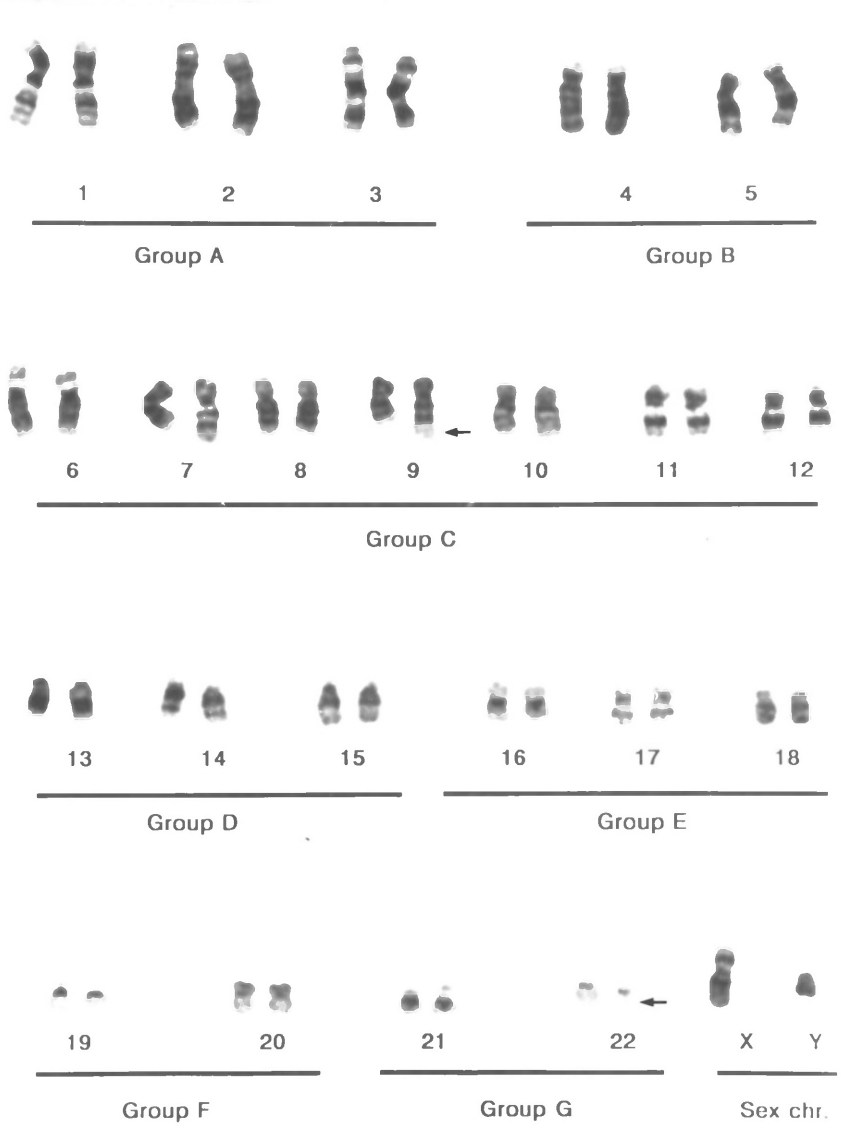


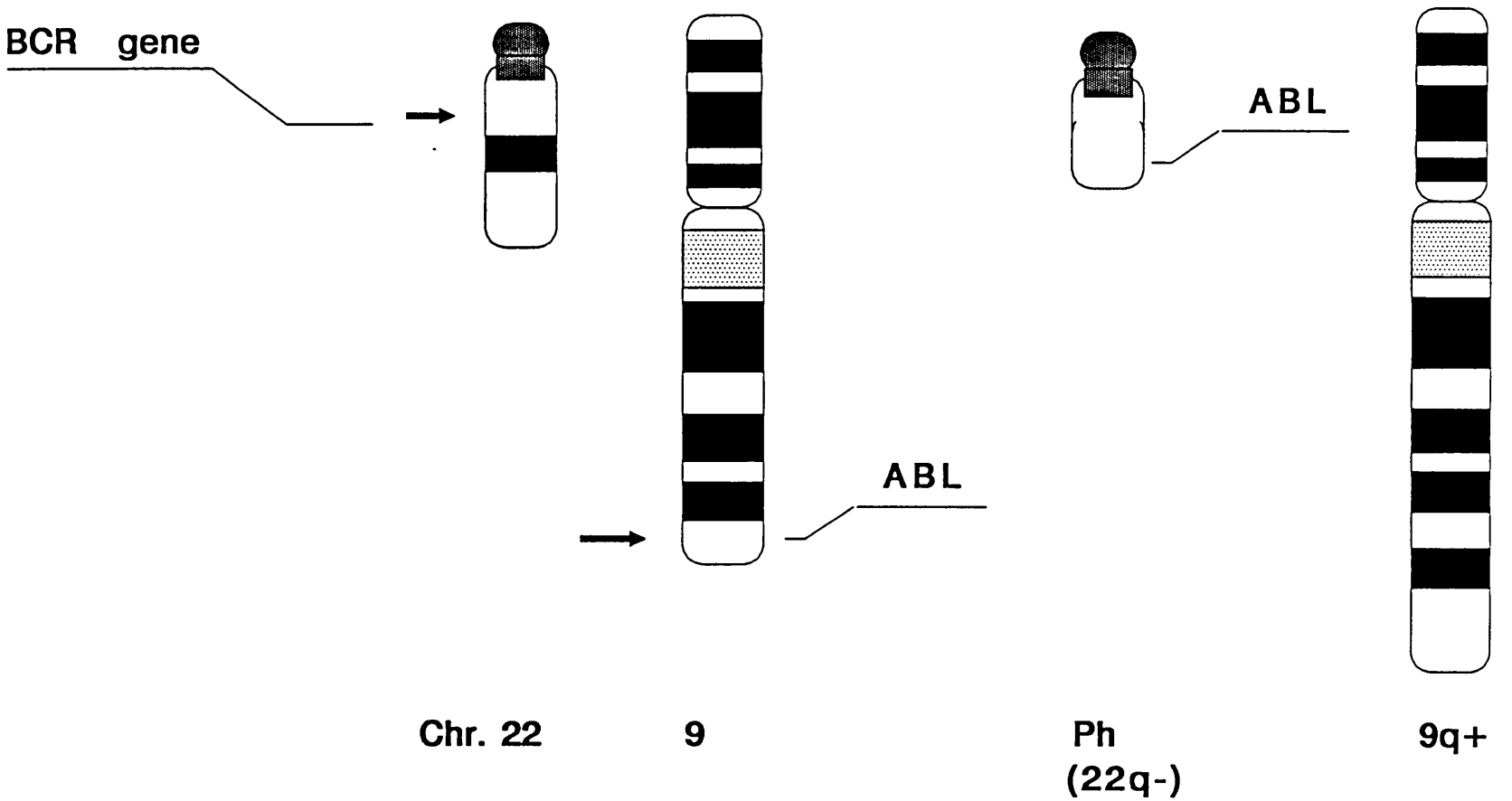
Fig. 1.1 The Philadelphia Chromosome and Its Formation.

(a). The karyotype from a male CML patient marrow cells showing 9q+ and 22q-, the Philadelphia chromosome, as indicated by the arrows.

(b). (next page) The diagram showing the mechanism of the formation of the Ph chromosome and the t(9;22)(q34;q11). The diagram also shows the localization of ABL proto-oncogene and the BCR gene on the chromosomes.

Fig. 1.1 (b). Formation of the Ph Chromosome

t(9;22)(q34;q11).



and erythroblastic precursor cells are Ph-positive (Fialkow et al, 1977). The Ph chromosome is also characteristically found in some B-lymphocytes (Martin et al, 1980). Peripheral blood T lymphocytes are usually Ph-negative, but there are reports of exceptional cases showing involvement of T-cell lineage in CML (see section 1.2.3 CLONALITY).

The Ph translocation, t(9;22)(q34;q11) is found in about 90% of CML cases (Canellos, 1976). Because of its consistent association with the leukaemic cells in CML, one assumes that it must be important in the pathogenesis of CML. Identification of the Ph chromosome has been widely used as a marker for the presence of CML cells in various studies and in the management of CML patients.

1.4.2 VARIANT TRANSLOCATIONS IN CML

The t(9;22)(q34;q11) is the standard Ph translocation. Variant forms of the Ph translocation are found in about 5% of cases. Three major cytogenetic variants have been described (Hagemeijer, 1987):

1. Simple translocations of the distal part of chromosome 22(22q11-qter) to another chromosome than 9, without apparent involvement of chromosomes 9.
2. Complex translocations involving chromosome 9 and 22 and at least one (sometimes more) additional chromosome.
3. Masked Ph chromosomes in which the Ph chromosome has lost

its typical 22q- appearance as a result of further translocations involving the q arm, or rarely the p arm. Simple or quite complex chromosomal rearrangements may give rise to a 'masked Ph chromosome' (Hagemeijer et al, 1985).

A fourth type of variant translocation, showing an apparently intact chromosome 22 and a breakpoint at 9q34, has also been reported (Bartram et al, 1985). These cases are considered to be Ph negative and will be discussed in the next section.

From a clinical point of view there seems to be no difference between cases with standard or variant Ph translocations; the actuarial curves for duration of chronic phase and survival overlap (reviewed by Hagemeijer, 1987).

1.4.3 CML PATIENTS LACKING THE Ph CHROMOSOME

CML can be divided into Ph-positive and Ph-negative leukaemia on the basis of presence or absence of Ph chromosome. Most cases of CML (90%) are Ph-positive and clinically typical. About 5-15% of CML cases are Ph-negative on cytogenetic study (Whang-Peng et al, 1968; Ezdinli et al, 1970; Kantarjian et al, 1986). However with higher resolution banding technology some of these patients were found to be CML with a masked Ph chromosome. These patients usually have a typical clinical picture resembling to the Ph-positive CML. In addition, a few cases of Ph-negative CML have other chromosome abnormalities

but not a masked Ph, such as a breakpoint at 9q34 with apparently intact chromosome 22, as mentioned above (Bartram et al, 1985). Other patients are karyotypically strictly normal. Generally speaking these 'true' Ph-negative CML patients constitute a group that is heterogeneous in their biological and clinical features. The atypical features are characterized by shorter chronic phase and shorter survival, lack of splenomegaly, and other aberrant morphological features (Ezdinli et al, 1970; Kantarjian et al, 1986). These include a striking excess of monocytes, a virtual absence of basophils, and the presence of prominent dysplastic features in the granulocytic cells from the myelocyte to more mature stages (Shepherd et al, 1987).

Molecular studies of BCR/ABL rearrangements (see Chapter 2) in this group of Ph-negative CML patients have provided a new basis for their classification (Shepherd et al, 1987; Gao et al, 1990a). For example, the CML patients with a masked Ph chromosome, some CML patients with a 9q34 breakpoint but apparently normal chromosome 22 and some cases of CML with a strictly normal karyotype, were found to have M-BCR rearrangement and to be clinically indistinguishable from typical CML (Bartram et al, 1985; Hagemeijer et al, 1985). However, the Ph-negative CML patients with atypical features are M-BCR non-rearranged. There is no evidence for BCR gene or ABL gene involvement (Wiedemann et al, 1988; Gao et al,

1990).

Yet another group of atypical patients should be mentioned briefly, i.e. the rare cases with late appearance of the Ph chromosome (Lisker et al, 1982) and cases with early disappearance of the Ph chromosome (Hagemeijer et al, 1979). Both types of event do not apparently affect the course of the disease. These cases are extremely rare.

1.4.4 FURTHER CHROMOSOMAL CHANGES IN ACCELERATED PHASE AND BLAST TRANSFORMATION (BT)

In the acute phase of Ph-positive CML, 20% of patients show only the original t(9;22)(q34;q11) translocation, while 80% show karyotypic changes in addition to the Ph coinciding with or preceding BT. Although some cases with persisting chronic phase have been observed to show additional karyotypic change, the appearance of additional chromosome changes on serial examinations is generally regarded as a sign of evolution to BT, especially when clonal expansion and increasing complexity of the karyotype are observed (Lawler, 1977; Hagemeijer et al, 1980). The principal new abnormalities in order of frequency are: an additional 8, an additional Ph and isochromosome 17q (reviewed by Richman et al, 1984). The isochromosome 17q [i(17q)] is formed as the result of p arm deletion and duplication of q arm. Myeloid and lymphoid blast transformations may have different additional changes: for

example, in myeloid BT a hyperdiploid karyotype is usually present with acquired non-random abnormalities such as +8, +19, i(17q) and +22q- (Ph) in various combinations. In lymphoid BT, the majority of the cases show only the Ph without additional changes (Yao, 1985). Whether specific cytogenetic markers correspond to the lymphoid phenotype is not yet clear. The i(17q) seems restricted to myeloid transformation; duplication of Ph and trisomy 8 are found in both myeloid and lymphoid transformations (Hagemeijer, 1987; Alimena et al, 1987). The mechanism and biological significance of these additional chromosome changes in BT are not clear. In most cases these additional changes have not been characterized at the molecular level. Only rare cases have secondary rearrangements of BCR. Inactivation of P53 gene or mutations of the RAS oncogenes have been found in BT. P53 gene is located in the p arm of chromosome 17 (Bartram et al, 1986a; Liu et al, 1988; Ahuja et al, 1989). The correlation between abnormalities of P53 gene with cytogenetic changes is yet to be studied.

1.5 TREATMENT

Conventional treatment for CML involves single-drug chemotherapy for controlling leucocytosis in chronic phase (CP) and combination chemotherapy for controlling blast cell

proliferation and leukaemia-related symptoms in blast crisis (Allan & Shepherd, 1987). Although there is no convincing evidence that conventional treatment for CML can prolong the duration of the CP or of survival, high dose chemoradiotherapy (or chemotherapy) combined with allogeneic bone marrow transplantation (BMT) does offer a possibility for eradicating CML (Fefer et al, 1979; Speck et al, 1984; Armitage et al, 1984; Thomas et al, 1986; Goldman, 1986; 1988; McGlave et al, 1987a). However, BMT is still associated with major problems such as relapse, engraftment failure and graft-versus-host disease (GvHD) (Mackinnon & Goldman, 1987). The use of interferon-alpha in the treatment of CML seems encouraging but it is too early to draw any useful conclusion about prolongation of survival (Talpaz et al, 1987).

1.5.1 CONVENTIONAL MANAGEMENT

The management of the chronic phase is designed to alleviate symptoms or to delay their onset. Busulphan may still be the drug of choice but hydroxyurea is a reasonable alternative (Galton, 1953; Bolin et al, 1982). Attempts to eradicate or reduce the number of malignant stem cells or to postpone blast crisis by aggressive multiple chemotherapy that includes cytarabine, daunorubicin, and/or cyclophosphamide have been generally unsuccessful and engender considerable toxic effects (Hester et al, 1984; Clarkson, 1985).

The management of accelerated phase of CML has no precise guidelines. Instead, the choice of treatment may best be tailored to the needs of the patient, e.g. splenectomy, higher doses of cytotoxic drugs, or antibiotics if patients develop infections (Goldman, 1986).

It is customary to treat patients with myeloid transformation of CML with combinations of cytotoxic drugs appropriate to the management of acute myeloid leukaemia. Thus some combination of an anthracycline with cytarabine with or without 6-thioguanine is the most usual initial approach (Coleman et al, 1980). Other combinations have included new drugs such as 5-azacytidine (Winton et al, 1981; Schiffer et al, 1982).

There is no universally agreed protocol for the management of patients with lymphoid transformation but the majority will respond to cytotoxic drugs that are usually effective in the management of ALL. Thus treatment may reasonably begin with vincristine and corticosteroids (Canellos et al, 1976).

1.5.2 α-INTERFERON TREATMENT

In 1983 the group in Houston reported that administration of partially purified alpha interferon controlled the leucocyte count in 20 of 27 patients with CML (Talpaz et al, 1983). More recently they treated 17 patients with recombinant human interferon alpha; 14 patients responded by entering complete

or partial haematological remission. Of great interest in this study was the observation that 8 of 17 patients showed evidence of suppression of Ph-positive haemopoiesis, although this was frequently not sustained (Talpaz et al, 1986). Clearly, in some patients interferon has the capacity selectively to suppress Ph-positive haemopoiesis. The Ph-negativity at cytogenetics level can be confirmed by Southern analysis for M-BCR rearrangement (Yoffe et al, 1987b). The possible benefit on the overall survival remains to be assessed (Allan & Shepherd, 1987).

1.5.3 BONE MARROW TRANSPLANTATION (BMT)

The treatment of CML with most cytotoxic drugs is limited in part by their toxicity to the patient's marrow. The availability of normal marrow for transplantation permits the administration of far higher and potentially curative antitumour doses of chemotherapy or chemoradiotherapy followed by restoration of the patient's haemopoietic and immunological functions by the donor cells.

In order to minimize the risk of graft rejection and of graft-versus-host disease (GvHD) marrow donors have hitherto been mainly syngeneic or genotypically HLA-identical siblings. However only about a third of patients in the western world have such matched marrow donors (Beatty et al, 1988). A small

number of transplants have been performed for patients with CML using HLA matched unrelated donors (Goldman et al, 1987; McGlave et al, 1987b). It is too early to draw firm conclusions but the use of such donors may eventually yield results similar to those using HLA identical siblings.

The initial results of BMT for CML have been encouraging, but the risk of severe GvHD is still considerable and may lead to transplant related death. Much effort has therefore been devoted to its prevention. T-cell depletion of donor marrow seemed at one time to be a practical solution. However, although the incidence of GvHD and the post transplantation death were reduced, the relapse rate increased very substantially. The use of monoclonal antibodies for T cell depletion is also associated with an increased risk of graft failure (Apperley et al, 1986a; Goldman, 1988).

Reports from the Seattle group indicate that about 75% of patients who receive syngeneic transplants during the chronic phase have remained in complete remission 24-89 months after BMT, but patients treated in blast transformation have a much less favourable response. Only one out of eight patients in BT who received syngeneic BMT is in complete remission 4.5 years after a second BMT (Fefer et al, 1982; Fefer et al, 1984). A survey of the results in different transplant centres with syngeneic donors for patients with CML has been carried

out. The actuarial disease-free survival for patients transplanted in CP was 65% at 6.6 years (Champlin et al, 1983). The results of allogeneic transplants for CML are basically in line with the above observations (Fefer et al, 1984; Goldman et al, 1986). The probability of relapse for the non-depleted transplant was estimated as 2-7% (Apperley et al, 1988; Thomas et al, 1986) and for the T-depleted was > 50% (Apperley et al, 1988).

1.5.4 ENGRAFTMENT AND RELAPSE AFTER BMT

A successful BMT must depend on the effective elimination of leukaemic cells and reconstitution of normal haemopoiesis by donor stem cells. Relapse after BMT can be haematological and/or cytogenetic. Haematological relapse is featured by the recurrence of leukaemic cells and the reappearance of a haematological and clinical picture of CML. Relapse may be more likely to occur in patients with cytogenetic abnormalities before transplant in addition to the standard Ph chromosome, especially +8, +Ph or variant Ph, than in those without evidence of cytogenetic evolution (Przepiorka and Thomas, 1988). If the patient was in chronic phase of CML at the time of the transplant and received unmanipulated donor cells relapse is uncommon but can still occur. If however the patient received a transplant with a donor marrow depleted of T cells, relapse is much more common. In either case relapse is usually recognized within 2 years of transplant but may

occasionally occur at intervals up to 4 years post transplant (Goldman et al, 1988). In relapse cytogenetic analysis will reveal the Ph chromosome in the majority of metaphases (Zaccaria et al, 1987; 1988; Arthur et al, 1988). The pattern of rearrangement in the BCR gene at relapse is identical to that seen before transplantation (Ganesan et al, 1987; Gao et al, 1988), a finding that supports the conclusion that relapse is due to failure to eliminate the original leukaemic clone and not to development of CML de novo on a second occasion.

In other cases the only evidence for relapse is the finding of Ph positive metaphases in the marrow cells after transplant. These cases we have called 'cytogenetic relapse' (Arthur et al, 1988). Most of these cases were identified within 1 or 2 years of transplant without progression to haematological relapse (Thomas et al, 1986; Apperley et al, 1986b; Sessarego et al, 1987; Zaccaria et al, 1987, 1988; Arthur et al, 1988). In some of these cases the proportion of Ph positive metaphases rose to a peak value and then declined. In other cases the finding of Ph positive marrow metaphases persisted for months or years without haematological relapse or proceeded to overt haematological relapse (Arthur et al, 1988; Hughes et al, 1989).

Two CML cases have been reported in which relapse after transplant occurred in cells that appeared to be of donor

origin (Marmont et al, 1984; Smith et al, 1985). Both patients were transplanted after having entered blastic transformation. Both received marrow from HLA identical siblings of the opposite sex and in both cases blast cells at relapse had the sex chromosome pattern of the donor. There are in theory many mechanisms by which relapse after BMT could be of donor origin (Fialkow et al, 1971; Editorial, 1984). There could exist a powerful microenvironmental influence that caused the original leukaemia in the host and later induced leukaemia in the donor cells. Alternatively the appearance of relapse in donor cells might be artefactual if exchange of sex chromosomes had taken place between the host's leukaemic cells and normal cells of donor origin. Perhaps the most likely interpretation is the transfer or 'transfection' of a 'transforming' sequence of DNA from residual leukaemic cells of host origin to the donor cells.

1.5.5 AUTOGRAFTING

The possibility of curing CML by autografting still seems remote. The majority of patients treated by high-dose chemotherapy and autografting for CML in transformation recover partial and transient Ph-negative haemopoiesis (Haines et al, 1984). There are anecdotal reports of patients rendered Ph-negative by high-dose busulphan or by autografting in chronic phase (Brito-Babapulle et al, 1987; 1989). Similarly a small minority of patients treated with -interferon become

Ph-negative. In such cases one could contemplate the use of Ph-negative autologous marrow in an autograft procedure designed to cure the patient, but the successful use of this approach has not yet been reported.

Perhaps the most promising future approach to autografting involves the use of peripheral blood or marrow cells collected from the patient at diagnosis and 'purged' in vitro to favour selective regeneration of Ph-negative, putatively normal, haemopoietic stem cells. How exactly this purging is best achieved, whether by immunological, pharmacological or cytokinetic means, is at present speculative (Mackinnon & Goldman, 1987; Barnett et al, 1989; Brito-Babapulle et al, 1989).

CHAPTER TWO MOLECULAR BIOLOGY OF CML

2.1 BCR/ABL CHIMERIC GENE

The Ph translocation brings the ABL oncogene located on the long arm (q34) of chromosome 9 to the long arm (q11) of chromosome 22, where it is juxtaposed to the upstream portion of the BCR gene. The position of the breakpoint on chromosome 22 is limited to a 5.8kb region in most of cases of CML (Groffen et al, 1984). This region has been called the major breakpoint cluster region (M-BCR) and hence the gene in which the M-BCR is situated is called BCR gene (Heisterkamp et al, 1985; Gale and Goldman, 1988). In the Ph translocation, ABL is joined at its 5' end to the 5' portion of BCR gene and the BCR/ABL chimeric gene is formed. This new gene is transcribed as a chimeric mRNA of 8.5kb and translated into a new protein P210. The P210 is capable of phosphorylating tyrosine residues on proteins, including itself, in vitro as well as in vivo (Konopka et al, 1984). This is believed to be important in the leukaemic transformation of haemopoietic cells. This rearrangement has been widely quoted as an example of oncogene activation underlying human malignancy.

2.1.1 ABL PROTO-ONCOGENE

Study of a transforming murine leukaemia virus, isolated by Abelson and Rabstein, in the late 1970's led to the discovery of the ABL oncogene (Abelson and Rabstein, 1970; Reynolds et al, 1978; Shields et al, 1979; Goff et al, 1980; Reddy et al, 1983; Wang et al, 1984). The Abelson variant of the Moloney murine leukaemia virus (M-MuLV) includes cellular sequences of mouse origin which, when occurring in the virus, have been designated v-abl. The v-abl sequences encode a protein with tyrosine-specific protein kinase activity in vitro and in vivo (Witte et al, 1980a). The intrinsic tyrosine kinase activity of the protein is closely associated with the ability of A-MuLV to transform cells. Mutants with reduced kinase efficiency have lower transforming efficiency and mutants which lack tyrosine kinase activity are transformation-deficient (Witte et al, 1980b). Tyrosine kinase activity has also been associated with the transforming proteins of other retroviruses and with the cellular receptors for several growth factors, e.g. epidermal growth factor, platelet derived growth factor and insulin (Ushiro and Cohen, 1980; Ek et al, 1982; Kasuga et al, 1982). Nucleotide sequence comparisons of the genes encoding these viral and cellular tyrosine kinases show that they are highly homologous in their catalytic domains (Reddy et al, 1983; Groffen et al, 1983).

The ABL proto-oncogene was identified in the human genome by

Southern blotting using a v-abl probe. The ABL gene has been highly conserved during evolution and is located on the long arm of chromosome 9 (9q34) in man (Heisterkamp et al, 1982; de Klein et al, 1982; Bartram et al, 1983). The normal product of the ABL gene is a 145kD protein that weakly phosphorylates tyrosine residues of proteins. This protein kinase activity can be easily demonstrated in vitro as the protein can act on its own substrate (in vitro autophosphorylation) (Konopka and Witte, 1985). The precise function of this protein is unknown.

Using a cosmid library of human carcinoma DNA, sequences homologous to v-abl in the human genome were characterized which were distributed discontinuously over a region of 32kb and dispersed over nine exons (Heisterkamp et al, 1983a; Shtivelman et al, 1985). Comparison of the sequences of v-abl, which may be regarded as an incomplete cDNA copy of the murine ABL oncogene, with the sequences of 6 and 7 kb human ABL mRNA revealed the existence of additional human ABL exons located 5' to the v-abl homologous exons. The exon located immediately 5' of the v-abl homologous exons has been designated exon II. There are two additional ABL exons present 5' of exon II, designated exons Ia and Ib (Fig. 2.1a). These two exons alternatively splice to exon II to produce the 6 and 7kb mRNAs respectively. If transcription is initiated from exon Ib then exon Ia is removed by splicing with the surrounding introns. As the two ABL mRNAs initiate at different promoters and these

Fig. 2.1 Physical Map of the Human BCR and ABL genes.

(a). Physical map of the ABL gene. The open box marked 'body exons' contains 11 exons but the details were omitted in this map. Vertical arrows indicate the common locations of the breakpoint in the Ph translocation.

(b). Physical map of the BCR gene with an enlarged restriction map of the M-BCR. Black boxes represent the known exons while the shaded boxes represent the possible uncharacterized exons. Vertical arrows indicate the common locations of the breakpoint in the Ph translocation. E = EcoRI, B = BamHI, Bg = BglII and H = HindIII. m-BCR stands for minor breakpoint cluster region.

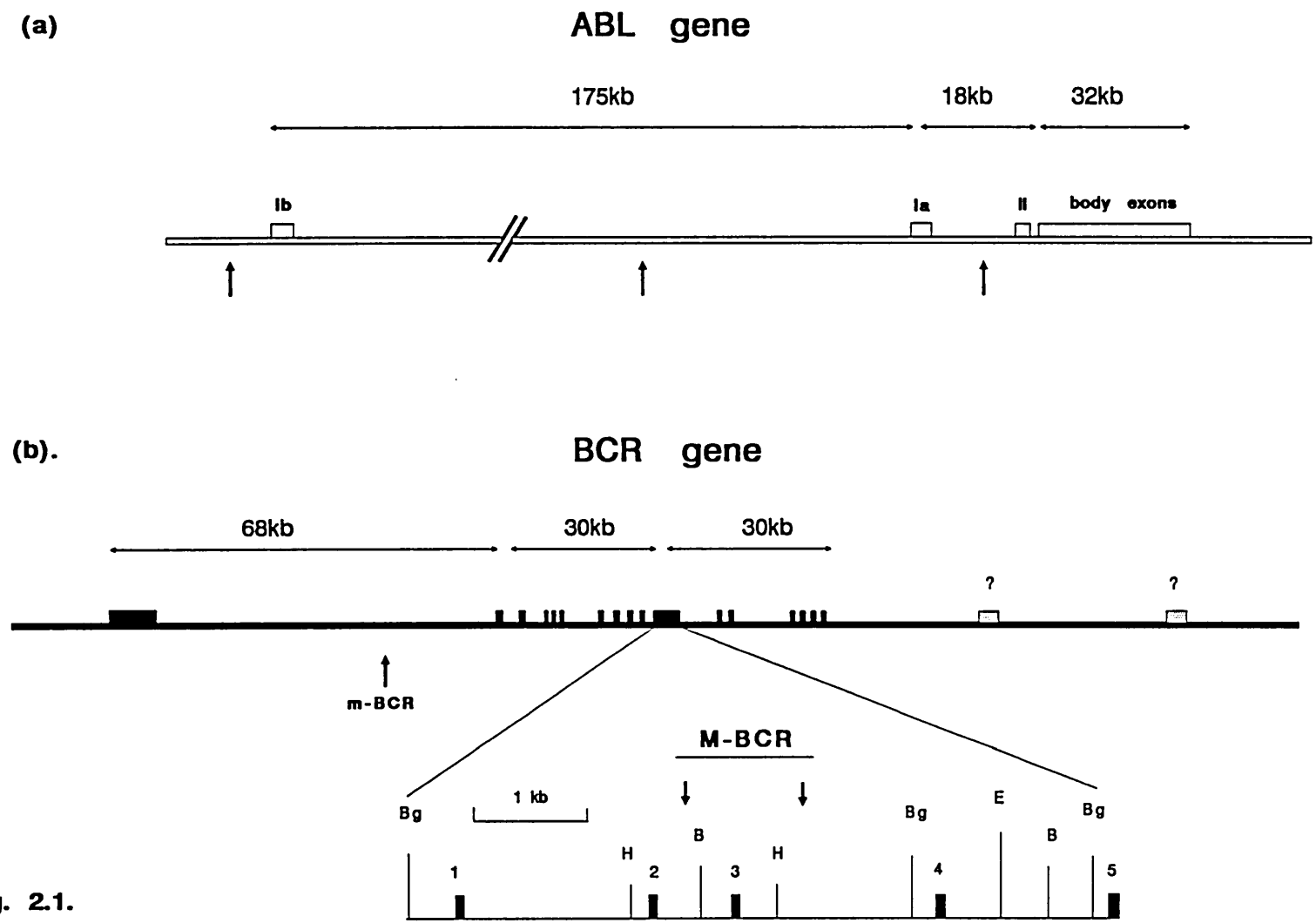

(a)
ABL gene
175kb
18kb
32kb
Ib
Ia
II
body exons
(b).
BCR gene
68kb
30kb
30kb
?
?
m-BCR
M-BCR
1 kb
Bg
Bg
Bg
E
B
B
H
H
1
2
3
4
5

Fig. 2.1.

exons contain a protein initiating site, they give rise to proteins that differ at their N-termini (Shtivelman et al, 1985; 1986). The ABL oncogene lacks TATA and CAAT boxes, and its two promoter regions are G+C-rich and contain multiple GCGCGC repeats. Together with the low expression in many tissues these features suggest that ABL might be a 'housekeeping' gene (Melton et al, 1986; Groffen et al, 1987).

To determine the real size and structure of ABL gene, molecular cloning was used. The v-abl homologous exons alone were found to be dispersed over a region of 32kb. ABL exon II is located very close to the v-abl homologous exons. Exon Ia was located 18kb 5' of the most 5' v-abl homologous exon. Although considerable stretches of DNA immediately 5' of this exon have been cloned, none of these cloned DNAs contain exon Ib (Shtivelman et al, 1985; 1986). Experiments using pulsed field gel electrophoresis (PFGE) have demonstrated that exon Ib is situated approximately 200kb 5' of exon II (the first common exon) (Westbrook et al, 1987). These data indicate that the ABL gene has some unusual features: splicing must occur over very long distances and when transcription initiates at exon Ib, exon Ia is spliced out and exon Ib is fused to exon II.

2.1.2 BCR GENE AND M-BCR

Heisterkamp et al (1983b) isolated a chimeric DNA fragment

from one CML patient containing sequences from chromosome 9 and 22. Studies of chimeric restriction fragments resulting from the Ph translocation in a group of CML patients led to the discovery of a region in 22q11 in which the breakpoints in the Ph chromosome were located in most of the cases (Groffen et al, 1984). This region, defined by two Bgl II sites 5.8kb apart, was named the "breakpoint cluster region" (bcr). The region is now known to be situated in the middle of a gene, whose function is as yet undefined (Heisterkamp et al, 1985; Gale and Goldman, 1988). The gene has been called the BCR gene and this terminology is at present widely accepted.

Nevertheless, some Ph-positive CML patients and some Ph-positive acute leukaemia patients have since been shown to have breakpoint outside this breakpoint cluster region. In about half of the Ph-positive acute leukaemias the chromosome 22 breakpoint was located in the first intron of the BCR gene (Bartram et al, 1987b; Hermans et al, 1987; Gao et al, 1990a). Therefore the name 'major breakpoint cluster region' (M-BCR) has been proposed for the classical breakpoint cluster region, and 'minor breakpoint cluster region' (m-BCR) for the region in the BCR gene first intron where breakpoints occur in about half of Ph-positive leukaemic patients (Gale and Goldman, 1988) (Fig. 2.1b).

The BCR gene is about 130kb in size and its coding sequence (the size of the full length cDNA) is about 4.5kb. The M-BCR is about 100kb downstream of the exon 1 of the gene and the first intron is about 68kb in size (Heisterkamp et al, 1985; 1988a; Lifshitz et al, 1988). Within the M-BCR there are five small exons (varying from 76 to 105 base pairs), which are numbered b1 to b5 (Heisterkamp et al, 1985). In most CML patients the breakpoint is located either between exons 2 and 3 or exons 3 and 4 (Fig. 2.1b). A restriction map of the M-BCR enables the location of the breakpoint in individual CML patients to be determined.

The orientation of the BCR gene on chromosome 22 has been determined: the 5' end of the BCR gene is directed towards the centromere of chromosome 22 and remains on the Ph chromosome, while the 3' end is directed towards the telomere and is translocated to chromosome 9 in the t(9;22) (Heisterkamp et al, 1985).

Computer banks have been searched to discover whether any homology could be detected between the BCR gene and recognized oncogenes, sequences coding for growth factors or other known proteins. However, no significant homology has been found, indicating that at present the BCR protein sequence yields few clues as to its normal cellular function or its possible role in CML (Hariharan and Adams, 1987).

2.1.3 BCR/ABL CHIMERIC GENE

The human ABL was localized to chromosome 9 using somatic cell hybrids and in situ hybridization. It is known that the ABL oncogene is translocated to the Ph chromosome in the t(9;22)(q34;q11) translocation (de Klein et al, 1982). In addition, the translocation of ABL in variant forms of CML was investigated. Employing in situ hybridization techniques, translocation of human ABL was not consistently found in patients of Ph-negative CML. However, in some patients with Ph-negative CML in which chromosome 22 was previously thought not to be involved, ABL is translocated to chromosome 22 (Bartram et al, 1983; 1985; Morris et al, 1986).

More direct evidence of ABL involvement in CML might have come from the discovery of a translocation breakpoint on chromosome 9 that occurred either within or in relatively close proximity to ABL. However, standard Southern analysis with various ABL probes usually fails to find the breakpoint in CML patients (Heisterkamp et al, 1983b). Molecular cloning of DNA from a few patients with Ph-positive CML showed that the breakpoints were distributed over a large area at various distances from the first common exon (exon II) (Groffen et al, 1984; Heisterkamp et al, 1985; de Klein et al, 1986a). Northern blot and protein studies demonstrating the BCR/ABL chimeric gene products provide direct evidence of ABL involvement (discussed

below).

In the Ph translocation the 3' portion of the ABL which contains the transcriptional sequence for protein kinase products (in all the cases, this portion presumably includes exon II and the following downstream 'body' exons) is translocated to the middle of the BCR gene (Heisterkamp et al, 1983b; Shtivelman et al, 1985). The 5' portion of the BCR gene is juxtaposed to the ABL and a BCR/ABL hybrid gene is formed (Fig. 2.2). This rearrangement is best detected by Southern analysis for M-BCR rearrangement, because the distribution of breakpoints in chromosome 9 over a large area makes Southern analysis for the ABL breakpoint very unrewarding. After rearrangement, the hybrid ABL product acquires some new characteristics, such as the capability to autophosphorylate its tyrosine residues (Davis et al, 1985; Konopka & Witte, 1985).

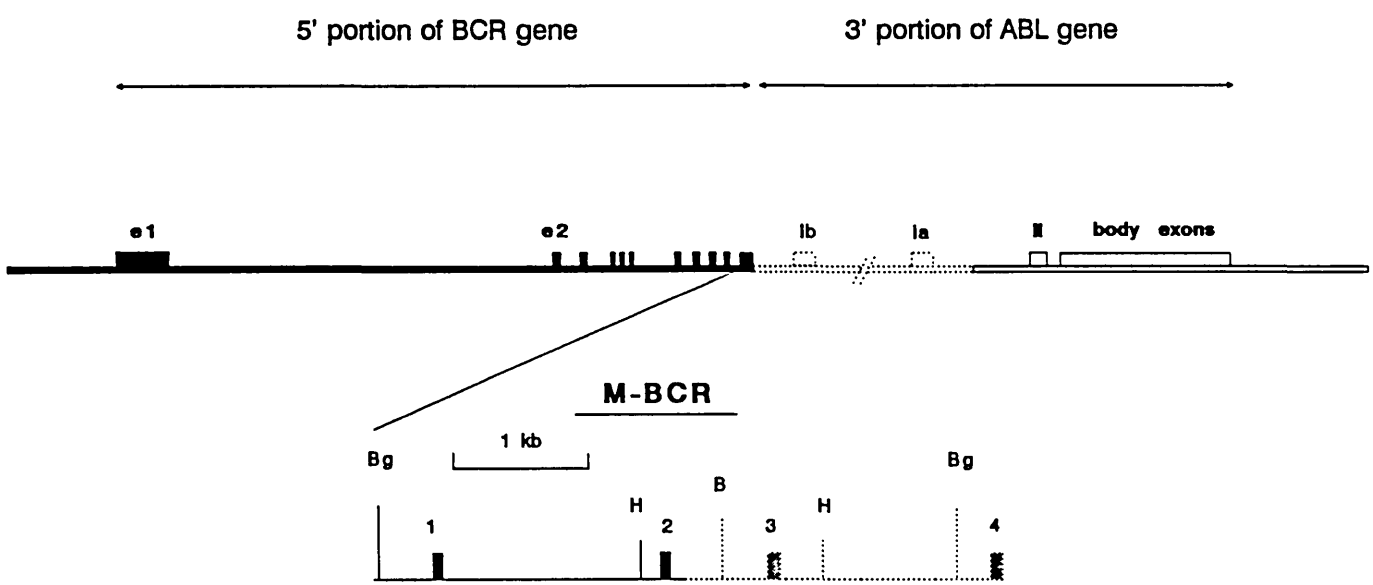
2.1.4 LOCATION OF THE CHROMOSOME 22 BREAKPOINT IN RELATION TO THE CLINICAL FEATURES OF LEUKAEMIA

Speculation that the precise location of the breakpoint in M-BCR (namely, 3' or 5' in the M-BCR) might be related to the phenotype and prognosis in individual patients began in 1987. Two studies of the locations of the breakpoint on chromosome 22 in patients with Ph-positive CML found a strong association between more distal breakpoints (near the 3' end of the M-BCR,

Fig. 2.2 Rearrangement of the BCR and ABL Genes.

The 5' portion of the BCR gene rearrange with the 3' portion of the ABL gene. The dotted lines reflect the zones in the ABL (above) and BCR (below) in which the alternative breaks may occur.

Fig. 2.2 **Rearrangement of the BCR gene and the ABL gene**



downstream of the third exon) and early blast transformation (Schaefer-Rego et al, 1987; Mills et al, 1988). These reports suggested that the location of the breakpoint involved in formation of the Ph chromosome may have a significant influence on the course of CML. This important question is now being studied prospectively. If a correlation between location of the breakpoint and survival were confirmed, this could be a major new prognostic feature. However, at least one recent report has failed to confirm the association (Jaubert et al, 1989).

2.2 M-BCR NON-REARRANGED CML

In a minority of cases of Ph-positive CML there is no evidence of M-BCR rearrangement (Selleri et al, 1987; Saglio et al, 1988). In some of these patients lacking evidence for M-BCR rearrangement were found to be due to a small deletion of the 3' section of M-BCR. In these cases the rearrangement can still be detected if a 5' M-BCR probe or a full length M-BCR probe is used (Popenoe et al, 1986; Hirosawa et al, 1988). Cases have been reported in which a breakpoint is located outside M-BCR but still within the BCR gene (Bartram et al, 1987b; Saglio et al, 1988).

One case of Ph-positive, M-BCR non-rearranged CML in whom a

breakpoint was found in the first intron of the BCR gene has been reported (Selleri et al, 1990). This case is very unusual because it is generally believed that a first intron breakpoint is confined to Ph-positive, M-BCR non-rearranged acute leukaemia (see below).

Some CML patients have clinical features closely resembling typical CML but lack the Ph chromosome. Some of them are M-BCR rearranged and the others are M-BCR non-rearranged (Morris et al, 1986; Wiedemann et al, 1988). The absence of M-BCR rearrangement in the above mentioned CML patients raises two fundamental questions: do these patients have BCR gene involvement (with or without the formation of Ph chromosome) and if so where is the breakpoint? It is also interesting to ascertain the biological significance of such 'deviation' from the conventional site of breakpoint.

2.3 BCR/ABL HYBRID mRNAs

2.3.1 NORTHERN ANALYSIS

There are two normal human ABL transcripts: 6.0kb and 7.0kb mRNAs. Exon Ia is transcribed in the 6.0kb mRNA. In exon Ib transcript, the 7.0kb ABL mRNA, exon Ia is spliced out. The two ABL mRNAs initiate at different promoters and give rise to proteins that differ in their N-termini (Shtivelman et al,

1986). There is no evidence that the two proteins are expressed differentially, as both ABL mRNAs have been detected in approximately equal quantities in all tissues analyzed (Westin et al, 1982; Groffen & Heisterkamp, 1987).

The BCR gene also has two normal transcripts, a 4.5kb and 6.5kb mRNA (Shtivelman, et al, 1985; 1986; Heisterkamp et al, 1985). The 4.5kb mRNA represents the transcript of all known exons of the BCR gene while the origin of the normal 6.5kb transcript remains unclear. The 6.5kb mRNA appears to contain most if not all of the sequences present in the 4.5kb mRNA. The additional sequences in the 6.5kb transcript might be located at its 5' end, internally, or at its 3' end (Groffen & Heisterkamp, 1987).

As the result of the Ph translocation hybrid BCR/ABL transcripts are produced. The abnormally sized 8.5kb BCR/ABL mRNA (the reported size of this abnormal transcript varies between 8 to 8.5kb in different studies) is characteristically detected in CML cells (Canaani et al, 1984; Collins et al, 1984; Gale and Canaani, 1984). It is chimeric and represents 5' BCR sequences fused to 3' ABL sequences. As predicted from the genomic DNA organization, it encompasses all the BCR 5' exons up to and including M-BCR exon 2 or 3 (fig. 2.3); the BCR sequence is then joined to ABL exon II in such a fashion that it is in frame (Shtivelman et al, 1985; 1986). Although

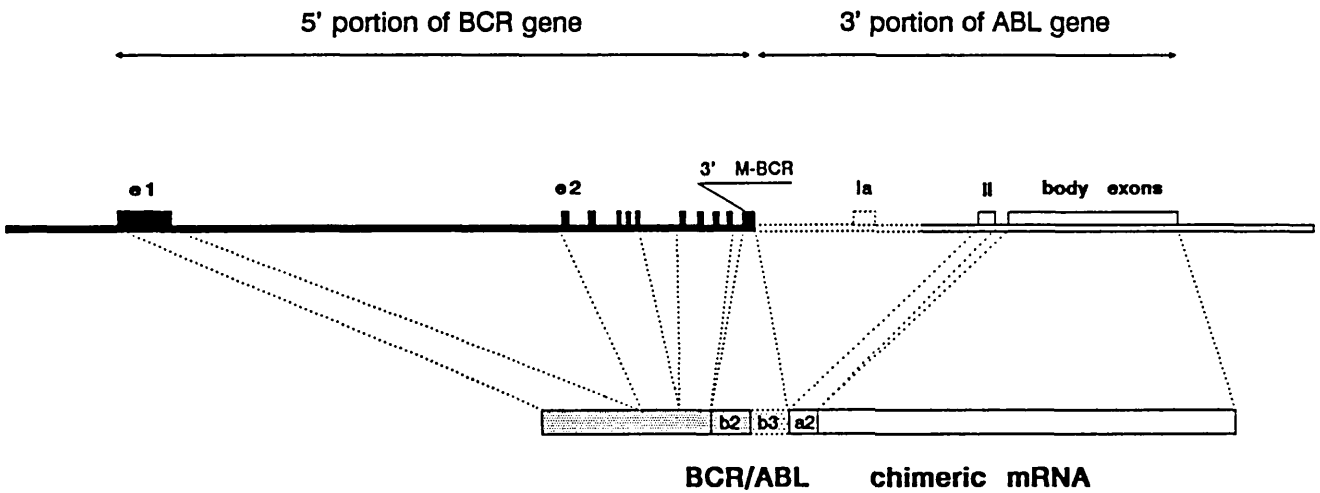


Fig. 2.3 The formation of the alternative BCR/ABL chimeric mRNA in CML

b2 and b3 represent M-BCR exons 2 and 3; a2 represents ABL exon II. When the M-BCR breakpoint is located between exons b2 and b3, a chimeric mRNA with b2a2 junction is produced (the solid boxes); if the M-BCR breakpoint is located between exons b3 and b4, a mRNA with b3a2 junction is produced (dotted line box).

one ABL exon 5' to exon II (exon Ia) is present in the genomic DNA of K562 after the Ph translocation, it is apparently deleted by splicing in the formation of mature chimeric mRNA; the CML cell line EM2 has a similarly structured chimeric mRNA, with M-BCR exon 3 joined to ABL exon II. Most Ph-positive CML cells seem to contain a similarly sized abnormal transcript of 8kb (8.5kb documented by some researchers). However two cases with an additional transcript of 9.0kb have been reported (Gale and Canaani, 1984). This indicates that in some cases additional BCR and/or ABL exons may be included in the transcript.

2.3.2 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction has been used to study the presence of the BCR/ABL hybrid mRNA and the type of junctions (Fig. 2.4). In patients with a M-BCR breakpoint located between M-BCR exons b2 and b3, a b2a2 junction mRNA is produced. However, in patients with breakpoint between b3 and b4, both mRNA of two junctions (b2a2 and b3a2) could be produced by alternative splicing of b3. There may or may not be a correlation between the type of junction and the prognosis and the phases (CP or BT) of the disease (Schaefer-Rego et al, 1987; Shtivelman et al, 1987; Dobrovic et al, 1988; Lange et al, 1989). In cases of Ph-positive leukaemia with a breakpoint in the first intron of the BCR gene, a junction of BCR gene exon 1 (e1) and a2 would be expected

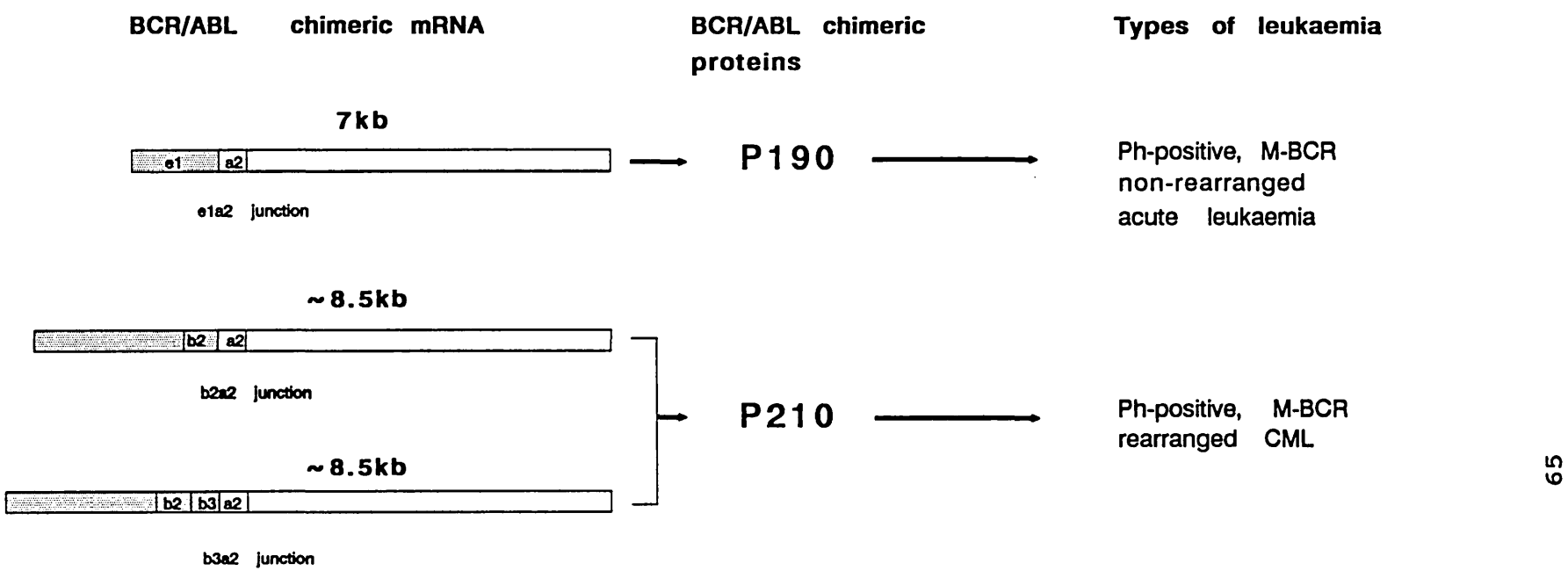


Fig. 2.4 Alternative junctions in different BCR/ABL chimeric gene products.

mRNA with alternative junctions due to different breakpoints in the BCR gene results in three gene products: the two types of P210 and the P190. This diagram also shows their relation with clinical features.

(Kawasaki et al, 1988; Hermans et al, 1988) (Fig. 2.4).

Because of the high sensitivity of this technology, PCR has been also used in the study of residual leukaemic cells after bone marrow transplantation for CML (Lee et al, 1988; Morgan et al, 1989). The collected data suggest that most longterm survivors have no evidence of leukaemia detectable by PCR; in contrast some of the patients transplanted within the last 5 years do have evidence of residual leukaemia. This suggests that in a significant number of patients the leukaemic clone may survive for several years after BMT before it is eradicated (or falls below the threshold for detection by PCR). If this is confirmed the finding of residual leukaemia by PCR in the first few years post BMT would have limited prognostic significance (Hughes and Goldman, 1990).

2.4 BCR/ABL FUSED PROTEIN (P210)

The normal human ABL protein product is a phosphoprotein with molecular weight of 145kd (P145) (Witte et al, 1979; Konopka and Witte, 1985). In the CML cell line K562 established in 1975 (Lozzio and Lozzio, 1975) v-abl antisera precipitate two abnormally sized phosphoproteins of 210 and 190kd (P210 and P190) in addition to P145 (Konopka et al, 1984). These results indicate that the chimeric BCR/ABL mRNA present in Ph-positive

CML cell lines and patient material is translated into a chimeric protein; the difference in molecular weight between P210 and the normal ABL P145 is due in part to the addition of a BCR moiety to the N-terminus of P145 (Ben-Neriah et al, 1986b).

