Characterisation of normal and leukaemic stem cells in chronic myeloid leukaemia

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Abstract

Previous studies have demonstrated a residual population of normal cells in the BM of some patients with CML and they are found in the CD34$^+$DR$^-$ fraction. My initial studies involved the in-vitro characterisation of residual normal stem cells in the BM and PB at diagnosis in CML. The CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions were assessed as these contain cells with properties of the haemopoietic stem cell. Progenitors from these populations showed variable levels of leukaemic involvement between patients. In contrast, the more primitive cells, defined as pre-progenitors, in both the BM and PB of these populations were predominantly BCR-ABL$^-$ by RT-PCR analysis of individual secondary colonies. Hence PB may be used as a source of normal cells at diagnosis in CML and potentially is a richer source of normal stem cells because larger volumes can be collected compared to BM. Growth of cells in vitro may select for certain cell populations and thus the leukaemic stem cell in CML may reside in the CD34$^+$DR$^-$ or CD34$^+$38$^-$ populations but not proliferate in an in-vitro culture system. To further characterise both normal and leukaemic stem cells in CML a model of CML was established in immunodeficient (NOD/SCID) mice. Infusion of $\geq 4 \times 10^7$ chronic phase CML PB cells results in engraftment levels of $\geq 1\%$ in the BM of 84% of mice by 4 to 6 weeks. Engraftment of the spleen was seen in 60% of mice with BM engraftment. Intraperitoneal injection of recombinant SCF produced a higher level of leukaemic engraftment without increasing Ph$^-$ engraftment. Assessment of differential engraftment of normal and leukaemic cells by FISH analysis with BCR and ABL probes showed that a median of 35% of engrafted cells present in the murine BM were leukaemic. BM engraftment was multilineage with myeloid, B and T cell engraftment whereas T cells were the predominant cell type in the spleen. Selected CD34$^+$ CML blood cells engrafted the BM of NOD/SCID mice with cell doses of 7 to 10×10^6 cells with differentiation into the myeloid and B cell lineages. CD34$^-$ cells did not engraft, thus normal and leukaemic stem cells are present exclusively in CD34$^+$ blood cells from CML patients at diagnosis.

Declaration of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and , to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Abbreviations

>	greater than
≥	greater than or equal to
<	less than
%	percentage
°C	degrees Celsius
Ab	antibody
BM	bone marrow
bp	base pair
CD	cluster of differentiation
cDNA	complementary DNA
CSF	colony stimulating factor
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide, either dATP, dCTP, dGTP, or dTTP
DTT	dithithreitol
dTTP	deoxythymidine-5'-triphosphate
FACS	fluorescent activated cell sorting
FISH	fluorescent in-situ hybridisation
FITC	fluoroscein isothiocyanate
g	gravity
Gy	Gray
HGF	haemopoietic growth factor
IL	interleukin
IP	intraperitoneal
IV	intravenous

kb	kilobase pair
kD	kilodalton
L	litre
lin	lineage
M	moles per litre
mAb	monoclonal antibody
ml	millilitre
MNC	mononuclear cell
mM	millimole per litre
mol	moles
mRNA	messenger RNA
MW	molecular weight
µg	microgram
µl	microlitre
µM	micromole per litre
neg	negative
NOD	non-obese diabetic
PB	peripheral blood
PE	phycoerythrin
pos	positive
ng	nanogram
nm	nanometre
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pg	picogram
RNA	ribonucleic acid
RT	reverse transcription
SCID	severe combined immunodeficient

SSC	standard saline citrate
U	units
UV	ultra violet

Publications and Presentations Arising

Publications

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Presentations

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Chapter 1

Introduction

1.1 Chronic myeloid leukaemia

Chronic myeloid leukaemia (CML) is a disorder of the pluripotent haemopoietic stem cell characterised by expansion of myeloid cells. The disorder was first described in 1845 when the prominent splenomegaly and "suppuration of the blood" were noted (Bennet, 1845; Craigie, 1845; Virchow, 1845). The Philadelphia (Ph) chromosome, the characteristic cytogenetic marker of CML, was discovered in 1960 (Nowell and Hungerford, 1960) with the reciprocal translocation between chromosomes 9 and 22 being recognised in 1973 (Rowley, 1973). In the last 15 years the consequences of the translocation at the molecular level have been well characterised with current studies focussing on the biochemical consequences of the BCR-ABL fusion protein.

1.2 Epidemiology

CML comprises 20% - 30% of all cases of leukaemia in the western world with an annual incidence of approximately 1 per 100, 000 (Goldman and Lu, 1982). It affects all age groups with a peak incidence between 40 and 60 years and there is a slight male preponderance (Spiers, 1977).

1.3 Aetiology

The incidence of CML is increased following exposure to high levels of radiation, including survivors of atomic bomb explosions (Lange et al., 1954; Heyssel et al., 1960) or following radiotherapy for ankylosing spondylitis (Court Brown and Abbatt, 1955) or cervical carcinoma (Boice et al., 1985). There is no evidence that alkylating agents, chemicals or viruses have a causative role in CML. Apart from isolated descriptions there is no significant hereditary tendency (Weiner, 1965; Goh et al., 1967).

1.4 Natural History

1.4.1 Clinical features

Common presenting features of CML include fatigue, bleeding, weight loss, splenic discomfort and sweats, but, with the wide availability of automated blood counts, up to 20% of cases are diagnosed incidentally. Splenomegaly and purpura are the most frequent clinical signs (Savage et al., 1997).

1.4.2 Laboratory features

The blood picture at diagnosis shows leucocytosis with 74% of patients having a leucocyte count >100 x 10^9/L. Anaemia and thrombocytosis are common. The blood smear shows characteristic increase in myelocytes with neutrophilia and basophilia (Spiers, 1977). Bone marrow (BM) examination shows typical hypercellularity with hyperplasia of myeloid and megakaryocyte lineages and increase in reticulin staining (Gralnick et al., 1971).

1.4.3 Clinical course

CML is a biphasic or triphasic disease. The initial chronic phase is usually well controlled by single agent therapy and is compatible with a good quality of life. However the malignant clone is inherently unstable and inevitably transforms to an acute leukaemia known as blast crisis which is generally resistant to therapy. Blast crisis usually involves myeloblasts but lymphoid cells with a pre-B cell phenotype account for up to 30% of cases (Rosenthal et al., 1977a; Griffin et al., 1983a). In the remainder of cases, megakaryoblasts (Bain et al., 1977), erythroblasts (Rosenthal et al., 1977b) or rarely T cells (Griffin et al., 1983b) are involved in the acute transformation. Blastic transformation may occur abruptly or more slowly through an accelerated phase. The timing of blast crisis determines overall survival in CML. The use of the Cox proportional hazard model has shown that age, spleen size, platelet count and circulating blast cells at diagnosis defines patients with low, intermediate or high risk disease (Sokal et al., 1984). Median survival ranges from 32 months for patients with high risk disease to 60 months for patients with low risk disease.

1.4.4 Therapy of CML

The conventional approach to therapy of CML is essentially palliative. A number of drugs are able to decrease the overproduction of granulocytes and platelets and maintain the patient sympton free. Busulphan and hydroxyurea are the two commonly used drugs with recent evidence showing hydroxyurea improves survival compared to busulphan (Hehlmann et al., 1993). However as these agents have minimal effect on the number of Ph positive (Ph^+) cells in the BM they do not prolong the course of the disease significantly.

Various forms of interferon alpha (α) or gamma (γ) have been used in the management of CML since 1981. Interferon α therapy results in haematological remission in up to 75% of patients with 20% of patients achieving a complete cytogenetic response (Talpaz et al., 1991a). Interferon improves survival in comparison to busulphan therapy but in comparison to hydroxyurea the results are conflicting (Hehlmann et al., 1994; The Italian Cooperative Study Group on Chronic Myeloid Leukemia, 1994). However, patients who have a complete cytogenetic response still have detectable BCR-ABL by reverse transcription-polymerase chain reaction (RT-PCR) (Lee et al., 1992; Malinge et al., 1992). The major disadvantages of interferon therapy are its cost and side effects.

Allogeneic bone marrow transplantation (BMT) from a related or unrelated donor is the only potential curative treatment for CML. This is only suitable for approximately 25% of patients because of age restrictions or lack of histocompatible donors (Griffin, 1986). Patients less than 60 years old transplanted within 12 months of diagnosis with BM from a fully matched relative have a 5 year survival rate reaching 80% (Clift et al., 1993). However, the majority of patients with CML are not suitable for allogeneic transplantation and are incurable. There is a need for different forms of therapy to be developed to try and improve survival in CML.

1.5 The cytogenetics of CML

The Ph chromosome was the first consistent chromosomal abnormality associated with a human cancer when Nowell and Hungerford described a small acrocentric chromosome in the leukaemic cells of 7 patients with CML (Nowell and Hungerford, 1960). The advent of chromosomal banding with quinacrine fluorescence and Giemsa staining showed that in addition to the deletion of the long arm of chromosome 22 (22q-) additional material was detected at the end of the long arm of chromosome 9 (9q+) raising the possibility that the Ph abnormality was a balanced translocation between the long arms of chromosomes 9 and 22 (Rowley, 1973). Subsequently the sub-band localization of the translocation was defined as t(9;22) (q34.1;q11.21) (Prakash and Yunis, 1984). In addition to the typical Ph translocation variant translocations have been described in approximately 4% of chronic phase CML cases (Bernstein, 1988). These variants are usually complex translocations which involve one or more chromosomes in addition to chromosomes 9 and 22. At diagnosis, up to 10% of patients have additional chromosomal abnormalities including duplication of Ph and trisomy 8 (First International Workshop on Chromosomes in Leukaemia, 1978) but the presence of these at diagnosis does not influence prognosis (Whang-Peng et al., 1968). Transformation to the accelerated or blastic phases of CML is associated with additional chromosomal abnormalities in 80% of cases. These may be extremely complex but duplication of Ph, trisomy 8, isochromosome 17, trisomy 19 and loss of the Y chromosome are most commonly observed (Bernstein, 1988).

The Ph chromosome is relatively specific for CML but is also found in approximately 25% of cases of adult acute lymphoblastic leukaemia (ALL) (Catovsky et al., 1979) and occasionally seen in acute myeloid leukaemia (AML) (Preudhomme et al., 1992), essential thrombocythemia (Martiat et al., 1989), multiple myeloma (van den Berghe et al., 1979), and non-Hodgkins lymphoma (Fujii et al., 1990).

1.6 Molecular biology of CML

1.6.1 History

The study of RNA transforming viruses, or retroviruses, led to the identification of unique transforming genes within the retroviral genome, termed viral oncogenes. These are derived from homologous genes present in normal eukaryotic DNA, termed proto-oncogenes or cellular oncogenes. Many of these genes play critical roles in the regulation of cell growth and differentiation. The viral oncogene, v-ABL, is the transforming component of the Abelson strain of Moloney murine leukaemia virus (Abelson and Rabstein, 1970) and transforms lymphoid cells in vitro and causes leukaemia in susceptible mice (Baltimore et al., 1979). The human homologue of v-ABL, c-ABL, was shown to be localised to chromosome 9 at band q34 and therefore possibly involved in the Ph translocation (Heisterkamp et al., 1982). This was confirmed by hybridisation studies in which specific probes to c-ABL and v-ABL localised to chromosome 22 in CML (de Klein et al., 1982).

The c-ABL gene spans approximately 230-kb. It contains two alternative 5' exons, 1a and 1b, spliced to a common set of ten 3' exons, to yield the two major RNA transcripts of 6-kb and 7-kb (Shtivelman et al., 1986). Breaks in ABL usually occur 5' to ABL exon 2 and typically in the long intron between exons 1a and 1b (Leibowitz et al., 1985) (Figure 1.1).

Figure 1.1.

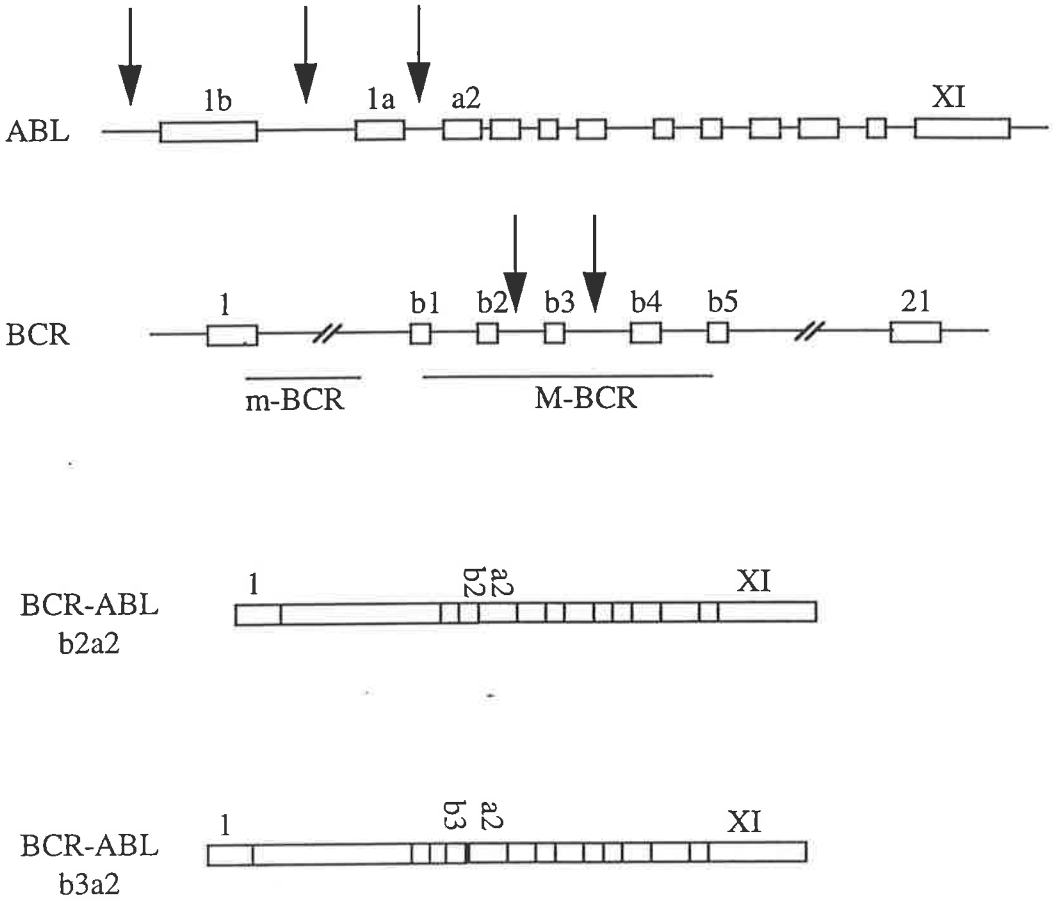
Schematic representation of the ABL, BCR and BCR-ABL fusion genes.

The ABL gene on chromosome 9 consists of 11 exons with 2 alternative 5' exons, 1a and 1b. Breaks in ABL usually occur 5' to ABL exon 2 in the regions represented by the arrows.

The BCR gene consists of 21 exons. Breakpoints are clustered in a limited region of up to 5.8-kb, the major breakpoint cluster region (M-BCR). Breakpoints occur either between exons b2 and b3 and result in a fused b2a2 BCR-ABL transcript or between exons b3 and b4 resulting in a b3a2 BCR-ABL transcript.

In Ph^+ ALL breakpoints can occur 5' to the M-BCR in the minor-breakpoint cluster region (m-bcr).

(Figure adapted from Gale and Goldman, 1988; and Melo et al., 1993)


ABL
1b
1a
a2
XI
BCR
1
b1
b2
b3
b4
b5
21
m-BCR
M-BCR
BCR-ABL
b2a2
1
b2
a2
XI
BCR-ABL
b3a2
1
b3
a2
XI

In contrast, the breakpoints on chromosome 22 are restricted to a limited region of up to 5.8-kb, originally designated the breakpoint cluster region (BCR) (Groffen et al., 1984), now known as the major - breakpoint cluster region (M-BCR). The M-BCR contains five exons, b1 to b5, which are exons 12 to 16 of the 70-kb BCR gene (Hariharan and Adams, 1987), and breakpoints occur between exons b2 and b3 or exons b3 and b4 (Heisterkamp et al., 1985) (Figure 1.1).

The result of this translocation is a fused BCR-ABL mRNA. Initial studies demonstrated the presence of an 8.5-kb ABL transcript in patients with CML and the Ph^+ cell lines K562 and EM2 which was distinct from the 2 normal ABL transcripts of 6-kb and 7-kb respectively (Gale and Canaani, 1984). Analysis with BCR and ABL probes confirmed the presence of chimaeric BCR-ABL mRNA (Shtivelman et al., 1985; Stam et al., 1985).

The product of this chimaeric BCR-ABL mRNA is a novel protein of MW 210-kD ($p210^{BCR\text{-}ABL}$) which has both ABL and BCR domains (Ben-Neriah et al., 1986). This protein is specific for CML being found in Ph^+ cell lines and patient samples (Konopka et al., 1985). The normal c-ABL protein, MW 145-kD ($p145^{ABL}$), lacks detectable tyrosine kinase activity in vitro (Konopka et al., 1984). The c-ABL domain of p210 has an altered structure consisting of phosphorylated tyrosine domains which activate tyrosine specific protein kinase activity and is thought to be important in the oncogenic potential of the BCR-ABL protein (Konopka et al., 1984). Further evidence that alteration in tyrosine kinase activity is important in leukaemogenesis is derived from studying Ph^+ ALL. The abnormal BCR-ABL protein in Ph^+ ALL is 190-kD ($p190^{BCR\text{-}ABL}$) (Fainstein et al., 1987) due to loss of BCR sequences compared to p210 (Lugo et al., 1990). This leads to more profound activation of ABL tyrosine kinase suggesting that quantitative changes in ABL kinase activity may be important in leukaemogenesis (Lugo et al., 1990).

The observation that breakpoints in the BCR gene are localised to M-BCR led to the analysis of DNA from patients who clinically appeared to have CML but were Ph-negative (Ph^-). The majority of these patients have rearrangement of M-BCR as detected by Southern analysis (Bartram, 1985; Ganesan et al., 1986; Kurzrock et al., 1986). BCR-ABL mRNA is produced with corresponding p210 protein but without translocation of the downstream portion of BCR, suggesting the mechanism of Ph^- CML is interstitial insertion of sequences from c-ABL into BCR (Dreazen et al., 1987; Dewald et al., 1993).

The Ph chromosome is also found in approximately 25% of cases of adult ALL (Catovsky et al., 1979). These can be divided into two diseases, patients with CML presenting in lymphoid blast crisis who express an 8.5kb BCR-ABL mRNA and p210 protein, and patients with de novo ALL. In these cases, c-ABL is translocated but BCR rearrangement is more variable than in CML, with breakpoints occurring both within the M-BCR and also in regions 5' to the M-BCR known as the minor-breakpoint cluster region (m-BCR) (de Klein et al., 1986a, Hermans et al., 1987, Heisterkamp et al., 1988). This results in a chimaeric 7-kb BCR-ABL mRNA which encodes a Ph^+ ALL specific $p190^{BCR-ABL}$ hybrid protein with increased tyrosine kinase activity (Fainstein et al., 1987).

1.6.2 Role of the BCR-ABL chimaeric gene in CML

Although the role of the Ph chromosome in the causation of leukaemia is still unclear the biochemical effects of the BCR-ABL chimaeric gene on cell function are being unravelled. Studies using retroviral gene transfection show that p210 enhances cell growth, transforms some cell lines and produces leukaemia when transplanted into mice. Transfection of murine BM cells with $p210^{BCR-ABL}$ results in selection and outgrowth of immature cells but does not confer growth factor independence (McLaughlin et al., 1987) but can transform pre-existing growth factor dependent lines to growth factor independence (Daley and Baltimore, 1988; Sirard et al., 1994). Transplantation of BM expressing $p210^{BCR-ABL}$ into lethally irradiated mice produces various haemopoietic disorders including a CML like myeloproliferative disorder and acute leukaemia of myeloid or lymphoid origin (Daley et al., 1990; Gishizky et al., 1993). In the transgenic mouse model genomic integration of either $p210^{BCR-ABL}$ or $p190^{BCR-ABL}$ produces acute leukaemia which is of long latency for p210 and short latency for p190 (Voncken et al., 1995).

The potential targets that may be involved in BCR-ABL mediated transformation of cells include enhancement of tyrosine kinase activity (Lugo et al., 1990), actin binding (McWhirter and Wang, 1991), Grb-2 protein (Pendergast et al., 1993; Tauchi et al., 1994), CRKL protein (ten Hoeve et al., 1994), and phosphatidyl-3 kinase (Skorski et al., 1995).

1.6.3 The ABL-BCR gene

The reciprocal hybrid gene of the BCR-ABL translocation, ABL-BCR, is expressed in approximately 70% of patients with Ph^+ CML, although the protein product has not been defined (Melo et al., 1993). The significance of this is unclear with conflicting reports on its role in determining response to interferon therapy (Yin et al., 1995; Melo et al., 1996).

1.7 Pathogenesis of CML

1.7.1 The involvement of the haemopoietic stem cell in CML

CML is an acquired defect that results from the malignant transformation of a single haemopoietic stem cell. The evidence that a pluripotent stem cell is involved comes from isoenzyme, cytogenetic and molecular studies of multiple haemopoietic lineages.

The Ph chromosome has been detected in precursors of neutrophils (Chervenick et al., 1971), monocytes (Golde et al., 1977), basophils (Denegri et al., 1978), erythrocytes (Rastrick, 1969), and megakaryocytes (Whang et al., 1963). Isoenzyme studies using glucose-6-phosphate dehydrogenase (G6PD) have shown restricted enzyme patterns in granulocytes, monocytes, erythrocytes, platelets (Fialkow et al., 1967; Fialkow et al., 1977), and eosinophils (Koeffler et al., 1980). In contrast fibroblast colonies are Ph$^-$ (Greenberg et al., 1978) and cultured marrow fibroblasts are heterozygous for G6PD (Fialkow et al., 1967) indicating that stromal elements in CML are not derived from the malignant clone.

The involvement of the lymphoid system in CML has been more difficult to characterise with a number of conflicting studies. Studies on lymphoid blast crisis have shown the majority to be of pre-B immunophenotype (LeBien et al., 1979; Bakhshi et al., 1983) although rare cases of T-cell blast crisis have been described (Stewart et al., 1991) and Ph$^+$ T cell lines established (Kuriyama et al., 1989). The clonal origin of B lymphocytes in CML was confirmed by immortalising B cells from a case of CML with nearly all derivative cell lines being Ph$^+$ and having a single G6PD isotype (Martin et al., 1980). A further study established B lymphoblastoid cell lines from 12 CML patients and 4 of these were Ph$^+$ (Nitta et al., 1985). The study of T cell invovlement in CML is complicated by the long half-life of T cells in the circulation and hence the majority of T cells may have arisen prior to the leukaemic event. Analysis of selected or cultured T cells have shown both Ph$^-$ (Kearney et al., 1982; Nitta et al., 1985) and Ph$^+$ (Fauser et al., 1985; Nogueira-Costa et al., 1986) clones. Similar studies using RT-PCR have also shown BCR-ABL-negative (BCR-ABL$^-$) (Ariad et al., 1993) and BCR-ABL-positive (BCR-ABL$^+$) (Jonas et al., 1992) results. The use of FISH to determine T cell involvement in CML has shown 12% to 39% BCR-ABL$^+$ cells in 4 of 25 patients in 3 studies (Garicochea et al., 1994; Torlakovic et al., 1994; Tefferi et al., 1995). It would therefore appear that the extent of clonal lymphoid invovlement in CML is variable.

In summary CML arises from a haemopoietic cell that differentiates into granulocytic, monocytic, erythroid, megakaryocyte and B lymphocyte progeny. Occasional involvement by T cells implies a variable level of differentiation capacity of the leukaemic stem cell.

1.7.2 The role of the Philadelphia chromosome in the pathogenesis of CML

The precise role of the Ph chromosome in the pathogenesis of CML remains uncertain. Indirect evidence suggests it is not the primary event in the causation of CML but a secondary phenomenon which results in the neoplastic clone gaining a proliferative advantage over normal cells. Sporadic cases of CML which are Ph^- at diagnosis but subsequently develop the Ph chromosome during the course of the disease suggest that the cells are leukaemic prior to the acquisition of the Ph chromosome (Lisker et al., 1980).

The study of immortalised B lymphoblastoid cell lines from a female CML patient heterozygous for G6PD showed 65 of 74 cell lines were Ph^- and 71% showed B type G6PD isoenzyme which was the enzyme type of the leukaemic clone (Fialkow et al., 1981). Some of these lines had chromosomal abnormalities which suggested they arose from an abnormal population which was Ph^- with the conclusion that the Ph chromosome was induced in one or more descendents of the clone. In a follow-up study, Ph^- B lymphoblastoid cell lines expressing a single G6PD enzyme type were established in 5 of 14 female CML patients (Raskind et al., 1993). Despite these findings little progress has been made in confirming the observation or in elucidating the nature of the initial event and there is the possibility that the results are due to pseudoclonality or artefactual skewing toward a single enzyme type in heterozygotes.

In contrast to these findings the Ph chromosome has been detected prior to the development of overt leukaemia (Canellos and Whang-Peng, 1972) and the recent demonstration of low level BCR-ABL transcripts being detected in apparently healthy individuals (Biernaux et al., 1995) further complicate the role of the Ph chromosome in leukaemia causation.

1.8 Haemopoiesis

1.8.1 Normal haemopoiesis

A small population of BM cells, the haemopoietic stem cells (HSC), are responsible for the maintenance of the haemopoietic system which involves the daily production of 210 billion erythrocytes, 60 billion neutrophils and 175 billion platelets (Finch et al., 1977). The haemopoietic system is organised in a hierarchy of haemopoietic cells ranging from pluripotent HSC through to lineage committed progenitors and their progeny.

Haemopoietic stem cells are defined by their ability to rescue lethally irradiated animals and reconstitute long term haemopoiesis. Therefore these cells must have the capacity to self-renew to maintain long term supply of progeny and also differentiate into progenitors of all blood cell lineages. Initial studies of murine BMT demonstrated the presence of cells with multilineage potential (Ford et al., 1956) and subsequent studies with serial transplantation showed the BM contained cells with self-renewal and multipotential differentiation capacity with the ability to sustain long-term haemopoiesis (Till and McCulloch, 1961). These properties have been confirmed with human BMT and demonstrate that both myeloid and lymphoid lineages arise from a common cell (Abramson et al., 1977).

The lack of specific markers for HSC has resulted in numerous functional and indirect assays to characterise them. Progenitor cells are progeny of more immature cells that have restricted lineage potential and minimal self-renewal capacity. Progenitor cells are relatively easily assayed in semi-solid media with appropriate growth factors. In these colony forming unit (CFU) assays individual progenitors give rise to distinct colonies which are defined by their cell content and include the CFU-granulocyte-macrophage (CFU-GM), CFU-megakaryocyte (CFU-Meg) and burst forming unit-erythroid (BFU-E) assays.

Colony forming assays of more immature progenitors include CFU-granulocyte erythroid macrophage megakaryocyte (CFU-GEMM) (Fauser and Messner, 1979), high proliferative potential progenitors (HPP-CFC) (McNiece et al., 1989) and blast cell forming progenitors (CFU-blast) (Leary and Ogawa, 1987). Characteristic of these assays is the need for multiple cytokines and the replating capacity of individual cells within each colony and it is probable that they measure closely related or overlapping populations.

Indirect assays of primitive haemopoietic cells are based on the observation that precursors to haemopoietic progenitors are not detected in clonogenic assays but will differentiate in the appropriate environment into progenitors. The generation of nascent CFU in secondary clonogenic assays serves as an index of precursors to CFU. The long-term bone marrow culture (LTBMC) assay (Dexter et al., 1977) reproduces the interaction between the HSC and bone marrow stromal cells thus recapitulating many of the in vivo conditions that regulate haemopoiesis. The cell responsible for initiating and maintaining LTBMC is the long term culture-initiating cell (LTC-IC) defined by its ability to produce CFU after 5 weeks of LTBMC (Sutherland et al., 1990). A stroma-free assay, the pre-CFU assay (Smith et al., 1991), also measures secondary output of CFU and can be manipulated to assess more directly the influence of haemopoietic growth factors on primitive cells.

Characterisation of HSC on the basis of surface membrane antigen expression, cell cycle status and metabolic state enables the study of the in-vitro properties of rare populations of cells. The CD34 antigen is present on 1% to 4% of human BM cells (Civin et al., 1984) including virtually all haemopoietic progenitors detected by in vitro assays (Andrews et al., 1986). Highly enriched populations of CD34 positive ($CD34^+$) cells are capable of restoring haemopoiesis when transplanted into lethally irradiated baboons (Berenson et al.,

1988) and this has been extended to autologous (Berenson et al., 1991) and allogeneic (Dreger et al., 1994) human BMT. Thus HSC capable of reconstituting long-term haemopoiesis reside within the CD34$^+$ population. Further characterisation of these CD34$^+$ cells shows that the more primitive cells have low expression of HLA-DR (Sutherland et al., 1989) and CD38 (Terstappen et al., 1991), are lineage negative (lin$^-$) (Andrews et al., 1990), express high molecular weight CD45 RA (Lansdorp et al., 1990), have low uptake of Rhodamine 123 (Udomsakdi et al., 1991), are Thy-1 positive (Thy-1$^+$) (Baum et al., 1992), and are transferrin receptor negative (Lansdorp and Dragowska, 1992). Transplantation studies have shown that CD34$^+$HLA-DR$^-$ (Srour et al., 1992), CD34$^+$Thy-1$^+$ (Sutherland et al., 1996) and CD34$^+$CD38$^-$ (Civin et al., 1996) populations are able to initiate and sustain chimaeric human haemopoiesis in fetal sheep suggesting they are functionally very similar populations.

A multitude of cytokines are involved in the regulation of haemopoietic cell proliferation and function. Individual cytokines often have multiple biological activities, can act directly or indirectly, are often synergistic, can be stimulatory or inhibitory, and promote cell survival by inhibiting apoptosis (Williams et al., 1990). Cytokines, or haemopoietic growth factors (HGF), that directly influence the proliferation and function of mature target cells include erythropoietin (EPO) (Eaves and Eaves, 1984), interleukin-5 (Campbell et al., 1987), macrophage-colony stimulating factor (M-CSF) (Stanley and Jubinsky, 1984) and granulocyte (G) -CSF (Nicola et al., 1985). EPO and M-CSF are relatively lineage specific but the majority of cytokines have activity on multiple lineages and act at different stages of committment and differentiation.

Cytokines that influence the proliferation of multipotential progenitors and more primitive haemopoietic cells often act in a synergistic way. These include IL-3 (Clark and Kamen, 1987; McNiece et al., 1991), GM-CSF (Sieff et al., 1985), IL-6 (Ikebuchi et al., 1987), IL-11 (Lemoli et al., 1993), G-CSF (Ikebuchi et al., 1988), stem cell factor (SCF) (Brandt et al., 1992; Heyworth et al., 1992), thrombopoietin (Kobayashi et al., 1996; Young et al., 1996), and FLT3 ligand (McKenna et al., 1995; Petzer et al., 1996a). Cytokines that have an inhibitory role on primitive haemopoietic progenitors include transforming growth factor β (TGF-β) (Cashman et al., 1990) and macrophage inflammatory protein 1α (MIP-1α) (Broxmeyer et al., 1990). The precise role of each cytokine in vivo is difficult to define based on in vitro studies because of the significant redundancy within the system.

The haemopoietic microenvironment plays a critical role in the regulation of haemopoiesis. Cells contributing to the microenvironment, known as the stroma, are of non-haemopoietic origin and include endothelial cells, fibroblasts, adventitial reticular cells and adipocytes (Dorshkind, 1990). The direct intimate interaction between stromal cell elements and primitive haemopoietic cells by cell-cell interaction and production and localisation of growth factors (Gordon et al., 1987a) is thought to be crucial for maintaining the quiesent and controlled proliferation of primitive cells (Verfaillie, 1993).

1.8.2 Leukaemic haemopoiesis

The striking abnormality in CML is the marked expansion of the leukaemic clone to very high cell densities with replacement of normal haemopoietic elements. This is most pronounced in the granulocyte and megakaryocyte lineages which retain the ability for terminal differentiation and generally function normally. The precise causes for the growth advantage of the leukaemic clone are unclear but as the regulatory controls of normal haemopoiesis are complex it is probable that there are multiple interacting processes responsible.

At diagnosis there is a large increase in circulating immature cells recognised as either progenitors, CFU-GEMM, CFU-GM, BFU-E or CFU-Meg (Moore et al., 1973; Moberg et al., 1974; Goldman et al., 1980; Eaves and Eaves, 1987), or CD34$^+$ cells (Banavali et al., 1991; Silvestri et al., 1991). There is some expansion of these cells in the BM compared to normal but this is not as dramatic as the peripheral blood (PB) findings. The increase in progenitor numbers in PB is linearly related to the white cell count (Moberg et al., 1974; Eaves and Eaves, 1987) and the ratio of CFU-GM to BFU-E is similar to that seen in normal haemopoiesis (Goldman et al., 1980; Eaves and Eaves, 1987). The gross morphology and size distribution of CML progenitors is similar to cultures of normal BM (Moore et al., 1973).

The suggested causes of this increase at the progenitor level include increased numbers of leukaemic stem cells, either by unregulated proliferation (Eaves et al., 1993a), increased self-renewal (Dowding et al., 1986), decreased apoptosis (Bedi et al., 1994a), or "discordant maturation" in which there is abnormal maturation and expansion in later maturational compartments with impaired regulation (Strife and Clarkson, 1988; Strife et al., 1988).

CML cells retain an absolute requirement for growth factors in vitro (Goldman et al., 1974; Metcalf et al., 1974), although occasional patients show low level autonomous production of colonies in factor free clonogenic assays (Moore et al., 1973; Siitonen et al., 1996). An increased sensitivity to stimulatory growth factors or impaired response to inhibitory factors may explain the increased proliferation of cells. While CML cells remain dependent on HGF their proliferative response to myeloid growth factors such as IL-3 and GM-CSF appears normal in clonogenic assays (Emanuel et al., 1991; Bedi et al., 1994b). There is no evidence of autocrine or paracrine production of HGF in the LTBMC system (Otsuka et al., 1991) but autocrine production of G-CSF by promyelocytes and myelocytes has been reported (Klein et al., 1990). Primitive cells in CML are defective in their response to the inhibitory cytokine MIP-1α but are inhibited normally by TGF-β (Cashman et al., 1992; Eaves et al., 1993b; Holyoake et al., 1993).

A number of defined defects in erythropoiesis may explain the expansion of BFU-E. Primitive cells in CML have an increased expression of erythropoietin receptors (Wognum et al., 1992) and thus may be more responsive to EPO. In vitro production of BFU-E is independent of exogenous EPO if exogenous SCF is supplied and all BFU-E produced under the influence of SCF are leukaemic by analysis of BCR-ABL expression (Issaad and Vainchenker, 1994). This suggests a linkage of the intra-cellular pathways of SCF and EPO unique to CML cells. Despite the expansion of BFU-E most patients with CML are anaemic at diagnosis. Defects in the terminal differentiation of BFU-E and CFU-E have been proposed to explain the ineffective erythropoiesis (Eaves and Eaves, 1979; Singer et al., 1981; Marley et al., 1996).

At diagnosis the relative number of LTC-IC in CML BM is reduced compared to normal but increased in the PB. However, the massive myeloid expansion in CML results in an increase in absolute numbers of LTC-IC in both BM and PB. The clonogenic output of CML LTC-IC is similar to normal but CML LTC-IC decline rapidly in secondary cultures compared to normal suggesting a defect in self-maintenance (Udomsakdi et al., 1992a). Most circulating LTC-IC in CML have features of proliferating cells such as expression of CD34, HLA-DR and rhodamine-123 (Udomsakdi et al., 1992b). Circulating CFU-GEMM also show a higher proportion in proliferative cycle (Hibbin et al., 1983).

The haemopoietic microenvironment is important in the regulation of normal haemopoiesis and defects in the interaction of primitive CML cells and the microenvironment may affect the control of haemopoiesis in CML resulting in excessive expansion. Defective adhesion to stroma by CML cells was first defined in a blast colony-forming assay system (Gordon et al., 1987b) and has been characterised as defective function of the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin receptors with impaired adhesion to normal stromal layers and fibronectin (Verfaillie et al., 1992a). Abnormal adhesive interactions between the BM microenvironment and primitive

CML cells may explain the unregulated proliferation and abnormal circulation of progenitors and more primitive cells.

