CLONALITY OF NORMAL AND MALIGNANT HEMOPOIESIS

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ABSTRACT

In the normal adult human, hemopoiesis appears to be maintained by the simultaneous activity of many stem cell-derived clones. Conversely, most examples of human myeloid malignancies have been shown to represent clonal populations arising as a result of the unregulated expansion of a single transformed hemopoietic stem cell. The limits of the proliferative capacity of normal hemopoietic stem cells in humans and their persistence in hemopoietic malignancies have, however, not been extensively investigated. One of the most likely reasons for this is the lack, until very recently, of a widely applicable method to analyze the clonality status of human cell populations. Methylation analysis of two polymorphic genes, HPRT and PGK, now allows such studies to be performed in approximately 50 % of females.

The possibility that normal human hemopoietic stem cells might have the capacity to mimic the behaviour of some transformed stem cells by generating clones of progeny that could dominate the entire hemopoietic system was then examined. Such a phenomenon has been well documented in animal models of marrow cell transplantation. I therefore undertook an analysis of all allogeneic marrow transplants performed over a 1 to 1-1/2 year period where the genotype of the donor made clonality analysis using the HPRT or PGK systems possible. Using this approach, I obtained evidence in two patients suggesting that a single or, at most, a very small number of normal primitive hemopoietic stem cells were able to reconstitute the hemopoietic system. In one case the data suggested that such reconstitution was likely to have derived from a stem cell with both lymphopoietic and myelopoietic potential. However, in all other cases hemopoiesis in the transplant recipient was found to be polyclonal. Such findings indicate that clonal dominance in the hemopoietic system is not sufficient to infer that a genetically determined neoplastic change has occurred. In addition, these findings have implications for the design of future gene therapy protocols.

The same methodology was also applied to investigate the clonality of different hemopoietic cell populations in patients with chronic myelogenous leukemia (CML) and essential thrombocytosis (ET). In both of these myeloproliferative disorders, the neoplastic clone produces terminally differentiated progeny that appear minimally different from normal. Data from the CML studies confirmed the non-clonal nature of the cells emerging in long-term CML marrow cultures. Similarly, patients transplanted with cultured autologous marrow were shown to undergo polyclonal and bcr-negative reconstitution of their hemopoietic system. Analysis of a series of patients with a clinical diagnosis of ET showed that polyclonal hemopoiesis in the presence of an amplified neoplastic clone is not a rare event in this disorder, and that clonality results do not always correlate with other neoplastic markers associated with myeloproliferative diseases in general. Another example of polyclonal hemopoiesis in the presence of an amplified neoplastic clone was demonstrated in a patient with Ph^1-positive ALL whose disease appeared to have originated in a lymphoid-restricted stem cell.

The studies described in this thesis reveal a level of complexity of normal and neoplastic stem cell dynamics not previously documented. They highlight the need for more precise information about the molecular basis of regulatory mechanisms that govern hemopoietic cell proliferation and survival at every level of differentiation. Finally they support the accumulating evidence that acquisition of full malignant potential requires several additive genetic changes first postulated many years ago as the somatic mutation theory of carcinogenesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

a2	second exon of abl gene.
AET	5-2-aminoethylisothiouronium bromide hydrobromide.
ALL	Acute lymphoblastic leukemia.
AML (M2)	Acute myeloid (myelogenous) leukemia, M2 subtype of French-American-British (FAB) classification.
AMMoL (M4)	Acute myelomonocytic leukemia, FAB M4 subtype.
AMoL (M5)	Acute monoblastic leukemia, FAB M5 subtype.
APL (M3)	Acute promyelocytic leukemia, FAB M3 subtype.
ATCC	American Type Culture Collection
B	BamH I
b1,2,3,4	Four exons of the breakpoint cluster region.
B4	CD19 (B-cell) positivity as recognized by antibody B4.
bcl	B-cell leukemia/lymphoma
BCNU	Bis-chloroethylnitrosourea.
bcr	Breakpoint cluster region
BCR	Breakpoint cluster region gene.
BFU-E	Burst forming unit-Erythroid.
BM	Bone marrow.
BMT	Bone marrow transplantation.
bp	base pair.
BSA	Bovine serum albumin.
c-abl	Cellular abl protooncogene.
CALLA	Common acute lymphoblastic leukemia associated antigen.
CCR	Clinical complete remission.
CD	Cluster of differentiation.
CFU-S	Colony forming unit-Spleen
CFU-GM	Colony forming unit-Granulocyte-macrophage.
CFU-E	Colony forming unit-Erythroid.
CFU-GEMM	Colony forming unit-Granulocyte-Erythrocyte-Monocyte-Megakaryocyte.
CFU-Mk	Colony forming unit-Megakaryocyte.
cGy	Centigray
CLL	Chronic lymphocytic leukemia.
CML	Chronic myeloid (myelogenous) leukemia.
Ci	Curie
CP	Chronic phase.
CsCl	Cesium chloride.
CSF	Colony stimulating factor.
CytIgM$^+$	Cytoplasmic immunoglobulin M positivity.
D+	Days after transplantation.
D-	Days before transplantation.
DMSO	Dimethylsulfoxide.
DNA	Deoxyribonucleic acid.
e1	First exon of BCR gene.
EBV	Epstein-Barr virus
ECM	Extracellular matrix.
EDTA	Ethylenediamine tetraacetic acid.
Epo	Erythropoietin.
ET	Essential thrombocythemia.
FCS	Fetal calf serum.

Fibr	Fibroblasts.
G	Granulocytes.
G-CSF	Granulocyte-colony stimulating factor.
G418	Geneticin.
GM-CSF	Granulocyte-macrophage colony stimulating factor.
G6PD	Glucose-6-phosphate dehydrogenase.
GVHD	Graft-versus-host disease.
GVL	Graft-versus-leukemia.
Hb	Hemoglobin.
HCl	Hydrochloric acid.
HL-60	Human promyelocytic cell line.
HGF	Hemopoietic growth factors.
Hh	Hha I restriction enzyme.
HLA	Human Leukocyte Antigen
H-2	Mouse histocompatibility complex.
Hp	Hpa II restriction enzyme.
HPLC	High performance liquid chromatography.
HPRT	Hypoxanthine phosphoribosyl transferase.
HPRT-p600	600 base pair HPRT probe.
HPRT-p800	800 base pair HPRT probe.
HS	Horse serum.
Ig	Immunoglobulin.
IgH	Immunoglobulin heavy chain.
IL	Interleukin.
Kd	Kilodalton.
Kb	Kilobase.
LAP	Leucocyte alkaline phosphatase.
LDC	Light density cells.
$L12^+$	CD19 antigen positivity as recognized by Leu12 antibody.
LTMC	Long-term marrow culture.
M-CSF	Monocyte/macrophage colony stimulating factor.
mCyt	Methyl-cytosine.
ME	Mercaptoethanol.
MLC	Mixed lymphocyte culture.
MPD	Myeloproliferative disorder.
N/A	Not available.
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NK	Natural killer.
OCT	Ornithine carbamyl transferase.
OD	Optical density.
PBMC	Peripheral blood mononuclear cells.
PBS	Phosphate buffered saline.
PCR	Polymerase chain reaction.
PGK	Phosphoglycerate kinase.
Ph^1	Philadelphia chromosome.
PHA	Phytohemagglutinine.
PIC	Polymorphism information content.
PV	Polycythemia vera.
PVSG	Polycythemia vera study group.
p145	145 kd normal abl gene product.
p190	190 kd protein resulting from first intron rearrangement in the BCR gene.
p210	210 kd protein resulting from rearrangement in the bcr.
R	Correlation coefficient from regression analysis.
RAEB	Refractory anemia with excess of blasts.

RE	Restriction enzyme.
RFLP	Restriction fragment length polymorphism.
RNA	Ribonucleic acid.
SD	Standard deviation.
SDS	Sodium dodecyl sulfate.
SEM	Standard error of the mean.
SRBC	Sheep red blood cells.
SSC	Sodium chloride-sodium citrate buffer.
SV-40	Simian virus-40.
T	T-cells.
TA	Tris-Acetate.
TCR	T-cell receptor.
TdT	Terminal deoxynucleotidyl transferase.
TE	Tris-EDTA
TGF	Transforming growth factor.
TNF	Tumor necrosis factor.
TNE	Tris-sodium chloride-EDTA buffer.
U	Unit
UPN	Unique patient number.
UV	Ultraviolet.
v-abl	Viral abl oncogene.
VNTR	Variable number of tandem repeats.
WBC	White blood cells.
XCI	X-chromosome inactivation.
Xmat	X-chromosome of maternal origin.
Xpat	X-chromosome of paternal origin.

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C H A P T E R I

INTRODUCTION

1- AN OVERVIEW OF HEMOPOIESIS

(A)- HEMOPOIETIC CELLS

Blood contains a variety of mature cell types of vital importance but of short life span. Although some blood cells such as memory T cells can persist in the body in a dormant state for many years, the majority die in a few days or weeks after their production. The continuous production of these cells throughout life is due to the proliferative activity of a small number of very primitive cells, designated as hemopoietic stem cells. During ontogeny, these cells arise in the yolk sac of the embryo (1) and then migrate into the developing liver (1). Subsequently, hemopoietic cells appear in the bone marrow and the spleen (1). Shortly after birth, the liver ceases to be a hemopoietic organ and in man most hemopoietic cells are produced thereafter in the bone marrow.

Within the hemopoietic system, a hierarchy of progenitor subsets have been defined, starting from the most primitive (stem cells) and leading up to their most mature progeny, including the granulocytes, monocytes, B and T- lymphocytes, platelets and erythrocytes in the circulation as well as a variety of mature cells found outside the circulation (e.g. mast cells, tissue macrophages). The hemopoietic system may be viewed as sequential compartments of cells of decreasing proliferative potential and increased differentiation towards a particular

mature blood cell type (Figure 1). Between the most primitive (stem) and the most differentiated compartments there are subsets of intermediate progenitors. These are distinguished from the stem cell population from which they derive, by their more restricted differentiative potentialities and finite proliferative capacity.

a- Stem cells and hierarchical structure

Stem cells are conceptually defined by their self-renewal ability and operationally, by their ability to generate pluripotent daughter cells with enormous proliferative capacity, i.e. sufficient to maintain blood cell production for many months. Stem cells are difficult to study as they represent only a small fraction of the cells in any hemopoietic tissue (2) and they can not be identified directly by any currently available technique. In 1961, Till and McCulloch developed the spleen-colony forming assay which detects and measures a very primitive hemopoietic cell in mice (3). In this procedure, the cells to be assayed are generally injected into lethally irradiated syngeneic mice and the macroscopic nodules that appear on the surface of the spleen are counted 7-14 days later. The cell that is able to generate these nodules is referred to as a colony- forming-unit spleen, or CFU-S. It was found that each spleen colony is derived from a single cell (4) at least some of which have the capacity to generate erythroid, granulopoietic and megakaryocytic cells (5) and may also undergo some self-renewal during their initial proliferation (6). The question of whether the CFU-S contains a lymphoid stem cell which is different from the pluripotent(myelo-lymphoid) stem cell was addressed in a study by Abramson et al (7) who showed that some secondary recipients of W/W mice, initially transplanted with cytogenetically marked cells, can have the marker only in their PHA-stimulated lymphocytes, as opposed to other recipients which have the marker both in their bone marrows and their spleen colonies. They concluded that mouse bone marrow must contain in addition to a pluripotent stem cell, a cell with only myeloid and another cell with only lymphoid commitment (7).