The normal ABL protein (P145) as well as the A-MuLV transforming protein P160 and the BCR/ABL hybrid protein P210 can autophosphorylate their own tyrosine residues in vitro but the sites of phosphorylation are different (Konopka & Witte, 1985). Furthermore both P210 and P160 were shown to contain phosphorylated tyrosines in vivo but the P145 did not (Konopka et al, 1984). The altered P160 and P210 have lost the first exon as a result of viral transduction and chromosomal translocation respectively. This suggests that removal of regulatory sequences contained within the first ABL exon gives rise to proteins with altered tyrosine kinase activities. It has been demonstrated that the transforming potential of the v-abl proteins is closely associated with the tyrosine kinase activity (Rosenberg et al, 1980). However, experiments using P210 have been unable to transform NIH 3T3 fibroblast cell line, although P210 can stimulate the growth of haemopoietic cells (Daley et al, 1987; McLaughlin et al, 1987). Some of the clonal cell populations obtained following retrovirus-mediated P210 infection can progress in their transformed phenotype to a fully malignant state characterized by increased cloning

efficiency in agar suspension and tumour induction in syngeneic mice. Nevertheless P210-infected lines inoculated into syngeneic mice show a low rate and long latency for tumour formation even though all infected cell lines have high levels of P210 expression. The oncogenic behaviour of P210 in these experiments is reminiscent of the long chronic phase observed in CML. One explanation for the low oncogenic potential of P210 is that additional genetic changes may be required for progression to the full oncogenic phenotype (Pendergast and Witte, 1987).

2.5 ATYPICAL VARIANTS OF CML

In more than 90% of the cases of CML the clinical and haematological features are remarkably uniform. As interest in the study of Ph-negative CML grows, more attention has been paid to the atypical features in a minority cases of CML. The typical picture of a classical Ph-positive CML has been described in Chapter 1; in this section the atypical clinical and haematological features and their associations with the cytogenetic and molecular events are discussed.

The minority of CML patients who lack a Ph chromosome tend to have lower leucocyte and platelet counts and inferior survival prospects (Ezdinli et al, 1970; Kantarjian et al, 1986). The

blood film appearances of these Ph-negative CML are markedly heterogeneous. In some cases they are indistinguishable from or very close to those of Ph positive CML, but in others there are major differences, in particular, a striking excess of monocytes, a virtual absence of basophils, and the presence of prominent dysplastic features in the granulocytic cells from the myelocyte to more mature stages (Pugh et al, 1985; Shepherd et al, 1987).

Shepherd et al (1987) classified the chronic myeloid leukaemias into three categories by morphological assessment in combination with cytogenetics and molecular studies. Those CML patients who present with the typical features described in Chapter 1 and are Ph-positive, M-BCR rearranged are classified as chronic granulocytic leukaemia (CGL). Those CML patients who present with the atypical features described above and are Ph-negative, M-BCR non-rearranged are classified as either chronic myelomonocytic leukaemia (CMML) or atypical CML (aCML) (Tab. 2.1).

Patients who are Ph-negative but M-BCR rearranged usually have typical features and are also classified as CGL. Furthermore, the classical BCR/ABL fusion protein P210 has been detected in these patients (Wiedemann et al, 1988). However, it is worth mentioning that in rare cases of Ph-positive, M-BCR rearranged CML, a somewhat atypical pictures can be seen (Gao

Table 2.1

(a) The salient morphological features of CMML:

(i) monocytosis and neutrophilia with a low neutrophil to monocyte ratio,

(ii) immature granulocytes account for < 15% of the total leukocyte count, and are almost always < 5%,

(iii) the absolute basophil count is almost always within normal range, and

(iv) the neutrophils may be normal or dysplastic.

(b) The salient morphological features of aCML:

(i) prominent dysgranulopoiesis (lacking in CGL),

(ii) monocytosis, and

(iii) basophil counts usually exceed the normal range only when monocytes exceed 3% of the total leukocyte count (features not found in CGL); note however that in CGL the monocyte count may not fall below 3% until the total leukocyte count exceeds 50 x 10^9/l).

(adapted from Shepherd et al, 1987)

et al, 1990a).

In about half of the Ph-negative CML patients the principal features that distinguished them from CGL were the high prevalence of dysgranulopoiesis, the raised monocyte counts and the low basophil counts. In a series of 35 cases of Ph-negative CML studied by Shepherd et al (1987) all the 17 cases of morphologically atypical CML were M-BCR non-rearranged. These Ph-negative M-BCR non-rearranged cases were subclassified as CMML and aCML based on morphology.

Since this subclassification of Ph-negative, M-BCR non-rearranged CML is not widely adopted, and since at this stage the molecular mechanisms for this group of diseases are not clear, I have not adopted this subclassification. However, the criteria for classification of typical and atypical features of CML are adopted. The classification and relationship of different types of CML are summarised in Figure 2.5.

2.6 Ph-POSITIVE ACUTE LEUKAEMIA

The Ph translocation is not restricted to CML but is also observed in 17-25% of cases of adult acute lymphoblastic leukaemia (ALL), 2-6% of childhood ALL and 1-2% of acute myeloid leukaemia (AML) (Chessells et al, 1979; Le Beau and

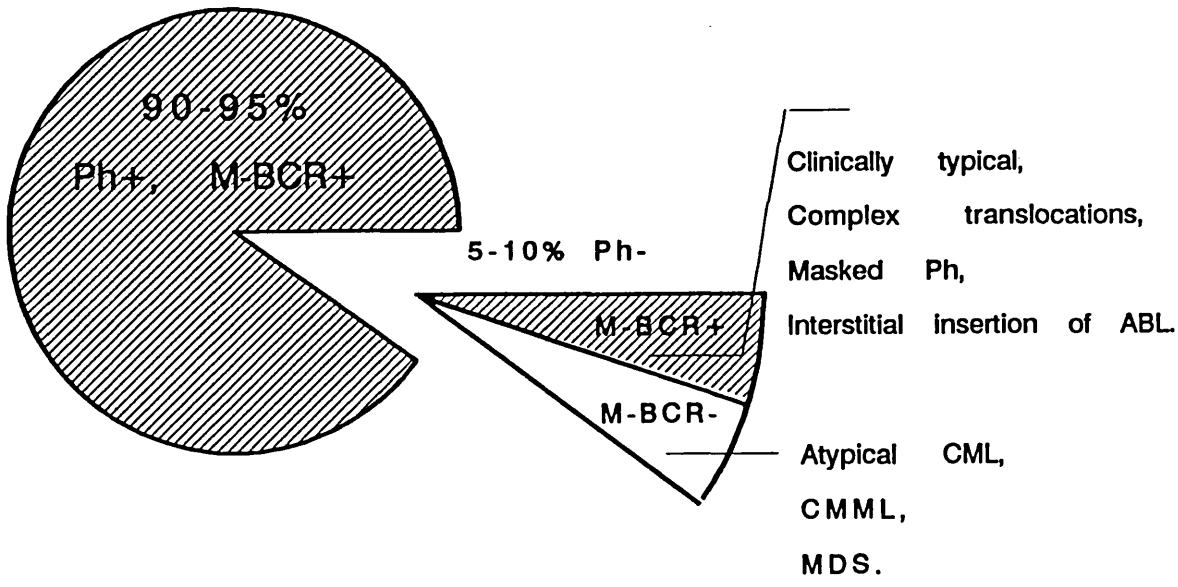


Fig. 2.5. Classification of CML.

The diagram shows the cytogenetic, molecular and clinical classification of CML and their inter-relationships. CMML = chronic myelomonocytic leukaemia; MDS = myelodysplastic syndromes.

Rowley, 1984). However, the clinical distinction between blast crisis of CML and de novo Ph-positive acute leukaemia is not always clear (Beard et al, 1976). Nevertheless, these two disorders have fundamental differences: the salient feature of CML is the rapid proliferation of mature myeloid cells, whereas that of ALL is a greatly increased number of immature lymphoid cells. Furthermore, the presence of some Ph-negative cells in the bone marrow during the acute phase and the elimination of Ph-positive cells from bone marrow during remission are typical features of cases presenting as Ph-positive acute leukaemias with no known prior CML. These different clinical manifestations of Ph-positive CML compared with ALL suggest discrete events in their pathogenesis (Groffen & Heisterkamp, 1987).

In about half the cases of Ph-positive acute leukaemia, the breakpoint on chromosome 22 cannot be mapped within the M-BCR by standard Southern analysis (de Klein et al, 1986b;). These Ph-positive, M-BCR non-rearranged acute leukaemias in fact have a breakpoint further upstream to the M-BCR, but still within the BCR gene. These breakpoints can be mapped within the first intron of the BCR gene by pulsed field gel electrophoresis (PFGE) and molecular cloning, and seem to cluster to a relatively defined region, the minor breakpoint cluster region (m-BCR) (Hermans et al, 1987; Rubin et al, 1987; Heisterkamp et al, 1988a; Gale & Goldman, 1988) (Fig.

2.6). Denny et al (1989) have demonstrated that these at breakpoints are all located the 3' end of the intron around an unusual restriction fragment length polymorphism caused by deletion of a 1kb fragment containing Alu family reiterated sequences. The proximity of the translocation breakpoints to this constitutive deletion may indicate shared mechanisms of rearrangement or that such polymorphisms mark areas of the genome prone to recombination.

A smaller BCR/ABL mRNA is produced by these leukaemic cells. The protein product from this BCR/ABL chimeric gene is substantially smaller than the classical chimeric gene product P210 because the large bulk of exons from exon 2 to M-BCR is not involved. The new product is 190 kd (P190) (Fig. 2.4; 2.6) (Fainstein et al, 1987; Chan et al, 1987; Kurzrock et al, 1987; Walker et al, 1987). This P190 is different from the ABL protein with similar molecular weight found in K562, which is felt to represent a proteolytic fragment of P210 (Konopka et al, 1984). PCR study on K562 showed that this cell line did not produce mRNA with e1a2 junction which was produced characteristically in the Ph-positive M-BCR non-rearranged acute leukaemia producing P190 (Kawasaki et al, 1988).

In the other half of patients with Ph-positive, M-BCR rearranged acute leukaemia, the classical BCR/ABL chimeric products found in CML cells, the 8.5kb mRNA and P210, were

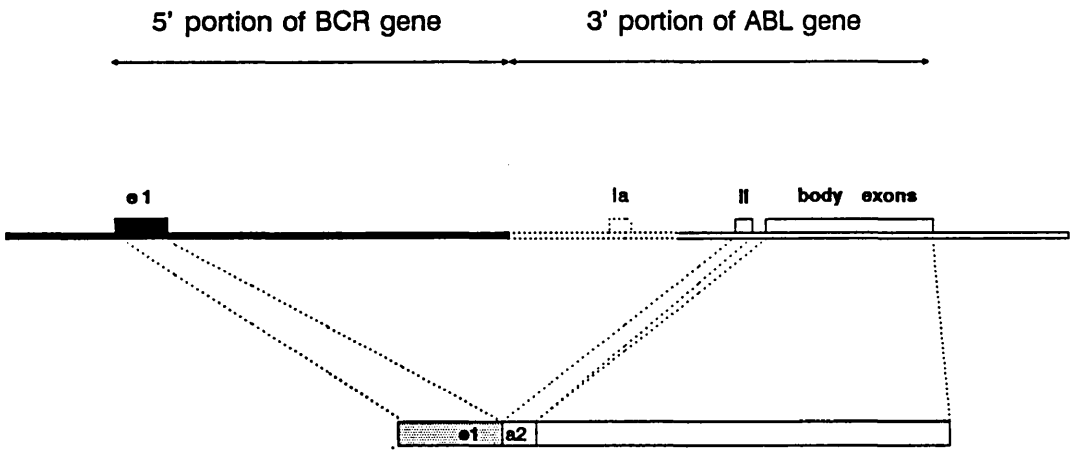


Fig. 2.6 Rearrangement of the BCR and ABL gene in Ph-positive, M-BCR non-rearranged acute leukaemia

This diagram shows the breakpoint in the first intron of the BCR gene and the formation of the BCR/ABL chimeric gene and its product found in Ph-positive M-BCR non-rearranged acute leukaemia.

produced (de Klein et al, 1986b). Both the P210 and the P190 found in these leukaemic cells have tyrosine-specific auto-phosphorylating activity.

2.7 SUMMARY

Figure 2.5 summarized the classification of CML based on haematology, cytogenetics and molecular biology. According to clinical and haematological features CML can be divided into CGL, CMML and aCML. By cytogenetic criteria CML can be divided into Ph-positive and Ph-negative. BCR/ABL rearrangement seems to be crucial in the pathogenesis of CML. In the majority of cases of CML the chromosome 22 breakpoints are located within the M-BCR, and can be demonstrated by standard Southern analysis. However in a small minority of CML cases and about half the cases of Ph-positive acute leukaemia the chromosome 22 breakpoint is located outside the M-BCR, so that the classification of M-BCR rearranged and M-BCR non-rearranged leukaemia is proposed. In all cases of Ph-negative, M-BCR non-rearranged CML (morphologically CMML and aCML) there is no suggestion that a chromosome 22 breakpoint can be mapped in m-BCR or that the remainder of the BCR gene is involved. However a thorough study has not been conducted. The distinction between Ph-positive and Ph-negative CML (in fact, between M-BCR rearranged and M-BCR non-rearranged in cases of

Ph negative CML) is not only semantic; it has important prognostic implications and may in the future be an indication for different therapeutic approaches.

There have been some reports on the Ph-positive M-BCR non-rearranged CML. In some cases the M-BCR negativity is due to deletion within the M-BCR so that the rearrangement escapes detection by conventional methods. In other cases the breakpoints are truly located outside M-BCR. The precise location of chromosome 22 breakpoint in these patients may be important in the understanding of the mechanism of BCR/ABL rearrangement.

CHAPTER THREE MATERIALS & METHODS

3.1 INTRODUCTION

Since the central topic of this thesis is the analysis of the BCR gene structure and BCR/ABL rearrangements in various leukaemias, molecular biology techniques form the core of the methodology. Methods which are described in Molecular Cloning: A Laboratory Manual (Maniatis et al, 1982; for convenience it will be called 'the Manual' in this text) were used throughout this work. Therefore in this section, only modifications from the standard protocols will be described. Methods which were not described in this Manual are also included in this section.

3.1.1 SOURCES OF CHEMICALS AND BIO-PRODUCTS

We purchased most of the chemicals and reagents from BDH, UK. The following chemicals and reagents were the exceptions: Agarose Type II Medium EEO; Agarose Type VII Low Gelling Temperature; ampicillin sodium salt; bovine serum albumin (BSA); caesium chloride (CsCl); dithiothreitol (DTT); ethidium bromide; magnesium sulphate ($MgSO_4$); phenylmethylsulphonyl fluoride (PMSF); potassium chloride (KCl) and sodium azide were all purchased from Sigma, USA. Absolute alcohol was

purchased from James Burrough Ltd, UK. Ficoll-Lymphoprep was purchased from Nyco Med Ltd, UK. Phenol (water saturated glass distilled grade) was purchased from Rathburn Chemicals Ltd, UK.

Most of the restriction enzymes were purchased from New England Biolabs, USA; SalI, SmaI and NarI were purchased from BRL, USA. Hybond-N membrane and ^{32}P-dCTP were purchased from Amersham, UK. Sephadex G-50 and oligolabelling kit were obtained from Pharmacia, UK. Proteinase K was obtained from Boehringer Manneim GMB H, West Germany.

3.1.2 SOURCES OF EQUIPMENT

All the sterilized plastic pipettes and centrifuge tubes were obtained from Sterilin, UK. Fluid filters for sterilization FlowPore D came from Flow Laboratories, West Germany. All the glassware was obtained from Pyrex, UK. Gilson pipetman and yellow tips were obtained from Gilson, France. All the centrifuges were purchased from Beckman (USA) except the Eppendorf Centrifuge (Anderman, West Germany). The Micro Centrifuge Tube was obtained from Elkay Products, USA. The pH meter Corning pH 106 was purchased from Corning, USA.

The electrophoresis apparatus for conventional Southern blotting was made by BRL, USA. The set of equipment for pulsed field gel electrophoresis (PFGE), Rotaphor was manufactured

by Biometra, West Germany. UV Transilluminator was made by UVP, Inc. USA. Polaroid MP4 Land Camera was made by the Polaroid Corp., USA. Water bath HAAKE D8, Shaking Water Bath and Magnetic Stirrer Hotplate 300 were made by Gallenkamp, UK. X-ray cassette, Saranwrap (Dow Chemical Company) and aluminium foil (Damar) were purchased from Genetic Research Instrumentation Ltd, UK. and X-ray film was obtained from Kodak, USA. The Southern transfer tray was made by the Medical Physics Department of the Royal Postgraduate Medical School.

3.1.3 STOCK SOLUTIONS

In order to prevent DNA degradation or contamination caused by bacterial growth, all stock solutions used in the molecular biology experiments were sterilized by autoclaving or filtration. In the following descriptions all the solutions were autoclaved unless specifically stated otherwise. For convenience most of the stock solutions were prepared in a concentrated form and were diluted to the required concentration immediately before use. The protocols to make most of standard stock solution have been described in the Manual. In this part I will only describe those solutions that do not appear in the Manual.

BSA A 10mg/ml BSA stock solution was prepared by adding 100mg of BSA to 10ml of double deionized (d.d.) water, mixed to dissolve and filtered through a FlowPore D. The stock solution

was stored in a 10ml plastic tube in -20°C.

PMSF was dissolved in absolute alcohol (A.R. Quality) to make a 100 mM stock solution and stored at 4°C.

PBS Phosphate Buffered Saline (PBS) was made by dissolving one PBS tablet (Oxoid, UK.) in 100ml d.d. water.

TBE 1M TBE stock solution was prepared by dissolving 121.1g Tris HCl, 61.8g boric acid and 7.4g ethylenediaminetetracetic acid (EDTA) in d.d. water up to the final volume of 1 L.

Agarose block store buffer (500mM EDTA, 10mM Tris HCl, 1% Sarkosyl, pH 9.5) 500ml of the store buffer was made as below: d.d. water was added to the correct amount of EDTA and Tris HCl up to total volume of 495ml; NaOH pellets were used to adjust the pH of the solution to 9.5. The solution was autoclaved and 5ml of Sarkosyl was added in a sterile manner.

Swelling buffer (10 mM Tris HCl pH7.5, 10 mM NaCl, 3 mM MgCl) This was made up with 1ml of 1M Tris pH 7.5, 1ml of 1M NaCl, and 0.3ml of 1M MgCl and 97.7ml of d.d water.

Lysis buffer (7M Urea, 0.3M NaCl, 10mM EDTA, 10mM Tris HCl pH 7.5) A 10x stock solution without urea was prepared; the correct amount of urea was added to 1x diluted buffer each

time before use. The full buffer was sterilized by filtration.

Phenol 250ml of water saturated phenol was put into a 500ml dark bottle and 200 ml of 1M Tris-HCl pH9.0 was added. The bottle was shaken and left until the layers separated. The upper aqueous layer was removed and the above procedures were repeated. The equilibrium procedures were repeated with 1M Tris-HCl pH8.5, until the pH of the upper aqueous was around 7.5. The aqueous layer was removed, 1 x TE pH 8.0 was added, the bottle was shaken and allowed to stand. This phenol was ready for use or stored at 4°C.

TY medium 16g Tryptone, 10g yeast extract and 7.5g NaCl were dissolved in 800ml of d.d. water. The final concentration was made up to 1 L with d.d. water after the solution was completely dissolved. 100 ml aliquots were made in screw top bottles and then autoclaved.

3.2 PATIENTS

Most of the patients selected for study were referred to the Hammersmith Hospital from other local hospitals with a diagnosis of CML based on haematologic and cytogenetic findings. All patients gave their verbal permission for the research studies described in this thesis. For cytogenetic

studies we usually collected bone marrow samples rather than blood cells because the probability of obtaining analyzable metaphases is greater with bone marrow. For molecular analysis of the DNA from the patients we collected peripheral blood because most of the peripheral blood cells in CML are leukaemic and have the BCR/ABL rearrangement. Furthermore, a comparative study of BCR rearrangement in marrow cells and peripheral blood cells showed no quantitative or qualitative differences (Fig. 3.1). Cytogenetic studies with G-banding were performed routinely for every admitted patient or outpatient. Only those cases with unusual haematological or atypical cytogenetic features (such as Ph negative CML) were selected for further cytogenetic analysis by synchronized culture G-banding. 10 patients were selected for follow-up study of possible further chromosomal changes in anticipation of blast crisis.

3.2.1 PATIENTS SELECTED FOR STUDIES OF ENGRAFTMENT AND RELAPSE AFTER BMT FOR CML

Forty-eight patients transplanted for CML were included in a serial cytogenetic study of engraftment and relapse (in collaboration with Dr CK Arthur). All patients were HLA-identical with their respective donors and had nonreactive mixed lymphocyte cultures. All received daunorubicin 60mg/m^2, cyclophosphamide 120mg/kg, and fractionated total body irradiation (TBI) to a total dose of 10 or 12Gy. Twenty-eight

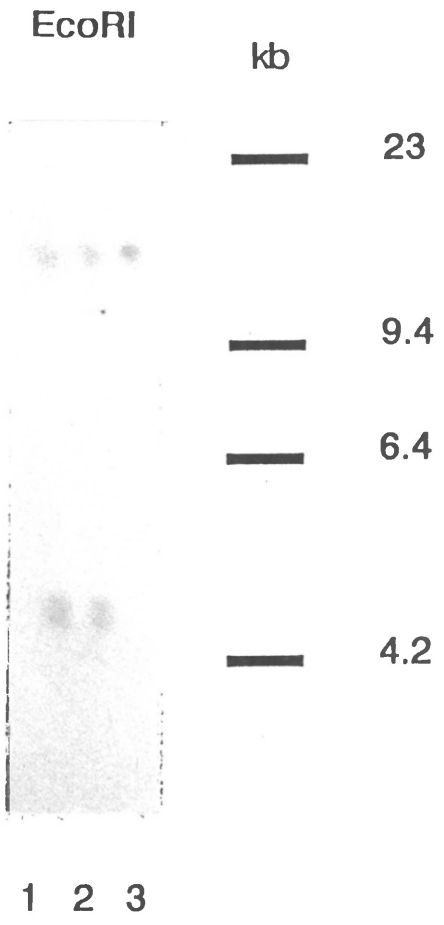


Fig. 3.1. Comparison of the M-BCR Rearrangement in DNA from Marrow and Peripheral Blood cells.

DNA from marrow cells (lane 1) and peripheral blood cells (lane 2) from a patient with Ph-positive CML was digested with EcoRI and hybridised to probe F (described later). Lane 3 is DNA from normal control. The rearranged band at 5 kb is observed in both patient samples. There is no visible difference between the two.

patients received donor bone marrow that was depleted of T cells with the monoclonal antibody Campath-1, and 20 received non-depleted marrow cells.

During the period when I studied marrow engraftment and relapse by DNA technology, I selected 9 CML and 1 ALL patients with haematological relapse, 9 CML and 1 AML patients newly transplanted or post-transplant followed-up. Most of them were among the above 48 patients and transplanted for CML with the T-depleted marrow. Another group of 21 patients selected in this study were transplanted for aplastic anaemia (in collaboration with Weitzel et al, 1988). A typical serial study started with the samples collected before BMT, and included samples collected at regular intervals post BMT (two weeks post BMT and then every two to four weeks) and controls from normal donors. In cases in which the donor DNA was not available, the pre-transplant DNA was used as controls for the post BMT engraftment and relapse or vice versa.

3.2.2 PATIENTS SELECTED FOR STUDIES OF BCR/ABL REARRANGEMENT

At the beginning of this study we included 52 patients with CML at presentation or soon after. Emphasis has been put on Ph-negative CML in the cases selected for study. Of 52 patients 19 had Ph-negative CML and 33 Ph-positive CML. All but one of the Ph-positive patients and 8 of the 19 Ph-

negative patients were haematologically and morphologically typical, while 11 out of 19 Ph-negative CML patients had some atypical features based on morphological and clinical criteria (Shepherd et al, 1987).

After carrying out Southern analyses we selected for further study by pulsed field gel electrophoresis (PFGE) 8 patients who lacked evidence of M-BCR rearrangement or the evidence were ambiguous. 3 of them were with Ph-positive and 5 with Ph-negative CML. Peripheral blood cells from 5 normal individuals were used to construct a long range map of the BCR gene and 6 patients with Ph-positive, M-BCR rearranged CML were used as controls for the PFGE study.

In addition 5 cases of acute leukaemia were studied for M-BCR rearrangement; one had Ph-positive acute leukaemia, 2 had Ph-negative ALL and the other two had Ph-negative AML. The last four patients were also used for controls in the studies.

3.2.3 IDENTIFICATION OF INDIVIDUAL PATIENTS

Because of the relatively large number of patients studied I have allocated to patients a number that corresponds to the chapter and section in which studies involving his or her leukaemic cells are first described (for example no. 422.1 indicates a patient first referred to in chapter 4, section 2 and subsection 2).

3.3 BLOOD CELL FRACTIONATION

Blood cell fractionation was used to study the engraftment of different cell populations and subpopulations after BMT, e.g. the T lymphocyte origin after graft failure or relapse. Blood cell fractionation was also used in PFGE in which the nucleated cell samples free of red cells, e.g. the mononuclear cell fraction, were needed.

3.3.1 NUCLEATED CELL FRACTIONATION

For peripheral blood samples with normal or low white cell counts 50ml of blood was required. From patients with high white counts only 10-20ml was needed. The peripheral blood specimens were diluted with an equal volume of PBS and centrifuged at 2000 rpm for 10 min.

The buffy coat (containing all the white cells) was collected with a wide mouth Pasteur pipette and washed again with PBS. After washing the supernatant was discarded and the cell pellet was resuspended in enough PBS to achieve the suspension containing cells in required number.

3.3.2 MONONUCLEAR CELL FRACTIONATION

A. 20-50 ml of peripheral blood was diluted with an equal volume of PBS and then layered onto an equal volume of Ficoll-

Lymphoprep and centrifuged at 2500 rpm for 20 min. All the greyish layer at the interface between the Ficoll and the plasma was collected.

B. The mononuclear cells in the greyish layer were washed twice with PBS in a plastic universal tube by centrifugation at 2500 rpm for 5 min. The supernatant was then poured off. The cell pellet was resuspended in PBS. The volume of PBS used to make the suspension depended on the cell concentration required.

3.3.3 T-LYMPHOCYTE FRACTIONATION

T-lymphocytes were fractionated by the E-rosette sedimentation method using 2-aminoethylisothiouronium bromide hydrobromide treated sheep red blood cells and further gradient centrifugation on Lymphoprep as described by Bartram et al (1987a).

3.3.4 CELL LINES

The cell line K562 was established originally from the pleural effusion of a patient in blastic crisis of CML. It has retained some specific cytogenetic changes as well as other malignant growth properties since its establishment after 175 passages of culture in vitro (Lozzio & Lozzio, 1975). Today this cell line still retains a chimeric chromosome 9/22 although the visible Ph chromosome has disappeared. We

obtained this cell line from Imperial Cancer Research Fund (London) and we maintained it by the methods described by Lozzio & Lozzio (1975).

3.4 CYTOGENETICS

Cytogenetic preparations were made from proliferating tissues, ie, bone marrow, or from cells capable of proliferation (mainly lymphocytes) in peripheral blood. For those tissues not proliferating actively, such as peripheral blood, culture was required to accumulate enough proliferating cells. Even for marrow cells a period of cell culture was often required to achieve good results (Rooney & Czepulkowski, 1986a; Watt & Stephen, 1986). Colchicine and its analogues were used to block the formation of the mitotic spindle so that the individual chromosomes were dispersed and analyzable (Tjio and Levan, 1956). In addition, the accumulation of cells arrested in metaphase provided many more divisions available for analysis. However, we tried not to expose the cells to colchicine excessively as it causes loss of detailed morphology due to contraction of chromosomes and separation of chromatids. Exposure of the cells to a hypotonic solution causes the cells to take up water, swell and lyse which further aids spreading in the final stages (Hungerford, 1965). The cells were then exposed to fixative, a mixture of glacial

acetic acid and absolute methanol, and finally dropped onto a microscope slide and flattened by air drying prior to staining and microscopy (Moorehead et al, 1960).

Synchronisation of cultures is intended to allow analysis of human chromosomes at earlier stages of cell division in order to gain additional information from more elongated chromosomes which permit high resolution banding. Arresting agents which specifically stop prophase and prometaphase have not been identified, but it is possible to introduce a chemical block at an earlier stage of the cell cycle, so that when cultures are subsequently released from the block, the cells proceed in synchrony to complete division (Yunis, 1976). Careful timing allows one to harvest cultures with a relatively high proportion of prophase, prometaphase or early metaphase cells as required. The exposure to colcemid should be brief (Watt and Stephen, 1986).

3.4.1 CONVENTIONAL & SYNCHRONIZED CULTURES OF MARROW AND BLOOD CELLS

In conventional cultures 1 ml of the marrow or peripheral blood sample was added to 10ml of culture medium. The composition of the culture medium was quite similar for marrow cultures and blood cultures; the only difference was the addition of 200ul phytohaemagglutinin (PHA) to the medium for blood culture to stimulate lymphocyte proliferation. 10ml of

culture medium was made up with 8ml of RPMI 1640, 2ml of fetal calf serum and 10ul (10 ug/ml) of penicillin and streptomycin. After mixing by inversion, the cultures were incubated at 37°C for 48-72 hours.

For synchronisation of cultures the chemical blocking agent methotrexate (MTX) was used. I followed the protocol for MTX block and release of the block by changing for culture medium containing thymidine as described by Watt and Stephen in Human Cytogenetics: A Practical Approach (Rooney and Czepulkowski, 1986b).

3.4.2 HARVESTING AND STAINING

Standard harvesting, staining and banding protocol were described by Watt et al and Benn et al in Human Cytogenetics: A Practical Approach. Two different banding techniques were used in this study: G-banding and C-banding. G-banding by trypsin treatment and Giemsa staining is the most widely used method for identification of chromosomes and its changes. C-banding is used for the identification of polymorphic markers in the centromere of the chromosome. This was useful when the position and the size of the centromere in the chromosome needed to be identified more clearly.

3.5 SOUTHERN BLOTS

The technique of Southern blotting is now used widely in the study of genomic rearrangements. The DNA is first extracted from cells and then 'cut' into smaller fragments which can then be separated according to their molecular weight. The restriction endonucleases used to cleave the DNA recognize specific sets of short sequences (restriction sites) and then produce discrete reproducible restriction fragments.

In most cases the molecular sizes of the germ line restriction fragments differ from those of the rearranged genes. After separation by electrophoresis on agarose gel matrix and then transfer to a membrane these fragments are identifiable by a labelled DNA fragment (a probe) comprising sequences homologous to the gene studied (Southern, 1975).

3.5.1 DNA EXTRACTION

There are various different methods for extracting DNA from cells, depending on the purity and the average size of DNA fragments required. For Southern analyses in this study the "quick" method for extracting DNA from blood cells was used.

A. Buffy coat cells were resuspended in 20-40ml of swelling buffer in a universal tube and left on ice for 10 min. Triton X was added to a final concentration of 2%. The tube was

vortexed for 10 seconds and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the pellet was used for DNA extraction or stored in a -20°C freezer.

B. 0.5ml of lysis buffer was added to the pellet and mixed vigorously using a glass rod. Successive 2ml aliquots of lysis buffer were added and mixed well until a total volume of 10ml was reached. 2ml of 10% SDS was added and mixed well, and then incubated for 10 min at 37°C.

C. The suspension was transferred to a 50ml screw-top polypropylene tube. 5ml of organic solvent (24:1 mixture of chloroform and isopropanolol) and 10ml of equilibrated phenol were added. These were mixed by inverting the tube repeatedly for about 1 min (the precise length of time for mixing depended on the viscosity of the liquid) and centrifuged for 15 min at 4000 rpm.

D. The supernatant (i.e. aqueous phase) was collected into another 50ml polypropylene tube with a wide-mouthed pasteur pipette. The interface containing whitish cell debris and proteins was left behind.

E. The above phenol extraction procedure was repeated twice. The residual phenol in the aqueous phase supernatant was removed by adding 5ml of chloroform mixture, mixing well and

then centrifuging at 4000 rpm for 15 min.

F. The aqueous phase was collected in a universal tube and DNA was precipitated by 2 volumes of -20°C ethanol. DNA precipitate was put into an eppendorf tube for centrifugation for 10 min at 10,000 rpm. The pellet was washed twice with 70% ethanol and left to dry.

G. The dried DNA pellet was dissolved in 200ul 1 x TE and the concentration of DNA was measured in a SP 500 Series 2 UV & Visible Spectrophotometer (Wright Scientific Ltd., London UK.)

3.5.2 DIGESTION WITH RESTRICTION ENZYMES

10ug of DNA was digested with 20-30 units of restriction enzyme in 1 x buffer for overnight or at least 2 hours. The total volume of the reaction is 50ul. The buffers and the temperatures were as recommended by the suppliers.

For DNA fingerprinting studies the restriction enzymes HinfI, Sau3AI and HaeIII were used. For the study of M-BCR rearrangement by Southern blotting restriction enzymes EcoRI, BglII, BamHI and HindIII were used.

3.5.3 DNA SEPARATION BY AGAROSE GEL ELECTROPHORESIS & SOUTHERN TRANSFER

After complete digestion the DNA was electrophoresed together

with a Lambda HindIII molecular weight marker on a 1% agarose Type II gel at 4 V/cm in 1 x TAE buffer overnight. The procedure for making the agarose gel for electrophoresis has been described in the Manual. After electrophoresis the gel was stained with 1ug/ml ethidium bromide for 30 min. A photograph of the ethidium bromide stained gel was usually taken to check the quality of the DNA digestion and the gel electrophoresis. The photograph was kept as a record of the position of the molecular weight markers. The DNA in the gel was then transferred to a Hybond-N membrane by alkali blotting methods as recommended by the supplier. After Southern transfer the DNA blots were fixed by baking in an oven at 80°C for 2 hours.

3.5.4 LABELLING OF DNA PROBES

DNA probes were labelled with α-^{32}P dCTP by the oligolabelling method (Feinberg et al, 1983). Random oligonucleotide primers were annealed to the probe DNA where they initiate the synthesis of the other strand using Klenow fragment of DNA polymerase I. The radio-labelled dCTP was incorporated into the growing complementary DNA strand during this process. The protocol is according to the recommendation of the supplier of the oligolabelling kit. After labelling overnight, the labelling mixture was purified by column chromatography through Sephadex-G50. There were two peaks of radioactivity of the elution, the leading peak contained labelled probe and

the second peak contained unincorporated ^{32}P. This procedure is described in detail in the Manual. I collected the radioactive fractions in the leading peak for immediate use or storage at -20°C. The radioactivity of the labelled probe usually ranged from 10^6 to 10^9 cpm/ug.

3.5.5 PRE-HYBRIDIZATION, HYBRIDIZATION & WASHING

Pre-hybridization, hybridization and washing were carried out in accordance with recommendations of the Hybond-N membrane supplier.

3.5.6 AUTORADIOGRAPHY

After washing the membrane filter was wrapped with Saran wrap and all the sides of the filter were sealed. The membrane was then exposed to X-ray film Kodak X-Omat AR with two intensifying screens in a X-ray cassette for 12 hours to 7 days. The films were developed in a Fuji RG 2 X-Ray Film Developing Machine (Japan).

3.6 PULSED FIELD GEL ELECTROPHORESIS (PFGE)

PFGE is a technique for analyzing very large DNA fragments, with a range of resolution of DNA fragments between 20 to 10,000kb. In PFGE the large DNA fragments are forced to reorient themselves while they move along the gel driven by

an electrical field that alternates at regular intervals from different directions in a predetermined pattern. The reorientation of the large DNA fragments provides additional size-dependent resolution (Carle et al, 1984, Schwartz et al, 1984).

For PFGE the chromosomal DNA must be almost intact before cutting with restriction enzymes. In order to avoid shearing and other mechanical damage to the DNA molecule the intact cells were first embedded in agarose. To avoid interfering with subsequent enzyme reactions by the non-agarose elements, ultrapure agarose was used. The cellular structures and proteins in the blocks were then digested with proteinase K. The almost intact DNA molecule remains in the agarose blocks.

In addition to the mechanical factors, bacterial infection that produces DNAse can damage the integrity of DNA molecule and cause degradation, so extra precautions were required to avoid bacterial contamination.

3.6.1 PREPARATION OF AGAROSE BLOCK CONTAINING HIGH MOLECULAR WEIGHT DNA

A. The mononuclear cell separation has been described above (3.3.2). The cell were suspended at concentration of 2 x 10^6/ 50 ul PBS.

B. The cell suspension was mixed with an equal volume of 2% ultra pure agarose in PBS at 50°C. The mixture was kept in a water bath at 50°C while the agarose blocks were made. 100ul of the mixture was loaded into each block former (supplied with the Rotaphor).

C. The block formers were then put on ice to allow the agarose blocks to set; the agarose blocks were then transferred sterilely into a plastic universal tube. 10ml of agarose block store buffer containing 10mg Proteinase K was added and the blocks in buffer were then incubated in a 50°C water bath for 3 days.

D. After 3 days the buffer containing proteinase K was changed for 10ml of fresh agarose block store buffer without proteinase K and the blocks were incubated in 50°C water bath for 30 min; this step was repeated twice. The agarose blocks in 10ml fresh agarose block store buffer were ready for restriction enzyme digestion or stored in a 4°C refrigerator.

3.6.2 DIGESTION WITH RESTRICTION ENZYMES

One of the most critical parts of the PFGE analysis is to ensure complete digestion of the DNA with the restriction enzyme selected. Thus an excess amount of enzyme was usually necessary to secure complete digestion.

A. To inactivate the residual proteinase K each agarose block was placed into 10ml of 0.1 mM PMSF dissolved in sterilized 1 x TE. This was mixed well and put on ice for 30 min. The procedure was repeated twice.

B. Each agarose block was placed into a sterile micro centrifuge tube and equilibrated in ice with 1ml 1 x enzyme buffer (recommended by the supplier) for 30 min.

C. Each agarose block was transferred to a sterile micro centrifuge tube containing 200ul of enzyme buffer. 40 units of the selected enzyme were added. After mixing well and brief spinning in a Eppendorf centrifuge the tubes were put into a water bath for at least 8 hours. The precise reaction temperature were set according to the recommendation of the supplier of the enzyme.

In PFGE studies of the structural maps and physical rearrangements of the BCR and ABL genes I used the rare cutting restriction enzymes BssHII, NarI, MluI, SalI, NotI, SmaI, NruI and SfiI.

3.6.3 ELECTROPHORESIS

After digestion with restriction enzymes the agarose blocks were placed into 0.025 M TBE pH 8.5 buffer for 15 min for equilibration.

The method of making agarose gel for PFGE was similar to that used for standard Southern blotting. The main differences were the buffer and the gel tray used. The buffer was 0.025 M TBE. The gel tray was supplied with the PFGE machine (Rotaphor). 200ml of 1% agarose type II was used to make the gel.

The agarose blocks were loaded into the holes in the gel and 1% agarose type VII (low gelling temperature) at 37°C was used to seal the tops of the holes.

The conditions for PFGE were as follows: the temperature was 9°C; the field angle was 120°C (step 160); the pulse intervals were between 10-60 seconds; the voltage was set between 220-180V. The duration of electrophoresis was 24-48 hours. The electrophoresis buffer was 0.025 M TBE pH 8.5. This was made from 1 M TBE stock solution. Bacteriophage lambda multimers (Clontech Lambda DNA Ladder, Cambridge Bio Science, UK) were used as the molecular weight markers. A setting of 10 second pulse intervals, 200V field strength and running for 24 hours resulted in better resolution of DNA of molecular sizes between 50 to 300kb. A setting of 30 second pulse intervals, 210V and for 24 hours resulted in better resolution of DNA of molecular sizes between 50-600kb. A setting of 60 second pulse intervals, 180V and for 48 hours resulted in average resolution of DNA of molecular sizes between 50-800kb. A

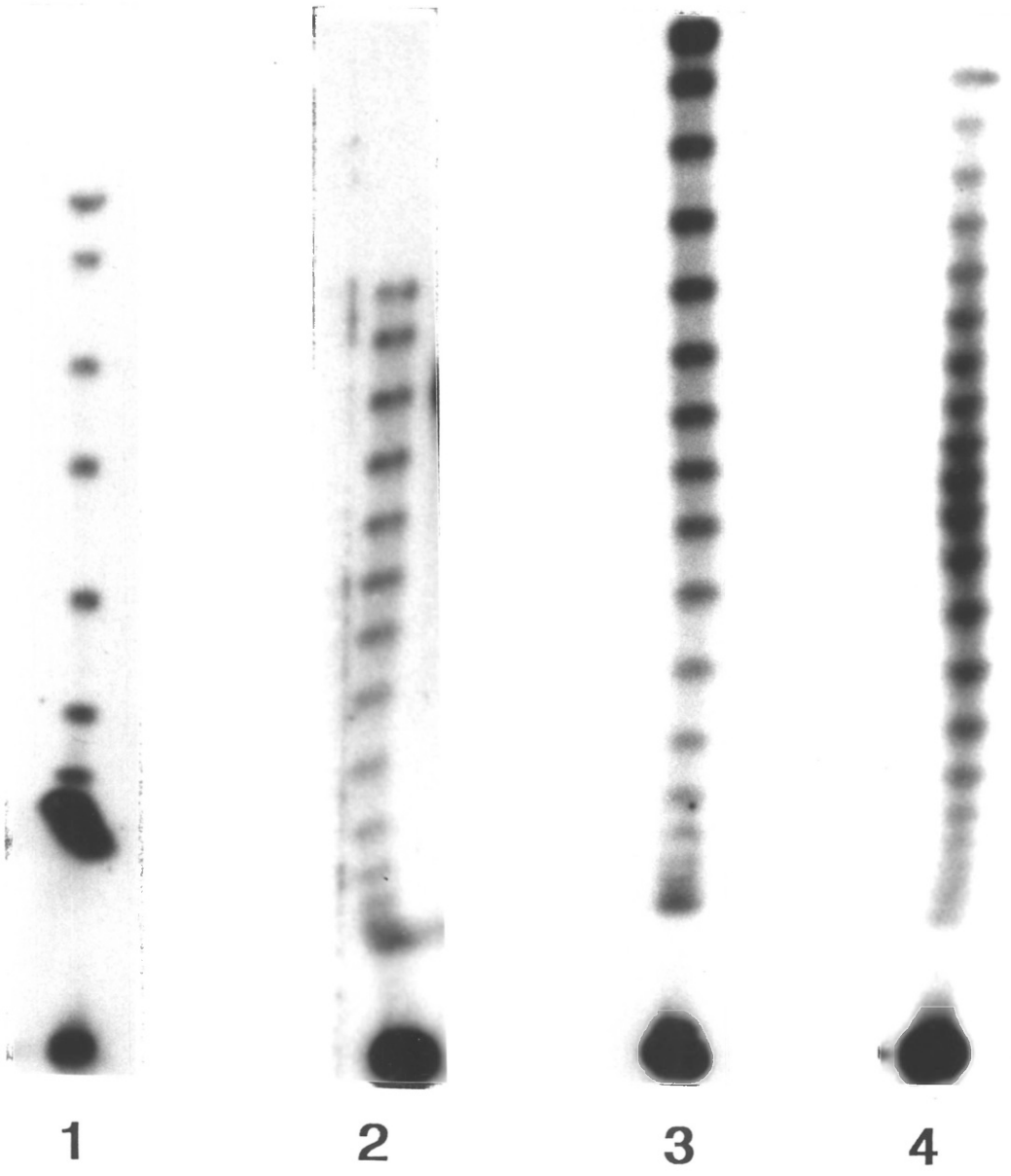


Fig. 3.2 Setting for PFGE for Different Ranges of Resolution of DNA Fragments.

All the lanes above are Lambda DNA multimer ladders run under different settings. The settings for lane 1 are 10 seconds, 200V and 24 hours; for lane 2, 30 seconds, 210V and 24 hours; for lane 3, 60 seconds, 180V for 48 hours, and for lane 4, 75 seconds, 180V for 48 hours.

setting of 75 second pulse intervals, 180V and for 48 hours resulted in better resolution of DNA of molecular sizes between 300-900kb (Fig. 3.2). I chose to use the setting of 60 seconds, 180V for 48 hours in most of the experiments.

3.6.4 SOUTHERN TRANSFER (as described in 3.5.3)

3.6.5 LABELLING OF THE PROBES (as described in 3.5.4)

3.6.6 PRE-HYBRIDIZATION, HYBRIDIZATION & WASHING (as described in 3.5.5)

3.6.7 AUTORADIOGRAPHY (as described in 3.5.6)

3.7 RE-PROBING FILTERS

The Hybond-N membrane can be used for re-probing for confirming studies or studying with other probes. For stripping the original probes, the following method was used:

0.1% SDS solution at 100°C was poured onto the membrane and allowed to cool to room temperature. The filter can then be pre-hybridized and hybridized with the new probe.

3.8 DNA PROBES

A 4kb cDNA probe for BCR (Lifshitz et al,1988), a 0.6kb

genomic DNA 3' and a 2kb genomic DNA 5' probe for M-BCR (Schaefer-Rego et al, 1987) were used in this study. A number of cDNA probes were derived from the restriction fragments of the 4kb cDNA probe. The positions of these probes are shown in Figure 3.3. One 0.85kb genomic exon Ib ABL probe pHabl5' (Bernards et al, 1987) and a 1.5kb PstI fragment of the ABL cDNA clones (ABL body probe, Ben-Neriah et al, 1986a) were used in PFGE. Jeffreys' probes 33.15 and 33.6 (Jeffreys et al, 1985a; 1985b) were also used in the study of cell origins after BMT.

The 4kb cDNA BCR probe (probe A, Fig. 3.3) is an approximately full length cDNA probe which contains most of the sequences of exon 1 (a small part of 5' sequence is excluded) together with other exons up to the 3' end of the BCR gene; a few of the exons immediately 5' of the M-BCR region are omitted. This probe is derived from a clone lambda-105 from a cDNA library. A 0.75kb BglII fragment from the most 5' portion of probe A contains the BCR exon 1 sequence and is used as probe B. A 1.1kb BglII fragment which is just 3' to probe B, contains some 3' sequence of exon 1 and the other following 3' exons, is used as probe C. The 1.5kb BglII fragment of the 3' end of probe A is used as probe D. The 2kb genomic DNA probe, a BglII and HindIII fragment located in 5' portion of the M-BCR region, is used as probe E. The 0.6kb genomic DNA probe, a PstI and BglII fragment located in 3' portion of the M-BCR

Fig. 3.3. Relative Position of Probes.

(a). Positions of various probes in the BCR gene and in M-BCR. Probes A, B, C and D are shown above map and hybridize to exons present in fragments indicated; probes B, C and D are the BglII fragments derived from a full length BCR gene cDNA probe (probe A). Probes E and F are genomic M-BCR probes. This BCR gene map is not to scale. Exons in this M-BCR are numbered 1 to 5 in accordance with standard nomenclature.

(b). Positions of a 1.5kb cDNA ABL probe and a 0.85kb genomic probe pHabl5' in the ABL gene. Black boxes represent the exons. B = BamHI; Bg = BglII; E = EcoRI; H = HindIII and Pst = PstI.

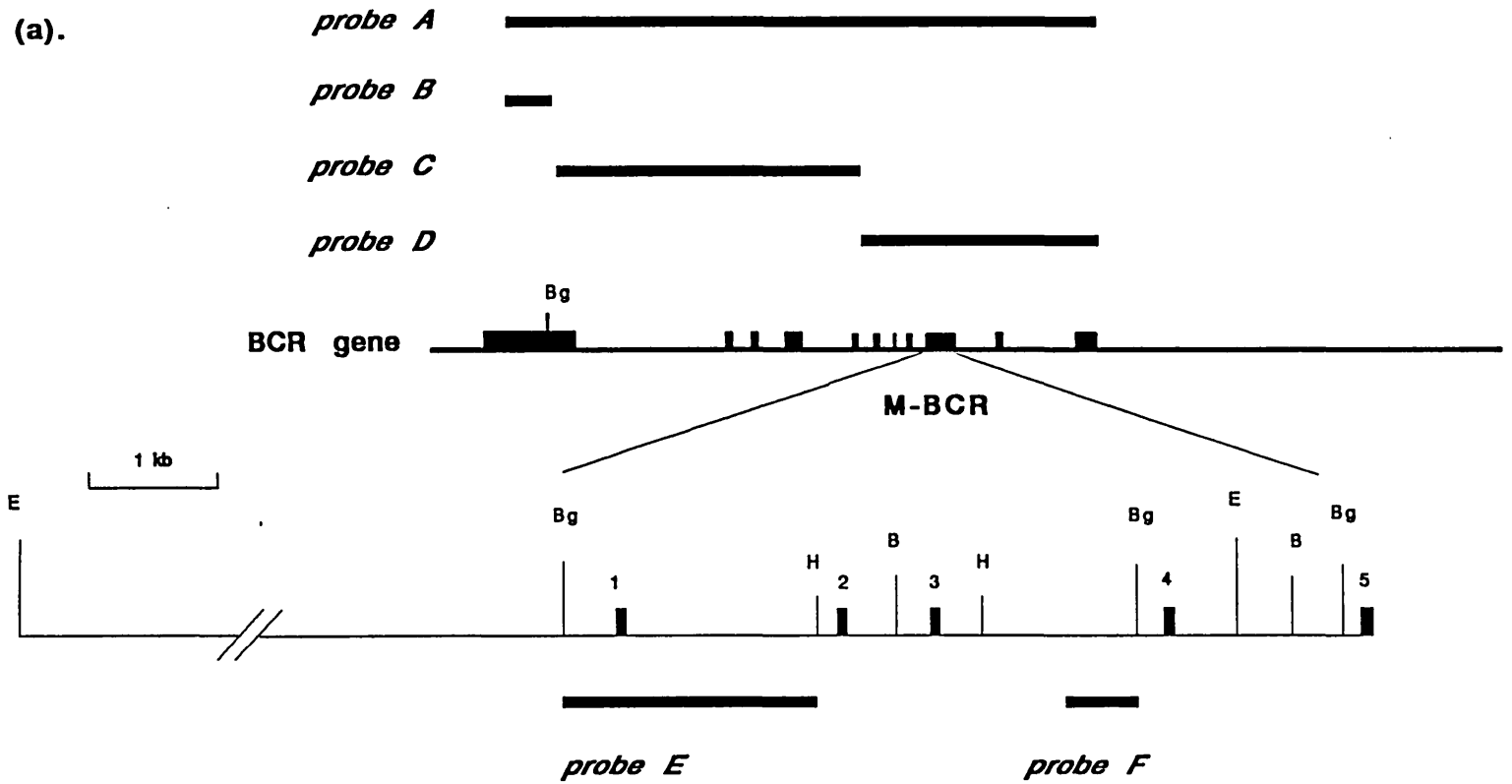

(a).
probe A
probe B
probe C
probe D
Bg
BCR gene
M-BCR
1 kb
E
Bg
H
B
H
Bg
E
Bg
B
1
2
3
4
5
probe E
probe F
(b).
ABL gene
0.85kb probe pHabl5'
1.5kb cDNA probe
Ib
Ia
II
body exons
B
H
Pst
Pst

Fig. 3.3

region, is used as probe F (Fig. 3.3).

3.9 PREPARATION OF DNA PROBES

The 4kb cDNA BCR probe was ligated with a small E. coli plasmid vector pUC 19 and then used to transform host cell LE 392. The transformed cells were plated onto a TY agar plate and incubated inverted for 24 hours at 37°C. Ampicillin was used for transformant selection. A single discrete colony of cells was isolated, plucked and inoculated into 10ml of TY medium. This was incubated in a orbital shaking incubator at 37°C overnight and then used for large scale isolation of plasmid DNA. The DNA insert in the plasmid was cut out with restriction enzyme EcoRI and purified by electrophoresis, electroelution and ethanol precipitation. The above procedures were performed according to the protocol in the Manual.

The Jeffreys' probes 33.15 and 33.6 in single-stranded M13 DNA were used to transform E. coli JM101 and double-stranded M13 RF DNA with the inserts was prepared (Messing & Vieira, 1982). The EcoRI/HindIII insert fragments of 33.15 and EcoRI/BamHI fragments of 33.6 were separated from the M13 vector by electrophoresis in low melting point agarose and stored at -20°C until use.