1.9 Murine models of human haemopoiesis and leukaemia

1.9.1 Murine models of human haemopoiesis

The use of in vitro assays has contributed enormously to our understanding of human haemopoiesis but to evaluate the complex processes involved in the regulation and organisation of haemopoiesis in vivo models are required. Initial attempts at transplanting human haemopoietic cells into immunocompetent mice were usually unsuccessful (Louwagie and Verwilghen, 1970) and it was realised that immune-deficient mice were necessary to prevent rejection of the graft. The fortuitous development of the severe combined immune deficient (SCID) mouse during experiments to develop immunoglobulin heavy chain mouse strains (Bosma et al., 1983) has enabled the development of models of human cell transplantation in these mice. The SCID defect is due to a mutation in a gene on chromosome 16 which prevents gene rearrangement and subsequent production of mature B and T cells. These mice also have a generalised radiation repair defect (Bosma and Carroll, 1991). The bg/nu/xid mouse strain has also been used to study human haemopoiesis and this strain was constructed by combining the 3 recessive mutations. Beige (bg) mice have deficiencies in cytotoxic T cells (Saxena et al., 1982) and natural killer (NK) cells (Roder, 1979). The nude mouse is athymic (Pantelouris, 1971) and the xid defect affects lymphokine activated killer cells as well as B cell responses to certain thymus antigens (Scher et al., 1975).

Initial studies showed low level engraftment in murine BM and spleen of human BM cells infused into sublethally irradiated bg/nu/xid mice treated with exogenous GM-CSF and IL-3 (Kamel-Reid and Dick, 1988). Higher engraftment levels were seen using the SCID mouse and it appeared that exogenous human cytokines were essential to promote engraftment

(Lapidot et al., 1992). Concerns that difficulties in establishing engraftment were related to the absence of human stromal cells led to the development of the SCID-hu mouse model in which small spicules of adult or fetal bone were subcutaneously implanted in the SCID mouse. Most of these studies show active human haemopoiesis at the site of human bone but detection of human cells in the murine blood or bone marrow has been low (Kyoizumi et al, 1992; Heike et al, 1995; Sandhu et al, 1996). In contrast to adult BM, umbilical cord blood progenitors engraft SCID mice without any requirement for exogenous cytokine (Vormoor et al., 1994).

A mouse strain with more profound immunodeficiency, the non-obese diabetic (NOD)/LtSz-SCID/SCID mouse was produced by backcrossing the SCID mutation onto the NOD/Lt strain resulting in a strain with profound defects in B cell, T cell, macrophage, NK cell and complement function (Shultz et al., 1995). Studies have shown that both cord blood (Lowry et al., 1996; Pflumio et al., 1996) and adult BM (Cashman et al., 1997) engraft at higher levels in NOD/SCID than in SCID mice. Furthermore, it has been shown that as few as 500 $CD34^+CD38^-$ cord blood cells will engraft NOD/SCID mice (Larochelle et al., 1996). Despite the improved engraftment levels there is an absolute need to pre-condition these mice with irradiation prior to transplantation (Cashman et al., 1997).

It is also posssible to transplant human lymphoid cells in immune deficient mice. Transplantation of human peripheral blood lymphocytes (PBL) in SCID mice resulted in recovery of functional human B and T cells from the spleen, lymph nodes and PB up to 26 weeks post-transplantation. However engraftment only occurred following intraperitoneal (IP) injection of cells, no human cells were detected following intravenous (IV) infusion (Mosier et al., 1988). Higher engraftment of human PBL of the spleen and PB following IP injection has been shown in the NOD/SCID mouse (Hesselton et al., 1995).

1.9.2 Murine models of human leukaemia

As well as studying normal haemopoiesis murine models can be used in the study of human leukaemia and can be used to study the leukaemic stem cell, assess residual disease, develop individual therapies and may be used as a prognostic indicator.

Engraftment of SCID mice with ALL cells of pre-B and T cell type is possible without exogenous cytokine (Cesano et al., 1991; de Lord et al., 1991) and this has been shown to be a poor prognostic sign in pre-B ALL (Kamel-Reid et al., 1991; Uckun et al., 1995). Engraftment of AML in SCID mice requires administration of exogenous cytokine (Lapidot et al., 1994; Terpstra et al., 1995).

Mouse models of CML have been more difficult to establish. Infusion of blast crisis CML cell lines, such as K562, EM-2, BV173, or KBM-5, cause disseminated leukaemia in SCID mice (Cesano et al., 1992; Sawyers et al., 1992; Skorski et al., 1992 Beran et al., 1993). Similarly, cells from CML patients in blast crisis engraft and disseminate in SCID mice (Cesano et al., 1992; Sawyers et al., 1992; de Lord et al., 1993). However these are really models of acute leukaemia and do not recapitulate the features of chronic phase CML.

Studies using chronic phase CML cells in SCID mice at doses of 1 to 5 x 10^7 given IV, IP or implanted under the kidney capsule resulted in local recovery of cells given IP only and after 3 weeks these were predominantly CD3 positive (Sawyers et al., 1992). The use of higher cell numbers, 8 to 14 x 10^7, resulted in mean engraftment of 0.02% to 10% following IV infusion of chronic phase CML cells, but cells from only 5 of 10 patients engrafted at mean levels of 1% or more (Sirard et al., 1996). A preliminary report suggests higher engraftment can be achieved in NOD/SCID mice (Wang et al., 1995).

Chapter 2

Current concepts in the characterisation of normal haemopoietic cells in chronic myeloid leukaemia.

2.1 Introduction

Allogeneic BMT is currently the only curative treatment for CML. However only 20% to 25% of patients are suitable for this procedure. New therapies are necessary to improve survival in the remaining patients. Evidence of the existence of Ph^- stem cells in CML has led to various methods to isolate and characterise these cells in the hope that they can be manipulated either in-vivo or ex-vivo to re-establish Ph^- haemopoiesis following autologous transplantation or other therapy.

2.2 Clinical Studies

2.2.1 Chemotherapy

The finding that approximately 10% of patients have some Ph^- metaphases detectable in the BM at diagnosis indicated a residual pool of potentially normal cells (Sokal, 1980). Ph^- haemopoiesis could also be detected in some patients following therapy. The alkylating agent busulphan was commonly used as single agent myelosuppressive therapy. Occasionally it produced BM hypoplasia with associated degrees of Ph^- haemopoiesis (Tough et al., 1963; Finney et al., 1972; Golde et al., 1976). The results of multiagent intensive chemotherapy in acute leukaemia led some investigators to trial this form of treatment in CML in an attempt to achieve remission. This resulted in variable degrees of reduction of the Ph^+ clone in 32% to 58% of patients treated, with a number of patients achieving complete restoration of Ph^- haemopoiesis (Cunningham et al., 1979; Sharp et al., 1979; Goto et al., 1982). However the duration of Ph^- haemopoiesis was usually brief and there was no demonstrable improvement in survival. Restoration of the Ph^- state following intensive chemotherapy can result in the re-emergence of polyclonal haemopoiesis (Singer et al., 1980).

2.2.2 Interferon

The interferons are a group of naturally occurring polypeptides with pleiotropic properties including growth regulation, immunomodulation and anti-viral properties. As single agent therapy they produce high rates of complete haematological remission in CML with 15% to 20% of patients achieving complete cytogenetic response (Talpaz et al., 1991b; Kloke et al., 1993). However patients who are Ph^- and M-BCR rearrangement negative usually have BCR-ABL detected by PCR indicating persistence of malignant cells in these patients (Opalka et al., 1991; Kloke et al., 1993). Patients with a complete cytogenetic response survive longer than non-responders (Allan et al., 1995) but whether this equates to cure is still unknown. Interferon induced cytogenetic remission has also been associated with the restoration of polyclonal haemopoiesis (Claxton et al., 1992).

2.2.3 Autologous transplantation

The ability of blood-derived chronic phase CML cells collected at diagnosis to restore a second chronic phase in patients transplanted with CML in transformation has led to further investigations into the use of autologous transplantation in chronic phase CML (Haines et al., 1984). Transplantation in chronic phase has resulted in superior survival compared to conventional therapy but the studies have been retrospective (Hoyle et al., 1994; McGlave et al., 1994). In these studies a number of patients have developed some degree of Ph^- haemopoiesis post transplantation but the majority of these have had recurrence of Ph^+ haemopoiesis within 12 months. This finding is intriguing given that the inoculum of cells infused is predominantly Ph^+. It suggests preferential engraftment of residual Ph$^-$ cells in the graft or restoration of haemopoietic activity from residual Ph$^-$ cells in the BM. The success of syngeneic transplantation in twins with about 50% long term cure (Butturini et al., 1990) means that the conditioning regimen prior to transplant is able to successfully eliminate the malignant clone in some cases in the absence of an allogeneic graft-versus-leukaemia effect. Thus if the inoculum can be manipulated to contain predominantly Ph$^-$ cells then prolonged Ph$^-$ haemopoiesis may be achieved following autologous transplantation.

2.3 Methods for analysis of primitive cells in CML

2.3.1 Cytogenetics

Cytogenetic examination of BM remains the principal tool in the diagnosis of CML. The major advantages are its relative simplicity together with the ability to detect additional karyotypic abnormalities which may be useful in determining disease progression. It is used to assess response to interferon therapy and monitoring recovery post BMT. However, compared to molecular methods it is relatively insensitive. Cytogenetics can generally detect residual leukaemia if >5% of the BM metaphases are Ph$^+$. The requirement for generating metaphases means that delay in culture, analysis of stored material or analysis of PB may be suboptimal. These limitations mean that cytogenetics is inappropriate to examine rare populations of primitive cells and cannot be used to quantify minimal residual disease. However it can be used to analyse individual haemopoietic colonies and therefore can be used to characterise individual cell lineages at the progenitor level.

2.3.2 Restriction enzyme analysis with Southern hybridisation

DNA extracted from BM or PB is cut by restriction enzymes and hybridised with specific probes to detect rearrangements within M-BCR. As the analysis is performed on DNA, stored material can be used and there is no requirement for proliferating cells. In a review of 253 published cases of Ph$^+$ CML 98% had a detectable rearrangement within M-BCR and so it is a sensitive test for the diagnosis of CML (Kurzrock et al., 1988). It can detect 1% leukaemic cells in a specimen (Blennerhassett et al., 1988). Appropriate molecular probes spanning 5' in addition to 3' regions must be used to avoid false negative results. Quantification can be performed by densitometry but it is not sensitive enough to detect early relapse post BMT (Westbrook, 1992).

2.3.3 Polymerase chain reaction

The polymerase chain reaction (PCR) is a method of oligonucleotide primer mediated amplification and detection of specific DNA sequences (Saiki et al., 1985). In CML, breakpoints in the 1st intron of ABL can arise in a region of up to 200-kb and therefore PCR amplification of CML DNA requires primers specific for each patient. The PCR technique is modified by using cDNA, obtained by enzymatic reverse transcription of RNA, as a template for the reaction. This is a very sensitive technique able to detect a 1:10^5 dilution of Ph$^+$ K562 RNA in Ph$^-$ RNA (Kawasaki et al., 1988). The two different mRNA products b2a2 and b3a2 can be distinguished as different sized products on gel electrophoresis. The use of different primers targeting the more proximal breakpoints in m-BCR allows the distinction between the p190 product seen almost exclusively in Ph$^+$ ALL and the p210 product seen in nearly all cases of CML and some cases of Ph$^+$ ALL.

The high sensitivity of PCR means strict precautions must be taken to avoid false positive results. The need for RNA requires care to prevent degradation and contamination. The recent development of a competitive PCR titration assay allows quantification of BCR-ABL mRNA which may be useful in the monitoring of residual disease post BMT (Cross et al., 1993a). Small numbers of cells, such as those in a single haemopoietic colony, are amenable to PCR analysis (Hernandez et al., 1990). In fact, analysis of single cells by PCR is possible (Maguer-Satter et al., 1996) but this is technically demanding and concerns about sensitivity and contamination are valid.

Furthermore, analysis of rare populations of primitive cells depends upon these cells expressing BCR-ABL mRNA. Bedi et al have shown that BM $CD34^+lin^-$ cells from 6 CML patients that were BCR-ABL gene rearrangement positive had no (4 of 6) or trace (2 of 6) amounts of mRNA detectable by RT-PCR (Bedi et al., 1993). Lack of BCR-ABL expression may also occur in haemopoietic colonies. A study by Keating et al comparing cytogenetics and RT-PCR of individual colonies from BM mononuclear cells (MNC) of 12 patients with CML showed 23% ± 18% of colonies were Ph^+ but $BCR\text{-}ABL^-$ despite having detectable RNA (Keating et al., 1994). This is in contrast to a study by Diamond et al which showed that BCR-ABL could be detected in all colonies that were Ph^+ if the PCR reaction was repeated up to 5 times if necessary (Diamond et al., 1995).

PCR of genomic DNA eliminates the problem of BCR-ABL expression but this requires cloning and characterisation of BCR-ABL breakpoints for each individual patient. A study of minimal residual disease post BMT comparing genomic PCR with RT-PCR showed high concordance (Zhang et al., 1996). Long-template-PCR uses multiple primers to produce patient specific amplification products without the need for direct sequencing and this may be an alternative strategy (Dennebaum et al., 1996).

In summary, PCR analysis in CML has many advantages but also some limitations. Provided care is taken to minimise risk of contamination and appropriate positive and negative controls are used it is a powerful technique that can be used to study rare cell populations and haemopoietic colonies.

2.3.4 In situ hybridisation

Direct detection of BCR-ABL fusion by in situ hybridisation of fluorescent labelled DNA probes (FISH) allows analysis of individual interphase cells and can be used to analyse rare populations of different cell lineages (White et al., 1995). It is not dependent on dividing cells or expression of BCR-ABL and can be used to assess cytogenetically inadequate specimens. Large numbers of cells can be analysed and FISH is particularly useful in the assesment of Ph^- CML, complex Ph translocations and duplication of Ph (Dewald et al., 1993). The background level of $BCR\text{-}ABL^+$ cells due to chance juxtaposition of probes has been reported to range from 0% to 6.5% in 3 studies using at least 25 normal controls (Dewald et al., 1993; Najfeld et al., 1996; Nolte et al., 1996). This level means that FISH is not a useful tool for assessing early relapse or minimal residual disease.

2.4 Studies of residual normal cells in CML

2.4.1 Background

The finding that some CFU-GM were Ph^- in CML patients confirmed that Ph^- cells co-existed with Ph^+ cells in CML (Chervenick et al., 1971). Subsequently it was shown that the adherent fraction of early chronic phase CML BM contained predominantly Ph^- progenitors after 4 weeks LTBMC (Coulombel et al., 1983). Further analysis of treated and untreated patients showed that only 3 of 6 treated patients contained Ph^- cells following LTBMC (Dube et al., 1984a). Also, direct cytogenetic analysis of individual haemopoietic progenitors showed some Ph^- colonies in 6 of 26 patients in chronic phase (Dube et al., 1984b). All patients in these studies were 100% Ph^+ by cytogenetic analysis of BM. Thus from an early stage two points were evident. Firstly, despite all analysable metaphases being Ph^+ some patients had BM progenitor cells that were Ph^-. Secondly, LTBMC selected for a population of cells which were almost exclusively Ph^- in patients at diagnosis but this was more variable in patients who were more advanced in the disease, suggesting a decrease in numbers of residual Ph^- cells in time. Further characterisation of these Ph$^-$ progenitors demonstrated they were non-clonal (Hogge et al., 1987; Turhan et al., 1990).

These findings led to a clinical trial using autologous BM maintained in culture for 10 days prior to infusion. Of 22 patients infused with cultured BM, 16 achieved haematological recovery and 13 of these developed exclusively Ph$^-$ haemopoiesis. Re-emergence of Ph$^+$ cells occurred in all but one patient 4 to 36 months post transplantation (Barnett et al., 1994).

2.4.2 Selection of cells based on immunophenotype

Analysis of cells by immunophenotype has shown that the CD34$^+$HLA-DR$^-$ (CD34$^+$DR$^-$) population, which contains cells with properties of primitive progenitors, was enriched for BCR-ABL$^-$ cells in the BM of patients with chronic phase CML. It may therefore be possible to select cells on the basis of cell surface characteristics, which are predominantly normal, for use in autologous transplantation.

Verfaillie et al collected CD34$^+$DR$^-$ and CD34$^+$HLA-DR$^+$ (CD34$^+$DR$^+$) cells by fluorescent activated cell sorting (FACS) after bone marrow mononuclear cells (BMMNC) were elutriated and lineage depleted. The number of CD34$^+$DR$^-$ cells was decreased in CML BM compared to normal. LTBMC was established and secondary haemopoietic colonies analysed at weeks 1, 5 and 8. In 6 of 7 patients cytogenetic analysis at each timepoint revealed exclusively Ph$^-$ colonies. This was confirmed by RT-PCR of colonies being negative for BCR-ABL in 4 patients at weeks 5 or 8. The CD34$^+$DR$^+$ population showed Ph$^+$ colonies in 6 of 7 patients following LTBMC (Verfaillie et al., 1992b). No analysis of directly sorted cells was performed and although Ph$^-$ and BCR-ABL$^-$ cells were demonstrated from secondary haemopoietic colonies derived from the CD34$^+$DR$^-$ fraction a selective effect of in-vitro culture may have over-estimated their frequency.

These findings were extended by Leemhuis et al who assessed similar populations of cells following elutriation and cell sorting. Analysis by RT-PCR on the sorted populations showed detection of BCR-ABL in the CD34$^+$DR$^+$ population in 11 of 12 patients and in the CD34$^+$DR$^-$ population in 6 of 9 patients. Individual colonies from directly sorted cells showed 36% to 90% BCR-ABL$^+$ colonies from the CD34$^+$DR$^+$ population and 0% to 62% BCR-ABL$^+$ colonies from the CD34$^+$DR$^-$ population. Cells derived from a stroma-free LTBMC remained BCR-ABL$^+$ in the CD34$^+$DR$^+$ population but BCR-ABL$^+$ cells were not detected in 3 of 5 patients from the CD34$^+$DR$^-$ population. These results confirmed the finding of BCR-ABL$^-$ cells within the CD34$^+$DR$^-$ population in some patients but the results on the directly sorted cells and progenitors show it is not a pure population of benign cells in most patients.

The CD34$^+$CD38$^-$ (CD34$^+$38$^-$) population of normal human BM is enriched for primitive progenitors (Rusten et al., 1994). In a 3 colour analysis of CD34, CD33, CD38 and HLA-DR expression in normal BM and CML PB and BM de Fabritiis et al suggested most CD34$^+$38$^-$ cells expressed HLA-DR. This was inferred on the basis that a smaller proportion of CD34$^+$ cells were HLA-DR$^-$ than CD38$^-$ and the majority of CD38$^-$ cells were HLA-DR$^+$. However, they did not evaluate the coexpression of CD34, CD38 and HLA-DR directly. Analysis of sorted CD34$^+$DR$^-$ and CD34$^+$38$^-$ cells for presence of the Ph chromosome following a 48 hour culture of sorted cells with HGF showed 22 of 114 Ph$^-$ metaphases in the CD34$^+$DR$^-$ fraction from 5 patients but only 1 of 123 were Ph$^-$ in the CD34$^+$38$^-$ population (de Fabritiis et al., 1993a).

A study of directly sorted cells for the presence of BCR-ABL by FISH using a BCR gene breakpoint specific probe was performed by Kirk et al. CD34$^+$ cells were sorted into CD38 low and high populations and HLA-DR high and low populations. There was no difference in the percentage of leukaemic cells by FISH in the CD34$^+$38$^+$ and CD34$^+$38$^-$ populations

(55% and 57% respectively) but the $CD34^+DR^-$ population had significantly less $BCR\text{-}ABL^+$ cells than the $CD34^+DR^+$ population (15% and 52% respectively). In a direct comparison in 2 patients 16% and 17% of cells were $BCR\text{-}ABL^+$ in the $CD34^+DR^-$ population compared to 46% and 66% in the $CD34^+38^-$ population (Kirk et al., 1995). These findings were further confirmation that BM derived $CD34^+DR^-$ cells are enriched for benign cells in early chronic phase CML.

The $CD34^+lin^-Thy\text{-}1^+$ population contains cells able to give rise to long-term cultures of both myeloid and B-lymphoid cell lines from single cells (Baum et al., 1992) and also initiate lymphopoiesis in human fetal thymus implanted in SCID-hu mice (Péault et al., 1991) and hence is a population that contains pluripotent cells (Baum et al., 1992). This population in CML BM has been shown to be $BCR\text{-}ABL^-$ in 5 of 6 cases (Negrin and Weissman, 1992) but these results have not been confirmed.

In an extension of their earlier study Verfaillie et al compared yield and BCR-ABL status of BM derived $CD34^+DR^-$ cells in early and late chronic phase (CP) CML and also accelerated phase (AP) CML. Three enrichment methods were used prior to FACS sorting. Two of these involved depletion of lineage positive cells by immunomagnetic beads or counterflow centrifugal elutriation, whereas the third method involved positive selection of $CD34^+$ cells by a Ceprate™ column. There was no difference in the recovery of $CD34^+DR^-$ and $CD34^+DR^+$ regardless of enrichment process chosen. The frequency of $CD34^+$ cells that were $HLA\text{-}DR^-$ was 0.5% to 3% in early CP CML compared with 5% to 10% in normal BM but the total number collected in 100 ml BM was no different because of the higher cellularity and yield of BMMNC from early CP CML BM. The studies of AP CML showed reduced numbers of $CD34^+DR^-$ cells, lower CFC yield from LTC-IC and also less than 50% of secondary colonies were $BCR\text{-}ABL^-$ (Verfaillie et al., 1996). Hence disease progression is associated with a lower frequency of $BCR\text{-}ABL^-$ cells.

2.4.3 Ex-vivo manipulation of leukaemic cells

An alternative approach to the selection of normal cells in CML is the depletion of Ph^+ cells by various purging strategies including culture techniques, pharmacological agents, or specific agents targeting the consequence of BCR-ABL translocation.

Carlo-Stella et al used a combination of $CD34^+$ selection, stroma adherence and mafosfamide incubation and assessed secondary generation of CFU-GM for the presence of Ph. The greatest number of Ph^- colonies were found in $CD34^+$ cells that had mafosfamide pre-incubation followed by stromal culture suggesting Ph^- progenitors adhered to stroma and were resistant to alkylating agent therapy (Carlo-Stella et al., 1994).

The role of specific HGF in promoting either Ph^+ or Ph^- growth remains unclear. Agarwal et al studied the effect of stroma and SCF on CFU-GM production by using combinations of SCF deficient or expressing murine cell lines with or without exogenous SCF. The SCF deficient stromal line produced 15% to 87% $BCR\text{-}ABL^-$ colonies in 8 of 9 patients following 3 to 7 weeks culture of CML BM $CD34^+$ cells. This preferential production of $BCR\text{-}ABL^-$ colonies was partially abrogated by exogenous SCF (Agarwal et al., 1995).

Novel therapeutic agents aimed at the specific consequences of BCR-ABL transformation in the cell offer promise as they target leukaemic cells. These include antisense oligodeoxynucleotides, ribozymes and tyrosine kinase inhibitors.

Synthetic antisense oligodeoxynucleotides (ODN) bind to specific DNA or mRNA sequences and suppress gene function. Specific ODN targeted to the BCR-ABL junction have been shown to inhibit leukaemic cell proliferation of CML blast crisis cells (Szczylik et al., 1991) and also CML cell lines (de Fabritiis et al., 1993b; Smetsers et al., 1994; Mahon et al., 1995) in vitro. In an extension of this work SCID mice were infused with a Ph^+ cell line, BV173. Mice treated with a specific ODN had reduced clonogenic leukaemic cells, BCR-ABL mRNA and increased survival compared to control mice given no ODN (Skorski et al., 1994). In a purging study, a single patient in accelerated phase post interferon therapy had BM harvested and incubated for 24 hours with specific ODN. The BM was then cryopreserved and reinfused following high dose therapy. Engraftment was achieved at day 15 and at day 180 only 10% of cells were BCR-ABL$^+$ by FISH and 8% Ph$^+$ by cytogenetics (de Fabritiis et al., 1995). This demonstrates that in vitro culture with the ODN does not affect haemopoietic engraftment but whether this contributed to the Ph$^-$ engraftment is speculative and will require controlled studies. It is possible that the ODN effect may be non-specific inhibition of cell growth rather than by a hybridisation dependent anti-sense mechanism (Kirkland et al., 1993; O'Brien et al., 1994).

Ribozymes are specific RNA sequences that have the ability to cleave other RNA molecules in a catalytic manner. The hammerhead domain contains a catalytic core with flanking arms that can be made complementary to sequences either side of the site specific targets. Ribozymes have a theoretical advantage over ODN because they can cleave multiple substrate molecules. A number of ribozymes have been developed with the major problem being non-specific cleavage of BCR and ABL mRNA (Wright et al., 1993; Pachuk et al., 1994; Kearney et al., 1995; Leopold et al., 1996) although there are reports of specific cleavage of the b3a2 mRNA molecule (Lange et al., 1993; Shore et al., 1993; James et al., 1996).

Selective inhibition of tyrosine kinase is a promising therapy with both naturally occurring and synthetic compounds showing activity against CML cells (Anafi et al., 1993). Various compounds such as herbimycin A, genistein and CGP 57148 have been shown to inhibit the growth of either Ph$^+$ cell lines or CML colony growth in-vitro (Okabe et al., 1992; Carlo-Stella et al., 1996; Druker et al., 1996).

A variety of other techniques are under investigation as potential purging procedures. These include incubation with interferon γ (McGlave et al., 1990; Becker et al., 1993), generation of antibodies to BCR-ABL (van Denderen et al., 1989) and a number of immunotherapy techniques such as generation of cytotoxic T cells (Choudhury et al., 1997) or activated NK cells (Cervantes et al., 1996; Silla et al., 1996) with anti-leukaemic activity. Whether any of these will ultimately have a role as a purging agent remains the subject of further study.

2.4.4 In vivo manipulation of normal and leukaemic cells

It is well established that stem cells can be mobilised into the peripheral blood of normal individuals and patients with haematological and non-haematological malignancies following cytotoxic therapy or the administration of HGF (To et al., 1997). Early work in CML has shown intensive chemotherapy followed by G-CSF mobilises exclusively Ph^- cells in some patients (Carella et al., 1993). Subsequent analysis shows that untreated patients in early chronic phase are more likely to mobilise exclusively Ph^- cells (Carella et al., 1996). Initial results of autologous BMT with Ph^- cells in 10 patients with early disease and no prior interferon therapy show 5 having persistent Ph^- haemopoiesis 2 to 10 months post-transplantation (Carella et al., 1996). In some of these cases, Ph^- haemopoiesis has been shown to be polyclonal (Bergamaschi et al., 1994). Different mobilisation regimens have been tried by other groups including cyclophosphamide and G-CSF (Hughes et al., 1997), cyclophosphamide and GM-CSF (McGlave et al., 1996), daunorubicin and high dose cytarabine (Kantarjian et al., 1995), fludarabine, high dose cytarabine and mitoxantrone (Kantarjian et al., 1995), and hydroxyurea and G-CSF (Johnson et al., 1996). In general, the degree of Ph^- mobilisation is greater earlier in the course of the disease and the more intensive the regimen but this is at a cost of increased toxicity.

The Upsala group has taken a different approach. Patients are initially treated with interferon and hydroxyurea and have a bone marrow harvest if they achieve a cytogenetic remission. Otherwise they proceed to up to 3 courses of differing regimes of intensive chemotherapy with BM harvest after cytogenetic response. Thirty patients have had a subsequent autologous BMT and 10 remain Ph$^-$ at 1 to 64 months (Simonsson et al., 1996).

2.5 Summary

Multiple techniques are being used in an attempt to characterise normal haemopoiesis in CML. Some methods are already being used clinically whereas others are still being evaluated in the laboratory. A number of issues need to be addressed.

Cells characterised as Ph$^-$ and BCR-ABL$^-$ may still be part of the leukaemic clone. The early work of Fialkow et al suggesting that the Ph translocation arises after the initial transforming event implies that there may be cells that are Ph$^-$ but leukaemic or pre-leukaemic (Fialkow et al., 1981). The re-establishment of polyclonal haemopoiesis following in vitro culture (Turhan et al., 1990), chemotherapy (Singer et al., 1980), interferon (Claxton et al., 1992) and following autologous transplantation (Bergamaschi et al., 1994) means it must be unlikely that there is a Ph$^-$ leukaemic population.

What is the optimal technique for isolating residual normal cells? Normal cells may be positively selected on the basis of certain characteristics or leukaemic cells may be depleted by various methods. The rarity of the normal cell in CML suggests that the optimal technique is one which isolates the majority of the target without undue cell loss and therefore cell manipulations should be minimised. Hence features that can discriminate normal and leukaemic cells need to be pursued. Lack of expression of HLA-DR, differential adhesion to BM stroma, and the use of specific pharmacological agents directed toward consequences of BCR-ABL translocation are different techniques which can be used to enrich for normal cells. Further study to determine the optimal method or combination of methods is required. Positive cell selection may prove superior to depletion techniques as a single approach to purging could be instituted. This is contingent upon the tumour cells not expressing the marker selected for and the purity of selected cells being sufficiently high to avoid contamination by tumour cells. On the other hand, cell depletion techniques may lack specificity and the dose, duration, delivery technique to the cell, stoichometry of reaction and influence of cellular compartmentalisation are all problems that need to be overcome for antisense oligodeoxynucleotides, ribozymes, and tyrosine kinase inhibitors.

The source of normal cells is important. Their low frequency means that large volumes of BM must be harvested to isolate sufficient cells for manipulation. In contrast large numbers of cells can be relatively easily obtained from the PB at diagnosis. However data on the characterisation of normal cells in the PB is lacking and needs further study. Diagnosis is the optimal time for harvesting because normal cells decrease in frequency as the disease progresses, although diagnosis is also the time when large numbers of leukaemic cells are present. The emergence of PBSC mobilisation offers a potentially exciting technique to collect large numbers of normal cells. It remains to be seen whether in vitro or in vivo manipulation produces a relatively pure cell product that can be used for autologous transplantation and reconstitute haemopoiesis in a timely fashion.

2.6 Statement of Aims

(1) To evaluate the $CD34^{+}HLA\text{-}DR^{-}$, $CD34^{+}HLA\text{-}DR^{+}$, $CD34^{+}38^{-}$ and $CD34^{+}38^{+}$ populations for the presence of $BCR\text{-}ABL^{-}$ progenitors and pre-progenitors obtained from the bone marrow and peripheral blood of patients with recently diagnosed CML.

(2) To establish a model of CML in the NOD/SCID mouse which may be used in the evaluation of new therapies in CML and the study of leukaemogenesis at the stem cell level and assess the characteristics of engraftment including lineage differentiation, cytokine response, organ infiltration and differential engraftment of normal and leukaemic cells.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Patient specimens

Patients with recently diagnosed CML had BM collected by aspiration from the posterior superior iliac spine (PSIS) and PB collected by apheresis. Normal volunteer donors had BM collected by aspiration from the sternum or PSIS and PB collected by venepuncture. All procedures and use of patient and volunteer material was approved by the Human Ethics Committee of the Royal Adelaide Hospital, Adelaide, South Australia. All patients and donors gave informed consent.

3.1.2 Tissue culture

Stroma-free liquid culture (pre-progenitor assay) was carried out in 24 well plates (Nunclon) in Iscove's Modified Dulbecco's Medium (IMDM; Gibco BRL) supplemented with 30% fetal calf serum (FCS; CSL), 1% bovine serum albumin (BSA; Cohn fraction V; Sigma), 3 mmol/L L-glutamine (Sigma) and 5×10^{-5} mol/L β-mercaptoethanol (Sigma) supplemented with 100 ng G-CSF, 100 ng SCF, 20 ng IL-6 and 10 ng IL-3 (Amgen).

CFU-GM assays were established in 35 mm petri dishes (Corning) in 0.9% methylcellulose (Methocel; Dow Chemical Co) in IMDM supplemented with 30% FCS and 3 mmol/L L-glutamine. Cultures were stimulated by 10 ng each of recombinant human IL-1, IL-3, IL-6, G-CSF, GM-CSF, and SCF (Amgen).

3.1.3 Antibodies

Directly conjugated monoclonal antibodies to CD3 (Leu 4), CD4 (Leu 3a), CD8 (Leu 2a), CD19 (Leu-12), CD33 (Leu M9), CD34 (HPCA-2), CD38 (Leu-17), CD45 (anti-HLe-1), CD56 (Leu 19) (Becton Dickinson), and HLA-DR (I3-RD1; Coulter) were used for cell labelling and sorting procedures. Isotype matched antibodies, mouse IgG1 FITC and mouse IgG1 PE (Dako), were used as controls.

Selection of $CD34^+$ cells was performed with Dynabeads M-450 CD34, 9C5 anti-CD34 antibody, Dynabeads M-450 and anti-Fab antiserum (DETACHaBEAD) (all supplied by Dynal).

3.1.4 Mice

A breeding colony of NOD/SCID mice was established at the University of Adelaide from animals originally obtained from the Animal Resource Centre, Perth, Western Australia. Experiments were carried out as approved by the Animal Ethics Committee of the University of Adelaide. Mice were kept in microisolator cages in a laminar flow room in specific pathogen free conditions. Mice were maintained on sterilised food and acidified water. Three days per week 100 ml water was supplemented with 60 mg trimethoprim and 300 mg sulfamethoxazole (Bactrim; Roche Products) to prevent opportunistic infections.

3.1.5 Solutions

DNA gel loading buffer:

5% glycerol, 1 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue (Bresatec)

DNA size markers:

pUC19 DNA restricted with Hpa II in 10 mM Tris-HCL, pH 7.6, 1 mM EDTA (Bresatec)

FACS Fix:

1 L PBS, 10 ml formalin, 20 G glucose, 0.2 G sodium azide

Fixative for cells for FISH (Carnoy's Fixative):

75% methanol, 25% acetic acid at -20°C

Hybridisation solutions for FISH:

Denaturation solution:

70% formamide, 2X SSC, pH 7.0

Post-hybridisation wash:

50% formamide, 2X SSC, pH 7.0

Hybridisation mixture:

10% dextran sulphate (Pharmacia), 2X SSC, 50% formamide (Kodak), 0.1% polyoxyethylenesorbitan monolaurate (Tween 20, Sigma)

Immunofluorescence buffer:

500 ml HBSS, 2% heat inactivated FCS, 0.2 G sodium azide

Isolation buffer:

$Ca^{++}Mg^{++}$ free PBS, 0.6% sodium citrate, 2% HSA

PBS:

140 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4

SSC (20X):

3 M NaCL, 0.3 M Na Citrate, pH 7.0

TAE (50X):

242 G Trizma base, 57.1 ml acetic acid, 100 ml 0.5 M EDTA pH 8.0

Thaw solution:

$Ca^{++}Mg^{++}$free HBSS, 10 mmol/L acid citrate, 2% BSA

3.2 Methods

3.2.1 Cell processing

3.2.1.1 Patient samples

PB or BM cells were diluted in Hanks Balanced Salt Solution (HBSS). Light density mononuclear cells were collected after centrifugation at 400*g* for 30 minutes over a Lymphoprep density gradient (1.077 g/dL; Nycomed) by aspirating the cells at the interface. Cells were washed twice in HBSS by centrifugation at 400*g* for 10 minutes. Cells were used fresh or cryopreserved. Cryopreserved cells were stored in autologous plasma at a concentration of 1 to 5 x 10^7/ml with 10% dimethylsulphoxide and 20% FCS. Controlled rate freezing was carried out and specimens stored in liquid nitrogen at approximately -195°C.

3.2.1.2 Selection of CD34 positive cells from cryopreserved samples

$CD34^+$ cells were selected from cryopreserved samples of CML PB for infusion into NOD/SCID mice. The technique used was immunomagnetic selection with Dynabeads. These are 4.5 µm spherical particles containing magnetic material (Fe_2O_3 and Fe_3O_4). For selection of $CD34^+$ cells these beads are coated with a mouse IgG2 monoclonal antibody (mAb 561) which is specific for a class III epitope on the CD34 antigen. These coated beads are applied to the cell suspension and during incubation the beads bind the target cells. After binding, the target cells are recovered by the use of a magnet. Dynabeads are removed from the target cells by detachment with a polyclonal antibody that reacts with the Fab-fragments of monoclonal antibodies (DETACHaBEAD). This technique allows up to 4 x 10^8 cells to be targeted, a much larger number than can be collected by fluorescent activated cell sorting. The method is relatively quick and simple and the recovered cells are of high purity.

For collection of CD34$^+$ cells from cryopreserved samples, the cells were thawed rapidly at 37°C in a waterbath and washed twice by centrifugation at 400*g* for 10 minutes in isolation buffer. Cells were resuspended at 1 to 2 x 10^7/ml in isolation buffer. Dynabeads were prewashed before use. The beads were mixed by gentle vortexing and the required volume transferred to a 5 ml plastic tube (Falcon 2063; Becton Dickinson). The washing tube was placed on the Dynal MPC magnet. The beads attached to the side of the tube and excess fluid was aspirated off. The tube was removed from the magnet and the beads resuspended in 3 ml of isolation buffer. The tube was placed on the magnet again and the fluid was aspirated off. This washing step was repeated and the beads resuspended in 1 ml of isolation bufer. Cells were rosetted with 4 x 10^7 Dynabeads M-450 CD34/ml of washed cells in a 5 ml plastic tube (Falcon 2063). The cell-bead mixture was briefly vortexed and then incubated at 4°C for 40 minutes with gentle rotation. Rotation was necessary to keep the cell-bead complexes in suspension. After incubation the cell-bead complex was resuspended by vortexing and then rosetted cells were selected by placing the tube on the magnet for 2 minutes and aspirating the supernatant containing the non-rosetted cells. The rosetted cells were resuspended in isolation buffer and separation procedure repeated 5 times. The non-rosetted cells, CD34 negative (CD34$^-$) were pooled. After the final wash the rosetted cells were resuspended in 100 µl of isolation buffer. Beads were detached from positively selected cells by incubating with 100 µl DETACHaBEAD per 4 x 10^7 Dynabeads at 37°C for 90 minutes using gentle rotation in a shaking waterbath. After incubation, 2 ml isolation buffer was added and the suspension briefly vortexed. The tube was placed on the magnet and the released cells aspirated after 2 minutes. This was repeated 3 times and the released cells were pooled. Released cells (CD34$^+$) and non-rosetted cells (CD34$^-$) were counted and purity determined by flow cytometry. In most cases they were used immediately for infusion into mice. Recovery of CD34$^+$ cells from cryopreserved samples ranged between 50% to 65% with purity levels of 88% to 94% (Table 6.5).