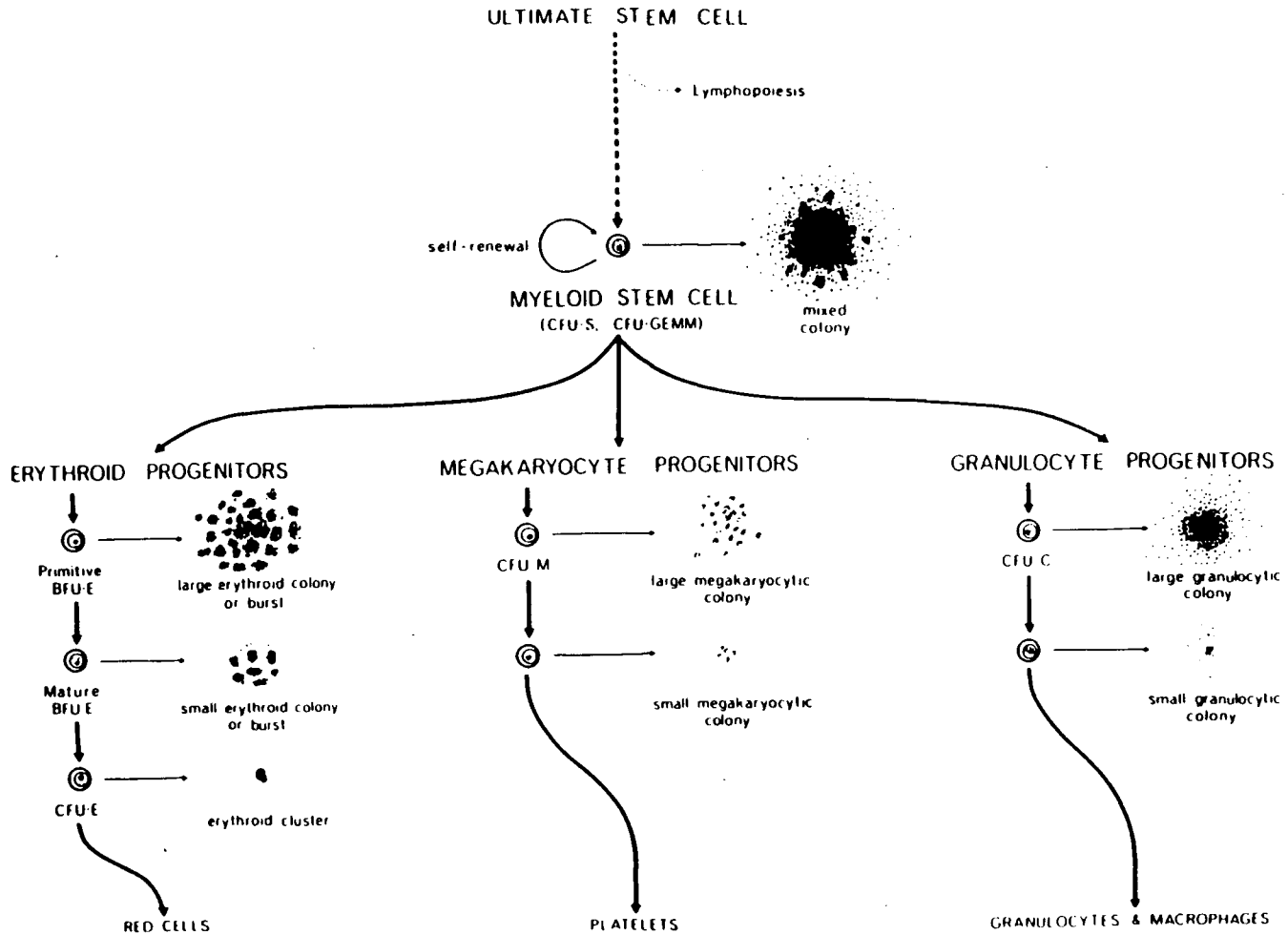


Figure 1. Diagram of Hemopoietic System as Defined by Clonogenic assays of Pluripotent and Committed Progenitors.

The concept of heterogeneity among the CFU-S, which would be supported by the possibility that restricted stem cells exist, has now been suggested by several findings including different CFU-S forming abilities of cells fractionated by velocity sedimentation (8) and sequential in vivo observation of CFU-S (9). In these latter experiments, it has been found that a 7-day CFU-S contains predominantly one cell type and did not produce CFU-S in secondary recipients, whereas the same primary recipients analysed at day-14 or later had larger spleen colonies in a location different from the early colonies which mostly had disappeared (9). This would indicate that early spleen colonies are the result of unipotent progenitors with limited proliferative capacity, whereas the late appearing colonies are the progeny of multipotent stem cells. Heterogeneity among CFU-S is also supported by recent findings that physical separation of a cell with higher self-renewal capacity than the CFU-S, i.e.,a pre-CFU-S is possible (10). Strategies for the purification and the quantification of primitive stem cells in murine systems have been extensively studied during the recent years with significant successes (11,12). It has also been possible to infect these highly purified cells with replication-defective retroviruses in order to follow their progeny after their transplantation to lethally irradiated mice (13). DNA extracted from different tissues of mice is then analysed by Southern blotting using retroviral specific probes. These experiments have shown that the same retroviral integration sites can be found in both myeloid and lymphoid tissues of a mouse, indicating their common origin from a primitive stem cell (13,14).

The repopulating ability of individual stem cell clones has been extensively debated (15). Micklem et al. first showed that a cytogenetically marked hemopoietic clone could undergo continuous expansion upon several serial transplantations (16). Serial transplantation experiments with retrovirally marked cells have subsequently confirmed that a single hemopoietic cell clone is able to reconstitute and maintain hemopoiesis for several months in both genetically defective W/W mice (13) and in lethally irradiated animals (14).

In humans, there is obviously no equivalent of the murine CFU-S assay; and clonal analyses of hemopoietic cell populations relied primarily on studies of G6PD (Glucose

6-Phosphate dehydrogenase) isoenzyme mosaicism in heterozygous females (17). Using this approach, Gandini and Gartler (18) have shown that the proportions of cells expressing each enzyme in erythrocytes, granulocytes and lymphocytes are strongly correlated, suggesting their origin from a common pluripotential stem cell. The most convincing evidence for the existence of such a pluripotent stem cell in human marrow has been obtained from the study of female G6PD heterozygotes with clonal hematologic disorders in which myeloid and lymphoid progeny belonging to the neoplastic clone has been demonstrated (19,20). However, interpretation of these studies relies on the perhaps valid, although untested assumption that the transformation event that conferred the capacity for clonal overgrowth, did not affect the differentiative capacities of the cell in which it occurred.

b- Hemopoietic progenitors

These cells are the progeny of hemopoietic stem cells.They represent a heterogeneous group which are separately defined according to different proliferative and differentiative capacities. They represent only 1-3% of hemopoietic cells and like stem cells, are not morphologically recognizable. The first such cells to be characterized are detected by methodologies introduced in 1965 by Pluznik and Sachs (21) and Bradley and Metcalf (22) who independently developed in vitro colony assays for progenitors in murine bone marrow that are committed to granulopoiesis. These granulocyte/macrophage colony forming cells are thus referred to as CFU-GM. In general, all hemopoietic colonies are obtained only in the presence of specific growth factors on which their growth depends (23) and they are categorized on the basis of their cellular content (which gives an idea about the potentiality of the original progenitor), their size and the time taken to generate mature progeny (which informs about the proliferative capacity of the original progenitor) and also their self-renewal ability by replating experiments (23). In order to be interpretable, colony assays can only be performed when the cells to be assayed can be obtained in a single cell suspension with retention of viability. These cells are then plated at adequately low cell concentrations. CFU-GM are defined

by their capacities to generate colonies containing at least 20 granulocytes and macrophages. Erythroid progenitors were first reported by Stephenson et al (24) in the mouse and later by Tepperman et al in studies of human cells (25). Three classes of erythroid progenitors have been characterized in both mouse and man by the time taken for their progeny to reach maturation and the ultimate size of the colonies they produce (26,27). CFU-E give rise to small, compact clusters of 8 to 100 hemoglobinized cells and they appear the most rapidly (within 10 days in assays of human cells). BFU-E (burst-forming-unit-erythroid) are more primitive erythroid progenitors that give rise to colonies consisting of groups of clusters which take longer to become recognizable as erythroid. Erythroid colonies consisting of 3-8 clusters are derived from " mature " BFU-E and those consisting of > 8 clusters are derived from " primitive " BFU-E). Other colony assays for progenitors committed to other differentiation lineages, e.g. for CFU-Mk (Megakaryocytes), CFU-Eosinophils and CFU-mast cells have also been described (2). If appropriate growth factors for multiple lineages are used in addition those required to stimulate primitive cells, colonies containing granulocytes, erythroid and monocytic cells, and megakaryocytes can be obtained in vitro (hence the term CFU-GEMM). More recently, a so-called blast-cell-colony which is also derived from a very primitive pluripotent cell as indicated by replating experiments has been described by Ogawa's group (28). A unique feature of these colonies is their delayed growth and their high content of clonogenic cells even after 2-3 weeks, suggesting that they are more primitive than most progenitors defined as CFU-GEMM by their ability to generate multilineage colonies within 2-3 weeks. In summary, in vitro colony assays exist for the detection of most if not all classes of committed and partially restricted progenitors in both mouse and and human tissues. Whether stem cells capable of sustaining hemopoiesis in vivo for extensive periods of time can be or are included amongst the populations currently defined by in vitro colony assays is not known and awaits further characterization and purification of repopulating stem cells.

c- Long-term marrow cultures (LTMC)

In 1977, Dexter et al described an in vitro system in which mouse bone marrow was seeded onto pre-established marrow stromal cell layers (29). The cells were diluted in a medium containing horse serum. Cultures were kept at 33°C, fed weekly by removal of half of the non-adherent supernatant and addition of an equal volume of fresh medium. These cultures allowed the establishment and maintenance of CFU-S for several weeks (30), the major site of CFU-S production being the adherent layer (31). Subsequently it was found that addition of a corticosteroid ensured the long-term maintenance of CFU-S in this system without the necessity of establishing an adherent layer beforehand (32). When the cycling status of the CFU-S in these cultures was assessed using the ^{3}H- thymidine suicide technique, it was found that within 1 day after feeding, the CFU-S had entered S-phase as defined by >35% of them being killed by a brief exposure to high specific activity tritiated thymidine. This high cycling activity of CFU-S returned to low levels (10%) within few days in these cultures, but was reinduced following the next medium change. In contrast, CFU-GM remained constantly in cycle (33). Thus, this system offers not only an approach to investigating CFU-S maintenance and differentiation but also their proliferative activity. Dependence on adherent stromal cells was demonstrated by early experiments showing the failure of hemopoiesis to be maintained in LTMC set up in siliconized glass bottles. This prevents cell attachment and thus inhibits the formation of a stromal cell layer. In addition when marrow from Sl/Sld mice (which have normal stem cells but a defective microenvironment) was used to set up LTMC, hemopoiesis was not maintained, indicating the importance of the stroma in the regulation of hemopoiesis in this system.

In 1980, Gartner and Kaplan described the equivalent of the murine LTMC system for human cells using identical culture conditions (34). An adherent layer develops during the first 3-4 weeks and in this meshwork of large confluent cells, populations of small, round cells are seen,representing areas of hemopoiesis. When the assays for clonogenic hemopoietic cells are performed weekly on both adherent and nonadherent layers, an initial rapid decline in the

number of these hemopoietic progenitors is found during the first 3 weeks, to 10% and 3% of the input values of CFU-GM and BFU-E, respectively (35). Granulopoiesis is supported in these cultures and significant numbers of granulocytic cells can be obtained during the half-medium changes for at least 8 weeks (36). In contrast, in the absence of erythropoietin, erythropoiesis is arrested at the BFU-E stage. The primitive cells which give rise to the non-adherent hemopoietic cells (mainly granulocytes and macrophages) are located in the adherent layer, as indicated by the larger size of the colonies obtained from progenitors in the adherent layer, and maintenance of non-adherent cell production despite complete removal of all non-adherent cells each week (35). Similar to the murine LTMC, the human LTMC system shows cycling changes in the turnover of primitive progenitors correlated with the weekly medium changes (37). In human LTMC, this occurs only in the adherent layer; progenitors in the non-adherent layer remain continuously in cycle, regardless of their state of differentiation or the time of assessment. The identities and functions of different positive and negative factors that mediate this phenomenon are now being delineated (38). The use of the LTMC combined with cycling assays is also affording important insights into the mechanisms of abnormal stem cell regulation in the myeloproliferative disorders as is discussed later in Section 3.