CHAPTER FOUR CYTOGENETICS OF CML

4.1 INTRODUCTION

Since the Philadelphia chromosome is present in 90 - 95% cases of CML, cytogenetic studies have been widely used in the diagnosis and management of patients with CML. CML is an excellent example of the process of multi-step carcinogenesis. Most patients with CML will, in time, transform from chronic phase to blast phase. This is usually accompanied by further chromosomal changes (Chapter 1), which have been found useful for predicting acceleration and/or blast phase (Lilleyman et al, 1977; Hagemeijer et al, 1980). Because these chromosomal changes may indicate alterations which are responsible for blast transformation of CML in at least some of the patients, it is important to study the mechanism of these changes. Ten patients with Ph-positive CML were analyzed by serial cytogenetic study for evidence of further chromosomal changes and their relationship to BT.

Patients with Ph-negative CML, referred to earlier, are generally regarded as a heterogeneous group whose disease is associated with poor prognosis, ie. relative resistance to

treatment, shorter chronic phase, shorter survival and atypical haematology (Ezdinli et al, 1970; Kantarjian et al, 1986). The cytogenetics of this group of CML patients are also heterogeneous. Some have abnormalities involving chromosome 9 and/or other chromosomes though not visibly no. 22. Others are cytogenetically strictly normal (Kantarjian et al, 1986; Hagemeijer, 1987). In an attempt to study the relationship between cytogenetic changes and clinical and haematological characteristics, I studied 19 patients with Ph-negative CML by conventional G-banding; 6 were studied in greater detail using chromosome preparations from synchronised cultures.

While these Ph-negative CML patients were undergoing clinical and haematological investigation, the concept of atypical CML was defined (Shepherd et al, 1987). Some of the Ph-negative CML patients resembled Ph-positive patients but at the same time had some features rarely or never present in typical Ph-positive CML. These have been described in detail in chapter 1. These criteria have been used in the study described in this chapter.

I also describe here cytogenetic studies of engraftment and relapse in post-transplant CML patients. The effects of the transplantation procedure on chromosomes were also studied. Since the Ph chromosome is a marker for CML cells, the discovery of a Ph-positive metaphase in the post-BMT

cytogenetic preparation suggests (but does not prove) the recurrence of proliferating leukaemic cells. This is called 'cytogenetic relapse' but it does not necessarily imply persisting recurrence of CML, since in some cases the Ph-positive cells can later no longer be identified on further analysis (Hughes et al, 1989). Efforts were also made to identify possible relapse in cells of donor origin and Ph negative haemopoiesis of host or recipient origin in sex mismatched transplants.

4.2 RESULTS

4.2.1 Ph-POSITIVE CML

Ten patients with typical Ph-positive CML, referred to us by other hospitals, were reexamined to confirm the cytogenetic findings, to monitor for possible further chromosomal changes and to serve as controls. 10-20 metaphases were analyzed each time and each patient was studied at 2-3 months intervals. When signs of acceleration or BT were observed, the patients were monitored more closely.

These ten patients all had the t(9;22)(q34;q11) translocation when they first presented to us, 6 in chronic phase and 4 in acceleration. Two patients later entered blast crisis. Further karyotypic changes (an additional Ph chromosome) were observed

in one patient (no. 421.1) one month before he entered lymphoid blastic transformation (8 out of 15 metaphases analyzed) (Fig. 4.1). Another patient (no. 421.2) in acceleration also had an additional Ph chromosome (3 out of 10 metaphases).

4.2.2 Ph-NEGATIVE CML

Nineteen patients with the clinical diagnosis of CML were found not to have a Ph chromosome either by us or by the hospital where they first presented. Conventional G-banding was performed for all and 6 cases had synchronized cultures. 10-20 metaphases were analyzed each time and each patient was studied at 2-3 months intervals. The enumeration and clinical features of these 19 patients is summarized in Table 4.1

The karyotypes of these Ph-negative CML patients were heterogeneous. By conventional G-banding there was no evidence of the Ph chromosome in the bone marrow metaphases analyzed from any of these patients. Thirteen patients showed apparently normal metaphases; 6 patients who appeared to have other abnormalities or who showed unsatisfactory banded metaphases were studied further by synchronised cultures. One of these patients (patient no. 422.1) had a masked Ph chromosome with a complex translocation involving chromosomes 9, 22, and 4 (Fig. 4.2a). Ten unsynchronised and a further 20 synchronized metaphases were analyzed; the karyotype was 46,

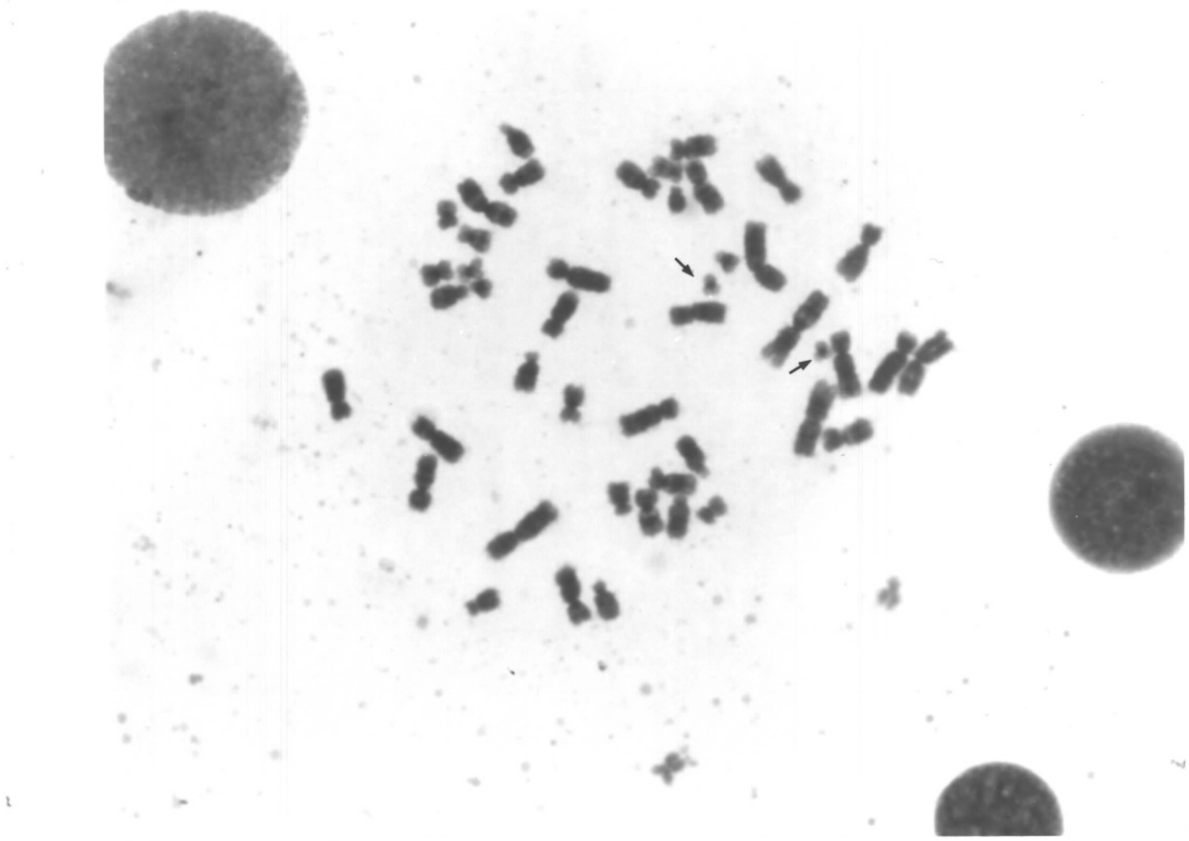


Fig. 4.1 Metaphase Showing an Additional Ph Chromosome in BT.

A metaphase from marrow cells of a patient (no. 421.1) one month before BT. The arrows indicate the two Ph chromosomes.

Table 4.1 Clinical and haematological features of the Ph-negative CML patients.

Patient no.	Age/ sex	Karyotype	Duration survival	At presentation								
			(months)	Spleen (cm)	Hb (g/dl)	WBC ($x10^9$/l)	Blast (%)	Baso (%)	Eos (%)	Neut (%)	Plts ($x10^9$/l)	Mono (%)
422.1	43/F	t(4;9;22)	13+	12	10.3	506	2	3	2	59	319	1
422.2	61/M	46XY	16	0	11.2	170	1	0.5	0	61	53	0.5
422.3	49/M	46XY	24+	12	12.9	170	0	4	2	61	283	1
422.4	24/M	46XY	24+	25+	8.3	174	6	14	6	41	941	0
422.5	28/F	46XX	108	13	5.6	650	5	2	-	50	280	0
422.6	81/M	46XY	24+	6	10.9	74	0	11	2	74	300	0
422.7	39/M	46XY	24+	0	11.3	298	1	0	4	35	210	0
422.8	48/M	46XY	13+	4	8.2	170	0	4	1	70	548	1
422.9	33/M	46XY	15+	10	18.0	175	0	2	1	82	137	0
422.10	59/M	46XY	11+	0	6.4	240	2	1	7	53	169	3
422.11	27/M	46XY	2	7	10.3	200	0	0	6	52	54	1
422.12	62/M	46XY	40+	0	15.7	23	0	1	0	83	290	4
422.13	4/F	46XX	15	8	10.9	29	1	2	5	39	384	16
422.14	63/M	46XY	41+	3	11	105	4	0	0	58	87	2
422.15	30/M	46XY	not available									
422.16	81/F	46XX	8*	0	12.4	65.4	0	0	0	64	294	12
422.17	73/F	46XX	28	7	5.4	24.9	0	0	0	63	228	4
422.18	34/M	46XY	not available									
422.19	34/F	46XX	118+**	0	10	77	3	0	9	54	492	3

* Accidental death.

** Duration of survival after BMT

\+ Still survive when data was collected.

Fig. 4.2 A Masked Philadelphia Chromosome.

(a). Synchronized culture and G-banded metaphases of marrow cell from patient no. 422.1 showing 4p-; 9q+ and 22q+ (masked Ph chromosome) as indicated by the arrows.

(b). Schematic presentation of the formation of t(4;9;22) (4p11;9q34;22q11).

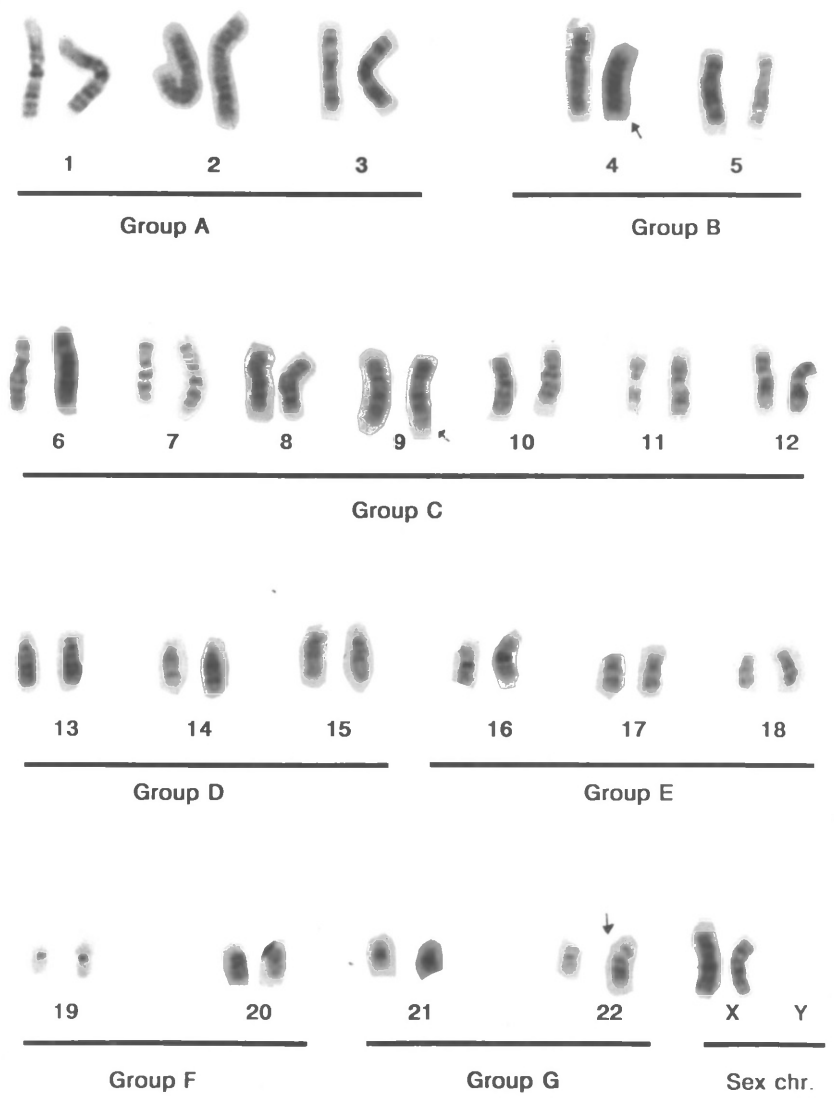

1
2
3
Group A
4
5
Group B
6
7
8
9
10
11
12
Group C
13
14
15
Group D
16
17
18
Group E
19
20
Group F
21
22
Group G
X
Y
Sex chr.

Fig. 4.2 (a)

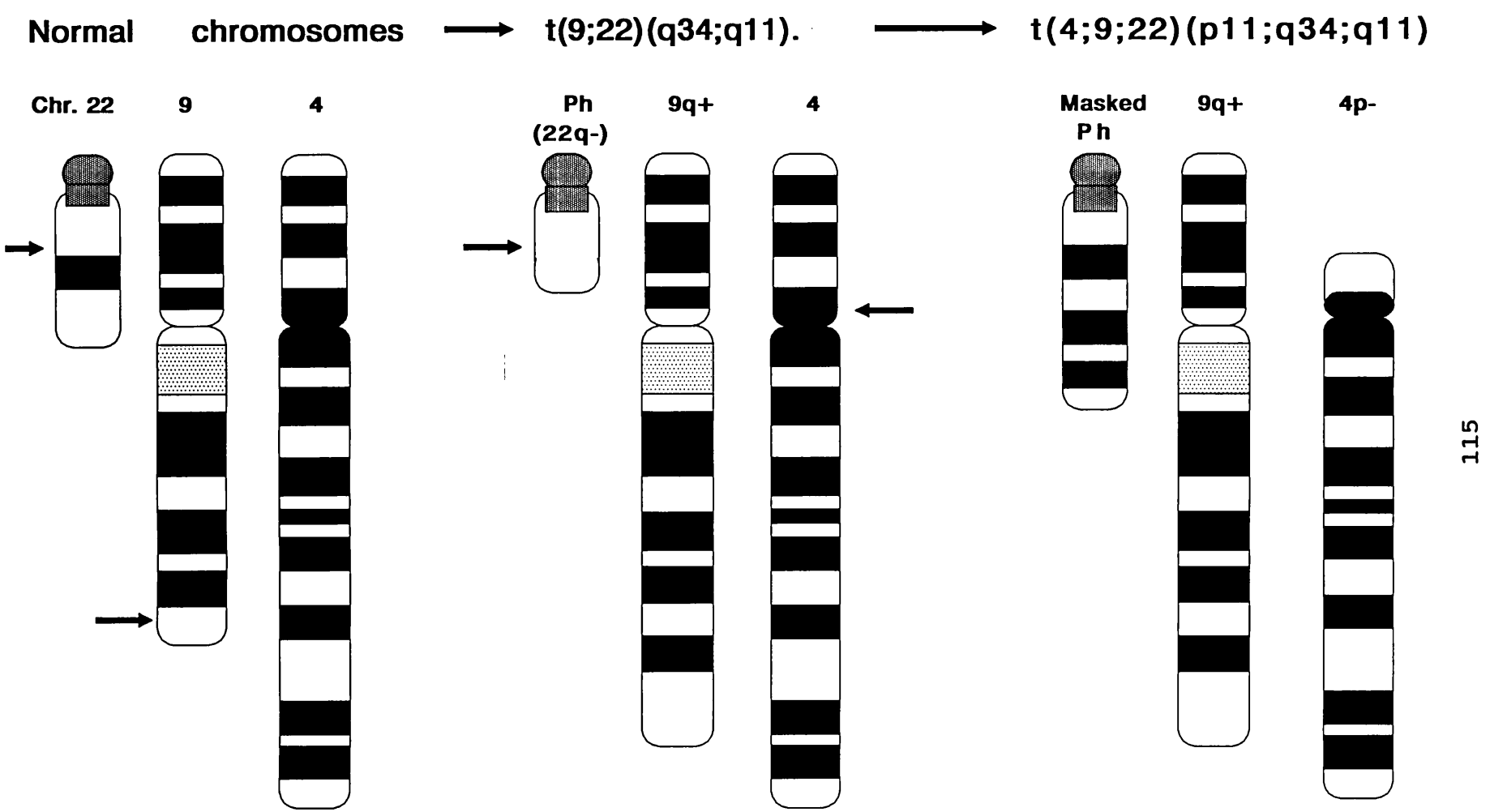


Fig. 4.2 (b). Formation of the Masked Ph Chromosome

XX, t(4;9;22)(4qter-> 4p11::9q34-> 9qter; 9pter-> 9q34::22q11-> 22qter; 22pter-> 22q11::4p11-> 4pter). These cytogenetic changes presumably began with the usual reciprocal translocation between the terminal parts of chromosomes 9 at q34 and 22 at q11 leading to the formation of the Ph chromosome; this was followed by a reciprocal translocation between the Ph and distal part of chromosome 4p11 that made the Ph chromosome appear much bigger (Fig. 4.2b). Clinically and haematologically this patient was indistinguishable from typical CML (Tab. 4.1).

In a second patient (no. 422.2), the karyotype was Ph-negative and seemed to have a small deletion of chromosome 16 long arm (16q-). The other chromosome 16 appeared normal (Fig. 4.3a). A more detailed study by synchronized culture G-banding, however, showed that the seemingly smaller 16 had no apparent deletion; instead it had a smaller centromere than its counterpart. This was confirmed by C-banding (Fig. 4.3b). Centromeric polymorphism is widely observed in normal individuals (Vogel and Motulsky, 1986). Nevertheless, this patient had some atypical features (Tab. 4.1): a relatively short chronic phase (4 months compared with 36-42 months which is the average for CML patients; Monfardini et al, 1973); a low platelet count and a small spleen. A rise of eosinophil count in the peripheral blood occurred 4 months after presentation; this coincided with blastic transformation. The

Fig. 4.3 Karyotype and C-banded Metaphase of Patient no. 422.2.

(a). G-banded metaphase showing small deletion of 16q.

(b). C-banded metaphses showing the apparent short 16q is due to centromere polymorphism (as indicated by arrows).

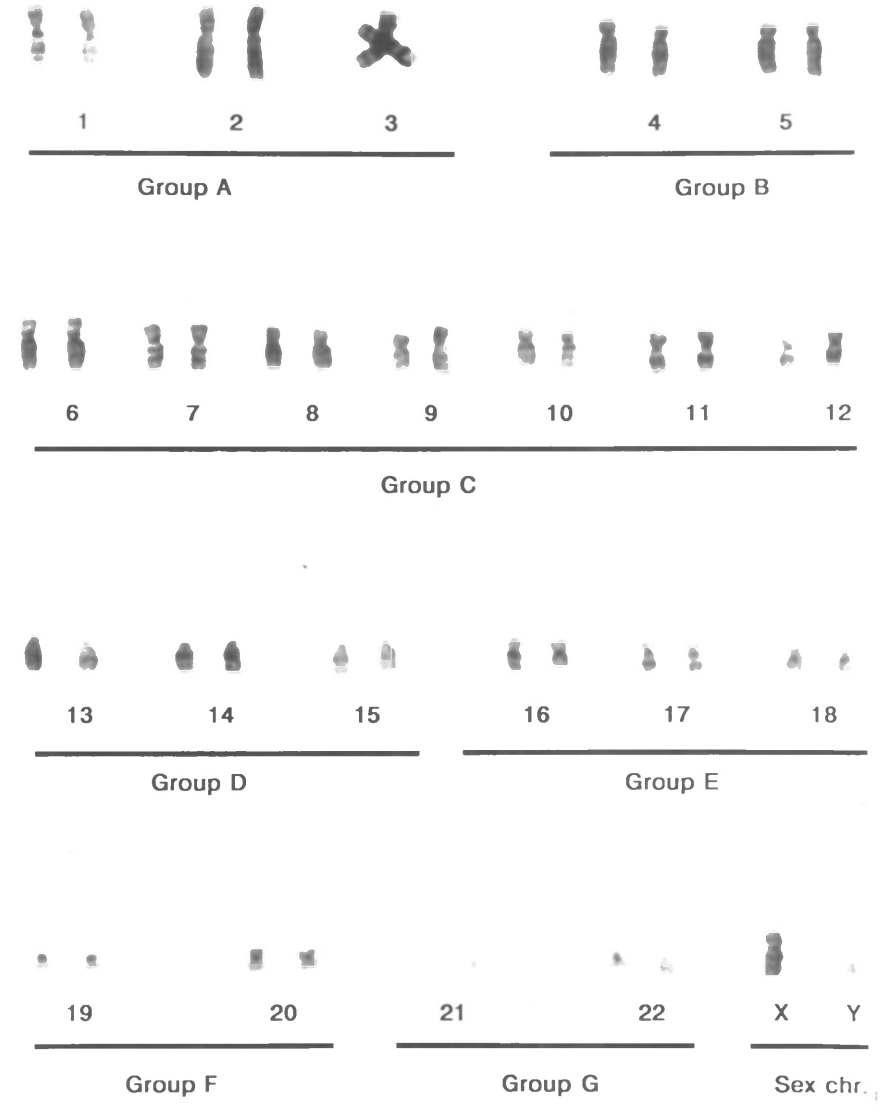

1
2
3
4
5
Group A
Group B
6
7
8
9
10
11
12
Group C
13
14
15
16
17
18
Group D
Group E
19
20
21
22
X
Y
Group F
Group G
Sex chr.

Fig. 4.3 (a)

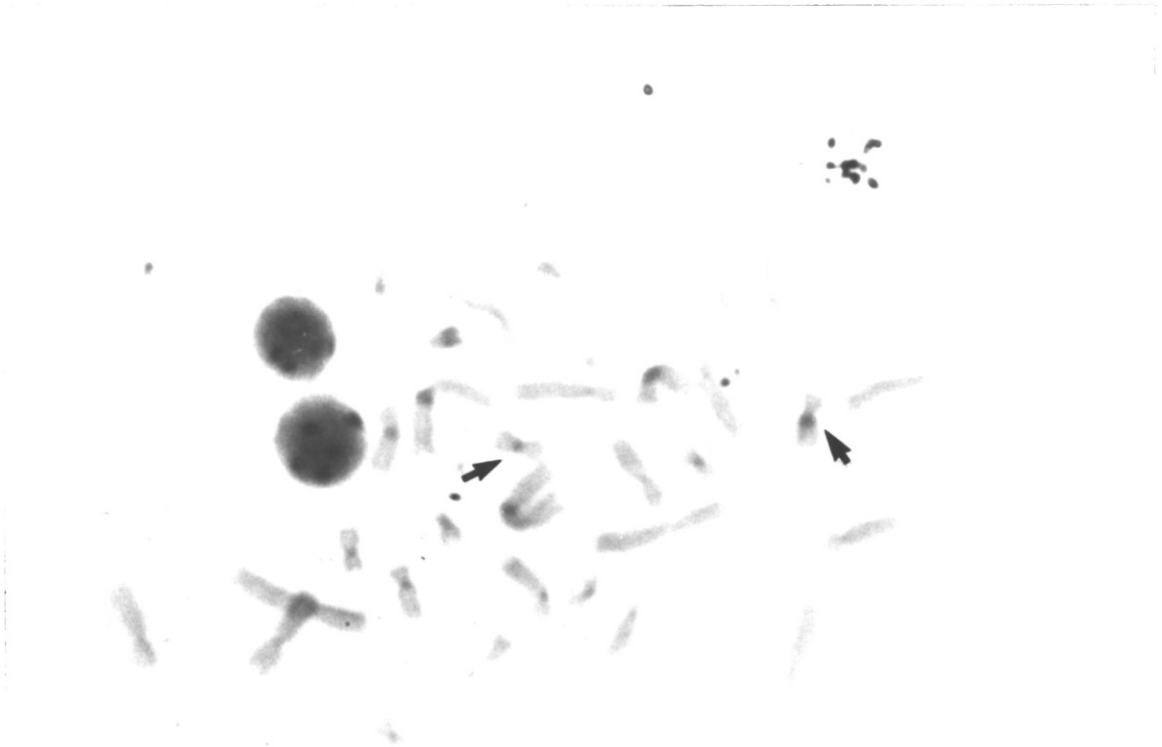


Fig. 4.3 (b)

peripheral blood eosinophil count fluctuated from 5 to 78% before his death. However, bone marrow aspiration at presentation and at the time when he was entering blast transformation showed no increase in eosinophils. It should be mentioned here that this patient had cardiac complications during the course of his disease.

Twenty to 50 metaphases were analyzed from further four Ph-negative CML patients (nos. 422.3, 422.4, 422.10 and 422.11); these were cytogenetically normal (Fig. 4.4). The clinical and haematological pictures of these cytogenetically normal CML patients varied; 2 (nos. 422.3 and 422.4) had typical CML and the other 2 (nos. 422.10 and 422.11) were atypical. The thirteen patients who had normal karyotypes by G-banded analysis also showed heterogenous haematological findings. Some atypical features were observed in 8 patients (from nos. 422.12 to 422.19) while the other 5 (from nos. 422.5 to 422.9) were indistinguishable from Ph-positive CML (Tab. 4.1).

4.2.3 CYTOGENETIC STUDIES IN BMT

Forty-eight patients treated by allogeneic bone marrow transplantation for Ph-positive CML in chronic phase had cytogenetic studies of marrow performed at intervals after BMT (in collaboration with Dr. Christopher Arthur). Out of these 48 patients 20 had received unmanipulated donor marrow and 28

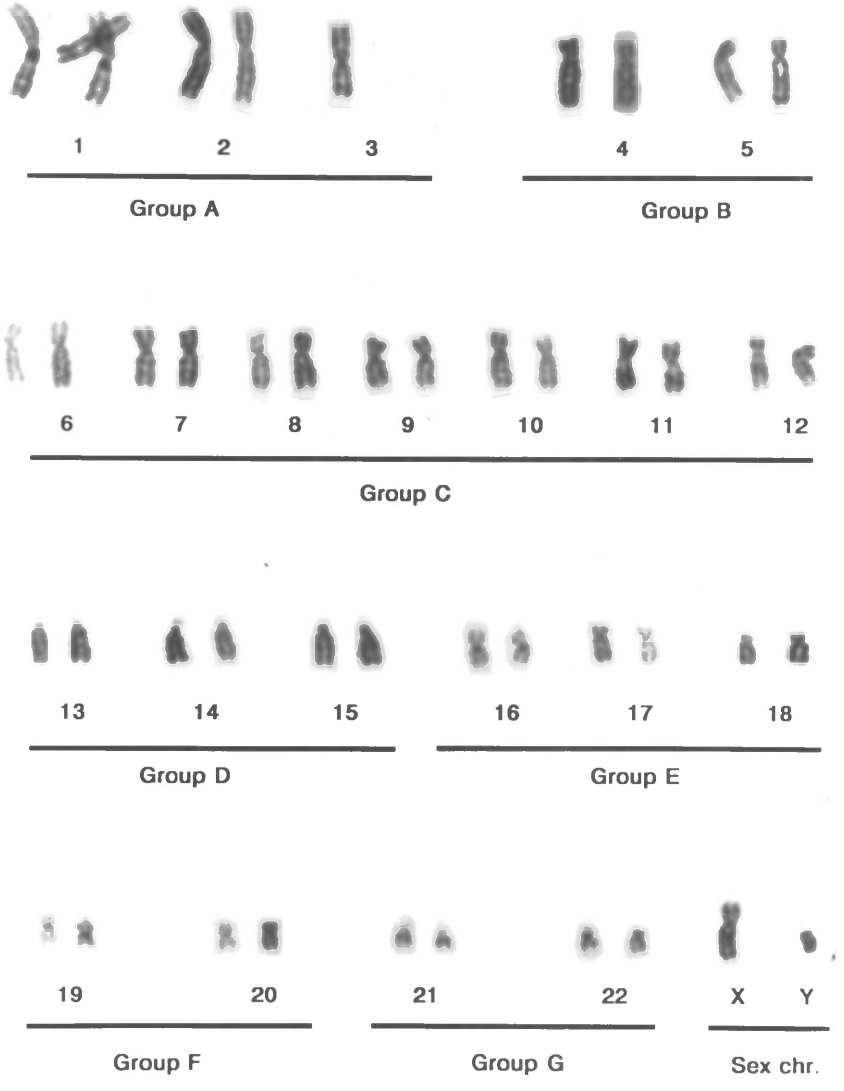


Fig. 4.4 Representative Metaphase Showing Normal Karyotype (46XY) in Ph-negative CML Patient (no. 422.4).

had received T cell-depleted donor marrow. Cytogenetic studies were attempted at approximately three month intervals post-BMT although not every study yielded analyzable metaphases. The number of metaphases successfully examined on each occasion varied from 2 to 34.

Of the 20 patients who received donor marrow cells that had not been depleted of T-cells, 18 had a Ph-negative marrow when first examined after BMT. Of these eighteen patients, 15 remained Ph-negative but 3 have since become Ph-positive. In one of the Ph-negative group, autologous recovery with Ph-negative host metaphases occurred. Two of the 3 Ph-positive group continued to show evidence of cytogenetic relapse while the other relapsed into blast transformation. The last two of these 20 patients were Ph-positive when first examined at 3 months and 12 months after BMT. Both subsequently became Ph-negative with clinical and cytogenetic follow-up at 9 months and 4 years post-BMT, respectively. This sequence of events we have called 'transient cytogenetic relapse'. The outcomes of these 20 patients have been summarized in Figure 4.5 (a).

The 28 patients who received T-depleted marrow cells showed a wider variety of cytogenetic and clinical patterns. Twenty patients achieved a Ph-negative marrow when first examined after BMT, but only nine remained entirely Ph-negative. Two of these had transient mixed chimerism with both donor cells

Fig. 4.5 Diagrams Illustrating the Variety of Cytogenetic Outcomes after BMT.

Sequential status of Ph-positivity in patients receiving BMT. Scheme shows evolution (left to right) of cytogenetic events starting immediately after BMT. Thin boxes represent patients without any detectable Ph-positive metaphases; thick boxes contain patients with some or all marrow metaphases showing the Ph chromosome. The thick shaded boxes contain patients in haematological relapse.

(a). 20 patients who received non-T cell depleted bone marrow transplants.

(b). 28 patients who received T-cell depleted marrow transplants.

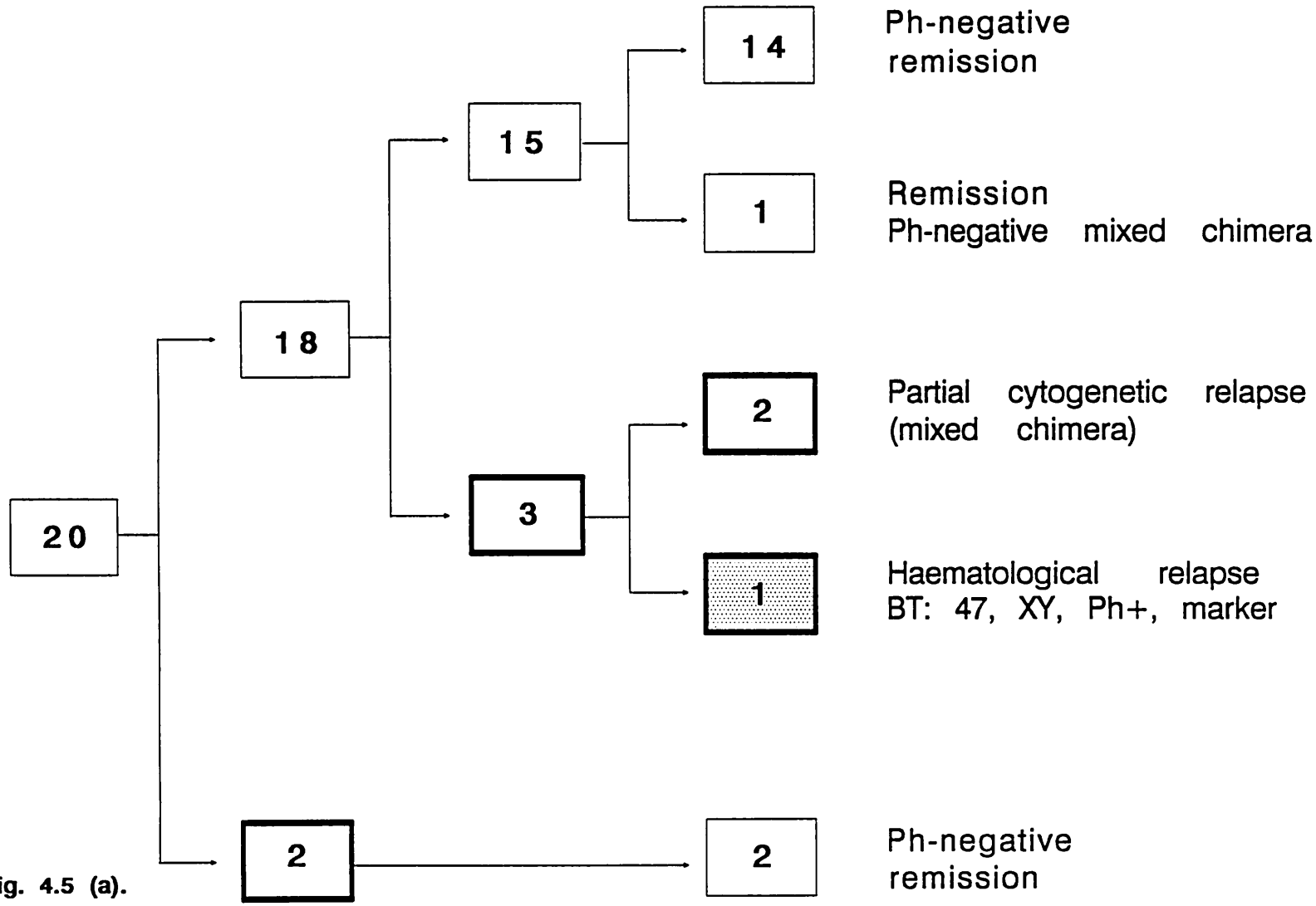

20
18
2
15
3
14
1
2
1
2
Ph-negative remission
Remission Ph-negative mixed chimera
Partial cytogenetic relapse (mixed chimera)
Haematological relapse BT: 47, XY, Ph+, marker
Ph-negative remission

Fig. 4.5 (a).

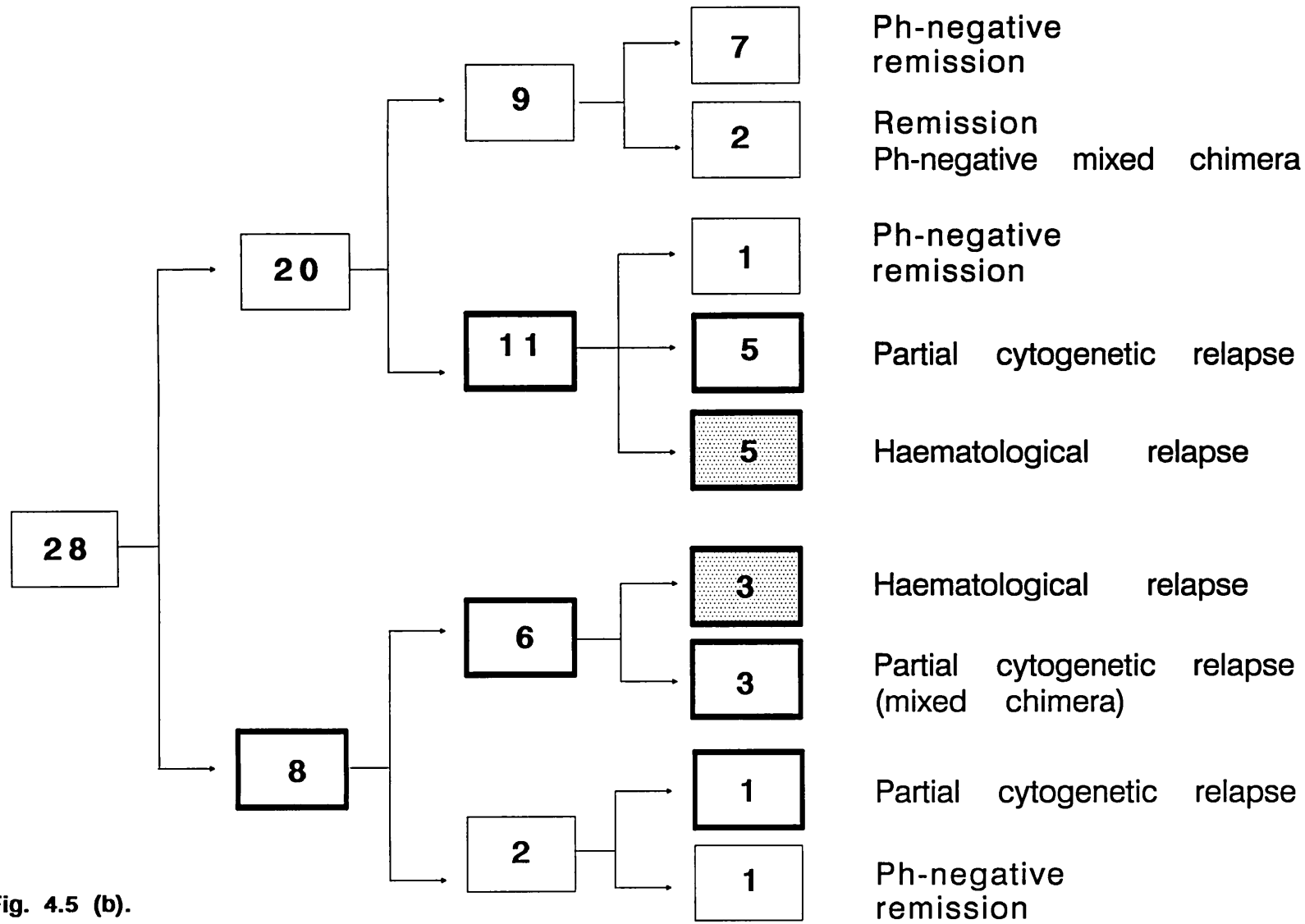

28
20
8
9
11
6
2
7
2
1
5
5
3
3
1
1
Ph-negative remission
Remission Ph-negative mixed chimera
Ph-negative remission
Partial cytogenetic relapse
Haematological relapse
Haematological relapse
Partial cytogenetic relapse (mixed chimera)
Partial cytogenetic relapse
Ph-negative remission

Fig. 4.5 (b).

and Ph-negative host cells present. Eleven of the Ph-negative group became Ph-positive on follow-up examination. This group includes 5 patients with cytogenetic relapse, 5 patients with haematologic relapse, and 1 patient who became Ph-negative again after a period of Ph positivity.

Eight patients were Ph-positive when first examined after BMT. Two of these 8 became Ph-negative on follow-up; this persisted in 1 patient but was transient in the other. Six remained Ph-positive. Two of these 6 patients have relapsed into chronic phase CML, one has accelerated and 3 showed cytogenetic relapse only. The outcomes of these 28 patients have been summarized in Figure 4.5(b).

Eleven of the patients who relapsed had marrow donors of the opposite sex. In each case, the metaphases showing the Ph chromosome had the sex chromosomal complement of the recipient. Two patients showed chromosomal abnormalities consisting of chromosomal fragments, chromatid breaks, and random chromosomal losses in the cells of donor origin. Both had received T cell-depleted donor bone marrow, but both had active chronic GVHD. They were receiving prednisolone and cyclosporin A but no cytotoxic agents. One patient was in remission with 100% donor cells. She had had disseminated infection with herpes varicella-zoster 6 months after transplantation that was successfully treated with acyclovir.

The other patient was a mixed marrow chimera with cytogenetic relapse. He had also had disseminated infection with herpes varicella-zoster at 5 months and oropharyngeal infection with herpes simplex at 6 months after BMT. He intermittently excreted cytomegalovirus (CMV) in the post transplantation period. In both patients cytogenetic studies were performed after the virus infections.

Two patients transplanted with T-cell depleted marrow and one with non-manipulated marrow had additional chromosomal changes. Both patients (one male and one female) who had T-depleted BMT had karyotypes 47,Ph plus a C group marker, the patient who had non-manipulated BMT had karyotypes 47,XY,Ph and a marker. These three patients all relapsed directly into accelerated or blastic phases.

4.3 DISCUSSION

4.3.1 ADDITIONAL CHROMOSOME CHANGES IN BLAST TRANSFORMATION

In this study 5 cases with additional chromosome changes were found coinciding with or preceding blast transformation. In one patient with Ph-positive CML, an additional Ph chromosome was found one month before lymphoid transformation. The other patient with Ph-positive CML in acceleration also had an additional Ph chromosome. Another three patients who were

transplanted relapsed directly into acceleration or blast transformation. These 3 patients showed marker chromosomes. The commonly recognised additional chromosomes 8 and 19 and the isochromosome 17q (Hagemeijer et al, 1980; 1987) were not observed. In our patients the additional chromosome changes associated with long continuing chronic phase have not been observed either. The number of patients with further chromosome changes in this series was not large enough to enable us to study the correlation between the types of chromosome changes and the sub-types of blastic transformation, namely the lymphoid or myeloid transformation.

4.3.2 Ph-NEGATIVE CML

Chromosomal abnormalities such as a masked Ph or other translocations were uncommon in our series of Ph-negative CML patients. Only one case was found to have cytogenetic abnormalities; the other 18 patients were apparently cytogenetically normal. Not surprisingly the patients with masked Ph chromosome and complex translocations were clinically and haematologically indistinguishable from typical CML, as they had in effect the Ph translocation. It seems that in complex translocations the cytogenetic events that occur after the formation of the Ph chromosome do not change the disease. In the karyotypically normal CML patients clinical and haematological features were quite heterogeneous. Ten of these patients were found to be atypical CML, while the other

8 were indistinguishable from typical CML.

Hagemeijer (1987) summarized 77 cases of Ph-negative CML from different centres, in which some of our patients were included. Sixty cases had a normal karyotype; seventeen presented with cytogenetic abnormalities. Six of these 17 had translocations involving 9q34 and a chromosome other than 22. Molecular analysis for BCR/ABL rearrangement of the karyotypically normal patients showed that one third had the rearrangement but the other two-thirds did not. In all the patients with translocations involving 9q34, with a masked Ph chromosome or a normal chromosome 22, BCR/ABL rearrangement were also found. A breakpoint in the M-BCR was found in only five patients with other cytogenetic abnormalities (Bartram et al, 1985; Bartram and Carbonell, 1986b; Kurzrock et al, 1986; Dreazen et al, 1987; Wiedemann et al, 1988).

In a retrospective analysis of the haematological features of 25 patients previously regarded as having Ph-negative CML, the authors concluded that all but one case could be better reclassified either as myelodysplastic syndrome or as a chronic myeloproliferative disorder other than CML (Pugh et al, 1985). However, in a morphological and haematological assessment as well as molecular study of 36 cases of Ph-negative CML, Shepherd et al (1987) concluded that about half of the Ph-negative CML patients were typical CGL and the other

half fell into four types: the first two, juvenile CML and chronic neutrophilic leukaemia, are excessively rare; the other two are chronic myelomonocytic leukaemia (CMML) and atypical CML.

As a result of studies by us and others, we suggest that Ph-negative CML patients form a group that is heterogeneous both in clinical-haematological features and in cytogenetics. There is no obvious indication from the karyotypes of Ph-negative patients of their likely clinical-haematological classification. However, in small numbers of patients the discovery of a masked Ph chromosome and possibly detectable translocations involving 9q34 is associated with the typical CML. It would be very interesting to study the BCR/ABL rearrangement in this group of cytogenetically atypical CML patients.

Patient no. 422.2 who seemed to have partial deletion of 16q had other atypical features plus peripheral blood eosinophilia. Arthur and Bloomfield (1983) reported 16q partial deletion associated with bone marrow eosinophilia in 5 cases of acute nonlymphocytic leukaemia (ANLL). In their study the patients had an acute leukaemia with a prognosis better than the average ANLL patient. Eosinophilia was strictly confined to the marrow. They did not do C-banding in all cases to confirm the del(16)(q22). I have observed some

differences in the case in my study. The patient had Ph-negative CML though his prognosis was worse than the average CML; the patient had eosinophilia in peripheral blood but not in the marrow; eosinophilia seemed to relate to the blast transformation. C-banding in this patient showed that the shortened chromosome 16 was due to a centromere polymorphism. It is not clear whether these two types of leukaemia with eosinophilia in blood or bone marrow are related. If they are, could eosinophilia be related to a chromosome 16 centromere polymorphism rather than to the suggested deletion? Furthermore, this patient would fit a diagnosis of eosinophilic leukaemia in many ways (Bentley et al, 1961). Krauss et al (1964) reported that two cases of Ph-negative CML were in fact eosinophilic leukaemia. However, Gruenwald et al (1965) reported one case of eosinophilic leukaemia with a Ph chromosome in the marrow metaphases. Taking the above data together CML characterized by marked eosinophilia can probably be divided into Ph-positive and negative cases.

The underlying molecular basis of the Ph translocation is the BCR/ABL rearrangement. This is associated with the haematological features typical of CML. Various types of Ph-negative CML have also been examined by molecular techniques for BCR rearrangement (see Chapter 6).

4.3.3 DIFFERING CYTOGENETIC PATTERNS AFTER BMT

A variety of cytogenetic patterns in the marrow of patients after BMT was observed. Cytogenetic relapse was more common than we expected since about 50% of the patients in our series had cytogenetic relapse. Of particular interest was the finding that the Ph chromosome appeared only transiently after transplantation in 4 patients. Similar transient relapses have been reported by others (Thomas et al, 1986; Zaccaria et al, 1987; Frassoni et al, 1988). In a multi-centre study of 100 patients receiving BMT for CML (4 received T depleted donor marrow), the Ph chromosome was observed after BMT in 22 patients who did not enter relapse during the observation time (10-1400 days - median 420 days) following initial detection of the chromosome. The Ph chromosome was present in 1-30% of the cells analysed. In 10 patients, Ph positive metaphases were detected only within the first 90 days after BMT, in 5 patients both before and after 90 days and in 7 patients only after 90 days (Zaccaria et al, 1988). Frassoni et al (1988) reported the cytogenetic and clinical course of three patients allografted for CML in whom they had observed competition between recipient Ph-positive cells and donor cells after BMT. At one stage the patients had Ph positive marrow metaphases and host type red cells ranging from 75% to 100% of the total cell population. However, these leukaemic cells of recipient origin were no longer identifiable in later studies.

Thus the finding of small numbers of Ph-positive metaphases in the marrow after BMT dose not necessarily mean that the transplant has failed. However, the failure to find Ph-positive metaphases on any one occasion must be interpreted with caution, since Ph-positive cells might escape detection if present in only small numbers or if none of the Ph-positive cells were in cycle. The recurrence of Ph-positive metaphases after an interval of Ph negativity or the persistence of Ph-positive metaphases after BMT may, however, have other outcomes. The detection of Ph-positive marrow metaphases may be followed by haematologic relapse. Such relapse may have features of chronic-phase disease, but three patients in this study relapsed directly into accelerated or blastic phases with additional chromosome abnormalities. In contrast, in 11 patients cytogenetic relapse did not progress to haematological relapse. Such progression might still occur if the patients are followed for longer periods, but one patient remained in 'stable' cytogenetic relapse without progression for 18 months.

After BMT the growth of residual leukaemic cells must be controlled by some mechanism. This could be by competition for growth between the normal donor cells and the host leukaemic cells, or an immunological mechanism such as a graft-versus-leukaemia (GVL) effect may operate to control or eliminate the leukaemic cells (Apperley et al, 1988). Strife and Clarkson

(1988) suggested a different explanation based on 'discordant maturation'. This hypothesis suggested that proliferation of the early leukaemic progenitors could still be subject to normal growth regulatory controls, but with asynchronous maturation and a greater proportion entering into the maturation compartments, the neoplastic population could expand in later maturational compartments not subject to regulatory control. For those patients who had an episode of cytogenetic relapse but then reverted to long term complete remission or those with long term cytogenetic relapse without producing full clinical and haematological picture of CML, the Ph positive metaphases might originate from residual leukaemic late progenitor cells such that they can transiently produce Ph positive metaphases but their life-span and proliferative capacity are limited. The most primitive Ph-positive progenitors may have been killed by conditioning treatment. Even though a few primary Ph-positive stem cells survived, it may require a very long period for them to become dominant as in the original evolution of the disease.

Autologous regeneration of Ph-negative host cells was observed in three of our patients. A similar case was reported by Vincent et al (1986). These patients displayed no CML features at the time of the studies. It is possible that these Ph-negative host cells could be leukaemic or 'pre-leukaemic'. This concept derives some support from study of a female Ph-

positive CML patient heterozygous for the somatic cell enzyme G6PD. The majority of Ph-negative B-lymphoid cell lines established from this patient's blood had a single type of G-6PD isoenzyme identical to the Ph-positive leukaemic clone; these Ph-negative cell lines had a relatively high incidence of chromosomal aberrations (Fialkow et al, 1981). However, we have assumed that the Ph-negative haemopoietic cells we observed in these 3 patients are normal because the patients had no CML features when the complete Ph-negative haemopoiesis resumed. These results indicate that some host Ph-negative haemopoietic cells can survive the conditioning regimens and proliferate after the Ph-positive leukaemic clones are suppressed by BMT.

4.3.4 CHROMOSOMAL DAMAGE AFTER BMT

Chromosomal damage such as chromosomal fragments, chromatid breaks and random chromosome losses were observed in cells of donor origin in two BMT patients who had received donor marrow depleted of T-cells. It seems that GVHD and/or viral infections may have been the cause because the donor cells had not been exposed to irradiation or cytotoxic agents. It has been reported that a variety of viruses including herpes simplex and varicella-zoster can cause chromosome damages (Sandberg et al, 1980). The role of GVHD is therefore speculative. The bone marrow of patients transplanted for leukaemia may show evidence of cell death and cellular debris

for many months after BMT, suggesting that an undefined microenvironmental factor or factors might damage developing haemopoietic cells (Lampert et al, 1987). These histopathological changes could be caused by the same factors that cause the chromosomal abnormalities we observed.

4.3.5 T CELL DEPLETION BMT ASSOCIATED WITH A HIGHER RATE OF RELAPSE

One prominent feature in this study was that patients who had received T-depleted marrow transplants had much higher rates of leukaemic cell recurrence and haematological relapse than those who had received transplants with unmanipulated cells [19 of 28 (68%) versus 5 of 20 (25%), $p < 0.05$]. This phenomenon has been observed by others. Maraninchi et al (1987) conducted a study of 70 patients transplanted for leukaemias (including 33 cases of CML in chronic phase). They found a significantly higher rate of relapse in the T-depleted transplants than in the unmanipulated marrow transplants. The higher rate of leukaemic recurrences in T depleted marrow transplants was observed in BMT in later phases of the disease as well as in chronic phase. In a report from Seattle, 90% of patients transplanted in accelerated phase with T-depleted marrow had evidence of recurrent leukaemia within 2 years (Clift et al, 1987); this contrasts with an actuarial risk of relapse of 55% for patients in acceleration transplanted with unmanipulated donor marrow (Speck et al, 1984). This

highlights the importance of a graft-versus-leukaemia (GVL) effect in preventing relapse in BMT patients (Apperley et al, 1988; Goldman et al, 1988).

CHAPTER FIVE MOLECULAR ANALYSIS OF DNA FROM PATIENTS TREATED BY BMT FOR CML AND SEVERE APLASTIC ANAEMIA

5.1 INTRODUCTION

5.1.1 TECHNIQUES THAT CAN DISTINGUISH DONOR AND RECIPIENT CELLS AFTER BMT

The study of relapse and engraftment by chromosome analysis has limitations. Patients must have some cytogenetic marker such as the Ph chromosome in CML or the sex chromosomes in sex mismatched transplants. Cytogenetic studies can only analyze dividing cells. Furthermore it is difficult to carry out quantitative studies of relapse and engraftment by cytogenetics (see chapter 4).