3.2.1.3 Selection of CD34 positive cells from fresh samples

Cells collected at diagnosis in 2 patients had CD34$^+$ cells selected with the Isolex 50 magnetic cell separation system (Baxter). This system is based on the Dynal technique but the design of the separation chamber allows up to 4 x 10^9 cells to be incubated, making it ideal for use on apheresis product collected at diagnosis when large numbers of cells are present. The method utilises an indirect capture technique. Cells are initially incubated with the primary antibody, anti-CD34, and then excess unbound antibody is removed by washing. Dynabeads, coated with a secondary antibody, sheep anti-mouse IgG, are added and these bind the cells which have primary antibody attached. Target cells are recovered by using a magnet and the beads removed by enzymatic cleavage.

Cells collected fresh by apheresis were washed twice in isolation buffer by centrifugation at 400*g* for 10 minutes and then 4 x 10^9 cells in a volume of 40 ml were placed in the isolation chamber and incubated with 2 mg 9C5 anti-CD34 antibody at room temperature for 30 minutes with gentle rotation. Cells were rosetted with 4 x 10^9 Dynabeads M450 and incubated at room temperature for 30 minutes with gentle rotation. The chamber was attached to the magnetic column and after 2 minutes the non-rosetted cells were drained. Rosetted cells were washed in 50 ml buffer and the separation procedure repeated 3 times. Non-rosetted cells were pooled. Rosetted cells were released by incubating with 100 pKat chymopapain (ChymoCell-R; Baxter) at room temperature for 15 minutes with gentle rotation. The chamber was placed on the magnet and the released cells were drained. The beads were resuspended in isolation buffer and the drainage procedure repeated twice. CD34 levels were evaluated in rosetted and non-rosetted cell fractions and cells were cryopreserved as described in 3.2.1.1. Recovery of CD34$^+$ cells from fresh samples ranged between 68% to 74% with purity levels of 94% to 98% (Table 6.5).

3.2.2 Antibody labelling

Cryopreserved cells were thawed rapidly in a waterbath at 37°C and washed twice in thaw solution. The phenotype of patient samples was assessed using directly conjugated monoclonal antibodies, anti-CD3, CD19, CD33, and CD34, used at a concentration of 2 μl per 5 x 10^5 cells. Samples were incubated for 40 minutes at 4°C and washed twice in immunofluorescence buffer and fixed in FACS fix. Analysis was performed on an EPICS profile analyser (Coulter). Antigen expression was compared to isotype matched negative controls.

Engraftment of NOD/SCID mice was assessed by immunolabelling BM and spleen suspensions of transplanted mice with directly conjugated anti CD45, a human specific pan leucocyte marker. Red cells were lysed prior to immunolabelling in 10X vol 0.83% ammonium chloride at 37°C for 10 minutes. Differentiation of engrafted cells was determined by dual colour labelling with anti-CD45 fluorescein isothiocyanate (FITC) and anti-CD3, CD4, CD8, CD19, CD33, CD34 and CD56 R-phycoerythrin (PE) respectively. Incubation, washing, cell fixing and analysis was identical to that for immunophenotyping of patient samples.

3.2.3 Fluorescent activated cell sorting

Fluorescent activated cell sorting (FACS) was performed on cryopreserved BM and PB of patients with early chronic phase CML. This procedure enables dual-labelled cells to be sorted into defined populations. It is ideal for collecting rare populations of primitive cells which may be present in frequencies as low as 0.1%.

Cryopreserved cells were thawed and washed as before. Cells were dual labelled with a FITC conjugated anti-CD34 antibody and a PE conjugated anti-HLA-DR or anti-CD38 antibody for 40 minutes at 4°C. Anti-CD34 and anti-CD38 antibodies were used at a concentration of 20 μl per 5 x 10^6 cells and anti-HLA-DR antibody was used at a concentration of 1 μl per 5 x 10^6 cells. After incubation cells were washed twice in HBSS supplemented with 5% FCS. Cells incubated with isotype-matched antibodies were used as controls.

Cells were sorted using a Becton Dickinson FACStar^{PLUS} laser flow cytometry system into HBSS supplemented with 5% FCS. Cells in the low-side scatter and low to intermediate forward light scatter windows were sorted into fractions expressing high density CD34 antigen (CD34$^+$) with either high or low expression of HLA-DR antigen (DR$^+$or DR$^-$) or CD38 antigen (CD38$^+$ or CD38$^-$). Sort windows were were based on isotype matched controls. After sorting, cell populations were washed once at 400*g* for 10 minutes in HBSS supplemented with 5% FCS. Cells were counted and appropriate assays established.

3.2.4 Pre-progenitor cell (pre-CFU) assay

The assay is an adaptation of the 4 day suspension culture system described by Iscove et al (Iscove et al., 1989) and Smith et al (Smith et al., 1991). The assay measures generation of nascent CFU-GM as an index of precursors to CFU-GM. It is based on the finding that precursors to CFU-GM do not proliferate in standard clonogenic assays but in a stroma-free suspension assay differentiate following treatment with HGF.

Sorted cells were resuspended at 1 x 10^3/ml in pre-CFU medium and placed in 24 well plates supplemented with 100 ng G-CSF, 100 ng SCF, 20 ng IL-6 and 10 ng IL-3. This combination of HGF was chosen as it has been shown to maximally stimulate production of nascent CFU-GM from suspension culture of normal BM CD34$^+$ cells in our laboratory (Haylock et al., 1992). Cells were incubated at 37°C in 5% CO_2 in a humidified atmosphere. At 7 day intervals cells were washed and resuspended with fresh medium and growth factor. Aliquots of the washed cells were plated in triplicate in the CFU-GM assay.

3.2.5 Progenitor (CFU-GM) assay

Triplicate 1 ml cultures were established. Sorted populations of $CD34^+$ cells were plated at 1000 cells/ml of methylcellulose. Cells harvested from the pre-progenitor assay were plated at various concentrations up to 1×10^5/ml. Increased cell numbers were necessary at later timepoints because as cells differentiate in the pre-progenitor assay the relative number of progenitors decreases. Cultures were stimulated by 10 ng each of IL-1, IL-3, IL-6, G-CSF, GM-CSF, and SCF.

In the murine transplantation experiments BM and spleen cells were plated at 1×10^5/ml, which is the appropriate cell concentration for culture of BMMNC. These cultures were stimulated by 10 ng each of IL-3, GM-CSF and SCF, a combination specific for human progenitors (Lapidot et al., 1996).

After 14 days incubation at 37°C in 5% CO_2 in a humidified atmosphere CFU-GM were scored as aggregates of >50 cells using an inverted microscope.

3.2.6 Cell lines

A number of human leukaemic cell lines were maintained in RPMI-1640 (Sigma) containing 10% FCS, glutamine, penicillin and streptomycin. These included K562 (Ph^+ erythroleukaemia), EM-2 (Ph^+ leukaemia) and KG1a ($CD34^+$ acute myeloblastic leukaemia). Cell lines were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cells were washed weekly and fresh media supplied.

3.2.7 RNA extraction

RNA was extracted from patient cells, cell lines and transplanted mice using a modification of the method of Chomczynski and Sacchi (Chomczynski and Sacci, 1987). Approximately 5 x 10^6 cells were lysed and homogenised with 1 ml RNAzol B (guanidinium thiocyanate and phenol, Tel-Test). Chloroform, 100 µl, was added and the mixture vortexed vigorously for 15 seconds and placed on ice for 5 minutes. This was followed by centrifugation at 12,000*g* for 15 minutes at 4°C, which results in the formation of two phases. The upper aqueous phase, containing the RNA, was transferred to a fresh tube in an equal volume of isopropanol, placed on ice for 15 minutes and then centrifuged at 12,000*g* for 15 minutes at 4°C, to precipitate the RNA. Supernatant was removed and the RNA pellet washed once in 75% ethanol and then centrifuged at 7,500*g* for 8 minutes at 4°C. The pellet was then dried briefly under vacuum and then dissolved in 50 µl diethylpyrocarbonate (DEPC, BDH) treated water and stored at -70°C. RNA was quantitated by measuring its absorbance in a spectrophotometer at a wavelength of 260 nm. Purity was checked by measuring absorbance at 280 nm.

This method was further modified for RNA extraction from haemopoietic colonies because of the lower cell numbers involved. Individual discrete colonies were harvested (Figure 3.1) and dispersed in 10 µl DEPC treated PBS. This was transferred to 100 µl RNAzol B to which 20 µg of glycogen (Boehringer) was added as a carrier for the RNA. Chloroform, 10 µl, was added and the mixture vortexed vigorously for 15 seconds and placed on ice for 5 minutes. This was followed by centrifugation at 12,000*g* for 15 minutes at 4°C. The aqueous phase was transferred to a fresh tube in an equal volume of isopropanol. Samples were stored overnight at -20°C to promote RNA precipitation and then centrifuged at 12,000*g* for 15 minutes at 4°C. Supernatant was removed and the RNA pellet washed once in 75% ethanol and then centrifuged at 7,500*g* for 8 minutes at 4°C. The pellet was then dried briefly under vacuum for 1 to 2 minutes and then dissolved in 25 µl DEPC treated

Figure 3.1.

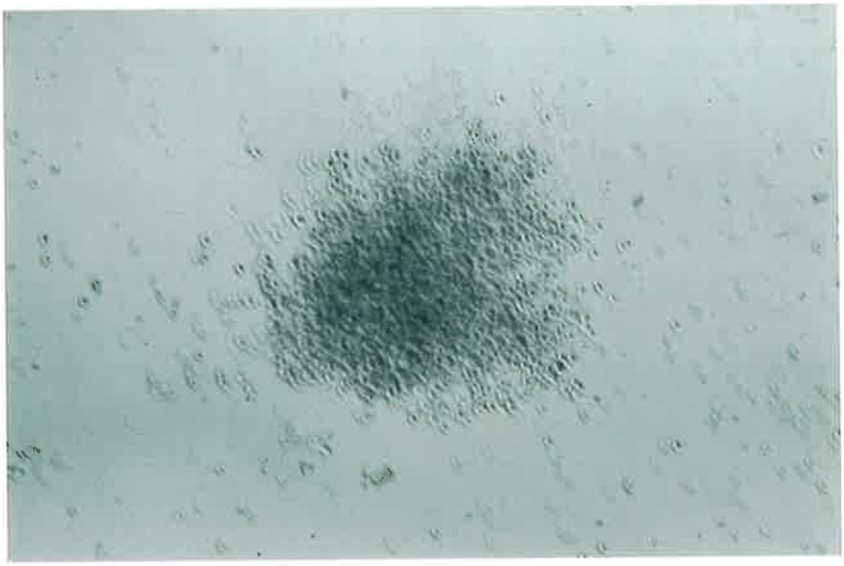
Harvesting CFU-GM for extraction of RNA or analysis of cells by FISH

3.1a. A single colony well separated from surrounding colonies is selected.

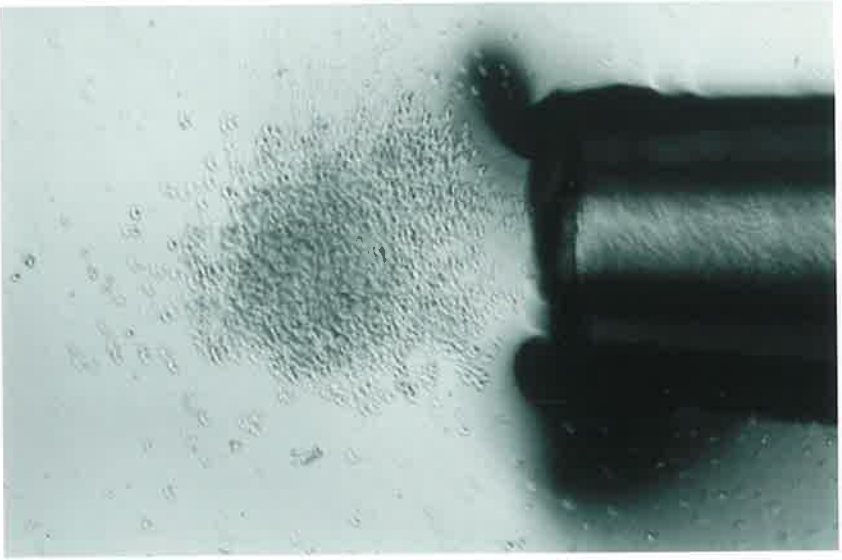
3.1b. The colony is gently aspirated using a 10 µl pipette. The contents are dispersed into either 100 µl RNAazol for RNA extraction (section 3.2.7) or 20 µl PBS for FISH analysis (section 3.2.11.6).

3.1c. A small number of residual cells remain in the methylcellulose after aspiration.

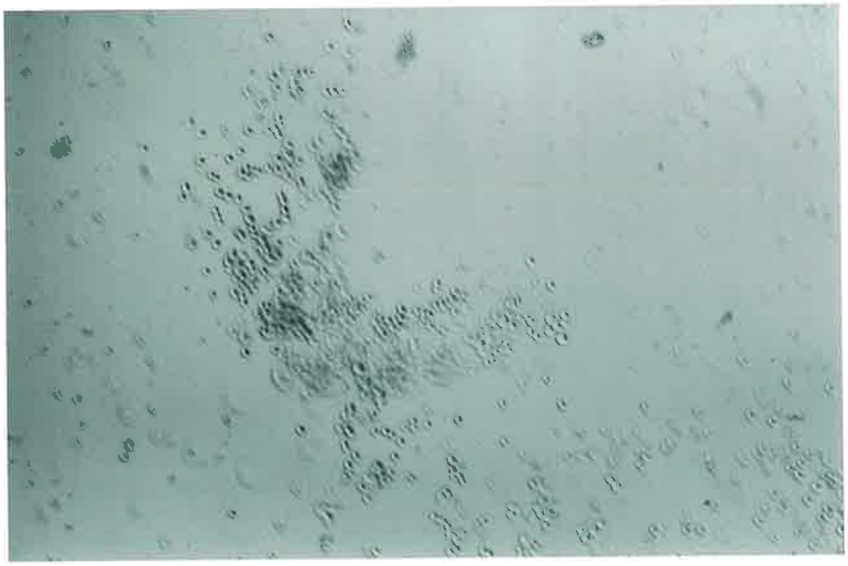
All steps performed using a dissecting microscope at magnification x 150.



a



b



c

water and stored at -70°C. Because of the low cell numbers being used RNA quantitation was not possible.

All stages were carried out in a dedicated RNA preparation room and sterile, RNAse free plasticware and filtered pipette tips were used throughout.

3.2.8 cDNA synthesis

Reverse transcription (RT) of mRNA was carried out by incubating RNA with an RT mixture. The RT mixture was made in a dedicated room free of DNA and RNA. RNA was added last in a different room to avoid contamination of reagents.

The following RT mixtures were made up:

"Standard" RT mixture

	concentration	volume (µl)
First Strand Buffer (Gibco BRL)	5X	40
DTT (Gibco BRL)	16 mM	10
Deoxynucleotides (dNTP; Pharmacia)	0.1 mM each	10
RNAguard (Pharmacia)	350 U	5
Random hexamers (pd(N)$_6$; Pharmacia)	1.2 µg	1.35
M-MLV Reverse Transcriptase (Gibco BRL)	2000 U	10
water		48.65

A modification of this RT mixture for reverse transcription of colony mRNA used lower volumes of RNAguard and reverse transcriptase because of the lower amount of mRNA product.

"Colony" RT mixture consisted of:

	concentration	volume (µl)
First Strand Buffer	5X	40
DTT	16 mM	10
Deoxynucleotides (dNTP)	0.1 mM each	10
RNAguard	175 U	2.5
Random hexamers (pd(N)$_6$)	1.2 µg	1.35
M-MLV Reverse Transcriptase	1000 U	5
water		56.15

For reverse transcription of mRNA from patient cells, cell lines and transplanted mice 2 µg RNA was diluted to 15 µl with water, heated to 65°C for 10 minutes to eliminate tertiary RNA structures, transferred to ice and then added to 25 µl of "standard" RT mixture. Reverse transcription of colony mRNA, with the lower amount of RNA present, used 12.5 µl of mRNA, heated and cooled as above, and added to 12.5 µl "colony" RT mixture. All samples were incubated at 37°C for 90 minutes. Following heating to 65°C for 10 minutes, to destroy reverse transcriptase enzyme activity, cDNA was stored at -20°C.

3.2.9 Polymerase chain reaction

Colony cDNA was assessed for the presence of leukaemia specific BCR-ABL by a nested PCR reaction. A nested PCR reaction was used because the increased sensitivity enables the detection of small amounts of cDNA. Integrity of RNA transcription was confirmed by performing PCR for the normal ABL gene. Oligonucleotide primers were produced by the Molecular Pathology Unit, IMVS. They were synthesized at 200 nm scale and provided in 1 ml concentrated ammonia solution. Oligonucleotides were precipitated by butanol extraction, dried under vacuum, resuspended in 100 µl sterile deionized water and quantified by UV spectroscopy at 260 nm. The following oligonucleotide primers were used (Figure 3.2):

Primer	Sequence (5' - 3')	Gene location	Reference
Nb1+	gagcgtgcagagtggagggagaaca	bcr (b1)	(Cross et al., 1993b)
Abl3-	ggtaccaggagtgtttctccagactg	abl (a3)	(Cross et al., 1993b)
B2a+	ttcagaagcttctccctgacat	bcr (b2)	(Cross et al., 1993b)
Ca3-	tgttgactggcgtgatgtagttgcttgg	abl (a3)	(Cross et al., 1993b)
Abl 1b+	cgctgagaattctggaagatcttgaa	abl (1b)	(Shtivelman et al., 1986)
C2-	ctccactggccacaaaat	abl (1a)	(de Klein et al., 1986b)
Ab+	cttctggaaaggggtacctatt	abl (1b)	(Melo et al., 1993)

PCR reaction mixtures were assembled in a dedicated room free of DNA and RNA. The first step PCR mixture were made up to the following concentrations in a total volume of 25 µl.

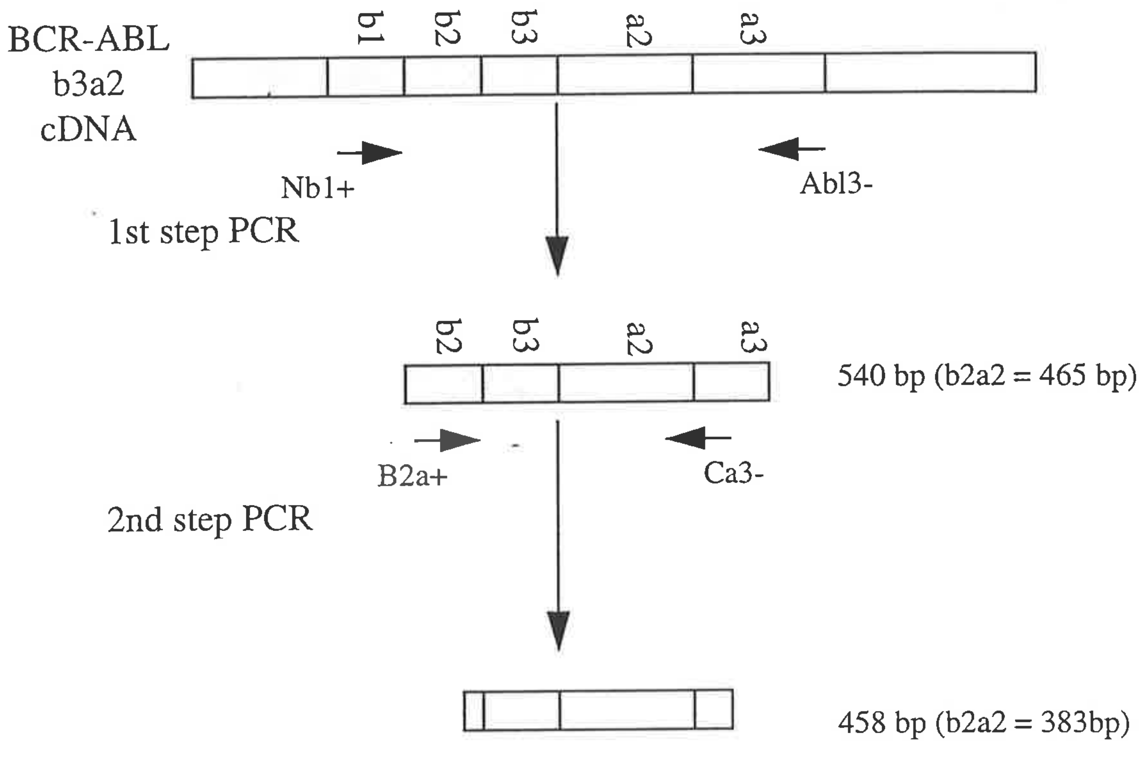
	Concentration
3' primer	0.625 µM
5' primer	0.625 µM
Tris pH 8.3	12.5 mM
$MgCl_2$	3.125 mM
KCl	62.5 mM
dNTP	0.25 mM each
AmpliTaq polymerase (Perkin Elmer)	0.75 U

For the first step PCR reaction, 5 µl of cDNA was added to 20 µl 1st step PCR mix, mixed well, overlayed with a drop of paraffin oil, to prevent evaporation whilst cycling, spun at 12,000*g* for 15 sec and placed in a thermal cycler (Perkin Elmer). First step primers for the

Figure 3.2.

Nested PCR reaction for the detection of BCR-ABL cDNA.

In the first step reaction primers Nb1+ and Abl3- amplify either a 540-bp (b3a2) or a 465-bp (b2a2) DNA fragment. A 1 µl aliquot of this reaction is then used in the second step reaction with internal primers B2a+ and Ca3- producing either a 458-bp (b3a2) or a 383-bp (b2a2) product.


BCR-ABL
b3a2
cDNA
b1
b2
b3
a2
a3
Nb1+
Abl3-
1st step PCR
b2
b3
a2
a3
540 bp (b2a2 = 465 bp)
B2a+
Ca3-
2nd step PCR
458 bp (b2a2 = 383bp)

detection of BCR-ABL were Nb1+ and Abl3- and for the detection of ABL were Abl 1b+ and Ca3-.

The second step PCR mixtures were made up to the following concentrations in a total volume of 20 µl.

	Concentration
3' primer	0.5 µM
5' primer	0.5 µM
Tris pH 8.3	10 mM
$MgCl_2$	1.5 mM
KCl	50 mM
dNTP	0.25 mM each
AmpliTaq polymerase	0.75 U

The second step PCR reaction was set up in a different room with dedicated pipettes. In this reaction, 1 µl of PCR product from the first step reaction was added to 19 µl second step PCR mix , mixed well, overlayed with a drop of paraffin oil, spun at 12,000*g* for 15 sec and placed in a thermal cycler (Perkin Elmer). Second step internal primers for the detection of BCR-ABL were B2a+ and Ca3- and for the detection of ABL were Ab+ and C2-.

PCR was performed on a Perkin Elmer Cetus DNA thermal cycler with an identical cycle program for 1st and 2nd step reactions. An initial denaturation of 95°C for 3 minutes was followed by 35 cycles of 95°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute followed by a 10 minute extension at 72°C. Reaction products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and viewed under UV light. BCR-ABL was seen as band sizes of 458-bp (b3a2) or 383-bp (b2a2) and ABL seen as 334-bp.

3.2.10 Infusion of NOD/SCID mice

Prior to cell infusion NOD/SCID mice 6 to 8 weeks old were irradiated with 3 Gy, delivered at 6 Gy per minute, by a CsCl blood cell irradiator. Cells were infused, in a volume of 500 µl to 1 ml of PBS, into a tail vein 24 hours after irradiation. Some mice received IP injections of recombinant HGF, either SCF (Amgen), G-CSF (Amgen) or GM - CSF (Sandoz), 5 µg 3 times per week. Mice were killed between days 28 and 50 by cervical dislocation. Prior to death PB was sampled from some mice. The BM from both femurs and tibias was flushed with HBSS supplemented with 5% FCS. The spleen was removed from the abdominal cavity and homogenised. Cells obtained from BM and spleen were washed once and counted prior to engraftment studies being performed.

3.2.11 Fluorescent in situ hybridisation (FISH)

3.2.11.1 Probe preparation

Human chromosome 8 was detected using a probe for the α satellite region at the D8Z2 locus (ATCC clone pJM128) which does not hybridise with mouse chromosomes. The probe was biotin labelled by standard nick translation with biotin-14-dATP (BioNick Labelling System; Gibco BRL) to 200-bp to 500-bp. Probe DNA, 2 µg, was mixed with 10 µl 10X dNTPs, 10 µl 10X enzyme mix and made up to 100 µl with water. The mixture was incubated at 15°C for 1 hour and terminated with 10 µl stop buffer. The DNA fragment was then purified with the GENECLEAN kit (Bio 101). The DNA solution was mixed with 3X vol sodium iodide and then 20 µl of GLASSMILK added and placed on ice for 5 minutes. The GLASSMILK/DNA complex was pelleted by brief centrifugation and washed 3 times with 800 µl cold NEW WASH. DNA was eluted into 500 µl water at 60°C for 5 minutes. Labelling was confirmed by running product on a 2% agarose gel and seeing a characteristic smear between 200-bp to 500-bp. Prior to hybridisation, 10 µl of hybridisation mixture was added to 50 ng to 100 ng of dessicated D8Z2 probe, denatured at 96°C for 4 minutes, cooled and then applied to the denatured slides.

3.2.11.2 Preparation of slides and cells for FISH

Glass microscope slides (76 x 26 mm; Objekttrager, Lomb Scientific Company) were methanol cleaned and allowed to air dry. They were then placed in a solution of poly-l-lysine (Sigma) diluted 1/10 with water for 5 minutes, removed and air dried. The poly-l-lysine coated slides enable cells to adhere to the surface. BM and spleen cells, approximately 1 x 10^4, from transplanted mice were washed twice in PBS and deposited on the slides. Cells were viewed under a microscope and one drop of 75 mM KCL was gently added to the cells. After 10 minutes cells were viewed again and if the nuclei were swollen, which enhances probe uptake, the cells were gently fixed three times and allowed to air dry.

3.2.11.3 Hybridisation

The technique used was a modified version of the methods described by Tkachuk (Tkachuk et al., 1990) and Lichter (Lichter et al., 1990). Cells were treated with RNAse A (Boehringer), 10 μg in 100 μl 2X SSC, for 1 hour at 37°C followed by 2 washes with 2X SSC. RNAse treatment destroys endogenous and nuclear RNA which may potentially hybridise with probe. Cells were then dehydrated with cold 70%, 90% and 100% ethanol. Cells were denatured, because the probe can only hybridise to single stranded DNA, in denaturation solution for 4 minutes at 70°C and then immediately dehydrated again with 70%, 90% and 100% ethanol. Probe was added to individual slides pre-heated to 37°C and incubated overnight in a humid atmosphere (Omni-Slide, Hybaid). After hybridisation slides were washed twice in post-hybridisation solution for 10 minutes at 42°C and then twice in 2X SSC and 1X SSC at room temperature to remove non-specifically bound probe.

3.2.11.4 Probe detection

Hybridised probe was detected using avidin-FITC (Vector), which binds to the hybridised biotin labelled probe. Slides were rinsed in 4X SSC/0.05% Tween 20 and pre-incubated for 10 minutes with 100 µl 4X SSC/3% BSA. Avidin-FITC diluted 1/400 with 4X SSC/1% BSA was added to individual slides and incubated in the dark at 4°C for 30 minutes. Slides were washed 3 times in 4X SSC/0.05% Tween 20, rinsed in distilled water and allowed to air dry in the dark. Nuclei were counterstained with propidium iodide (PI) and slides mounted in antifade (DABCO, 1,4 diazabicyclo[2.2.2.]octane; Sigma). Cells were examined at 1000X magnification under oil immersion using an Olympus BH2 microscope (Olympus) with fluorescence attachment. Cells in which 2 distinct green fluorescent signals were evident were scored as human. Cells stained with PI but with no fluorescent signal evident were scored as murine. A minimum of 300 cells were examined. Engraftment was determined by calculating the number of cells with 2 fluorescent signals as a percentage of the total number of PI stained cells. With each batch of slides control slides of normal human cells and normal murine cells were set up to confirm hybridisation and exclude cross-reactivity of probe.

3.2.11.5 FISH Analysis for leukaemic engraftment

The hybridisation protocol is similar to that described in 3.2.11.3. Prior to RNAse treatment slides were treated with 0.01 pg/ml proteinase K (Merck) , for 30 minutes at 37°C and then washed with 2X SSC. Proteinase K was used to permeabilise the cell nucleus. LSI bcr SpectrumGreen/abl SpectrumOrange dual color DNA probe mixture (Vysis) was prepared by mixing 1 µl probe, 2 µl water and 7 µl hybridisation mixture, denatured at 96°C for 4 minutes, cooled in ice and then added to slides. Dual-labelled cells were examined using a dual band-pass filter for both FITC and Texas red. Cells which contained 2 distinct green BCR signals and 2 distinct red ABL signals were defined as normal. Leukaemic cells were defined as cells in which a green BCR signal colocalised with a red ABL signal to produce a distinct colour shift with a resultant yellow signal. A single normal ABL and BCR signal should also be evident in the leukaemic cells.

3.2.11.6 FISH on individual haemopoietic colonies

Individual haemopoietic colonies were harvested, placed into 20 µl PBS and vortexed to disperse cells. A further 500 µl PBS was added. Following centrifugation at 400*g* for 10 minutes supernatant was removed and contents resuspended in 500 µl PBS and centrifugation repeated. Supernatant was removed gently leaving a residual volume of <50 µl. Cells were added to poly-L-lysine treated slides and treated with 75 mM KCl and fixed as previously described. Hybridisation with dual labelled probes to BCR and ABL was carried out as in 3.2.11.5.

3.2.12 Morphologic Assessment

Cytocentrifuge slides were prepared from BM and spleen of transplanted mice, air dried, and then stained with Jenner-Giemsa (BDH).

Conventional 4 µm histological sections of spleen and decalcified tibia were cut from formalin fixed, paraffin embedded material and stained with hematoxylin and eosin.

3.3 List of Suppliers

Amgen, Thousand Oaks CA, USA
ATCC, American Type Culture Collection, Manassas VA, USA
Baxter, Baxter Healthcare Corporation, Irvine CA, USA
BDH, BDH Laboratory Supplies, Poole, UK

Becton Dickinson, Becton Dickinson Immunocytometry Systems, San Jose CA, USA

Bio 101 Inc., La Jolla CA, USA

Boehringer, Boehringer Mannheim, Mannheim, Germany

Bresatec, Thebarton, SA, Australia

CSL, Commonwealth Serum Laboratories, Melbourne, Victoria, Australia

Corning, Corning Glass Works, Corning NY, USA

Coulter, Coulter Corporation, Hialeah FL, USA

Dako, Dako A/S, Glostrup, Denmark

Dow Chemical Co, Midland MI, USA

Dynal, Dynal A.S, Oslo, Norway

Gibco BRL, Life Technologies, Gaithersburg MD, USA

Hybaid, Hybaid Ltd, Teddington, Middlesex, UK

Kodak, Kodak Eastman Co, Rochester NY, USA

Lomb Scientific Company, Taren Point, NSW, Australia

Merck, Kilsyth, Victoria, Australia

Molecular Pathology, IMVS, Adelaide, SA, Australia

Nunclon, Nunc A/S, Roskilde, Denmark

Nycomed Pharma AS, Oslo, Norway

Olympus, Tokyo, Japan

Perkin-Elmer Corporation, Roche Molecular Systems, Inc., Branchburg NJ, USA

Pharmacia, Pharmacia Biotech, Uppsala, Sweden

Roche, Roche Products Pty Ltd, Dee Why NSW, Australia

Sandoz, Sandoz Pharma Ltd, Basle, Switzerland

Sigma, Sigma Chemical Company, Sigma-Aldrich Pty Ltd, St. Louis MO, USA

Tel-Test, Inc., Friendswood, TX, USA

Vector, Burlingame CA, USA

Vysis, Vysis, Inc., Downers Grove IL, USA

Chapter 4

In vitro studies of bone marrow derived normal and leukaemic stem cells in early chronic phase chronic myeloid leukaemia

4.1 Introduction

The majority of patients with CML are not suitable for allogeneic BMT and hence have little prospect of cure with current treatment modalitites. It is well established that residual normal haemopoietic cells are present in the BM of most patients at diagnosis. Manipulation of autologous BM for transplantation in CML to enrich for normal cells is an approach that may improve survival for patients not suitable for allogeneic transplantation.

Phenotypic characterisation shows that normal primitive cells are enriched in the $CD34^{+}DR^{-}$ fraction of CML BM (Verfaillie et al., 1992b; Leemhuis et al., 1993). In normal BM this fraction is enriched for primitive progenitors such as CFU-blast (Brandt et al., 1988) and HPP-CFC (Srour et al., 1993), which are capable of maintaining LTBMC (Verfaillie et al., 1990). However there is concern that HLA-DR may be an activation marker rather than a lineage marker. Recent studies have shown that the $CD34^{+}38^{-}$ population is enriched for primitive progenitors (Rusten et al., 1994) and contains cells with both in vitro replating potential (Terstappen et al., 1991) and the capacity to produce multilineage engraftment of fetal sheep (Civin et al., 1996). This fraction of normal adult BM therefore contains cells with features of haemopoietic stem cells. There are few studies on this population in CML and given its role in normal haemopoiesis it is critical to characterise these cells in CML.

To address this issue the BM at diagnosis from 3 CML patients was sorted into $CD34^{+}DR^{-}$ and $CD34^{+}DR^{+}$ populations and in separate experiments into $CD34^{+}38^{-}$ and

$CD34^+38^+$ populations to determine whether they were a source of primitive $BCR-ABL^-$ progenitors. These populations were analysed to evaluate the frequency of $BCR-ABL^-$ progenitors and pre-progenitors in these populations. Progenitor analysis was performed by RT-PCR of individual haemopoietic colonies from a standard CFU-GM assay. Analysis of pre-progenitors involved RT-PCR of secondary colonies derived from a stroma-free pre-CFU assay. These selected subsets of marrow derived $CD34^+$ cells are shown to be a source of normal pre-progenitors.

4.2 Methods

The approach taken is summarised in Figure 4.1. BM samples were collected from patients with recently diagnosed CML and processed as described in 3.2.1.1. Cells were labelled with appropriate antibody and sorted by FACS into defined populations. Sorted cells were used to establish pre-CFU and CFU-GM assays if numbers were sufficient. Secondary CFU-GM assays were established from the pre-CFU assay as described in 3.2.4. Individual CFU-GM were harvested, RNA was extracted, and the presence of leukaemia-specific BCR-ABL determined by RT-PCR.

4.3 Patient samples

Three patients with recently diagnosed chronic phase CML were studied (Table 4.1). Cytogenetic studies on BM showed 100% of metaphases positive for the Ph chromosome. All patients were studied at diagnosis and had not received any therapy.

4.4 Cell sorting

Cells were gated on the lymphocyte fraction (Andrews et al., 1989). The sort windows used are shown in Figure 4.2. The mean number of sorted cells which were $CD34^+DR^-$ and $CD34^+38^-$ were 0.1% (range 0.01% to 0.2%) and 0.1% (range 0.1% to 0.2%) respectively.

Figure 4.1.

Summary of methods used for analysis of progenitors and pre-progenitors in early chronic phase CML.

Peripheral blood or bone marrow was immunolabelled with anti-CD34 FITC and either anti-HLA-DR PE or anti-CD38 PE. Cells were sorted by FACS into populations of $CD34^+DR^-$ and $CD34^+DR^+$ or $CD34^+38^-$ and $CD34^+38^+$. Progenitor assays were established with 1000 cells per ml of methylcellulose and supplemented with 10 ng each of IL-1, IL-3, IL-6, G-CSF, GM-CSF and SCF. The pre-progenitor assay was established by plating 1000 cells in 1 ml pre-CFU medium and 10 ng IL-3, 20 ng IL-6, 100 ng G-CSF and 100 ng SCF. At 7 day intervals cells were counted and given fresh medium and growth factors. Aliquots of cells were used to establish secondary CFU-GM assays. After 14 days culture, CFU-GM were counted and individual discrete colonies harvested. RNA was extracted from individual colonies and RT-PCR performed for the presence of BCR-ABL and ABL. Some colonies were analysed by FISH for the presence of BCR-ABL.