(B)- REGULATION OF HEMOPOIESIS

The hierarchical structure of the hemopoietic system requires the existence of complex mechanisms of regulation to control blood cell production at multiple levels. Each stem cell has the capacity to produce very large numbers of differentiated progeny and the system must therefore be protected in some way, from producing too many differentiated cells. In addition, the body's needs for new blood cells may be more rapid than can be achieved by activation of stem cells suggesting a role for mechanisms that may act on committed progenitors and even

later. How these mechanisms may be explained both in terms of released growth factors and cell-cell interactions is now emerging.

a- Stem cell regulation

It has been estimated that normal hemopoiesis in the adult results from the proliferative activity of many stem cell clones (39). It is also known that the majority of more primitive hemopoietic cell types are normally in a quiescent G_0 state (40). These cells can undergo active cycling under the influence of stress situations such as following irradiation (41), or cytotoxic chemotherapy (42,43), or the administration of endotoxin (44). This would indicate that in cases of increased demand, more stem cells can be recruited into a mode where they generate differentiating progeny to restore the deficiency in blood cell levels. How this is sensed by stem cells is unknown, although experimental evidence from both in vivo (45) and in vitro (37) studies suggest that local activation of stem cells, perhaps by marrow stromal cells may be important. Mechanisms that may be involved in the initiation of differentiation within a given stem cell, thus causing it to irreversibly leave the stem cell pool have been extensively debated. Several have been proposed, and experimental evidence in support of each has been obtained. In the stochastic model developed by Till et McCulloch, the decision of a stem cell to renew itself or to be committed for differentiation is described by a probability function (46). Initially based on the analysis of the distribution of CFU-S progeny in individual spleen colonies in vivo, this model was later supported by similar analyses of daughter CFU-S and CFU-GEMM in mixed colonies generated in vitro (47,48). A corollary of this model is that the particular lineage to which a pluripotent stem cell becomes restricted and the sequence of restrictive decisions it undergoes will also not be rigid and will therefore also be described by a probability function. Considerable evidence of this has been reported by the extensive analyses of progeny types in individual mixed colonies (48,49). The alternative model is that these differentiative decisions are determined by the type of extrinsic influences to which the stem cell is exposed (50). Initially deduced from experiments using histologic studies of spleen colonies in mice, this

theory failed to discriminate between restriction of differentiative potential and later expression of that potential, two processes that were later shown to be not necessarily linked (51). Nevertheless, it has become clear that the microenvironment may exert important regulatory actions on stem cell proliferation, and the possibility of modulating the probability of self-renewal or differentiation has also some valid in vitro support (discussed next).

b- Micro-environmental regulation

Early experiments including those involving transplantation of bone particles under the kidney capsule (52) or under the skin (53) of syngeneic animals demonstrated that cells of recipient origin could colonize these bone particles and initiate hemopoiesis within them. Transplantation experiments using Sl/Sl^d animals as recipients revealed their reduced ability to support the growth of normal or even Sl/Sl^d marrow grafts, clearly implying a major role for a stromal-mediated regulation (54). As described above, this defect could also be reproduced in LTMC: Adherent layers prepared from Sl/Sl^d mice did not support the maintenance of CFU-GM, whereas adherent layers derived from W/W^v mice (which have a stem cell defect but normal stromal function) maintained CFU-GM production by added Sl/Sl^d marrow cells (55). LTMC thus represents an excellent system to study interactions between different stromal cell components and hemopoietic cells. The use of the LTMC system has shown that bone marrow mesenchymal cells, which are part of the LTMC adherent layer, can produce hemopoietic growth factors in culture (56). In addition to the production of regulatory molecules, there is evidence indicating that contact of primitive hemopoietic cells with particular components of the extracellular matrix (ECM) also produced by stromal cells may be an important part of how stromal cells regulate hemopoiesis. Adhesive properties of ECM proteins may change the shape of stem cells and modulate their responsiveness to growth factors (57). Another mechanism of regulation has been recently proposed by Gordon et al (58). They have shown that Granulocyte-Macrophage Colony Stimulating Factor(GM-CSF) can bind to glycosaminoglycans synthesized by marrow stroma cells and hence concentrate this molecule in the vicinity of

nearby hemopoietic cells. Some components of ECM could have a specific role for attachment of specific types of cells, such as hemonectin, which appears to be an important attachment protein for cells of the granulocytic lineage (59), or fibronectin for B-cell progenitors (60).

c- Growth factors

In vitro colony assay systems have been a major experimental tool for the discovery and development of a number of hemopoietic growth factors (HGF's). Thus, because of their initial identification as colony-stimulating factors (CSFs), they were named accordingly, e.g. GM-CSF, M-CSF (CSF-1), G-CSF and multi-CSF or Interleukin-3 (IL-3). HGFs appear to be essential for the survival, growth and differentiation of hemopoietic progenitors in vitro. Their exact role in vivo, under normal physiological conditions is unknown.During recent years, all 4 of these growth factors as well as others with CSF activity have been characterized in both murine and human systems, their cDNAs cloned and the recombinant product synthesized (61). Table 1 summarizes the names, some of the sources and spectrum of actions of these molecules (62). The availability of pure GM-CSF from recombinant sources has allowed its multi-lineage CSF activity to be discovered (63). G-CSF, M-CSF and erythropoietin appear to be lineage restricted in their action as CSFs; as opposed to IL-3 which is the major multi-lineage CSF (61). All of the known HGFs are glycoproteins and are active in stimulating cell proliferations at very low concentrations. There appear to be unique membrane receptors for each CSF (64). Several examples of CSF synergisms among various HGFs have also been reported (65-67).

Inhibitory factors seem also to be involved in the regulation of hemopoiesis. The exact nature of many inhibitory "activities" has not been elucidated. However, recently it has been shown that TGF-β (38,68), TNF-α and β (69) and some interferons (70) may inhibit hemopoietic cell proliferation after binding to specific cell surface receptors.

Table 1. Hemopoietic Growth Factors (CSFs and Interleukins)

Name	Abbreviations	Other Common Names	Major Hemopoietic Lineage Stimulated
Erythropoietin	Epo		Erythroid
Macrophage colony-stimulating factor	M-CSF, CSF-1		Monocyte/macrophage
Granulocyte colony-stimulating factor	G-CSF, CSF-α		Neutrophil
Granulocyte-macrophage colony-stimulating factor	GM-CSF, CSF-β	Pluripoietin	Neutrophil/macrophage
Interleukin-1 alpha	IL-1 α	Hemopoietin-1 (H-1)	Co-stimulator of early cells, T cells
Interleukin-1 beta	IL-1 β	Lymphocyte activating factor	Co-stimulator of early cells, T cells
Interleukin-2	IL-2	T cell growth factor	T cells
Interleukin-3	IL-3	Multi-lineage colony stimulating factor (multi-CSF), persisting cell factor, hemopoietin-2 (H-2), hemopoietic cell growth factor (HCGF), mast cell growth factor (MCGF)	Most myeloid lineages
Interleukin-4	IL-4	B cell stimulating factor-1 (BCSF-1), B cell differentiation factor	B cells, T cells
Interleukin-5	IL-5	T cell replacing factor, B cell growth factor-2 (BCGF-2), B cell differentiation factor	eosinophil differentiation
Interleukin-6	IL-6	B cell stimulating factor-2 (BCSF-2), hybridoma growth factor, plasmacytoma growth factor	B cells, co-stimulator of early cells
Interleukin-7	IL-7		Pre-B cells, T cells
Interleukin-8	IL-8	Monocyte-derived neutrophil chemotactic factor (MDNCF), monocyte-derived neutrophil-activating peptide (MONAP), NAP-1	Neutrophils, T cells
Interleukin-9	IL-9		Early erythroid

(C)- HEMOPOIETIC REGENERATION AFTER INTENSIVE THERAPY

a- Background

Considered as an experimental therapeutic modality as recently as 25 years ago, bone marrow transplantation (BMT) is now a well-established treatment procedure for a number of hematologic as well as non-hematologic disorders. The rationale for giving a marrow transplant is to allow hemopoietic recovery following the administration of treatments that eradicate the host's hemopoietic system.The purpose and hence the design of the conditioning treatment is essentially to kill more malignant cells than it would otherwise be possible, or to allow correction of an abnormal hemopoietic condition (e.g. thalassemia). Since most BMT involves the use of allogeneic marrow, the conditioning regimen must also be sufficiently immunosuppressive to the host to prevent graft rejection (host-versus-graft disease). Despite the matching of donor and recipient HLA antigens, an immunologic reaction of the graft towards the tissues of the host (graft-versus-host disease, GVHD) occurs in approximately 50% of cases and represent an important cause of morbidity and mortality in recipients of allogeneic marrow (71). Since the identification of the donor immunocompetent cells as the cause of GVHD, T-cell depletion of grafts prior to infusion has been performed (72). Although successful in reducing the risk of GVHD, this method has been found to be associated with an increased risk of graft failure, either due to the failure of initial engraftment or initial engraftment followed later by graft failure (73). An increased risk of relapse in T-cell depleted transplants has also been reported in a randomized trial (74), suggesting that the presence of T-cells in the graft may exert an anti-leukemic effect (graft-versus-leukemia, GVL). More selective T-cell depletion procedures to allow infusion of only those cells with a GVL effect may be an option, but clinical applications will probably have to await proper characterization of the cells responsible for this effect before the value of such developments can be assessed.

Autologous BMT refers to the use of the patient's own marrow as the cells to be transplanted. The advantage of this is the fact that it circumvents the problem of finding a suitable donor and is not associated with any major immunologic conflicts seen in allogeneic transplants. In hematological malignancies, especially in leukemias, but possibly in other cancers also, autologous BMT has the potential disadvantage that the marrow graft may be contaminated by neoplastic cells. To circumvent this, several ex-vivo marrow purging procedures have now been developed (75,76).

b- Hemopoiesis post-BMT : Studies in experimental animals

The classic experiments of Ford et al (77) and of Vos et al (78) have clearly demonstrated that hemopoiesis after BMT in heavily treated recipients is derived at least initially, from donor stem cells. The lympho-myeloid potentiality of repopulating cells has also been demonstrated (79). Several critical factors have been identified as determinants of a successful outcome. These include the number of cells infused, the origin of the graft (bone marrow, spleen cells or blood cells), and T-cell depletion prior to BMT (73). Because of the scarcity of the stem cells in the hemopoietic tissues, there is a direct relationship between the number of cells transplanted if limiting numbers are injected and chances of engraftment. In mice, 10^5 nucleated marrow cells, (which correspond approximately to 35 CFU-S) is considered as a requirement. Grafts derived from spleen cells protect irradiated hosts less well than bone marrow cells do, suggesting that there may also be qualitative differences between repopulating cells obtained from different sites (80).

For equivalent numbers of CFU-GM infused, dogs grafted with autologous peripheral blood show a significantly early regeneration of hemopoietic cells as compared to animals autografted with marrow (81). However, reasons for this difference might be related to the possible stimulatory role of lymphocytes infused along with the blood derived-grafts. Also it should be noted that this rapid engraftment can be followed by a rapid failure (81). In the allogeneic setting, T-depletion procedures may reduce the number of repopulating cells

transplanted, leading to graft failure (81). One mechanism of graft failure after T-cell depletion, might be related to absence of donor-T-cell derived hemopoietic growth factors in these grafts. Recently, T-cell depleted bone marrow transplants from histoincompatible donor mice have been shown to induce highly successful engraftments, if the bone marrow is incubated prior to infusion, with recombinant G-CSF (82).

Another mechanism of T-cell-depletion-induced graft failure might be the abolition of the GVL effect, allowing suppression of donor hemopoiesis by residual leukemic cells (72). It is known that leukemic cells secrete inhibitory molecules such as lactoferritins and isoferritins which may inhibit donor-derived hemopoietic cells after BMT (83). Engraftment also depends obviously on the degree of MHC matching between donor and recipient, as illustrated by the increased numbers of bone marrow cells needed to transplant H-2 incompatible mice (84). Finally, in addition to these factors, the presence in the host, of a healthy microenvironment able to support donor hemopoiesis (73) and the level of receptor expression for ECM proteins on the infused cells probably influence engraftment.

Analysis of the dynamics of hemopoietic reconstitution and the number of clones contributing to it, have been extensively studied in mice. Serial transplantation experiments have identified an early phase of engraftment, which appears to be the result of a progenitor with finite proliferative potential, followed by a second phase, thought to be the result of a stem cell with the capacity to sustain blood cell production for many months but which may be "run down" by serial transplantation performed at short intervals (85). The loss of grafting ability is not the result of a permanent loss of the proliferative capacity of the stem cell, as suggested by the fact that with increasing intervals between transfers, long-term repopulating ability of the graft can be restored (86). Although these experiments might suggest that small numbers of hemopoietic stem cell clones can also not sustain long-term engraftment, studies using the W/W mice as recipients and PGK isoenzymes as markers, have shown that a single hemopoietic stem cell can produce all of the red blood cells required for at least 8 weeks (87). Similar results were obtained when granulocyte and lymphocytes were evaluated (87). The

more recent use of retrovirally marked cells have shown that a single hemopoietic cell clone can reconstitute most if not all of the hemopoietic cells in a mouse for several months (13). In many cases however, hemopoiesis seems to be maintained by the successive activity of multiple, long-lived clones (14,88). These findings support the hypothesis that mouse bone marrow contains a very primitive stem cell with very extensive proliferative capacity, although the mechanisms that determine the number of these that are activated at any given time and their ability to maintain themselves remain unknown.

c- Hemopoiesis post-BMT in humans

In contrast to animal studies, the reconstitution of hemopoiesis in humans after BMT has less well been studied. During the second week post-BMT, reticulocytes and immature myeloid cells can be identified in the peripheral blood. A good correlation between the number of CFU-GM, BFU-E, CFU-E infused and the time to hemopoietic recovery is found essentially for smaller grafts where the number of repopulating cells has presumably been limiting (89). For unknown reasons, platelet counts almost always normalize last (after 1-3 months), although a moderate thrombocytopenia persists in a significant proportion of patients.