Apart from cytogenetics there are many other ways to distinguish donor from recipient cells, such as red cell antigen typing, HLA-typing, study of immunoglobulin isotypes, leucocyte isoenzymes or genetic polymorphisms in DNA (Sparkes et al, 1977; Blume et al, 1980; Reinherz et al, 1982; Boyd et al, 1982). These have been reviewed by Ginsburg et al (1985). Erythrocyte antigen and immunoglobulin isotype studies can be difficult to interpret after transfusion and are rarely useful

in the first few months after transplantation. Both techniques provide information relevant to only one cell lineage. HLA-typing can only be used in HLA mismatched transplants.

Two types of polymorphism are recognised in the human genome: variations at a single site, a few of which can abolish or create a restriction enzyme recognition site, or variation in the number of copies of a specific repetitive sequence. Both forms of polymorphism can alter the pattern of restriction enzyme digests and can be demonstrated by Southern blotting. These are referred to as DNA restriction fragment length polymorphisms (RFLPs). Both types of polymorphism are inherited in a Mendelian fashion (Botstein et al, 1980). In the first type a certain restriction enzyme could reveal two or more polymorphic alleles at a single locus. Since BMT is usually performed between closely related individuals the chance of identical alleles at one locus at both donor and recipient is very high. This method usually requires a panel of different probes and enzymes recognizing different polymorphic loci in the human genome (Ginsburg et al, 1985; Blazar et al, 1985; Minden et al, 1985).

5.1.2 DNA 'FINGERPRINTING'

The identification of the origin of a cell population could be simplified considerably by the availability of probes for hypervariable regions of human DNA showing multiallelic

variation and correspondingly high heterozygosities. In 1984 Weller et al (1984) found a short 'minisatellite' region consisting of four tandem repeats of a 33-base pair (bp) sequence in an intron of the human myoglobin gene. This myoglobin 33-bp repeat could be used to detect other tandem-repetitive 'minisatellite' regions dispersed throughout the human genome. This detection is based on shared 10-15 base pair 'core' sequence similar to the generalized recombination signal (x) of Escherichia coli. The copy numbers of the core sequence repeat are highly variable in some loci. When genomic DNA are digested with an enzyme which does not cut within the minisatellite sequence, a series of DNA fragments result. These are of different in length due to allelic variation of copy number in each locus and will be recognized by the core sequence probe(s) in Southern analysis. These produce band patterns that are unique for each individual in a manner similar to fingerprints (Jeffreys et al, 1985a;b).

Jeffreys et al (1985a) cloned a series of different minisatellite core sequence probes. Two of them, 33.6 and 33.15, were selected for DNA fingerprint studies. Probe 33.15 detects about 15 resolvable hypervariable fragments per individual in the 4-20kb size range, whereas probe 33.6 detects another 11 fragments in this size range not detected by probe 33.15. Most of these large fragments are unlinked and segregate independently in pedigrees.

The minisatellite band patterns in DNA fingerprinting are highly specific to an individual and few fragments are shared amongst randomly selected individuals. The probability of shared bands increases for smaller minisatellite fragments, probably resulting from lower genetic variability (higher allele frequencies) of these loci combined with the fortuitous co-migration of unrelated minisatellite fragments. For probe 33.15, the probability that all 15 resolved fragments in the 4-20kb size range in an individual A are also present in a unrelated individual B is $3x10^{-11}$; the probability that the band patterns of A and B are completely identical is therefore less than $3x10^{-11}$. The probability that A and B have identical DNA fingerprints for both probes 33.15 and 33.6 is much less than $5x10^{-19}$. However, for closely related individuals the chance of fingerprint identity is increased. In an individual half the bands are inherited from the father and the other half from the mother (stably inherited in a Mendelian fashion). For unrelated marriages the probability that a hypervariable fragment in sib A is present in sib B is about 1/2, thus the probability that all 15 resolved bands detected by probe 33.15 in sib A are present in sib B is about 2^{-15} or $3x10^{-5}$ (about 10^{-8} for both probes 33.15 and 33.6). These DNA fingerprints are therefore almost totally individual-specific, even within a single family (except of course for identical twins) (Jeffreys et al, 1985b).

5.1.3 SEVERE APLASTIC ANAEMIA (SAA)

Aplastic anaemia is the term applied to a group of disorders characterized by a reduction in all the cellular elements of the blood (pancytopenia) and associated with hypocellularity of the bone marrow (Brain, 1979). Aplastic anaemia may be due to a fundamental defect in haemopoietic stem cell proliferation and differentiation. The observation that the hypocellular bone marrow in aplastic anaemia patients can maintain normal haemopoiesis after successful bone marrow transplantation supports this suggestion (Storb et al, 1974). It seems likely that the disorder of the stem cells is due to one or more somatic mutations which alter the controlling mechanisms governing cell replication and differentiation. These changes may reflect both genetic predisposition and the impact of environmental factors (Brain, 1979). Alternatively aplastic anaemia may in some cases be due to an immunologic mechanism; immunosuppressive therapy for BMT has led to recovery of autologous bone marrow function when a graft has been rejected (Ascensao et al, 1976; Territo et al, 1977). Some patients with aplastic anaemia respond well to immunosuppressive therapy without BMT (Baran et al, 1976).

In general the greater the degree of pancytopenia the worse the prognosis, which has led to many attempts to define that group of patients with the worst prognosis. This has led to

the concept of 'severe aplastic anaemia' (SAA), the criteria for which are shown in Table 5.1. Patients with this degree of pancytopenia have about 10% chance of surviving for 1 year when treated only with conventional measures (Gordon-Smith and Lewis, 1989).

The treatment of aplastic anaemia has changed radically in the past ten years as a result of the use of BMT. The success of this procedure depends on: (a) avoiding or preventing graft rejection; and (b) by minimizing or preventing GvHD. However, less than one fifth of SAA patients are eligible for BMT and the majority of patients must still rely on conventional

Table 5.1 Criteria for diagnosis of severe aplastic anaemia

Peripheral blood: two out of three values
a) Granulocytes < 0.5 x 10^9/l
b) Platelets < 20 x 10^9/l
c) Reticulocyte < 1% (corrected for haematocrit)
Bone marrow trephine:
a) Markedly hypocellular, < 25% normal cellularity
b) Moderately hypocellular, 25-50% normal cellularity with <30% remaining cells haemopoietic

(Gordon-Smith & Lewis, 1989).

treatment consisting of supportive therapy, use of drugs that stimulate haemopoiesis and immunosuppressive therapy (Brain, 1979). The clearest indication for transplant is the younger patient (less than 20 years old) with SAA, particularly if the neutrophil count is $<0.2x10^9/l$. For such patients, BMT gives a better long-term survival than immunosuppressive treatment. For other patients, immunosuppression offers a similar survival rate and there is a case for offering immunosuppression first with BMT reserved for those patients who fail to respond (Gordon-Smith and Lewis, 1989).

Successful engraftment of bone marrow occurs in 75-90% of recipients and these patients are then regarded as complete haemopoietic chimeras, with all circulating haemopoietic cells derived from the donor (Storb et al, 1983). Early graft failure defined as rejection of a partially established graft within the first 3 months occurs in up to 25% of recipients and usually results in a rapidly fatal course. However, in some cases, graft failure after BMT has been followed by recovery of autologous bone marrow function (Territo et al, 1977; Tsoi et al, 1983). Autologous recovery may also occur after late graft failure, defined as recurrence of pancytopenia and marrow hypoplasia after a period of satisfactory engraftment with freedom from blood product support (Hows et al, 1985; Anasetti et al, 1986). Recent studies have focused on the occurrence and significance of

mixed lympho-haemopoietic chimerism after BMT (Hill et al, 1986). It is suggested that mixed chimerism is associated with higher rate of rejection and lower rate of Grade II to IV acute GVHD. Mixed chimerism is defined here as the finding of recipient cells coexisting with donor derived haemopoietic cells after allogeneic BMT. In animal models, stable mixed chimerism has been associated with a decreased incidence of GvHD (Ildstad & Sachs, 1984).

In this chapter I describe studies of long-term engraftment in 21 multiply transfused patients transplanted for severe aplastic anaemia (SAA) 2-7 years previously from HLA identical sibling donors. The studies were performed in collaboration with Dr Jeffrey Weitzel.

5.2 RESULTS

5.2.1 SENSITIVITY OF DNA FINGERPRINTING IN IDENTIFYING DNA OF DIFFERENT ORIGINS

In order to determine the sensitivity of DNA fingerprinting in identifying DNA of different origins, we either mixed the blood cells and then extracted DNA or directly mixed DNA derived from different individuals in different proportions.

We first selected one patient transplanted for CML and his

donor for the mixing experiments. The DNA mixture ratios ranged from 10:0 to 0:10 in 10 steps. The intensity of the bands increased or decreased to reflect the increasing or decreasing proportions of DNA from donor and recipient respectively. For example, when probe 33.15 and enzyme HinfI was used, the band of recipient origin visible at 10kb diminished in intensity but was still readily detectable when the recipient's DNA constituted only 10% of the mixture (Fig. 5.1 a & b). Further studies reducing the amount of recipient DNA and increasing the amount of donor DNA showed that DNA fingerprinting can detect as little as 2% of DNA from the recipient (Fig. 5.1c).

In collaboration with Dr Weitzel, I also compared the results of studies of DNA preparations made by first mixing cells (from the human cell lines HeLa and K562) in varying ratios and then extracting the DNA, with results obtained by first preparing the DNA and then making the mixtures. Using probe 33.15 and enzyme HinfI we found that the minimum detectable individual DNA was 2-5% of the total DNA in the mixtures. There was no apparent difference between the two kind of mixing experiments. This indicated that in DNA fingerprint analysis the degree of DNA mixture can reflect the true proportion of mixed cell populations.

The actual sensitivity with which cell chimerism can be

Fig. 5.1 DNA Fingerprinting Showing DNA from Patient in Relapse Post-BMT and Mixing Experiment Showing the Sensitivity of the Method.

(a). DNA from donor and recipient mixed at differing ratios. Band patterns from recipient (patient no. 522.12) pre-BMT (lane 1), recipient in relapse after BMT (lane 2) and from donor (lane 3) are shown. Lanes 4-12 inclusive show band patterns from mixtures of donor and recipient DNA with ratio differing from 9:1 to 1:9 in 9 steps. The band patterns of the recipient in relapse are the same as those before transplant, while the bands of the donor DNA are not seen in DNA from the recipient in relapse. The arrow indicates two 10kb size bands of recipient origin that are clearly visible in lane 4 even though recipient-derived DNA accounted for only 10% of total DNA. DNA was hybridized with probe 33.15.

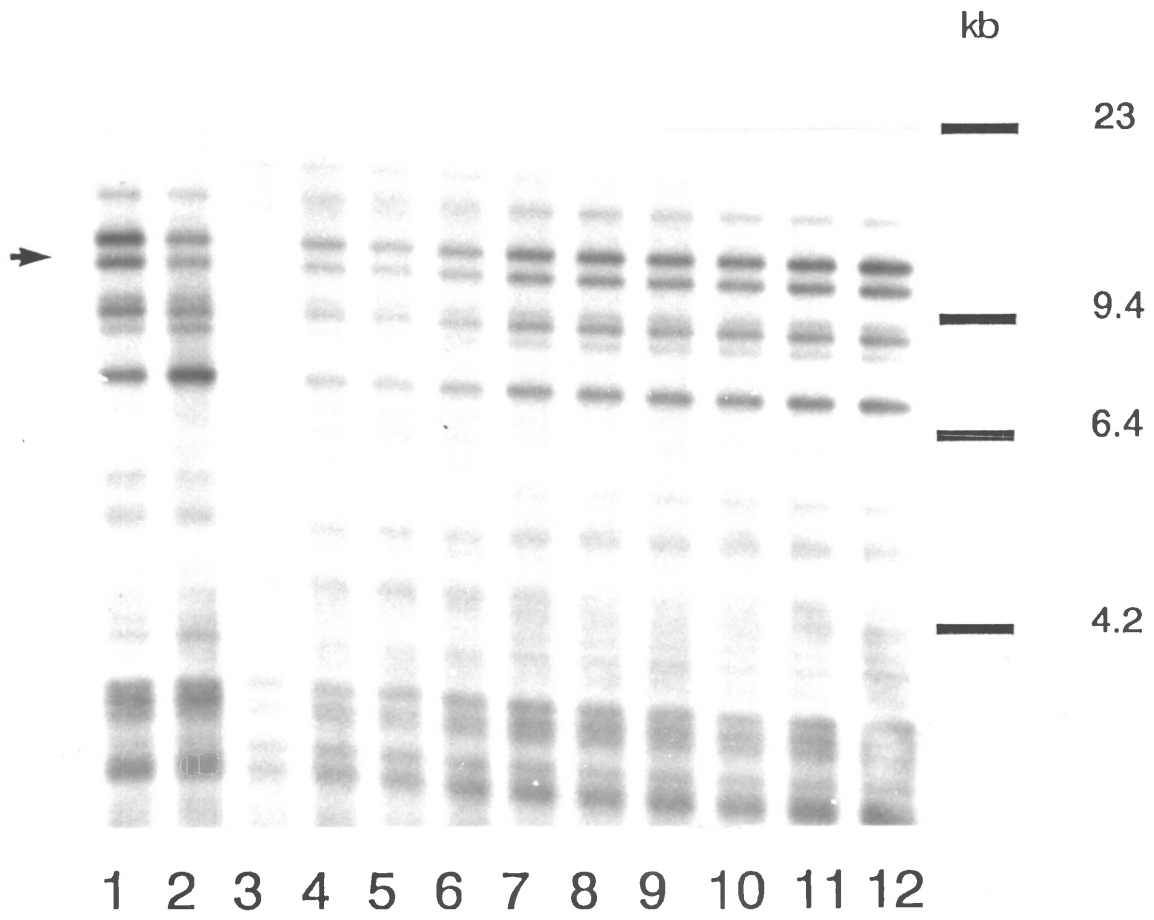


Fig. 5.1(a)

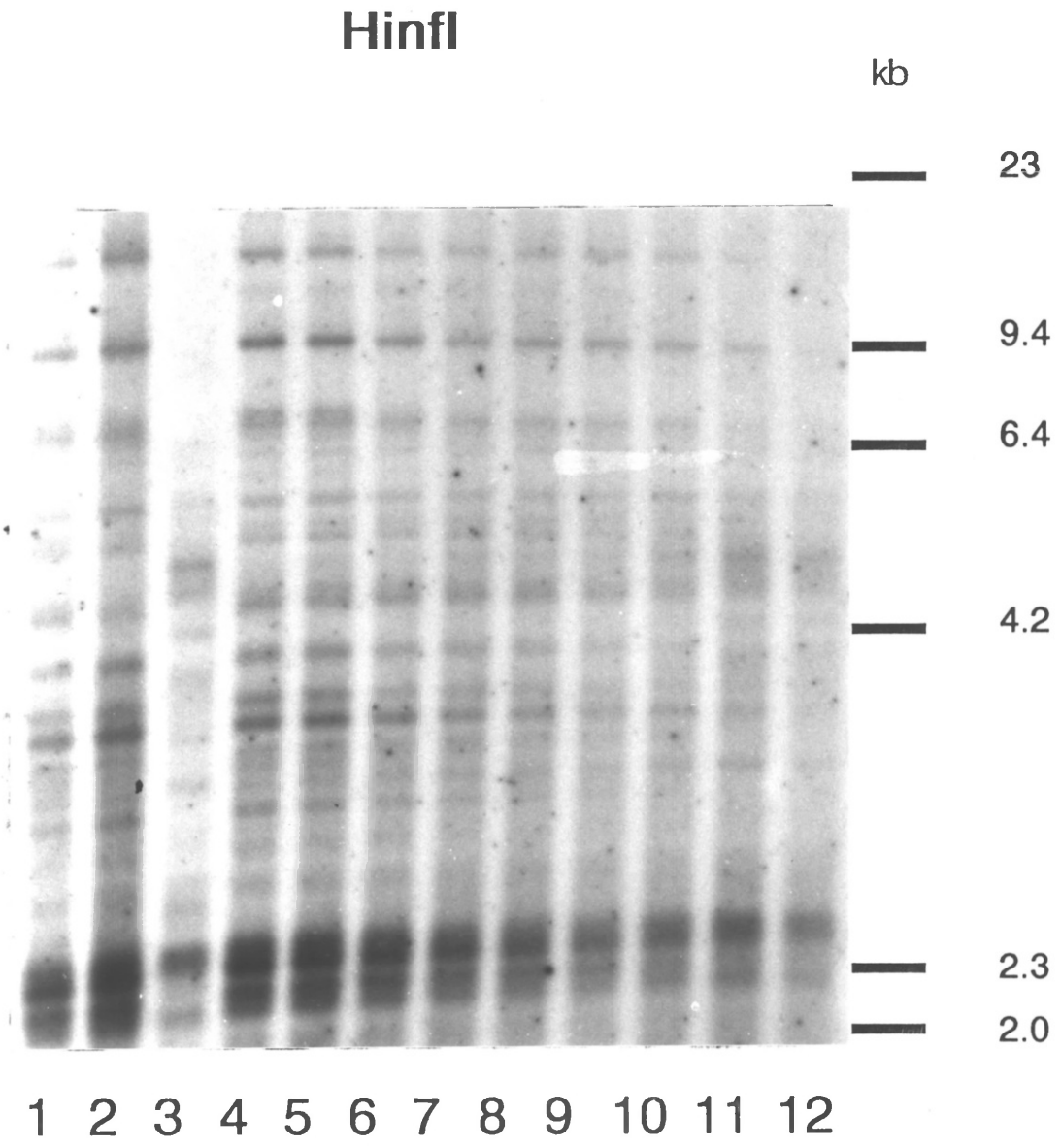


Fig. 5.1 (b). The DNA from the same donor/recipient combination as in Fig. 5.1 (a) were hybridized with probe 33.6. DNA in relapse (lane 2) is identical to recipient pre-transplant (lane 1). Mixture of DNA from donor/recipient (lanes 4 to 12) showing the sensitivity in this blot is 20% (the recipient band at 9.4kb can still be seen in lane 11).

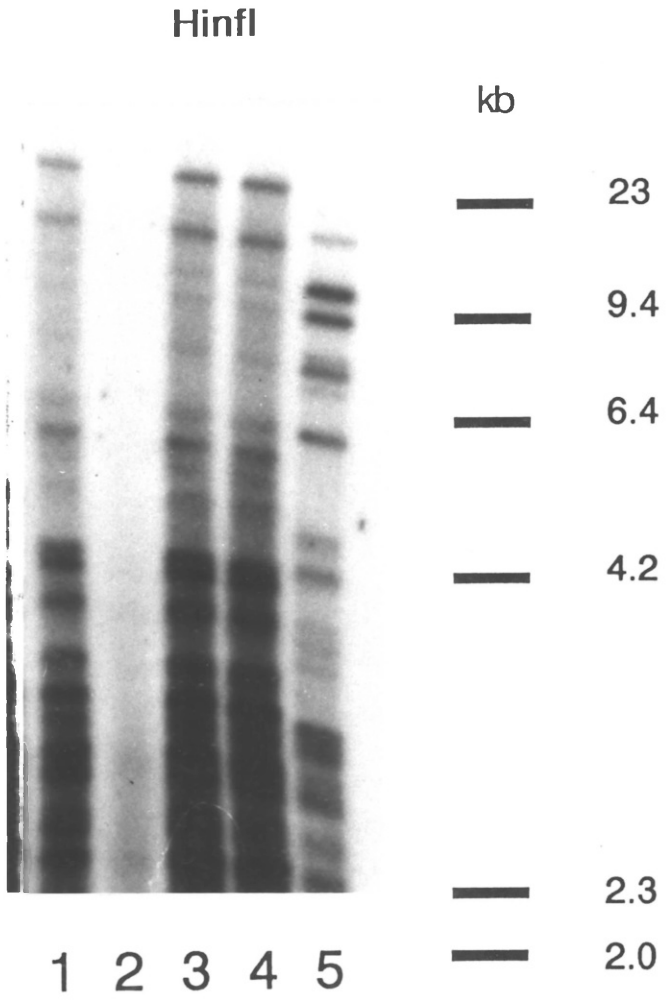


Fig. 5.1 (c). Further mixing experiment by reducing the proportion of recipient DNA to 5, 4, 3 and 2% showed the sensitivity of DNA fingerprinting. It could detect as little as 2% DNA (the two 10kb bands can still be traced in lane 1). Lane 5 is the recipient control; lane 4 accounts for 5% recipient DNA; lane 3 for 4%, lane 2 is blank due to sample degradation.

detected will of course depend on the choice of probes, enzymes, the quality of DNA and the precise fingerprint patterns of each donor and recipient combination. In general the sensitivity of DNA fingerprinting in our experiments was comparable to that reported for the use of other DNA probes (Knowlton et al 1986).

5.2.2 ENGRAFTMENT AFTER BMT FOR CML AND AML

I studied the DNA from nine recipients (8 CML and 1 AML) and their respective HLA-identical sibling marrow donors before BMT and at 3-8 week intervals for a total of 7-15 months after BMT. Samples taken from the patients 1 to 2 weeks post transplant did not usually contain enough white cells or DNA for meaningful analysis. Most of the studies therefore began at 3 weeks post transplant.

Engraftment with donor marrow could usually be identified as early as 3 weeks post-transplant, at which time DNA from blood cells was shown to be of donor origin (Fig. 5.2, patient no. 522.1). Sometimes bands of recipient origin in faint intensity could be traced that may represent the residual recipient cells that still survive in the peripheral blood for a while. When the transplant procedure appeared clinically to be successful, patient-derived DNA studied at intervals of 1-2 months post-transplant showed only donor type band patterns without any trace of recipient-derived patterns. In the

Fig. 5.2 DNA Fingerprinting Demonstrating Engraftment after BMT.

Early engraftment showing prominent bands of donor origin at 3 weeks post-transplant. DNA from the recipient (patient no. 522.1) post-BMT shows a series of bands at around 6.4 kb (arrow A, HinfI digests) corresponding to DNA of donor origin. Lane 1 is donor DNA; lane 2 is recipient post-BMT; lane 3 is recipient pre-BMT. The DNA digests were hybridized with probe 33.15.

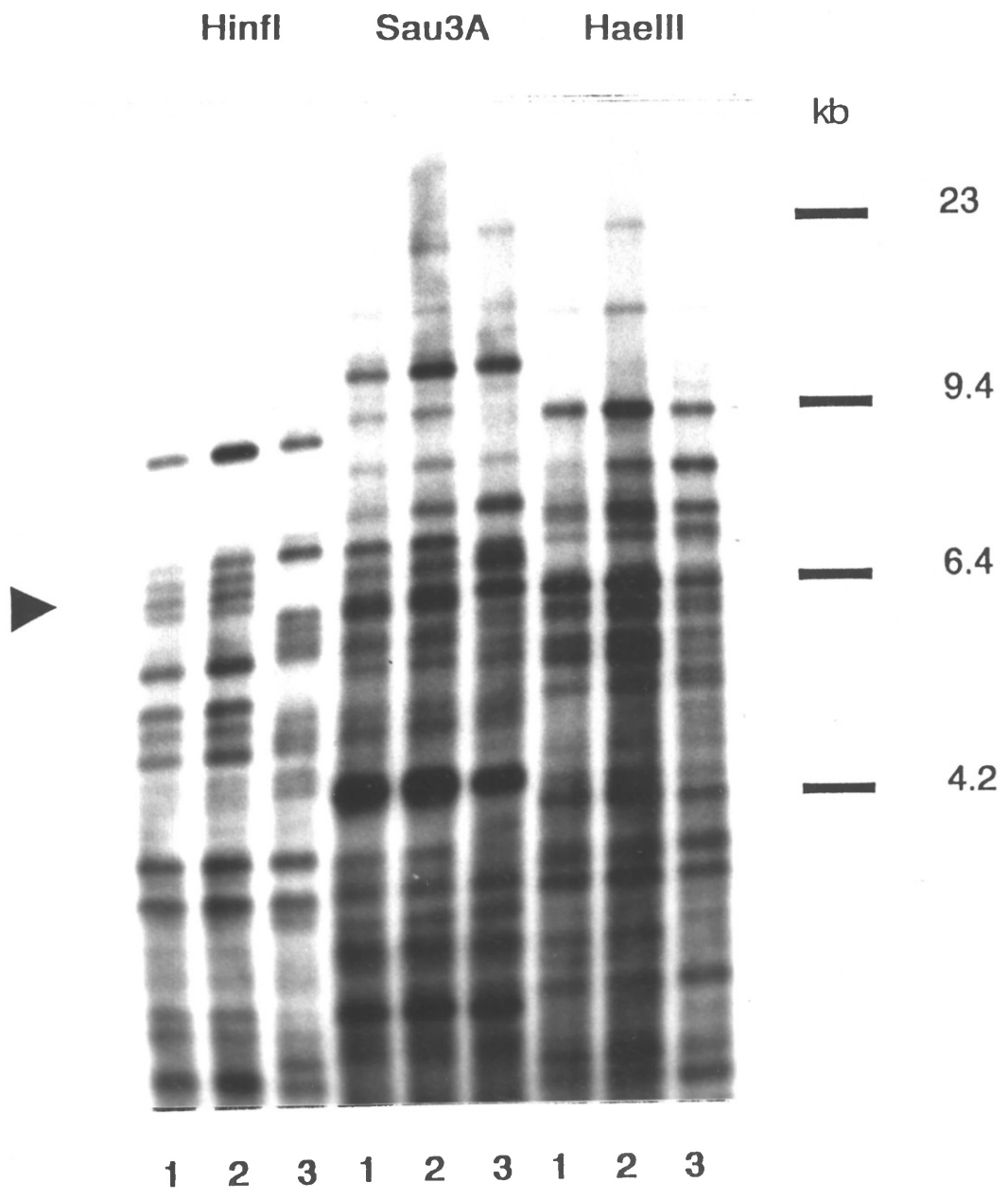


Fig. 5.2

majority of the cases the patterns of engraftment were sustained during follow-up for up to 15 months. Of these nine patients, three (nos. 522.2, 522.3 and 522.4) showed evidence of cytogenetic relapse during the study period and also showed reappearance of recipient type bands on DNA analysis. One patient (no. 522.5) whose graft failed at 3 months showed a DNA pattern of recipient type. In this patient graft failure was accompanied by cytogenetic relapse but clinical relapse was not detectable until 9 months.

5.2.3 RELAPSE AFTER BMT FOR CML AND ALL

Thirteen patients with CML who had evidence of cytogenetic or haematological relapse and one patient with ALL who relapsed after transplant were studied with the minisatellite probes (Table 5.2). Eight of the patients were also studied with the M-BCR probe to identify the reappearance of the Ph-positive cell population.

In seven of these patients I was able to examine samples from the recipient pre-transplant, from the donor and from the recipient after transplant. In four cases the band patterns were clearly of recipient origin (Fig. 5.1). No bands of donor origin were seen in any of these post-transplant samples. These results demonstrated the complete repopulation of the marrow by relapsed CML cells. All these four patients had full clinical relapse at the time of analysis. The other three

Table 5.2. Summary of the clinical features, cytogenetics, M-BCR rearrangement and DNA fingerprinting studies in leukaemic patients post-BMT.

Patient no.	Age (yr)	Sex (R/D)	Disease status at BMT	Time to: Cytogen. relapse (months)	Time to: Haemat. relapse (months)	Type of HR	Interval to recurrence of host cells (months)	Interval to recurrence of M-BCR rearrangement (months)
Chronic myeloid leukaemia:								
522.1	53	M/F	CP	9	NO	-	3WK*	3WK*
522.2	28	F/F	CP	9	25	CP	13	13
522.3	35	F/F	CP	NO	NO	-	NO	-
522.4	27	M/M	CP	NO	NO	-	NO	-
522.5	18	F/M	CP	GRAFT FAILURE AT 3 MONTHS			3	3
522.6	29	F/F	CP	13	13	AP	24 (T-cells)	-
522.7	29	F/F	CP	18	18	AP	18	18
522.8	21	M/F	CP	NO	NO	-	NO	-
522.9	34	M/M	CP	NO	NO	-	NO	-
522.10	34	M/F	CP	6	NO	-	7	7
522.11	38	M/F	BC	12	12	BC	12	-
522.12	17	F/F	AP	2	2	CP	1.5	1.5
522.13	39	M/M	CP	4	4	BC	25	-
522.14	32	M/M	CP	6	10	AP	14	-
522.15	22	F/M	CP	16	16	CP	17	17
522.16	42	F/F	2nd CP	18	18	CP	14	14
522.17	32	M/M	2nd CP	54	54	BC	52	52
Acute leukaemia:								
522.18	18	M/M	1st CR	NO	4	ALL	4	-
522.19	16	M/M	1st CR	NO	NO	-	NO	-

Table 5.2 (continual)

Abbreviations: ALL = Acute lymphoblastic leukaemia; CP = Chronic phase; AP = Accelerated phaes; BC = Blast crisis; 2nd CP = Second chronic phase after treatment of transformed leukaemia; R/D = Recipient/donor; HR = Haematological relapse; CR = Complete remission; NO = Not (yet) occurred.

* The recipient leukaemic cells detected at this early stage post-BMT are likely to be the residual cells still circulating in peripheral blood. They may be damaged by the BMT procedure but still survive for a while.

patients (nos. 522.2, 522.3 and 522.4) were described above. In four patients I was unable to study the DNA from donors, but the pattern post-transplant was identical to the recipient pre-transplant in these patients (Fig. 5.3). In three patients I was unable to study the DNA recipient before transplant, but the pattern post-transplant was clearly different from the donor in these patients (Fig. 5.4). These last seven patients were also cases of haematological relapse.

In one case (no. 522.2) study of peripheral blood 7 months post BMT was consistent with complete engraftment without any signs of DNA of recipient origin (Fig. 5.5). However, a cytogenetic study of marrow cells performed 1 month later showed that all metaphases had the Ph chromosome. This sequence could be explained if the proportion of Ph-positive marrow metaphases 1 month earlier had been low and all cells then in the peripheral blood had still been of donor origin. At 13 months post BMT DNA from peripheral blood cells showed a mixed pattern of recipient and donor bands with donor-derived bands predominating. Cytogenetic studies of the marrow cells at that time showed that the percentage of Ph positive metaphases had fallen. In the follow-up study of this patient by DNA fingerprinting 18 months post-transplant peripheral blood showed mixed chimerism with the proportion of donor's cell population diminishing. The patient entered overt clinical relapse 25 months post-BMT.

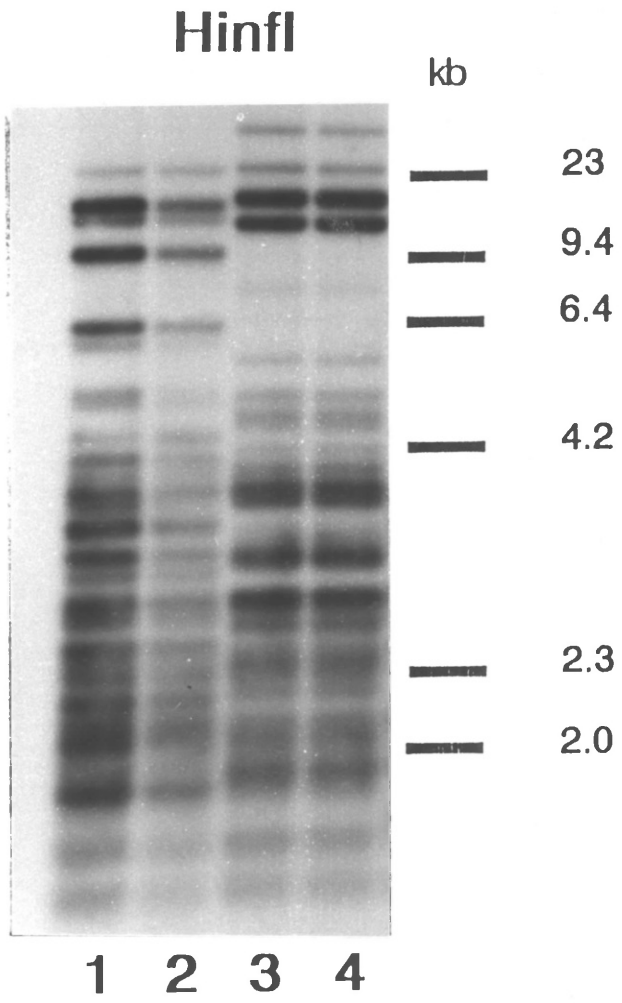


Fig. 5.3 DNA Fingerprinting Demonstrating Relapse after BMT. Relapse was demonstrated when the band patterns post-BMT were identical to the pre-BMT recipient in case the DNA from donor was not available. Lane 1 is the DNA from patient no. 522.16 pre-transplant; lane 2 is post-transplant in relapse; lane 3 is from patient 522.17 pre-transplant; lane 4 is post-transplant in relapse.

Fig. 5.4 DNA Fingerprinting Demonstrating Mixed Chimerism of T-Cells in Relapse after BMT.

Band patterns are shown from two different patients (patient no. 522.6, lanes 1 to 3; and patient no. 522.15, lane 4 and 5) who relapsed after BMT. DNA prepared from T cells of patient 522.6 (lane 2) shows basically band patterns of donor with some extra bands (arrows) not present in donor DNA (lane 1) or after apparently successful subsequent second transplant (lane 3).

For patient 522.15 band patterns from donor (lane 4) and recipient in relapse after BMT (lane 5) are clearly different. DNA digests were hybridized with probe 33.15.

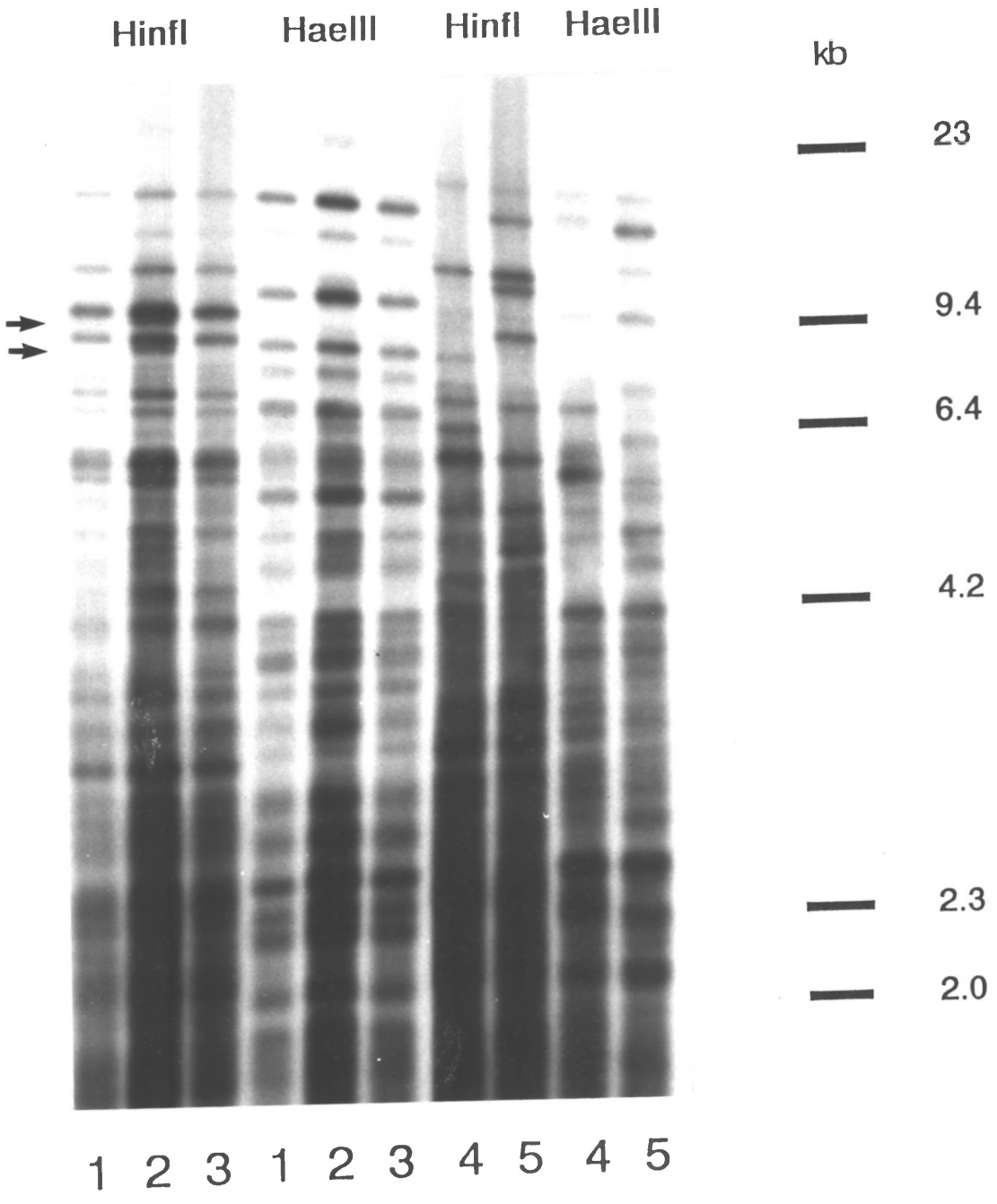


Fig. 5.4

Fig. 5.5 DNA Fingerprinting Demonstrating sequentially Engraftment, Mixed Chimerism and Relapse after BMT. Relapse after BMT showing reappearance of recipient type DNA band pattern. The 23kb band (arrow) in the DNA from the recipient (lane 1) has disappeared at 7 months post-transplant (lane 3) but has reappeared at 13 months (lane 4). The band patterns at 7 months post-transplant are identical to those of donor DNA (lane 2) but study at 13 months showed a mixture band patterns, the former predominating. The patient was no. 522.2 and DNA digests was hybridized with probe 33.15.

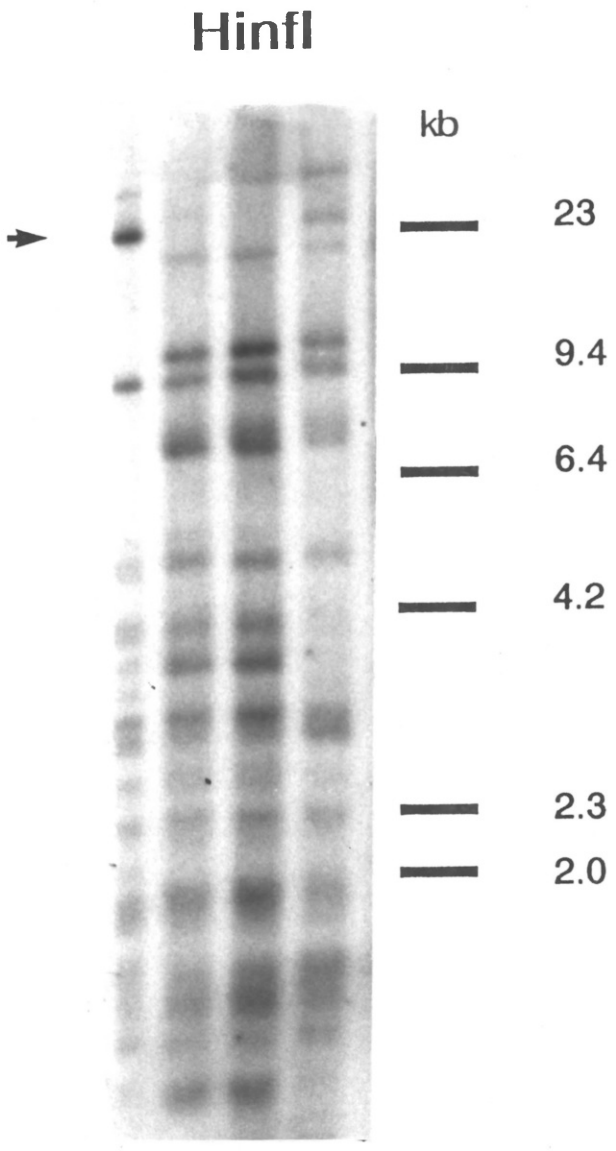


Fig. 5.5

In another patient (no. 522.6) who relapsed after transplantation with T-cell depleted donor marrow, DNA fingerprinting study of DNA from different peripheral blood leucocyte fractions showed that the T-lymphocytes shared patterns of both donor and recipient origin (Fig 5.4). The band patterns post-BMT in relapse were predominantly of donor type, but some other bands not seen in donor DNA were observed as well. These new bands were relatively weak in intensity. The result suggested a 'mixed chimerism' of the T-cell populations in this post-transplant relapsed patient with donor type cells predominant.

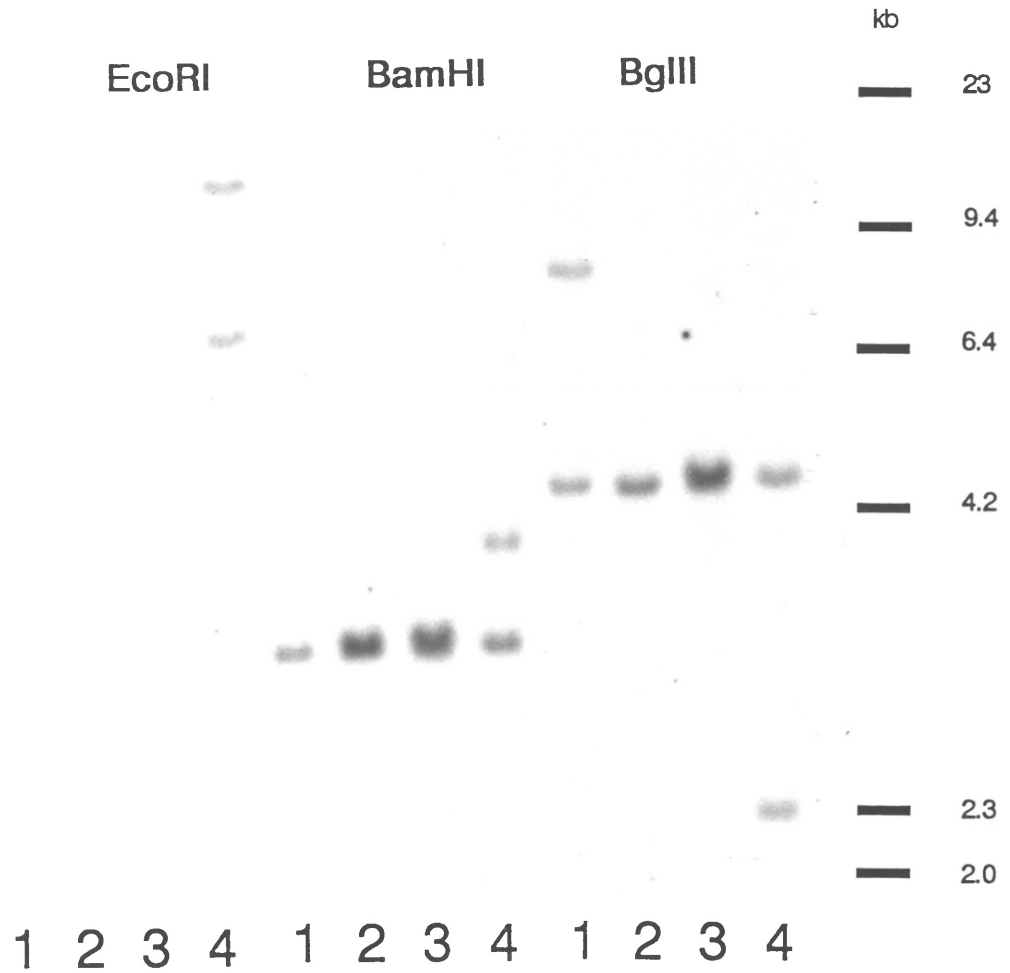
5.2.4 M-BCR REARRANGEMENT AS A MARKER FOR LEUKAEMIC RELAPSE IN CML

Rearrangement of genomic DNA within M-BCR is characteristic of cells from patients with Ph-positive CML (see chapter 2). To confirm that the recipient type of DNA was from the leukaemic cell population, I used the M-BCR 3' genomic probe (probe F, chapter 3) to study 5 patients at a time when they had no cytogenetic or clinical evidence of relapse after bone marrow transplantation. The rearranged band noted before transplant was not detected (Fig. 5.6a). One of these patients (no. 522.7) was studied again 18 months post-BMT and then found to have M-BCR rearrangement. In this patient relapse was also confirmed by cytogenetic studies and haematology as well

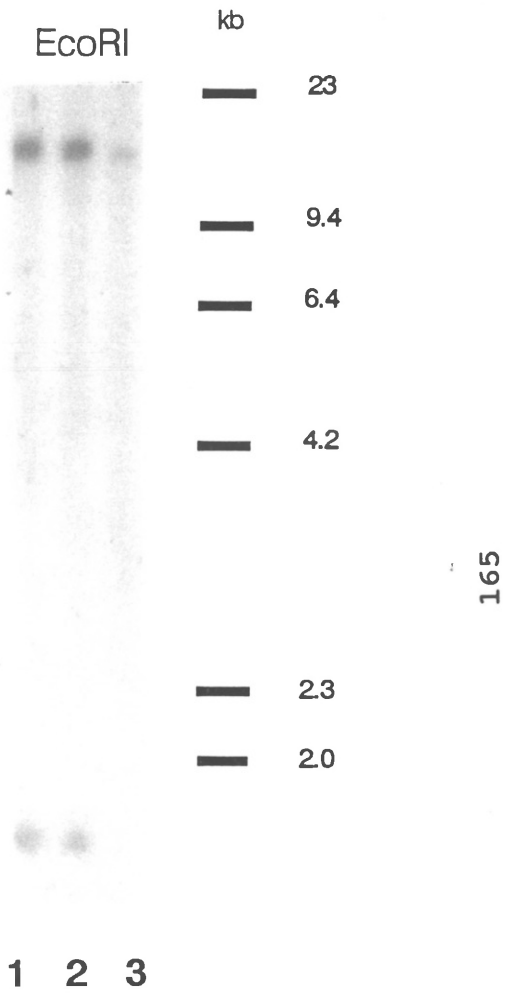
Fig. 5.6 Study of M-BCR Rearrangement in Patients Transplanted for CML.

(a). DNA from patient no. 522.6 digested with EcoRI, BamHI and BglII and hybridized with M-BCR probe F. DNA band patterns are shown from recipient before transplant (lane 1), from recipient 1 month post-transplant (lane 2), donor (lane 3) and a Ph-positive patient as control (lane 4). The rearranged bands at 8kb (BamHI) and 7kb (BglII) have disappeared after transplant. Lane 2 contains degraded DNA.

(b). DNA from patient 522.11 digested with EcoRI and hybridized with probe F. The rearranged band at 1.5kb present before transplant (lane 1) is visible in the same position at relapse 12 months post-transplant (lane 2). DNA from the donor was run in lane 3.


EcoRI
BamHI
BglII
kb
23
9.4
6.4
4.2
2.3
2.0
1 2 3 4 1 2 3 4 1 2 3 4

(a)


EcoRI
kb
23
9.4
6.4
4.2
2.3
2.0
1 2 3

(b)

Fig. 5.6

as by DNA fingerprinting at that time. Eight other relapsed patients were found to have M-BCR rearrangement. In all these patients the rearranged band reappeared in the same position as the corresponding one before transplant (Fig. 5.6b). These findings confirm that in these cases the DNA with fingerprinting patterns of recipient origin were derived from cells with M-BCR rearrangement.

5.2.5 AUTOLOGOUS RECOVERY AND MIXED CHIMERISM AFTER BMT FOR SAA

In a series of 51 consecutive patients with severe aplastic anaemia (SAA) transplanted with HLA identical sibling donors at the Hammersmith Hospital (Hows et al, 1987), 13 (33% of the 40 evaluable patients) developed chronic GVHD. In ten cases GVHD was transient and required no treatment; none of the long-term survivors had active disease. This study was designed to evaluate the incidence of either previously undetected autologous recovery or mixed haemopoietic chimerism and its possible relation to the absence of ongoing chronic GVHD. DNA fingerprinting was used to study 21 of these 51 patients.

The 21 patients were selected on the basis of blood sample availability from 35 designated as long-term survivors following BMT for SAA (between 1979 and 1985). The pretransplant conditioning and the GVHD prophylaxis regimens

were described previously (Hows et al, 1982). GVHD was diagnosed clinically and verified histologically where possible according to previously published criteria (Glucksberg et al, 1974). The patients were well, had normal haematological parameters and had been free from blood product support for at least 1 year at the time of sample collection.

Seventeen of 21 patients studied were shown to have 'complete' engraftment of donor elements. Three patients were shown to have autologous recovery and 1 had mixed chimerism by DNA fingerprinting. Two of the three patients shown to have autologous recovery by DNA fingerprinting 7 years post-BMT had had various degrees of acute GVHD. In the patient whose DNA fingerprint showed mixed chimerism 4 years post-BMT no significant acute or chronic GVHD was observed. The patient has subsequently been clinically well with normal haematological parameters.

5.3 DISCUSSION

5.3.1 COMPARISON OF DNA FINGERPRINTING WITH OTHER IDENTIFICATION METHODS FOR CELL PATERNITY

Although I made no direct comparison of DNA fingerprinting with other methods for detecting the origins of cells or DNA, I showed that the technique could be used successfully in

situations where other methods cannot be adequately applied. It was simpler than some other RFLP studies. As discussed above, RBC phenotyping, isoenzyme analysis and immunoglobulin allotyping cannot easily be applied to patients who are multiply transfused prior to referral for BMT because of the long life span of RBC and of some lymphocytes. While DNA technology uses nucleated blood cells, most of these are granulocytes with a short life span. This means that DNA fingerprinting will not easily be vitiated by transfusion.

In this study the results of cytogenetic analysis did not always parallel the DNA analyses. While cytogenetic analysis examines the proliferating marrow cells, DNA analysis examines the total nucleated cells in the peripheral blood. Because the more mature CML progenitor cells proliferate more actively (Strife & Clarkson, 1988), they are more likely to be detected in cytogenetic analysis. However they may not truly represent the extent of donor cells and CML cells haemopoiesis after BMT. It seems that DNA analysis may more accurately reflect the extent of mixed chimerism after BMT. DNA technology can be applied not only to the analysis of total cell populations in blood or in marrow but also to the analysis of different fractions of blood cells, such as determining the origin of T-cells after BMT. However, the polymerase chain reaction (PCR) is currently the most sensitive method for identifying small numbers of residual leukaemic cells after BMT for CML

(Hughes and Goldman, 1990).

5.3.2 ENGRAFTMENT AND RELAPSE AFTER BMT FOR CML

Using minisatellite probes I was able to document the existence of cells of donor origin as early as 3 weeks after BMT. Thereafter the band pattern became entirely consistent with DNA of donor origin in the majority of patients not destined to relapse. These findings are very similar to those reported in previous studies using other DNA RFLPs (Ginsburg et al, 1985). In this group of patients the recurrence of mixed chimerism, represented by the coexistence of band patterns of donor and recipient origin, was comparatively common and usually heralded or coincided with leukaemic relapse. The high frequency of relapse in this cohort of patients seemed to be associated with the use of T-cell depletion of donor marrow in an attempt to abrogate GVHD (Apperley et al, 1986a; also see Chapter 4). It must be emphasized, however, that mixed chimerism without leukaemic relapse has been documented in other patients studied by cytogenetic methods, both by us (see Chapter 4) and by others (Vincent et al, 1986).