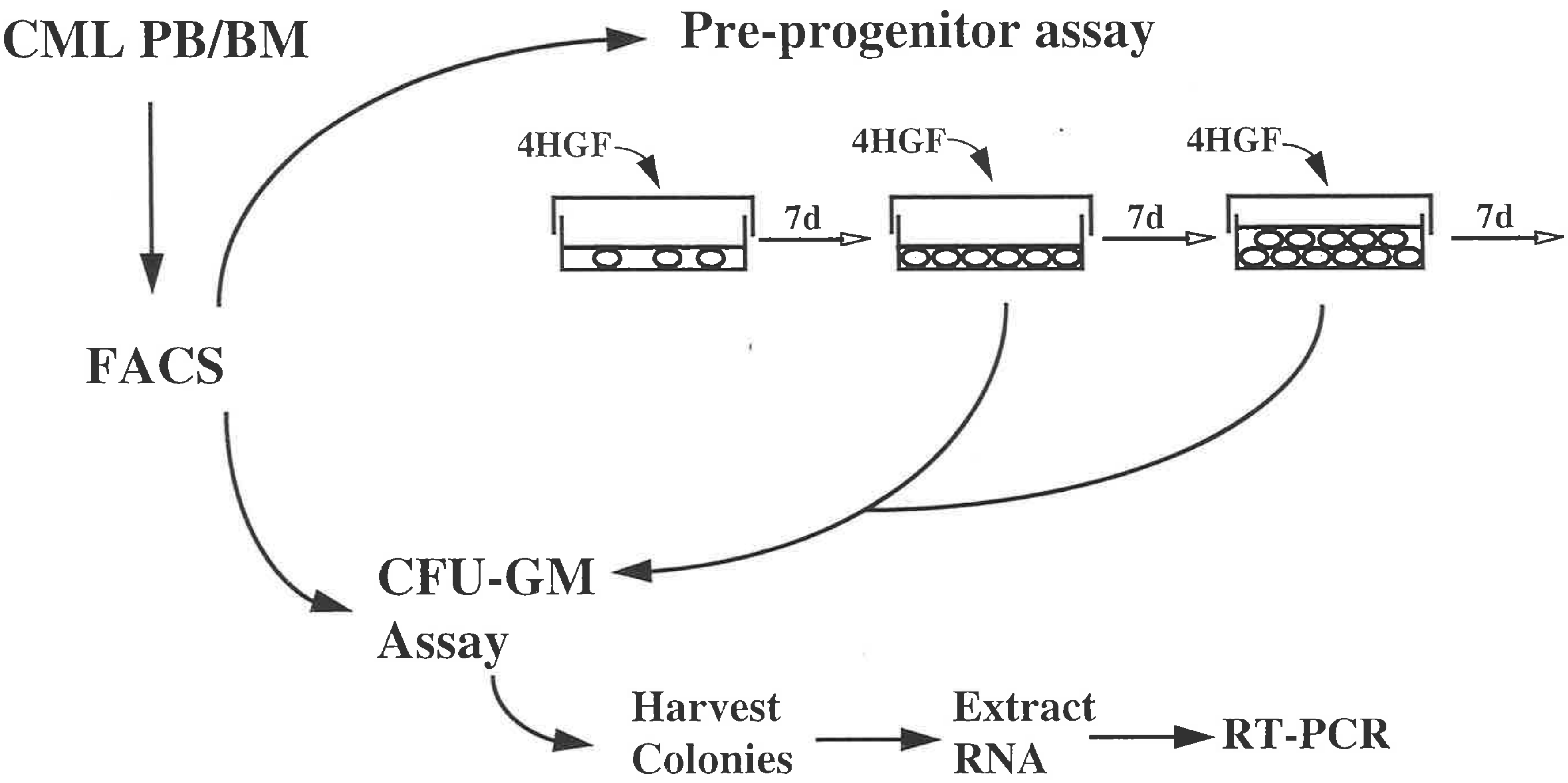

CML PB/BM
Pre-progenitor assay
4HGF
4HGF
4HGF
7d
7d
7d
FACS
CFU-GM
Assay
Harvest
Colonies
Extract
RNA
RT-PCR

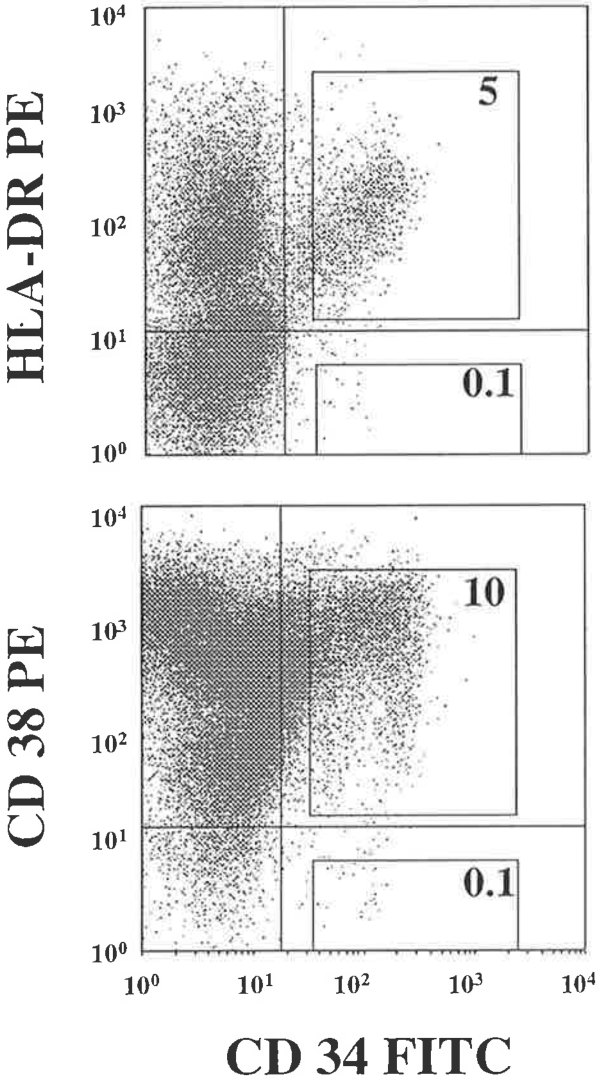
Table 4.1.
Demographic details of patients studied

Patient	Age	Sex	WCC	Ph+ metaphases/ total metaphases	Prior treatment
1	65	M	208	30/30	Nil
2	64	M	84	15/15	Nil
3	45	F	157	15/15	Nil

Abbreviation: WCC = white cell count x 10^9/L

Figure 4.2.

Cell sorting of CML BM into defined populations. Cells in the low side-scatter and low to intermediate forward light scatter windows were sorted into fractions expressing high density CD34 antigen and either high or low expression HLA-DR antigen (top diagram) or the CD38 antigen (bottom diagram). It can be seen that the $CD34^{+}DR^{-}$ and $CD34^{+}38^{-}$ populations make up only 0.1% of gated cells or approximately 1% to 2% of $CD34^{+}$ cells.


HLA-DR PE
10^4
10^3
10^2
10^1
10^0
5
0.1
CD 38 PE
10^4
10^3
10^2
10^1
10^0
10
0.1
10^0
10^1
10^2
10^3
10^4
CD 34 FITC

The low frequency of these populations resulted in insufficient cells being collected to enable CFU-GM assays to be established on all samples.

4.5 Cell proliferative capacity

The pattern of cell proliferation in the pre-CFU assay is shown in Figure 4.3. The $CD34^{+}DR^{-}$ and $CD34^{+}38^{-}$ populations showed marked expansion to days 49 to 56. There was heterogeneity between patients. The $CD34^{+}DR^{+}$ and $CD34^{+}38^{+}$ populations showed early proliferation with maximal cell numbers by days 14 to 28 with decline in cell numbers after day 28 in most samples. These growth patterns are similar to those seen in normal marrow (data not shown). The $CD34^{-}$ population did not proliferate.

The pattern of CFU-GM production reflected the cell proliferation responses (Figure 4.4). Total CFU-GM numbers were calculated by multiplying the total cell number in the pre-CFU assay by the ratio of CFU-GM per cells plated. In the $CD34^{+}DR^{-}$ and $CD34^{+}38^{-}$ fractions peak numbers of CFU-GM occurred at day 35 with the $CD34^{+}DR^{-}$ fraction producing more CFU-GM. The $CD34^{+}DR^{-}$ fraction produced CFU-GM at day 42 but the $CD34^{+}38^{-}$ fraction produced no colonies at this timepoint. The $CD34^{+}DR^{+}$ and $CD34^{+}38^{+}$ fractions had minimal colony production at days 7 and 14 and none at later timepoints.

4.6 Assessment of the sensitivity of RT-PCR

A nested PCR technique was used because of the small amount of RNA present in individual haemopoietic colonies. In this method an aliquot of PCR product from the 1st step reaction is used as a template for the second step reaction and amplified by primers internal to those used in the 1st step reaction. The sensitivity of this nested PCR reaction was assessed by serial dilution of K562 RNA. The standard reverse transcription reaction uses 2 μg of K562 RNA in a volume of 15 μl added to 25 μl of cDNA reaction mixture

Figure 4.3.

Proliferation of different populations of CML BM in the pre-progenitor assay. Cells were initially plated at 1000 cells per well and counted at 7 day intervals. The CD34$^+$DR$^-$ and CD34$^+$38$^-$ populations show marked expansion of cells up to day 49. The CD34$^+$DR$^+$ and CD34$^+$38$^+$ populations do not expand to the same degree with maximal cell numbers seen at day 28.

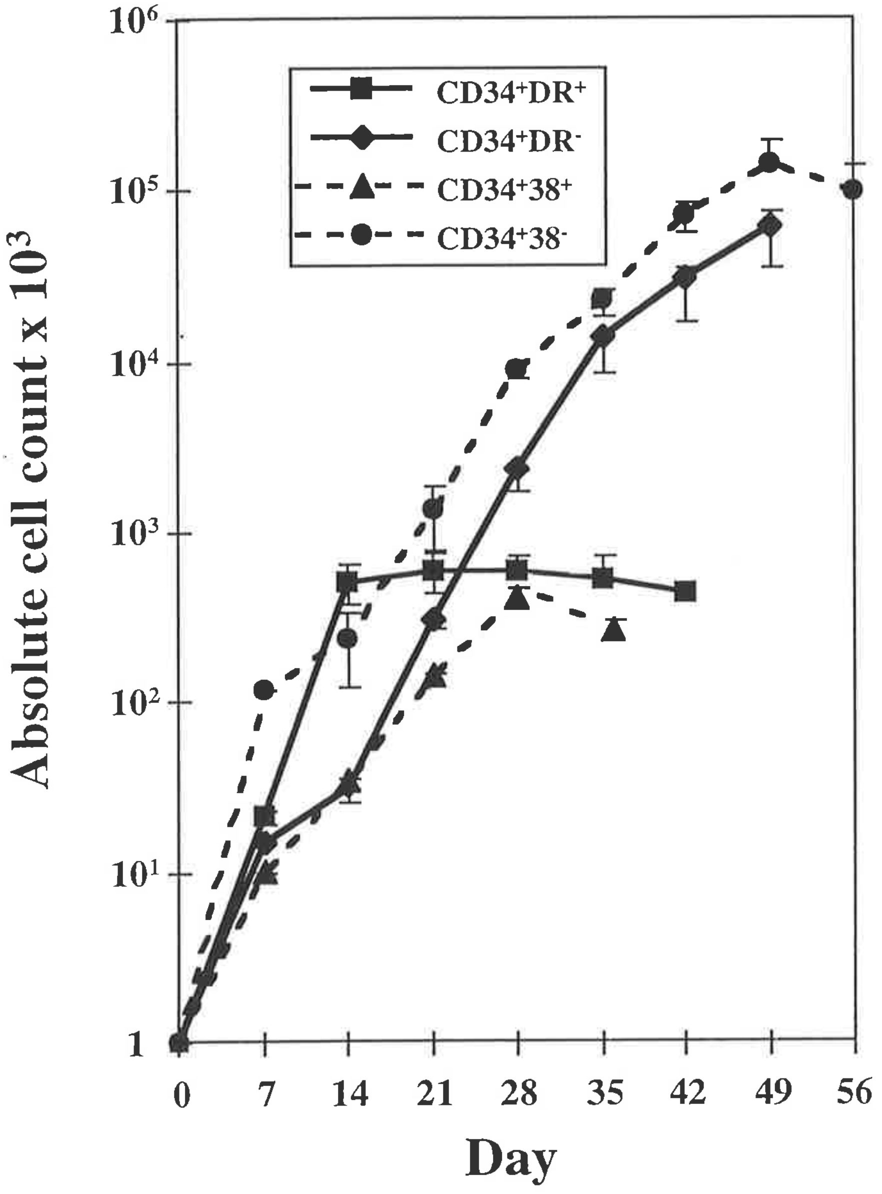
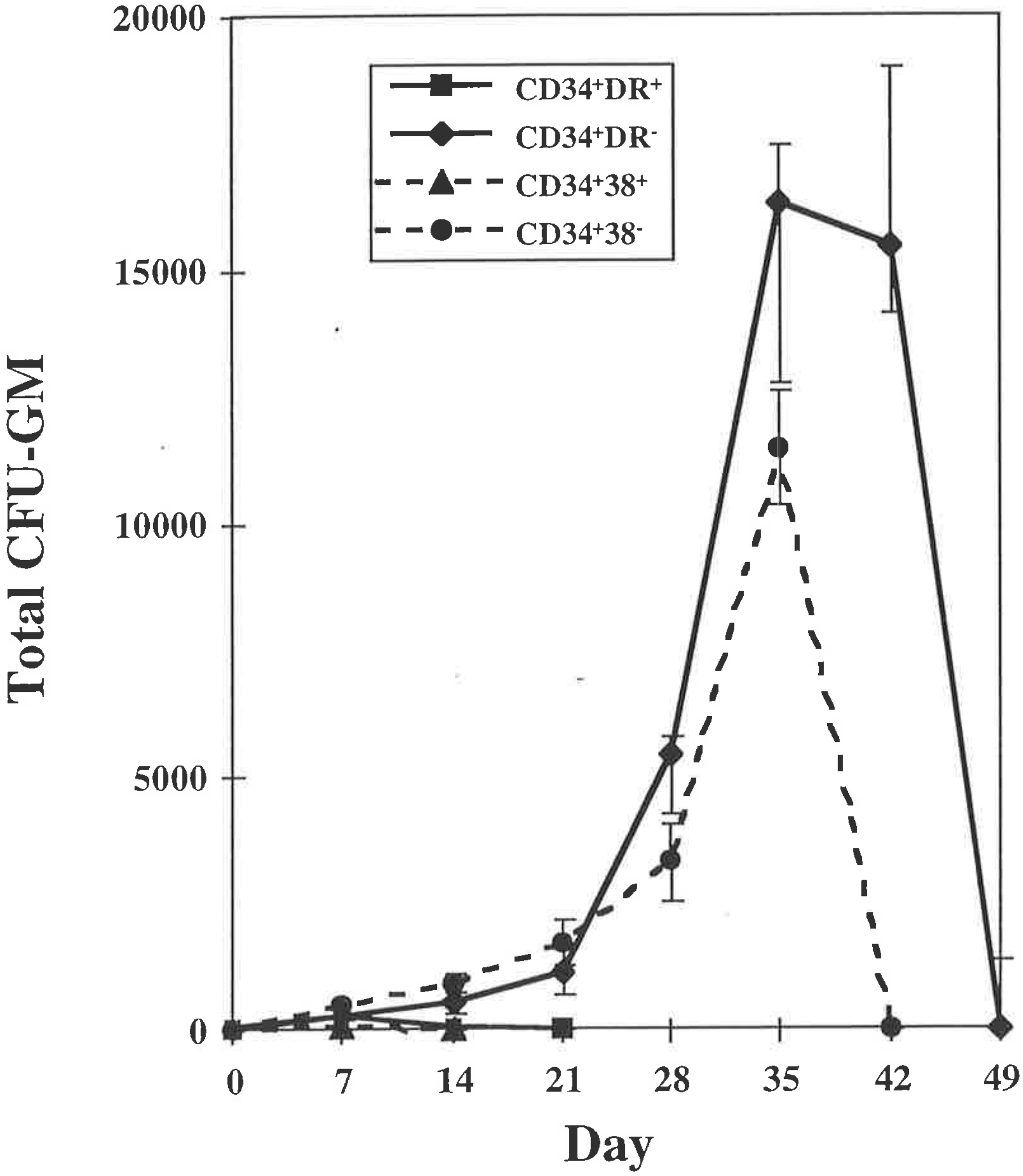

Absolute cell count x 10^3
10^6
10^5
10^4
10^3
10^2
10^1
1
0
7
14
21
28
35
42
49
56
Day
CD34+DR+
CD34+DR-
CD34+38+
CD34+38-

Figure 4.4.

Total CFU-GM production of different populations of CML BM. Numbers are calculated by counting CFU-GM per number of cells plated and multiplying by total cell numbers as counted in the pre-progenitor assay. The appearances reflect the results of the pre-progenitor assay with the $CD34^+DR^-$ and $CD34^+38^-$ populations producing large numbers of CFU-GM up to day 42 and 35 respectively. In contrast the $CD34^+DR^+$ and $CD34^+38^+$ populations produce few CFU-GM.


CD34⁺DR⁺
CD34⁺DR⁻
CD34⁺38⁺
CD34⁺38⁻
Total CFU-GM
20000
15000
10000
5000
0
0
7
14
21
28
35
42
49
Day

(total volume = 40 μl) and then 5 μl of cDNA product is used for the PCR reaction. Hence PCR is carried out on cDNA derived from approximately 0.25 μg of RNA.

To assess sensitivity, 2 μg of K562 RNA in an initial volume of 15 μl was serially diluted 1/10 in DEPC treated water to a final dilution of $1/10^6$. Each dilution of RNA was reverse transcribed using 15 μl of dilution mixture with 25 μl of cDNA reaction mixture. Nested PCR was carried out as described in 3.2.9. Products from the 1st step and 2nd step were electrophoresed on 2% agarose gels and the results are shown in figure 4.5. The 1st step reaction produces a BCR-ABL product of 540-bp and it can be seen in figure 4.5a that this reaction will detect a $1/10^2$ dilution of 2 μg K562 RNA. Using 1 μl of product from this 1st step reaction a 2nd step PCR is performed. In figure 4.5b the results of the second step PCR are shown. A BCR-ABL product of 458-bp is produced and the sensitivity of the reaction increases to a $1/10^5$ dilution of 2 μg K562 RNA.

Hence this nested PCR reaction can detect approximately 2.5 pg of K562 RNA. The cellular content of RNA is approximately 20 pg per cell. Therefore this technique is suitable for assessing individual haemopoietic colonies for the presence of BCR-ABL.

4.7 Analysis of haemopoietic colonies for BCR-ABL

Strict precautions were adhered to at all stages of colony processing to avoid contamination. Separate dedicated rooms were used for RNA preparation, 1st step PCR preparation, 2nd step PCR preparation, and analysis of products on agarose gels. Integrity of RNA was confirmed by performing a nested PCR reaction for the presence of the normal ABL gene. Colonies in which BCR-ABL was detected were defined as leukaemic. BCR-ABL$^-$ colonies in which ABL was detected were defined as normal. Colonies in which neither product was detected were not included in the results. All BCR-ABL$^-$ colonies were tested in duplicate. Examples of colony PCR studies are shown in Figure 4.6. The positive

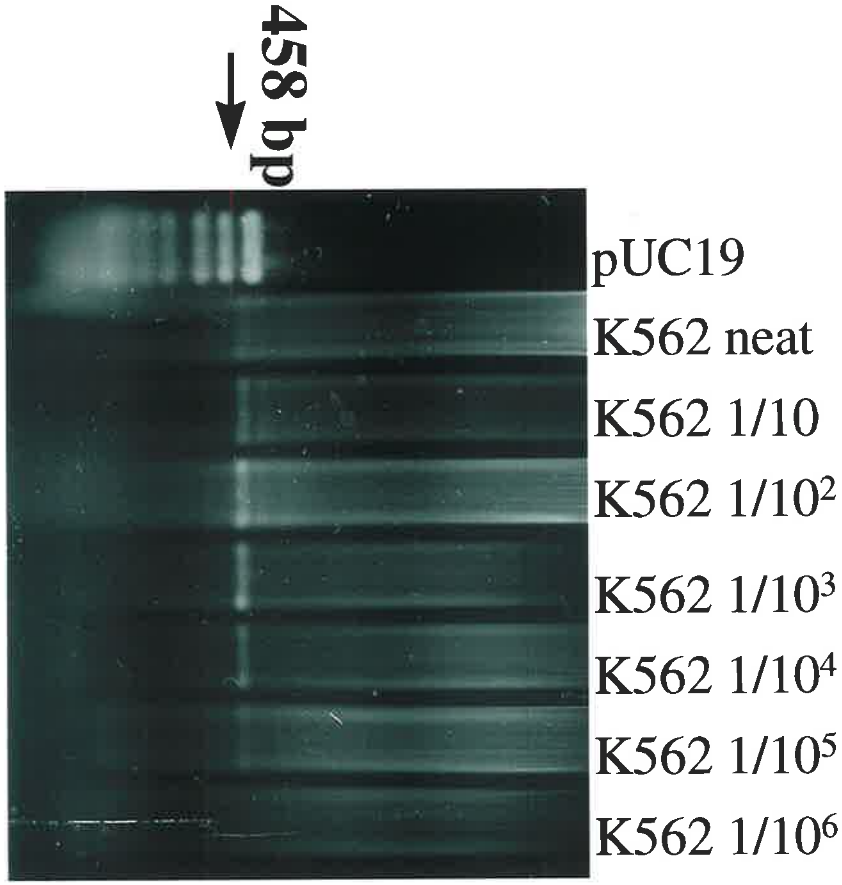
Figure 4.5.

Assessment of sensitivity of nested RT-PCR reaction for the detection of BCR-ABL.

K562 RNA, 2 µg in 15 µl, was serially diluted 1/10 to a final dilution of $1/10^6$. Each dilution had cDNA made by reverse transcription and then 1st and 2nd step PCR reactions were carried out. Products from 1st step and 2nd step reactions were electrophoresed on a 2% agarose gel, stained with ethidium bromide, viewed under UV light and photographed.

Diagram a shows the result of 1st step PCR on all dilutions. The 540-bp band of BCR-ABL is detected by neat K562 and the 1/10 and $1/10^2$ dilutions.

In the 2nd step reaction, 1 µl of 1st step PCR product was used as the template for the reaction. This results in an increase in the sensitivity of the reaction as seen in diagram b with the 458-bp second step BCR-ABL product being detected at a dilution of $1/10^5$.


458 bp
pUC19
K562 neat
K562 1/10
K562 1/10^2
K562 1/10^3
K562 1/10^4
K562 1/10^5
K562 1/10^6

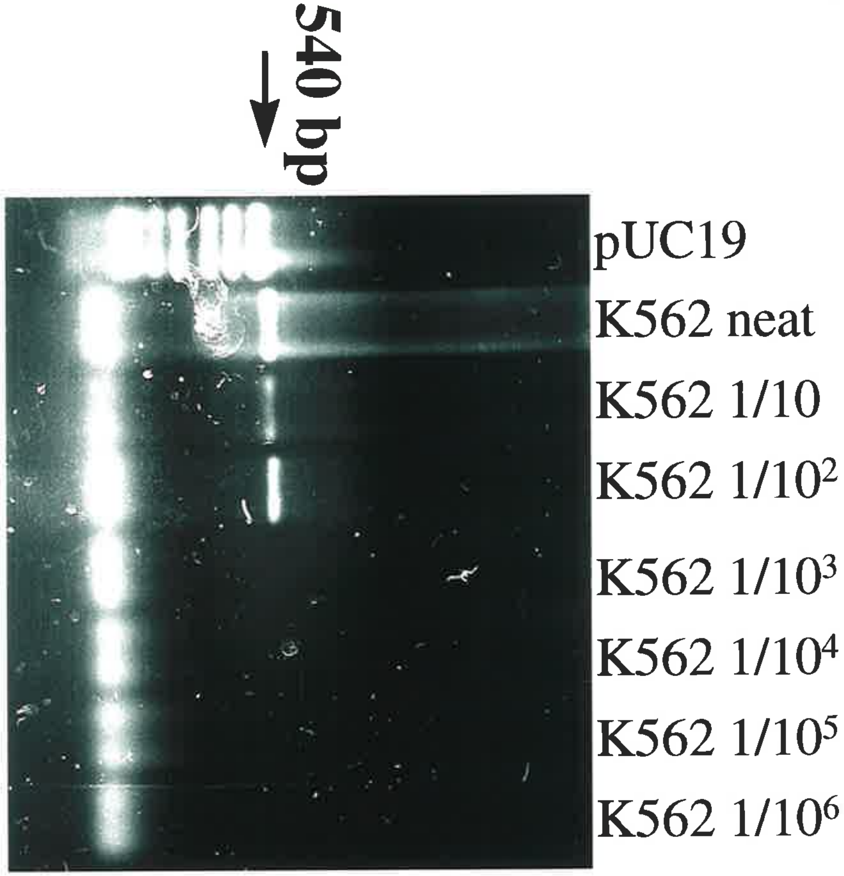

540 bp
pUC19
K562 neat
K562 1/10
K562 1/10^2
K562 1/10^3
K562 1/10^4
K562 1/10^5
K562 1/10^6

Figure 4.6.

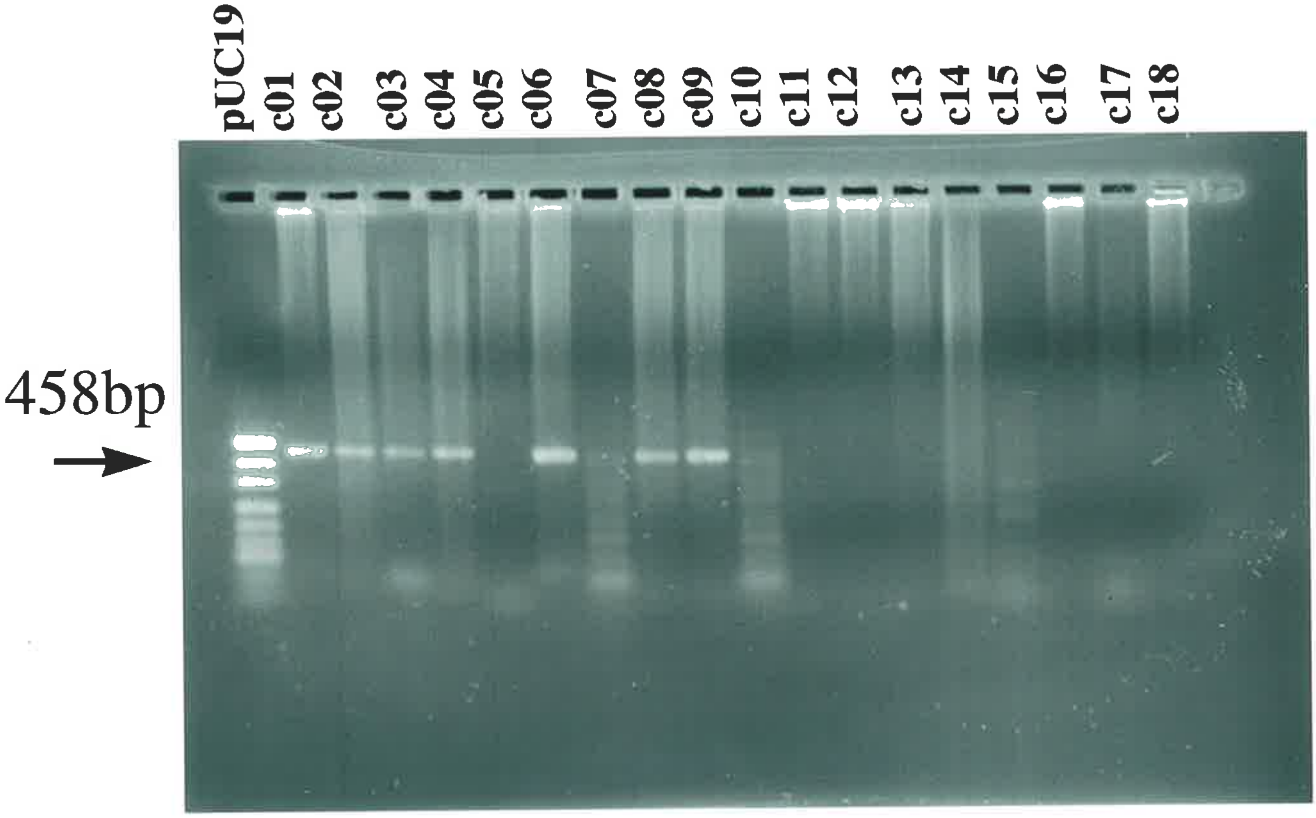
Examples of colony PCR studies.

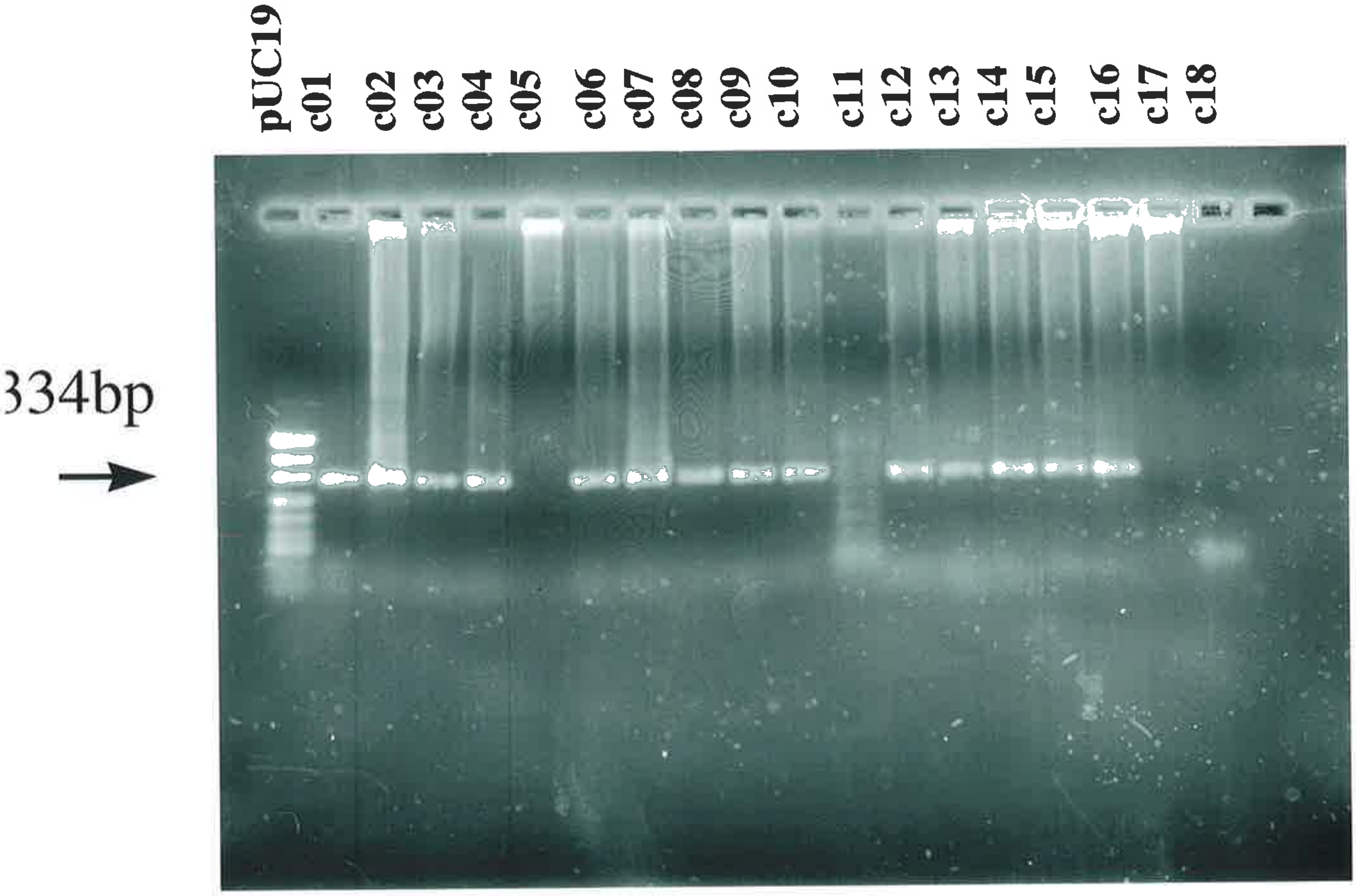
Colonies c01 - c06 are from day 0 CFU-GM. Colonies 01, 02, 03, 04 and 06 have both BCR-ABL and ABL detected and are therefore leukaemic. Colony 05 has neither product detected and is discarded from the results.

Colonies c07 - c11 are from day 14 pre-progenitors. Colonies 08 and 09 have BCR-ABL detected and are therefore leukaemic. Colonies 07 and 10 are BCR-ABL$^-$ but ABL$^+$ and are defined as normal. Colony 11 has neither BCR-ABL or ABL detected and is discarded from the results.

Colonies c12 -c16 are from day 28 pre-progenitors. No colonies have BCR-ABL detected but all have ABL detected and are classified as normal.

C17 is an RNA negative control, being KG1a for BCR-ABL and EM-2 for ABL. C18 is a water control.


pUC19
c01
c02
c03
c04
c05
c06
c07
c08
c09
c10
c11
c12
c13
c14
c15
c16
c17
c18
458bp


pUC19
c01
c02
c03
c04
c05
c06
c07
c08
c09
c10
c11
c12
c13
c14
c15
c16
c17
c18
334bp

controls used in this reaction were a 1/10^5 dilution of K562 RNA and known positive colonies from previous reactions. Negative RNA controls were the cell lines KG1a for BCR-ABL and EM2 for ABL. Water controls were used in both reactions. Multiple positive and negative controls were run in each batch of reactions to ensure validity of results. A further control was the use of plucked methylcellulose devoid of colonies to test for false results due to contaminating non-clonogenic cells and these were negative in the majority of reactions.

4.8 Analysis of BM progenitors for BCR-ABL

Progenitor analysis of the CD34$^+$DR$^-$ and CD34$^+$38$^-$ populations was only possible in one specimen because of the failure to sort sufficient cells from these rare populations. The one BM sample analysed from the CD34$^+$DR$^-$ fraction had 1 of 6 (17%) BCR-ABL$^-$ colonies (Patient 2).

Progenitors from the CD34$^+$DR$^+$ fraction showed 0 of 8 (0%), 1 of 5 (20%) and 1 of 5 (20%) BCR-ABL$^-$ colonies from patients 1, 2 and 3 respectively. Progenitors from the CD34$^+$38$^+$ fraction showed 8 of 17 (47%) and 3 of 9 (33%) BCR-ABL$^-$ colonies from patients 1 and 2 respectively.

4.9 Analysis of BM pre-progenitors for BCR-ABL

Pre-progenitors were analysed by assessment of secondary colonies at days 14 and 28 of pre-CFU culture. Where possible a minimum of 15 colonies were examined at each timepoint.

The majority of colonies derived from the CD34$^+$DR$^-$ fraction were BCR-ABL$^-$ by PCR analysis of individual colonies at days 14 and 28. Analysis of BM at day 14 showed 80%

to 100% (mean 96%) of colonies were BCR-ABL$^-$. At day 28, 89% to 93% (mean 90%) of colonies were BCR-ABL$^-$ (Table 4.2).

Analysis of colonies from the CD34$^+$38$^-$ fraction at day 14 showed 70% to 100% (mean 90%) BCR-ABL$^-$ colonies. Analysis at day 28 showed 100% of colonies were BCR-ABL$^-$ in 2 patients (Table 4.2). No analysable colonies were derived from the CD34$^+$DR$^+$ and CD34$^+$38$^+$ at either day 7 or 14.

4.10 Discussion

The results described here show that pre-progenitors derived from the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions of BM from patients with early chronic phase CML are predominantly BCR-ABL$^-$. Analysis of progenitors from these populations was limited because insufficient cells were sorted from these populations. Progenitors from the CD34$^+$DR$^+$ and CD34$^+$38$^+$ populations were predominantly leukaemic. These populations produced few pre-progenitors which reflects the relative mature nature of these cells compared to the more primitive CD34$^+$DR$^-$ and CD34$^+$38$^-$ populations.

Normal haemopoietic progenitors were demonstrated to coexist in CML BM following disappearance of the Ph$^+$ clone during long term stromal culture (Coulombel et al., 1983). Subsequently normal progenitors have been demonstrated in the CD34$^+$DR$^-$ fraction of CML BM. Verfaillie isolated CD34$^+$DR$^-$ BM cells and showed 4 of 5 patients had BCR-ABL$^-$ colonies following LTBMC (Verfaillie et al., 1992b). The study of Leemhuis showed a median of 50% of CD34$^+$DR$^-$ progenitors were normal (Leemhuis et al., 1993). The data presented on the analysis of pre-progenitors from the CD34$^+$DR$^-$ fraction showed that the majority derived from the BM are BCR-ABL$^-$. This is similar to previous results achieved following LTBMC. Stromal based assays may select for the Ph$^-$ clone independently because of a defect in cell adhesion of Ph$^+$ progenitors but this bias is not present in a

Table 4.2.