Abnormalities in the normalization of several hemopoietic progenitor cell compartments have been documented after transplantation. Gale et al (90) and Li et al (91) reported significantly diminished CFU-GM concentrations in the bone marrow following BMT in patients with aplastic anemia and acute leukemia. In some patients these persisted for up to 8 years after transplantation. In the series of patients reported by Arnold et al (89), an incomplete recovery of CFU-GM, BFU-E and CFU-E concentrations occurred in 7 cases, persisting for up to 3 years post-BMT in the presence of normal peripheral cell counts. Interestingly, a large majority of the marrow CFU-GEMM have been found to be in active cycle post-BMT even after normalization of the blood cell counts (92) suggesting a permanent BMT-induced abnormality in engrafted stem cell regulation that might be compensated for by later acting mechanisms. Abnormalities of immunocompetent cells after transplantation have been extensively studied

(93). Decreased CD4 lymphocyte numbers in the presence of increased CD8 numbers (94), depressed MLC reactivity (95), loss of delayed hypersensitivity reactions (94) and decreased NK cell activity (94) are commonly found. Humoral immunity is also significantly depressed for many months, but afterwards, most patients acquire adequate circulating immunoglobulin levels and normal peripheral blood B-cell numbers in the absence of GVHD (94). In patients with chronic GVHD, both cellular and humoral immunity often remain impaired, which could explain the persisting high susceptibility of these patients to infections (94).

d- Origin of cells post-BMT

After allogeneic or syngeneic BMT, hemopoietic cells are usually of donor origin (96,97) although partial chimerism or complete reversion to host-derived hemopoiesis can also be encountered (98,99). Sometimes different cell lineages appear to be contributed by donor and host-cells. The most typical example of this is due to the ability of some host T-cells to survive the conditioning therapy (100) and then to contribute to T-cell mediated immunity after transplantation (100). Several methods have been used to detect differences between donor and recipient cells, including immunoglobulin allotypes (101), red cell antigens (102), cytogenetic analysis (98,103) and more recently, recombinant DNA techniques to detect sex-linked differences or DNA restriction fragment length polymorphisms (RFLP's) (104). Although RFLP analysis can not be used to analyse red cells and platelets, its power resides in the extreme polymorphism of certain DNA sequences in the human genome. The use of appropriate probes for these sequences allows a distinction to be made between sibling cells with a certainty close to 100% (104-106). When a cytogenetic marker is available, it may complement the RFLP analysis, although cytogenetic studies can clearly only be used to analyze dividing cells or cells that can be stimulated to divide. The combined use of these methods have established that partial chimerism after transplantation is a common event (103,105) and is more frequently observed in recipients of T-cell depleted grafts (107,108) although it does not seem to be correlated with an increased relapse rate (109). In several cases, RFLP analysis combined with

cytogenetic markers, has been instrumental in detecting preclinical relapses (105,110) and recipient-derived hemopoietic recoveries (99) in children receiving allogeneic transplants for non-malignant constitutional diseases.

e- Clonal remissions in AML patients

In the majority of patients with AML, karyotypic and G6PD data indicate that chemotherapy-induced remissions are accompanied by non-clonal hemopoiesis (111,112). However, in 1981, Fialkow et al described a cytogenetically normal but clonal remission in a patient with AML (112). Subsequently, several other cases of clonal hemopoiesis persisting during remission have been reported (113-115). In the later series of Fialkow et al, this phenomenon was documented in 5 cases, with no evidence of clustering to a particular age group, and occurred in patients with both multipotential and lineage-restricted disease (115). Five additional cases have been documented using RFLP and methylation analysis in two different centres (114,116), bringing the total number of clonal remissions to 26 % of studied cases. This percentage can, however be an underestimation because of the relative insensitivity of the used techniques and the small number of patients analysable by this technology. The true frequency of clonal remissions is unknown at the present time. Whether clonal remissions have a prognostic significance is also unknown but in the small number of patients studied to date, no difference in survival parameters has been found to be emerging. The significance of clonal remissions is controversial. The generally accepted view is that the clonal remission in these patients is related to the presence of a preleukemic state, supporting the hypothesis of multistep pathogenesis in AML (115). The disappearance, during the clonal remission, of the complex cytogenetic abnormalities present in the blast cells at presentation in a case reported by Jacobson et al (113) is in favor of this view. The fact that in all clonal remission patients studied to date, the remission clone had the same phenotype as the original leukemia cells (113,115) also supports this hypothesis. However, it can not necessarily be assumed that normal stem cell do not have sufficient proliferative capacity to dominate the human

hemopoiesis with their progeny. In cats, the possibility of clonal overgrowth originating from normal stem cells, during recovery from chemotherapy-induced aplasia has been demonstrated (117). Recently, oligoclonal hemopoietic recovery from presumably normal stem cells has also been demonstrated in two patients with breast cancer after myeloablative chemotherapy (118). Clearly, more studies are needed for a better understanding of this phenomenon, which may then allow direction of additional therapies for patients in clonal remissions.

2) CLONALITY MARKERS

(A) CHROMOSOMAL ABNORMALITIES

Theoretically, any stable chromosomal abnormality could be used as a marker of the clonal progeny of the cell in which the abnormality first appeared. Chromosomal markers induced by radiation in mice were in fact extensively used for this purpose in the hemopoietic system (4). The possibility of detecting a chromosomal abnormality in a cell population, depends directly on the size of the clone and the number of metaphases available for cytogenetic analysis. Since the identification of the Philadelphia chromosome (119), which is an exquisitely specific chromosomal marker for CML, improved cytogenetic technology has allowed the characterization of many other specific chromosomal abnormalities in different human cancers. In fact, the data base catalog for cancer cytogenetics in 1988 contains 9069 cases of human cancer reported to be associated with a cytogenetic abnormality (120). However, in the majority of the cases (86%), the abnormalities reported are for hemopoietic cancers, which reflects current difficulties in obtaining chromosome preparations from solid tumors. In addition, the majority of the abnormalities reported are not consistent and they may represent nonspecific cytogenetic "noise", not related to the underlying malignancy. The significance of

even consistent chromosomal abnormalities still have to be interpreted cautiously, for they may not be directly related to events associated with the initiation of neoplastic proliferation. Nevertheless their detection can indicate the presence of a neoplastic clone, which can be followed by cytogenetic studies. However, molecular consequences of chromosomal abnormalities are being increasingly well explored and activation , disturbance or "rearrangement" of functionally important domains of the genome are thus being identified, indicating the crucial role that certain changes may play in the pathogenesis of neoplasia. Table 2 summarizes major specific chromosomal abnormalities found to be associated with hematological malignancies (120,121). In addition to the chromosomal abnormalities identified during the course of a disease, constitutional chromosomal mosaicisms such as 47,XXY/46,XY or 46,XX/45,X0 can be used as clonal markers. In such an individual with a malignant disease, the presence of one of the mosaic components in all tumor cells indicates a clonal development from a single cell.

TABLE 2

COMMON CHROMOSOMAL CHANGES IN HEMOPOIETIC NEOPLASIAS

TYPE	NUMERICAL ABNORMALITIES	REARRANGEMENTS
CML		
Chronic phase		t(9;22)(q34.q11)
Blast crisis	+8, +Ph^1, -7 (Rare)	t(9;22),i(17q)
ANLL		
AML (M2)	+8, -7, -5	t(8.21)(q22;q22)
APL (M3)		t(15;17)(q22;q11-12)
AMMoL (M4)	+8 -5	inv(16)(p13q22)
	+22	t(16;16).del(16q)
AMoL (M5)		t(9;11)(p22;q23)
		t(11q).del(11q)
M2/M4		t(6;9)p23;q34)
M4		t(3;3)(q21;q26).
		inv(3)
MYELODYSPLASIA	-7, +8, -5	del/t(11q)
		del/t(12p)
		5q-,7q-7p-
CLL		
B-cell	+12	14q+(q32)
T-cell		t(8;14)(q24.q11)
		inv(14)(q11q32)
ALL		
Early B-precursor		t(4;11)(q21;q23)
Common	+21, +6 Rare	t(9;22).del(6q)(q15-q21),near haploid
pre-B		t(1;19)(q23.p13)
B-cell		t(8;14)(q24;q32)
		t(2;8)(p12;q24)
		t(8;22)(q24;q11)
BURKITT		t(2;8)(p12;q24)
		t(8:14)(q24;q32)
		t(8;22)(q24;q11)
NON-HODGKINS LYMPHOMA	+3	t(11;14)(q13;q32)
		t(14;18)(q32;q21)
		t/del(6p)
		t/del 6q
		structural changes 1,3

(B) GENE REARRANGEMENTS

DNA analysis using Southern blotting (122) allowed major advances to be made in our understanding of the molecular changes occurring in human genome during differentiation and neoplasia. Some of these changes occur naturally in specific cell lineages, such as rearrangement of the Ig heavy (H) and light (L) chain gene loci and T-cell receptor (TCR) gene loci in the B- and T- cell lineages, respectively. Other changes may occur as a result of pathological processes in specific diseases. In both cases, but for different reasons, clonal cell populations can be detected using specific gene probes.

a- Rearrangement of Ig gene and TCR gene loci.

The chromosomal organization and DNA sequence of the Ig H and L chain genes are now known in great detail. In humans, sequences encoding the Ig H chain are located on chromosome 14 whereas those for κ and λ reside in 2 and 22 respectively. In 1976 Hozumi and Tonegawa demonstrated that in embryonic cells and in non-B cells these genes are organized in a discontinuous fashion and that their rearrangement is required to create functional Ig genes (123). This rearrangement occurs during the early B-cell development so that all daughter B-cells derived from a given pre-B cell end up synthesizing the same unique Ig molecule. During this process, IgH genes are first rearranged, followed by κ and λ chain genes. A similar rearrangement of the TCR gene loci involving TCR-β (chromosome 7), TCR-α (chromosome 14), TCR-γ (chromosome 7) and TCR-δ (chromosome 14) genes occurs during T-cell ontogeny in order to create a functional TCR (124-127). Functional and ontogenetic relationships between T-cells bearing α/β receptors and those bearing γ/δ receptors are not well known at the present time (128).

In both Ig and TCR gene rearrangements, the assembly of functional genes alters the germline configuration of DNA and these changes can be detected by restriction enzyme analysis of DNA and Southern blotting. In polyclonal cell populations, each individual B- or T-cell is expected to rearrange its DNA in a different manner, leading to a large number of individual rearrangements beyond the limit of separate discrimination by Southern analyses and therefore all that is seen is a smear. However, in the presence of a monoclonal B or T-cell expansion, all cells will have the same Ig or TCR configuration and this can be seen as a unique rearrangement pattern specific for this cell population. The concomitant comparison of non-lymphoid DNA allows identification of a germline hybridization band for a given restriction enzyme. This technique has allowed major advances to be made in our understanding of lymphoproliferative disorders and lymphoid cell ontogeny. In a given cell population, 1-2% of cells with clonal rearrangements can be detected, against a background of polyclonally rearranged or non-rearranged cells. This method can thus be extremely useful for detecting minimal residual disease in ALL (129), and several types of chronic lymphoproliferative disorders (130). The recent introduction of PCR technology with direct sequencing of TCR-δ V-J junction sequences increases considerably the power of this method (131). However, detection of a clonal T or B- cell lymphoproliferations is not always synonymous of malignancy as benign clonal proliferations for both lineages can occur (132,133).

b- Other clonal rearrangements

Some chromosomal translocations that involve breakpoints within or in vicinity of functionally important regions of the genome can be detected by molecular methods if the appropriate gene probes are available. The first and the best characterized example of this is the DNA rearrangement that results from the 9;22 translocation giving rise to the Philadelphia (Ph^1) chromosome. The **bcr-abl** rearrangement resulting from this translocation allows detection of clonal cell populations in virtually all patients with Ph^1-positive CML (134) and approximately 25% of patients with Ph^1- negative CML (135,136). (This method will be described in detail in a later section on CML.) Two other examples of structural chromosomal

changes leading to molecularly detectable breakpoints are those involving bcl-1 and bcl-2. The t(11;14) translocation that occurs in patients with small lymphocytic lymphomas and CLL, create a breakpoint in the IgH locus, leading to the juxtaposition of a putative oncogene from the 11q13 (**bcl-1** rearrangement) with 14q32 sequences (137). Similarly, in follicular lymphomas with the 14;18 translocation, one break occurs in the 14q32 , within the IgH locus, whereas the second breakpoint occurs in two different clusters on chromosome 18; the major breakpoint within the second exon (138) and the minor breakpoint approximately 20 kb downstream of this exon (139). As a result of this translocation, 14q+ sequences are fused to chromosome 18 sequences (**bcl-2** rearrangement). Using specific probes, a bcl-2 rearrangement can be demonstrated in a majority of patients with follicular lymphomas (140). Recently it was reported that clonal lymphoma cells could be demonstrated in the blood of patients with follicular lymphoma in remission, using the polymerase chain reaction (PCR) to detect rearranged bcl-2 sequences (141,142).