In the study of 9 cases of established CML relapse (cytogenetic and clinical) and 1 case of ALL relapse DNA fingerprinting showed that peripheral blood cells were completely of recipient origin in 7 cases. In 3 cases I could

not rule out the possibility of mixed chimerism because DNA from recipient pre-transplant was not available (see below). The relapses were confirmed by the use of M-BCR probe for BCR/ABL rearrangement in 8 cases. Four relapsed patients from our hospital were studied with Jeffreys' probes and M-BCR probe in collaboration with Dr T. Ganesan. The result is in line with our study. In addition, a Y chromosome specific probe was used in two male patients who had a female donor to confirm the DNA fingerprinting (Ganesan et al, 1987). My results provided no evidence for relapse in cells of donor origin in the patients. In overt relapse, most, if not all, of the patients had peripheral blood cells of recipient origin. The donor cells were not detectable in peripheral blood samples with DNA fingerprinting with a reasonable sensitivity. However, in one patient who received donor marrow cells depleted of T-cells in vitro DNA from a highly enriched T-cell fraction showed T-lymphocytes in the peripheral blood at relapse which were mixed-chimeric with donor type T-cells predominating. Hughes et al (1989) had studied the origin of the lymphoid population after relapse in 4 CML patients with haematological relapse by cytogenetics. They showed all 4 had almost exclusively donor-type T and B cells in the peripheral blood but recipient-type myeloid metaphases in the marrow.

After successful BMT DNA probed with a M-BCR probe (see Chapter 3 and Chapter 6) typically shows only a germline

configuration. Rearrangement of M-BCR is thus a highly specific indicator of the presence or absence of leukaemic cells in patients with CML. By use of this probe I was able to show in 8 patients temporal concordance of DNA of recipient origin recognized with minisatellite probes and the reappearance of a rearranged pattern within the BCR gene. The pattern of M-BCR rearrangement differed between patients but was identical before transplant and in relapse in individual patients. This result confirms the result reported in one patient by Zalcberg et al (1986). These findings imply that relapse after transplantation is due to re-emergence of the original leukaemic clone and not to reintroduction of a new leukaemia into haemopoietic stem cells.

When DNA from the recipient before BMT is not available, a result showing that the band patterns in relapse post-BMT differ from those of the donor must be interpreted with caution. If the BMT donor is a close relation of the patient, the chance of donor and recipient sharing bands will be 50%. In this situation it may be difficult to distinguish mixed chimerism from complete host haemopoietic regeneration or leukaemic relapse.

5.3.3 AUTOLOGOUS RECOVERY AND MIXED CHIMERISM IN LATE GRAFT FAILURE AFTER BMT FOR SAA

Twenty-one post-BMT SAA patients who were alive and well were

selected for study of long-term graft status. One was shown to be mixed chimeric and 3 had complete autologous recovery. 17 had complete engraftment. The Seattle group studied a cohort of sex-mismatched HLA identical sibling pairs transplanted for SAA using sequential cytogenetic analyses and reported that 58% of the patients became transient mixed chimerism (Hill et al, 1986). Most developed complete chimerism (100% donor metaphases) at a median of 54 days post BMT (range 17-395 days) and mixed chimerism could not be detected beyond 395 days. Acute GVHD (grade 2-4) occurred less frequently in patients who developed mixed chimerism ($p=0.03$) but there was no difference in the incidence of chronic GVHD or survival. Only sex mismatched pairs were evaluated and the analysis was restricted to cells capable of mitosis (usually T-lymphocytes in peripheral blood). Although these data cannot be compared directly with ours because we studied long-term survivors while they studied mainly recently transplanted patients, mixed lympho-haemopoietic chimerism (or 'occult' autologous recovery) does not appear to be a major factor in the observed low incidence of chronic GVHD in our group of patients. The finding of autologous recovery in a minority of patients after late graft failure in patients treated with cyclosporin and the occurrence of stable mixed chimerism are interesting observations which may contribute to our understanding of the immunobiology of aplastic anaemia and bone marrow transplantation.

CHAPTER SIX BCR/ABL REARRANGEMENT IN CML

6.1 INTRODUCTION

The genomic organization of the ABL and BCR genes has been outlined in Chapter 2. Although the location of the breakpoint in the ABL gene can occur over a large area, it is almost always located 5' to the ABL exon II and the Ia or Ib exons are spliced out during the formation of BCR/ABL mRNA, so that the fusion gene product consists of ABL exon II and the following 3' exons encoding kinase domain (Fig. 2.3). Since a variety of breakpoints in the ABL gene are observed in clinically homogeneous CML patients it seems that this variability of the location of the ABL breakpoint does not have biological significance.

M-BCR rearrangement has been used as an index of formation of the BCR/ABL chimeric gene (see Chapter 2). However, the location of the breakpoint on chromosome 22 can be distributed over a much larger area outside M-BCR than was originally thought (Bartram et al, 1987b; Saglio et al, 1988; Selleri et al, 1990; also see Fig. 2.4; 2.6). In contrast to variation in position of the breakpoint in the ABL gene, the variation

in the BCR gene occurs in a fashion that may result in the inclusion or exclusion of internal exon(s) in the formation of chimeric gene. It is interesting to study whether the variation of breakpoint on chromosome 22 has biological significance.

There are two major types of BCR/ABL rearrangements resulted from different breakpoints on chromosome 22. While typical CML patients almost always have an M-BCR/ABL rearrangement which produces a chimeric gene product P210, over 50% of Ph-positive acute leukaemia patients have a Ph chromosome resulting from a different BCR/ABL rearrangement in which M-BCR is not involved. These Ph-positive M-BCR non-rearranged acute leukaemias (including ALL, AML and undifferentiated acute leukaemia) have breakpoints in the first intron of the BCR gene, the so called 'minor breakpoint cluster region' (m-BCR) (see Chapter 2). This rearrangement leads to production of a substantially smaller chimeric protein (P190), because a number of the internal exons of the BCR gene are excluded, only exon 1 being retained (Fig. 2.6). These observations suggest that there is a relationship between the types of BCR/ABL rearrangement and the biological features of leukaemia. However, the BCR gene first exon breakpoint can be detected in rare examples of Ph-positive M-BCR non-rearranged CML patients (Selleri et al, 1990).

Through study of M-BCR rearrangement, CML can be classified as M-BCR rearranged or M-BCR non-rearranged. There are cases of Ph-positive CML in which the chromosome 22 breakpoint can be mapped neither in M-BCR nor in m-BCR, but must be located elsewhere inside or outside the BCR gene (Bartram et al, 1987b; Selleri et al, 1987, 1990; Saglio et al, 1988). Since CML can present typical or atypical features (Chapter 2), one would be tempted to ask, do these deviations of breakpoint from M-BCR correlate with any phenotypic alteration?

Furthermore, some Ph-negative CML patients present atypical features while others are indistinguishable from the Ph-positive CML (Chapter 4). About half Ph-negative CML are M-BCR rearranged and the other half are non-rearranged. One may ask whether the variations of BCR/ABL rearrangement contribute to the heterogeneity in these CML patients who seem not to have a Ph translocation ?

Among patients who have a chromosome 22 breakpoint within the M-BCR, its precise location within the M-BCR is still heterogeneous. Some authors have used a central HindIII restriction site to divide the M-BCR into 3' and 5' regions and suggested that the location of breakpoints within 3' portion of the M-BCR is associated with shorter chronic phase and shorter survival than those with 5' breakpoints (Schaefer-Rego et al, 1987; Eisenberg et al, 1988; Mills et al, 1988;

also see Chapter 2). Other studies have not confirmed this observation (Przepiorka, 1988; Jaubert et al, 1990).

The physical variations in the position of chromosome 22 breakpoints and their significance in terms of clinical and haematological features will be discussed in the following chapters. In this chapter I begin with Southern analysis for M-BCR rearrangement in Ph-positive and Ph-negative CML as well as other Ph-positive acute leukaemia. The possible secondary changes of M-BCR rearrangement during blast transformation (Bartram et al, 1986b; Shtalrid et al, 1988) is also referred to in this chapter.

6.2 RESULTS

6.2.1 Ph-POSITIVE CML

Of the 33 patients with Ph-positive CML, 30 showed evidence of breakpoints located within the M-BCR (Table 6.1). This was demonstrated by digestion of DNA from these patients with restriction enzymes EcoRI, BglII, BamHI and hybridization to a 3' genomic M-BCR probe F (Fig. 3.3). Twenty of the 30 patients had rearranged bands detected by all 3 enzymes (Fig. 6.1). Seven patients showed no rearranged band in BamHI digests suggesting that the breakpoints were located 5' to the BamHI site in the M-BCR (probably between M-BCR exons 2 and

Table 6.1. Results of Southern analysis in 52 patients with Ph-positive and negative CML using a 0.6kb 3' genomic M-BCR probe (probe F).

Number of patients	Rearrangements with		
-	BglII	BamHI	EcoRI
-Ph-positive CML (33 cases):			
20	+	+	+
7	+	-	+
3	-	+	+
3	-	-	-
Ph-negative CML (19 cases):			
7	+	+	+
1	+	+	-
1	-	+?	-
1	-	-	+
9	-	-	-

+ indicates that DNA digested with given enzyme showed rearranged bands.

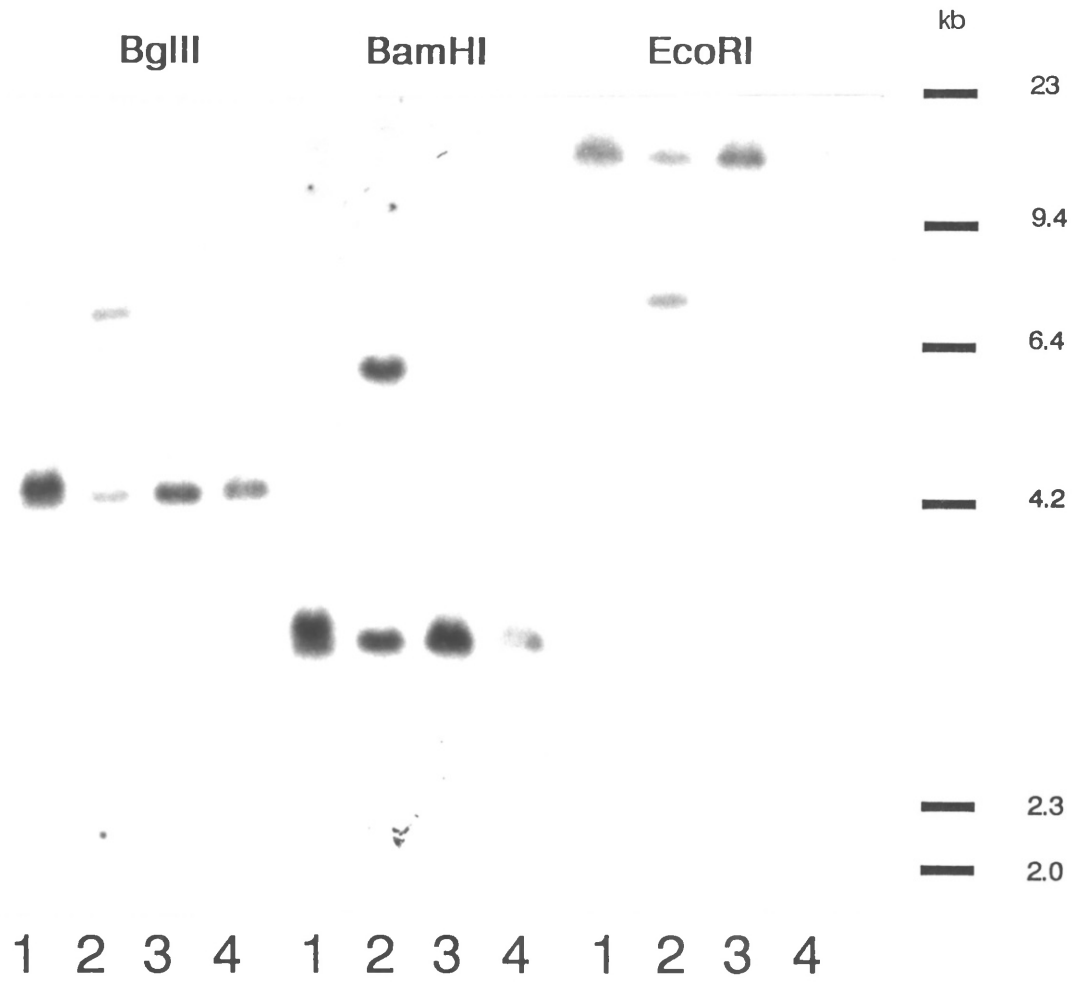


Fig. 6.1 Representative Southern Blots from DNA of Patients with CML.

Lane 1 contains DNA from normal control; lane 2 shows the typical M-BCR rearrangement found in majority of cases of CML; lane 3 shows DNA from a patient with Ph-positive CML without M-BCR rearrangement; lane 4 contains DNA from a patient with Ph-negative CML without M-BCR rearrangement. The DNA membrane was hybridized to probe F (see Fig. 3.3).

3). Three patients without rearranged bands in the BglII digests showed rearrangement in BamHI and EcoRI digests which would be compatible with breakpoints located 3' of the BglII site in the middle of the M-BCR. However, this is unlikely because the distance between BglII site and exon b4 is small (Fig. 2.1) and the breaks must be 5' of exon b4. A b4a2 linkage would produce an out of frame combination. Alternatively it is possible that aberrant BglII bands co-migrated with germ-line bands.

In 3 patients (nos. 621.1, 621.2 and 621.3) I failed to detect the M-BCR rearrangement in DNA digested with any of the three enzymes (Fig. 6.1). The absence of M-BCR rearrangement was confirmed by use of probes A, E (Fig. 3.3; also see Chapter 3) and a 4.8kb M-BCR full length genomic probe phl/bcr 3 (Oncogene Science Inc., Manhasset, NY, USA) in 1 patient (no. 621.1). The hybridization of DNA digests to probe phl/bcr 3 were carried out by Dr Leanne Wiedemann. In another patient (no. 621.2), however, faint abnormal bands were observed in BglII digests when probed with probe E (Fig. 6.2). There was also the possibility of another faint band very close to the germ line band in EcoRI digests. These were also observed using the phl/bcr 3 probe in this patient. Similar findings were observed in patient no. 621.3. Thus the difficulty in detecting M-BCR rearrangement in the latter two patients might have been due to deletion of a portion of the M-BCR sequence

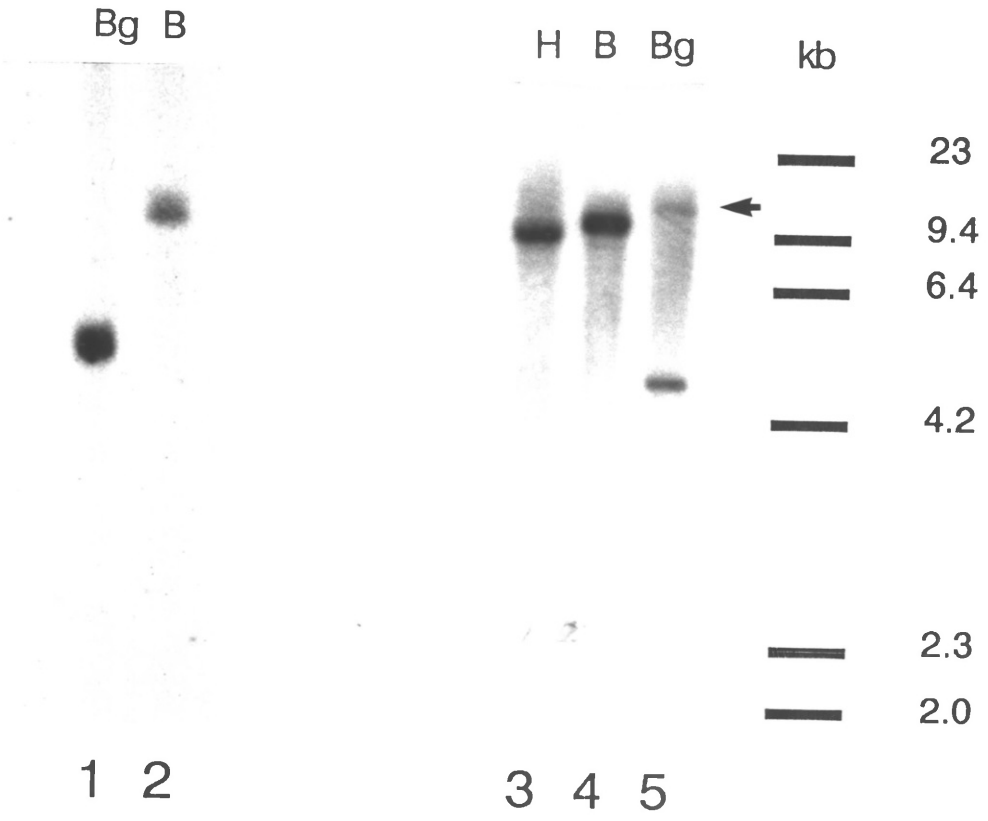


Fig. 6.2 Further Classification of M-BCR Rearrangement Using Probe E.

DNA digests from patient no. 621.1 (lane 1 and 2) and no. 621.2 (lane 3, 4 and 5) were hybridized to probe E. A faint rearranged band (arrow) were observed in the BglII digests of the DNA from patient no 621.2 (lane 5). The other lanes show germ line configuration only. B = BamHI; Bg = BglII, H = HindIII.

detected by the probe first used (Hirosawa et al, 1988). As the faint intensity of these bands made interpretation difficult, I decided to analyze these cases further by PFGE (see Chapter 8).

Four patients who were studied in chronic phase and then entered blast transformation were monitored for M-BCR rearrangement. One patient (no. 421.1) had an additional Ph chromosome during blast transformation (see Chapter 4). In all 4 patients the M-BCR rearranged bands during BT were in precisely the same positions as during their chronic phase. There were no change of band patterns at all (Fig. 6.3).

I have observed the clinical and haematological features of these 33 patients. The observations include duration of survival, response to chemotherapy, size of the spleen and haematological parameters. The clinical and haematological criteria for typical and atypical CML have been discussed in chapter 2.

The majority of these patients had typical CML; occasional cases presented one or more atypical features but no patient had the full picture of atypical CML. Patient no. 621.2, in whom a M-BCR rearranged band of faint intensity was detected with probes E and phl/bcr 3, had a somewhat atypical clinical picture with a marked thrombocytosis refractory to treatment

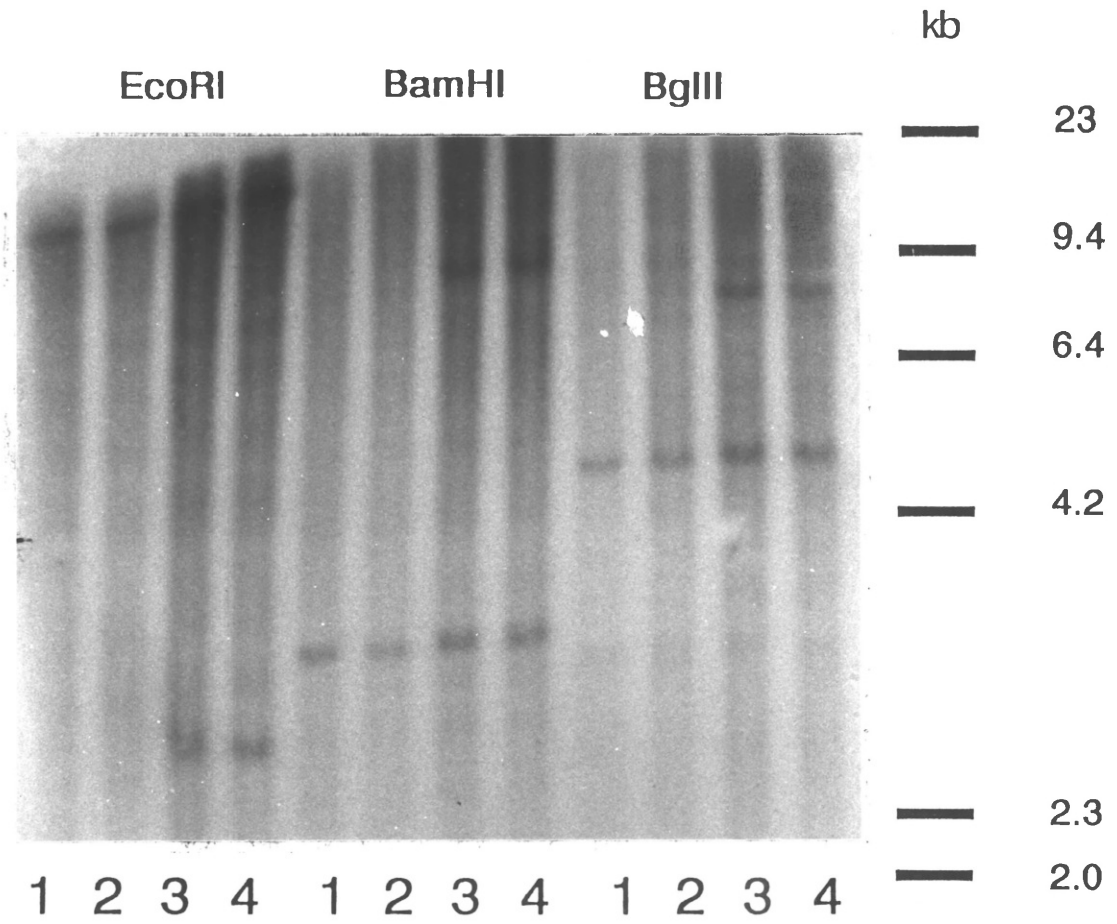


Fig. 6.3 Comparison of the Patterns of M-BCR Rearrangement in CP and BT.

DNA digests from patient no. 421.1 in chronic phase (lane 3) and in blastic transformation (lane 4) were digested with BglII, BamHI and EcoRI and hybridized with probe F. There is no difference in the patterns of M-BCR rearrangement in DNA from CP to BT. Lanes 1 and 2 are normal controls.

and no splenomegaly. However, the other patient (no. 621.3) with similar molecular features and the CML patient (no. 621.1), who was Ph-positive but M-BCR non-rearranged, had clinical pictures indistinguishable from typical CML (Table 6.2).

Most patients in this group received bone marrow transplants so a direct comparison of survival between the patients with 5' and 3' M-BCR breakpoint was inappropriate. However, some of the patients in this group who had not been transplanted, together with other patients who had not been transplanted and had cryopreserved blood cells available for DNA analysis, were studied further with restriction enzymes HindIII to determine the relationship of 3' or 5' breakpoints in M-BCR to the prognosis of the patients (Jaubert et al, 1990). I will discuss the results of this study below.

6.2.2 Ph-NEGATIVE CML

I studied the DNA from leukaemic cells from 19 patients with CML who lacked the Ph-chromosome. The cytogenetic findings in these 19 patients have been presented in Chapter 4. The results of the Southern analyses are summarized in Table 6.1. In 7 patients (no. 422.1 and nos. 422.3 to 422.8) I found evidence for a typical M-BCR rearrangement with rearranged bands in all three enzyme digests (Fig. 6.4). In another patient (no. 422.9) BamHI and BglII digests revealed

Table 6.2 Clinical and haematological features of three selected Ph-positive CML patients.

Patient no.	Age/ sex	Karyotype	Duration survival	At presentation								
			(months)	Spleen (cm)	Hb (g/dl)	WBC ($x10^9/l$)	Blast (%)	Baso (%)	Eos (%)	Neut (%)	Plts ($x10^9/l$)	Mono (%)
Ph-positive, M-BCR non-rearranged:												
621.1	37/F	t(9;22)	28+	8	8.8	328	1	5	1	60	292	8
Ph-positive, M-BCR equivocal:												
621.2	67/M	t(9;22)	26+	0	9.4	19.8	0	28	2	53	2900	0
621.3	61/F	t(9;22)	36+	12	8.3	507	8	2	0	34	417	1

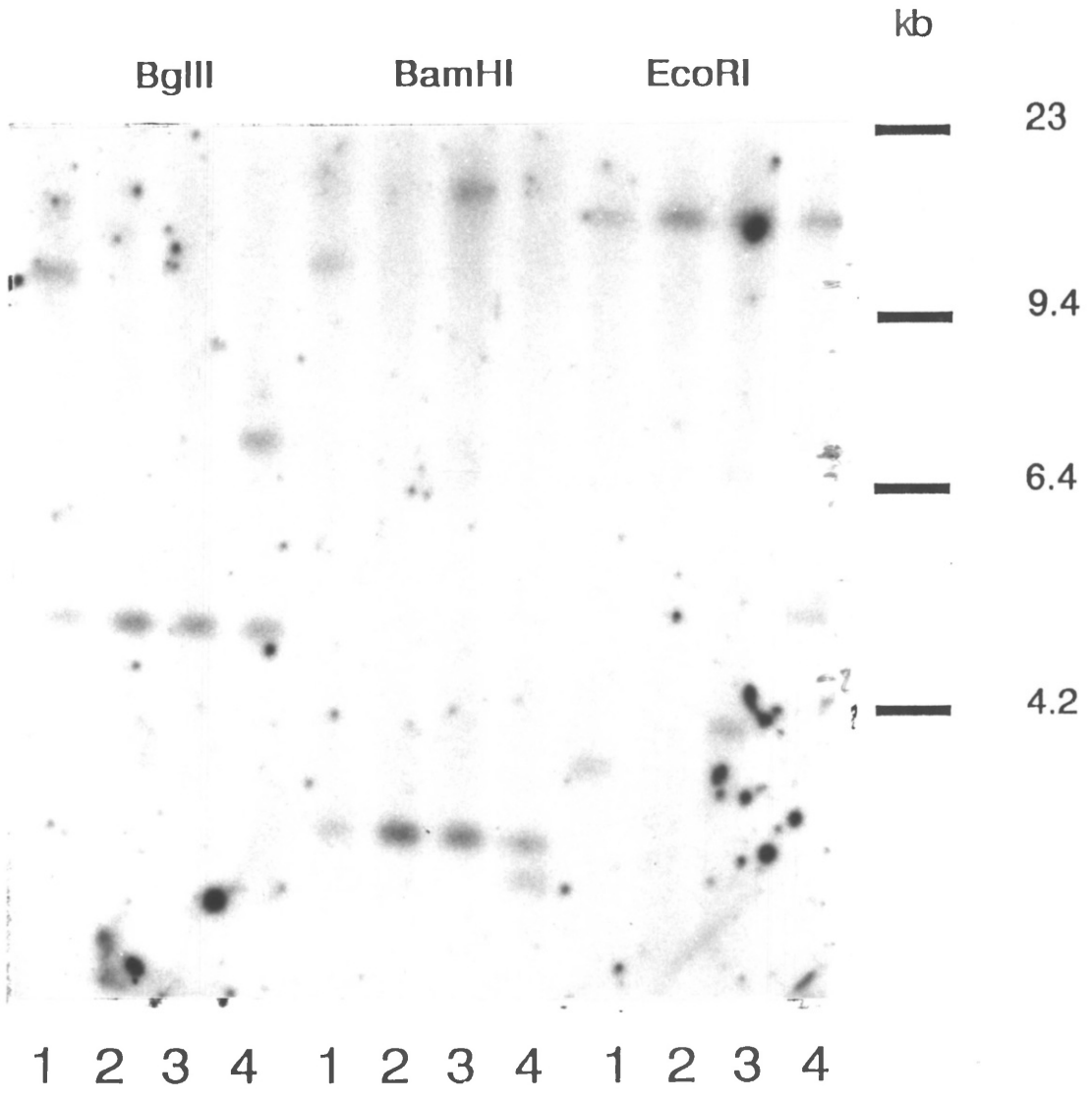


Fig. 6.4 Southern Blot Showing M-BCR Rearrangement in DNA from Ph-negative CML Patients.

DNA digests from patients nos. 422.1; 422.3 and 422.7 (lanes 1; 3 and 4 respectively) were digested with BglII, BamHI and EcoRI and hybridized with probe F. Lane 2 contains DNA from normal control. M-BCR rearrangement are observed in lanes 1; 3 and 4.

rearrangements but EcoRI did not. One explanation for this is that a rearranged fragment was present also in EcoRI digests but that it co-migrated with the germline fragment. In 9 cases (from nos. 422.10 to 422.18) DNA digested with EcoRI, BamHI and BglII and hybridized to probe F showed no M-BCR rearrangement (Fig. 6.5). In one patient (no. 422.2) an abnormal band could only be detected in EcoRI digests; this may have been due to a breakpoint 5' to M-BCR (Fig. 6.6). In the last patient (no. 422.19) abnormal bands could only be detected in BamHI digests at positions 2.2kb and 1.6kb (Fig. 6.5); this could be due to a breakpoint located between the EcoRI and BamHI sites in the 3' portion of the M-BCR (between M-BCR exons 4 and 5), but the b4a2 linkage would constitute an 'out of frame' combination. In fact, adding the sizes of these two bands gave a value equivalent to the size of the germ line fragment; these data are consistent with the presence of a BamHI site polymorphism in one of the alleles of M-BCR in this patient (Benn et al, 1988).

Probe E, a 2kb 5' M-BCR genomic probe that can detect M-BCR rearrangement even when there is a small deletion at the 3' portion of M-BCR, was used to hybridize the DNA from those patients with Ph-negative, M-BCR non-rearranged CML in order to confirm the absence of M-BCR rearrangement. No additional M-BCR rearranged patients were detected with this probe.

Fig. 6.5 Southern Blot Showing that Some Ph-negative Patients Lack M-BCR Rearrangement.

DNA from patients nos. 422.17; 422.19; 422.18 and 422.16 (lanes 1; 2; 3 and 4 respectively) were digested with BglII, BamHI and EcoRI and hybridized with probe F. There is no M-BCR rearrangement in lanes 1, 3 and 4. However, lane 2 shows abnormal bands at 2.2 and 1.6kb in the BamHI digest.

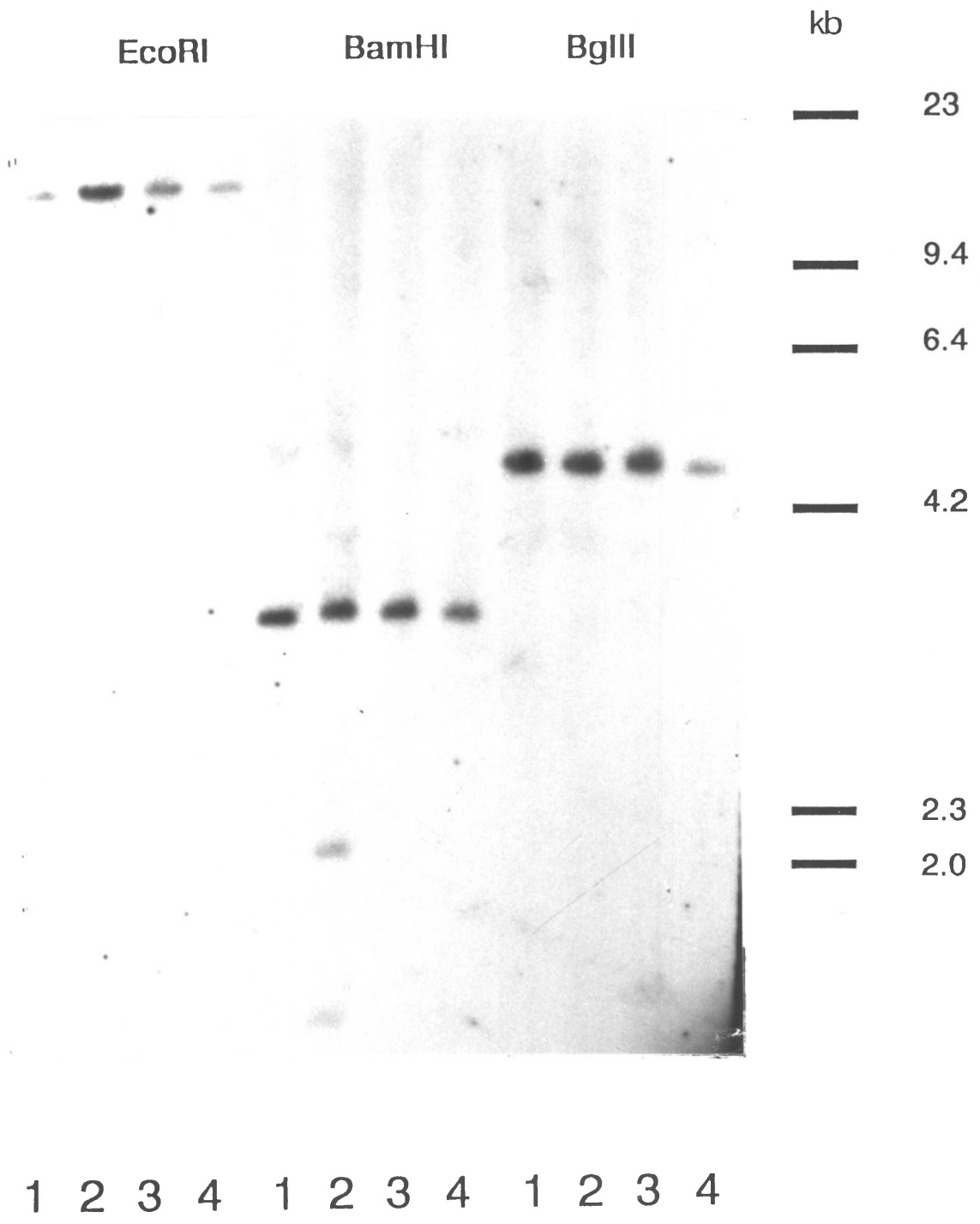


Fig. 6.5

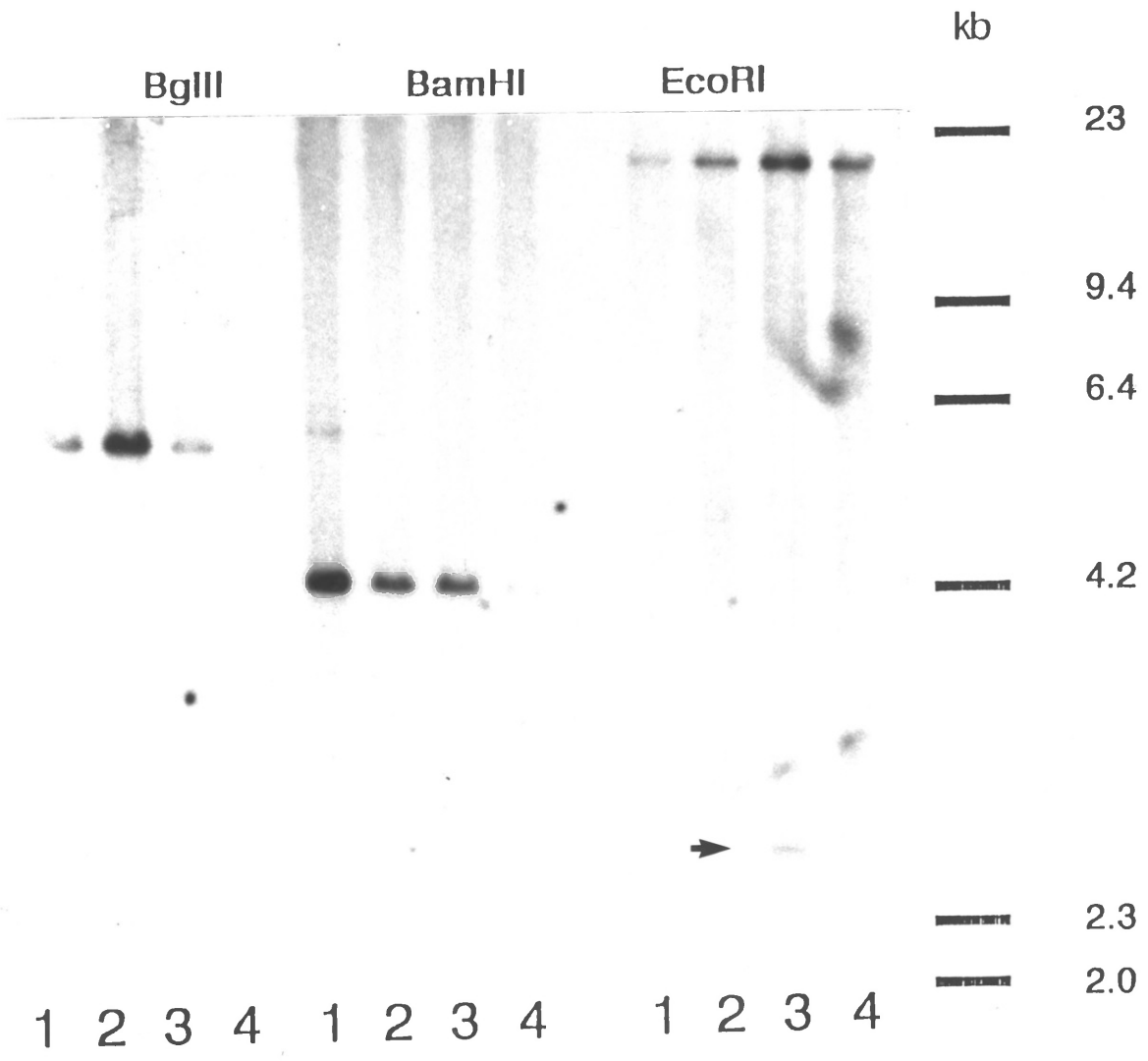


Fig. 6.6 Southern Blot Showing a Breakpoint 5' to M-BCR in One Patient with Ph-negative CML.

DNA digests from patients nos. 422.10; 422.2; 422.11 and 422.12 (lanes 1; 2; 3 and 4 respectively) were digested with BglII, BamHI and EcoRI and hybridized with probe F. There is no M-BCR rearrangement in lanes 1; 2 and 4. However, lane 3 shows one abnormal band at 3kb in the EcoRI digests (arrow).

I then assessed the clinical and haematological features of these Ph-negative CML patients (Table 4.1). Among these 19 patients 8 (nos. 422.1 and 422.3 to 422.9) were indistinguishable from typical CML while other 9 evaluable patients had various atypical features. All the 8 clinically typical but Ph-negative CML had M-BCR rearrangement. In the patients in whom I was unable to demonstrate M-BCR rearrangement by three enzymes atypical features were observed. Median survival in CML is generally 36-45 months (Monfardini et al, 1973; Canellos, 1976). In my Ph-negative M-BCR non-rearranged CML group one patient who received BMT has a duration of survival beyond 118 months while 3 others died within 15 months after presentation. Absence of splenomegaly was observed in 5 out of 9 evaluable patients in the latter group. The absolute basophil count in the latter group was generally below $0.14 \times 10^9/l$ (Shepherd et al, 1987). The ratios of percentage of granulocytes to monocytes were very low (between 2 to 52, mean 19). In the patient (no. 422.10) in whom abnormal bands were observed only in BamHI digests atypical features were also present. In this study I did not classify the Ph-negative, M-BCR non-rearranged CML further into CMML, aCML and MDS.

In a further patient (no. 422.2) in whom EcoRI digests suggested a breakpoint 5' outside M-BCR chronic phase and survival were shorter than average and there was no

splenomegaly (Table 4.1). Interestingly this patient presented an unusual syndrome of eosinophilia in the peripheral blood and cardiac complications, which resembled eosinophilic leukaemia (Chapter 4).

6.2.3 M-BCR REARRANGEMENT IN ACUTE LEUKAEMIA

5 patients with acute leukaemia were studied for M-BCR rearrangement. One patient had Ph-positive acute leukaemia with mixed monocytic and B cell morphology as well as mixed lymphoid and myeloid surface markers (Rassool et al, 1988). Two patients had acute lymphoblastic leukaemia without a Ph chromosome. The other 2 patients had acute myeloid leukaemia without a Ph chromosome. The 4 patients with acute leukaemia without the Ph chromosome were used as negative controls. In all 5 cases there were no history of CML in chronic phase.

To determine whether M-BCR rearrangement occurred in the leukaemic cells DNA from these patients were analyzed with probe F in EcoRI, BamHI and BglII digests. No M-BCR rearrangement was detected in any of these patients (Fig. 6.7). Further Southern analysis with a full length genomic M-BCR probe phl/bcr 3 on the Ph-positive, M-BCR non-rearranged acute leukaemic patient found no evidence of rearrangement (performed by Dr Leanne Wiedemann). By protein tyrosine kinase (PTK) assay this patient was found to have a P190. Polymerase chain reaction study showed an amplified product resulting

which

Fig. 6.7 Southern Blot of DNA from Acute Leukaemia Patients.

DNA digests from patients no. 623.1 (lane 1); two Ph-positive CML controls (lane 2 and 3) and a patient with AML (lane 4) were digested with BglII, BamHI and EcoRI and hybridized with probe F. There is no M-BCR rearrangement in the two acute leukaemia patients (lanes 1 and 4).

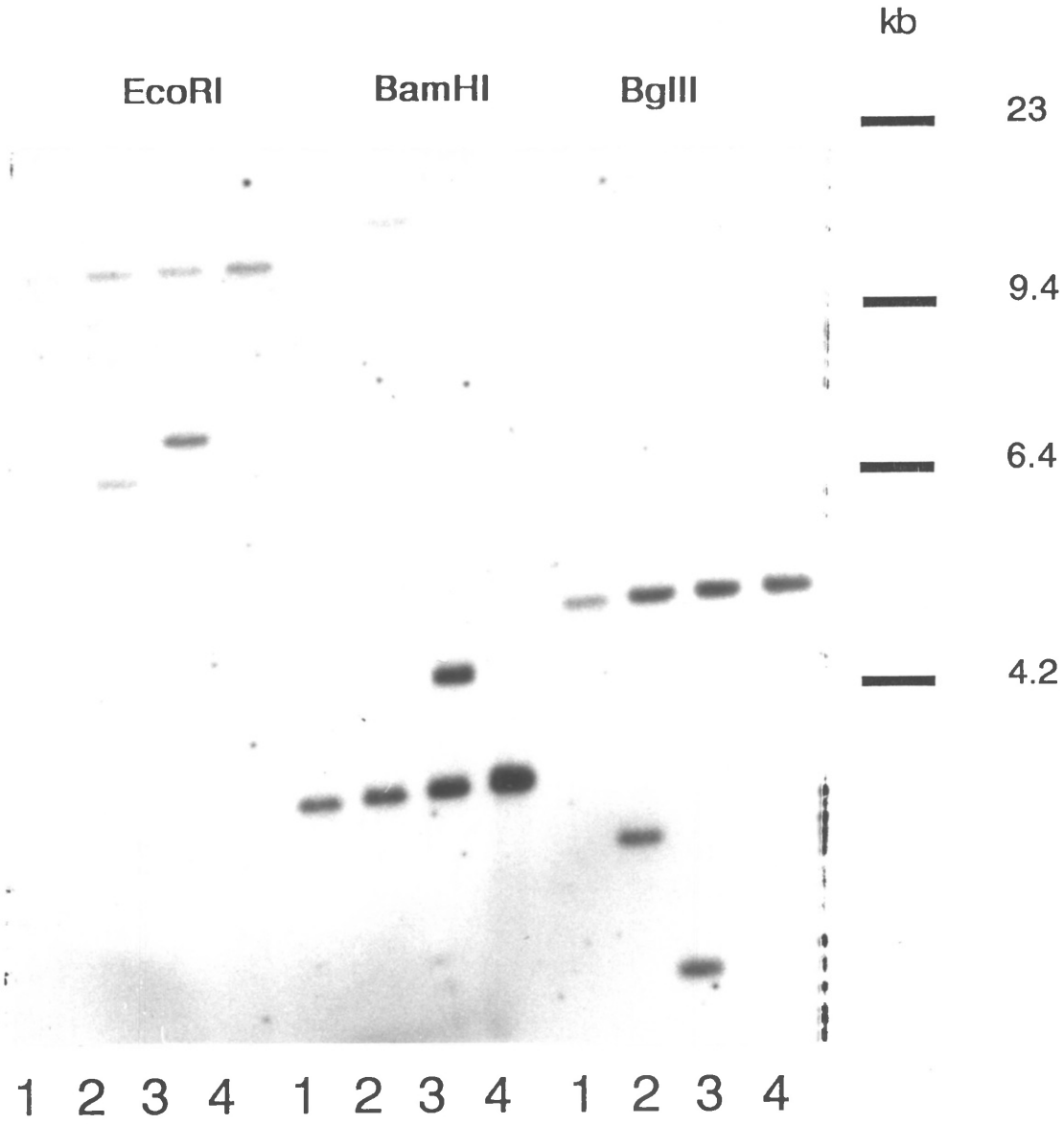


Fig. 6.7

from the fusion of the BCR gene first exon and the ABL gene starting from exon II (Wiedemann, personal communication).

6.3 DISCUSSION

6.3.1 THE BREAKPOINT CAN BE OUTSIDE M-BCR IN CML PATIENTS, BUT VARIATION IN ITS LOCATION DOES NOT AFFECT CLINICAL FEATURES OF CML

The results of our study of M-BCR rearrangement in CML using Southern blotting are in general agreement with previous reports (Groffen et al, 1984; Saglio et al, 1988; Wiedemann et al, 1988). The majority of patients with Ph-positive CML have genomic breakpoints within M-BCR but there are rare cases in which the breakpoint cannot be mapped within M-BCR even with use of multiple probes or the rearrangement within M-BCR is doubtful. In our 33 patients with Ph-positive CML 30 had a breakpoint that mapped within M-BCR. With the restriction enzymes EcoRI, BamHI and BglII I could not locate very precisely the locations of the breakpoint, i.e whether the breakpoint was between exons b2 and b3 (5' portion) or b3 and b4 (3' portion) in each patient because none of these enzymes cleave the sequences within the above exons. However, 7 patients in whom studies for the M-BCR rearrangement were negative in BamHI digests (hybridized to probe F) were likely to have breakpoints located between b2 and b3, unless the

rearranged band had co-migrated with the germline band or the rearranged band was so small that it had run off the gel. Leaving aside these possibilities I located the breakpoint in 3 patients in whom the M-BCR rearrangement was negative in BglII digests to between exons b3 and b4. For the other 20 patients in whom rearrangement were shown in all three enzyme digests I did not pursue the precise location of the breakpoint further. The main arguments for defining the precise locations of the breakpoint within the M-BCR are their possible implications for the prognosis of CML patients, but the group of patients that I studied was not suitable for assessment of prognosis because most of them had been transplanted. There were no atypical clinical or haematological features in these 30 patients irrespective of the location of the breakpoint.

Jaubert et al (1990) studied 67 cases of Ph-positive CML for correlation of breakpoints and prognosis. They used cryopreserved blood samples from those patients who were not transplanted and had adequate follow-up for prognosis assessment. Instead of EcoRI they used HindIII together with BamHI and BglII. They used the HindIII site between b3 and b4 as the point to divide M-BCR into 3' and 5' portions. 8 of the patients in my study were included in Jaubert's study. 3 had rearranged band in EcoRI, BamHI and BglII digests; 1 was negative in BglII digests and 4 were negative in BamHI

digests. The HindIII digests indicated that 4 patients had a 3' breakpoint and 4 had a 5' breakpoint. Together with other patients, 38 of 67 had a 5' breakpoint within M-BCR and 28 had a 3' breakpoint within M-BCR. One patient had a breakpoint apparently outside the M-BCR.

In contrast to some other studies Jaubert et al (1990) found no correlation between the positions of breakpoint within the M-BCR and the prognosis of the patients. The study by Przepiorka (1988) also found no evidence of this correlation. In general, the methods used to define the position of the breakpoint in most of the studies were imprecise, especially when using it as an index for the possible involvement of M-BCR exons b3 or b4. Furthermore if the breakpoints were located between the b3 and b4, mRNA with the b2a2 junction can be produced by the alternative splicing mechanism. These facts diminish the significance of the location of breakpoint within the M-BCR.

Among the Ph-positive CML patients one had no M-BCR rearrangement and 2 showed equivocal results. In the first patient Southern analysis indicated the breakpoint of the Ph chromosome was outside the M-BCR, though it could be still within the BCR gene; but it could also be outside the BCR gene. The possible BCR gene involvement in this patient is of interest. Furthermore this patient presented a clinical and

haematological picture indistinguishable from typical CML. In the other 2 patients who showed equivocal M-BCR status one had unusual features including marked thrombocytosis which was resistant to therapy. These 3 patients warranted further study of the locations of their chromosomal breakpoints.

Among the Ph-positive M-BCR rearranged CML patients I chose 4 patients for follow-up study for possible changes of M-BCR rearrangement after the patients entered blast transformation. The results showed no further changes. The majority of the CML patients in BT studied by others showed similar results (Andrews et al, 1987; Hagemeijer, 1987; Mills et al, 1988). Only rare cases of additional rearranged bands in BT have been reported (Bartram et al, 1986a; Shtalrid et al, 1988). In most instances the further molecular changes underlying BT are not related to further M-BCR rearrangement. The mechanisms for BT have recently been studied intensively. Alterations of the P53 gene by point mutations, deletions or rearrangements have been observed in significant number of CML patients in blast transformation. The P53 gene in these patients were usually functional during chronic phase (Ahuja et al, 1989). Another gene that may be responsible for blast transformation is the RAS oncogene. Liu et al (1988) reported greater frequency of RAS mutations in BT than in chronic phase. A third approach is to attempt to link the secondary chromosome changes in BT with possible oncogene or cancer related gene aberrations. A

possible example is the correlation of isochromosome 17q and abnormalities of the P53 gene (Kelman et al, 1989), since i(17q) is one of the common secondary chromosomal changes in BT and P53 gene is located on the short arm of chromosome 17 (Miller et al, 1986; Isobe et al, 1986; also see Chapter 1).

6.3.2 ABSENCE OF M-BCR REARRANGEMENT ASSOCIATED WITH ATYPICAL FEATURES OF Ph-NEGATIVE CML.

Of the 19 patients with Ph-negative CML 8 showed evidence of M-BCR rearrangement and one had evidence of a breakpoint 5' to the M-BCR. Nine patients had DNA that showed only germ-line bands. This is in agreement with other studies. Dreazen et al (1988) suggested that about 50% of the patients with Ph-negative CML have the same molecular changes as Ph-positive patients; the other 50% have no evidence of M-BCR involvement. The patients with Ph-negative M-BCR rearranged CML are clinically and haematologically indistinguishable from other Ph-positive CML patients. In situ hybridization analysis of some of these patients showed that in some cases Ph negativity was due to interstitial insertion of chromosome material from chromosome 9 to 22; others were due to complex chromosomal translocations that made the Ph chromosome lose its typical appearance (Rassool et al, 1990). In fact this group of Ph-negative CML patients had Ph translocations.

In one Ph-negative patient (no. 422.2) a breakpoint could be

mapped 5' to the M-BCR. This phenomenon has been reported previously in Ph-positive CML patients (Bartram et al, 1987b; Saglio et al, 1988). The location of the breakpoint 5' to M-BCR in these patients was demonstrated by M-BCR probe hybridization to the EcoRI digests, which indicated that the breakpoint in these patients was located within the 17kb EcoRI fragment. The end result of this kind of BCR/ABL rearrangement would be the exclusion from the chimeric gene and its products of several exons 5' to M-BCR. It is interesting that in this patient some atypical features were observed. However in those Ph-positive CML patients who also had a 5' breakpoint outside M-BCR the authors did not indicate the presence of atypical features. To what degree the 5' breakpoint can affect the clinical outcome of the patients is difficult to judge. Furthermore this patient had some features of eosinophilic leukaemia. It has been reported that eosinophilic leukaemia could be Ph-positive or Ph-negative (Krauss et al, 1964; Gruenwald et al, 1965). This case may represent an eosinophilic leukaemia variant of Ph-negative but M-BCR 'positive' (a breakpoint with short distance 5' to M-BCR) CML.

In the other 9 patients using Southern analysis I cannot at this stage rule out the involvement of the BCR gene but I was able to rule out the possibility of M-BCR rearrangement. The atypical appearance of this group of patients with Ph-negative, M-BCR non-rearranged CML underlines the relationship

of M-BCR rearrangement to the typical pictures of CML. Clearly a different molecular mechanism is responsible for this kind of CML. Similar results have been observed by other researchers (Morris et al, 1986; Wiedemann et al, 1988). Pulsed field gel electrophoresis, in situ hybridization and polymerase chain reaction analysis were used to study further the molecular mechanisms of these M-BCR non-rearranged CML patients. These will be discussed in the next three chapters.

6.3.3 Ph-POSITIVE ACUTE LEUKAEMIA

Chronic phase CML eventually transforms into blast phase. There is much evidence suggesting the BT of CML is a completely different form of leukaemia from de novo acute leukaemia (Chapter 2). However it is difficult sometimes to make a clear distinction, especially in leukaemias with a Ph chromosome. As discussed, if M-BCR rearrangement is the marker for the molecular change during the formation of Ph chromosome in CML, can the same change be found in Ph-positive acute leukaemia?