Results of colony analysis of pre-progenitors derived from the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions of BM at diagnosis. Results are expressed as number of BCR-ABL negative colonies per total analysable colonies.

Patient	CD34$^+$DR$^-$			CD34$^+$38$^-$	
	d0	d14	d28	d14	d28
1	NA	56/58 (97%)	NA	28/28 (100%)	5/5 (100%)
2	1/6 (17%)	4/5 (80%)	33/37 (89%)	12/12 (100%)	NA
3	NA	10/10 (100%)	14/15 (93%)	14/20 (70%)	26/26 (100%)

NA = no analysable colonies

stromal free system. However, the leukaemic pre-progenitor may not proliferate in liquid culture thus explaining the finding of predominant BCR-ABL$^-$ colonies in both the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions.

The demonstration of BCR-ABL$^-$ pre-progenitors in the CD34$^+$38$^-$ fraction of BM of patients with chronic phase CML is a new finding. In a study of sorted BM cells in patients with chronic phase CML treated with hydroxyurea or interferon the CD34$^+$38$^-$ population showed no enrichment for BCR-ABL$^-$ cells compared to the CD34$^+$38$^+$ population by FISH analysis (Kirk et al., 1995). Another report of cytogenetic evaluation of PB or BM of 6 patients with chronic phase CML showed 1 of 123 normal metaphases in the CD34$^+$38$^-$ fraction compared to 22 of 114 normal metaphases in the CD34$^+$DR$^-$ fraction (de Fabritiis et al., 1993a). The more primitive cells in the CD34$^+$38$^-$ population, defined by the pre-progenitor assay, show BCR-ABL$^-$ colonies with a mean of 90% at day 14 and 100% at day 28. This suggests that the CD34$^+$38$^-$ population contains pre-progenitors that are not leukaemic. Studies in both normal and CML BM have shown that less than 15% of the CD34$^+$DR$^-$ population are CD38$^-$ and similarly less than 15% of the CD34$^+$38$^-$ population is HLA-DR$^-$ (Rusten et al., 1994; Kirk et al., 1995). Given these findings it would be of interest to know what the expression of HLA-DR and CD38 is on the CD34$^+$38$^-$ and CD34$^+$DR$^-$ populations respectively. This may better define the phenotypic characteristics of the population that contains BCR-ABL$^-$ pre-progenitors. However, for technical reasons it was not possible to perform three colour sorting in this study, and therefore these populations could not be investigated.

In summary it has been demonstrated that pre-progenitors of both the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions are predominantly BCR-ABL$^-$ in the BM of patients with early chronic phase CML. If these populations are to be used as a source of BCR-ABL$^-$ cells for manipulation as an autologous transplantation strategy two concerns must be addressed. Firstly, the rarity of these populations may mean insufficient cells can be collected from the BM at diagnosis for use in autologous transplantation. Secondly, the failure to detect leukaemic pre-progenitors may be an artefact due to the in vitro culture conditions and therefore these populations may contain significant numbers of leukaemic pre-progenitors. These issues have been addressed by analysing peripheral blood as a potential abundant source of $BCR\text{-}ABL^-$ pre-progenitors and establishing a murine model of CML which potentially could be used as an in vivo assay to study normal and leukaemic stem cells present in CML.

Chapter 5

In vitro studies of normal and leukaemic stem cells in early chronic phase chronic myeloid leukaemia: Peripheral blood is a source of BCR-ABL-negative primitive progenitors (pre-progenitors)

5.1 Introduction

A major technical challenge in autologous transplantation of purified normal cells in CML is the low frequency of these cells in BM and the difficulties in obtaining sufficient BM for manipulation. Safe transplantation with limited numbers of cells may not be feasible and ex-vivo manipulation of rare cells may be difficult. Abundant $CD34^+$ cells can usually be collected from PB of CML patients at diagnosis but the presence of normal primitive progenitors in PB has not been established.

To address this issue the apheresis product was compared with BM at diagnosis as a source of primitive $BCR\text{-}ABL^-$ progenitors. The $CD34^+DR^-$ and $CD34^+38^-$ populations in 6 CML patients were analysed to evaluate the frequency of $BCR\text{-}ABL^-$ progenitors and pre-progenitors in these populations. These selected subsets of blood-derived $CD34^+$ cells, like their marrow-derived counterparts, are shown to be a source of normal pre-progenitors. The apheresis product collected at diagnosis is a more abundant source of $BCR\text{-}ABL^-$ pre-progenitors than BM and could potentially be utilised as a source of $BCR\text{-}ABL^-$ stem cells in CML.

5.2 Methods

The approach taken was identical to the methods summarised in chapter 4.2 for the analysis of BM specimens. In addition to the initial 3 patients studied a further 3 patients had studies performed exclusively on PB. To confirm the RT-PCR results a number of colonies were examined by FISH for the presence of the BCR-ABL translocation.

5.3 Patient samples

Six patients with recently diagnosed chronic phase CML were studied (Table 5.1). Cytogenetic studies on BM showed 100% of metaphases positive for the Ph chromosome. All patients were studied at or shortly after diagnosis and one patient had received hydroxyurea for 3 months.

5.4 Cell sorting

Cells were gated and identical sort windows used as for collection of the BM specimens (section 4.4). The PB appearances by flow cytometry were similar to the BM (Figure 5.1). The mean number of sorted cells which were $CD34^+DR^-$ and $CD34^+38^-$ were 0.25% (range 0.03% to 0.5%) and 0.2% (range 0.1% to 0.4%) respectively. Hence more cells in the PB were present in these populations compared to the BM but the differences were not significant ($p = 0.13$, paired t-test). More cells were able to be collected from these populations in the PB compared to the BM. This was a result of starting with higher cell numbers and the higher frequencies of the critical populations. Because of this, progenitor assays were able to be established from sorted populations of most specimens.

5.5 Cell proliferative capacity

The pattern of cell proliferation in the pre-CFU assay was similar between PB and BM (Figure 5.2) although expansion was greater from the BM derived cells. There was heterogeneity between patients. Peripheral blood from patient 1 continued to expand up to day 56 in both $CD34^+DR^-$ and $CD34^+38^-$ fractions. In contrast patient 5 showed no proliferation in the $CD34^+DR^-$ fraction but showed limited proliferation in the $CD34^+38^-$

Table 5.1.
Demographic details of patients studied

Patient	Age	Sex	WCC	Ph+ metaphases/ total metaphases	Prior treatment
1	65	M	208	30/30	Nil
2	64	M	84	15/15	Nil
3	45	F	157	15/15	Nil
4	54	F	32	15/15	OH 3 months
5	42	M	232	15/15	Nil
6	57	M	27	15/15	Nil

Abbreviations: WCC = white cell count x 10^9/L
OH = hydroxyurea

Figure 5.1.

Cell sorting of CML PB of the same patient shown in figure 4.2. Cells in the low side-scatter and low to intermediate forward light scatter windows were sorted into fractions expressing high density CD34 antigen and either high or low expression HLA-DR antigen (top diagram) or the CD38 antigen (bottom diagram). In comparison to the BM there are more total $CD34^+$ cells in the PB in this patient but the relative frequencies of the $CD34^+DR^-$ and $CD34^+38^-$ populations remain very low at 0.3% and 0.1% of gated cells respectively.

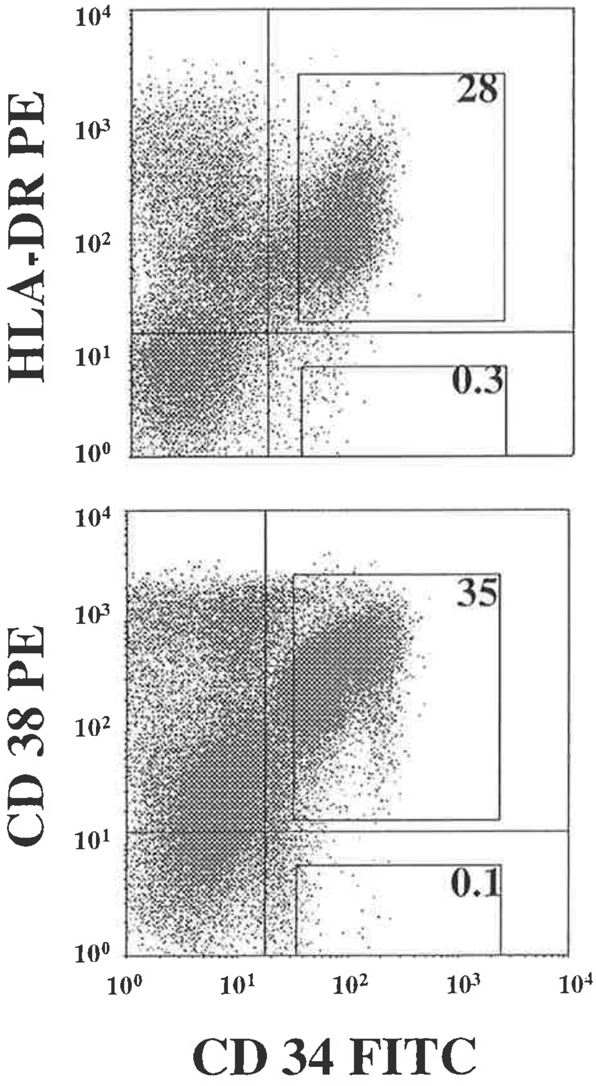
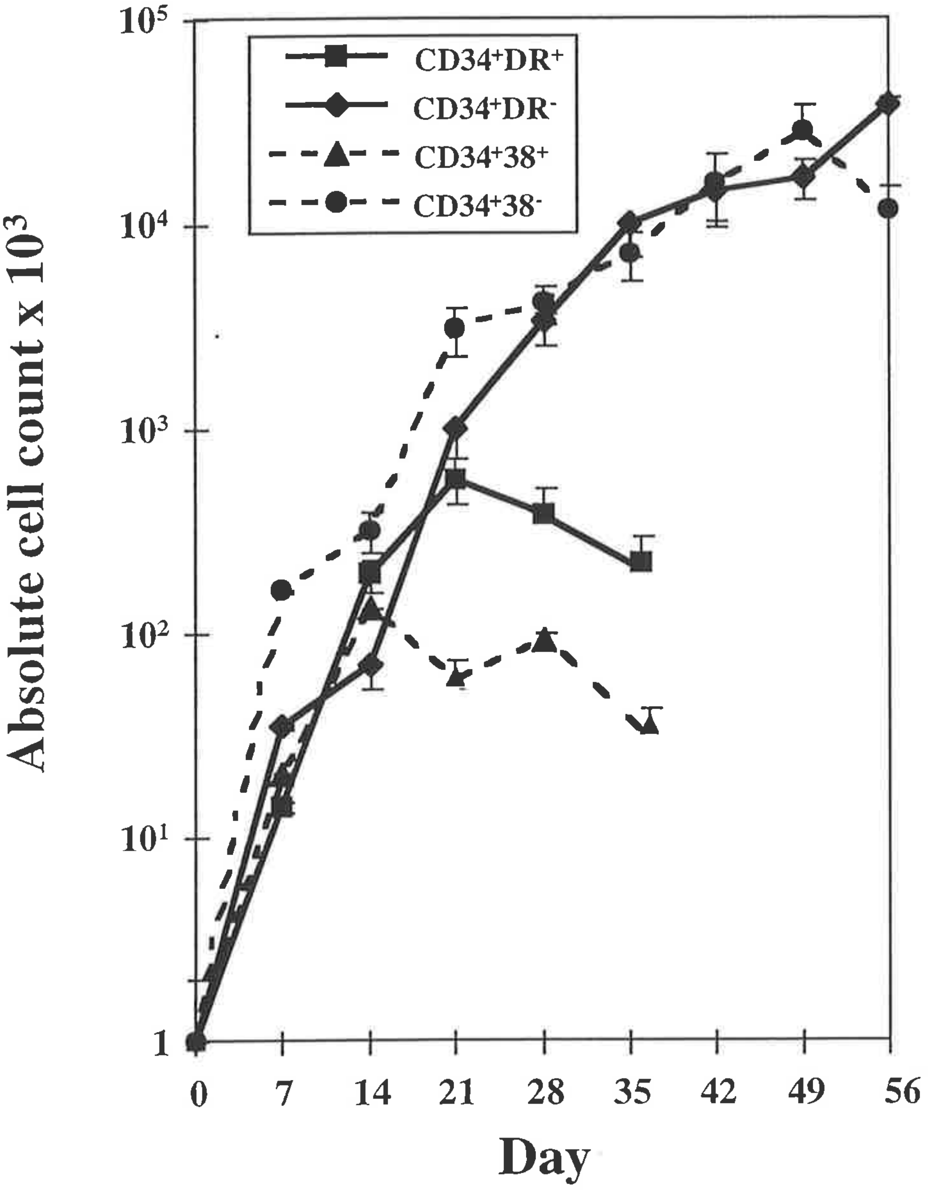

HLA-DR PE
10^4
10^3
10^2
10^1
10^0
28
0.3
CD 38 PE
10^4
10^3
10^2
10^1
10^0
35
0.1
10^0
10^1
10^2
10^3
10^4
CD 34 FITC

Figure 5.2.

Proliferation of different populations of CML PB in the pre-progenitor assay. Cells were initially plated at 1000 cells per well and counted at 7 day intervals. The $CD34^+DR^-$ and $CD34^+38^-$ populations show marked expansion of cells up to day 56 and 49 respectively. However the degree of expansion is not as marked as seen in the BM. The $CD34^+DR^+$ and $CD34^+38^+$ populations show similar expansion to their BM counterparts with maximal cell numbers seen at day 28.



CD34+DR+
CD34+DR-
CD34+38+
CD34+38-
Absolute cell count x 10³
10⁵
10⁴
10³
10²
10¹
1
0
7
14
21
28
35
42
49
56
Day

fraction. The $CD34^+DR^+$ and $CD34^+38^+$ populations showed early proliferation with maximal cell numbers at days 14 to 28 with decline in cell numbers after day 28. Proliferation of total $CD34^+$ cells from PB was also assessed in patients 1 and 5. The pattern of growth was similar to the $CD34^+DR^+$ population in both cases. The $CD34^-$ population did not proliferate.

Differentiation patterns of each population were similar for PB and BM. This is shown in Figures 5.3 to 5.6. Low power views of the $CD34^+DR^+$ and $CD34^+38^+$ populations show early expansion at days 7 and 14 with a plateau at day 21 and decline at 28 (Figure 5.3). The high power views of these populations show early differentiation occurring with the expansion with large numbers of granular cells and macrophages evident by day 14 and by day 28 the majority of cells are macrophages (Figure 5.4). In contrast the $CD34^+DR^-$ and $CD34^+38^-$ populations show little expansion by day 14 but then proliferate extensively by day 28 (Figure 5.5). The majority of cells at day 14 remain small and agranular and even though granular cells and macrophages are prominent at day 28 there is still a number of these small agranular cells present (Figure 5.6).

The pattern of CFU-GM production in the PB was similar to the BM but the total numbers were lower because of the lower cell proliferation in the PB (Figure 5.7). The number of clonogenic cells at day 0 in the PB of the $CD34^+DR^-$ fraction was 7 ± 3 and in the $CD34^+38^-$ fraction 8 ± 1 per 1000 cells plated. The number of clonogenic cells in the $CD34^+DR^+$ and $CD34^+38^+$ fractions were 25 ± 6 and 9 ± 2 respectively per 1000 cells plated. But, the total CFU-GM production, which reflects the number of CFU-GM produced from cells derived from the pre-progenitor assay, showed maximal production in the $CD34^+DR^-$ and $CD34^+38^-$ fractions with peak numbers of CFU-GM seen at day 28. The $CD34^+DR^+$ and $CD34^+38^+$ fractions had minimal colony production at days 7 and 14 and none at later timepoints.

Figure 5.3.

Growth of CD34^{+}DR^{+} and CD34^{+}38^{+} CML PB populations in the pre-progenitor assay. This low power view (original magnification x 40) shows maximal proliferation of cells at days 7 and 14 with cell numbers static at day 21 and a decline seen at day 28.

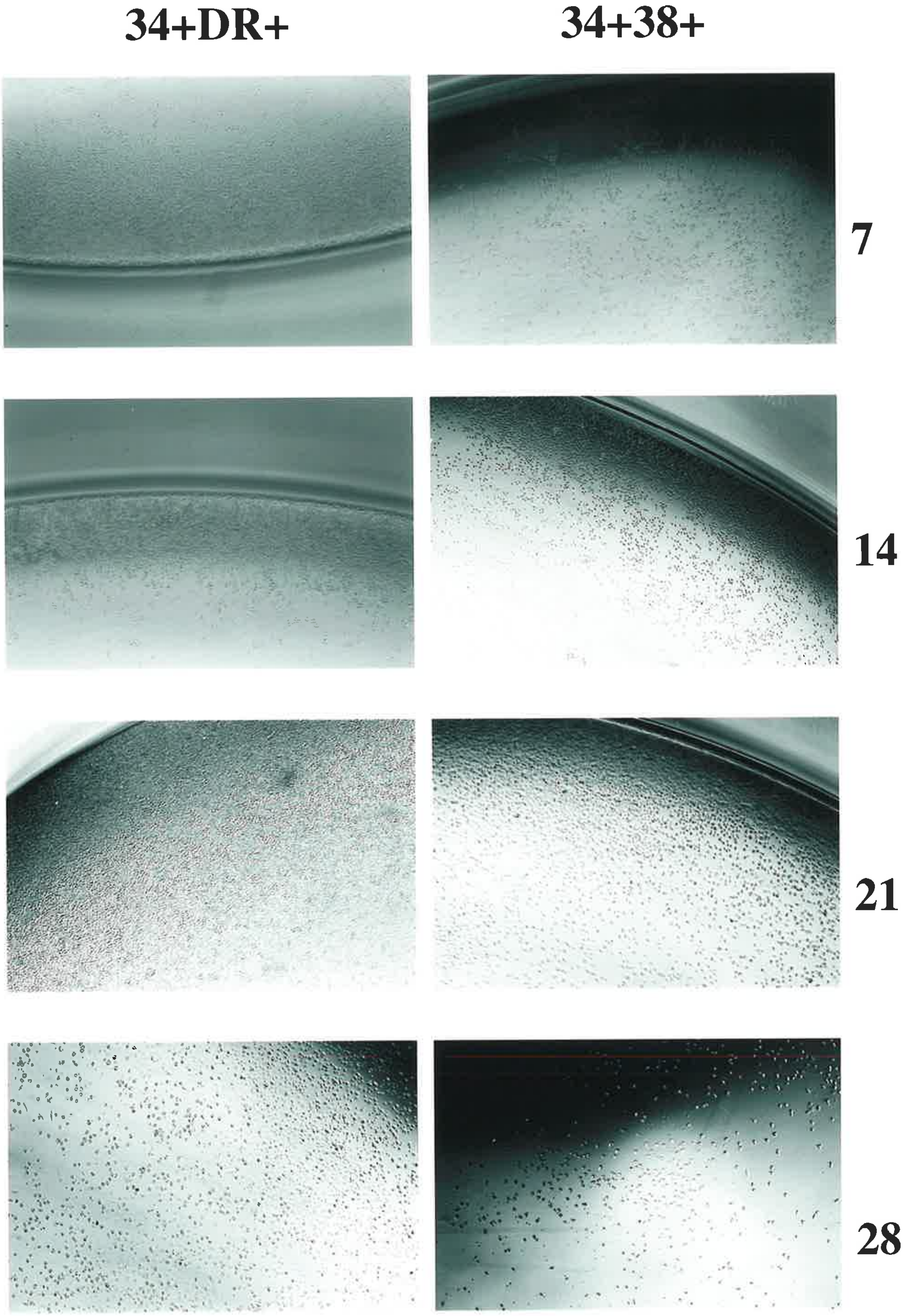

34+DR+
34+38+
7
14
21
28

Figure 5.4.

Growth of CD34$^+$DR$^+$ and CD34$^+$38$^+$ CML PB populations in the pre-progenitor assay. This high power view (original magnification x 200) shows the morphological appearance of cells. At day 7 small agranular cells are admixed with larger more granular cells. By day 14 the majority of cells are large and granular and these persist through days 21 and 28 when more adherent macrophage like cells are evident.

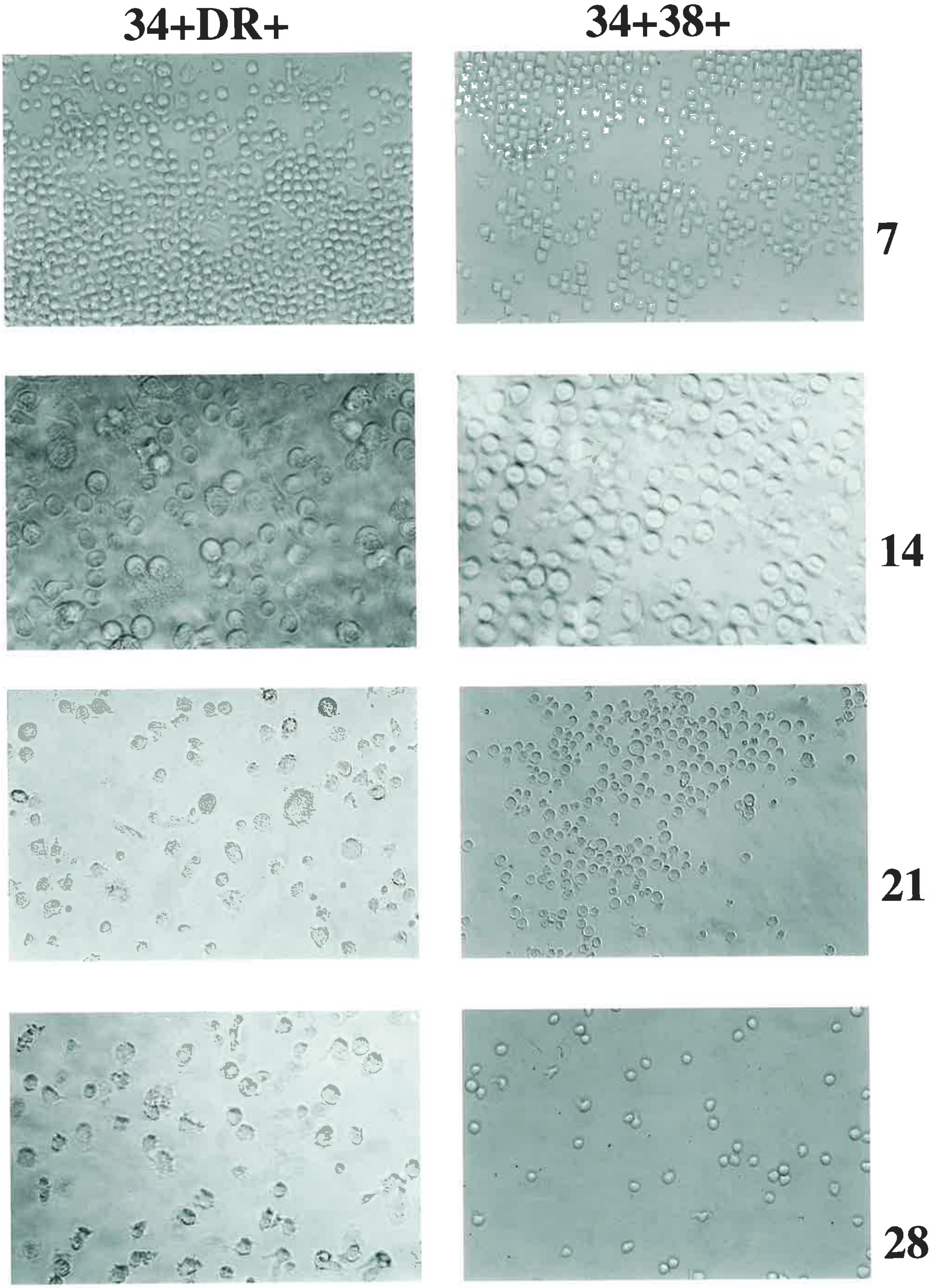

34+DR+
34+38+
7
14
21
28

Figure 5.5.

Growth of CD34$^+$DR$^-$ and CD34$^+$38$^-$ CML PB populations in the pre-progenitor assay. This low power view (original magnification x 40) shows little proliferation of cells at days 7 with marked expansion occurring at days 14 and 28. Cell numbers begin to decline at day 42.

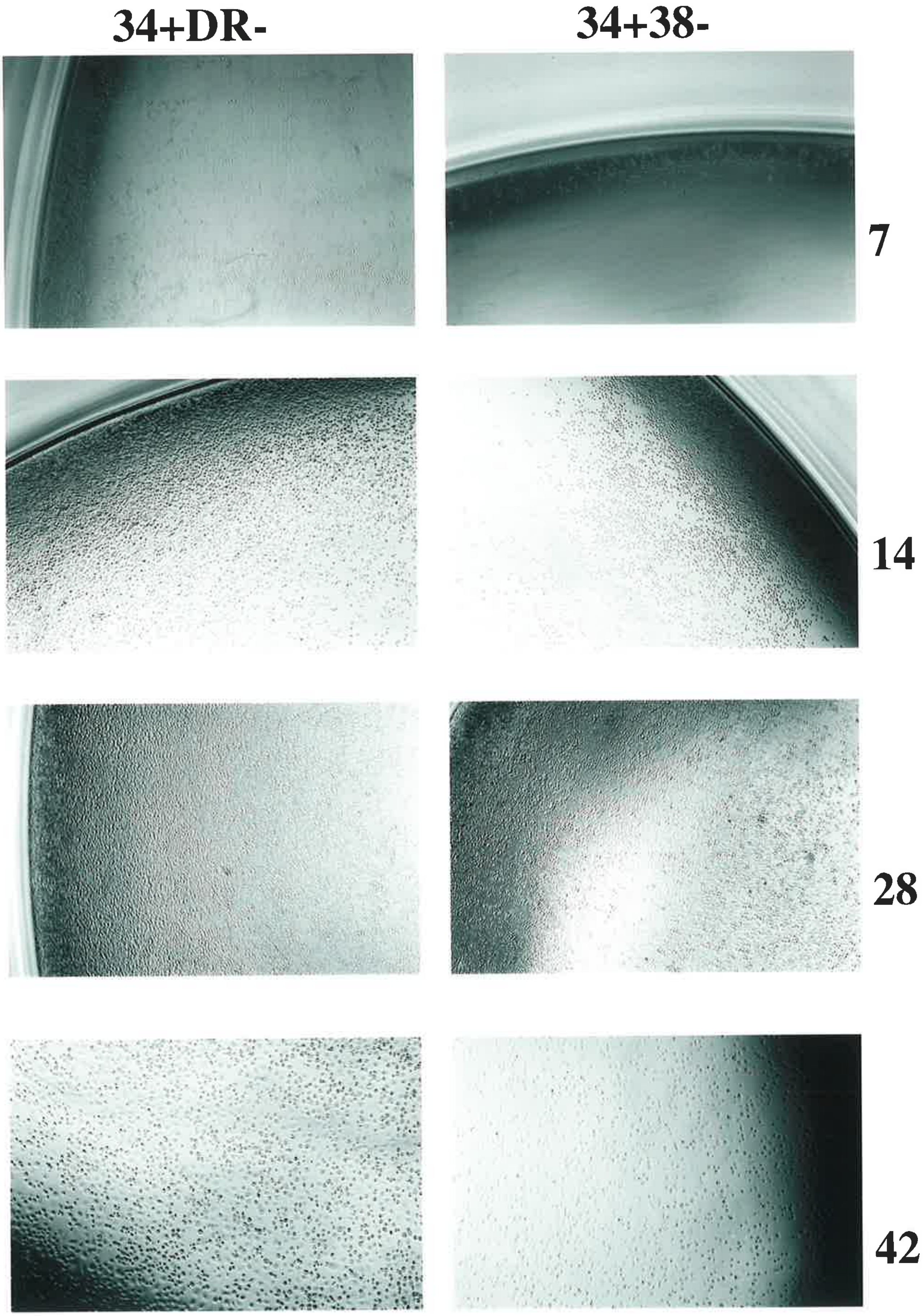

34+DR-
34+38-
7
14
28
42

Figure 5.6.

Growth of CD34^{+}DR^{-} and CD34^{+}38^{-} CML PB populations in the pre-progenitor assay. This high power view (original magnification x 200) shows large numbers of small agranular cells at days 7 and 14. At day 28 these cells are still evident but larger granular cells are also prominent. By day 42 the majority of cells have the appearance of macrophages.

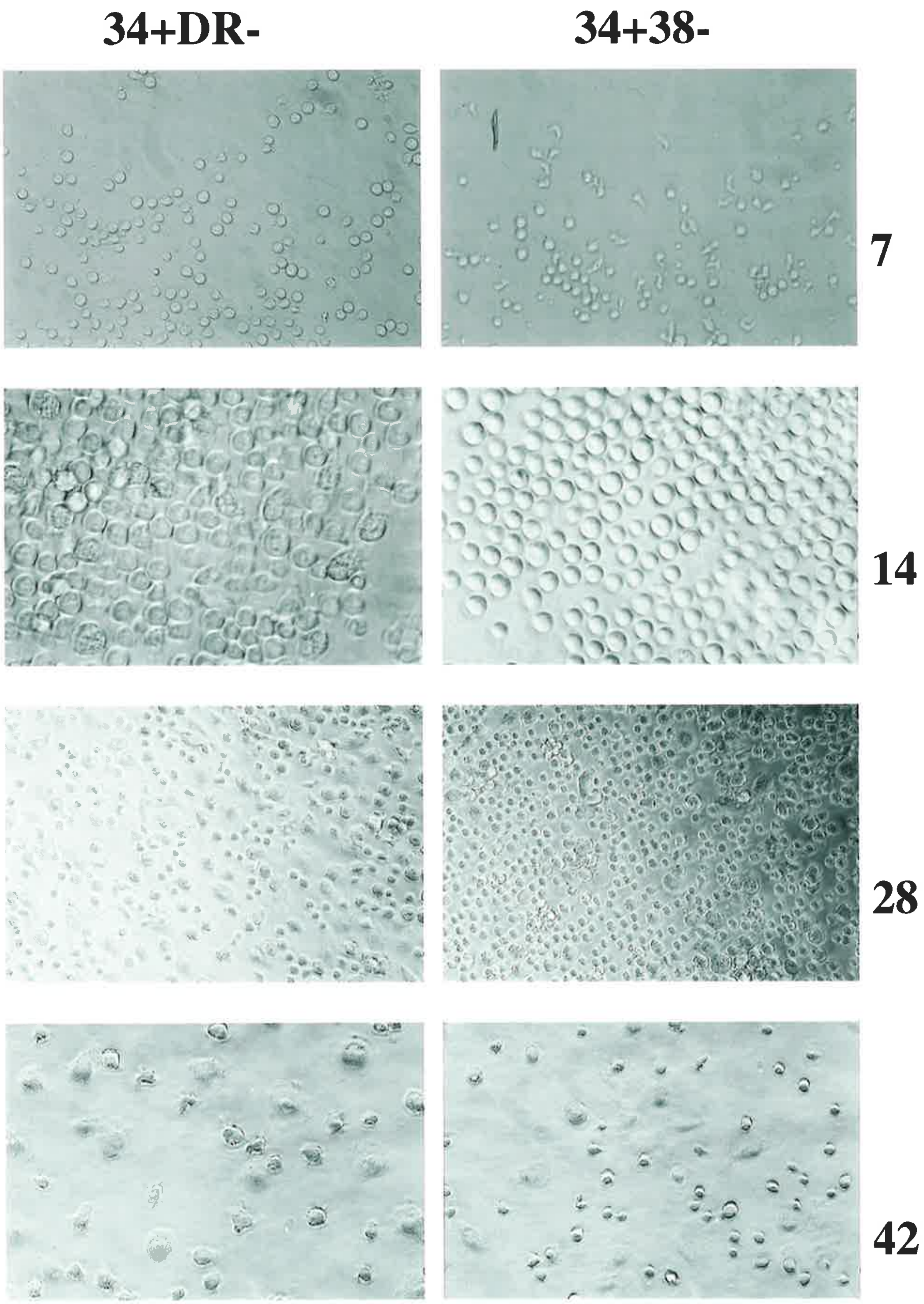
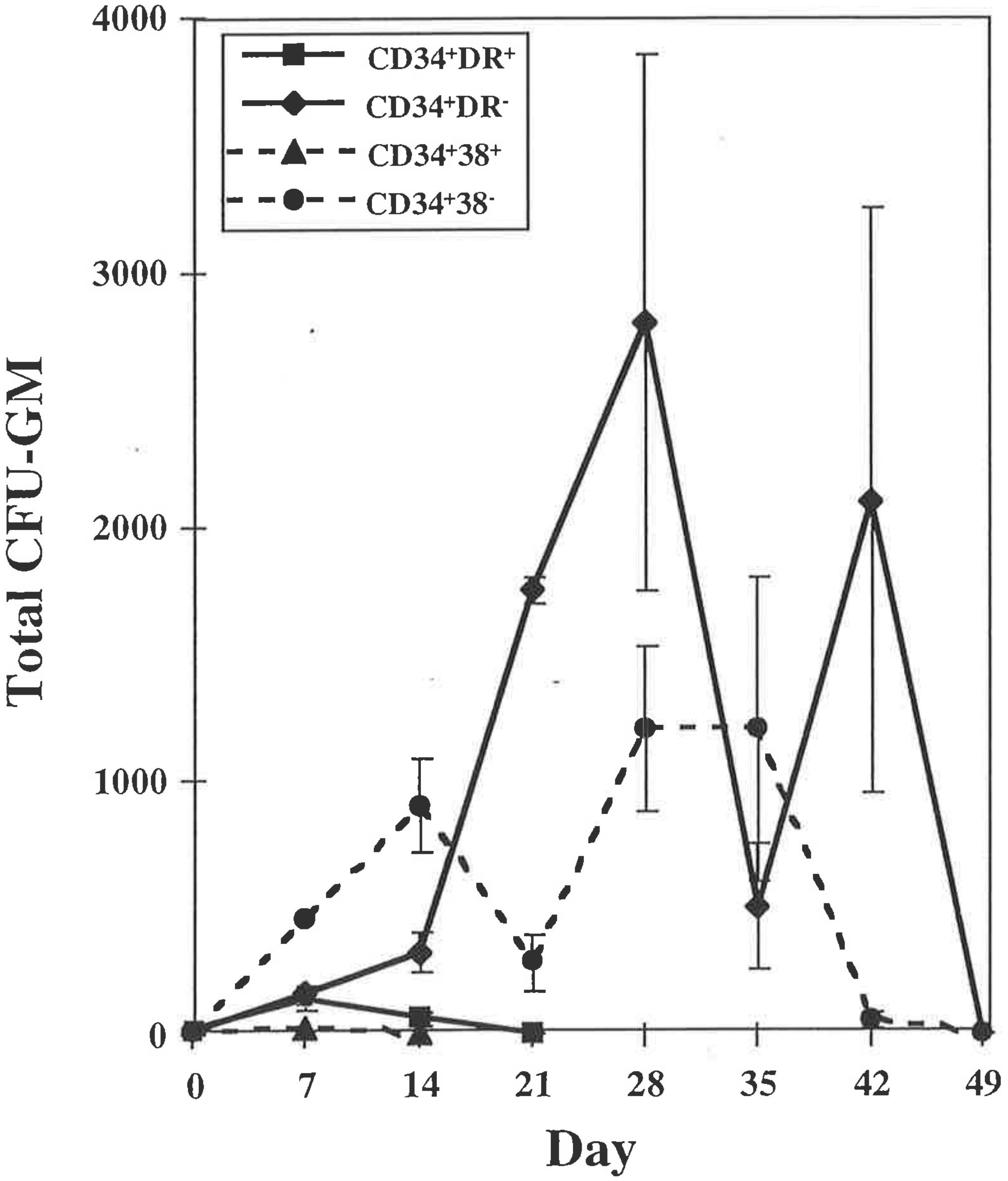

34+DR-
34+38-
7
14
28
42

Figure 5.7.

Total CFU-GM production of different populations of CML PB. Numbers are calculated by counting CFU-GM per number of cells plated and multiplying by total cell numbers as counted in the pre-progenitor assay. The appearances are similar to those seen in the BM but the total numbers of CFU-GM are lower which is due to the lower proliferation of these cells in the pre-CFU assay.



4000
3000
2000
1000
0
Total CFU-GM
CD34+DR+
CD34+DR-
CD34+38+
CD34+38-
0
7
14
21
28
35
42
49
Day

5.6 Analysis of PB progenitors for BCR-ABL

The PB progenitors from the CD34$^+$DR$^-$ fraction showed predominantly BCR-ABL$^-$ colonies with levels ranging from 0% to 100% (mean 78%). A total of 77 colonies were informative from 5 patients. In contrast 0% to 90% (mean 29%) of 109 progenitors examined from the CD34$^+$DR$^+$ fraction of 6 patients were BCR-ABL$^-$ (Figure 5.8).