(C) METHODS BASED ON DETECTION OF INACTIVATED X-CHROMOSOME LINKED GENES

These methods exploit a natural mosaic system that occurs in all females and therefore offers a more general approach than other methods for clonal analysis, where it is not possible to establish whether the unique molecular (or cytogenetic) change being monitored was the first or a later event in the evolution of the neoplasm.

a- X-Chromosome inactivation

In mammalian cells, the equivalence of dosage of expression of X-linked genes between females and males is achieved by inactivation of one X-chromosome in females at an early stage of embryogenesis (143). The initiation of inactivation does not start at the same time in all cells in the embryo. The earliest cell types to undergo inactivation of one X-chromosome are

those in the trophectoderm and the primitive endoderm, at 3.5 and 4.5 days of gestation, respectively (144). In both of these lineages, there is a preferential inactivation of the X-chromosome of paternal origin, Xpat (145). The reason for the non-randomness of this phenomenon is not known, but an autosomal "imprinting", influencing the choice of the Xpat has been suggested (146). At 5.5 to 6.5 days of gestation, at which time the primitive ectoderm (from which most tissues of the embryo are later derived) has developed, one X-chromosome in each ectodermal cell is randomly inactivated (147). The inactive X then becomes late-replicating (148). Thereafter, the inactive Xpat or the inactive Xmat is transmitted faithfully to the progeny of each ectodermal cell throughout all the somatic cell divisions that subsequently take place in this female (Figure 2). All females are therefore natural mosaics in their somatic tissues; and approximately 50% of the cells in any particular tissue, will have an inactive Xpat; with the remainder of their cells carrying an inactive Xmat (maternally derived X-chromosome). However, because of the randomness of the initial inactivation process, the ratio of cells with Xpat vs Xmat inactivated in a population of females fits a normal distribution. The majority of females will exhibit a ratio close to 50:50, although the extremes of 10:90 are also found, albeit very rarely (149). Under physiological conditions, reactivation of the inactive X is known to occur only in germ cells prior to meiosis (150). However recently, an X-linked gene, ornithine carbamyl transferase (OCT) has been shown to be reactivated with increasing age in mice (151). The significance of this latter phenomenon is not known. Several genes reside on the X-chromosome and they have been extensively used to characterize the X-chromosome inactivation. Such genes include G6PD, HPRT, PGK and OCT. The exact mechanisms of the signal(s) which initiate, spread and maintain the X-chromosome inactivation are unknown, but DNA methylation clearly has some role, as is discussed below.

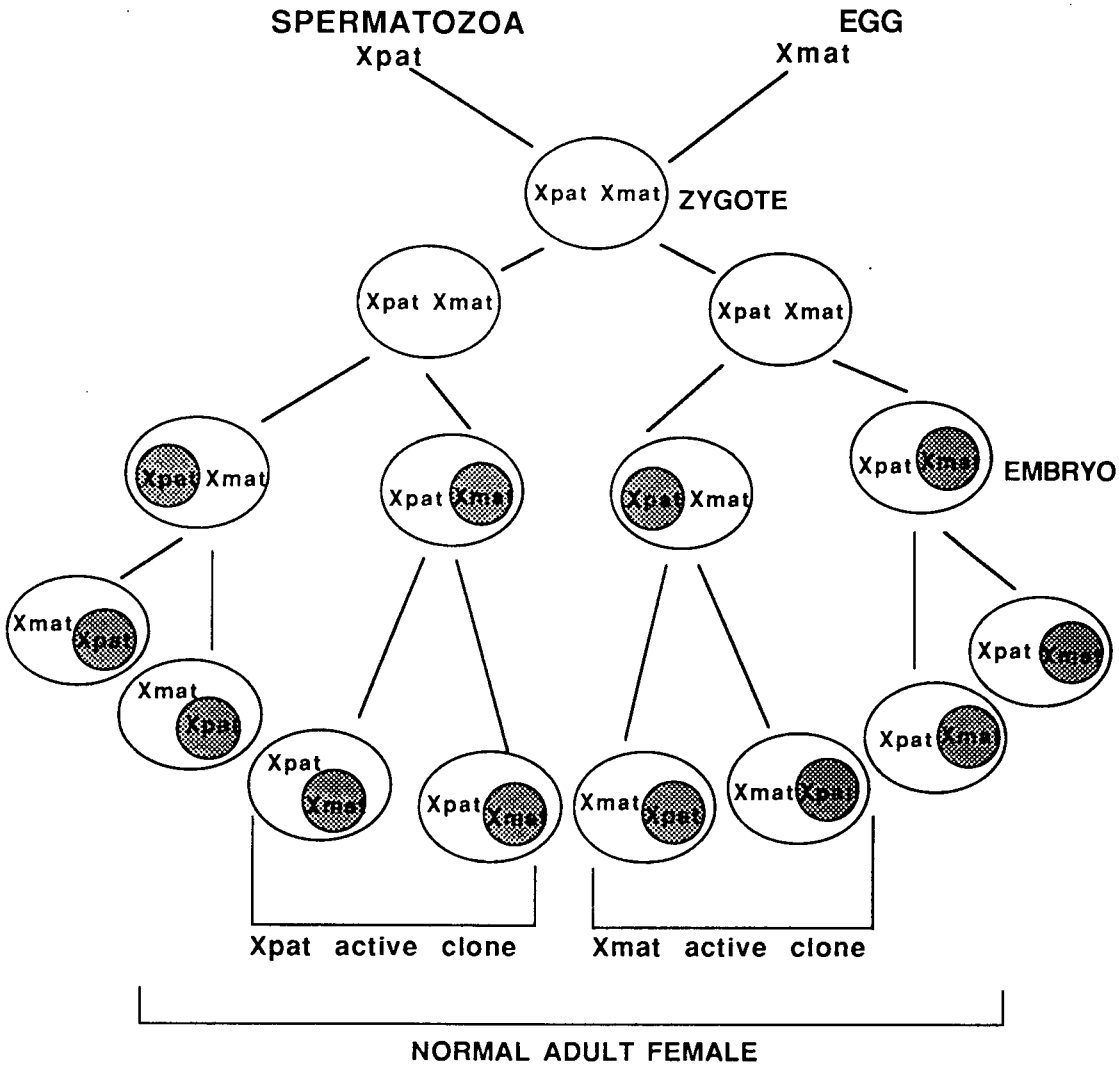


Figure 2. Schematic Representation of X-Chromosome Inactivation Mosaicism in Females. The inactivation occurs randomly in cells of the primitive ectoderm, resulting in the inactivation of an X-chromosome of maternal (Xmat) or paternal (Xpat) origin.

b- DNA methylation and gene activity

In mammalian cells, DNA undergoes a post-synthetic modification by methylation of cytosine residues, giving rise to methyl-cytosine, mCyt (152). Methylcytosine represents 2.5% of the total cytosine residues. This change occurs under the enzymatic control of methyltransferases which transfer a methyl group to C5 of the cytosine (152). Quantitation of total mCyt in DNA can be achieved by biochemical methods such as chromatography, mass spectrophotometry and HPLC; as well as by immunological methods using specific anti-mCyt monoclonal antibodies (152). Restriction endonucleases (RE) which recognize specific methylated sequences in the DNA can also be used to study DNA methylation. The majority of the mCyt in mammalian cells are in the CpG dinucleotide sequence which is recognized by several RE. These enzymes in general do not cleave a DNA sequence containing the CpG motif if the internal cytosine is methylated. For example HpaII, which recognizes the sequence CCGG does not cleave this sequence if the internal cytosine is methylated, whereas methylation of the first cytosine residue in this sequence does not interfere with its action (153). Msp I recognizes the same sequence as Hpa II, but cuts DNA regardless of the methylation status of the internal CpG sequence, whereas methylation of external cytosine residue renders Msp I inactive (153) (Figure 3). Thus using both Hpa II and Msp I digestion, it is possible to study tissue-specific gene methylation patterns by comparing test samples with DNA from a different "control" tissue (154). Other useful methylation sensitive RE are Hha I (GCGC), Xho I (CTCGAG), Sal I (GTCGAC), and Sma I (CCCGGG).

Several studies have shown that, the symmetry of DNA methylation is maintained after DNA replication by virtue of a maintenance methylase (155) which does not methylate previously non-methylated sequences (156). Methylated gene transfer experiments in mice (157) and methylation analysis of cells from human pedigrees (158) indicate that methylation patterns are stably inherited.

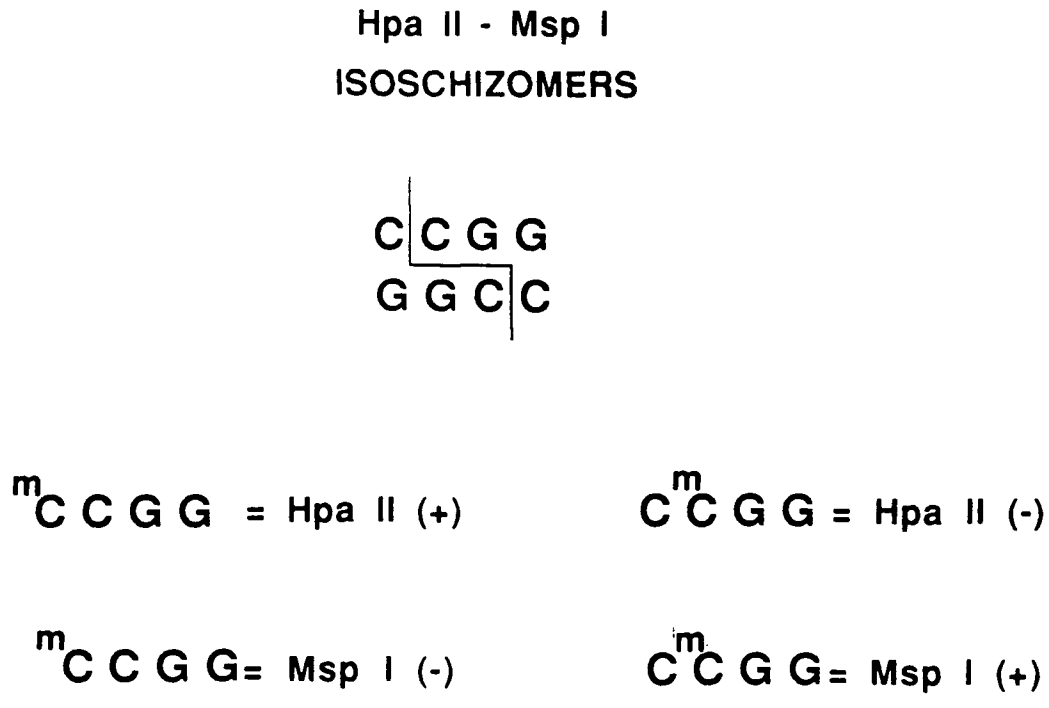


Figure 3. Methylation-sensitive restriction enzymes Hpa II and Msp I and their ability to cleave DNA as a function of the methylation status of the internal cytosine at the CCGG sequence. m= methyl group.

DNA methylation has been found to decrease in cultured normal mice, hamster and human fibroblasts, but not on immortal mouse cell lines (159). A random "clonal drift" phenomenon has also been documented in human lymphoblast cultures initiated with cells from G6PD heterozygous females (160). Similarly, instability of methylation patterns at the γ-globin locus has been demonstrated after serial passages of human fibroblasts (161).

The widespread occurrence of DNA methylation in mammalian genomes and the distribution of unmethylated regions (designated as Hpa II tiny fragments or HTF islands) in the promoter domains of many genes suggest an important function for methylation in regulating gene activity (162). Specific examples of a regulatory role of gene methylation have been described for many gene systems. In 1979 two groups independently reported a positive correlation between hypomethylation of chicken β-globin (163) and ovalbumin (164) genes and their transcriptional activity. This has also been confirmed for the human β-globin gene (165). The use of 5-azacytidine,(which can be incorporated into DNA in place of cytosine but can not be methylated) in a mouse 10T1/2 cell line has demonstrated that this cell line can be induced to differentiate to adipocytes, muscle and chondrocytes in the presence of this hypomethylating agent (166). This effect was apparent even after a short exposure to the drug, suggesting a clonally inheritable change. The role of DNA methylation is now under extensive investigation in transcriptional processes (167), in the formation of active chromatin (168), genomic imprinting (158,169) and neoplasia (170). A good correlation between hypomethylation and gene activity has been generally found but is not always the rule (152,171).