I present one case of Ph-positive acute leukaemia without evidence of M-BCR rearrangement. Further studies by PCR, in situ hybridization and protein assay showed a break in the first intron of the BCR gene, and the corresponding chimeric protein P190. The studies of M-BCR rearrangement in Ph-positive acute leukaemia by others showed that about 50% of

them were Ph-positive, M-BCR rearranged, while the other 50% were Ph-positive, M-BCR non-rearranged and had a breakpoint in the first intron of the BCR gene (Hermans et al, 1987; Rubin et al, 1987; Heisterkamp et al, 1988a). Although the first intron break can be found in rare cases of Ph-positive CML, experiments using in vitro transformation of lymphoid cells showed that the BCR/ABL chimeric products resulting from the first intron break were more effective than those resulting from M-BCR break (McLaughlin et al, 1989). These findings underline the more 'malignant' nature of P190 than P210 and the association of P190 with de novo Ph-positive acute leukaemia.

CHAPTER SEVEN LONG RANGE MAP OF THE BCR GENE

7.1 INTRODUCTION

The majority of patients with Ph-positive CML have a chromosome 22 breakpoint located within the M-BCR. However, in a minority of cases there is no evidence of M-BCR rearrangement (Selleri et al, 1987; Saglio et al, 1988) and the breakpoints must lie outside the M-BCR. One patient in my study also lacked evidence for a breakpoint within the M-BCR and two patients were equivocal (see Chapter 6). Some of these patients may have a breakpoint in the vicinity of the M-BCR. Breakpoints within 20kb 5' or 3' of M-BCR can be analyzed relatively easily by Southern blotting (Saglio et al, 1988) but breakpoints further away from the M-BCR are more difficult to detect. Ph-positive patients who lack evidence for M-BCR rearrangement when analyzed with multiple probes and enzymes must therefore have breakpoints some distance from the area designated as M-BCR. This raises two fundamental questions: do these patients have BCR gene involvement at all, and, if they do, where exactly is the breakpoint? Clarifying the BCR gene involvement in these patients may better define the role of BCR gene in ABL activation and the role of BCR/ABL

rearrangements in CML.

Other CML patients have clinical features closely resembling typical CML but lack the Ph chromosome. Some of these patients are M-BCR rearranged and others M-BCR non-rearranged (Morris et al, 1986; Wiedemann et al, 1988; also see chapter 6). Study of BCR gene involvement in this group of Ph-negative, M-BCR non-rearranged CML patients is important, at least to answer one question: What degree of molecular similarity is there between these two groups of patients who are clinically similar but have their own features? Some researchers have reported the absence of an BCR/ABL mRNA and aberrant ABL protein, and no evidence for ABL translocation in these patients (Wiedemann et al, 1988; Dobrovic et al, 1989). However, these findings do not entirely rule out the possibility of some other form of BCR/ABL rearrangement. The question is still open.

In addition to the classical BCR gene, there are 3 other DNA sequences homologous to the 3' structure of the BCR gene in the same chromosomal region, 22q11.2. These 4 related loci are designated BCR1, BCR2, BCR3 and BCR4 respectively. BCR1 is the original BCR gene and the others have been called BCR-related genes (Croce et al, 1987; Heisterkamp & Groffen, 1988b). The order of loci on chromosome 22 is: Centromere--> (BCR2, BCR4, IGL)--> BCR1--> BCR3. The biological significance and the

possible involvement of these sequences in CML are not clear.

All these points suggest the need for a technology that can examine large DNA fragments extending up to the size of a whole gene. Since both the BCR and ABL genes are very large, about 130 and 230kb respectively (see Chapter 2), I decided to use pulsed field gel electrophoresis (PFGE). PFGE maintains the integrity of large fragments of DNA during preparation and permits size dependent fractionation of DNA fragments measuring from 20 to 10,000kb. The technique was therefore used to study BCR gene involvement in various types of leukaemia in which the use of Southern analysis is unhelpful (Hooberman et al, 1989; Gao et al, 1990a).

In this chapter I describe the construction of a long range map of the BCR gene and the BCR-related genes using DNA from normal subjects. I made use of the rare cutting restriction enzymes BssHII, MluI, NarI, NotI and SalI. This map provides a basis for study of possible aberrant involvement of BCR gene in leukaemia. I also describe briefly my version of a long range map of the ABL gene, although comparable maps have been constructed by others (Westbrook et al, 1987; Bernards et al, 1987). The results of study of the location of breakpoints in chromosome 22 in various types of leukaemia will be introduced in next chapter.

7.2 RESULTS

7.2.1 LONG RANGE MAPPING OF THE NORMAL BCR GENE

Since a long range map of the ABL proto-oncogene had already been constructed with rare cutting restriction enzymes NotI, SfiI and SacII (Bernards et al, 1987), I first selected these enzymes to map the normal BCR gene. However, NotI digests resulted in a very large BCR fragment of 450kb (Fig. 7.1), which was hybridized to 5' BCR cDNA probes B, C and genomic M-BCR probe E. When full length cDNA probe A was used two more fragments of 270 and 550kb presumed to represent the BCR-related genes were detected. Since the 450kb fragment hybridized to a range of probes spanning the whole coding sequence of the gene, this fragment should encompass the whole BCR gene. However, this NotI fragment is so much larger than the BCR gene itself that it was difficult to determine the position of the NotI sites relative to the gene. A computer search of the published BCR gene cDNA sequences (Lifshitz et al, 1988) did not reveal any NotI site.

In contrast the enzymes SfiI and SacII cut very frequently within the BCR gene and therefore produced many small fragments. I decided not to attempt localize these fragments.

BssHII The restriction enzyme BssHII was therefore used to study the BCR gene. A computer search of the published 5' BCR

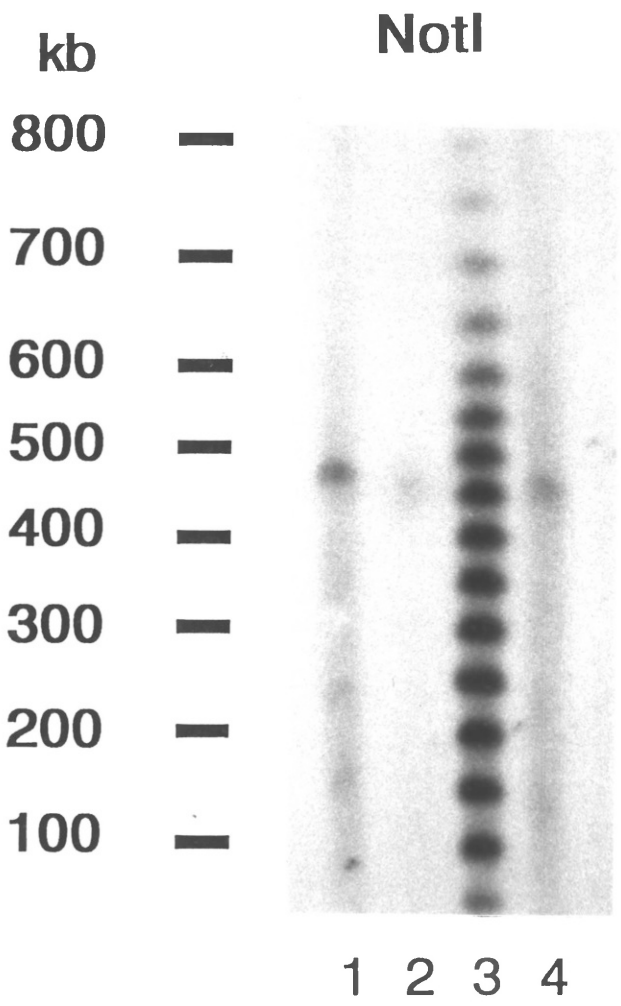


Fig. 7.1 Normal DNA Digested with NotI Hybridized to Probe C.

Lanes 1, 2 and 4 contain DNA from normal blood samples and show the germline band at 450kb. Two other faint bands at 150 and 250kb in lanes 1 and 4 which contain larger quantities of DNA are also observed. This may indicate the presence of heavily methylated NotI sites within the 450kb fragment. Lane 3 contains a molecular weight marker (concatenated λ ladder).

cDNA sequences revealed seven BssHII sites within exon 1, 6 of them located 5' to the coding sequences. The seventh lay inside the long open reading frame and was located 240bp upstream of the 5' boundary of probe A. Because these 7 BssHII sites are grouped relatively closely I treated them as a single site, as if located inside the long open reading frame. Digestion of DNA from normal cells resulted in two fragments hybridizing to BCR probe C. These two fragments measured 120 and 230kb respectively (Fig. 7.2). The 120kb band was very faint but the 230kb band was always very strong. Since probe C contains a repetitive sequence which is also present 5' to the BCR gene, the 120kb fragment could be either 3' or 5' to the BssHII site in exon 1. Therefore, in order to identify its origin the DNA membrane was reprobed with probe E, which detects only one M-BCR band in normal DNA by Southern blotting (see Chapter 6). Probe E identified both the 120kb and 230kb fragments. When a 3' BCR cDNA probe D was used these two bands as well as other bands that probably represent BCR-related genes (see below) were identified (Fig. 7.3). These results ruled out the possibility that the 120kb fragment could be located 5' of the exon 1 BssHII sites. Thus the two new BssHII sites are mapped respectively 120kb downstream (encompassing the M-BCR sequence) and 230kb downstream of exon 1. The 120kb site appears highly resistant to cleavage so its presence can be ignored for practical purposes, while the 230kb site is efficiently cleaved by BssHII. Because the 3' end of the 4.5kb

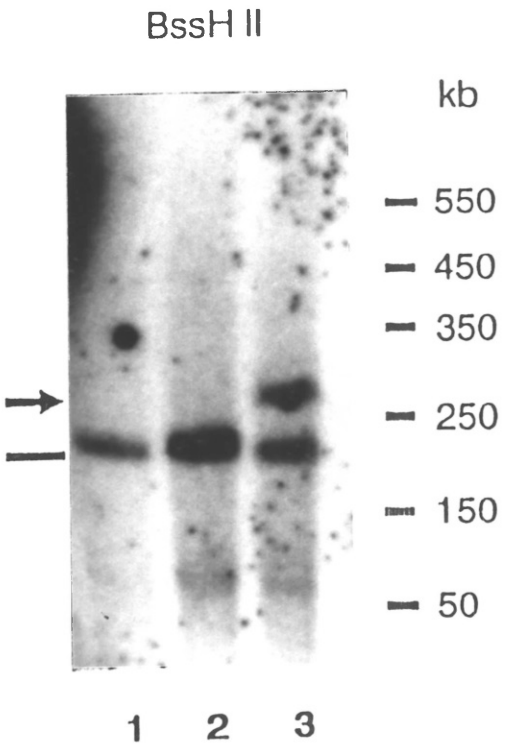


Fig. 7.2 DNA Digested with BssHII Hybridized to Probe C. Lanes 1 and 2 contain DNA from normal blood samples; lane 3 contains DNA from a control patient with Ph-positive, M-BCR rearranged CML. Two germline bands at 120 and 230kb are seen; the 120kb band is very faint as compared with the 230kb band. A rearranged band is present at 300kb in lane 3.

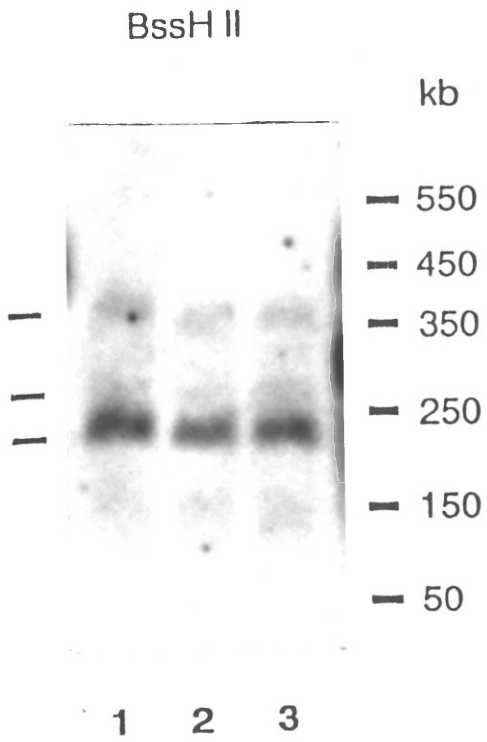


Fig. 7.3 Normal DNA Digested with BssHII Hybridized to Probe D.

Lane 1, 2 and 3 contain BssHII digests of DNA from normal blood samples. After hybridization with the 3' portion of BCR gene cDNA probe D, two additional germline bands are observed at 280 and 430kb in addition to those seen in Figure 7.2.

BCR gene transcript is thought to be located about 130kb downstream of exon 1 (Heisterkamp et al, 1988a), the 230kb BssHII fragment should encompass the whole BCR gene, apart from an upstream portion of exon 1 (Fig. 7.4).

Since I and others have studied some cases of Ph-positive leukaemia in whom BCR gene rearrangement cannot be found in BssHII digests (Hooberman et al, 1989; Gao et al, 1990a), I sought additional enzymes to complement results obtained with BssHII.

NarI Digestion of normal DNA with NarI produced a 230kb fragment which hybridized to probes B, C and E respectively. A computer search identified four NarI recognition sites in BCR exon 1. As in the case of BssHII, for practical purposes these NarI sites can be regarded as a single site. There is a fifth NarI site located in the 3' untranslated region of the gene, but this was not detected by the above probes. I presume therefore that this site is methylated. Since the NarI sites in exon 1 are very close to the BssHII sites and both digests produce fragments of similar size, NarI probably cuts at sites very close to the BssHII sites in and around the BCR gene. These sites may be located in the GC rich islands which usually signal the 5' end of a gene (Bird, 1986).

MluI When normal DNA was digested with MluI and hybridized

Fig. 7.4 Long Range Map of the BCR Gene and the BCR-related Genes.

Long range map of BCR gene and BCR-related genes showing sites recognized by rare cutting restriction enzymes. Sa = SalI; Bs = BssHII; Na = NarI; Bg = BglII; No = NotI and Ml = MluI. The data suggest that there is one MluI site in the region covered by probe C in the middle of the gene; the precise position of this site is 40kb downstream of the 5' BssHII site. One MluI site is mapped 500kb 3' and another one 250kb 5' to the BCR gene. The dashed lines represent sites where cutting is somewhat inefficient and the dotted lines represent sites where cutting is even more inefficient, perhaps due to methylation, such as at the SalI sites in the middle of the gene. This map is not exactly to scale and the exon structure of the gene is simplified.

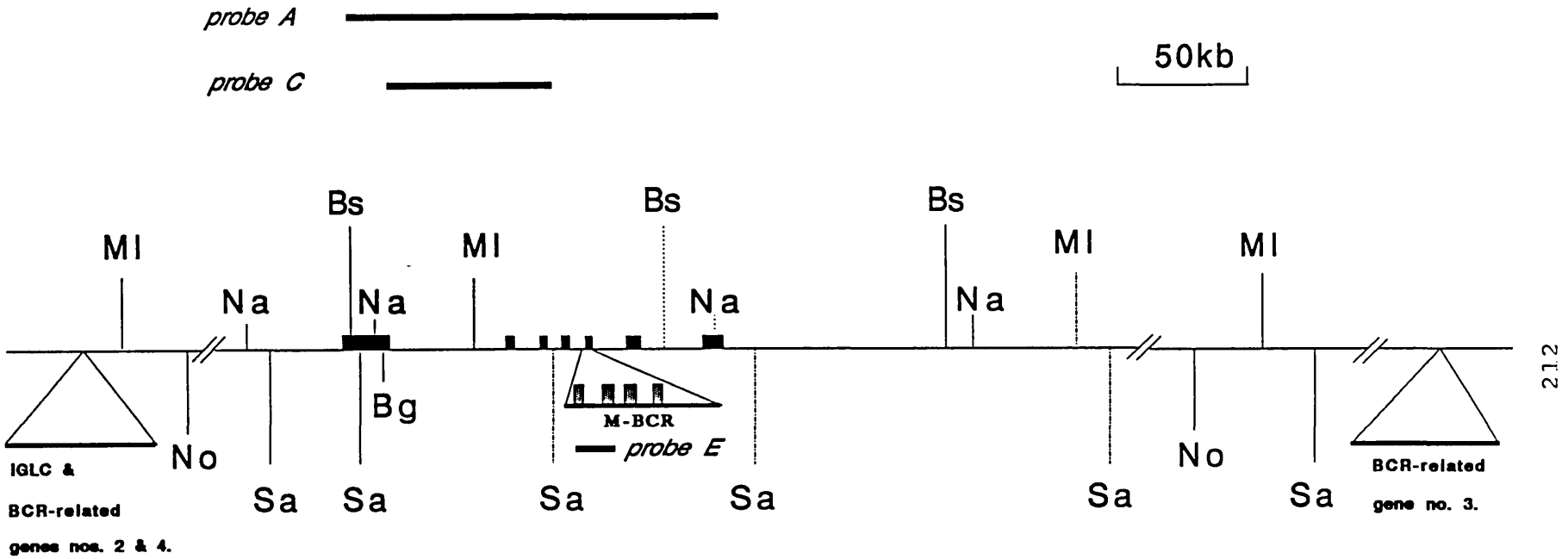

probe A
probe C
50kb
Bs
Bs
Bs
Ml
Ml
Ml
Ml
Na
Na
Na
Na
Na
Bg
M-BCR
probe E
No
No
Sa
Sa
Sa
Sa
Sa
Sa
IGLC &
BCR-related
genes nos. 2 & 4.
BCR-related
gene no. 3.
212

Fig. 7.4

with probes C and F two fragments of sizes 250 and 500kb were identified (Fig. 7.5a). When the filter was re-hybridized with probe D, two additional larger fragments presumably representing BCR-related genes were recognized. When the MluI digests were hybridized to probe B, the 250 and 500kb fragments were not identified. Instead, a faint 200kb band was observed. A computer search of BCR gene exon 1 did not reveal any MluI site. These findings indicate that both 250 and 500kb fragments contain the bulk of the BCR gene, but not the first exon. There is one MluI site within the BCR gene but downstream of the first exon. Another site is located 250kb 3' and this site is partially methylated. A third site is about 250kb further downstream. The faint 200kb band detected by probe B may be due to a methylated site at 200kb upstream (Fig. 7.4).

In order to localize the MluI site in the middle of the gene more precisely, double digests with MluI and BssHII were performed. After hybridization with probe C two prominent bands of 40kb and 180kb were observed (Fig. 7.5b). This indicated that the MluI site in the middle of the BCR gene was located 40kb downstream of the 5' BssHII site(s) (Fig. 7.4). This must be within the first intron of the BCR gene. This site cannot be located close to the 3' BssHII site because that would be outside region covered by probe C. It is known now that the size of first intron of the BCR gene is 68kb

Fig. 7.5 MluI Digests.

a. Normal DNA Digested with MluI Hybridized to Probe C.
Lanes 1, 2 and 3 contain DNA from normal blood leucocytes. Germline bands are observed at 250 and 500kb. MluI digestion is usually very difficult and the intensity of MluI bands is faint.

b. Normal DNA Double Digested with MluI and BssHII Hybridized to Probe C.
Lanes 1 and 2 contain DNA from normal blood samples double digested with MluI and BssHII. Two bands at about 40 and 180kb were observed. The sizes of these bands are different from those observed with MluI or BssHII alone.

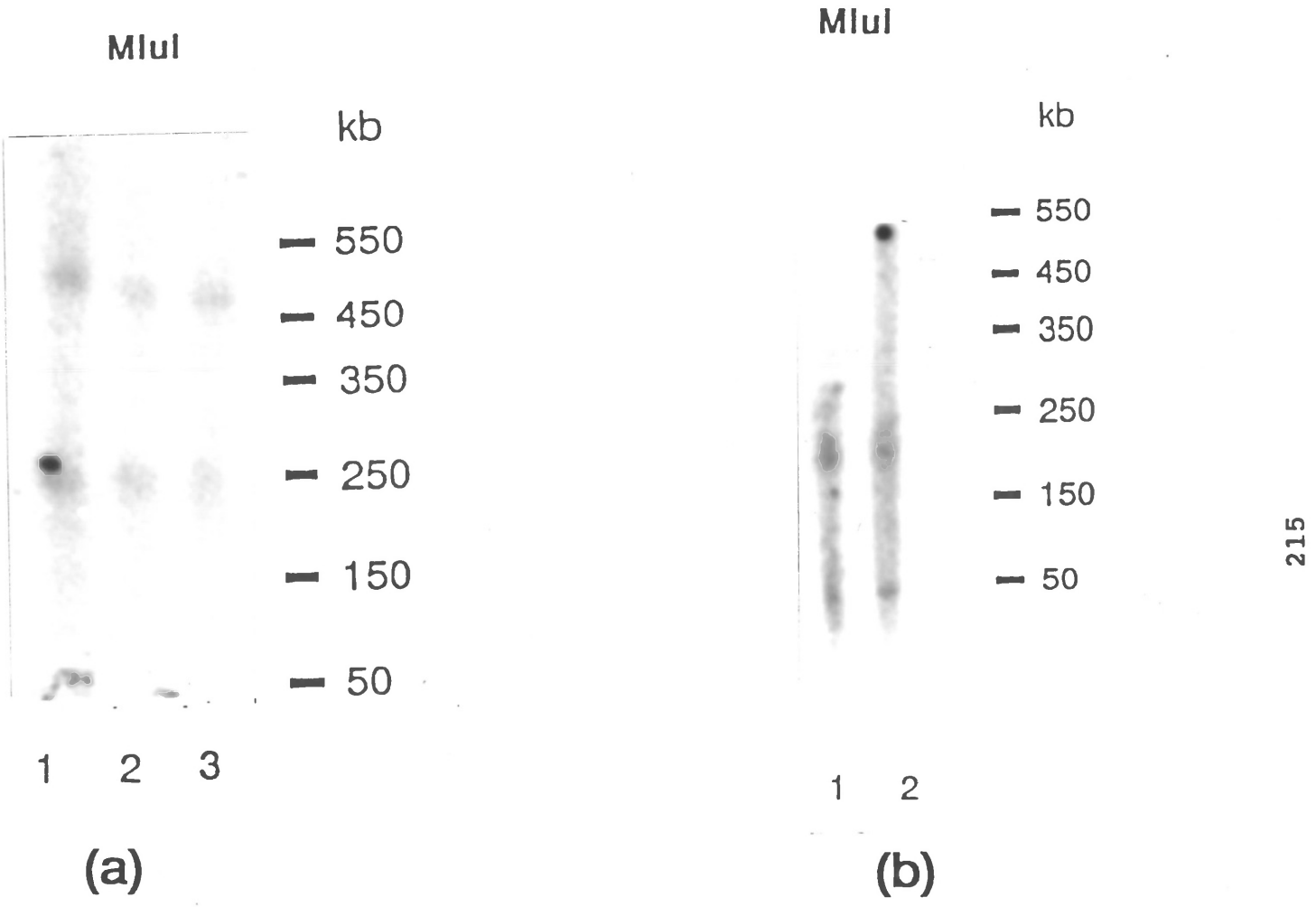

MluI
kb
550
450
350
250
150
50
1
2
3
(a)
MluI
kb
550
450
350
250
150
50
1
2
(b)

Fig. 7.5

(Heisterkamp et al, 1988a).

It must be stressed that DNA digestion with MluI is difficult. Twice as many units of the enzyme and longer digestion times are required. Even then, the bands are faint.

SalI PFGE studies with DNA digested with the restriction enzyme SalI were unrewarding because of the variable degrees of methylation at many sites downstream of the first exon of the BCR gene. Hybridization of the SalI digests with probe B identified 8 fragments sized 50, 75, 200, 300, 350, 450, 500 and 600kb respectively (Fig. 7.6). However, when SalI digests were hybridized to probe C only 7 fragments remained; the 50kb fragment was not recognized. A computer search for SalI sites in first exon of the BCR gene revealed a site 500bp upstream to the probe C fragment. From these data I deduced that there is a SalI site 50kb 5' of exon 1. The SalI site 75kb downstream of exon 1 is located 5' to the M-BCR region. The digestion of this site is usually inefficient because this band appeared with different intensities in different individuals. The 200kb band is very faint and did not appear consistently suggesting an even higher degree of protection from cleavage by SalI. The 300 and 600kb bands were usually strong in DNA from normal cells suggesting efficient digestion at these sites. The 300 and 600kb fragments starting from exon 1 are long enough to encompass the whole BCR gene. When DNA

Fig. 7.6. Normal DNA Digested with SalI Hybridized to Probe

Lanes 5 and 6 contain DNA from normal blood samples and lanes 2 to 4 contain DNA from Ph-positive CML patients. Germline bands are present at 50, 75, 200, 300, 350, 450, 500 and 600kb. The germline bands at 75, 300, 450 and 600kb appear consistently in all the lanes but the others are faint and do not consistently appear. A band at 130kb appears in all 3 CML samples (lanes 2 to 4) but not obviously in normal samples. Only one rearranged band (at 200kb) can be observed in lane 3. The SalI digest was hybridized to probe C.

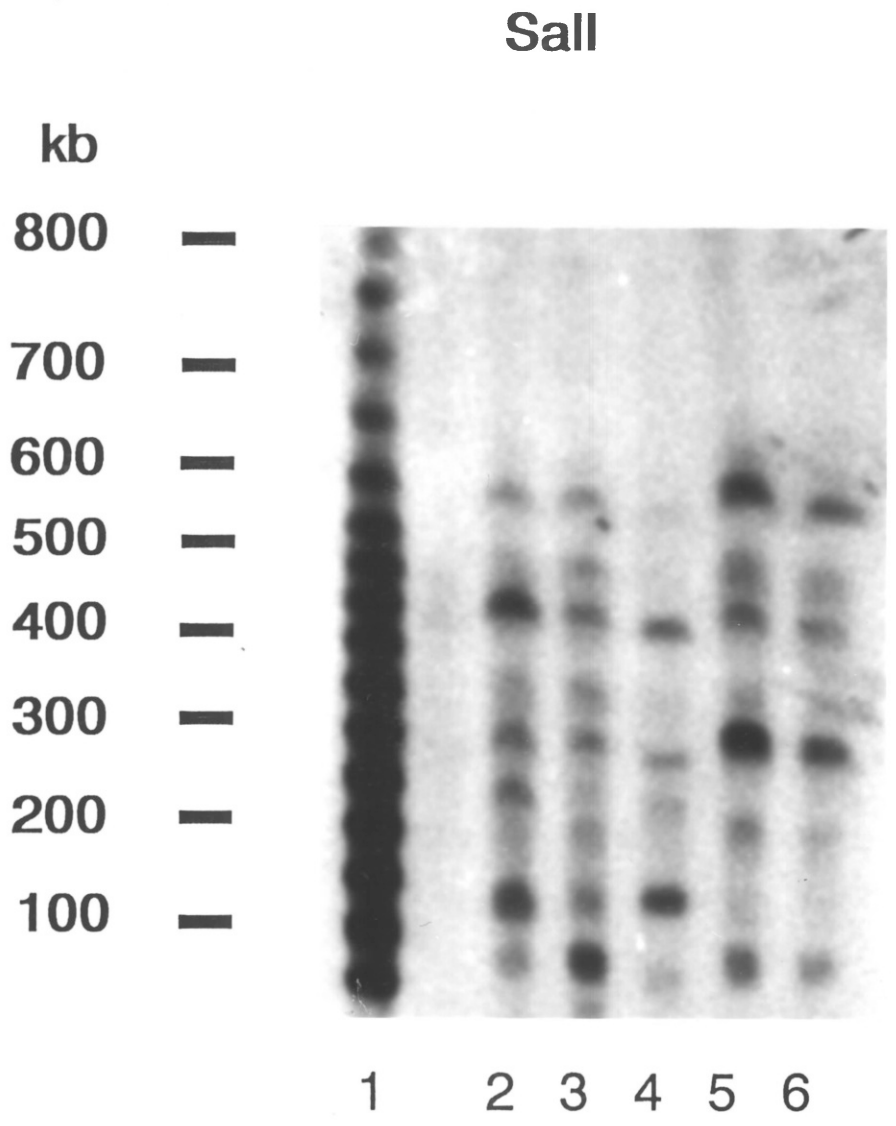


Fig. 7.6

from 3 CML patients were digested with SalI and hybridized to probe B, an additional fragment of 130kb were observed. This site may be demethylated to some degree in CML cells (Fig. 7.6).

Thus the long range map of BCR gene constructed with SalI can be summarized as follows: there is a Sal I site within the exon 1, and other sites at about 75, 130, 200, 300, 450 and 600kb downstream. Because cleavage at these many sites is inefficient, hybridization with BCR probes resulted in many bands that makes study of BCR gene rearrangements difficult, especially when the breakpoint is located 3' of the first partially cleaved SalI site 75kb downstream of exon 1 (Fig. 7.4; 7.6). A band at 130kb appears in all CML samples but not obviously in normal samples. This may represent a site in the BCR gene (it could also be outside) which is de-methylated in the CML cells.

7.2.2 LONG RANGE MAPPING OF BCR RELATED GENES

When normal DNA was digested with BssHII and hybridized with probes A and D, three fragments measuring 280, 380 and 430kb respectively were identified in addition to the 120 and 230kb fragments. The intensity of the 280 and 380kb bands was weak while the 430kb band was very strong. The 380kb band did not consistently appear in all individuals (it did not appear in Figure 7.3, but it appeared in other BssHII digests, see

Figure 8.1b in Chapter 8). From these results and those obtained by hybridizing with probes B and C I concluded that these fragments must reflect sequences homologous to the 3' section of the BCR gene located at minimum distances of 280, 430 and possibly 380kb respectively from the BCR gene. These are the so-called BCR-related genes (Croce et al, 1987; Heisterkamp & Groffen, 1988b). The 380kb band could be due to a methylated site around one of the BCR-related gene, or it could be a BCR related gene sharing less homology. Specific bands representing BCR-related genes were also observed in MluI and NotI digests (above).

7.2.3 LONG RANGE MAPPING OF THE ABL GENE

Since the BCR gene was mapped with BssHII and NarI, I also mapped the ABL gene with these enzymes. The BssHII digests of normal DNA were hybridized to a 1.5kb cDNA ABL body probe (Chapter 3, Fig. 3.3). Two bands at 30 and 230kb were recognized (Fig. 7.7). Using an ABL body probe that excluded exon II Selleri et al (1990) detected a single germline band of 150kb. This difference could be due to the fact that molecular weight estimations between different laboratories can differ; another possible explanation for the difference in fragment size between my study and Selleri's could be the presence of a restriction site at the 3' end of 230kb fragment that is methylated to variable degrees. As a result of my findings and the molecular cloning and PFGE results by Selleri

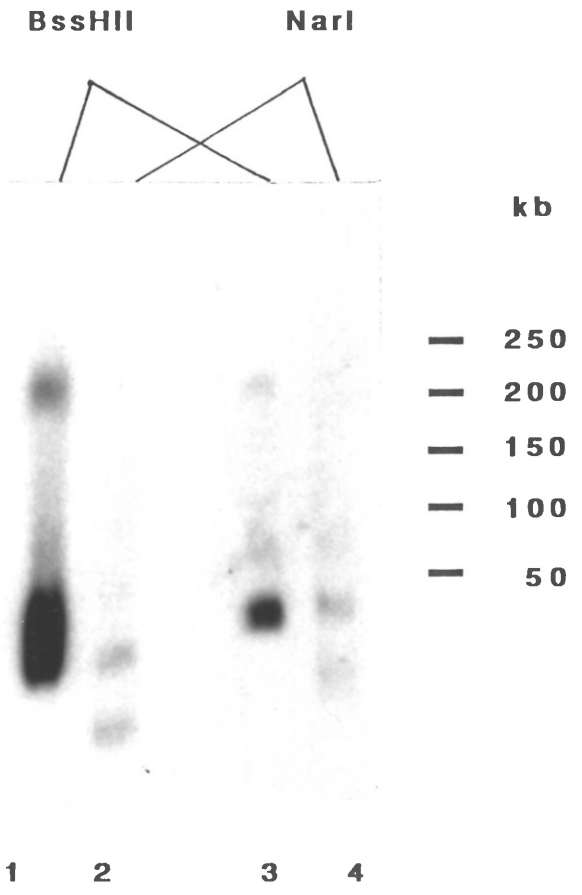


Fig. 7.7 Normal DNA Digested with BssHII and NarI Hybridized to the ABL Probe.

DNA digests with BssHII (lanes 1, 3) and with NarI (lanes 2, 4) were hybridized with 1.5 kb cDNA ABL body probe. BssHII germline fragments of ABL gene are 30 and 230 kb while the NarI germline fragments are at about 10 and 30 kb (estimated) respectively. Lane 1 and 2 contain the DNA from two normal controls, lane 3 and 4 contain the DNA from patient no. 621. 1. No rearranged bands were observed.

et al, I postulate that there should be one more BssHII site within the ABL gene in the map suggested by Selleri. This site should be within the v-abl homologous region covered by the 'body' probe and should be 3' to exon II. Starting from the site just 5' to exon Ib, there are 4 sites within and around the ABL gene. There is one just 5' of exon Ia (in fact there are 4 sites there, for practical reason I treat it as if a single site). This site is methylated in CML cells (see Chapter 8). The one next is 30kb downstream (within the v-abl homologous region). The last is 230kb further downstream (Fig. 7.8).

NarI was also used to construct an ABL map. Normal DNA digests showed two bands at about 10kb and 30kb respectively when hybridized with the ABL body probe (Fig. 7.8). A computer search of the published ABL sequences (Shtivelman et al, 1986) revealed two NarI sites in exon Ia. Assuming that these sites are not methylated the other NarI sites in the ABL gene should be 30kb and a further 10kb downstream respectively. From these data it seems that there are two NarI sites in the ABL gene which are also close to the BssHII sites (Fig. 7.8).

7.3 DISCUSSION

To construct a long range map of the BCR gene, I selected the

Fig. 7.8 Long Range Map of the ABL Gene.

Long range map of ABL gene showing sites recognized by rare cutting restriction enzymes. Bs = BssHII; Na = NarI; B = BamHI; H = HindIII and P = PstI. The data suggest that there is one BssHII site just in front of exon Ib. There are four BssHII sites just in front of exon Ia; for practical reasons I regard them as a single site. These sites are methylated in CML cells. The next site is 30kb downstream, located within the v-abl homologous region. The last site is 230kb further downstream. There are two NarI sites within Ia and I treat them as a single site. The next two sites are 10 and 30 kb downstream respectively. The dotted lines represent sites where cutting is inefficient, perhaps due to methylation, such as at the BssHII sites in the middle of the gene. This map also is not to scale and the exon structure of the gene is simplified.

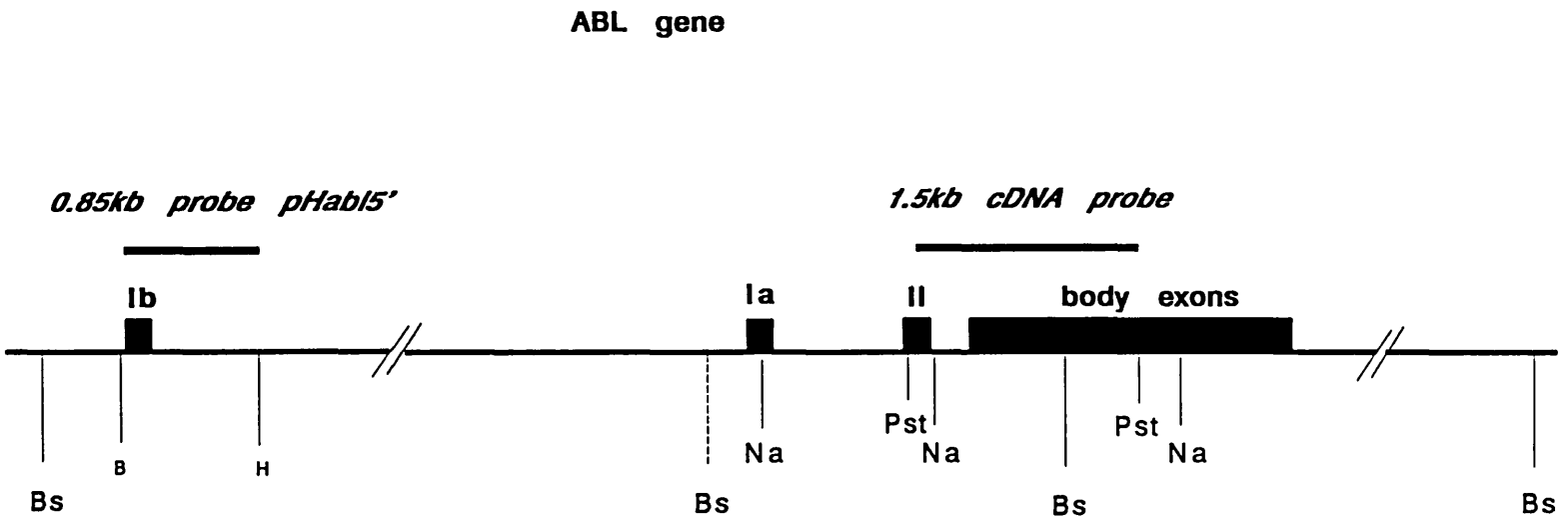

ABL gene
0.85kb probe pHabl5'
1.5kb cDNA probe
Ib
Ia
II
body exons
B
H
Pst
Pst
Na
Na
Na
Bs
Bs
Bs
Bs

Fig. 7.8

infrequent cutting restriction enzymes BssHII, NarI, SalI, NotI and MluI. There is a known BssHII restriction site in the first exon of the BCR gene. Digestion of DNA with BssHII produced a BCR gene fragment of 230kb size which probably encompasses the whole gene; the additional 120kb fragment identified with probes C, D and E could be due to an additional methylated BssHII site. Since the BssHII fragment encompasses the BCR gene, BssHII should be valuable for further study of BCR gene involvement in various leukaemias, especially CML patients without evidence of M-BCR rearrangement (Selleri et al, 1990; Gao et al, 1990). I also constructed a BssHII map of the ABL gene. Together with the results reported by Selleri et al I mapped a BssHII site at the 5' end of the gene (just upstream of exon Ib). Another 4 clustered sites were mapped just upstream of exon Ia. These 4 sites are all methylated in CML cells. One site is about 30kb downstream and the last one should be 230kb further downstream.

I found that the value of SalI in this study was limited because some of the restriction sites were methylated to varying degrees. The same observations were reported by Heisterkamp et al (1988a). My results in CML patients were difficult to interpret. For patients with Ph-positive, M-BCR rearranged CML whose DNA was heavily methylated at the internal sites (the sites 75, 130 and 200kb 3' to exon 1

site), a rearranged band could be detected, while in those patients whose DNA was methylated to variable degrees, no obvious rearranged band could be seen. However, some investigators who used this enzyme for study of Ph-positive, M-BCR non-rearranged acute lymphoblastic leukaemia found BCR gene rearrangement (Hermans et al, 1987). This rearrangement was later found to involve a breakpoint in the first intron of the BCR gene. After rearrangement the portion of BCR gene which contains these partially methylated SalI sites will be replaced by the ABL gene which may not have this problem. This could explain the differing results using the SalI enzyme.

The NarI restriction sites are located close to the BssHII sites in the BCR gene. Although the sizes of the BssHII and NarI fragments generated by digestion of the BCR gene do not differ greatly, the restriction sites are different in the ABL gene. Thus hybridization with both BCR and ABL probes of DNA fragments digested with these two enzymes and separated by PFGE should be complementary under certain circumstances. This depends on whether the two NarI sites in the ABL exon Ia are methylated (the BssHII sites around exon Ia are methylated in CML cells, Selleri et al, 1989), or whether the ABL breakpoint occurs in the intron between exons Ia and II.

Even though MluI is difficult to work with, use of MluI digests enabled us to study BCR gene rearrangements that

occurred over a wider distance. The MluI fragment contains more DNA sequences downstream of the BCR gene than the BssHII fragment. I have found MluI useful for determining aberrant involvement of the BCR gene (see Chapter 8).

I also used NotI to map the BCR gene. I found a 450kb fragment which may encompass the whole BCR gene. This is inconsistent with the findings by Westbrook et al (1988) who reported a NotI BCR gene fragment larger than 1200kb. This discrepancy may due to the fact that there is a partially methylated site in the middle of the 1200kb fragment. This site may be methylated to different degrees in different DNA specimens.

The use of PFGE in this study confirmed the existence of the BCR-related genes. The intensities of the three bands representing the BCR-related genes in the BssHII digests were different. This may reflect different degrees of 3' homology of the two BCR-related genes to the original copy. These BCR-related genes were distributed over a relatively large distance.

CHAPTER EIGHT BCR/ABL REARRANGEMENTS IN Ph-NEGATIVE AND OTHER ABERRANT TYPES OF CML

8.1 INTRODUCTION

In chapter 6 I described the pattern on BCR rearrangement in 52 patients with CML by Southern blotting. Of 33 Ph-positive patients 30 had evidence of M-BCR rearrangement, two cases were difficult to interpret and one clearly lacked evidence of M-BCR rearrangement. Of 19 Ph-negative patients 9 showed M-BCR rearrangement, 9 showed no rearrangement and one result was uncertain. Here I classify those patients with M-BCR non-rearranged CML or those who lack clear evidence for M-BCR rearrangement as CML with atypical molecular features. I searched for evidence for BCR gene involvement in this group of patients. I also tried to map the position of the breakpoints. I compared the molecular changes with the clinical features in these patients.

After constructing the long range maps of the BCR and ABL genes I selected the restriction enzymes BssHII, NarI and MluI for PFGE studies of these patients with atypical molecular

features. Other technologies such as polymerase chain reaction (PCR, in collaboration with Dr Phillippe Martiat) and in situ hybridization of marrow metaphase (in collaboration with Mrs Guo Ai Pu) were also used to complement the PFGE results. PCR was used to study the presence of BCR/ABL chimeric mRNA and the types of junction between these two genes, namely b2a2, b3a2 or e1a2 (Chapter 2). In situ hybridization was used to characterize the precise location of breakpoint and gene translocations in metaphase chromosomes.

8.2 MATERIAL & METHODS

8.2.1 PATIENTS

After standard Southern analysis I selected for further study by PFGE one case of Ph-positive, M-BCR non-rearranged CML (no. 621.1), 2 cases with doubtful M-BCR status (nos. 621.2 and 621.3) and 4 of the 9 cases Ph-negative, M-BCR non-rearranged CML (from nos. 422.10 to 422.13). I also studied the Ph-negative CML patient who had abnormal bands in the BamH I digestion (no. 422.19). The selection of the above patients was based in part on the availability of further blood samples. Of the 3 Ph-positive CML patients, no. 621.2 had atypical clinical features with no splenomegaly, marked thrombocytosis and poor response to busulphan. All the Ph-negative CML patients selected for further study had some

atypical features (Shepherd et al, 1987), while the other 2 cases of Ph-positive patients were clinically and haematologically indistinguishable from typical CML. The clinical and haematological features are summarized in Table 4.1 and 6.2. Six patients with Ph-positive, M-BCR rearranged CML were used as controls.

8.2.2 PULSED FIELD GEL ELECTROPHORESIS

The methodology is described in detail in Chapter 3.

8.2.3 POLYMERASE CHAIN REACTION

Total RNA was isolated from mononuclear cells using the method of Chomczynski (1987). cDNA was transcribed from the mRNA with reverse transcriptase (BRL, USA). Three oligonucleotides were made using an Applied Biosystems DNA synthesizer. The sequences of the oligomers were:

Oligo A: 5' GAAGAAGTGTTTCAGAAGCTTCTCCC 3' (b1b2 sense strand);

Oligo B: 5' TTCAGCGGCCAGTAGCATCTGACTT 3' (a2 sense strand);

Oligo C: 5' GACCCGGGAGCTTTTCACCTTTAGTT 3' (a2a3 antisense strand).

The PCR was performed as previously described (Hermans et al, 1988) except that a programmable heating block was used for the amplification steps. This was set at 93°C for 4 minutes, 43°C for 2 minutes and 72°C for 5 minutes. A total of 30 cycles was carried out. Combinations of two primers (A/C or B/C) were

used. The combination A/C amplifies a region of 304 or 379 base pairs (bp) reflecting either the b2a2 or the b3a2 junction of the BCR/ABL mRNA. The B/C combination was used as internal control, since this amplifies a 185bp fragment corresponding to the normal ABL transcript.

8.2.4 IN SITU HYBRIDIZATION

In situ hybridization was carried out according to the method of Bartram et al (1983) with slight modifications. Cytogenetic preparations were prepared by standard methods. The probe was labelled with [^{3}H] dNTP's to a specific activity of $3x10^{8}$ cpm/ug and resuspended to a concentration of 0.2ug/ml in hybridization buffer containing 50% formamide, 2 x SSC, 10% dextran sulphate, and 1000-fold excess of alkali-denatured salmon sperm DNA. The denatured probe was hybridized with denatured chromosomal DNA for 15 hours at 37°C. The slides were rinsed thereafter in a standard manner. After dipping in Ilford L4 nuclear emulsion the slides were stored in the dark for 7 days at 4°C, developed in D19 for 8 minutes at 15°C, and then banded with the Wright's stain by the method of Harper and Saunders (1981). Hybridization of complementary DNA sequences in situ was seen as one or more black grains over individual chromosome(s). The number of grains per unit length of chromosome was scored for each metaphase.

8.3 RESULTS

8.3.1 RESULTS OF PFGE STUDIES IN Ph-POSITIVE, M-BCR REARRANGED CML PATIENTS

DNA from 6 cases of Ph-positive, M-BCR rearranged CML, digested with BssHII, all showed BCR gene rearrangement when probed with probes B and C. The rearranged bands were located in positions between 270 to 400kb (Fig. 7.2 & 8.1a). Based on the long range map of the BCR gene which I constructed (see chapter 7 and also Fig. 7.4), the variability in the size of this rearranged fragment must reflect the variability of the breakpoint in the ABL proto-oncogene, which may be located anywhere from 170kb to about 300kb 5' to exon II. These distances were estimated by comparing the size of BCR and ABL germline fragments and were based on the suggestion that the distance between the first exon of the BCR gene and M-BCR is about 100kb (Heisterkamp et al, 1988a). I also assumed that there were no major deletions or insertions as a result of the translocation, although these may be present in a small number of cases (de Klein et al, 1986a). MluI digests of three of these control samples showed analogously rearranged bands.

8.3.2 LOCALIZATION OF CHROMOSOME 22 BREAKPOINTS IN Ph-POSITIVE, M-BCR NON-REARRANGED CML PATIENTS

Table 8.1 summarizes the results of PFGE studies in these patients. DNA specimens from one Ph-positive, M-BCR non-

Fig. 8.1 Representative Blot of BssHII Digests Hybridizing to Probes C and D.

(a). Representative PFGE studies on various CML patients with BssHII digestion and probe *C* hybridization. Lane 1 contains DNA from patient no. 422.11; lane 2 contains the mol wt markers of bacteriophage lambda multimers. Lane 3 shows DNA from patient no. 621.2. Lane 4 shows DNA from patient no. 621.1. Lanes 5 and 6 contain DNA from Ph-positive, M-BCR rearranged CML controls. The bands at the 230kb position are in germline configuration. Rearranged bands are observed in patient no. 621.2 and in the Ph-positive, M-BCR rearranged controls.

(b). When the filter shown in (a) was reprobed with probe D, only the 4 normal germline bands (similar bands to those shown in Fig. 7.3) were observed in patient no. 621.1 (lane 4). The reciprocal translocation product could not be detected in this patient. On the other hand the reciprocal translocation product may be present at position about 140kb in patient no. 621.2 (lane 3) who had a breakpoint within the BCR gene. This reciprocal band overlaps the faint normal 120kb band.

BssHII

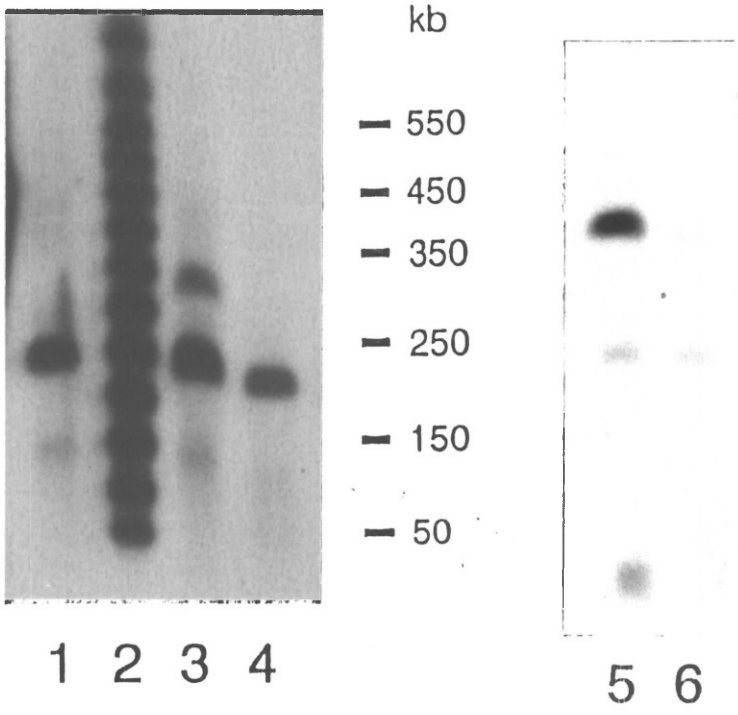


Fig. 8.1 (a)

BssHII

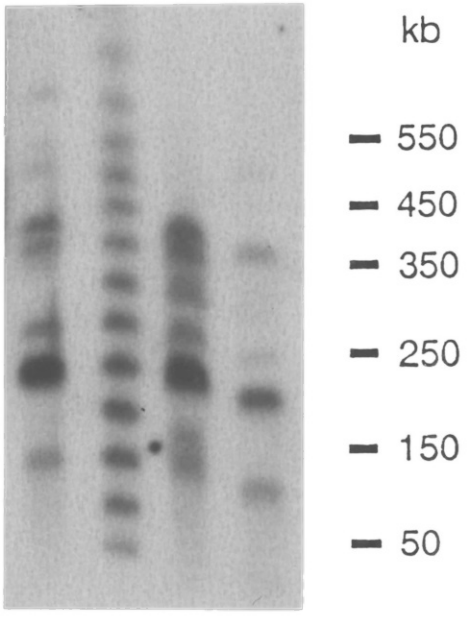


Fig. 8.1 (b)

Table 8.1 Results of PFGE analysis in 6 CML patients with M-BCR negative and 2 patients with doubtful M-BCR status.

Patient number:	PFGE		
	BssH II	Nar I	MluI
Ph-positive, M-BCR non-rearranged:			
621.1.	G	G	320*
Ph-positive, M-BCR equivocal:			
621.2.	330	350	550
621.3.	350	370	ND
Ph-negative, M-BCR non-rearranged:			
422.10.	G	G	G
422.11.	G	G	G
422.12.	G	ND	G
422.13.	G	ND	ND
422.19.	G	ND	ND

* The size of the rearranged bands is given in kilobase pairs.

G = Only germline band present.

ND = Not done.

rearranged CML patient and two with doubtful M-BCR status were digested with BssHII and hybridized to probes B, C and E separately. Rearranged bands were found in the two patients who could not be classified by Southern analysis (nos. 621.2 and 621.3). The intensities of the rearranged bands in PFGE were lower than the germline bands in a proportion similar to those in Southern blotting, but the rearranged bands were clearly visible in BssHII digests. In the third patient (no. 621.1) no rearranged band was found (Fig. 8. 1a). When NarI digests of DNA from the above patients were hybridized to probe C, rearranged bands were shown in DNA from patients nos. 621.2 and 621.3. The absence of rearrangement in BssHII digests in patient no. 621.1 was confirmed by NarI digestion and hybridization with probes C and E separately (Fig. 8.2). The NarI sites in the BCR gene are very close to the BssHII sites (Fig. 7.4). Since one BssHII fragment encompasses all the known exons of the BCR gene (see chapter 7), the absence of rearrangement in BssHII digests suggests that the breakpoint in this case lay outside the BCR gene.