Analysis of the CD34$^+$38$^-$ fraction in 4 PB samples showed the majority of progenitors were leukaemic. The number of BCR-ABL$^-$ colonies was 11% to 50% (mean 17%) of 75 examined. Analysis of the CD34$^+$38$^+$ fraction from 5 patients showed 0% to 73% (mean 23%) of 71 colonies examined were BCR-ABL$^-$ (Figure 5.8).

The number of BCR-ABL$^-$ colonies from the total CD34$^+$ cells was 2 of 12 (17%) in patient 1 and 1 of 16 (6%) in patient 5 which was no different to the results obtained from the CD34$^+$DR$^+$ fraction.

5.7 Analysis of PB pre-progenitors for BCR-ABL

Pre-progenitors derived from the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions of PB were predominantly BCR-ABL$^-$, similar to their BM derived counterparts. Analysis of the CD34$^+$DR$^-$ fraction of PB from 4 patients at day 14 showed 90% to 100% (mean 95%) of colonies were BCR-ABL$^-$. At day 28, 85% to 100% (mean 93%) of colonies were BCR-ABL$^-$ (Table 5.2). Analysis of colonies from the CD34$^+$38$^-$ fraction at day 14 showed 23% to 100% (mean 49%) BCR-ABL$^-$ colonies in 4 patients. Analysis at day 28 showed 42% to 100% (mean 74%) of colonies were BCR-ABL$^-$ in 4 patients (Table 5.2).

The CD34$^+$DR$^+$ fraction gave rise to very few colonies at day 7 and 14. Analysis in patient 1 showed 14% and 56% BCR-ABL$^-$ colonies at these timepoints respectively. No analysable colonies were produced from the CD34$^+$38$^+$ fraction at day 7 or 14. In patient 1,

Figure 5.8.

Analysis of PB progenitors from the CD34$^+$DR$^-$, CD34$^+$DR$^+$, CD34$^+$38$^-$ and CD34$^+$38$^+$ populations for the presence of BCR-ABL. Results are expressed as the percentage of colonies which are BCR-ABL$^-$. These are colonies in which BCR-ABL has not been detected following duplicate nested RT-PCR reactions but the normal ABL gene has been detected confirming the integrity of RNA extraction and reverse transcription.

The results shown are variable with the majority of BCR-ABL$^-$ progenitors being found in the CD34$^+$DR$^-$ population. Patients 4 and 6 have high levels of BCR-ABL$^-$ progenitors found in all populations.

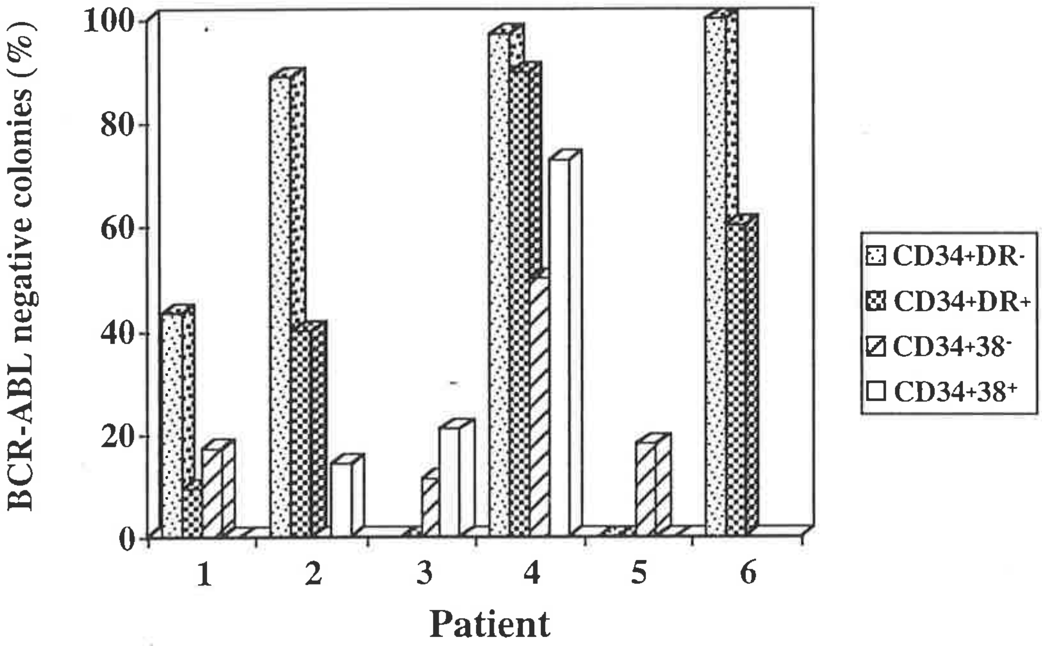

BCR-ABL negative colonies (%)
100
80
60
40
20
0
1
2
3
4
5
6
Patient
CD34+DR-
CD34+DR+
CD34+38-
CD34+38+

Table 5.2.

Results of colony analysis of pre-progenitors derived from the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions of PB at diagnosis. Results are expressed as number of BCR-ABL negative colonies per total analysable colonies.

Patient	CD34$^+$DR$^-$			CD34$^+$38$^-$		
	d0	d14	d28	d0	d14	d28
1	9/21 (43%)	11/12 (92%)	13/15 (87%)	1/6 (17%)	10/10 (100%)	NA
2	8/9 (89%)	NA	NA	NA	3/10 (30%)	NA
3	NA	18/20 (90%)	11/13 (85%)	3/27 (11%)	15/24 (63%)	23/23 (100%)
4	30/31 (97%)	11/11 (100%)	15/15 (100%)	2/4 (50%)	NA	NA
5	NA	NA	NA	7/38 (18%)	6/26 (23%)	8/19 (42%)
6	13/13 (100%)	15/15 (100%)	12/12 (100%)	NA	NA	NA

NA = no analysable colonies

the unseparated CD34$^+$ cells gave rise to few colonies at day 7 and no colonies at day 14. At day 7, 1 of 3 (33%) of colonies were BCR-ABL$^-$. Patient 5 produced more colonies from unseparated CD34$^+$ cells and at day 14, 6 of 28 (21%) of colonies were BCR-ABL$^-$.

5.8 Fluorescent in situ hybridisation of haemopoietic colonies for BCR-ABL

Selected colonies from patient 1 were analysed by FISH for the presence of BCR-ABL$^+$ cells. Five colonies from the CD34$^+$DR$^+$ population at day 0 were examined and showed 99% of cells were leukaemic (Table 5.3, Figure 5.9). This is in agreement with the PCR findings and demonstrates that FISH can detect leukaemic colonies. Analysis of 3 colonies at day 14 from this population showed only 7% leukaemic cells. This is around the background level of BCR-ABL detection by FISH and suggests that these colonies were derived from BCR-ABL$^-$ pre-progenitors. Analysis of the CD34$^+$38$^-$ population at days 14 and 28 showed 96% and 99% of cells were BCR-ABL$^-$ and hence these colonies have arisen from BCR-ABL$^-$ cells (Table 5.3, Figure 5.9).

5.9 Mobilised peripheral blood contains BCR-ABL-negative progenitors and pre-progenitors

Mobilisation of haemopoietic stem cells into the peripheral circulation and subsequent collection provides a source of material suitable for reconstituting haemopoiesis following high dose chemo/radiotherapy. In CML this technique is being increasingly used as a method of in vivo purging of leukaemic cells. Chemotherapy is used to deplete leukaemic cells and it is thought that normal haemopoietic stem cells recover and are mobilised before leukaemic cells thus providing an opportunity to collect an apheresis product enriched for normal cells. Conventional assessment of the apheresis product shows variable numbers of Ph$^-$ metaphases with some patients having 100% Ph$^-$ cells in their collections. There is some evidence that patients with 100% Ph$^-$ cells mobilise Ph$^-$ LTC-ICs (Podestà et al.,

Table 5.3.

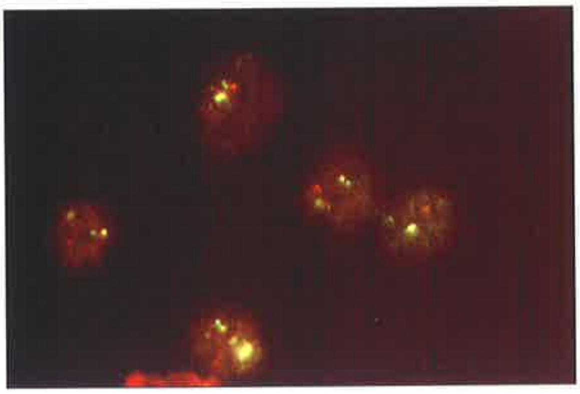
FISH analysis of harvested colonies from the CD34$^+$DR$^+$ and CD34$^+$38$^-$ populations for the detection of the BCR-ABL translocation. Individual colonies were harvested and analysed by FISH as described. Results are expressed as the pooled total number of cells that were leukaemic or normal from the number of individual colonies (n) analysed.

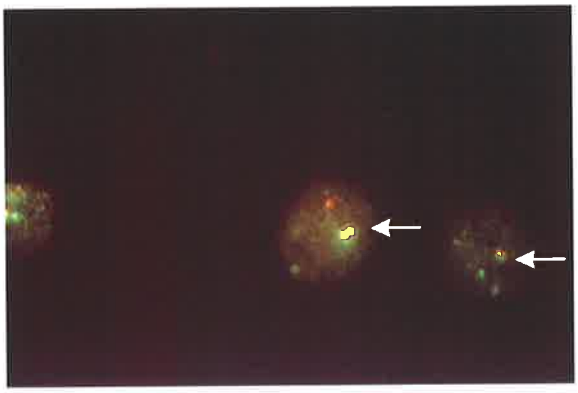
Sample	n	Normal	Leukaemic
CD34$^+$DR$^+$			
day 0	5	2	144
day 14	3	64	5
CD34$^+$38$^-$			
day 14	3	53	2
day 28	4	71	1

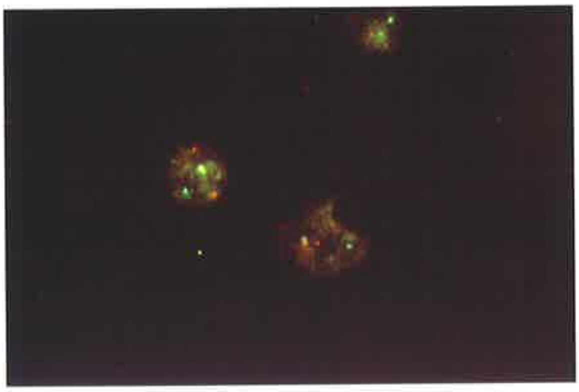
Figure 5.9.

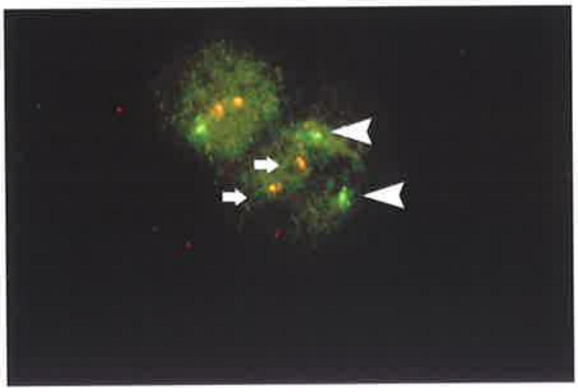
FISH analysis of colonies.

Cells in the top 2 photos are from progenitors from the $34^{+}DR^{+}$ population. All cells have evidence of the BCR-ABL translocation with co-localisation of the red ABL signal and green BCR signal producing a single yellow signal (⟶). A single normal red ABL signal (➡) and green BCR signal (►) are also seen. Cells in the bottom 2 photos are from day 28 pre-progenitors from the $34^{+}38^{-}$ population. All cells are normal with two red ABL signals and two green BCR signals.









1995) and it is the effect of mobilisation on recruitment of primitive BCR-ABL$^-$ progenitors that is critical if this technique is going to be a useful therapy in CML.

Within 6 months of diagnosis, patients 1, 2, 4, and 6 received high dose cyclophosphamide followed by G-CSF and susequently had PBSC harvested. All had 4 or 5 collections and mobilised variable numbers of Ph$^-$ cells with the best results for each patient being 12%, 32%, 100% and 87% respectively.

Progenitor analysis for the presence of BCR-ABL was performed on the CD34$^+$DR$^-$ and CD34$^+$DR$^+$ populations of each patient. Of 74 colonies from the CD34$^+$DR$^-$ population 69 (93%) were BCR-ABL$^-$. Patient 1 had more BCR-ABL$^-$ progenitors in the mobilised specimen compared to steady state but the other patients had similar numbers (Figure 5.10a). Analysis of the CD34$^+$DR$^+$ progenitors showed 23 of 51 (45%) were BCR-ABL$^-$ which is similar to the overall number of BCR-ABL$^-$ progenitors from the steady-state specimens of these patients (Figure 5.10b). It is of interest to note that patients 4 and 6 who mobilised the greatest number of Ph$^-$ cells had relatively high numbers of BCR-ABL$^-$ progenitors in the CD34$^+$DR$^+$ population suggesting they had biologically favourable disease. However, given the low patient numbers this is speculative and requires further investigation.

Pre-progenitor assays were established from the mobilised PB of 3 of these patients. At days 14 and 28, 98% and 92% of pre-progenitors from the CD34$^+$DR$^-$ population were BCR-ABL$^-$ respectively. This is a similar result to the steady-state data. Analysis of the CD34$^+$DR$^+$ population at day 14 showed 53% BCR-ABL$^-$ pre-progenitors.

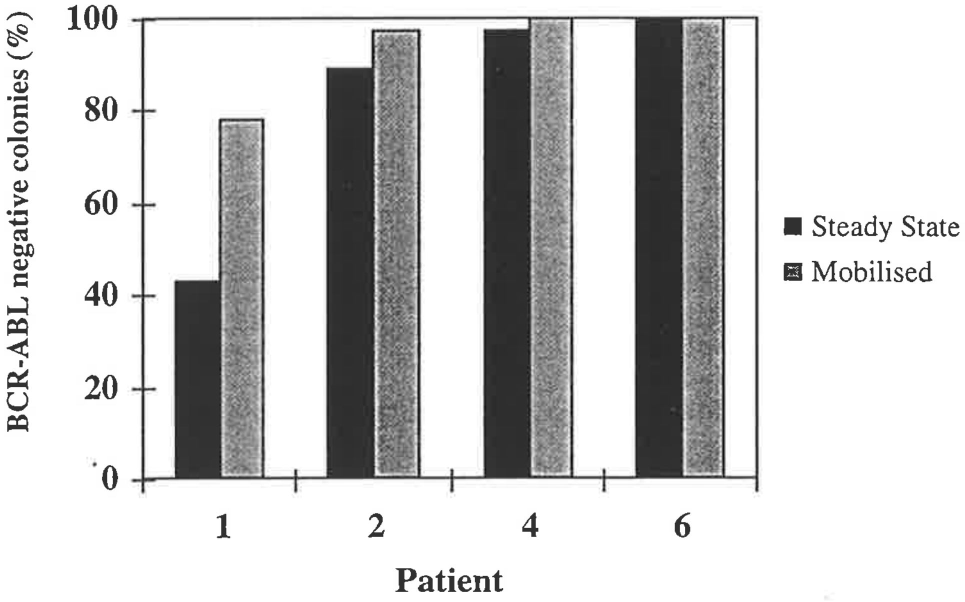
Figure 5.10.

Effect of mobilisation on the production of BCR-ABL$^-$ progenitors. Patients were treated with cyclophosphamide and G-CSF and following recovery of WCC they underwent leukapheresis. Progenitors were analysed as in figure 5.8

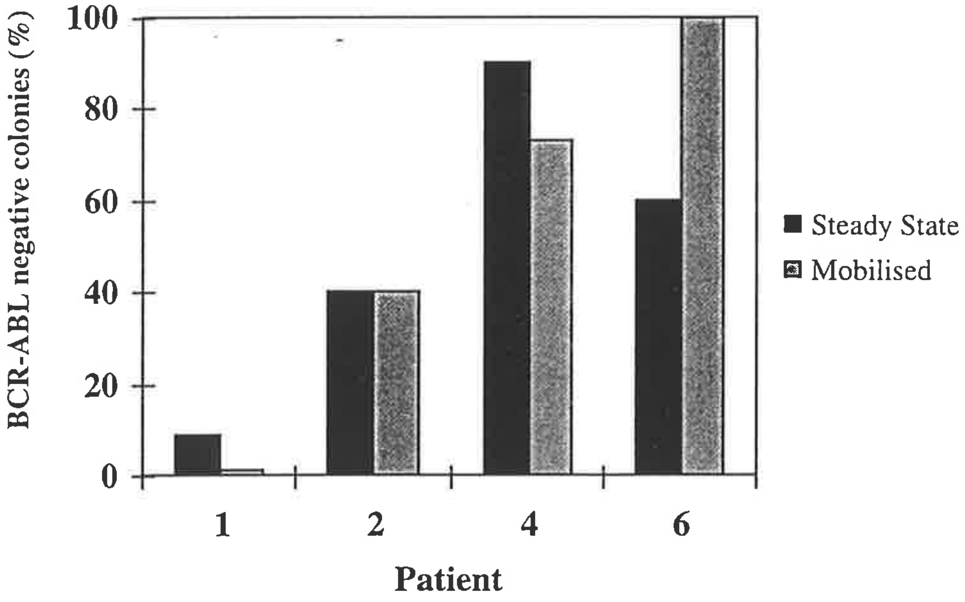
5.10a. CD34$^+$DR$^-$ population. Patients 2, 4, and 6 had high levels of BCR-ABL$^-$ progenitors in steady state peripheral blood at diagnosis. A slight increase in BCR-ABL$^-$ progenitors was seen following mobilisation in patients 2 and 4. Patient 1 had an increase in BCR-ABL$^-$ progenitors from 42% to 78% following mobilisation.

5.10b. CD34$^+$DR$^+$ population. Mobilisation only produced an increase in BCR-ABL$^-$ progenitors in patient 6. Patients 4 and 6 with relatively high numbers of BCR-ABL$^-$ progenitors in steady state had mobilisation of 100% and 85% Ph$^-$ cells by cytogenetics.

a


BCR-ABL negative colonies (%)
100
80
60
40
20
0
1
2
4
6
Patient
■ Steady State
▨ Mobilised

b


BCR-ABL negative colonies (%)
100
80
60
40
20
0
1
2
4
6
Patient
■ Steady State
▨ Mobilised

5.10 Discussion

The results described here show that pre-progenitors derived from the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions of peripheral blood from patients with early chronic phase CML, like their bone marrow derived counterparts, are predominantly BCR-ABL$^-$. In contrast, progenitors from these populations showed variable levels of BCR-ABL$^-$ colonies from the CD34$^+$DR$^-$ population. In comparison, progenitors of the CD34$^+$38$^-$ population were predominantly leukaemic. Thus, leukaemic involvement at the progenitor level does not reflect leukaemic involvement of the more primitive pre-progenitor.

Analysis of progenitors in this study for the presence of BCR-ABL show some heterogeneity betwen patients but this is consistent with previous findings (Verfaillie et al., 1992b; Leemhuis et al, 1993). The main finding is that the CD34$^+$DR$^-$ population contains normal progenitor cells but they co-exist with leukaemic progenitors. In 4 of 6 patients analysed in this study the CD34$^+$DR$^-$ population contained the greatest number of normal progenitors. In 2 individual patients, the CD34$^+$38$^-$ and CD34$^+$38$^+$ populations had the greatest number of normal progenitors detected but they were at low levels compared to the numbers seen in the other patients. Patients with more advanced CML are known to have less BCR-ABL$^-$ progenitors (Verfaillie et al., 1996). Thus the pattern of leukaemic involvement within progenitors of different phenotype may reflect the stage of disease evolution within individual patients, although all were recently diagnosed, or differences in the expression of the HLA-DR or CD38 antigen between leukaemias. Heterogeneity between patients occurs and it is difficult to draw conclusions from individual patients. Generally, progenitors from the CD34$^+$38$^-$ fraction of PB showed high levels of leukaemic involvement which is in agreement with previous studies (de Fabritiis et al., 1993a; Kirk et al., 1995). Patient numbers were low so further studies are necessary to assess the degree of this heterogeneity between the four cell populations studied.

The data presented on the analysis of pre-progenitors from the CD34$^+$DR$^-$ fraction show similar results to the BM derived cells with predominant BCR-ABL$^-$ pre-progenitors at days 14 and 28. Furthermore, BCR-ABL$^-$ pre-progenitors are also found in the CD34$^+$38$^-$ fraction of PB, confirming the BM findings. Interestingly, there are more BCR-ABL$^-$ pre-progenitors at day 28 compared to day 14 in the CD34$^+$38$^-$ population. This may indicate that the more primitive cells in this population are normal with progressive leukaemic infiltration in the more mature cell. Leukaemic progenitors are detected in the standard progenitor assay but the leukaemic pre-progenitor is not detected in the pre-progenitor assay. It is possible that stroma-free liquid culture selects for BCR-ABL$^-$ cells in both PB and BM. Leukaemic pre-progenitors are detected in this assay system but they still may have a growth disadvantage. Analysis of patient 5 is of interest. The CD34$^+$DR$^-$ population from this patient failed to grow in the pre-CFU assay but the CD34$^+$38$^-$ population did. Analysis of pre-progenitors showed 77% leukaemic colonies at day 14 and 58% leukaemic colonies at day 28. Hence leukaemic pre-progenitors do grow in this assay system. However, the numbers of leukaemic pre-progenitors may be relatively reduced compared to normal pre-progenitors, and the massive expansion of cells in CML may be due to the enormous proliferative potential of these cells. However, it is the finding of populations enriched for BCR-ABL$^-$ pre-progenitors, which may be targets for a positive selection strategy in CML, that is important.

An alternative explanation to the finding of BCR-ABL$^-$ pre-progenitors may be that these primitive cells have the BCR-ABL gene rearrangement but do not express BCR-ABL mRNA. It has been reported that some Ph$^+$ haemopoietic colonies from CML BM do not transcribe the aberrant gene (Keating et al., 1994). However, in another study Ph$^+$ BCR-ABL$^-$ colonies were virtually non-existent (Diamond et al., 1995). These conflicting results may be due to the sensitivity of the RT-PCR reaction in detecting small quantities of mRNA in haemopoietic colonies. It has also been demonstrated that CD34$^+$lin$^-$ cells with the BCR-ABL gene rearrangement do not express BCR-ABL mRNA (Bedi et al., 1993).

The $CD34^+DR^-$ and $CD34^+38^-$ populations are found within the $CD34^+lin^-$ subset and therefore may not transcribe BCR-ABL. The use of FISH to visualise individual cells within each colony is a method that overcomes the problems of lack of gene expression and analysis of small quantities of mRNA by RT-PCR. The data presented on the analysis of colonies by FISH is limited but it does demonstrate some important points. Individual colonies were analysed and the results from each population pooled. The analysis of progenitors from the $CD34^+DR^+$ population shows that they were all leukaemic, as predicted, and demonstrates the suitability of FISH to detect leukaemic colonies. In contrast, the day 14 pre-progenitor colonies from this population were $BCR\text{-}ABL^-$ suggesting they originated from a normal cell. Very few day 14 colonies were produced from the $CD34^+DR^+$ population and analysis of those from a single patient by RT-PCR showed 56% $BCR\text{-}ABL^-$ colonies which is consistent with the FISH findings and suggests that the more immature cells in this population contain pre-progenitors which have variable levels of normal and leukaemic involvement. Pre-progenitors from the $CD34^+38^-$ population were analysed at day 14 and day 28. All colonies analysed by FISH were $BCR\text{-}ABL^-$. This suggests the RT-PCR findings are valid and that normal pre-progenitors are present in the $CD34^+38^-$ population. The background level of $BCR\text{-}ABL^+$ cells detected is probably due to chance co-localisation of signal because all cells in the colony derive from a single progenitor. Although the number of colonies analysed by FISH was low the results are consistent with and confirm the RT-PCR findings.

Current therapeutic strategies in CML for patients lacking an HLA-matched donor, including interferon and autologous transplantation, aim to exploit the presence of normal stem cells. However, restoration of Ph^- haemopoiesis following unmanipulated autologous transplantation does not occur in all patients and is usually only of short duration in those patients who do achieve this (McGlave et al., 1994). Potential strategies to produce long term Ph^- haemopoiesis following ABMT include positive selection of Ph^- cells or purging of Ph^+ cells. Methods under investigation for depletion of Ph^+ cells include depletion of

$CD34^+DR^+$ cells, incubation with antisense oligonucleotides, ribozymes or tyrosine kinase inhibitors or incubation on stroma to exploit the differential adherence of Ph^- and Ph^+ progenitors. There is evidence that leukaemic cells present in autologous grafts contribute to relapse in CML (Deisseroth et al., 1994) and also the percentage of Ph^+ cells in an autologous graft predicts the outcome of the transplant (Talpaz et al., 1995). Therefore for optimal results transplanted cells must be enriched for normal cells and depleted of leukaemic cells. Syngeneic transplants in CML cure approximately 50% of patients (Butturini et al., 1990) suggesting that conditioning therapy and reinfusion of purified Ph^- autologous cells could achieve similar results. However, the low frequency of normal cells in CML means that large scale selection of low frequency subsets may not produce sufficient cells to reconstitute haemopoiesis. We have demonstrated $BCR\text{-}ABL^-$ cells exist in both the $CD34^+DR^-$ and $CD34^+38^-$ fractions of PB in CML at diagnosis. Blood is more accessible than bone marrow and this enables the collection of much larger numbers of cells. It has been estimated that 1 to 3 x 10^5 $CD34^+DR^-$ cells/kg can be recovered following a 2.5 L bone marrow harvest and this number may be sufficient for autologous transplantation (Verfaillie et al., 1996). This number is based on 60% cell recovery following $CD34^+$ selection with a further 60% recovery following fluorescent activated cell sorting. The mean number of cells collected from newly diagnosed CML patients in our institution is 8.4 x 10^8 cells/kg (range 6.9 to 9.9, n = 5) from 350 ml (range 230ml to 400ml) of apheresis product. On average, 0.25% of these cells will be $CD34^+DR^-$. Allowing for a final recovery of 36% following selection and sorting this product contains 7.6 x 10^5/kg (range 6.2 to 8.9) $CD34^+DR^-$ cells. Therefore in an apheresis product of less than 500 ml we can collect more than twice the number of critical cells than a 2.5 L BM harvest. This may allow selection of a subset which contains Ph^- cells in sufficient numbers for transplantation.

Autologous transplantation with mobilised PBSC enriched for Ph^- cells is another strategy which is currently being investigated as a therapy to improve survival in CML. The data presented here, although limited, shows that $BCR\text{-}ABL^-$ progenitors and pre-progenitors are mobilised and predominantly found in the CD34$^+$DR$^-$ fraction of blood. However, the frequency of BCR-ABL$^-$ cells does not differ significantly from the frequency of these cells in steady state blood. This may be a reflection of the intensity of the chemotherapy used for mobilisation. It should also be noted that the 2 patients who had low numbers of Ph$^-$ metaphases in their apheresis specimens still had readily detectable BCR-ABL$^-$ progenitors and pre-progenitors. This area requires further investigation with the critical issues including the predictors of Ph$^-$ mobilisation, which may determine the intensity of the conditioning regimen, and also which techniques should be applied to assess the apheresis product, or subpopulations within it, such as cytogenetics, RT-PCR or FISH.

In summary it has been demonstrated that pre-progenitors of both the CD34$^+$DR$^-$ and CD34$^+$38$^-$ fractions are predominantly BCR-ABL$^-$ in the PB and BM of most patients with early chronic phase CML. Both subsets may be suitable for selection strategies in CML depending upon the results of pre-progenitor studies. However there is heterogeneity between patients and thus no single selection strategy is likely to be successful in all cases. Importantly, blood collected at diagnosis is a more abundant source of normal stem cells than BM, which may be manipulated for therapeutic purposes.

Chapter 6

Establishment of a reproducible model of chronic phase chronic myeloid leukaemia in NOD/SCID mice using blood derived mononuclear or CD34 positive cells

6.1 Introduction

The co-existence of normal haemopoietic stem cells with leukaemic stem cells in the BM of patients with early CP CML is well established. The presence of normal haemopoietic pre-progenitors in the PB of patients with CML has been detailed in chapter 5. Critical biological differences between Ph^+ and Ph^- primitive progenitors have been identified which could be exploited to develop effective cell selection or purging strategies. These differences have only been identified at the progenitor and pre-progenitor level and it is therefore critical to develop assays for transplantable normal and leukaemic cells which may allow potential differences to be explored at the stem cell level. The ability of human haemopoietic cells to repopulate immunodeficient mice provides a new approach to the development of assays for transplantable normal and leukaemic stem cells in CML.

The aim of this study was to establish a model of CML in non-obese diabetic/LtSz scid/scid (NOD/SCID) mice. This strain is profoundly immunodeficient with defective T and B cell function and marked impairment of macrophage, natural killer cell and haemolytic complement activity (Shultz et al., 1995). The dose of CML blood cells which would reliably achieve engraftment was determined and the engrafting human cells were characterised in terms of lineage and BCR-ABL expression. Peripheral blood was used exclusively as it has been shown to engraft SCID mice at equivalent rates to BM (Sirard et al., 1996) and my previous studies have identified CML blood as an abundant source of normal pre-progenitors. Ex-vivo manipulation of CML blood is a promising approach for autologous transplantation so a detailed assessment of the engraftment potential of CML blood is a necessary precedent to studies of purging strategies.

6.2 Methods

An outline of the approach taken is shown in Figure 6.1. PB was collected by apheresis from patients with recently diagnosed CML and processed as described in chapter 3.2.1.1. The phenotype of patient samples was assessed by standard technique using directly conjugated monoclonal antibodies to CD34, CD33, CD3 and CD19. CD34$^+$ cells were selected by immunomagnetic selection as described in chapters 3.2.1.2 and 3.2.1.3.

Prior to cell infusion NOD/SCID mice were irradiated with 3 Gy, delivered at 6 Gy per minute, by a CsCl blood cell irradiator. Cells were infused into a tail vein 24 hours after irradiation. Some mice received IP injections of recombinant human HGF, either SCF, G-CSF, or GM-CSF, 5 µg 3 times per week. Peripheral blood was analysed prior to death in some animals. Mice were killed between days 28 and 50 by cervical dislocation. The BM from both femurs and tibias was taken and the spleen homogenised.

Engraftment of human cells was assessed by FISH analysis for human chromosome 8 and immunophenotype of cells for expression of human specific CD45. Engrafting cells were characterised for the presence of leukaemia specific BCR-ABL by FISH. Differentiation of cells was assessed by immunophenotype and morphology, and engraftment of human progenitors assessed by CFU-GM assay.

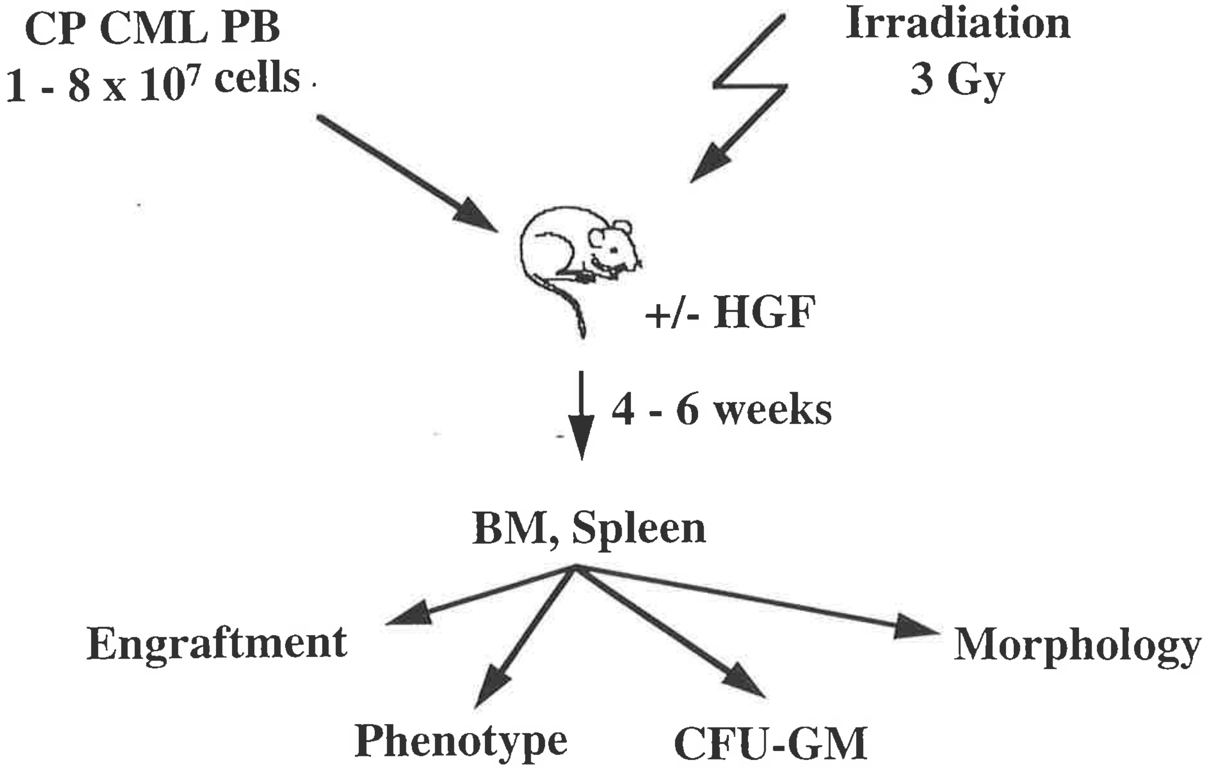
6.3 Establishment of a safe radiation dosage

The requirement for conditioning of immunodeficient mice with radiation prior to transplantation of human cells has been established in a number of studies (Fulop and Phillips, 1986; Cashman et al., 1997). This is thought to facilitate engraftment by impairing

Figure 6.1.

Summary of methods for analysis of NOD/SCID mice transplanted with chronic phase CML cells.

NOD/SCID mice, 4 - 6 weeks old, were irradiated with 3 Gy by a CsCl blood cell irradiator. Cells were infused 24 hours later by tail vein injection. Mice were sacrificed 4 - 6 weeks later and the BM and spleen examined. Engraftment was assessed by FISH analysis for human chromosome 8 and CD45 expression. Engrafting cells were characterised for leukaemic engraftment, immunophenotype, progenitor production and morphology.


CP CML PB
1 - 8 x 10^7 cells
Irradiation
3 Gy
+/- HGF
4 - 6 weeks
BM, Spleen
Engraftment
Phenotype
CFU-GM
Morphology

any residual immune function in the mouse and providing "space" in the marrow cavity for cells to lodge. Reported doses of radiation sufficient to facilitate engraftment in SCID or NOD/SCID mice range from 3.5 to 4 Gy. In our institution radiation was delivered by a CsCl blood cell irradiator at a relatively rapid rate of 6 Gy per minute. This dose rate resulted in a high mortality of mice when doses of 3.5 or 4 Gy were given. A dose of 3 Gy was determined as the maximal dose safely tolerated by the colony of NOD/SCID mice. Even at this dose approximately 15% of mice died between days 10 and 17 of radiation toxicity. Attempts to increase the dose given by reducing the dose rate were tried by using a linear accelerator in the radiotherapy department. This delivered radiation at approximately 0.75 Gy per minute. However, this resulted in high mortality at total doses of 3Gy, 3.5Gy and 4 Gy.

6.4 Patient characteristics

Cryopreserved PB from 15 patients with newly diagnosed CP CML was infused into 163 NOD/SCID mice in different experiments. The WCC at presentation ranged from 39 to 376 (median 133) x 10^9/L (Table 6.1). Twelve patients had exclusively 100% Ph$^+$ metaphases in the BM at diagnosis with one patient having 82% Ph$^+$ metaphases, one patient having a complex translocation and one patient having an additional minor abnormality (Table 6.1). Analysis of thawed material by FISH showed 37% to 51% (median 48%) BCR-ABL$^+$ cells in 5 samples analysed. The immunophenotype of thawed cells was quite variable. CD34 levels ranged from 3% to 31% (median 18%). Myeloid cells, as assessed by CD33 expression, ranged from 7% to 60% (median 30%), and T lymphocytes, as assessed by CD3, ranged from 12% to 49% (median 27%). The level of B cells was low, <10% in all cases (Table 6.1). Trypan blue estimation of cell viability prior to infusion into mice showed 66% to 88% viability.

Table 6.1.