The role of gene methylation in different phases of X-chromosome inactivation and reactivation remains unclear. Most in vitro studies of the HPRT gene, showed clearly that methylation interferes with the activity of this gene : 1) Studies using hybrid somatic cell lines have demonstrated a differential methylation pattern of HPRT genes on active and inactive X-chromosome (172,173). 2) The HPRT gene on the active X-chromosome, but not on the inactive X, can transmit the HPRT+ phenotype to HPRT- recipient cells (174). 3) The potent methylation

inhibitor, 5-azacytidine, can reactivate HPRT and other genes on the inactive human X-chromosome that is contained in certain somatic cell hybrids (173). 4) After 5-azacytidine-induced reactivation, previously incompetent HPRT genes acquire their ability to function in a gene transfer assay (175). Although clearly significant, these in vitro data do not demonstrate a clear-cut in vivo role of methylation in the initiation of X-chromosome inactivation. In fact, recent data indicate that methylation of the HPRT gene on the inactive X occurs after the inactivation process indicating that methylation may be an important, but not the primary event (176). Nevertheless, for the several X-linked genes that exhibit differentially methylated cytosines on the active and inactive X-chromosome, this methylation pattern seems to be faithfully transmitted from one cell to its progeny in vivo.

c- G6PD analysis of clonality

The gene for G6PD is located on the long arm of the X-chromosome and its 3' region (as opposed to the 5' region of HPRT or PGK) is differentially methylated in active and inactive X-chromosomes (177). The G6PD gene possesses more than 200 allelic forms and several variant protein products have been reported (178). This protein is a key enzyme for NADPH production and hematological diseases related to its deficiency are well known (178). It occurs in two major isoenzyme forms, designated as G6PD A and B. In females heterozygous at this locus, each adult somatic cell synthesizes either type A or type B G6PD (but not both) according to the origin of the X-chromosome which has remained active in this cell: Type A and B isoenzymes can be easily distinguished by electrophoresis because of their different electrophoretic mobilities (179). The A- variant that has been found in a minority of females has the same electrophoretic mobility as A. In cell populations originating from more than one cell, according to the binomial equation, the likelihood of finding the same enzyme will decrease as the number of founder cells increases. Thus in a G6PD heterozygous A/B female, both types of products, A and B will be found in normal tissue samples, indicative of their polyclonal origin. In contrast, a tumor arising from a single cell, will exhibit a single enzyme phenotype,

consistent with its monoclonal origin. This method was first used by Linder and Garter in 1965, to study the clonality of uterine leiomyomas (180). It has been extremely useful in establishing the single cell origin of many solid tumors (181). It has also been widely used in studying normal hemopoiesis (18) and hematological malignancies (182) to determine the number of stem cells from which such cell populations have arisen. G6PD analysis has also been instrumental in demonstrating clonal origin of certain human atherosclerotic plaque lesions (183,184). The sensitivity of the assay is approximately 5% and results are compared with the A/B enzyme ratio obtained from normal control tissue taken from the patient. Several potential pitfalls must be considered in interpreting G6PD data. For example, a double enzyme pattern will be seen if the monoclonal sample is contaminated with stromal or other non-clonal cells. In females with an unequal Xpat/Xmat inactivation ratio, a false interpretation of monoclonality or clonal overgrowth may be ruled out by demonstration of the same skewed pattern in all somatic tissues (39). Approximately 1 in 200 females will have a pronounced skewing of the A/B ratio (149). The presence of a monoclonal pattern could also theoretically be due to the neoplastic overgrowth of more than one cell with the same inactive X-chromosome, but binomial distribution analysis can be used to determine the statistical limits of this possibility. Another possible explanation of a single enzyme pattern, more difficult to demonstrate, is a post-inactivation selection phenomenon, analogous to a situation found in HPRT gene. In female carriers of a mutant form of HPRT, erythroid cells have been found to contain normal levels of HPRT enzyme (as opposed to the expected 50% level typically found in their skin fibroblasts) and in one such family also heterozygote for G6PD polymorphism, erythrocytes and granulocytes were found to contain a single G6PD B isoenzyme (185). Thus, it is thought that in such females, the mutant allele is present in skin fibroblasts and other tissues along with the normal allele, whereas only the normal allele is found in their hemopoietic cells due to the selection of this allele during hemopoietic development (185). The existence of an X-linked gene, other than G6PD or HPRT, resulting in such a selection phenomenon has also been suggested (186). The equivalent of this selection process could

explain the apparent monoclonality pattern of blood granulocytes in 2 of 241 G6PD heterozygous females studied by Fialkow et al (39) in the presence of a double enzyme phenotype of their fibroblasts. One other potential problem is related to the fact that not all cells have been found to contain the same amounts of G6PD enzyme (macrophages contain 30 to 100-fold increased levels, compared to T-cells or granulocytes) and this can make the interpretation difficult for mixed cell populations (187).

The main drawback of the G6PD analyses is, however, the low prevalence of heterozygosity at this locus in the general female population in North America (only 3% of Caucasian females are heterozygous). A more powerful approach from this point of view is the recently developed methylation analysis of certain polymorphic X-linked genes.

d- RFLP and methylation analysis of X-linked genes

A restriction fragment polymorphism (RFLP) is defined as an inherited difference in the restriction enzyme cleavage site found between two alleles of a gene or region of DNA, leading to differences in the size of the fragments resulting from digestion with that enzyme (188). It is estimated that on average, a single base difference can be found between two strands of DNA, for every 250-500 base-pairs (189). In the majority of cases, these are neutral mutations without any consequences other than the creation or abolition of a specific restriction site in one allele of a gene and/or a region of DNA. Other mechanisms of RFLP production is insertions or deletions of large fragments of DNA (190). The majority of neutral mutations give rise to a double allelic RFLP, whereas RFLP's resulting from insertions or deletions are more likely to give rise to several alleles. These allelic differences can usually easily be detected by Southern blot analysis (Figure 4). RFLP's are identified by analysis of DNA from a large number of unrelated individuals. Definitive confirmation of the existence of an RFLP requires both demonstration of its Mendelian inheritance pattern and an estimation of allele frequencies, in order to determine if genotype distributions are consistent with those expected from a Hardy-Weinberg equilibrium (190). RFLP's can occur in genes with a known function or in anonymous regions of DNA. The first human RFLP's characterized were at the

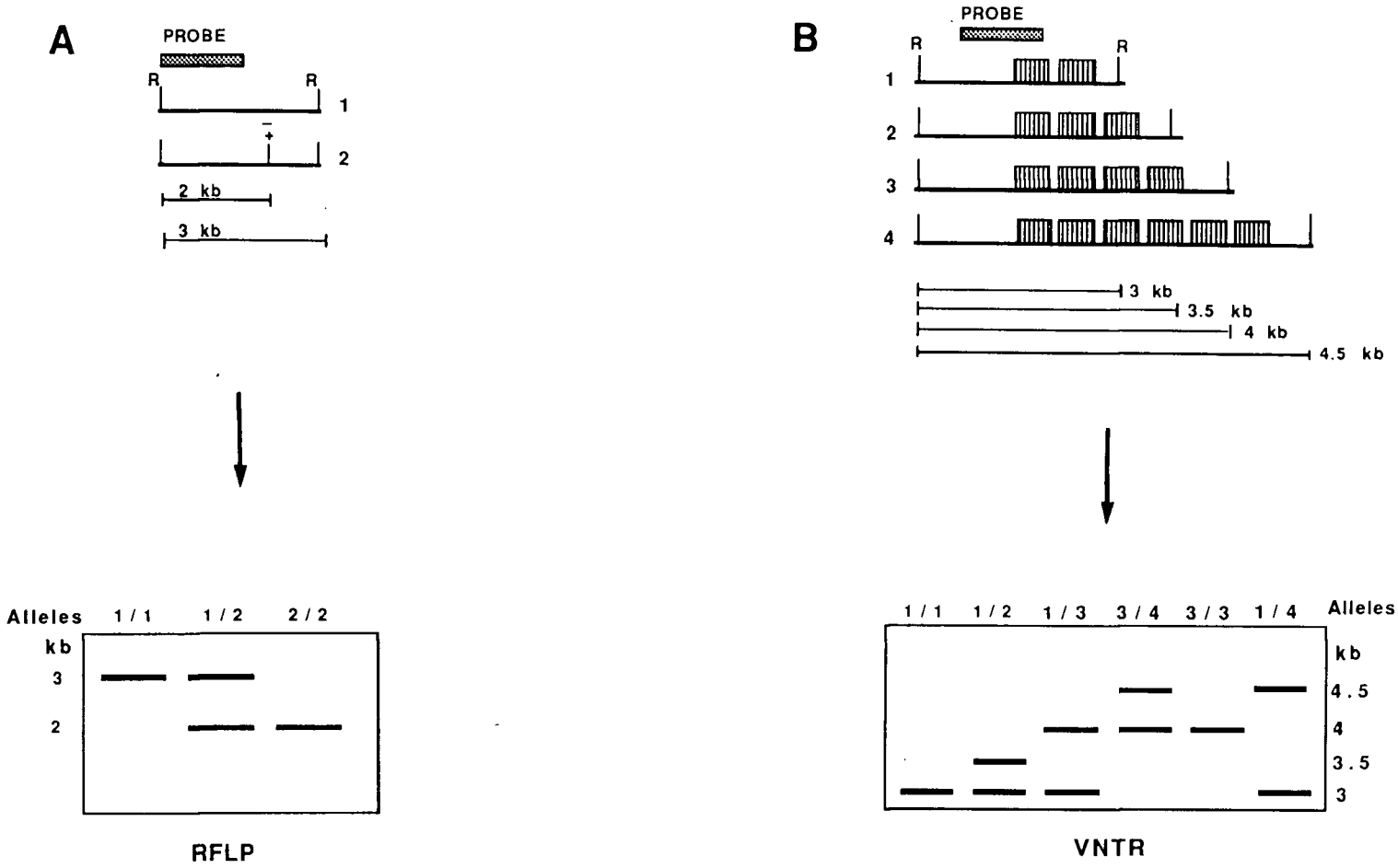


FIGURE 4. RFLP MECHANISMS

A- Creation of a restriction site by single base mutation giving rise to a bi-allelic RFLP. Using an appropriate probe, one can detect heterozygosity for this site of restriction by Southern blotting (alleles 1 and 2). Absence of a polymorphic restriction site or presence of a polymorphic site in both chromosomes give rise to a single band after Southern blotting (alleles 1/1 and alleles 2/2, respectively). R= Hypothetical restriction site.

B- Creation of a multi-allelic RFLP due to the presence of a varying number of repeated sequences in each allele.

insulin (191) and globin (192) loci. Many other RFLP's were found by chance, using total human DNA screened with several restriction enzymes and a variety of cloned DNA segments.

RFLP's have had a major importance in genetic linkage studies, by the fact that they allow two allelic copies of the same gene to be distinguished and these may display informative segregation patterns in families with specific genetic disorders. The use of chromosome-specific libraries has allowed topographic assignments to be made for specific RFLP's. Today several hundred polymorphic probes are available as genetic markers, each detecting a polymorphic site at a specific chromosomal site. They are being used extensively in genetic linkage analysis of inherited diseases (189), construction of the human gene linkage map (193), analysis of genetic losses in human tumors (194) and determination of the origin of cell populations after marrow transplantation (110). In this latter setting, RFLP analysis has also been used for HLA-matching of donors and recipients (195); and in one case, in determining a third-party rejection of a marrow transplant in a heavily transfused recipient (196).

For practical purposes, the usefulness of an RFLP resides in its capacity to distinguish accurately two siblings. This is directly related to the Polymorphism Information Content (PIC) value of a probe (188) which is close to 1 for most polymorphic probes. For example, RFLP's resulting from single base mutations typically have two alleles and they show low PIC values. Nakamura et al developed another approach of RFLP detection, based on the different numbers of repetitive sequences(variable number of tandem repeats or VNTR) present between two allelic copies of many genes, at their 5' or 3' region (197). These probes with high PIC values allow detection of a very large number of allelic fragments and they therefore have the capacity to distinguish DNA from most siblings (Figure 4). An extreme form of the polymorphism detection approach takes advantage of the presence of many unlinked hypervariable loci in autosomal DNA. These hypervariable probes reveal a series of bands unique to each individual, representing a " DNA fingerprint " (198-200). Initially applied in forensic medicine (201), this method was recently used to analyse clonality of certain solid tumors, by detection of clonally amplified somatic mutations (202). Its usefulness after allogeneic BMT has been suggested

(106) but because of the large number of alleles present, interpretation of these blots has not been practical.