To investigate the possibility that a BCR/ABL gene had been formed but could not be demonstrated in BssHII digests, for reasons such as co-migration of a rearranged band with the germline, DNA from the same patient was digested with BssHII and hybridized to a 0.85kb genomic ABL exon Ib probe and a 1.5kb cDNA ABL probe. When the first ABL probe was used only

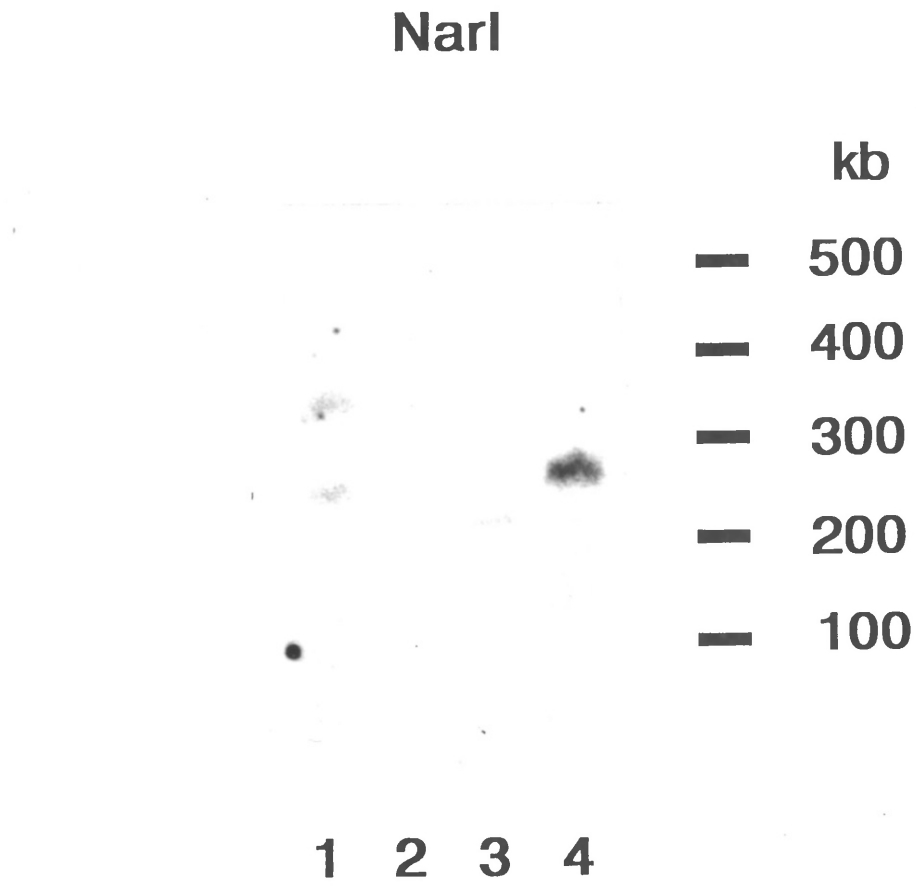


Fig. 8.2 DNA Digested with NarI Hybridizing to Probe E.
DNA samples were digested with NarI and hybridized to probe E. Lane 1 contains DNA from a Ph-positive M-BCR positive control and shows a rearranged band at about 320kb; lane 2 contains DNA from a normal control; lane 3 contains DNA from patient no. 621.1; lane 4 contains DNA from patient no. 422.11. Only germline bands are observed in lanes 2 to 4.

one faint germline band at about 200kb was observed. Because of the weak hybridization of this probe to the BssHII digests this result was not very informative. When the second probe was used two germline bands were found, a 30kb band and a 230kb band respectively (Fig. 8.3 and also see Chapter 7); unfortunately the 230kb ABL germline fragments in BssHII digests migrated at the same position as the BCR gene fragment. However, when NarI digests of DNA from patient no. 621.1 were hybridized to the second ABL probe, the germline fragments measured about 10 and 30kb (estimated); no band was observed at the BCR gene germline position (also 230kb). There are 2 NarI sites in the ABL exon Ia. If these two sites were not methylated NarI would sever the ABL hybridizing sequences from the BCR sequences unless the breakpoint in ABL occurred downstream of the NarI site in ABL exon Ia (Fig. 7.4). Thus the use of ABL probe can still not rule out the possibility of co-migration. I then used probes D and F respectively hoping to detect the reciprocal translocation product if the breakpoint occurred within the BCR gene. The use of these probes failed to detect any rearranged band in patient no. 621.1 (Fig. 8.1b). The failure of these 3' BCR probes to detect a reciprocal translocation product provides further evidence that the breakpoint is located outside the BCR gene in this patient.

DNA specimens from these three patients were studied further

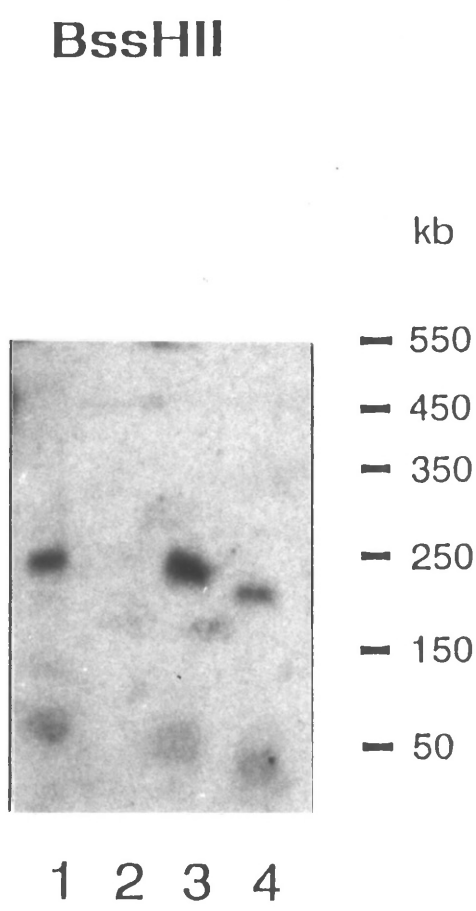


Fig. 8.3 Representative Blot of BssHII Digests Hybridizing to an ABL Probe.

When filter shown in Fig. 8.1 (a) was reprobed with 1.5kb ABL body probe, only the two germline bands at 30 and 230kb were present.

by digestion with other enzymes. In patient no. 621.1, whose DNA showed no rearrangement with BssHII and NarI digestion, a rearranged band was found when DNA was digested with MluI and hybridized with probes C and D (Fig. 8.4). The size of the rearranged fragment was about 320kb which suggests that the chromosome 22 breakpoint was located within a range of 320kb downstream of the MluI site in the middle of the BCR gene (see chapter 7 and also Fig. 7.4). To rule out the possibility that this band was due to MluI site polymorphism, a 1.5kb cDNA ABL body probe was used to hybridize to the MluI digests. A rearranged band at the same position was observed (Fig. 8.5).

8.3.3 USE OF PCR IN PATIENT NO. 621.1

When mRNA from patient no. 621.1 was studied by PCR using the primer pair A/C, an amplified fragment of 304bp was seen; this is consistent with the presence of mRNA with a b2a2 junction typical of Ph-positive M-BCR rearranged CML (Fig. 8.6).

8.3.4 IN SITU HYBRIDIZATION IN PATIENT NO. 621.1

Since I could not rely entirely on interpretation of the BssHII digest results to determine the position of the breakpoint, studies with in situ hybridization were performed. Probe A was hybridized to metaphases of cultured marrow cells. 71 metaphases were examined. The full length cDNA BCR probe hybridized to the Ph chromosome and chromosome 22, but not significantly to chromosome 9 or to other chromosomes (Fig.

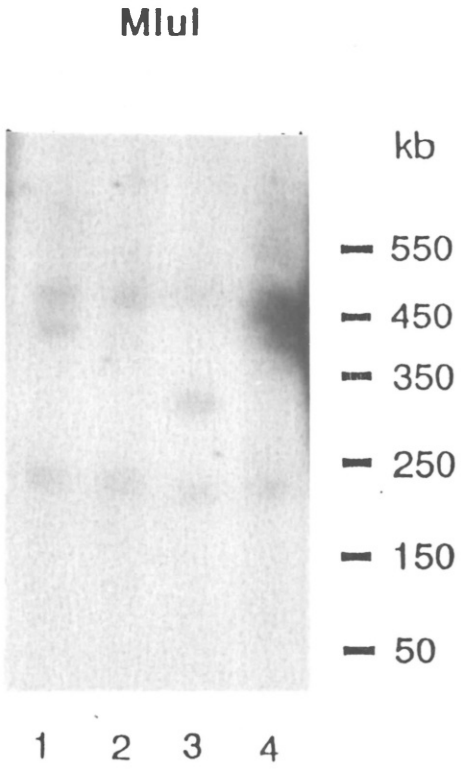


Fig. 8.4 Representative Blot of MluI Digests Hybridizing to Probe C.

MluI digests of DNA from a Ph-positive, M-BCR rearranged CML patient (lane 1), patient no. 422.11 (lane 2), patient no. 621.1 (lane 3), and a normal subject (lane 4) were hybridized to probe C. Two germ line bands were observed at positions 250kb and 500kb respectively. A rearranged band at 320kb position was observed in the DNA from patient no. 621.1 and a rearranged band at 450kb position was observed in the DNA from the Ph-positive, M-BCR rearranged patient.

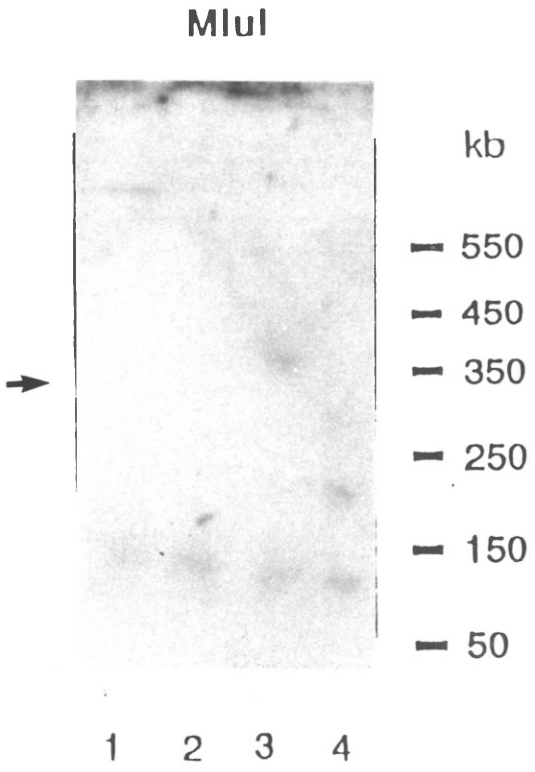


Fig. 8.5 Representative Blot of MluI Digests Hybridizing to ABL Probe.

When the filter shown in Fig. 8.4 was reprobed with the 1.5kb ABL body probe, one germline band at 70kb was present. A rearranged band at 320kb (arrow, the same position as in the BCR probe hybridization, Figure 8.4) is observed in lane 3.

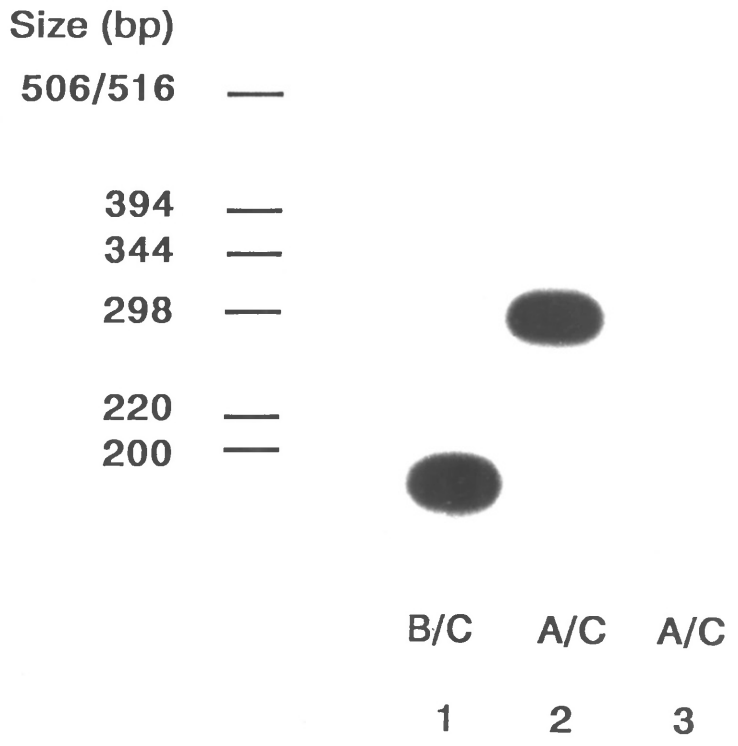


Fig. 8.6 PCR

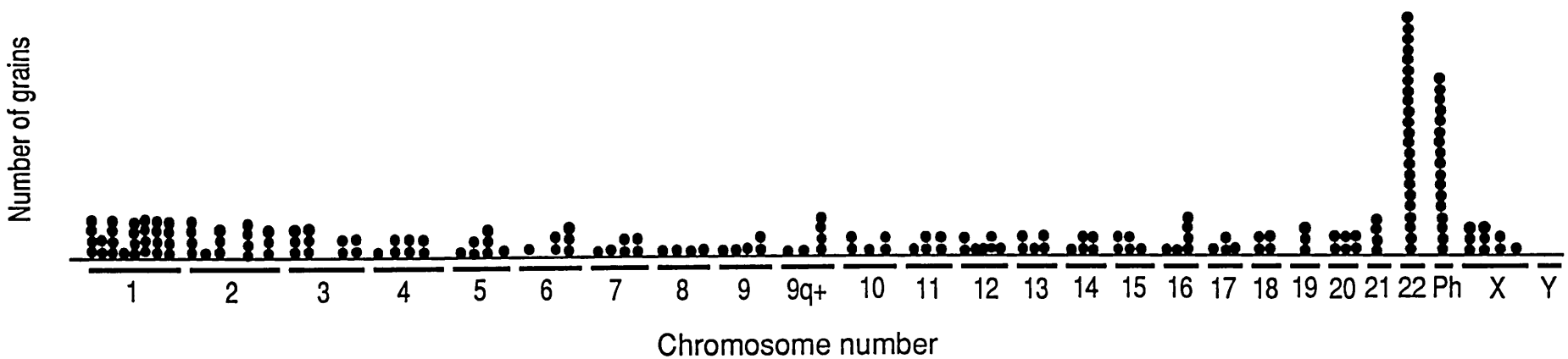
PCR analysis for presence of BCR/ABL mRNA in patient no. 621.1. PCR product hybridized with A2 universal probe. Lane 1 contains normal ABL PCR product amplified by the primers B and C. Lane 2 contains BCR/ABL (P210 type) product amplified by the primers A and C. The size of the product is 304 bp which is consistent with a b2a2 junction of BCR/ABL mRNA. Lane 3 is the negative control (PCR product amplified from mRNA of normal blood cells using primers A and C).

Fig. 8.7 In Situ Hybridization.

Composite diagram showing grain distribution in 71 metaphases for the full length cDNA BCR probe A in Ph-positive metaphases. The X axis represents the chromosomes in proportion to their size. The Y axis shows the number of silver grains. The normal chromosome 22 and the Ph chromosome have very large amounts of labelling in comparison with nonspecific background grains found on all other chromosomes. The number of grains on the 9q+ was consistent with nonspecific background.

In situ hybridization with probe A (Patient No 621.1)

Total number of metaphases analyses : 71
Total number of grains : 239
Mean number of grains per metaphase : 3.4



8.7). This result is consistent with a chromosome 22 breakpoint downstream of the BCR gene and accords with the results of PFGE. When a 1.5kb cDNA ABL body probe was used, this probe hybridized to Ph chromosome and chromosome 9, suggesting the ABL gene translocated to Ph chromosome.

8.3.5 STUDY OF BCR GENE INVOLVEMENT IN Ph-NEGATIVE, M-BCR NON-REARRANGED CML PATIENTS BY PFGE

In the 4 cases of Ph-negative, M-BCR non-rearranged CML no rearranged bands were observed when the DNA from these patients were digested with BssHII and hybridized to probe C (Fig. 8.1). This suggests that there is no rearrangement of the BCR gene in these patients. This was confirmed by study of NarI digests in two patients and MluI digests in three respectively (Tab. 8.1). In one case of Ph-negative CML (no. 422.19) in whom two abnormal bands were detected in BamHI digests analyzed with probe F in conventional Southern blots, BssHII digests demonstrated no rearrangement. This suggests that the additional BamHI fragments were probably due to a polymorphism rather than to M-BCR rearrangement (Benn et al, 1988; also see Chapter 6).

8.3.6 ABL INVOLVEMENT IN PATIENTS WITH M-BCR NON-REARRANGED CML

The above patients with Ph-negative, M-BCR non-rearranged CML were studied for evidence of the ABL gene rearrangement by

PFGE. DNA from these patients were digested with BssHII and hybridized with two ABL probes: the 1.5kb cDNA body probe and the 0.85kb genomic exon Ib ABL probe pHabl5'. There was no evidence of involvement of the ABL gene in any of these patients (Fig. 8.3).

8.4 DISCUSSION

Two of the Ph-positive patients who could not be classified by Southern analysis had rearranged bands when the BCR gene was studied by pulsed field gel electrophoresis (PFGE). The breakpoint on chromosome 22 in these two patients were clearly localized within the BssHII fragment encompassing the whole BCR gene. The lower intensities of the rearranged bands in both Southern blotting and PFGE may reflect a low proportion of leukaemic cells carrying the rearrangement in the peripheral blood. This was somewhat unexpected as one would assume that in most CML patients with high counts the majority of peripheral blood cells were leukaemic and therefore carried the rearrangement.

The third Ph-positive patient (patient no. 621.1) is of particular interest. By conventional Southern analysis with a range of probes I failed to detect rearrangement within or immediately downstream of M-BCR. However, study of the

patient's mRNA by PCR showed a typical b2a2 junction. This result effectively rules out the possibility of a breakpoint on chromosome 22 upstream of M-BCR. Furthermore, there was no evidence of rearrangement in the BCR gene in DNA digested with BssHII or NarI. There was however a rearranged band in DNA digested with MluI. These findings would be consistent with a breakpoint on chromosome 22 downstream of the 3' end of the BCR gene as conventionally defined. This 3' location of breakpoint outside the BCR gene was confirmed by in situ hybridization of the full length cDNA BCR probe to chromosome 22q- in this patient. For such a breakpoint to result in a typical BCR/ABL mRNA one would have to assume that a relatively large number of 3' BCR exons were excluded in the splicing process. Although long range splicing and differential use of (upstream) exons is an accepted feature of ABL gene expression (Groffen and Heisterkamp, 1987), such events have not been described in the BCR gene involved in the Ph translocation. An alternative explanation is the possibility that the breakpoint is still within the BCR gene but the gene extends further downstream than has conventionally been recognized. It is notable in this context that the 6.5kb transcript which is one of the alternative products of the normal BCR gene could result from alternative splicing involving uncharacterized 3' sequences of the BCR gene (Groffen and Heisterkamp, 1987).

This patient (no. 621.1) with atypical molecular findings had haematological features and a clinical course thus far that do not differ from Ph-positive M-BCR rearranged CML. This observation underlines the importance of the BCR/ABL mRNA and P210 in determining the principal phenotypic features underlying the chronic phase of CML and downgrades the importance of the precise details of the genomic changes that underlie formation of the chimeric mRNA. This general conclusion is supported by two other observations: Ph-negative, M-BCR rearranged patients are generally indistinguishable on clinical ground from Ph-positive, M-BCR rearranged patients (Wiedemann et al, 1988; Dreazen et al, 1988) and our own studies (in contrast to those of others) provide no support for the concept that Ph-positive patients with b2a2 mRNA junctions and patients with b3a2 junctions have different clinical courses (Jaubert et al, 1990).

My PFGE studies of DNA from patients with Ph-negative, M-BCR non-rearranged CML showed no evidence of formation of a BCR/ABL chimeric gene and indeed no evidence of involvement of the BCR gene. This means that a totally different molecular mechanism must be responsible for the pathogenesis of the leukaemia in such patients.

My studies designed to detect ABL oncogene rearrangement in the absence of BCR involvement in patients with Ph-negative

M-BCR non-rearranged CML showed no evidence of involvement of the ABL gene. This is in consistent with other studies using PCR and mRNA analysis (Wiedemann et al, 1988; Dobrovic et al, 1989).

CHAPTER NINE SUMMARY AND CONCLUSIONS

The normal function of the ABL gene product is unknown. Mutational inactivation of ABL gene in mouse embryo stem cells does not significantly affect embryogenesis nor does it bring about obvious histological changes in many systems of the mouse (Tybulewicz, personal communication). However, 5' alterations of the ABL gene can alter the pattern of protein phosphorylation and stimulate cellular growth or induce the capacity for cellular transformation. The altered ABL protein may phosphorylate tyrosine residues on the proteins which are part of the signal transduction pathway. The 5' alterations which can activate the ABL gene include viral transduction in the formation of Abelson murine leukaemia virus and association with the BCR gene in the Ph translocation. Recently Jackson & Baltimore (1989) demonstrated that specific 5' deletions in the ABL gene expressed in a retroviral vector can activate its leukaemogenic potential.

The normal functions of the BCR gene and its role in ABL activation in leukaemia are likewise unclear. Since 5' deletion alone can activate the leukaemogenic potential of the

ABL gene, the role of the BCR gene in the activation of ABL in the BCR/ABL rearrangement is questionable. If the 5' sequences of the BCR gene are not essential for ABL activation, could the BCR gene be responsible for the selection of different target cells which would result in different phenotypes of leukaemia? A major part of the work in this thesis is the analysis of BCR gene involvement in CML in different phases and in different cytogenetic and haematological classifications.

9.1 FURTHER GENETIC CHANGES IN BLAST TRANSFORMATION OF CML

I studied the presence of further chromosomal changes in blast transformation (BT) in six Ph-positive patients. These patients were followed from CP to acceleration or BT. Two of the 6 had further changes characterized by the appearance of an additional Ph chromosome. Three transplanted cases who relapsed directly into BT after BMT also showed additional changes. It appeared that further changes were rather common in our series; this was consistent with reports by others (Lawler, 1977; Hagemeijer et al, 1980; 1987). However, the small number of cases I studied did not enable me to conduct a meaningful statistical analysis of the overall frequency of further cytogenetic changes and their association with the sub-type of BT.

Four of these patients with additional chromosomal changes in BT were studied for possible further changes in BCR/ABL rearrangement. I found no further changes. The majority of the CML patients in BT studied by others showed similar results (Andrews et al, 1987; Hagemeijer, 1987). Only rare cases of additional rearranged bands appearing in BT have been reported (Bartram et al, 1986a; Shtalrid et al, 1988). The significance of these secondary M-BCR rearrangements is reduced because it is found only on occasion in some of the patients with an additional Ph chromosome but not in the majority of cases. The new M-BCR rearrangement may represent a new clone of CML cells developed in BT. At present the molecular mechanism underlying additional chromosomal changes in BT is unknown. Another possible molecular mechanism is inactivation of the P53 gene which is located in the p arm of chromosome 17 (Kelman et al, 1989). The i(17q) is one of the common secondary chromosomal changes in BT. A third proto-oncogene that may be responsible for the blast transformation is RAS. Liu et al (1988) reported a greater frequency of RAS mutations in BT than in CP. However, RAS mutations can hardly be associated directly with the non-random secondary chromosome changes in BT.

9.2 Ph-POSITIVE CML

The results of my studies of M-BCR rearrangement in CML using Southern blotting are in general agreement with previous reports (Groffen et al, 1984; Saglio et al, 1988; Wiedemann et al, 1988). The great majority of patients with Ph-positive CML have genomic breakpoints within M-BCR but there are rare cases in which the breakpoint cannot be mapped within M-BCR even with use of multiple probes and other cases in which the rearrangement within M-BCR is doubtful. In our 33 patients with Ph-positive CML 30 had a breakpoint mapped within M-BCR. In 2 patients, although 3' M-BCR probe did not detect the rearranged band, a 5' M-BCR probe did. These data suggested that the breakpoint still mapped within the M-BCR, but that the 3' portion of M-BCR was deleted during the translocation. The rearrangement of BCR gene in these two patients was confirmed by PFGE analysis. A 3' partial deletion in M-BCR was also reported by Hirosawa et al (1988). In one patient I was unable to locate the breakpoint within the M-BCR by Southern analysis nor locate it within the BCR gene by PFGE. However, study of the patient's mRNA by PCR showed a typical b2a2 junction. Furthermore, additional evidence consistent with a breakpoint on chromosome 22 downstream of the 3' end of the BCR gene as conventionally defined was obtained with PFGE and in situ hybridization analysis. For such a breakpoint to result in a typical BCR/ABL mRNA one would have to assume that

a relatively large number of 3' BCR exons were excluded in the splicing process. This patient with atypical molecular findings had haematological features and a clinical course thus far that do not differ from Ph-positive M-BCR rearranged CML.

I did not localize the precise position of the M-BCR breakpoint in the Ph-positive patients. Although there is controversy as to whether the precise location of the breakpoint within the M-BCR relates to prognosis in CML patients (Schaefer-Rego et al, 1987; Mills et al, 1988; Jaubert et al, 1990), the patients in this study were not suitable for conventional assessment of survival after diagnosis because most of them had undergone allogeneic bone marrow transplantation. As regards the clinical and haematological features I observed no atypical features in these 30 patients regardless of the locations of breakpoint.

9.3 COMPLEX TRANSLOCATIONS AND Ph-NEGATIVE CML

Chromosomal abnormalities such as a masked Ph or other translocations were uncommon in our series of Ph-negative CML. Only one case was found to have cytogenetic abnormalities; the other 18 patients were apparently cytogenetically normal. Not surprisingly the patients with a masked Ph chromosome and

complex translocations were clinically and haematologically indistinguishable from typical CML, because they had de facto the Ph translocation. The biological significance of these complex translocations is not clear. They do not usually produce any significant changes in the disease. Furthermore, except for the chromosome 9 and/or 22, most of the other chromosome aberrations found in these complex translocations are non-specific. This may indicate an event which destabilizes the genetic constitution of cells before the cells go into leukaemic transformation; some such events may be relevant to the leukaemia such as the Ph translocation, while others probably have no significance.

In the karyotypically normal CML patients the clinical and haematological features were heterogeneous. Eight of these patients had atypical CML, while the other 10 were indistinguishable from typical CML.

Because of the heterogeneity in cytogenetic findings and clinical features of Ph-negative CML patients, many researchers have tried to explain the common as well as the unique features of this group of leukaemias and to compare them with the Ph-positive CML (Pugh et al, 1985; Bartram et al, 1985; Bartram and Carbonell, 1986b; Kurzrock et al, 1986; Hagemeijer, 1987; Dreazen et al, 1987; Shepherd et al, 1987; Wiedemann et al, 1988). Taken together the results of studies

by us and by others suggest that Ph-negative CML constitutes a group of leukaemias that are heterogeneous both in clinical-haematological features and in cytogenetics. Apart from the presence or absence of Ph chromosome or of a detectable masked Ph chromosome, the cytogenetic findings of Ph-negative CML usually do not indicate their likely clinical-haematological classification. However, the discovery of a masked Ph chromosome in a small number of patients is associated with typical CML.

Various types of Ph-negative CML were examined by molecular techniques for BCR gene involvement. Of the 19 patients with Ph-negative CML 9 showed evidence of M-BCR rearrangement and one had evidence of a breakpoint 5' to the M-BCR. Nine patients had DNA that showed only germ-line bands. This is in agreement with other studies. Dreazen et al (1988) suggested that about 50% of the patients with Ph-negative CML have the same molecular changes as Ph-positive patients; the other 50% have no evidence of M-BCR rearrangement. The patients with Ph-negative M-BCR rearranged CML are clinically and haematologically indistinguishable from other Ph-positive CML patients. In situ hybridization analysis of these patients showed that in some cases the Ph negativity were due to interstitial insertion of chromosomal material from chromosome 9 to 22 (Rassool et al, 1990); others were due to complex chromosomal translocations that made the Ph chromosome lose

its typical appearance. This group of Ph-negative CML patients do in fact have a Ph translocation. These events indicate that there must be a specific molecular mechanism which facilitates the BCR/ABL gene rearrangement, and that these events can occur independently of an obvious chromosomal translocation.

Although I could not rule out involvement of the BCR gene by Southern analysis in the other 9 patients, I could rule out the possibility of M-BCR rearrangement. The association of atypical features with this group of Ph-negative M-BCR non-rearranged CML patients underlines the relationship of M-BCR rearrangement to the typical picture of CML. Clearly a different molecular mechanism is responsible for this kind of CML. Similar results have been observed by others (Morris et al, 1986; Wiedemann et al, 1988). Pulsed field gel electrophoresis, in situ hybridization and polymerase chain reaction analysis have been used to study further the molecular mechanisms in these M-BCR negative CML patients.

My PFGE studies of DNA from patients with Ph-negative, M-BCR non-rearranged CML showed no evidence of formation of a BCR/ABL chimeric gene and indeed no evidence of involvement of the BCR gene. I conducted studies designed to detect possible ABL oncogene rearrangement in the absence of BCR involvement in these patients by PFGE, but found no evidence of ABL involvement. A group in Australia have studied the

BCR/ABL chimeric gene products by PCR. In patients with Ph-negative, M-BCR non-rearranged CML they found no BCR/ABL mRNA of any kind (Dobrovic et al, 1989). This means that a molecular mechanism other than the BCR/ABL rearrangement must be responsible for the pathogenesis of the leukaemia in such patients.

It is generally believed that development of CML involves multiple steps, and that more than one oncogene is probably involved. P53 gene, RAS oncogene and a X transforming gene may be associated with CML (Liu et al, 1988; Ahuja et al, 1989; Cogswell et al, 1989). Despite the fact that Ph-negative, M-BCR non-rearranged CML patients have atypical features they do still resemble Ph-positive CML. Although the BCR/ABL rearrangement play no part in these CML, it remains possible that some of the other genetic aberrations associated with Ph-positive CML could be involved. This should be analysed in the future.

9.4 Ph-POSITIVE ACUTE LEUKAEMIA

Chronic phase CML eventually transforms into a blast phase. However, the BT of CML is a completely different form of leukaemia from de novo acute leukaemias. Some patients present with acute leukaemia also have Ph chromosome. Are these de

novo acute leukaemia or BT of CML? Do these patients with Ph-positive acute leukaemia have similar BCR/ABL rearrangement found in CML? With this questions in mind I also studied the M-BCR rearrangement of patients with acute leukaemia.

All the acute leukaemia patients without the Ph chromosome were M-BCR non-rearranged. One case of Ph-positive acute leukaemia also lacked evidence of M-BCR rearrangement. Further studies by PCR, in situ hybridization and PTK assay on this patient showed a first intron break in the BCR gene and the corresponding chimeric protein P190. Other studies of M-BCR rearrangement in Ph-positive acute leukaemia showed that about 50% were rearranged, while the other 50% were non-rearranged and probably had a breakpoint in the first intron of the BCR gene (Hermans et al, 1987; Rubin et al, 1987; Heisterkamp et al, 1988a). As in most cases of CML, the leukaemic cells from patients with M-BCR rearranged acute leukaemia produce P210, while cells from patients with a rearrangement in the first intron of the BCR gene produce P190. Experiments using in vitro transformation of lymphoid cells showed that the P190 type of cDNA constructs were more effective in growth stimulation than those of P210 (McLaughlin et al, 1989). These findings suggest a more aggressively malignant nature of P190 than P210. It has been suggested that Ph-positive acute leukaemia with M-BCR rearrangement is in fact CML in blast transformation, i.e. that M-BCR rearrangement is the primary

event that induced an occult CML and other genetic changes are responsible for presentation in blast transformation. In contrast Ph-positive acute leukaemias with rearrangement in the first intron of the BCR gene are de novo acute leukaemias.

9.5 ROLE OF BCR GENE IN CML

Deletion of certain sequences at the N-terminal of ABL cDNA constructs can activate fibroblast transforming ability (Jackson & Baltimore, 1989). This implies that the involvement of the BCR gene is not always necessary for ABL activation. However, when the ABL gene is linked at its 5' end with the BCR gene, an alternative type of BCR gene involvement may render different cellular transforming ability, e.g. P190 transforms cells more efficiently than P210 (McLaughlin et al, 1989; also see Chapter 2). It is therefore interesting to study the clinical features in patients with types of BCR gene involvement other than the M-BCR or m-BCR rearrangements.

The first intron breakpoint in BCR gene is associated with acute leukaemia while the M-BCR breakpoint is associated with typical CML. This may suggest that there is one or more specific region(s) in the BCR gene which is important in determining the phenotype of leukaemia. This region should be close to but 3' of exon 1 of the BCR gene. However, except for

one case in my study which had features of eosinophilic leukaemia and a 5' breakpoint outside M-BCR, other reports of breakpoints outside M-BCR in Ph-positive CML are not associated with atypical features. The inclusion or exclusion of exons around M-BCR may not be important.

There is no conclusive evidence as yet to confirm that variation in the position of breakpoints within M-BCR relates to the prognosis of CML patients. Furthermore the use of Southern analysis to define the positions of the breakpoint in most reported studies is rather crude, especially when using it as an index for the possible inclusion of M-BCR exons b3 or b4. For example, if the breakpoint is located between the exons b3 and b4, mRNA with the b2a2 junction could still be produced by alternative splicing. These facts complicate interpretation of studies designed to assess the prognostic significance of the location of the breakpoint within the M-BCR. The use of PCR analysis for different types of junctions in larger numbers of patients should help to elucidate this problem.

I studied a patient with a 3' breakpoint probably outside the BCR gene. A typical BCR/ABL chimeric mRNA was produced and typical clinical features were present. This observation emphasizes the importance of the BCR/ABL mRNA and P210 in determining the principal phenotypic features underlying the

chronic phase of CML and downgrades the importance of the precise details of the genomic changes that underlie formation of the chimeric mRNA.

Whatever the role of the BCR gene in activating the leukaemogenic potential of the ABL gene in the Ph translocation, the BCR gene promoter must control the transcription of BCR/ABL hybrid gene. The use of an antisense oligonucleotide against 5' BCR mRNA can reduce the level of BCR/ABL gene expression in vitro and indeed kill CML cells in vitro (Taj et al, 1990).

9.6 BMT FOR CML

Bone marrow transplantation (BMT) is the only curative therapy for CML at present. Relapse, GVHD and graft failure still constitute the major threat to survival after transplant. Various methods have been used to study these problems. I used cytogenetics and DNA analysis to study relapse and engraftment after BMT for CML.

About 50% of the patients in our series had evidence of cytogenetic relapse. It was more common than had been expected. Patients who received T cell depleted marrow transplants had a higher incidence of relapse than those who

received non-depleted transplants. Transient cytogenetic relapse after transplantation is another interesting feature. Similar transient relapses have been reported by others (Thomas et al, 1986; Zaccaria et al, 1987; Frassoni et al, 1988). Cytogenetic relapse can appear at any time. Apart from the transient relapse, some patients had other outcomes. Cytogenetic relapse may be followed by haematologic relapse. Such relapse may have features of chronic-phase disease, but three patients in this study relapsed directly into accelerated or blastic phases. However, in the majority of patients cytogenetic relapse has not yet progressed to haematologic relapse. Such progression might still occur in some if the patients are followed for longer periods.

Thus the finding of Ph-positive metaphases in the marrow after BMT does not necessarily mean that the transplant has failed. However, the failure to find Ph-positive metaphases on any one occasion must be interpreted with caution, since Ph-positive cells might escape detection if present in only small numbers or if they were not in cycle.

DNA analyses have also been used for the study of relapse and engraftment after BMT. I used DNA fingerprinting to examine cellular origin and M-BCR analysis to identify the presence of CML cells in peripheral blood. The majority of patients who are not destined to relapse usually have band patterns

consistent with complete engraftment. These findings are very similar to those reported previously using other DNA RFLPs (Ginsburg et al, 1985). In my group of patients (all of whom received T-cell depletion of donor marrow in an attempt to abrogate GVHD) the recurrence of mixed chimerism (the coexistence of cells of donor and recipient origin) was comparatively common and usually heralded or coincided with haematologic relapse. In contrast, less than half of these T depleted BMT patients with cytogenetic relapse developed haematologic relapse (see Chapter 4). This may suggest that DNA analysis represents more accurately the true level of the residual leukaemic cells which are likely to cause relapse.

The high frequency of relapse in this cohort of patients seemed to be associated with the use of T-cell depletion (Apperley et al, 1986a; also see Chapter 4). This was also observed in cytogenetic analyses for relapse. It must be emphasized, however, that mixed chimerism without leukaemic relapse has been documented in other patients studied by cytogenetic methods, both by us (see Chapter 4) and by others (Vincent et al, 1986).

My results (both cytogenetic and DNA analysis) provided no evidence for relapse occurring in cells of donor origin. In overt relapse, most, if not all, of the patients studied had peripheral blood cells of recipient origin. Donor cells were

not usually identifiable in peripheral blood samples using DNA fingerprinting with reasonable sensitivity. However, in one patient who received donor marrow cells depleted of T-cells in vitro, analysis of DNA from a highly enriched T-cell fraction showed that T-lymphocytes in the peripheral blood at relapse were mixed-chimeric with the donor type T-cells predominating. Another report also showed similar findings (Hughes et al, 1989). This may suggest that the T-cell population is more sensitive to the pretransplant conditioning regimen than the in vitro T-cell purging treatment. After in vitro depletion some T cells may still survive. It would be interesting to study why these residual donor T cells exert little GVHD or graft versus leukaemia effects.

DNA analysis for M-BCR rearrangement is a highly specific indicator of the presence or absence of leukaemic cells in patients with CML. Temporal concordance of DNA of recipient origin recognized with minisatellite probes and the reappearance of a rearranged pattern within the BCR gene were shown in all the patients studied who relapsed. The pattern of M-BCR rearrangement was identical before transplant and in relapse. These results confirm results reported by Zalcberg et al (1986). They imply that relapse after transplantation is due to re-emergence of the original leukaemic clone and not to reinduction of a new leukaemia in haemopoietic stem cells.

After BMT the growth of residual leukaemic cells must be controlled through a specific mechanism. This might be competition for growth between the donor cells and host leukaemic cells (Frassoni et al, 1988), or an immunological mechanism, such as a GVL effect, may operate to control or eliminate residual leukaemic cells (Apperley et al, 1988). The high relapse rate in our patients transplanted with marrow depleted of T-cells is the obvious example of lack of GVL effect. Since T cell depletion is associated with a high relapse rate, both the GVHD and GVL effects must be mediated through T cell populations. It is important to study whether these effects are mediated by different T cells populations. A more selective purge of T cells to abrogate GVHD but retain the GVL effect may be the way for successful BMT for leukaemia in the future. At present some centres are trying to use monoclonal antibodies against T cells in vivo in the hope that some such 'selectivity' may be achieved. It is too early to judge the results.

With regard to the various outcomes of relapse Strife and Clarkson (1988) suggested a different explanation based on the 'discordant maturation' hypothesis. For those patients who had an episode of cytogenetic relapse but then reverted to long term complete remission or those with long term cytogenetic relapse without producing full clinical and haematological picture of CML, the Ph positive metaphases could be derived

from residual leukaemic late progenitor cells so that they can transiently produce Ph positive metaphases but their proliferative capacity is limited. The primitive Ph-positive stem cells may have been killed by conditioning treatment. Alternatively a few primitive Ph-positive stem cells may have survived, but it might require a very long period for them to become dominant as in the original evolution of the disease. According to this hypothesis we can presume that the larger the amount of residual CML cells surviving after BMT, the more CML stem cells remain alive and the more likely they are to cause relapse. This may be the reason why DNA analysis seems more accurate in predicting haematologic relapse than cytogenetic studies, which can only examine the cells in cycle. PCR has recently been used to study residual CML cells after BMT. Because PCR is too sensitive and not yet standardized, it seems at present unreliable in predicting relapse. In the future a more systemic study comparing these different methods is necessary to decide their true value and the criteria for predicting relapse.

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Cytogenetic Events After Bone Marrow Transplantation for Chronic Myeloid Leukemia in Chronic Phase

By C.K. Arthur, J.F. Apperley, A.P. Guo, F. Rassool, L.M. Gao, and J.M. Goldman

Forty-eight patients treated by allogeneic bone marrow transplantation (BMT) for Philadelphia (Ph) chromosome-positive chronic myeloid leukemia in chronic phase had serial cytogenetic studies of marrow performed at intervals after transplant. Twenty patients received marrow cells from donors of opposite sex. Ph+ marrow metaphases were identified in 24 of 48 (50%) of patients after BMT; they were first seen early (within 1 year) in 16 cases and late (>1 year after BMT) in eight cases. Ph-positivity after BMT occurred more commonly in recipients of T-depleted than nondepleted marrow (19 of 28 *v* 5 of 20). In 4 cases the Ph+ metaphases were found only transiently after BMT; in 11 cases the Ph+ metaphases have persisted but hematologic relapse has not ensued; in 9 cases the finding of Ph+ metaphases coincided with or preceded hematologic relapse. Chromosomes in cells of donor origin had morphological abnormalities in two cases. No relapses were identified in cells of donor origin. Our data suggest that the relationship between cells of recipient and donor origin is complex: cure of leukemia may depend on factors that operate for some months or years after BMT.

INCREASING NUMBERS of patients with chronic myeloid leukemia (CML) have been treated by chemoradiotherapy and allogeneic bone marrow transplantation in recent years.[1-3] The probability of disease-free survival at 4 years for patients transplanted in chronic phase is 55%.[4] The probability of relapse at 4 years is relatively low (9%), however, in patients transplanted with unmanipulated marrow cells,[4] which may mean that the standard transplant procedure (and ensuing events) are generally more effective at controlling (and possibly curing) CML in chronic phase than acute myeloid leukemia in first remission. The precise mechanism by which transplantation is effective is unclear.

For patients with CML, the Philadelphia (Ph) chromosome is a marker for leukemic cells and its finding during the evolution of the disease is the first evidence of leukemia.[5] One might assume that the persistence or reappearance of Ph+ cells in the marrow after BMT would indicate that the attempt to eradicate the disease had failed and that clinical relapse would soon follow.[3,6] We report the results of serial cytogenetic analysis of marrow cells from patients transplanted for CML. The pattern of findings is complex and casts doubt on the notion that cure of leukemia by BMT depends exclusively on the immediate effects of the chemoradiotherapy used.

MATERIALS AND METHODS

Patients. The 48 patients reported in this study were drawn from a series of 73 patients with Ph-chromosome positive CML in first chronic phase treated by allogeneic BMT between May 1981 and July 1986 (Tables 1 and 2). Patients were included if they survived >100 days and were available for serial cytogenetic studies. All patients gave their informed consent to bone marrow aspiration carried out for this purpose. Twenty-five patients were excluded for the following reasons: survival <100 days, 9 patients; technically unsatisfactory cytogenetic studies on more than one occasion, 4 patients; alive but unavailable for cytogenetic studies, 9 patients; and graft failure, 3 patients. All patients were HLA-identical with their respective donors and had nonreactive mixed lymphocyte cultures. All received daunorubicin 60 mg/m^2, cyclophosphamide 120 mg/kg, and fractionated total body irradiation (TBI) to a total dose of 10 or 12 Gy.[2] Twenty-eight patients received donor bone marrow that was T cell depleted with the monoclonal antibody Campath-1,[7] and 20 received non-depleted marrow cells. Each patient was identified by a leukemia transplant number (LTN) allocated in sequence. Survival was assessed on March 1, 1987.

Cytogenetic studies. Cytogenetic analysis of bone marrow cells was performed on each patient immediately before the transplant procedure and was attempted after BMT at intervals that varied between 3 and 12 months. Mononuclear cells were separated from the bone marrow by centrifugation over Ficoll Hypaque and incubated at a concentration of 10^6/mL in RPMI 1640 (GIBCO-Biocult, GIBCO, Paisley, Scotland), 20% fetal calf serum (FCS) (GIBCO), penicillin, and streptomycin. Direct preparations were not routinely performed. Cells were harvested after incubation for 24, 48, or 72 hours. Colcemid (10 μg/mL) was added to the cell suspension for 1 hour, after which the cells were washed and resuspended in 0.075

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Table 1. Results of Cytogenetic Analysis of Marrow Metaphases in 20 Recipients of Unmanipulated Donor Marrow

Patient No.	LTN	Sex (Donor Match)	Interval Post-transplant (mo) 3	6	9	12	18	24	36	48	60	Evidence of Relapse	Survival (mo)
1	5	M (S)	25/25* Ph− (1 mo)	16/16 Ph−	—	5/5 Ph−	—	5/5 Ph−	—	10/10 Ph−	15/15 Ph−	Nil	70+
2	6	M (S)†	14/14 Ph− (1 mo)	—	—	10/10 Ph−	—	—	3/3 Ph−	15/15 Ph−		Nil	68+
3	12	M (MM)	—	—	—	—	—	14/14 Ph−D	—	10/10 Ph−D		Nil	63+
4	16	M (S)	—	—	—	—	—	15/15 Ph−	—	30/30 Ph−		Nil	50(D)
5	18	F (MM)	9/9 Ph−D (1 mo)	—	—	—	—	—	—	1/21 Ph+H 20/21 Ph−D		Cyto	58+
6	21	F (S)	—	—	—	10/10 Ph−	—	—	—	—		Nil	57+
7	22	F (S)	—	—	—	—	—	15/15 Ph−	—	—		Nil	55+
8	23	F (S)	—	5/5 Ph−	10/10 Ph−	—	—	—	10/10 Ph−	2/2 Ph−		Nil	54+
9	24	F (MM)	12/12 Ph−D	—	—	—	—	10/10 Ph−D	—	20/20 Ph−D		Nil	54+
10	26	F (MM)	—	—	1/11 Ph−H 10/11 Ph−D	—	—	—	16/23 Ph−H 7/23 Ph−D			Nil	53+
11	27	M (S)	—	—	—	1/5 Ph+ 4/5 Ph−	1/20 Ph+ 19/20 Ph−	20/20 Ph−	20/20 Ph−	20/20 Ph−		Cyto (transient)	52+
12	34	M (S)	10/10 Ph− (2 mo)	3/3 Ph−	—	—	15/15 Ph−	—	20/20 Ph−†			Nil	48+
13	39	F (S)	7/7 Ph−	—	—	—	—	—	—			Nil	46+
14	44	F (MM)	—	15/15 Ph−	—	—	—	14/14 Ph−	—			Nil	44+
15	47	M (S)	5/5 Ph−	—	15/15 Ph−	—	—	5/5 Ph+ (with additional abnormalities)				Hemat	29(D)
16	48	F (S)	—	—	9/9 Ph−	—	—	—	—			Nil	42+
17	50	F (S)	—	—	—	10/10 Ph−	—	6/6 Ph−	10/10 Ph−			Nil	41+
18	57	M (S)	—	8/8 Ph−	—	—	—	3/20 Ph+ 17/20 Ph−				Cyto	25(D)
19	106	M (S)	—	15/15 Ph−	—	—	—					Nil	17+
20	125	M (S)	1/5 Ph+ 4/5 Ph−	15/15 Ph−	—							Cyto (transient)	9+

MM, sex mismatch donor; S, same sex donor; D, donor; H, host; NM, no metaphases found; dash, cytogenetic analysis not done or study technically unsatisfactory; cyto, cytogenetic relapse; hemat, hematologic relapse; (+), patient surviving at date of analysis; (D), patient dead at interval specified posttransplant.

Time of analysis appears at the top of the column except where stated.

*Total number of metaphases examined appears on right-hand side of slash mark; number of Ph− or Ph+ metaphases appears on left-hand side.

†Three of 20 metaphases with a marker chromosome.

mol/L KCl for 20 minutes at 37°C. After being washed, the cell slides were prepared and fixed in 1:3 acetic acid/methanol solution. G banding was performed by Seabright's trypsin-Giemsa technique.[8]

Definition of relapse. The finding of even a single definite Ph+ metaphase after BMT was classified as "relapse." If on subsequent analyses no further Ph+ metaphases were identified, the relapse was classified as "transient." If further examinations showed persisting Ph+ metaphases but the patient developed no clinical or hematologic features of CML, the relapse was classified as "cytogenetic." If the finding of Ph+ metaphases coincided with or progressed to hematologic disease (eg, leukocytosis, basophilia), the relapse was classified or reclassified as "hematologic."

RESULTS

Frequency of cytogenetic analyses and numbers of metaphases examined. The frequency of successful cytogenetic analyses and the number of metaphases analysed on each occasion for each patient in this series are shown in Tables 1 and 2. On 137 occasions, 1,826 metaphases were analyzed from the 48 patients. The number of metaphases examined on each occasion varied from 2 to 34 (mean 13).

Timing of relapse. Sixteen patients had evidence of relapse within 1 year of BMT (Tables 1-3). Such "early" relapses were more common in recipients of T-depleted marrow cells than in recipients of unmanipulated marrow. The proportion of patients whose marrow cytogenetics were sucessfully studied within 1 year of BMT was higher, however, in the former than in the latter group. Eight patients have relapsed >1 year after BMT (late relapses). This figure may increase further with passage of time.

Patients receiving unmanipulated donor marrow cells. In the 20 patients who received nondepleted donor marrow cells, a variety of cytogenetic and clinical sequences occurred with at least five different eventual outcomes (Fig 1). Eighteen patients had a Ph− marrow when first examined after BMT; 15 patients remain Ph−, and 3 have since become Ph+. In one (LTN 26) of the Ph− group, autologous recovery with Ph− host metaphases has occurred (Table 4).

Three of the 18 Ph− group became Ph+ on follow-up examination. Cytogenetic relapse has occurred in two patients, and the third patient relapsed directly into blast crisis 21 months after BMT. He had no evidence of a preceding chronic phase and at relapse had a clonal abnormality with 47,XY and a marker chromosome in addition to the Ph chromosome. Finally, two patients (LTN 27 and 125) in this group were Ph+ when first examined at 3 months and 12 months after BMT. Both subsequently became Ph− with clinical follow-up of 9 months and 4 years posttransplant, respectively (Table 5).