**Presenting features of patients whose cells were cryopreserved at diagnosis.
After thawing, FISH and immunophenotype were performed prior to infusion into mice.**

Patient	WCC* (x 10^9/L)	Ph^+ metaphases*	FISH** BCR-ABL$^+$ (%)	Immunophenotype** CD34	CD33	CD3	CD19
01	222	100%	ND	24	60	17	3
02	142	100%	48	22	28	12	3
03	112	100%	50	7	30	49	2
04	133	100%	ND	18	27	48	4
05	71	100%	ND	9	ND	ND	ND
06	370	100%#	37	5	54	26	9
07	192	100%	ND	ND	ND	ND	ND
08	284	100%	51	21	37	27	4
09	214	100%	ND	3	26	49	3
10	98	100%	ND	3	ND	ND	ND
11	70	82%	ND	20	ND	ND	ND
12	109	100%##	ND	25	50	15	1
13	35	100%	43	31	52	18	2
14	376	100%	ND	20	ND	ND	ND
15	39	100%	ND	3	7	49	1

* at diagnosis

** prior to infusion into mice on thawed PBMNC

t(9;22)[73%]t(9;22), del(20p)[27%]

t(3;9;22)[100%]

ND - not determined

6.5 Sensitivity of FISH to assess engraftment of human cells

The use of human specific probes to assess engraftment of human cells into murine hosts allows direct visualisation of human cells with relatively simple enumeration. Only 1000 cells are required, much lower than are required for flow cytometry. All viable, nucleated cells are seen and counted. The accuracy and sensitivity of this technique was assessed by a mixing experiment in which human cells were mixed with murine cells in defined proportions. Two blinded observers analysed 300 cells from each dilution. The results are shown in Table 6.2. It can be seen that at low levels of human cells the concordance rate between observers and the actual level of human cells is high, therefore the method is suitable for analysing engraftment to the 1% level. This dilution experiment was repeated for CD45 analysis and shows similar sensitivity at low levels of engraftment (Table 6.2).

6.6 Engraftment

In separate experiments 115 mice were infused with 1.1 to 7.6 x 10^7 unmanipulated PB cells from 13 patients. Some mice received HGF. Eighteen mice (16%) became ill between days 10 to 18 with lethargy, ruffled fur and wasting suggesting radiation toxicity and were killed. A further 6 mice (5%) became ill between days 42 to 50 with features of lethargy and wasting. Analysis of these mice did not show evidence of overwhelming leukaemia and they may have succumbed to a graft versus host disease (GVHD) type illness as has previously been described in NOD/SCID mice occurring at this timepoint (Lowry et al., 1996).

6.6.1 BM engraftment

Human cells ≥1% were detected by FISH in 69 of the 91 mice (76%) analysed between days 28 to 51 with a range of engraftment of 1% to 87% (median 9%) (Figure 6.2). BM engraftment ≥10% was seen in 32 of the 69 engrafted mice (46%). Engraftment was correlated with cell dose infused. Eighteen mice were given less than 4 x 10^7 cells; 8 mice

Table 6.2.

Assessment of FISH and CD45 for analysis of engraftment of human cells in NOD/SCID mice. A known dilution of human cells and mouse cells were tested.

% Human cells diluted in mouse cells	FISH(D8Z2)(%)*		CD 45 (%)
	scorer 1	scorer 2	
0	0	0	0
0	0	0	0.1
0	0	0	0
1	1	0.5	1.2
2	2	3	3.3
3	2.7	3	1.8
5	5	4	4.6
10	7.3	4	6.9
25	29	27	22.3
50	36	46	49.9
75	70.3	72	69.4
100	98	99	88.3

*300 cells counted in a blinded fashion

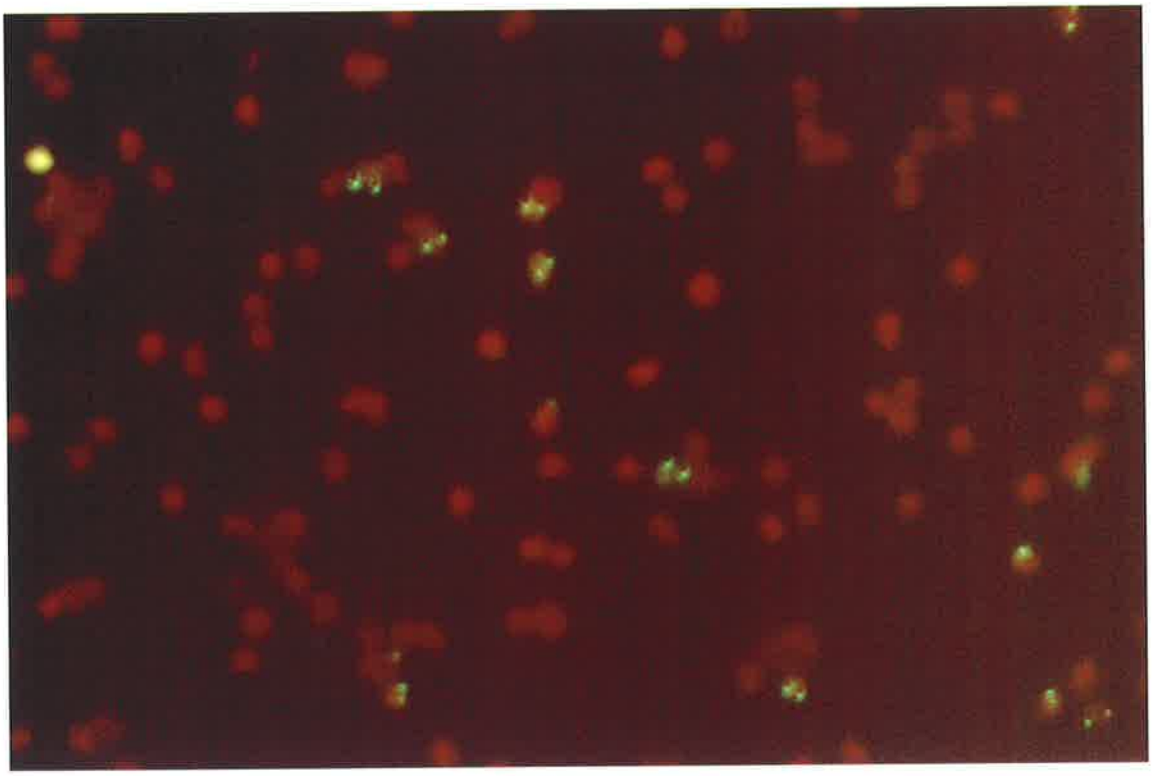
Figure 6.2.

Analysis of engraftment by FISH.

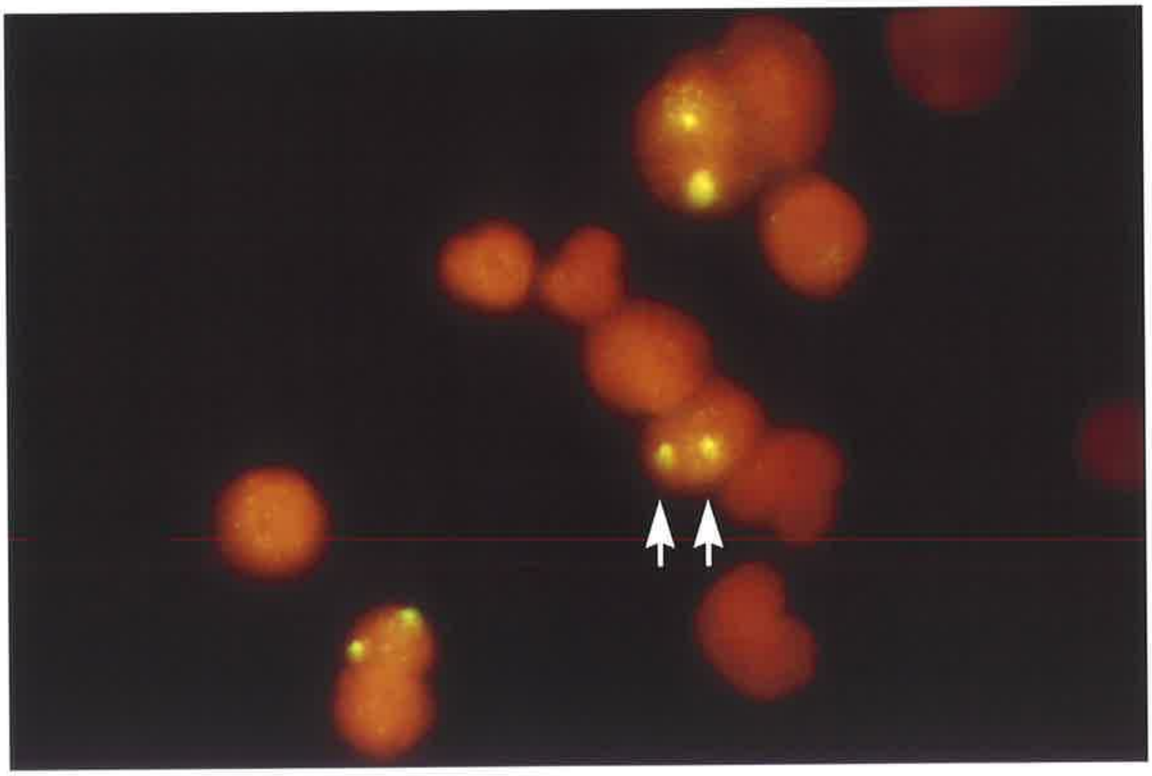
Human chromosome 8 was detected using a probe for the α satellite region at the D8Z2 locus.

6.2a. Low power view (original magnification x 200) shows human cells detected by D8Z2 probe with 2 bright green fluorescent signals in contrast to PI stained murine cells with no fluorescent signal.

6.2b. High power view (original magnification x 1000) shows human cells with 2 distinct fluorescent signals (arrow) and no background staining of murine cells.



a



b

showed no engraftment, and 10 mice had a median engraftment of 3% (range 1% to 7%). In the 73 mice receiving $\geq 4 \times 10^7$ cells 61 mice engrafted with a level of 20% ± 3% (median 10%) (Figure 6.3) The effect of cell dose is further demonstrated in Figure 6.4. Different cohorts of mice were infused with different cell doses from 4 patients. In each case higher engraftment was seen at the higher cell dose. Analysis of predictors of engraftment showed that the cell dose infused and the donor ($p<0.01$, analysis of variance) were independent predictors. Engraftment was confirmed by analysis of human specific CD45, a pan leukocyte marker (Figure 6.5). All mice that had engraftment ≥1% by FISH had $CD45^+$ cells detected. The level of engraftment by CD45 was slightly lower compared to FISH. This may have been due to the exclusion of some human cells by the gate applied or failure to lyse all mouse red blood cells resulting in a "dilution" of human cells.

The ability of engrafted cells to produce CFU-GM was assessed in 17 mice with engraftment levels of 1% to 87%. CFU-GM were detected in 15 of 17 cases with a level of 35 ± 7 (range 1 to 94) colonies per 10^5 BM cells plated. The total number of human CFU-GM present in 2 femurs and 2 tibias was 7 to 2690 (mean 697). As these 4 long bones contain approximately 20% of the total mass of murine BM (Boggs, 1984), the total number of human CFU-GM in murine BM was 35 to 13450 (mean 3485). The mean number of infused CFU-GM was 61 000 per mouse.

Morphologic assessment showed hypercellularity of BM with increased numbers of megakaryocytes and eosinophilia in highly engrafted animals but there was no fibrosis (Figure 6.6). Assessment of the peripheral blood white cell count in 6 mice showed a normal white cell count and differential.

To establish that the engrafting cells were transplantable into secondary recipients serial engraftment experiments were performed. Four mice received 1.2 to 8×10^6 cells each, being the contents of the 4 long bones, from 4 mice that had engrafted with 28% to 63% human

Figure 6.3.

Results of engraftment by FISH of 19 cohorts of 2 or 3 mice given different doses of chronic phase CML cells collected from patients at diagnosis. Results are mean ± sem engraftment for each cohort of mice.

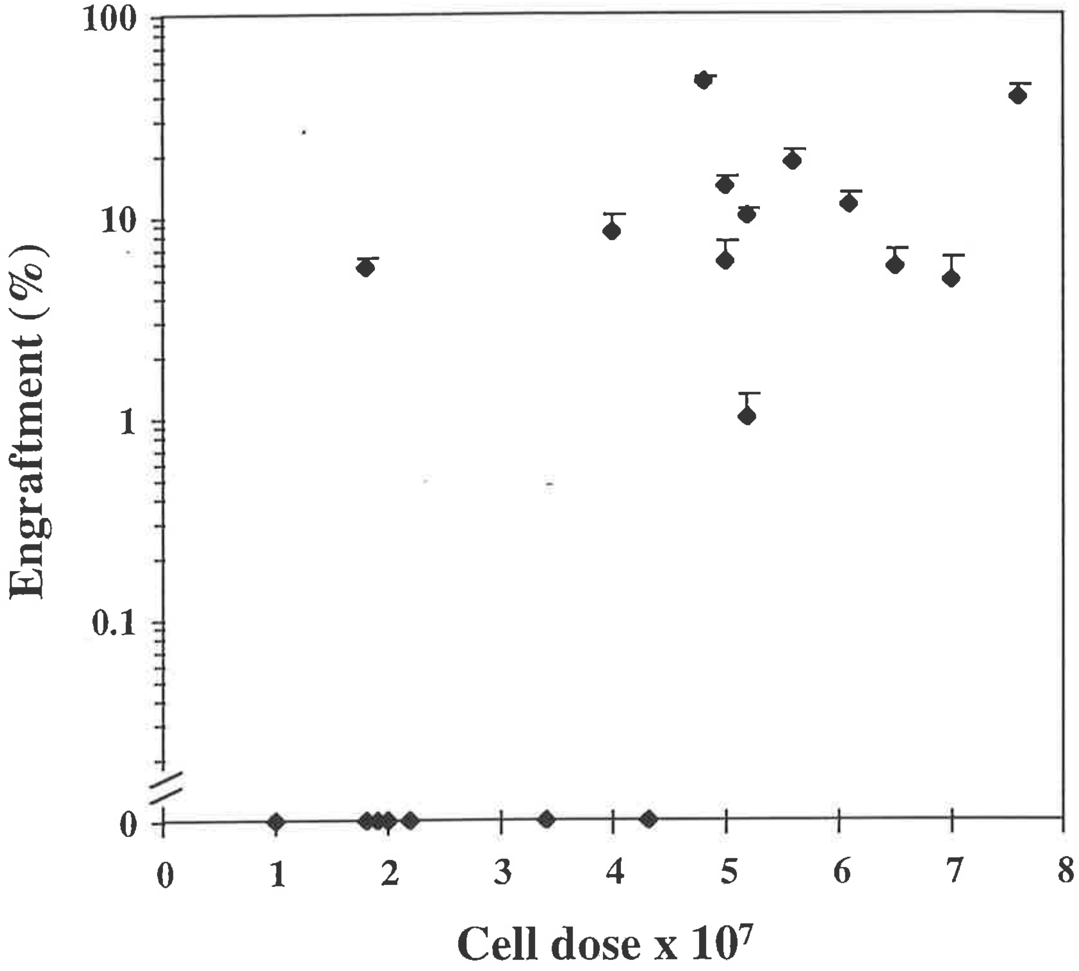

100
10
1
0.1
0
Engraftment (%)
0
1
2
3
4
5
6
7
8
Cell dose x 10^7

Figure 6.4.

Effect of cell dose on engraftment. Graph shows increased engraftment in cohorts of mice given different cell doses from the same patient sample. Results are mean BM engraftment for each cohort.

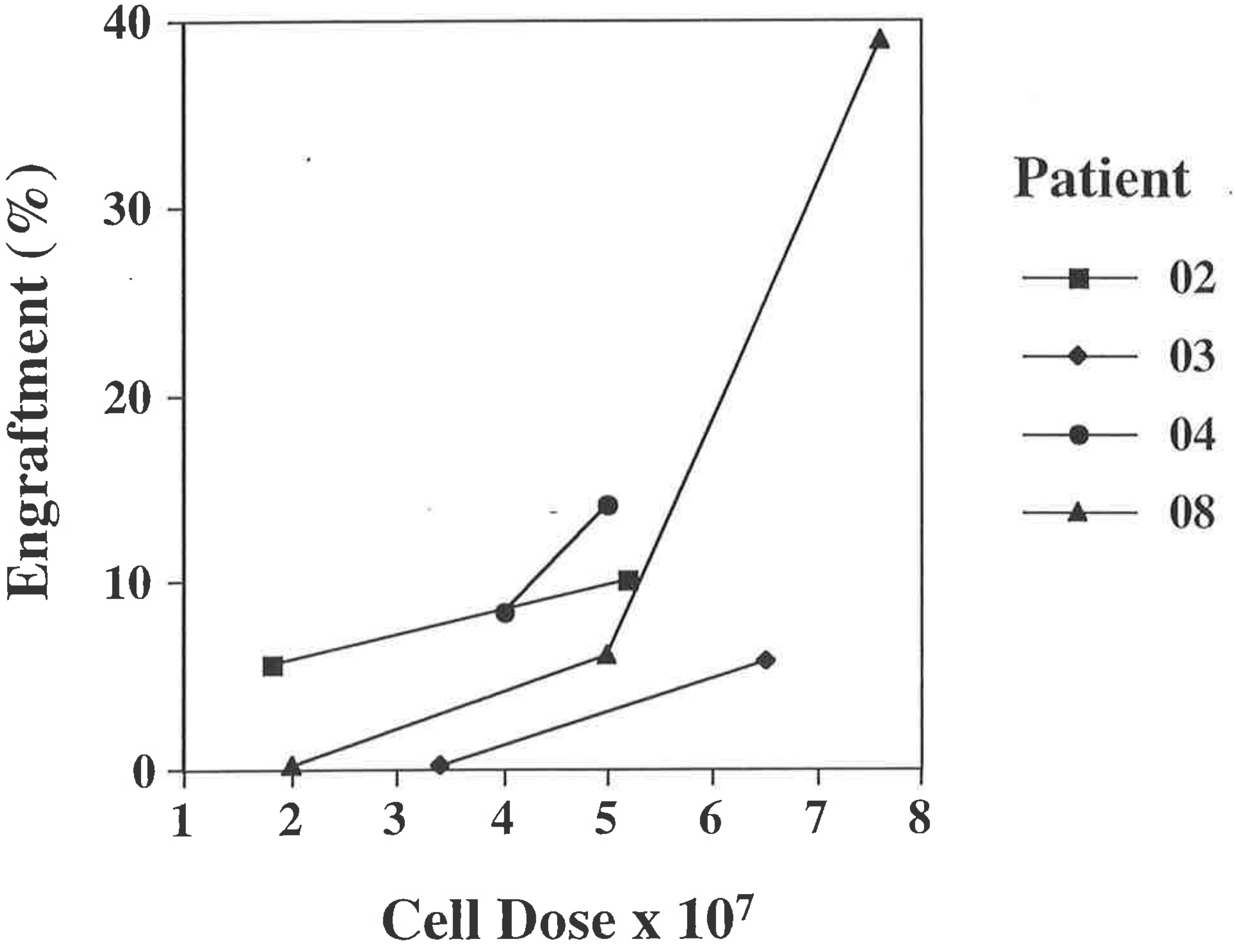
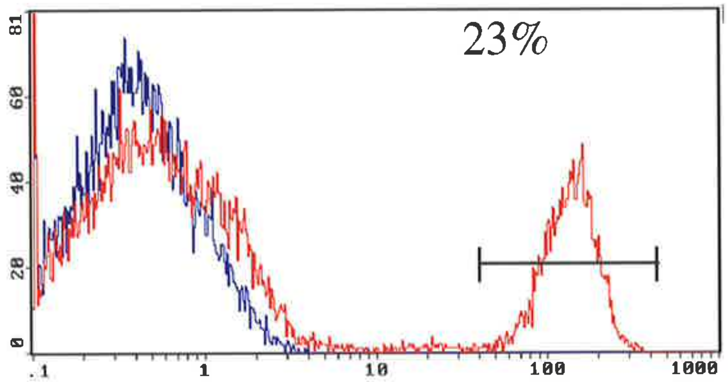
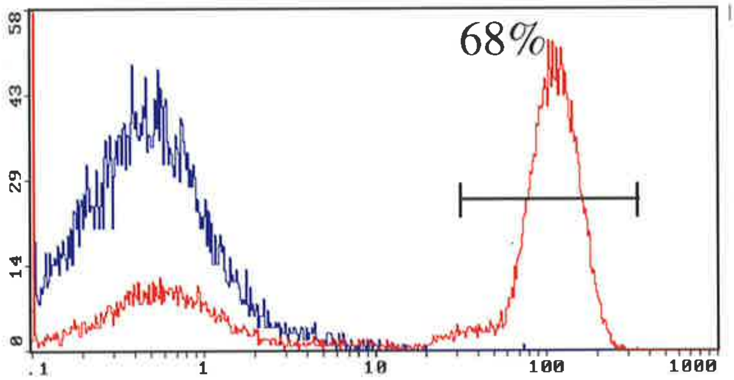

Engraftment (%)
0
10
20
30
40
1
2
3
4
5
6
7
8
Cell Dose x 10⁷
Patient
02
03
04
08

Figure 6.5.

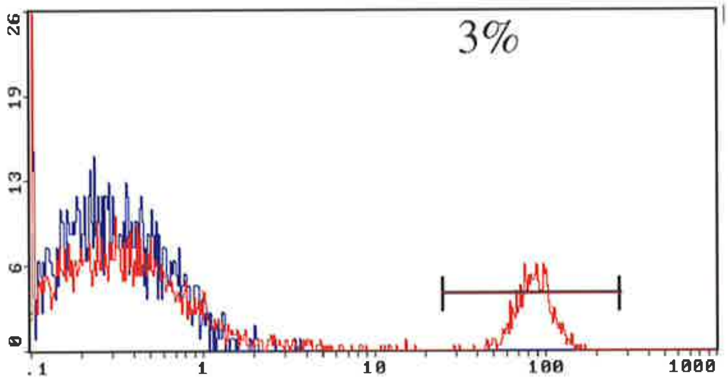
Analysis of engraftment by CD45. Cells from transplanted mice were washed, red cells lysed and then immunolabelled with anti-CD45 FITC, a human specific pan leucocyte marker. Expression of CD45 from the BM, spleen and blood of a representative mouse is shown in the red histogram compared to the isotype matched control cells in the blue histogram.


23%
81
60
40
20
0
.1
1
10
100
1000

BM


68%
58
43
29
14
0
.1
1
10
100
1000

SPL


3%
26
19
13
6
0
.1
1
10
100
1000

PB

Figure 6.6.

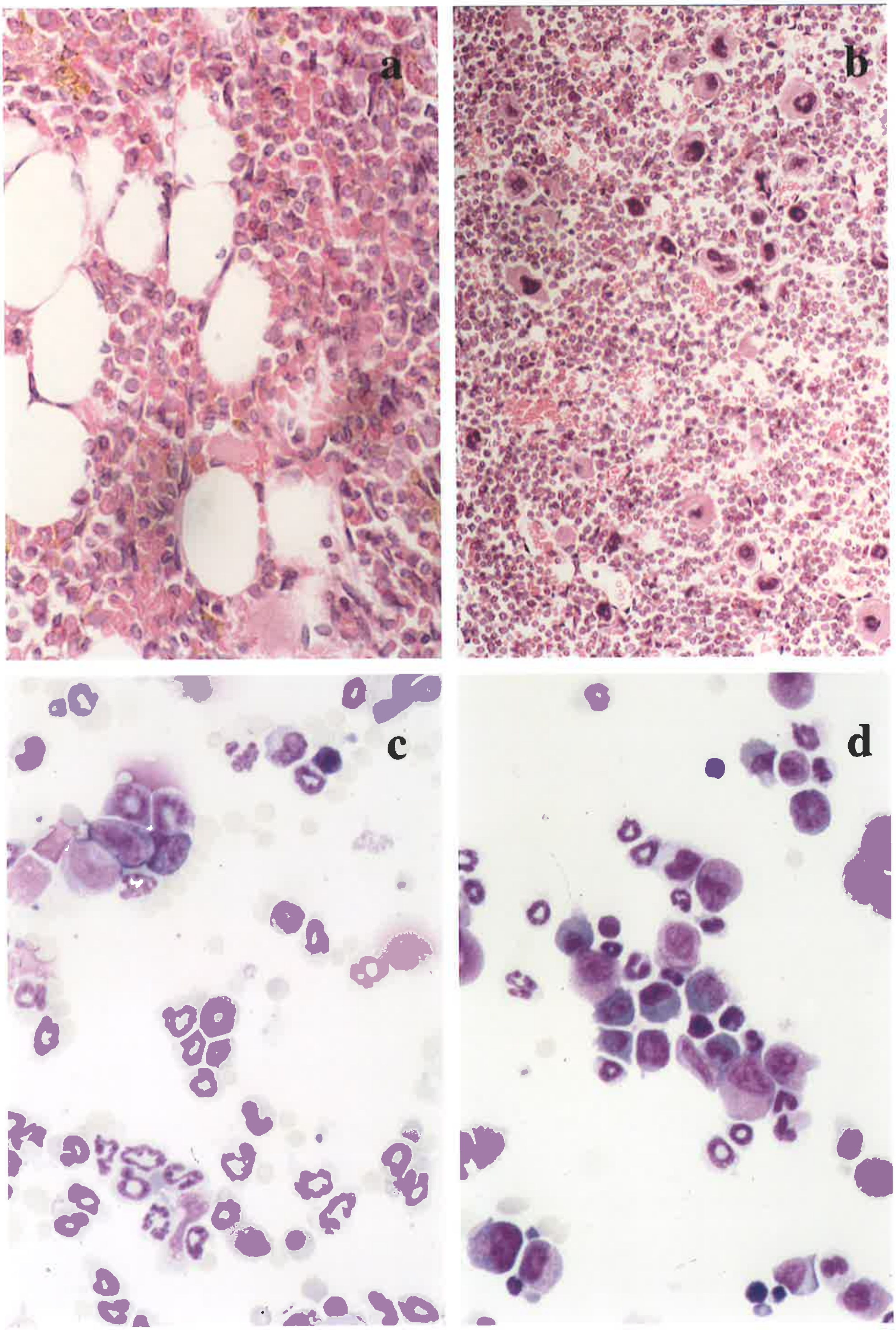
Morphologic assessment of murine BM engrafted with CML cells

6.6a. Histological section of tibia of NOD/SCID mouse irradiated but not infused with human cells at day 42 showing a normocellular marow (original magnification x 400).

6.6b. Histological section of tibia of NOD/SCID mouse with 28% human cells detectable at day 42 showing a hypercellular marrow with proliferation of megakaryocytes and eosinophils (original magnification x 200).

6.6c. Cytospin preparation of BM from NOD/SCID mouse irradiated but not infused with human cells at day 42 showing prominence of murine ring neutrophils (original magnification x 400).

6.6d. Cytospin preparation of BM from NOD/SCID mouse with 44% human cells detectable at day 42 showing developing myeloid cells with some cells showing eosinophilic granules (original magnification x 400).


a
b
c
d

cells. None of the secondary recipient mice had evidence of human cells at day 28 which is probably due to the relatively low cell dose given.

6.6.2. Splenic engraftment

Out of 64 mice that had BM engraftment, 42 (66%) had detectable human cells in the spleen with engraftment of 16% ± 3% (range 1% to 92%). The donor was a predictor of spleen engraftment ($p < 0.01$, analysis of variance), mice infused with cells from patients 02, 03 and 04 had higher levels of splenic engraftment, whereas mice infused with cells from patients 06, 11 and 12 had low splenic engraftment despite quite high BM engraftment (Figure 6.7). In 16 mice the level of splenic engraftment exceeded the level of BM engraftment. No mice had isolated splenic engraftment. Mice with high splenic engraftment had moderate splenomegaly. Histologic examination of the spleen showed infiltration by megakaryocytes in most engrafted mice (Figure 6.8).

6.6.3. Leukaemic engraftment

The differential engraftment of leukaemic and normal human cells was assessed by FISH analysis with directly conjugated probes for BCR and ABL (Figure 6.9). There was no cross reactivity of the probes with murine cells. In 25 mice with BM engraftment ≥9% the level of BCR-ABL$^+$ cells detected was 39% ± 5% (range 5% to 91%) of the engrafted human cells. Analysis of 6 spleen specimens, which had 29% to 87% engraftment, for the presence of leukaemic cells showed a lower level of BCR-ABL$^+$ cells compared to BM, with 11% ± 2% (range 4% to 17%) of human cells being leukaemic.

6.6.4. Use of HGF to improve engraftment

The role of HGF in promoting engraftment of human cells was assessed in 9 experiments with 59 mice. Intraperitoneal injections of 5 μg of SCF, G-CSF or GM-CSF were given

Figure 6.7.

Correlation between BM engraftment and splenic engraftment of 61 mice who received unmanipulated chronic phase CML PB from 8 patients.

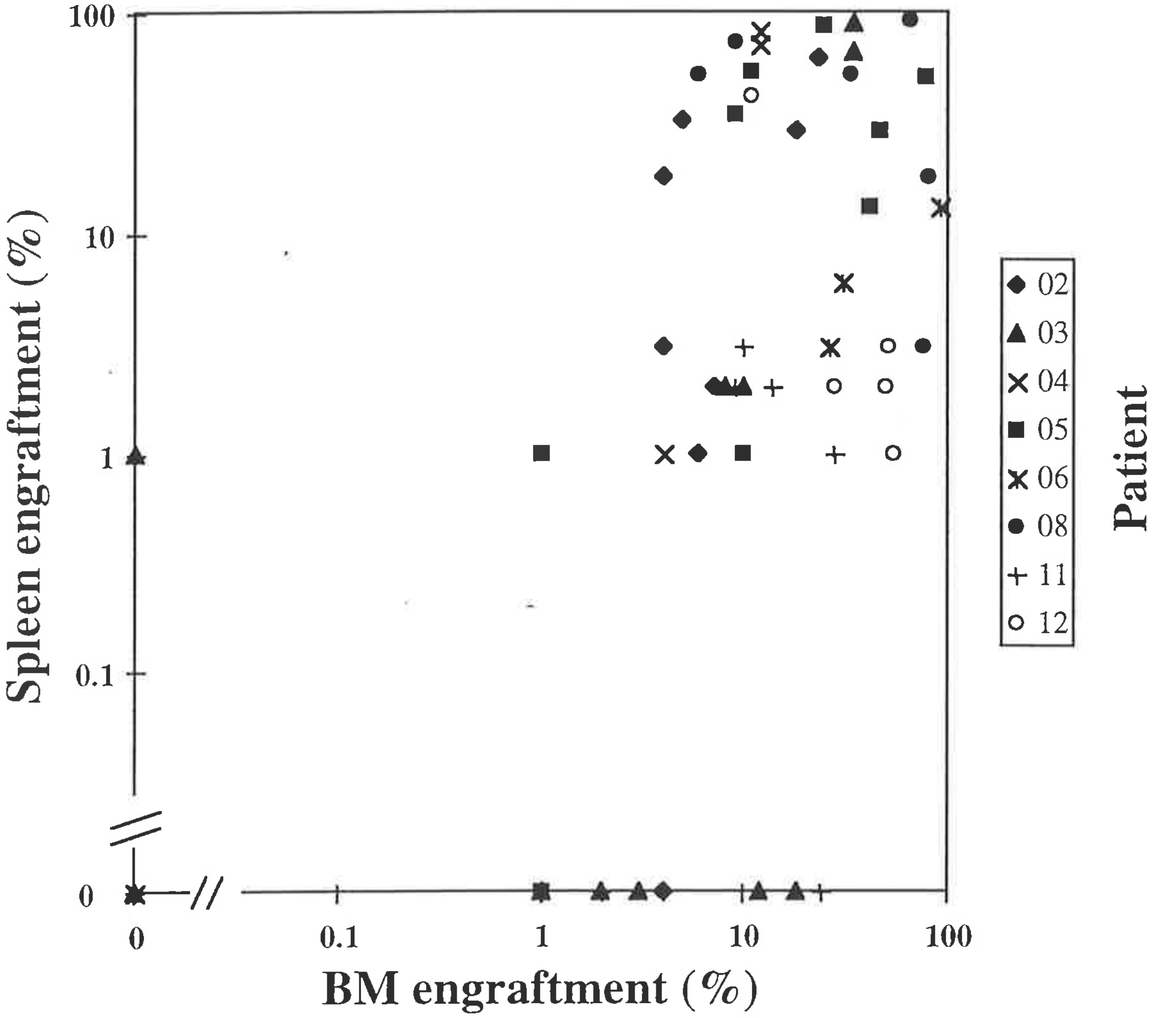

Spleen engraftment (%)
100
10
1
0.1
0
BM engraftment (%)
0
0.1
1
10
100
Patient
◆ 02
▲ 03
× 04
■ 05
✱ 06
● 08
+ 11
○ 12

Figure 6.8.

Morphologic assessment of murine spleen engrafted with CML cells

6.8a. Histological section of spleen of NOD/SCID mouse irradiated but not infused with human cells at day 42 showing normal splenic histology with prominent lymphoid cells (original magnification x 400).

6.8b. Histological section of spleen of NOD/SCID mouse with 33% human cells detectable at day 42 showing infiltration of megakaryocytes (original magnification x 400).

6.8c. Cytospin preparation of spleen from NOD/SCID mouse irradiated but not infused with human cells at day 42 showing polymorphous cell population consisting of myeloid, lymphoid and erythroid cells (original magnification x 400).

6.8d. Cytospin preparation of spleen from NOD/SCID mouse with 56% human cells detectable at day 42 showing infiltration by megakaryocytes (original magnification x 400).

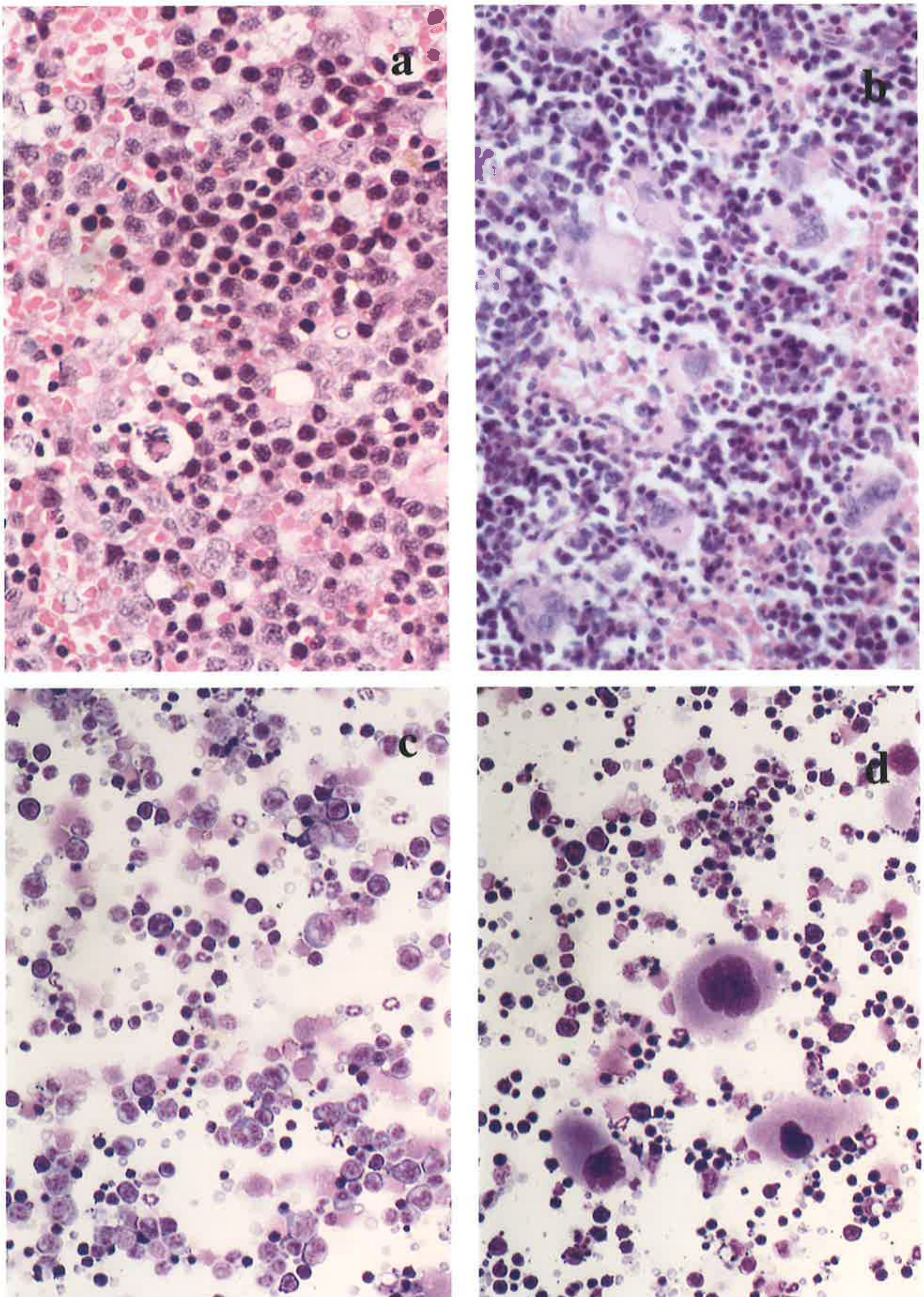
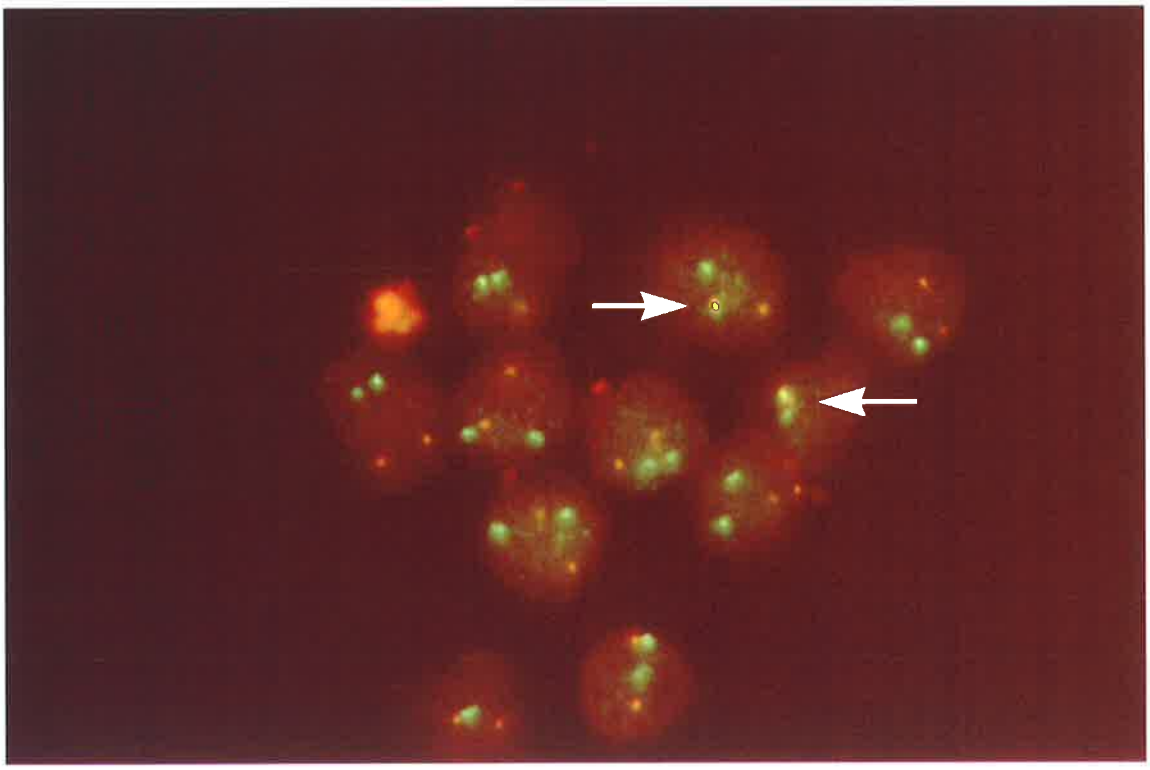

a
b
c
d

Figure 6.9.