A molecular strategy for **methylation analysis of polymorphic X-linked genes** was first described by Vogelstein et al. in 1985 (203). This method takes advantage of a polymorphic site in an X-linked locus, allowing a distinction to be made between the paternal and maternal copies of the gene. A second restriction enzyme, which is methylation-sensitive (HpaII or HhaI) is then used to assess methylation patterns of the active and inactive copies. In cell populations originating from a single cell, active and inactive alleles are clustered to the same parental chromosome (Xpat or Xmat) but not to both. An identical methylation patterns must therefore be present, associated with either the Xpat or the Xmat (Figure 5). When the sample is treated with Hpa II or Hha I, all the unmethylated sites are cleaved identically, whereas all the methylated sites are Hpa II or Hha I resistant. An X-linked probe of appropriate size hybridizes therefore to only one polymorphic band, the other being totally digested, giving rise to smaller fragments of < 8kb. Thus, detection of this hybridization pattern is indicative of a monoclonal cell population (Figure 5). In polyclonal cell populations, active and inactive alleles are expected to be distributed equivalently to both Xpat and Xmat; the fragments resulting from methylation-sensitive enzyme treatment hybridize equivalently to X-linked probes, with reduction of the intensity of both polymorphic bands by approximately 50%, depending essentially on the Xpat/Xmat inactivation ratio of the individual (204). This balanced pattern of hybridization is indicative of a polyclonal cell population (Figure 5). Two genes, both located on the long arm of the X-chromosome, have now been successfully used for this type of analysis: HPRT and PGK.

Hypoxanthine Phosphoribosyl Transferase,(HPRT) is a well characterized gene which has been cloned, mapped and sequenced in both mice and humans (205,206). The product of this gene is a major enzyme of the purine salvage pathway and its deficiency, always symptomatic in males, leads to gouty arthritis(partial deficiency) or to Lesch-Nyhan syndrome (in cases of total deficiency). Using a number of restriction enzymes to examine DNA from

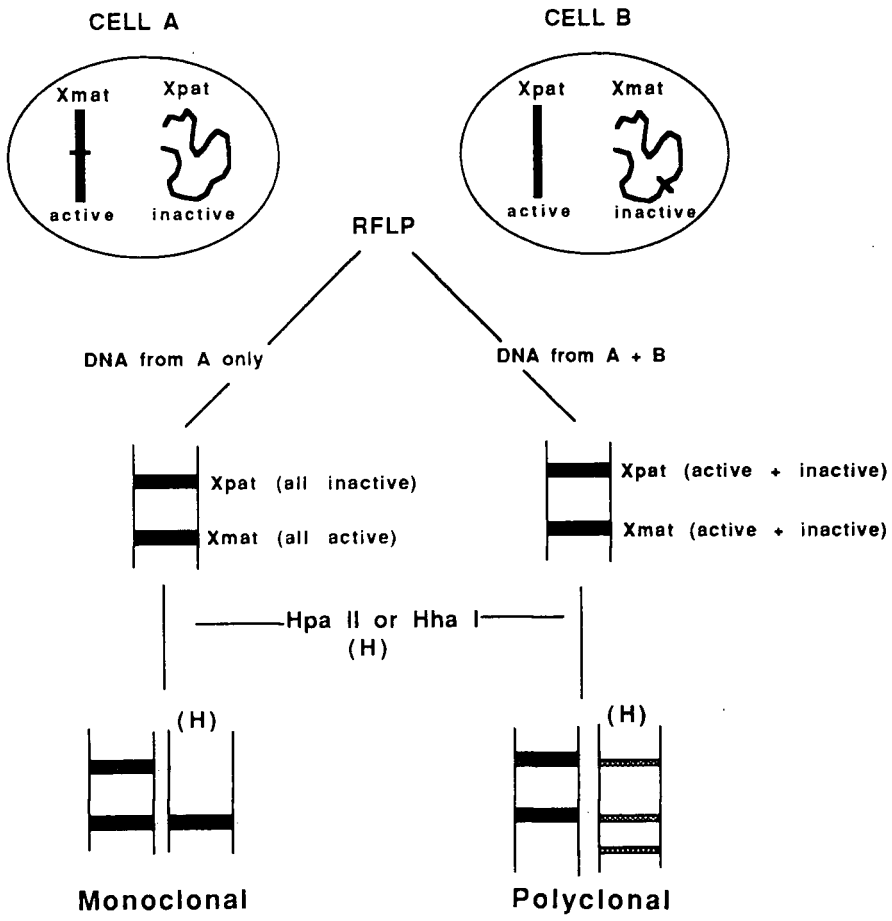


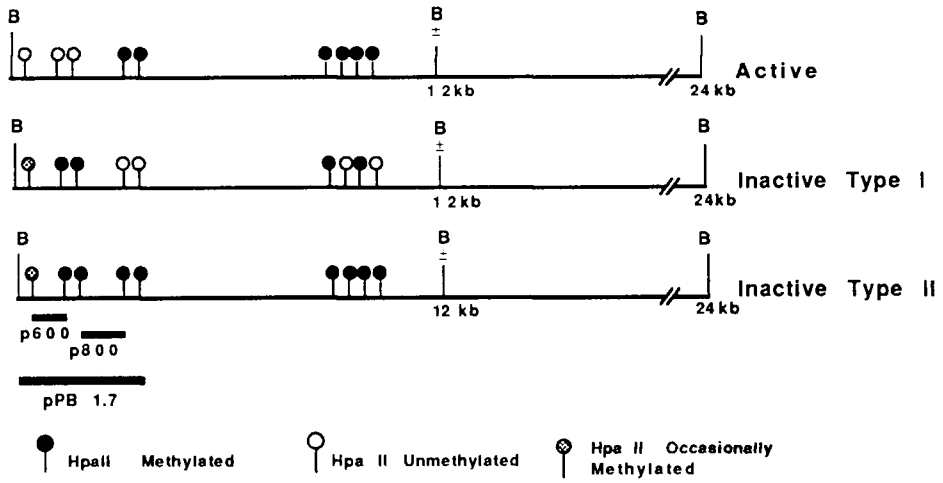
Figure 5.

Schematic Representation of the Basic Concepts for Clonality Analysis of Polymorphic X-Linked Genes. A polymorphic site (as indicated by a bar in the middle of the chromosome) can be present either on a paternal (Xpat) or maternal (Xmat) X-chromosome which might be active or inactive. In clonal cell populations arising from only one type of cell (Cell A), methylation-sensitive enzymes (Hpa II or Hha I, indicated by H) alter only one hybridizing band (monoclonal pattern) whereas in polyclonal cell populations (Cells A+B) both bands are cleaved by Hpa II or Hha I (polyclonal pattern). In H-digested lanes, bands smaller than the original polymorphic bands are digestion products.

a large unrelated female population, a polymorphic BamH I site was found at the 5'region of the gene, within the first intron, in 27% of females (204). When DNA digested with BamH I was hybridized to a 1.7 kb probe obtained from the 5'region of the gene, (Figure 6) two hybridizing bands of 24 kb and 12 kb were identified in heterozygous females. There are 9 HpaII sites and 6 HhaI sites in this region, all within a 24 kb fragment bracketed by two BamH I sites, and their methylation pattern have been extensively studied (172). The fact of major interest for clonality analysis purposes, is the demonstration that there is a differential pattern of methylation of these sites between active and inactive X-chromosomes. Two clusters of differential methylation have been identified for Hpa II cleavage sites. The first occurs at sites 1 to 3 and the second at sites 4 to 9 (Figure 6). Hpa II site 1 is unmethylated when carried by an active X-chromosome and occasionally methylated on inactive X-chromosomes. Hpa II sites 2 and/or 3 are unmethylated when carried on the active X-chromosome, and always methylated when carried on the inactive X (therefore HpaII resistant). The second cluster of differential methylation has been identified at sites 4-6, which are constantly methylated on active X-chromosomes, whereas two types of methylation patterns occur on inactive X-chromosomes at the HPRT loci. The most common pattern is the presence of at least one unmethylated (therefore cleavable) Hpa II sequence between sites 4 to 9 (Type I inactive alleles) whereas in the least common type, all sites 2 to 9 are methylated (type II inactive alleles). In individual females, a variable proportion of these two types of inactive alleles is present in all tissues and the proportion of cells with type II alleles has been found to vary from 0% to 40% (204). Theoretically, a monoclonal cell population can arise from a cell with either type I inactive or type II inactive allele. In the first case, a 1.7kb probe (pP1.7) from the 5' region of the gene, can successfully be used to demonstrate a typical monoclonal pattern because of the fact that it hybridizes to all large active fragments resulting from Hpa II digestion, whereas it can recognize only small digestion products originating from the type I inactive alleles. The final result is therefore the complete disappearance of one band (type I inactive allele) with retention of the other polymorphic band (active allele). However, if the monoclonal cell population originates

from a cell with type II inactive allele, the pP1.7 probe hybridizes to both active and inactive fragments, which are theoretically clustered to either Xpat or Xmat. Thus, the use of the large pP1.7 probe does not allow demonstration of monoclonal pattern in these samples, because it can not make a distinction between active and inactive alleles (Figure 6). An 800 bp subfragment of the pP1.7 (p800) later generated by Vogelstein et al. to study methylation patterns of sites 3 and 4 has also been found to be unable to demonstrate monoclonality in tumors with type II inactive alleles (204).

To circumvent this problem, a 600 bp subfragment of the same 1.7 kb probe was designed in order to explore methylation patterns of the first Hpa II sites of the gene. This probe has been found to have two essential advantages. The first is the absence of repetitive sequences, leading to a considerable improvement of the specific hybridization, and the second is its ability to demonstrate typical clonal pattern in tumors with type II inactive alleles (204). In clonal expansions arising from this latter type of cell, p800 hybridizes to both active (which are slightly shortened from 24 or 12 kb to 23.2 or 11.2 kb, respectively, because of the cleavage at the site 1) and inactive alleles and therefore does not distinguish between monoclonality and polyclonality. However, the p600, located 5' to the unmethylated sites of the active allele does not detect any full length fragment originating from active alleles whereas inactive alleles are fully recognized because all sites are methylated, eg. uncleaved by Hpa II. Consequently, the p600 is able to discriminate between the active and inactive alleles due to the fact that type II inactive alleles are always detected by this probe (Figure 6). If a tumor originating from a cell with type I inactive allele (therefore showing a monoclonal pattern with p800) is analysed with p600, both bands disappear in the Hpa II digested lanes, because p600 fails to hybridize to any significantly sized fragments originating from either active or inactive alleles. In this case, it is the 800 bp probe that detects a single band indicative of monoclonality.



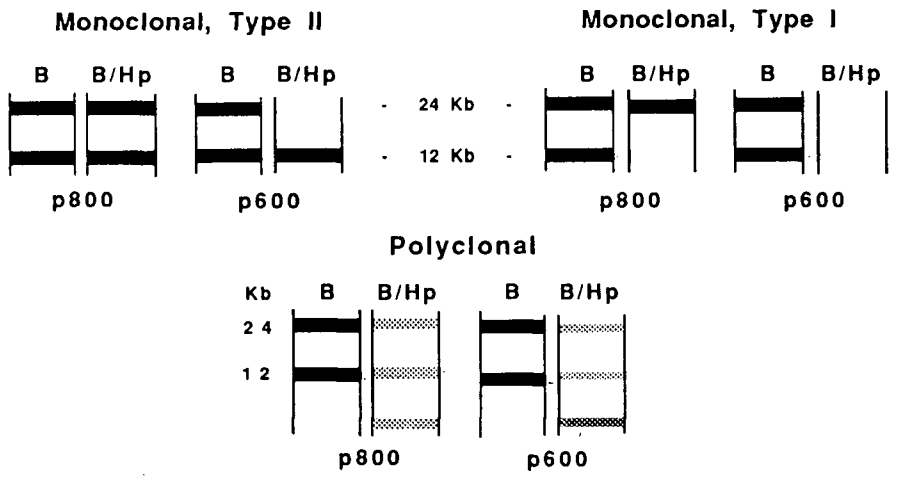


FIGURE 6.