Patients receiving T cell-depleted donor marrow cells. In the 28 patients who received T-depleted marrow cells a wider variety of cytogenetic and clinical sequences occurred. There were nine different eventual outcomes (Fig 2). Twenty patients achieved a Ph− marrow when first examined after BMT. Only nine remain entirely Ph−. Two of these (LTN 75 and 93) had transient mixed chimerism with both donor cells and Ph− host cells present (Table 4). Eleven of the Ph− group became Ph+ on follow-up examination. This group includes 5 patients with cytogenetic

Table 2. Results of Cytogenetic Analysis of Marrow Metaphases in 28 Recipients of T Cell-Depleted Donor Marrow

Patient No.	LTN	Sex (Donor Match)	Interval Post-transplant (mo) 3	6	9	12	18	24	36	Evidence of Relapse	Survival (mo)
1	58	M (MM)	10/10* Ph−D	15/15 Ph−D	10/10 Ph−D	10/10 Ph−D	—	10/10 Ph−D	20/20 Ph−D	Nil	38+
2	63	M (S)	5/5 Ph−	6/6 Ph−	5/5 Ph−	10/10 Ph−	—	15/15 Ph−	15/15 Ph−	Nil	36+
3	64	F (MM)	—	—	—	—	—	20/20 Ph−D		Nil	35+
4	68	M (S)	—	8/8 Ph−	—	—	14/30 Ph+ 16/30 Ph−	15/15 Ph+	15/15 Ph+ (30 months)	Cyto → hemat	33+
5	69	F (S)	—	8/8 Ph−	10/10 Ph−	—	—	13/15 Ph+ 2/15 Ph−		Hemat	28(D)
6	70	F (S)	—	3/3 Ph−	—	—	20/20 Ph−	24/24 Ph−		Nil	32+
7	72	F (MM)	4/4 Ph−D	—	—	—	1/25 Ph+H 24/25 Ph−D	5/14 Ph+H 9/14 Ph−D		Cyto (sustained)	31+
8	73	F (MM)	5/5 Ph−D	—	—	—	—	4/5 Ph+H 1/5 Ph−D		Hemat	30+
9	75	F (MM)	—	—	8/9 Ph−D 1/9 Ph−H	—	9/9 Ph−D	11/11 Ph−D		Nil	29+
10	77	M (MM)	—	—	—*	2/3 Ph−D 1/3 Ph+H	16/17 Ph−D 1/17 Ph+H			Cyto (sustained)	28+
11	80	M (S)	—	11/11 Ph−	—	10/10 Ph−	22/22 Ph−	20/20 Ph−		Nil	27+
12	83	M (MM)	—	—	—	8/15 Ph+H 7/15 Ph−D	25/25 Ph+H	15/15 Ph+H		Cyto → hemat	27+
13	84	M (S)	—	—	3/3 Ph−	—	—			Nil	26+
14	86	F (S)	—	—	5/5 Ph−	—	—	10/10 Ph+ (additional abnormalities)		Hemat	24+
15	93	F (MM)	—	6/8 Ph−D 2/8 Ph−H	—	15/15 Ph−D	20/20 Ph−D			Nil	22+
16	94	M (S)	—	4/8 Ph+ 4/8 Ph−	13/20 Ph+ 7/20 Ph−	10/10 Ph+ (additional abnormalities)	10/10 Ph+			Cyto → hemat	21+
17	97	F (MM)	—	—	2/34 Ph+H 32/34 Ph−D	4/4 Ph−D	18/20 Ph+H 2/20 Ph−D	15/15 Ph+D		Cyto (sustained)	20+
18	102	M (MM)	—	4/13 Ph+H 9/13 Ph−D	1/13 Ph+H 12/13 Ph−D	1/20 Ph+H 19/20 Ph−D	15/15 Ph−D	—		Cyto (transient)	18+
19	105	F (MM)	—	10/10 Ph−D	1/9 Ph+H 8/9 Ph−D	4/15 Ph+H 11/15 Ph−D	—			Cyto → hemat	17+
20	107	F (S)	10/25 Ph+ 15/25 Ph−	(died at 4 months)						Cyto	4(D)
21	108	F (S)	19/19 Ph−	11/11 Ph−	5/25 Ph+ 20/25 Ph−	7/10 Ph+ 3/10 Ph−	5/5 Ph+			Cyto (sustained)	17+
22	109	M (S)	—	4/23 Ph+ 19/23 Ph−	—	20/20 Ph+				Cyto → hemat	16+
23	112	F (S)	4/16 Ph+ 12/16 Ph−	20/20 Ph−	—	2/23 Ph+ 21/23 Ph−				Cyto (sustained)	15+
24	116	F (MM)	20/20 Ph−D	1/15 Ph+H 14/15 Ph−D	15/15 Ph−D	10/10 Ph−D				Cyto (transient)	12+
25	118	F (MM)	5/5 Ph−D	21/21 Ph−D	12/12 Ph−D					Nil	11+
26	119	M (MM)	11/11 Ph−D	3/10 Ph+H 7/10 Ph−D	4/15 Ph+H 11/15 Ph−D					Cyto (sustained)	11+
27	120	M (MM)	NM	2/15 Ph+H 13/15 Ph−D	2/17 Ph+H 15/17 Ph−D					Cyto (sustained)	10+
28	123	M (S)	20/20 Ph− (5 weeks)	NM	1/10 Ph+ 9/10 Ph−					Cyto	9+

MM, sex-mismatched donor; S, same sex donor; D, donor; H, host; NM, no metaphases found; dash, cytogenetic analysis not done; cyto, cytogenetic relapse; hemat, hematologic relapse; +, patient surviving at date of analysis; (D), patient dead at interval specified posttransplant.

Time of analysis appears at the top of the column except where stated.

*Total number of metaphases examined appears on right-hand side of slash mark; number of Ph− or Ph+ metaphases appears on left-hand side.

Table 3. Details of Patients and Timing of Relapse

	Recipients of: Unmanipulated Donor Marrow	Donor Marrow T Depleted	Total
n	20	28	48
Age, *median*	29	31	31
Range	(13-43)	(15-53)	(13-53)
Sex (M/F)	10/10	13/15	23/25
Donor/recipient sex match (%)	15 (75)	13 (46)	28 (58)
Duration of follow-up, median (mo)	53	23	33
(Range)	(9-70)	(9-39)	(9-70)
Total number with any Ph+ metaphases after BMT (%)	5 (25)	19 (68)	24 (50)
No. adequately studied within 12 mo of BMT (%)	13 (65)	27 (96)	40 (80)
No. with Ph+ metaphases first detected:			
Within 3 mo	1	2	3
From 3 to 12 mo	1	12	13
After 12 mo	3	5	8

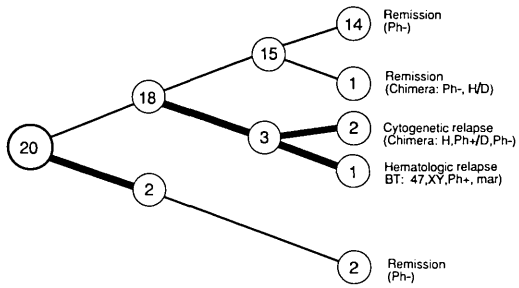

Fig 1. Sequential status of Ph positivity in patients receiving non-T cell-depleted bone marrow. Scheme shows evolution (left to right) of cytogenetic events starting immediately after BMT. Thin lines represent evolution of patients without any detectable Ph+ metaphases; thick lines reflect patients with some or all marrow metaphases showing the Ph chromosome. H, host; D, donor; BT, blast transformation; Mar, marker chromosome. Host and donor metaphases are specified where sex marker differences allowed identification.

relapse, 5 patients with hematologic relapse, and 1 patient (LTN 116, Table 5) who became Ph− again after a period of Ph positivity. One of the patients (LTN 86) in hematologic relapse has additional chromosomal markers consistent with accelerated phase, ie, a clone with 47,XX,Ph plus a C group marker.

Eight patients were Ph+ when first examined after BMT. Six remained Ph+. Two of these have developed chronic phase CML, 1 (LTN 94) had accelerated phase disease with additional chromosomal abnormalities (ie, 47,XY Ph plus a C group marker), and 3 had cytogenetic relapses. One patient in the last category died 4 months after BMT from septicemia. Two of these 8 became Ph− on follow-up; this has persisted in 1 patient (LTN 102, Table 5) but was transient in the other.

Transient relapse. In four patients, Ph+ metaphases were present on first examination or appeared on subsequent analysis but were not identified in later studies (Table 5). Two of these patients (LTN 27 and 125) had grade I to II acute graft-*v*-host disease (GVHD). The third (LTN 116) had both acute and chronic GVHD. The fourth (LTN 102) had no GVHD. Patient LTN 27 has been described in detail elsewhere.[6]

A Ph− clonal abnormality developing posttransplant. One patient (LTN 34) who had received unmanipulated donor marrow cells developed a clonal abnormality in Ph− marrow cells first recognized 36 months after transplant (Table 6). The peripheral blood was normal. He was suffering from mild chronic GVHD. Because patient and donor were of the same sex, sex chromosome differences could not be used to determine the host or donor origin of the clone.

Table 4. Appearance of Ph− Recipient Cells After Transplant

	No. of Recipient/Donor Metaphases at Intervals After BMT (mo)					
Patient	6	9	12	18	24	36
LTN 26	—	1/10	—	—	—	16/7
LTN 75	—	1/8	—	0/9	0/11	—
LTN 93	2/6	—	0/15	0/20		

LTN, leukemia transplant number of patient.

Table 5. Spontaneous Disappearance of Ph Chromosome

	No. of Ph+ Marrow Metaphases at Various Intervals After BMT (mo)							
Patient	3	6	9	12	18	24	36	48
LTN 27	—	—	—	1/5 (20%)	1/20 (5%)	0/20 (0%)	0/20 (0%)	0/20 (0%)
LTN 102	—	4/13 (30%)	1/13 (10%)	1/20 (5%)	0/20 (0%)			
LTN 116	0/20 (0%)	1/15 (7%)	0/15 (0%)	0/10 (0%)				
LTN 125	1/5 (20%)	0/15 (0%)	0/10 (0%)					

LTN, leukemia transplant number of patient.

Donor or recipient origin of relapse. Eleven of the patients who relapsed had donors of the opposite sex. One patient had received unmanipulated and ten T-depleted marrow cells. In each case, the metaphases showing the Ph chromosome had the sex chromosomal complement of the recipient.

Chromosomal abnormalities occurring in cells of donor origin. Two patients who had received marrow cells from donors of opposite sex had chromosomal abnormalities in cells of donor lineage (Table 7). The abnormalities consisted of chromosomal gragments, chromatid breaks, and random chromosomal losses (Fig 3A and B). Both had received T cell-depleted donor bone marrow, but both had active chronic GVHD. They were receiving prednisolone and cyclosporin A but no cytotoxic agents. One patient (LTN 118) was in remission with 100% donor cells. She had had disseminated infection with herpes varicella-zoster 6 months after transplantation that was successfully treated with acyclovir. The other patient (LTN 120) was a mixed chimera with cytogenetic relapse. He had also had disseminated infection with herpes varicella-zoster at 5 months and oropharyngeal infection with herpes simplex at 6 months after BMT. He intermittently excreted cytomegalovirus (CMV) in the posttransplantation period. Both patients had abnormalities at the time of the first cytogenetic study, and in each case these were still present 3 months later.

DISCUSSION

We have seen a variety of cytogenetic patterns in the marrow of patients followed sequentially after BMT for

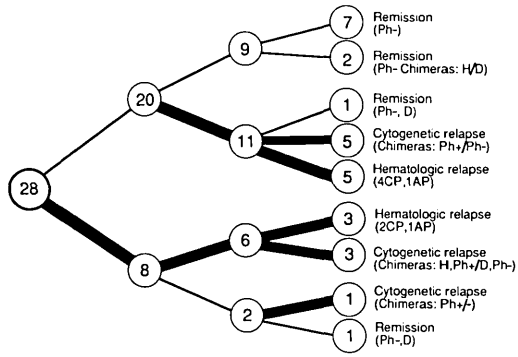

Fig 2. Sequential status of Ph positivity in patients receiving T cell-depleted bone marrow. CP, chronic phase; AP, accelerated phase. Symbols as in legend to Fig 1.

Table 6. Development of a Ph− Clone in Patient LTN 34

Cytogenetic Findings				
	Interval After BMT (mo)			
Before BMT	3	6	18	36
46,XY,Ph	46,XY (10)	46,XY (3)	46,XY (14) 44,XY,−D,−F (1)	46,XY (17) 46,XY,+D (3)

Numbers of metaphases showing each pattern are given in parentheses.

CML. The Ph chromosome may be present when the marrow is first examined, it may appear later, or it may never be identified after transplantation. Of particular interest is the finding that the Ph chromosome may appear only transiently after transplantation. Four patients in this series who had some Ph+ metaphases in their marrow after transplantation subsequently became Ph−, and one has now been followed for 4 years since transplantation without subsequent relapse. Similar transient relapses have been reported by others.[3,9,10] Thus, the finding of small numbers of Ph+ metaphases in the marrow within 2 years of BMT does not necessarily mean that the transplant has failed. Although the mechanisms leading to eradication of the Ph+ clone, and presumably to cure of CML, are unknown, they apparently act for months or even years after BMT. Patients with acute leukemia who sustain GVHD are known to have a lower incidence of relapse,[11] and GVHD might play a role in suppressing the leukemia in such patients. Three of the 4 CML patients referred to above had acute GVHD but 1 did not; 1 patient had chronic GVHD. Against the role of GVHD is the fact that the loss of the Ph chromosome in two patients occurred from 6 to 18 months after BMT, long after the acute GVHD had resolved.

The failure to find Ph+ metaphases on any one occasion must be interpreted with caution, since Ph+ cells might escape detection if present in only small numbers or if none were in cell cycle. The various confidence limits for excluding cytogenetic mosaicism for any given number of metaphases analyzed have been calculated.[12]

The recurrence of Ph+ metaphases after an interval of Ph negativity or the persistence of Ph+ metaphases after BMT may, however, have other outcomes. Thus, the detection of Ph+ marrow metaphases may be followed by hematologic relapse. Such relapse may have features of chronic-phase disease, but three patients in this study relapsed directly into accelerated or blastic phases with additional chromosomal abnormalities; no preceding chronic phase had been detected. In contrast, in 11 patients cytogenetic relapse has not progressed to hematologic relapse. Such progression might still occur if the patients are followed for longer periods, but one patient has remained in "stable" cytogenetic relapse without progression for 18 months.

The finding of Ph− recipient cells in three patients who were Ph+ before BMT raises further questions regarding the eradication of leukemia and the interactions between recipient and donor hematopoiesis. We assume for the purposes of this discussion that these Ph− cells of recipient origin were normal hematopoietic cells although in other patients treated conventionally for Ph+ CML there is evidence that some Ph− cells may be leukemic or "preleukemic."[13] Autologous recovery of marrow function in patients transplanted for CML, if it occurred at all, might have been anticipated to involve regeneration of Ph+ leukemic cells or perhaps a mixture of leukemic and normal cells. The recipient metaphases observed in these three patients were exclusively Ph−, however, and no Ph+ metaphases have been identified with a maximal follow-up of 36 months. This suggests that in these cases some component or components of the BMT procedure suppressed or eliminated the Ph+ clone but failed to eliminate all normal hematopoietic stem cells. Autologous regeneration of Ph− host cells has been observed in other patients after BMT.[14,15] These findings suggest that leukemic cells are at times more sensitive to radiotherapy than are normal hematopoietic cells.[16]

In one patient (LTN 26) with Ph− host cells, the chimerism persisted from 9 months to 3 years, but in the other two cases it was transient. This implies that there may be a competition between donor and host nonleukemic cells and that its outcome is unpredictable. Because such autologous nonleukemic (Ph−) regeneration may be transient, it may occur more frequently than has hitherto been recognized.

In one patient, a clonal abnormality developed 3 years after BMT. The significance of a new chromosomal abnormality after transplantation is unknown, although trisomy 8 has been reported in donor cells in a patient who relapsed after transplantation for acute lymphoblastic leukemia (ALL).[17] The abnormal clone disappeared when the relapse was successfully treated. Stable and unstable clonal abnor-

Table 7. Fragments and Deletions in Donor Cells

Patient (Sex)	Abnormalities	Clinical Status
LTN 118 (F)	At 6 and 9 mo after BMT: frequent chromosomal breaks in all donor (male) metaphases	In remission, chronic GVHD
LTN 120 (M)	At 6 mo: 2/15 metaphases 46,XY,Ph, 13/15 metaphases of donor sex: 4-46,XX + fragments, 2-44,XX,−D,−C, 7-46,XX	Cytogenetic relapse, chronic GVHD
	At 9 mo: 2/17 metaphases 46,XY,Ph, 15/17 metaphases 46,XX, some with fragments	Cytogenetic relapse, chronic GVHD (mild)

Both patients had received marrow from donors of opposite sex.

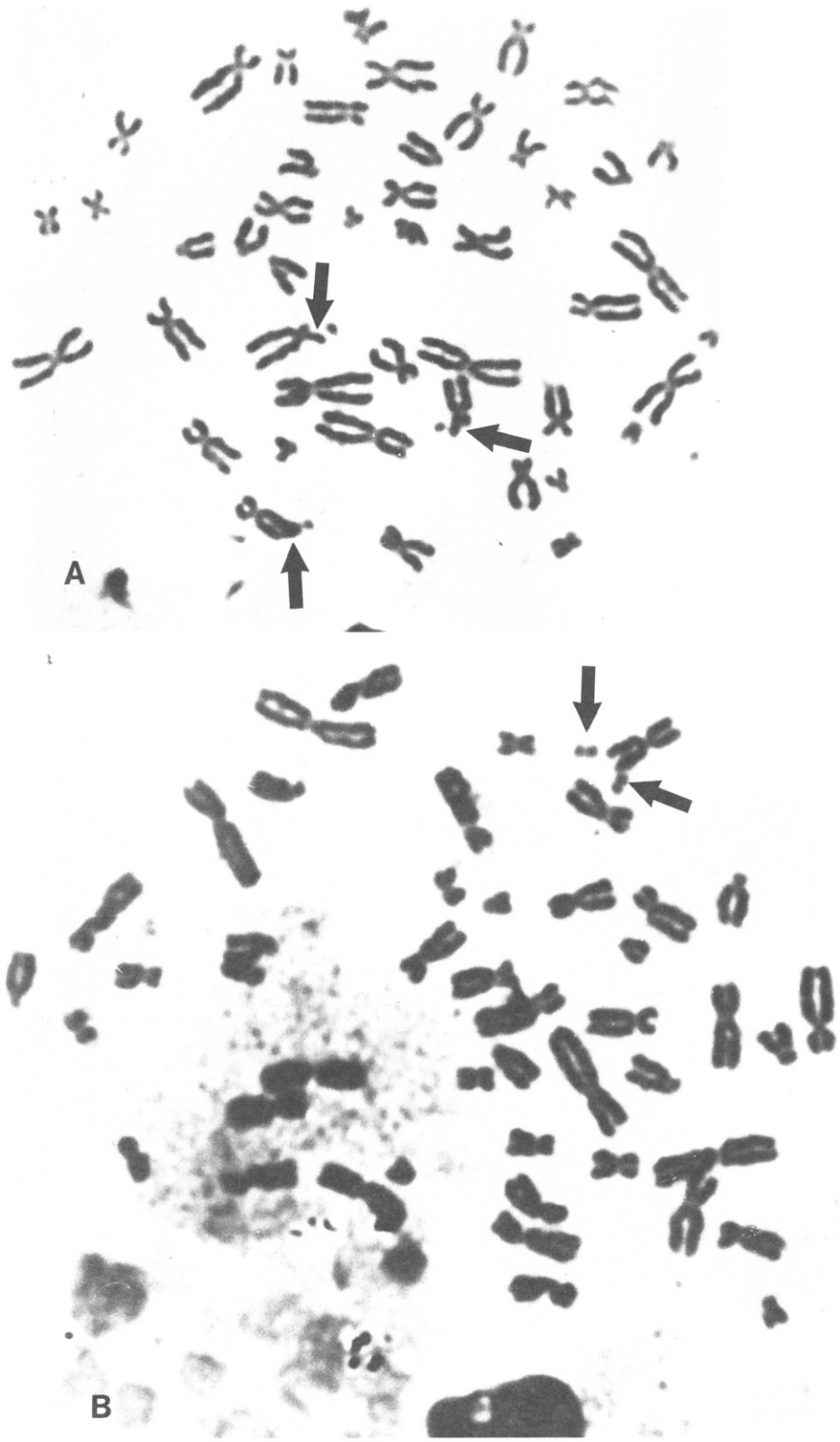

Fig 3. A: A "male" donor metaphase from female patient LTN 118 showing chromatid breaks (arrows). B: A "female" metaphase from male patient LTN 120 showing chromosome fragments (arrows).

malities have also been noted in host cells regenerating after BMT and show typical changes of radiation damage presumably incurred at the time of conditioning.[18]

Two cases have been reported in which leukemia recurred in donor cells after BMT for CML in second chronic phase. In one case, the relapse occurred in lymphoid blast cells bearing the Ph chromosome.[19] In the other, relapse occurred with a picture of ALL with Ph− blast cells.[20] In the present series, the study of sex chromosome markers in 11 patients with donors of opposite sex was consistent with relapse occurring in cells of host origin in each case. Thus, relapse in donor cells is probably uncommon in CML and indeed has not yet been reported after BMT in first chronic phase.

In two sex-mismatched patients, morphological abnormalities were detected in chromosomes from cells of donor origin. Similar abnormalities have been reported in recipient cells after BMT, but recipient cells have clearly been exposed to radiation and chemotherapy.[18] Our patients had received no irradiated blood products of donor origin. The reason for these chromosomal abnormalities in donor cells is unknown. The patients were taking no cytotoxic agents known to cause chromosomal damage, although they were receiving corticosteroids and cyclosporin A for treatment of chronic GVHD. A variety of viruses including herpes simplex and varicella-zoster can cause chromosomal breaks and gaps in various in vivo and in vitro models.[21] Both patients had had viral infections several months before the abnormal cytogenetic findings. The bone marrow of patients transplanted for leukemia may show evidence of cell death and cellular debris for many months after BMT, suggesting that an undefined microenvironmental factor or factors might damage developing hematopoietic cells.[22] These histopathologic changes could be caused by the same factors that cause the morphological chromosomal abnormalities described above. GVHD, viral infections, or other acquired abnormalities of marrow stroma are the main candidates.

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British Journal of Haematology, 1988, 68, 195–201

Use of minisatellite DNA probes for recognition and characterization of relapse after allogeneic bone marrow transplantation

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Summary. Restriction fragment length polymorphisms can be used to distinguish blood and marrow cells from close relatives. We used two probes that recognize a series of dispersed and highly polymorphic tandem-repetitive minisatellite regions in the human genome that can be detected via a shared 10–15 base pair core sequence similar to the generalized recombination sequence (χ) of *E. coli*. We have studied the resulting individual-specific DNA fingerprints in 15 patients before and after allogeneic bone marrow transplantation performed for chronic myeloid leukaemia and in two patients transplanted for acute leukaemia. Early engraftment could be demonstrated at 3 weeks post-transplant based on the recognition of cells of donor origin. One patient who failed to engraft had only recipient type marrow cells 3 months post-transplant. Nine patients who relapsed after transplantation had only cells of recipient origin. In one patient who relapsed after transplantation with T-cell depleted donor marrow, fractionation studies showed that his T-cells at relapse were of recipient origin. We conclude that these minisatellite probes are valuable for characterizing the origin of different cell populations after marrow transplantation and could be useful for characterizing relapse when donor and recipient are of the same sex.

The identification of the donor or recipient origin of haemopoietic cells after allogeneic bone marrow transplantation (BMT) can be valuable for assessing the speed of engraftment, for the definition of any residual population or sub-population of normal host cells and for the early recognition of relapse. When recipient and donor are of opposite sex, analysis of sex chromosomes in dividing cells will provide the necessary information. When dividing cells are not available or when donor and recipient are of the same sex, techniques that exploit erythrocyte antigen typing, HLA-typing, immunoglobulin isotypes, leucocyte isoenzymes or genetic polymorphisms in DNA to identify differences between closely related individuals may be valuable (Ginsburg *et al*, 1985; Blazar *et al*, 1985; Minden *et al*, 1985; Knowlton *et al*, 1986).

We report here the use of restriction fragment length polymorphisms (RFLPs) occurring in dispersed tandem repetitive minisatellite regions of genomic DNA (Jeffreys *et al*, 1985a, b; Thein *et al*, 1987) to identify the donor or recipient origin of haemopoietic cells after allogeneic BMT for leukaemia.

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MATERIALS AND METHODS

Patients. We selected for study 15 patients with Philadelphia (Ph) chromosome positive chronic myeloid leukaemia (CML) and two patients with acute leukaemia treated by allogeneic BMT. Sixteen patients received marrow cells from HLA-identical siblings and one patient received marrow from an HLA-matched unrelated donor. In five cases the patient and donor were of opposite sex. The details of conditioning and of the marrow transplant procedure have been reported elsewhere (Goldman *et al*, 1986). The introduction of T-cell depletion of donor marrow by incubation *in vitro* with the monoclonal antibody Campath-1 has resulted in a high relapse rate for patients transplanted for CML in chronic phase (Apperley *et al*, 1986). We studied seven patients with techniques described below for evidence of engraftment over a period of up to 12 months post-transplant. Three of these patients subsequently relapsed. Including these three, we studied a total of 13 patients in relapse, 12 with CML and one with AML, over a period of 1–52 months post-transplant.

Collection of blood. We collected 10–50 ml of peripheral blood, the precise quantity depending on the peripheral blood leucocyte count. In eight cases we collected the samples from the patient before and after bone marrow transplantation as well as from the corresponding donor. In other cases we

compared DNA from the patients' cells post-transplant with DNA from autologous cryopreserved cells or with DNA from donor cells because either the corresponding donor samples or DNA from fresh pre-transplantation samples were not available. In one patient who relapsed post-transplant T-cells were separated from the mononuclear cell fraction of peripheral blood by E-rosette sedimentation (Catovsky *et al*, 1981).

Sensitivity studies. In preliminary experiments we mixed DNA from a single donor and corresponding recipient in varying ratios to establish the lowest concentration of DNA that could be detected in the mixture. We also made mixtures in various ratios of the cell lines HeLa and K-562. We compared results of studying DNA preparations made by first mixing the cells and then preparing DNA with results obtained by first preparing DNA and then making the various mixtures.

Preparation of DNA. Nuclei were prepared from peripheral blood by lysing cells with ice-cold buffer solution (10 mM Tris pH 7·4, 10 mM NaCl, 3 mM $MgCl_2$). The white cell nuclei were pelleted by centrifugation at 3000 rpm for 10 min. DNA was extracted from nuclei by standard methods using phenol extraction and cold ethanol precipitation. After the extraction DNA was dissolved in 10 mM Tris-HCl, 1 mM EDTA pH 7·5 and stored at −20°C before use. From each DNA preparation we took three samples of 8 μg. Each sample was digested with DNA restriction endonuclease HinfI, Sau3A or HaeIII (Anglian Biotechnology Limited, Essex, U.K.). After complete digestion the DNA was electrophoresed on a 1% agarose Type II gel (Sigma) at 4 V/cm in 1 × Tris-acetate buffer overnight by which time DNA fragments smaller than 1·5 kb had run off the gel. The DNA was then transferred to nitrocellulose membrane filters (Schleicher & Schull, Dusseldorf, F.G.R.) by standard methods (Southern, 1975).

DNA probes. The probes 33·15 and 33·6 in single-stranded M13 DNA (Jeffreys *et al*, 1985a) were used to transform *E. coli* JM101 (Messing & Vieira, 1982) and double-stranded M13 RF DNA with the inserts was prepared. The EcoRI/HindIII insert fragments of 33·15 and EcoRI/BamHI fragments of 33·6 were separated from the M13 vector by electrophoresis in low melting point agarose (Sigma). The inserts were cut out from the low melting point agarose and stored at −20°C until use. The double-stranded probes were labelled with α^{32}P-dCTP by the oligolabelling method of Feinberg *et al* (1983) using reagents supplied by Pharmacia (Bucks., U.K.). The activity of the probes ranged from 10^6 to 10^9 cpm/μg. The labelled probes were hybridized with the nitrocellulose filter in 3 × SSC, 0·1% SDS, 1 × Denhardt's solution, 0·05 mg/ml double-stranded herring sperm DNA (Boehringer, Sussex, U.K.) and 10% dextran sulphate buffer at 65°C overnight. (Single-stranded herring DNA competitor was avoided as this hybridizes to minisatellite probes 33·6 and 33·15 and reduces the Southern blot hybridization signal (A.J.J., unpublished data).) The filter was washed at 65°C with 3 × SSC and 0·1% SDS for 30 min and with 1 × SSC and 0·1% SDS for 30 min at 65°C and exposed to a Kodak X-ray film overnight with an intensifier screen.

We used a 0·6 kb 5′ genomic probe for the breakpoint cluster region (*bcr*) gene (Ganesan *et al*, 1986) (kindly provided by Dr H. White, St Bartholomew's Hospital, London) to detect rearrangement on chromosome 22 characteristic of leukaemic cells from patients with Ph-positive CML.

RESULTS

Sensitivity of the method

With probes 33·6 and 33·15, which detect multiple hypervariable loci and produce individual-specific DNA 'fingerprints', we could recognize the presence of less than 10% of cells of different parentage by the appearance of bands specific to that individual. DNA from a recipient and a donor was mixed in ratios from 10:0 to 0:10 in 10 equal steps. The intensity of the bands increased or decreased to reflect the increasing or decreasing proportions of DNA from donor and recipient respectively (Fig 1). For example, the band of recipient origin visible at 10 kb diminished in intensity but it was still readily detectable when the recipient's DNA constituted only 10% of the mixture.

When material from the HeLa and K-562 cell lines was

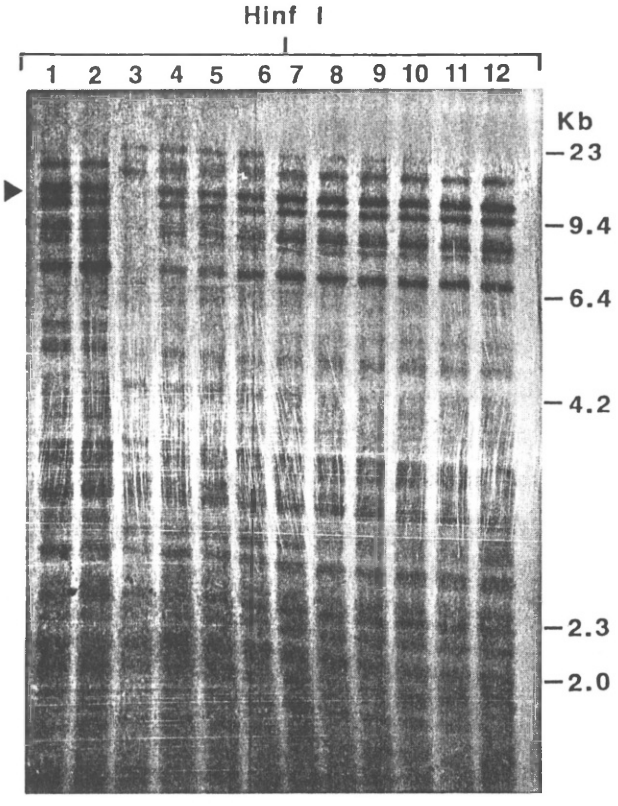

Fig 1. DNA fingerprints from donor and recipient mixed at differing ratios. Band patterns from recipient (patient 2) pre-BMT (lane 1), recipient in relapse after BMT (lane 2) and from donor (lane 3) are shown. Lanes 4–12 inclusive show band patterns from mixtures of donor and recipient DNA differing ratio from 9:1 to 1:9 in equal steps. The band patterns of the recipient in relapse are the same as those before transplant, while the bands of the donor DNA are not seen in DNA from the recipient in relapse. The arrow indicates two 10 kb size bands of recipient origin that are clearly visible in lane 4 even though recipient-derived DNA accounted for only 10% of total DNA. DNA was digested with HinfI and hybridized with probe 33·15.

mixed in various concentrations, we could detect as little as 2% minority DNA when the DNA was prepared separately from each cell line and when it was prepared from the cell mixtures (data not shown).

The actual sensitivity with which cell chimaerism can be detected will of course depend on the precise fingerprints of each donor and recipient combination.

Speed of engraftment

We studied the DNA from seven recipients and their respective HLA-identical sibling marrow donors before BMT and at 3–8 week intervals for a total of 7–12 months after BMT. Engraftment with donor marrow could be identified as early as 3 weeks post-transplant at which time DNA from blood cells was shown to be of donor origin (Fig 2). When the transplant procedure appeared clinically to be successful, patient-derived DNA studied at intervals of 1–2 months post-transplant showed only donor type band patterns without any trace of recipient-derived patterns. Of these seven patients two (nos. 10 and 12) showed evidence of cytogenetic relapse during the study period and both showed reappearance of recipient type bands on DNA analysis. One patient whose graft failed at 3 months showed a DNA pattern of recipient type.

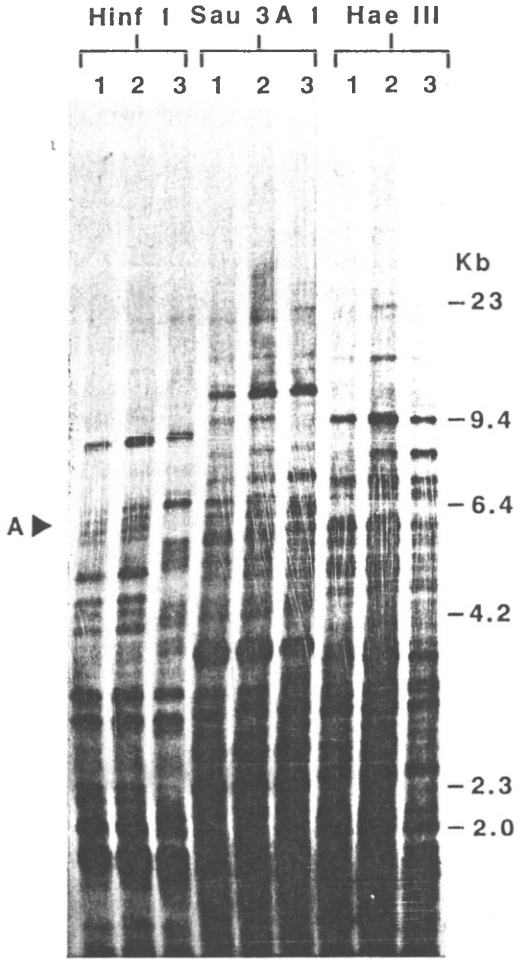

Fig 2. Early engraftment showing prominent bands of donor origin at 3 weeks post-transplant. DNA from the recipient (patient 11) at 3 weeks post-transplant shows a series of bands at 6·4 kb (arrow A, HinfI) corresponding to DNA of donor origin. Lane 1 = donor DNA; lane 2 = recipient post-transplant; lane 3 = recipient pre-transplant. DNA was digested with HaeIII, Sau3AI and HinfI and hybridized with probe 33·15.

Recognition of relapse

Twelve patients with CML who relapsed with cytogenetic or haematological evidence of leukaemia and one patient with AML who relapsed post-transplant were studied with the minisatellite probes (Table I). Six of the CML patients were also studied with the *bcr* probe to identify the reappearance of the Ph-positive cell population. In six of these patients we were able to examine samples from the recipient pre-transplant, from the donor and from the recipient after transplant. In four cases the band patterns were clearly of recipient origin (Fig 1). No bands of donor origin were seen in any of these post-transplant samples. The other two patients are described above. In eight patients we were unable to study either the donor or the recipient DNA before transplant but the pattern post-transplant was identical to the recipient pre-transplant in three patients or clearly different from the donor in five patients (Fig 3, lanes 4 and 5).

In one case (no. 12) a study of peripheral blood 7 months post BMT was consistent with complete engraftment without any signs of DNA of recipient origin (Fig 4). However, a cytogenetic study of marrow cells performed 1 month later showed that all metaphases had the Ph chromosome. This sequence could be explained if the proportion of Ph-positive marrow metaphases 1 month earlier had been low and all cells then in the peripheral blood had still been of donor origin. At 13 months post BMT DNA from peripheral blood cells showed a mixed pattern of recipient and donor bands with donor-derived bands predominating. Cytogenetic studies of the marrow cells at that time showed that the percentage of Ph positive metaphases had fallen.

In another patient (no. 3) who relapsed after transplantation with T-cell depleted donor marrow, study of DNA from different peripheral blood leucocyte fractions showed that the T-lymphocytes were of recipient origin (Fig 3, lanes 1–3).

We used the *bcr* probe to study three patients at a time when they had no cytogenetic or clinical evidence of relapse after bone marrow transplantation. The rearranged *bcr* band noted before transplant was not detected (Fig 5A). One of these patients (no. 1) was studied later and found to have *bcr* rearrangement. In this patient and in five other patients studied in relapse the rearranged band reappeared in the same position as the corresponding one before transplant (Fig 5B). These findings confirm that in these cases the DNA patterns of recipient origin were derived from cells with *bcr* rearrangement.

DISCUSSION

A variety of methods may be used to characterize the donor or recipient origin of cells in the peripheral blood and marrow of patients after allogeneic bone marrow transplantation

Table I. Clinical course of 15 patients with CML and two patients with acute leukaemia included in this study

Patient no.	Age (yr)	Sex (R/D)	Disease status at BMT	Time to: Cytogenetic relapse (months)	Time to: Haematological relapse (months)	Type of HR	Interval to recurrence of host cells (months)	Interval to recurrence of *bcr* rearrangement (months)
Chronic myeloid leukaemia								
1	38	M/F	BC	12	12	BC	12	–
2	17	F/F	AP	2	2	CP	1·5	1·5
3	29	F/F	CP	13	13	AP	24 (T-cells)	–
4	39	M/M	CP	4	4	BC	25	–
5	32	M/M	CP	6	10	AP	14	–
6	29	F/F	CP	18	18	AP	18	18
7	22	F/M	CP	16	16	CP	17	17
8	42	F/F	2nd CP	18	18	CP	14	14
9	32	M/M	2ry CP	54	54	BC	52	52
10	34	M/F	CP	6	NO	–	7	7
11	53	M/F	CP	9	NO	–	3 wk	3 wk
12	28	F/F	CP	9	NO	–	13	13
13	35	F/F	CP	–	NO	–	–	–
14	27	M/M	CP	–	–	–	–	–
15	18	F/M	CP	Graft failure at 3 months			3	3
Acute leukaemia								
16	18	M/M	1st CR	–	4	ALL	4	–
17	16	M/M	1st CR	–	–	–	–	–

Abbreviations: CML = Chronic myeloid leukaemia; CP = Chronic phase; AP = Accelerated phase; BC = Blast crisis; 2nd CP = Second chronic phase after treatment of transformed leukaemia; 2ry CP = Secondary chronic phase after presentation with acute lymphoblastic leukaemia (ALL); R/D = Donor/Recipient; HR = Haematological relapse; CR = Complete remission; NO = Not (yet) occurred.

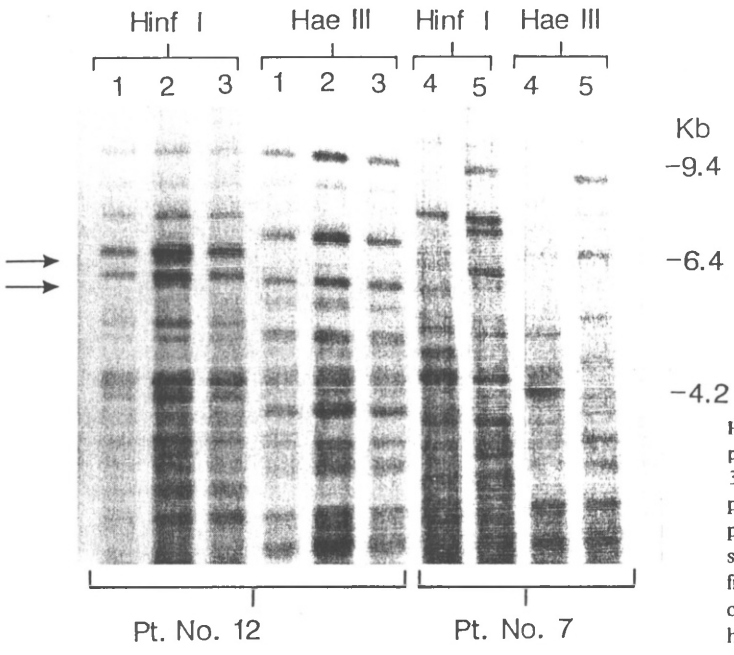

Fig 3. Relapse after BMT involving cells of recipient origin. Band patterns are shown from two different patients (patient 3, lanes 1–3; and patient 7, lanes 4 and 5) who relapsed after BMT. DNA prepared from T cells of patient 3 shows extra bands (lane 2) not present in donor DNA (lane 1) or after apparently successful subsequent second transplant (lane 3). For patient 7 band patterns from donor (lane 4) and recipient in relapse after BMT (lane 5) are clearly different. DNA was digested with HinfI or HaeIII and hybridized with probe 33·15.

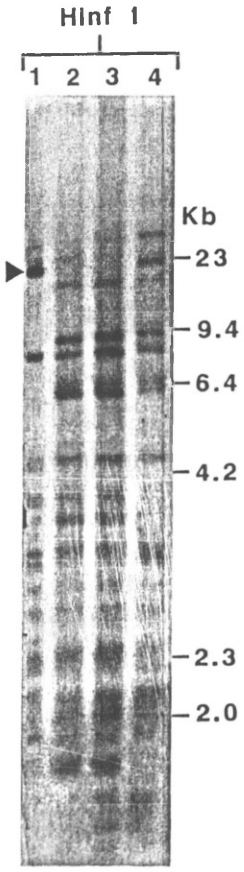

Fig 4. Relapse after BMT showing reappearance of recipient type DNA band pattern. The 23 kb band (arrow) in the DNA from the recipient (lane 1) has disappeared at 7 months post-transplant (lane 3) but has reappeared at 13 months (lane 4). The band patterns at 7 months post-transplant are identical to those of donor DNA (lane 2) but study at 13 months show a mixture and recipient band patterns, the former predominating. The patient was no. 12 and DNA was digested with HinfI and hybridized with probe 33·15.

(Sparkes *et al*, 1977; Blume *et al*, 1980; Reinherz *et al*, 1982; Gale *et al*, 1978; Boyd *et al*, 1982). In general, studies designed to take advantage of restriction fragment length polymorphisms have involved the use of a panel of up to 10 different probes recognizing polymorphic loci in the human genome. With such panels of probes specimens of DNA from two related individuals can be differentiated in perhaps 95% of cases. In contrast the minisatellite probes used in this study detect large numbers of distinct polymorphic sites in the human genome and thus give rise to a series of restriction fragments of differing size for any one individual. The number and precise location of these fragments are so variable that each blot may be regarded as having the same degree of individual specificity as do fingerprints. These probes may therefore be especially useful to differentiate the DNA from two closely related individuals, as is usually the case in clinical bone marrow transplantation.

The sensitivity of methods designed to distinguish two different DNA populations on the basis of differences in RFLP patterns is not well defined, partly because different probes vary in their ability to hybridize to low concentrations of DNA. In some studies artificial mixtures of DNA from two sources suggested that a concentration of DNA below 5% might be difficult to detect (Ginsburg *et al*, 1985). We have not defined the lower limit of detection of a minor quantity of DNA with the minisatellite probes but our preliminary experiments suggest that it may be well below 5%.

Cytogenetic methods have been used to characterize the donor or recipient origin of cells in the marrow and blood cells after bone marrow transplantation (Lawler *et al*, 1984, 1987). Such methods have the possible limitations, however, that they selectively examine cells capable of undergoing mitosis *in vitro* and they are based on the existence of a specific cytogenetic difference between donor and recipient, such as a sex chromosome. Cytogenetic methods have shown however that myeloid and lymphoid cells are usually both of donor origin after successful bone marrow transplantation using unmanipulated marrow cells. Such findings have been confirmed by others using techniques that exploit restriction fragment length polymorphisms (Ginsburg *et al*, 1985).

Using minisatellite probes we were able to document the existence of cells of donor origin as early as 3 weeks after BMT. Thereafter the band pattern became entirely consistent with DNA of donor origin in the majority of patients not destined to relapse. These findings are very similar to those reported previously (Ginsburg *et al*, 1985). In this group of patients the recurrence of mixed chimaerism, represented by the coexistence of band patterns of donor and recipient origin, was comparatively common and usually heralded or coincided with leukaemic relapse. The high frequency of relapse in this cohort of patients seemed to be associated with the use of T-cell depletion of donor marrow in an attempt to abrogate graft-versus-host disease (Apperley *et al*, 1986). It must be emphasized, however, that mixed chimaerism without leukaemic relapse has been documented in other patients studied by cytogenetic methods, both by us (Arthur *et al*, 1988) and by others (Vincent *et al*, 1986).

Using minisatellite probes we have shown that the majority of patients without cytogenetic or haematological evidence of relapse after marrow transplantation for leukaemia have only cells with DNA of donor origin in their peripheral blood. In one patient, however, who received donor marrow cells depleted of T-cells *in vitro* T-lymphocytes in the peripheral blood at relapse were of host origin.

Internal rearrangement of genomic DNA within the *bcr* is characteristic of cells from patients with Philadelphia chromosome positive chronic myeloid leukaemia. After successful bone marrow transplantation DNA probed with a *bcr* homologous sequence typically shows only a germline configuration. The *bcr* probe is thus a highly specific indicator of the presence or absence of leukaemic cells in patients with CML. By use of this probe we were able to show in six patients temporal concordance between haematological evidence of relapse, the reappearance of DNA of recipient origin recog-

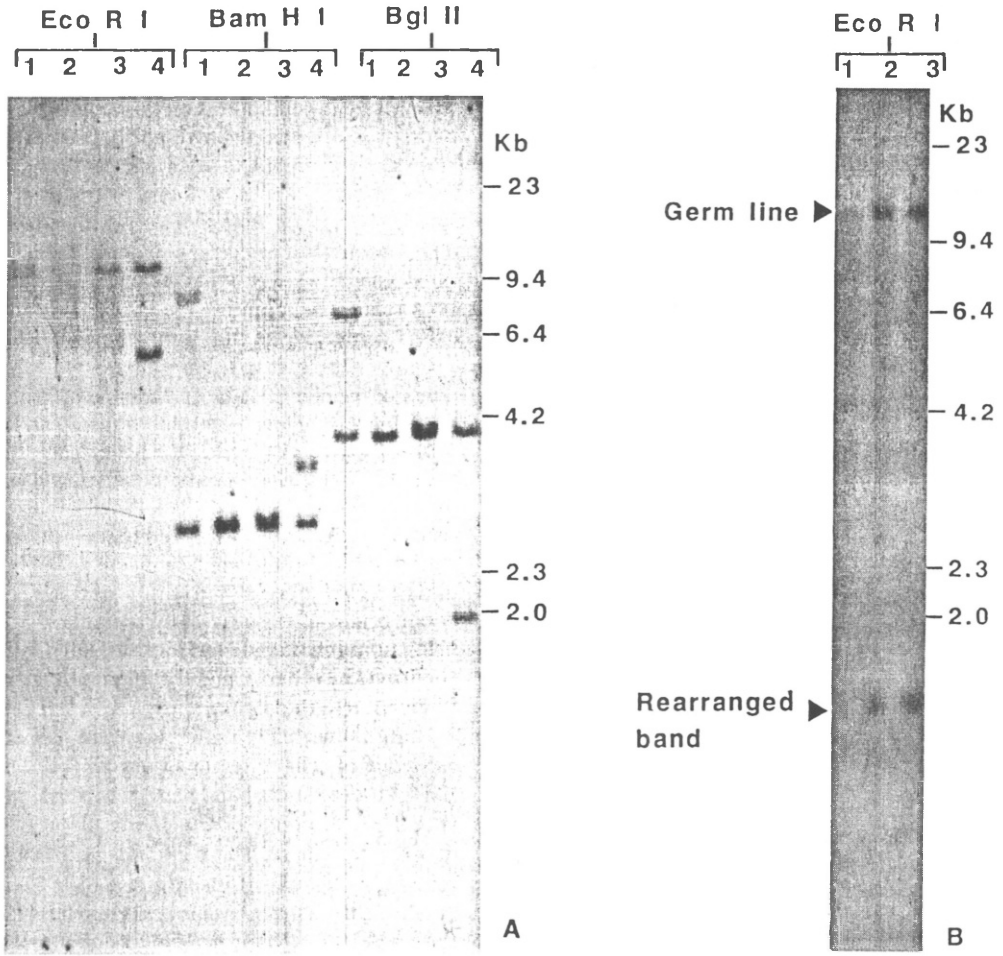

Fig 5. Disappearance of rearranged *bcr* band after transplant and reappearance at relapse. (A) DNA from patient 3 digested with EcoRI, BamHI and BglII and hybridized with *bcr* probe. DNA band patterns are shown from recipient before transplant (lane 1), from recipient 1 month post-transplant (lane 2), donor (lane 3) and a Ph-positive patient as control (lane 4). The rearranged bands at 8 kb (BamHI) and 7 kb (BglII) have disappeared after transplant. (Lane 2* contains DNA incompletely digested with EcoRI.) (B) DNA from patient 1 digested with EcoRI and hybridized with *bcr* probe. The rearranged band at 1·5 kb present before transplant (lane 2) is visible in the same position at relapse 12 months post-transplant (lane 3). DNA from the donor was run in lane 1.

nized with minisatellite probes and the reappearance of a rearranged pattern within the *bcr* gene. The pattern of *bcr* rearrangement differed between patients but was identical before transplant and in relapse in individual patients. This result confirms the results reported in one patient by Zalcberg *et al* (1986) and those obtained in some of the patients reported here using *bcr* and Y-chromosome specific probes (Ganesan *et al*, 1987). These findings imply that relapse after transplantation is due to re-emergence of the original leukaemic clone and not to reinduction of leukaemia in haemopoietic stem cells.

ACKNOWLEDGMENTS

We thank Professor Rodney Harris (Manchester) who provided advice and laboratory facilities for the senior author while he gained familiarity with the use of the minisatellite probes. We thank Professor Lucio Luzzatto and Dr Letizia Foroni for advice and encouragement with these studies. We thank also the various haematologists who provided samples. A.J.J. is a Lister Institute Research Fellow. The minisatellite probes are the subject of patent applications. Commercial enquiries should be addressed to the Lister Institute of Preventive Medicine, Brockley Hill, Stanmore, Middlesex HA7 4JD, U.K.

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SUPPLEMENT TO THESIS SUBMITTED BY DR GAO LING MIN

I would like to add the following points as suggested by the examiners during my oral examination.

(1) Lack of Evidence of ABL Involvement in M-BCR Rearranged CML. There is a single report of a case where M-BCR was rearranged yet ABL was not apparently involved[1]. This was a Ph-negative CML patient. Southern blot analysis of the DNA from this patient showed M-BCR rearrangement. The evidence for absence of ABL involvement was derived from in situ hybridization and Northern blot analysis. It would have been interesting to study this patient by techniques not available in 1985, for example PFGE, PCR, immunoprecipitation with ABL specific antibodies and molecular cloning and sequencing of the rearranged BCR gene, to confirm the absence of ABL involvement.

(2) BCR Gene Involvement in Ph-negative ALL. There is a report (in press) of acute lymphoblastic leukaemia cases in which both chromosomes 22 are apparently normal yet m-BCR was evidently rearranged[2]. This molecular change is identical to that found in about half the cases of Ph-positive acute leukaemia, especially in children. This suggests that BCR gene involvement in leukaemias may be commoner than has hitherto been recognized.

(3) RAS Involvement in CML. Cogswell et al recently

reported that 54% (7/13) of the Ph-negative, M-BCR non-rearranged atypical CML patients had RAS mutations; in contrast no case of Ph-positive typical CML in chronic phase and only 6% (1/18) of the Ph-positive CML patients in blastic phase had RAS mutations[3]. This high incidence of RAS mutations in Ph-negative atypical CML, together with the absence of BCR/ABL rearrangement, suggests that it is an entity molecularly distinct from typical CML. The clinical characteristics and the high frequency of RAS mutations suggest that atypical CML may constitute a subset of the myelodysplastic syndrome and may be best classified as a variant of chronic myelomonocytic leukaemia (CMML).

Though the mechanisms responsible for the differences between typical and atypical CML have been explored fairly extensively, it is still not clear why these two diseases have certain features in common. There could still be a common molecular mechanism that has not yet been identified. To address this question one should seek possible ABL mutations in Ph-negative, M-BCR non-rearranged atypical CML.

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Table 9.1. The Relationship between Cytogenetic, Molecular and Clinical Findings in CML and Other Leukaemias

Cytogenetics	M-BCR	m-BCR	Clinical	Number studied In this thesis
Ph-pos	**R**	**G**	**'Classical CML'**	**32**
	G	**G**	**Rare variant of CML[1, 2]**	**1**
	G	**R**	**1. Rare variant of CML[3]**	**0**
			2. About 50% of Ph-pos acute leukaemias	**1**
Ph-neg	**R**	**G**	**About 50% of Ph-neg typical CMLs**	**9**
	G	**R**	**Some cases of acute leukaemia[4]**	**0**
	G	**G**	**1. About 50% of Ph-neg CML patients with atypical features have no evidence of BCR (or ABL) gene involvement.**	**10**
			2. Other leukaemias.	**4**

Table 9.1 The Relationship between Cytogenetic, Molecular and Clinical Findings in CML and Other leukaemias.

'R' = DNA rearranged; 'G' = DNA not rearranged (germline configuration in Southern blot); 'pos' = positive; 'neg' = negative.

REFERENCES

(1) Selleri et al, 1987.
(2) Saglio et al, 1988.
(3) Selleri et al, 1990.
(4) Bartram et al, 1990 (in press).