Differential engraftment of normal and leukaemic CML cells in NOD/SCID mice detected by dual probes for BCR and ABL. Normal cells show 2 red ABL signals and 2 green BCR signals. Leukaemic cells show a single red and green signal representing normal ABL and BCR genes and a yellow signal representing fusion of ABL and BCR genes (arrowhead) (original magnification x 1000).



every second day except in 4 mice who received SCF and G-CSF or SCF and GM-CSF. The use of HGF did not produce an increase in engraftment compared to control mice who received the same dose of CML cells but no growth factor. Mice receiving no growth factor had levels of engraftment of 22% ± 6% ($n = 22$), compared to 19% ± 5% for SCF (alone or in combination, $n = 28$), 26% ± 8% for GM-CSF ($n = 7$) and 6% ± 3% for G-CSF ($n = 8$).

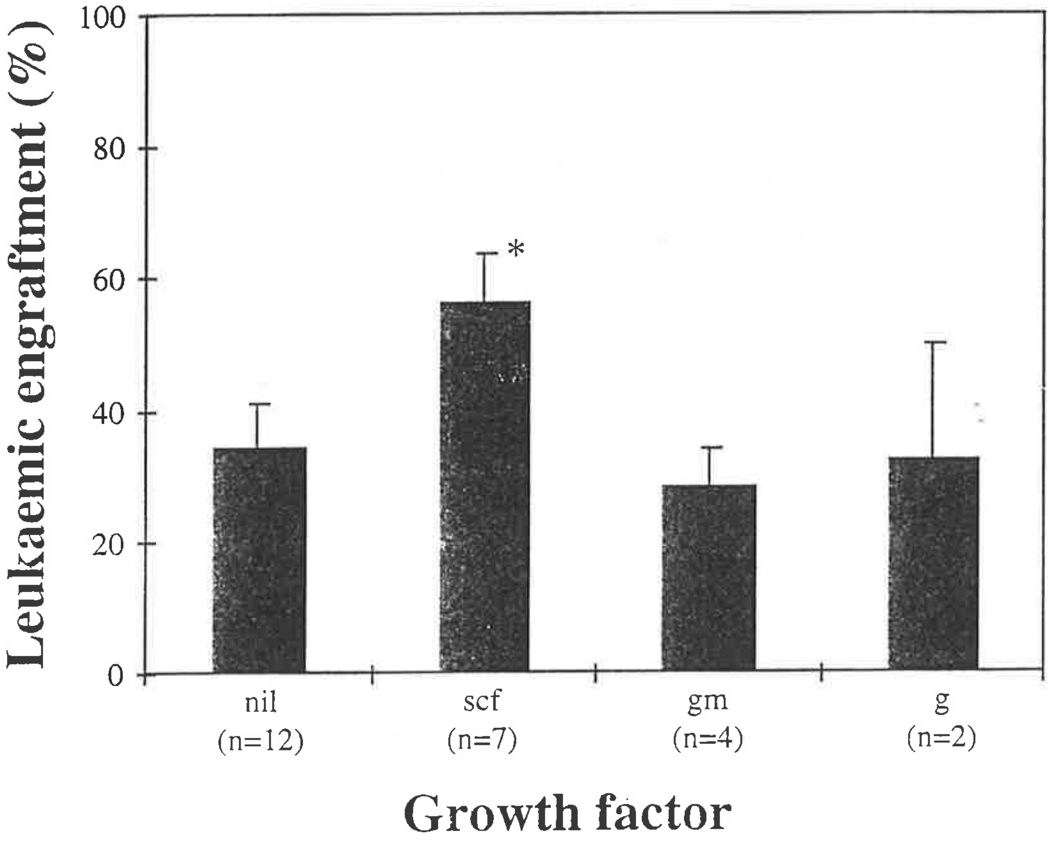
The influence of HGF on leukaemic cell engraftment was also assessed and there was a trend for mice receiving SCF alone or in combination to have higher levels of BCR-ABL$^+$ cells detected ($p = 0.02$, analysis of variance). In mice receiving SCF ($n = 7$) the level of leukaemic engraftment was 56% ± 8% compared to 34% ± 7% for mice receiving no HGF ($n = 12$), 28% ± 6% for mice receiving GM-CSF alone ($n = 4$) and 32% ± 18% for mice receiving G-CSF alone ($n = 2$) (figure 6.10).

6.6.5 Phenotype of engrafted cells

The immunophenotype, as assessed by dual colour immunofluorescence, of the engrafted human cells in the BM is shown in Table 6.3. The engrafted cells were predominantly myeloid in origin as shown by expression of CD33 (Figure 6.11). Primitive cells, defined by expression of CD34, were detected in 12 of 24 samples analysed at levels of 1% to 14% of engrafted cells. The total number of CD34$^+$ cells recovered calculated on cell numbers recovered from the 4 long bones was 0.12 to 6.2×10^6 (mean 2.1×10^6). This compares with the total numbers infused being 2 to 16×10^6. In no mouse were more CD34$^+$ cells recovered than were infused. CD19$^+$ cells were rare in the BM with 1.9% ± 1.2% of human cells positive. Analysis of CD3 showed predominant expression in the BM of 2 mice, with mean levels of 54% and 58%. The samples infused into these mice contained 12% and 49% CD3$^+$ cells respectively. These CD3$^+$ cells consisted of distinct populations of CD4$^+$ and CD8$^+$ cells with a mean number of 67% expressing CD8. Analysis of sorted CD3$^+$ cells by FISH for BCR-ABL showed only 2% leukaemic cells which is within the background level

Figure 6.10.

Influence of haemopoietic growth factors on leukaemic engraftment. Results are the mean ± sem of BCR-ABL positive cells by FISH as a proportion of total human cells. (scf = stem cell factor, gm = gm-csf, g = g-csf, nil = no growth factor).


Leukaemic engraftment (%)
100
80
60
40
20
0
*
nil
(n=12)
scf
(n=7)
gm
(n=4)
g
(n=2)
Growth factor

*p = 0.02

Table 6.3:

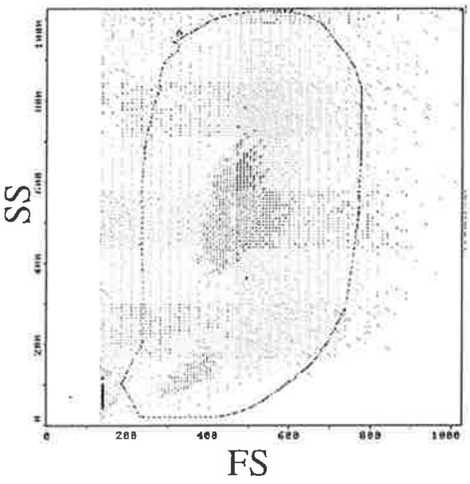
Phenotype of engrafted cells in mouse BM. Results are percentage of CD45+ cells expressing cells expressing the denoted antigen (mean +/- sem).

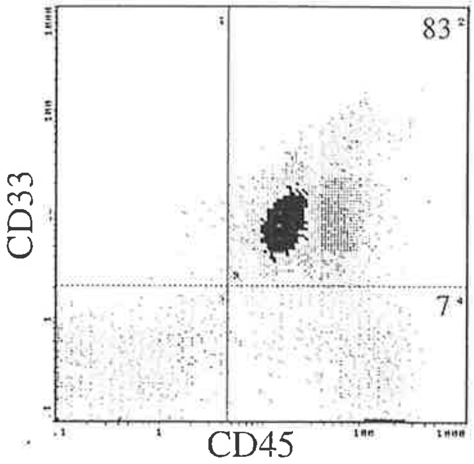
Patient	Phenotype of engrafted cells in BM			
	CD 34	CD 33	CD 3	CD 19
02	0	17 ± 7.3	54.2 ± 12.1	1.8 ± 0.6
03	0	0.7 ± 0.7	58 ± 10.5	0.3 ± 0.3
04	0.3 ± 0.3	49.7 ± 18.2	18.3 ± 18.3	ND
06	3.3 ± 1.9	75.3 ± 6.1	2 ± 1.5	7.3 ± 7.3
08	5.3 ± 2.8	ND	ND	ND
11	8.8 ± 2.6	70.8 ± 11.7	ND	ND
12	0	51.3 ± 14.3	2.7 ± 2.7	ND
mean ± SEM	2.7 ± 0.9	42.8 ± 7.2	30.2 ± 7.7	1.9 ± 1.2

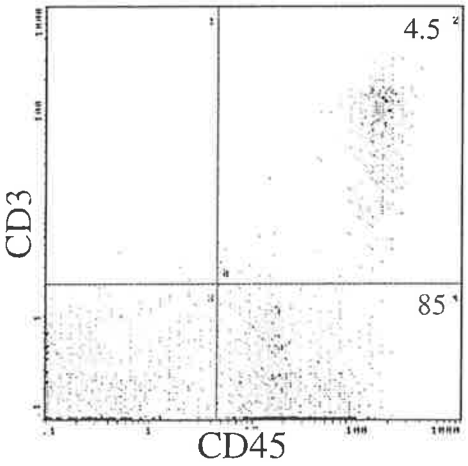
ND - not determined

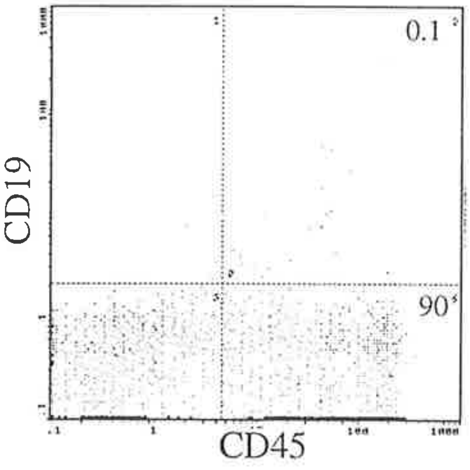
Figure 6.11.

Immunophenotype analysis of BM specimen at day 42. Analysis of forward and side scatter shows a population of cells suggestive of myeloid origin. This is confirmed with a high proportion of $CD45^+$ cells expressing CD33 with lower levels of $CD3^+$ and $CD19^+$ cells.


SS
FS
0
200
400
600
800
1000


CD33
CD45
83
7
.1
1
100
1000


CD3
CD45
4.5
85
.1
1
100
1000


CD19
CD45
0.1
90
.1
1
100
1000

of BCR-ABL detection suggesting that virtually all the $CD3^+$ cells were Ph^-. Expression of CD56 was assessed to evaluate the role of natural killer cells in engraftment. In 6 mice evaluated, 6% ± 3% of human cells expressed this antigen.

Analysis of cell surface phenotype of human cells in the spleen showed a different picture (Figure 6.12). No $CD34^+$ cells were detected and $CD33^+$ cells were only 9.3% ± 6.3% of engrafted cells. The predominant cell type expressed CD3 with 72.8% ± 7% positive (Table 6.4). These $CD3^+$ cells expressed either CD4 or CD8 and were 95% $CD45RO^+$. $CD19^+$ B lymphocytes were also more frequent in the spleen with 6.1% ± 2.2 % cells detected (Table 6.4).

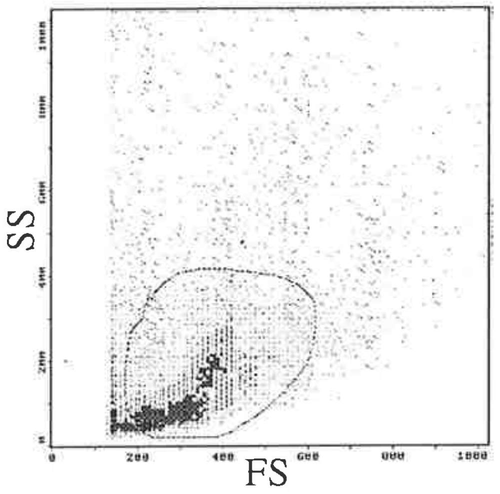
To assess the kinetics of engraftment 6 mice were infused with 5×10^7 cells and 2 were killed at days 14, 28, and 42. The mean BM engraftment was 38%, 13% and 30% at each of these timepoints respectively. At days 14 and 28 $CD33^+$ and $CD34^+$ cells were detected but these fell by day 42 with an associated increase in $CD3^+$ cells. No engraftment of the spleen was noted at day 14 or 28 but 33% human cells were detected at day 42, with 94% of these being $CD3^+$.

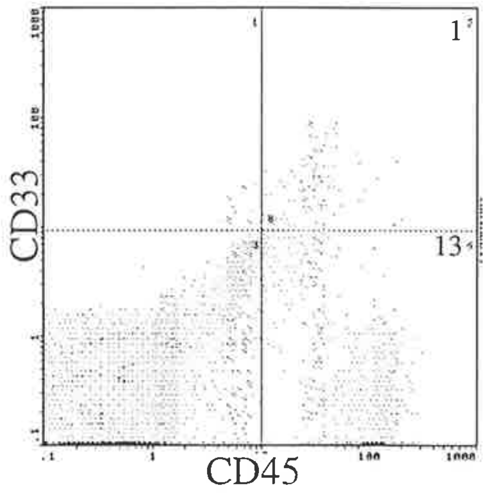
6.7 Engraftment of $CD34^+$ cells

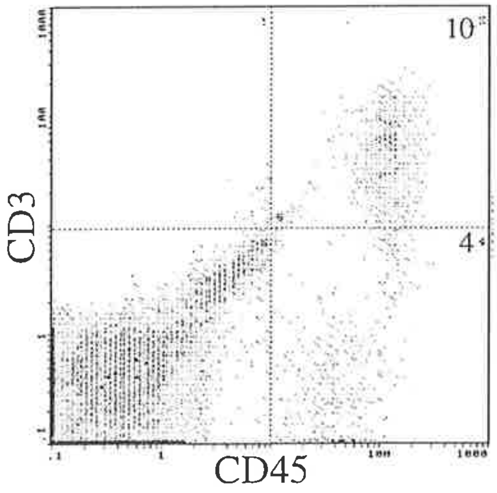
$CD34^+$ cells were selected from 4 patient samples (05, 08, 10, and 11) by immunomagnetic selection on thawed material. Two patient samples (01 and 07) had large scale selection of $CD34^+$ cells by an Isolex column. One of these samples (01) had $CD34^+$ cells selected at diagnosis on leukapheresis product which were then cryopreserved prior to use. The mean purity of recovered cells was 93% (range 88% to 98%). Cell doses of 1×10^4 to 1×10^7 were infused from these 6 patient samples into 36 mice (Table 6.5). Mice were killed at day 28 (13) or day 42 (25). Engraftment was seen in 2 mice at day 28 with levels of 2% and 63% respectively. At day 42, 7 mice engrafted with levels from 0.7% to 54%. Eight

Figure 6.12.

Immunophenotype of spleen cells from the same mouse. Analysis of forward and side scatter shows predominantly lymphoid type cells. The CD45$^+$ cells are predominantly CD3$^+$ with very few CD33$^+$ and CD19$^+$ cells detected.


SS
FS
0
200
400
600
800
1000


CD33
CD45
1
13
.1
1
100
1000


CD3
CD45
10
4
.1
1
100
1000

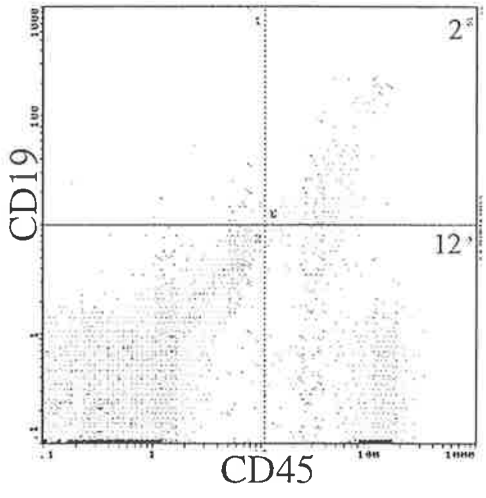

CD19
CD45
2
12
.1
1
100
1000

Table 6.4.

Phenotype of engrafted cells in mouse spleen. Results are percentage of CD45$^+$ cells expressing the denoted antigen (mean +/- sem).

Patient	Phenotype of engrafted cells in spleen			
	CD 34	CD 33	CD 3	CD 19
02	0	1.8 ± 1.2	81.8 ± 8.7	1.8 ± 0.5
03	0	1.5 ± 1.5	73.7 ± 17.3	6 ± 4
04	0	0	94	10
06	0	27.7 ± 18.9	50 ± 11	10.7 ± 6.3
mean ± SEM	0	9.3 ± 6.3	72.8 ± 7.0	6.1 ± 2.2

Table 6.5.

Bone marrow engraftment of selected CD34 positive and negative cells

Patient	CD 34 Positive Cells; Dose Given	Purity (%)	% BM Engraftment(#)	CD 34 Negative Cells; Dose Given	% BM Engraftment(#)
01	1 X 10^7	94	63	ND	ND
			2		
			54		
05	5 x 10^4	89	0 (5)	5 x 10^7	0 (3)
	5 x 10^5	89	0 (5)		
	7 x 10^6	89	7		
07	2 x 10^5	98	0 (2)	1.2 x 10^7	0
	5 x 10^6	98	0 (7)	3 x 10^7	0 (3)
	1x 10^7	98	0		
			0.7		
			3		
			4		
08	1 X 10^7	94	0	5 x 10^7	0 (2)
10	1 X 10^4	88	0 (3)	5 x 10^7	0 (2)
	1 X 10^5	88	0 (4)		
11	1 X 10^7	93	33	5 x 10^7	0

(#) number of mice tested. Results with no number are from single mice.
ND - not determined

out of 10 mice receiving $\geq 6 \times 10^6$ cells showed engraftment but none of the 26 mice receiving lower cell numbers did. Only 2 samples had engraftment in the spleen at levels of 1% and 4%. The 2 mice that had the highest engraftment levels, 54% and 63%, were given CD34$^+$ cells selected from fresh leukapheresis material.

CFU-GM production was assessed in the 2 mice with 63% and 54% engraftment. The number of CFU-GM produced was 108 and 104 per 10^5 cells plated (Table 6.6), or a total number of 1.5×10^5 and 1.9×10^5 respectively. The total number of CFU-GM infused was 8×10^5 per mouse.

The engrafted cells from the 3 mice with maximal engraftment were phenotyped and showed myeloid and B lymphoid cells in the BM. The mean level of CD34 expression was 10%. The total number of CD34$^+$ cells recovered from these mice, which had all received 1×10^7 CD34$^+$ cells, was 0.2 to 5.3×10^6. It is unlikely that all CD34$^+$ cells infused homed to the BM and so there has been some expansion of CD34$^+$ cells in these mice. The mean level of CD33$^+$ cells was 80% and CD19$^+$ cells was 10.7%. CD3$^+$ cells were not detected (Table 6.6).

Assessment of leukaemic cell engraftment by FISH was performed on the 3 samples with the highest engraftment. The number of BCR-ABL$^+$ cells was variable, being 23%, 35% and 64% (Table 6.6).

To confirm that the engrafting cell in CML is CD34$^+$ parallel experiments with the infusion of CD34$^-$ cells were carried out. Cells were infused from 5 patients (05, 07, 08, 10, and 11) at doses ranging from 1.2 to 5×10^7 cells into 12 mice. No engraftment by either FISH or CD45 analysis was seen in any of the mice infused (Table 6.5).

Table 6.6.

Characterisation of engrafted CD34 positive cells in bone marrow of NOD/SCID mice

Patient	Engraftment (%)	BCR-ABL$^+$ cells by FISH (%)*	Phenotype				CFU-GM /10^{5}cells
			CD 34	CD 33	CD 3	CD 19	
01	63	64	11	88	0	7	108
01	54	35	15	71	0	20	104
11	33	23	4	82	0	5	ND

* refers to percentage of human cells that are BCR-ABL positive.
ND - not determined.

6.8 Discussion

A model of CML in NOD/SCID mice has been established by infusing large numbers of cryopreserved CP CML PB into sublethally irradiated mice. The major determining predictor of engraftment was the cell dose infused with 84% of mice given $\geq 4 \times 10^7$ cells showing evidence of human cells 28 to 42 days post infusion. Of these, 46% of mice had >10% human cells detected in their BM. SCF, G-CSF or GM-CSF alone or in combination failed to improve overall engraftment rates. In addition human cells were detected in the spleen of 60% of mice that had BM engraftment. Previous studies of murine models of CML have also shown that the cell dose infused is critical for engraftment and that HGF do not improve engraftment levels. The study of Sirard et al infused 8 to 14×10^7 fresh PB or BMMNC from newly diagnosed CML patients into SCID mice and showed the majority of mice achieving 0.1% to 10% engraftment. Mice receiving 2 to 6×10^7 cells achieved negligible engraftment (Sirard et al., 1996). This study shows higher engraftment in the NOD/SCID mouse using lower cell doses. This may be the result of using cryopreserved material because of the selective loss of the more mature, non-engraftable cells during cryopreservation. However, the more immunosuppressed NOD/SCID mouse has been shown to improve engraftment of T-lymphoblastoid cells and human cord blood compared to SCID mice (Shultz et al., 1995; Pflumio et al., 1996) and this may account for the lower doses required. Morphological evidence of a CML like disease in the BM and spleen of engrafted mice was observed but no leukocytosis was seen, suggesting that this model recapitulates some of the features of chronic phase CML.

The ability to assess engraftment of leukaemic and normal cells is critical if the application of cell selection or purging techniques as therapeutic modalities in CML are to be tested in an animal model. FISH was used to detect BCR-ABL$^+$ cells in engrafted mice as a measure of leukaemic engraftment. This allows direct visualisation and enumeration of cells and avoids the reliance on proliferating cells necessary for cytogenetic analysis. Analysis of the CML cells prior to infusion from 5 patients showed 37% to 51% of cells were BCR-ABL$^+$

by FISH indicating the inoculum has equivalent numbers of leukaemic and non-leukaemic cells. The high number of non-leukaemic cells may be accounted for by the high levels of T cells in some samples. Analysis of 25 specimens of murine BM by FISH for BCR-ABL showed that of the human cells a mean level of 39% were leukaemic with a range of 5% to 94%. Thus, there is a wide range in the level of leukaemic engraftment in these mice but the majority of cases show preferential engraftment of normal cells.

The role of HGF is relevant as they are used clinically to mobilise stem cells or post transplantation to increase the speed of engraftment and hence may modulate normal or leukaemic engraftment. The finding that SCF has a role in promoting the engraftment and proliferation of leukaemic cells in vivo is consistent with in vitro findings. Erythroid colonies from patients with CP CML grow in the presence of SCF alone without the requirement for erythropoietin and these colonies all have BCR-ABL present (Issaad and Vainchenker, 1994). In LTBMC, SCF deficient stroma produces lower numbers of BCR-ABL^+ progenitors compared to stroma expressing transmembrane SCF or cultures in which exogenous SCF has been added (Agarwal et al., 1995). Therefore SCF may specifically favour the growth of leukaemic cells in CML.

The immunophenotype of engrafted cells shows differential engraftment of myeloid and lymphoid cells between the BM and spleen. Myeloid cells, defined by expression of CD33, were confined to the BM. This is not dependent on exogenous myeloid growth promoting factors as it was seen in mice who received no growth factor as well. The engraftment of primitive cells was confirmed in some mice by detection of CD34. The levels were low but in keeping with the frequency of these cells in CML BM and also consistent with the SCID model of CML (Sirard et al., 1996). In each case, fewer $CD34^+$ cells were detected compared to total numbers infused. In the study by Sirard et al 23 mice had a mean recovery of 2.9×10^4 $CD34^+$ cells per 4 long bones which is approximately 1.5×10^5 total $CD34^+$ cells per mouse. These mice received 6 to 14 x 10^7 cells and if only 1%

of these were $CD34^+$ then each mouse received a minimum of 6×10^5 $CD34^+$ cells (Sirard et al., 1996). Direct comparisons are difficult but the mean recovery of 2.1×10^6 $CD34^+$ cells per mouse reported here is greater than that of Sirard et al.

In the spleen, B cell lymphoid development of some mice was demonstrated but levels of B cells within BM were negligible. The finding of almost exclusive T cell involvement of engrafted murine spleen and high levels of T cells in the BM of some mice is intriguing. The early study of Sawyers et al showed recovery of predominantly $CD3^+$ cells from the peritoneal cavity of SCID mice given 1 to 5×10^7 CP CML cells IP (Sawyers et al., 1992). The analysis of engrafted human cells in the SCID model of CML demonstrated myeloid differentiation with CD13 expression and B cell differentiation with CD19 expression (Sirard et al., 1996). Human peripheral blood lymphocytes given by IV infusion do not engraft SCID mice (Mosier et al., 1988; Lapidot et al., 1992) but IP infusion of lymphocytes engrafts both SCID (Mosier et al., 1988) and NOD/SCID (Hesselton et al., 1995) mice. This results in engraftment of T and B cells with the spleen being the primary site of engraftment and very few cells detected in the BM. The results presented here show engraftment of polyclonal T cells in the spleen and BM of some mice. The engrafting T cells have the CD45RO phenotype of activated/memory cells with a higher number of CD8 cells compared to CD4 cells. These findings are similar to the T cell characteristics of IP infused normal lymphocytes in NOD/SCID mice (Hesselton et al., 1995). The kinetics of T cell engraftment showed maximal engraftment at day 42 compared to earlier timepoints which is similar to the pattern of engraftment of T-lymphoblastoid cells in NOD/SCID mice (Shultz et al., 1995). These are likely to be derived from long lived recirculating T cells present in the inoculum. This is confirmed by the finding of no T cell engraftment in mice infused with $CD34^+$ cells. However, mice infused with $CD34^-$ cells., which contain large numbers of T cells, did not show any engraftment. The mechanism of T cell engraftment in mice receiving unmanipulated PB is unclear. It is possible that the T cells require either

CD34^{+} cells to facilitate their engraftment or a growth factor produced by these cells. This is speculative and requires further investigation.

This study demonstrates that infusion of 7 to 10 x 10^{6} purified CML CD34^{+} cells engraft immunodeficient mice. The demonstration of BCR-ABL^{+} cells by FISH indicates engraftable leukaemic cells reside in the CD34^{+} population. Experiments with CD34^{-} cells showed no engraftment, confirming this finding. Splenic engraftment was only seen in 2 mice at low levels. There was a high level of CD34^{+} cells detectable in the BM 6 weeks post transplantation and high numbers of myeloid progenitors. Furthermore these cells differentiate into myeloid cells and B lymphocytes but no T cells were detected. Other studies show engraftment of purified CD34^{+} cells from cord blood (5 x 10^{4} cells) (Larochelle et al., 1996), AML (5 to 7 x 10^{6}) (Lapidot et al., 1994) and juvenile chronic myeloid leukaemia (JCML) (2 to 3 x 10^{5}) (Lapidot et al., 1996). Thus in CML higher doses of CD34^{+} cells are necessary for engraftment, although the studies in AML and JCML required HGF. The role of HGF in engraftment of selected CD34^{+} CML cells was not assessed in this study but they may allow a lower cell dose to be given. Functional differences in the CD34^{+} cell in each of these examples may explain the requirements for differing cell dose to achieve engraftment. In CML it is known that the majority of primitive cells have phenotypic characteristics of proliferating cells (Udomsakdi et al., 1992b) and there is evidence to suggest cycling cells do not efficiently engraft Balb/c mice (Ramshaw et al., 1995), although this is in an unirradiated model. Also, the frequency of the pluripotent HSC as defined by the phenotypes CD34^{+}DR^{-} or CD34^{+}38^{-} is reduced in chronic phase CML compared to normal haemopoiesis. In normal haemopoiesis the frequency of HLA-DR^{-} and CD38^{-} cells is 3% to 8% and 3% to 7% of the CD34^{+} population respectively (Huang and Terstappen, 1994; Rusten et al., 1994). In CML it has been demonstrated in chapters 4 and 5 that these populations are rarer, comprising 1% each of the CD34^{+} population. Another possibility is the interaction of the CML cell and the murine BM stroma. For successful BM engraftment homing of infused cells to BM stroma is critical. Homing is integrin dependent (Papayannopoulou et al., 1995) and CML cells have an integrin mediated defect in adhesion (Lundell et al., 1996) which may impair their homing ability. Hence the rarity of engraftable leukaemic stem cells and defective homing ability may both contribute to the high cell requirement of CML cells in the murine model and the preferential engraftment of normal cells.

The major concern with this model is whether cells with long-term repopulating potential have engrafted or are the results seen a reflection of more mature cells engrafting. Definitive evidence for this would come from secondary transplant studies showing evidence of serial engraftment in secondary recipients. This was attempted but no secondary recipient mice engrafted. This is probably because of the requirement for high cell numbers to achieve primary engraftment. The infusion of equivalent numbers of human cells into secondary recipients will require pooling of cells from multiple mice that have achieved primary engraftment. Engraftment of secondary recipients has not yet been achieved in a murine model of CP CML. An alternative approach is to leave the mice for longer periods of time to assess "long-term" engraftment. This was also attempted but all mice observed beyond 42 days became acutely unwell and either died or were sacrificed. No evidence of overt leukaemia was noted in these mice. A GVHD type illness has been reported in NOD/SCID occurring at this timepoint (Shultz et al., 1995). No histological analysis was performed on these mice but the pattern of T cell engraftment is consistent with a GVHD type effect. Indirect evidence for the engraftment of primitive cells is the detection of CFU-GM and CD34$^+$ cells as late as day 42 together with differentiation of cells into myeloid and B lymphoid lineages. The total numbers of CFU-GM and CD34$^+$ cells detected were lower than the numbers infused even in the experiments using pure CD34$^+$ cells. However this does not exclude the engraftment of long-term repopulating cells as there is not necessarily a correlation between this and expansion of either CFU-GM or CD34. This also assumes that all infused CD34$^+$ cells and CFU-GM home to the BM and this is probably unlikely.

In summary engraftment of mononuclear and CD34$^+$ selected cells from the PB of CML patients into NOD/SCID mice has been shown as evidenced by detection of human cells in the BM and spleen by FISH and immunophenotype. Good levels of engraftment with lower cell doses and recovery of greater numbers of CFU-GM and CD34$^+$ cells have been achieved compared to the SCID mouse model of CML (Sirard et al., 1996). This model recapitulates the characteristic features seen in the BM with megakaryocytic and myeloid overgrowth. This model is suitable for evaluating purging strategies and novel therapies in the treatment of CML.

Chapter 7

Concluding Remarks

There is a great deal of interest and research into alternative treatment strategies to improve survival in CML. Residual normal cells found in the BM of many patients at diagnosis mean that new therapies that target the leukaemic stem cell may allow residual normal stem cells to gain a proliferative advantage and repopulate the marrow. Autologous transplantation is an approach that can be used in many patients who are not suitable for allogeneic transplantation. Studies with unmanipulated blood or bone marrow show restoration of Ph^- haemopoiesis in some patients but current data do not demonstrate a significant survival advantage. Stem cell rescue with exclusively or predominantly Ph^- autologous cells may lead to prolonged Ph^- haemopoiesis and a corresponding prolongation of survival. Post-autograft immunotherapy may further enhance the prospects of prolonged Ph^- haemopoiesis. Manipulation of autologous marrow and blood using techniques such as culture on stroma, incubation with cytotoxics, interferon or antisense oligonucleotides, and mobilisation with chemotherapy and haemopoietic growth factors are all being investigated. Definitive conclusions cannot be made because of the lack of suitable controls but most patients engraft and some develop Ph^- haemopoiesis.

The best technique for isolating normal cells in CML has not been defined. Positive selection of normal cells based on cell phenotype is attractive because it may limit the number of manipulations required to obtain a pure population and therefore decrease cell loss. Evidence presented here shows that both the $CD34^+DR^-$ and $CD34^+38^-$ populations of blood and marrow of patients in early chronic phase CML contain $BCR\text{-}ABL^-$ pre-progenitors and are therefore potential targets for positive selection in an autologous transplant program. The major concerns with this approach are whether sufficient cells can be collected to produce engraftment and also potential leukaemic contamination of the graft.

The BM has been the most studied source for the presence of Ph$^-$ cells in CML because of the high numbers of leukaemic progenitors and LTC-IC found in the PB at diagnosis (Goldman et al., 1980; Udomsakdi et al., 1992a). However, it is difficult to collect sufficient BM for manipulation in CML. In this study PB is shown to be an abundant source of BCR-ABL$^-$ pre-progenitors. This finding has recently been confirmed by other investigators who show that in the PB at diagnosis Ph$^-$ LTC-IC can be detected in some patients (Petzer et al., 1996b; Podestà et al., 1997). Therefore PB at diagnosis is a potential source of normal primitive cells in CML. Furthermore, I have shown that equivalent numbers of CD34$^+$DR$^-$ cells can be isolated from 500 ml of blood at diagnosis compared to 2.5 L of BM.

Which population should be targeted for positive cell selection? Previous studies have shown the CD34$^+$DR$^-$ population is enriched for BCR-ABL$^-$ cells whereas the CD34$^+$38$^-$ is not. I have demonstrated that the more primitive cells in the CD34$^+$38$^-$ population, the pre-progenitors, are also enriched for BCR-ABL$^-$ cells and hence may be as suitable a target for manipulation as the CD34$^+$DR$^-$ population. These findings are not reflected in the progenitor populations, particularly the CD34$^+$38$^-$ population, where most progenitors are leukaemic. However, as the long-term engrafting cells are more primitive than progenitors, it is the nature of these cells that is important. In-vitro assays of normal and leukaemic progenitor cells have shown biological differences exist between these populations but a better assay of the leukaemic stem cell is necessary if in vitro findings are to be applied clinically. An animal model of CML may help to better to define these differences at the stem cell level.

Murine models of CP CML have been difficult to establish. The model of CML that I have established in NOD/SCID mice recapitulates some of the features of CP CML. Importantly I have shown that the leukaemic stem cell resides within the CD34$^+$ population. The use of FISH to analyse engraftment of normal and leukaemic cells is an ideal tool to assess the effects of in-vitro manipulations on leukaemic haemopoiesis. This can currently be applied to assess the effects of different therapies on leukaemic engraftment. Currently the high cell numbers required to achieve reproducible engraftment limit the usefulness of the model to study the engraftment of rare populations of cells. This should be the aim of future investigations. Transplantation of the $CD34^{+}DR^{-}$ and $CD34^{+}38^{-}$ populations and assessment of the characteristics of engraftment will enable the results of in-vitro studies to be clarified. Mechanisms that improve engraftment must be sought and confirmation that long-term repopulating cells engraft is necessary. Potential areas include the assessment of the interaction between the murine stromal cell and the human haemopoietic cell and more extensive assessment of the role of haemopoietic growth factors in engraftment.

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