Clonality Studies Using Methylation Analysis of Hpa II Sites at the HPRT Locus. A polymorphic BamH I site (as indicated by a + or -) may be present on either active or inactive X. In clonal cell populations, only one type of inactive X allele is expected to be present (Type I or Type II but not both). In tumors with Type II inactive alleles, HPRTp800 probe can not demonstrate a clonal pattern as it hybridizes to both active and inactive fragments. In these cases, HPRTp600 probe is useful as it hybridizes only to Type II inactive alleles, demonstrating monoclonality. In tumors with Type I inactive alleles, HPRTp800 is able to demonstrate a monoclonal pattern whereas HPRTp600 can not hybridize to any full-length fragments. In both monoclonal examples shown here, the allele lacking BamH I site is active. B: BamH I; B/Hp: BamH I + Hpa II.

Methylation patterns of HhaI sites at the 5' region of HPRT have been found to be simpler (Figure 7). There are 6 HhaI sites for which a differential methylation pattern between active and inactive X chromosomes has been demonstrated (204). Site 1 is unmethylated when carried on the active X-chromosome and methylated when carried on the inactive X-chromosome. Sites 2 to 6, are methylated in most active X-chromosomes, whereas inactive chromosomes have at least one of these sites in an unmethylated state. It is noteworthy that some tumors showing a polyclonal pattern using HhaI digests, can be shown to be monoclonal using an HpaII digest and vice-versa (204). It is therefore important to use both enzymes and both 600 and 800 bp probes for clonality analysis using HPRT polymorphism.

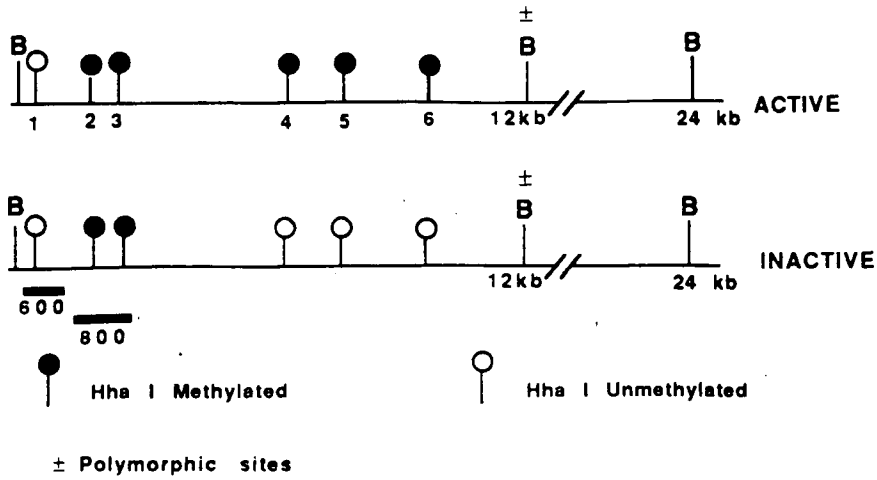
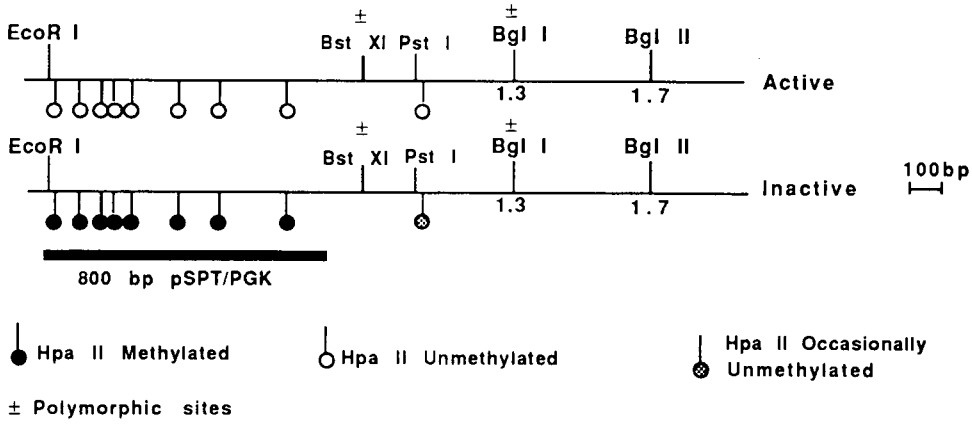


Figure 7.

Hha I Cleavage Sites and their Methylation at the HPRT Locus
B: BamH I

Phosphoglycerate Kinase (PGK) , is another X-linked gene located on the long arm of the X-chromosome. Differential methylation patterns of HpaII sites between active and inactive copies of the gene have been demonstrated, showing the presence of 8 sites which are unmethylated on the active and methylated on the inactive X (207). One additional site is present downstream, which is unmethylated in 20-40% of inactive X-chromosomes (Figure 8). Vogelstein et al (204) have identified a BglI site and a BstX I site 500 bp and 250 bp downstream respectively, from the differentially methylated region.In order to obtain fragments of DNA with sizes allowing satisfactory digestion and transfer, they digested DNA successively with EcoR I, Bgl II to bracket the differentially methylated region and then with BglI to reveal polymorphic alleles of 1.7 and 1.3 kb in heterozygous females. The polymorphic BglI site was found in 72 of 217 females tested (33 %) and appeared to be in a strong linkage disequilibrium with BstX I polymorphism (204). This latter polymorphism offers the advantage of eliminating site 9 when DNA is digested concommitantly by Pst I and therefore simplifying the interpretation of methylation patterns. This differentially methylated region has also an Eco 109 polymorphic site which is found in 5 % of females (204). Finally, an additional X-linked probe , pXUT23-2.1, derived from the short arm of the chromosome was found to detect a BglI polymorphism in 30 % of females studied by Vogelstein et al (204). This probe seemed to detect methylation differences between inactive and active sequences, but the overall complexity of the hybridization patterns rendered its routine use difficult.

Clonality analysis can routinely be performed using this method in approximately 50 % of the female population, which represents overall a 15-fold improvement of the heterozygosity prevalence over G6PD polymorphisms. However, several pitfalls of the method need to be recognized. As for G6PD analysis, contamination of a neoplastic clone with normal cells will give rise to a false impression of polyclonality, and likewise an apparent monoclonal pattern will be seen in rare females with an unequal Xpat/Xmat ratio, stressing the importance of analysing, whenever possible, normal control DNA from the same patient. Finally,



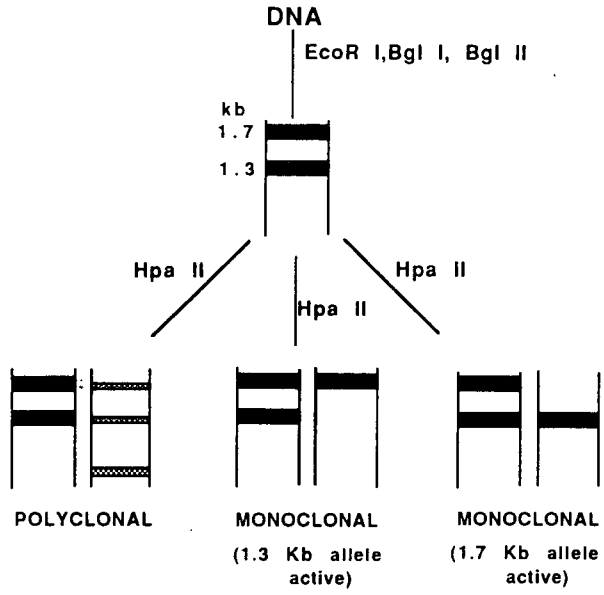


Figure 8

Clonality Studies Using Methylation Analysis of the PGK Gene. Polymorphic Bgl I and BstX I sites are indicated by + and -. In order to bracket the differentially methylated region, DNA is first cut by EcoR I, Bgl II, and then by Bgl I. To reveal a BstX I polymorphism, DNA is concomittantly digested with Pst I and BstX I. Methylation analysis is then performed in both situations using Hpa II, as detailed in the previous Figures. In the polyclonal example shown, a digestion product of < 1.3kb is seen.

aberrant methylation patterns may be present in some tumor cells, precluding methylation analysis (208).

(D)- VIRAL MARKERS

a- EBV Termini Analysis

This method is based on the fact that following the Epstein-Barr viral (EBV) infection of the host cell, the EBV DNA which is linear, becomes circularized by fusion of the terminal portions of the virus in a unique and specific way for each virus according to the number of tandem repeat sequences present at each terminus (209). In a given cell infected with EBV, only a single type of fusion is found and this episomal pattern is transmitted to the progeny of this cell. When a Southern blot analysis is performed using DNA obtained from different tissues, the use of an EBV terminus specific probe yields a single band of integration in monoclonal cell populations and several bands in polyclonal cell populations (209). This method has been used to analyse the clonality of B-cell lymphoproliferative disorders in recipients of T-cell depleted allogeneic transplants (210), and in other immunosuppressed patients receiving organ transplants (211). The stability of the viral genome following the host infection renders this method attractive although whether the EBV integration event is related to the mechanism of clonal amplification of the infected B-cell in different situations unknown. Interestingly, however, recent data combining EBV termini analysis and in situ hybridization using with EBV probes, suggest that EBV may be involved in the pathogenesis of Hodgkin's disease (212).

b- Retroviruses as clonal markers

Retroviruses integrate into the DNA of eukaryotic genomes randomly and their unique integration site is transmitted from one cell to all its progeny (213). These unique integration sites are detectable by Southern blot analysis. In order to prevent viral spread from one cell to

another after the integration of the retrovirus, replication-deficient viral constructs have been developed (214). These constructs typically contain a drug resistance gene which allows a selection to be made of the cells that have successfully integrated and express the introduced construct. In mice, incubation of marrow cells with retrovirus producing cells or released retrovirus allows marking of the various cell types within the marrow cell suspension. In vitro colony assays in the presence of G418 have been used to detect and quantitate clonogenic cells which have integrated the retrovirus along with the drug resistance gene. Similarly, transfer of the infected marrow cells into W/W^V recipients has allowed tracking of the progeny of infected CFU-S and stem cells capable of clonal repopulation of thymus, spleen and bone marrow (13). Methods for retrovirally infecting cells capable of initiating long-term hemopoiesis in human LTMC are now also being developed (215). In addition to their use as clonal markers, retroviral vectors are believed to hold considerable promise for future gene therapy (214).

(E)- COMPARATIVE ANALYSIS OF CLONALITY MARKERS

From the above chapters, it appears that the combined use of several clonality markers, when applicable, represents the most comprehensive way of studying clonal origin and differentiative capacities of cell populations. The usefulness of a given clonal marker can change according to the cell lineages studied; for example G6PD analysis is suitable for clonal analysis of red cells or platelets, whereas RFLP analysis is not applicable to study these cell populations. Similarly, G6PD analysis can be performed in single hemopoietic colonies, whereas it is impossible to obtain sufficient amounts of DNA from such colonies for RFLP and methylation analysis. On the other hand, RFLP analysis has the potential for wider application, related to the fact that approximately 50 % of an unselected female population can be expected to be heterozygous and therefore analysable by this technique, using available HPRT and/or PGK probes (204), whereas only 3 % of females are heterozygous for G6PD (39).

One problem which is inherent to X-linked markers is contamination with normal tissues, which would then give a false positive result of polyclonality. Results with both G6PD and HPRT or PGK analyses indicate that 3 to 5 % of contaminating polyclonal enzyme or DNA can be detected by these techniques. Contamination could explain some discrepancies appearing in the literature. In early studies, adenomas associated with inherited polyposis were found to be polyclonal using G6PD analysis (216) whereas a recent survey of 30 similar cases using the RFLP technique demonstrated monoclonality in all cases (217). Similarly, early studies with G6PD demonstrating multicellular origin of parathyroid adenomas were not confirmed by RFLP and methylation analysis (218).

The X-linked markers represent a general approach to study clonality because of their early appearance in cell ontogeny. However, they can not be used as markers for minimal residual malignant disease, as opposed to the methods based on chromosomal abnormalities or gene rearrangements. The latter correlate at least in time, if not causally with the initial malignant change and they can be extremely useful in detecting an abnormal clone marked by one of these events (such as Ph^1-chromosome, bcr rearrangements or Ig or TCR gene rearrangements). However, one disadvantage of Ig or TCR gene markers is represented by a very high rate of somatic mutations occurring in these genes, which then can lead to a false impression of polyclonality. An example of this is provided by the study of bi- or multi-clonal lymphomas which have been reported in immunosuppressed patients, especially after solid organ transplantation (219). Although some of these patients might have genuine bi- or multiclonal disease, the use of an independent marker of clonality such as 14;18 breakpoint sequencing in these "bigenotypic" populations has demonstrated that they have identical 14;18 breakpoints and that they therefore represent genetically diverged subclones originating from the same parental neoplastic clone as a result of a somatic mutation of an Ig gene (220). However, the reliability of breakpoint sequencing as a clonality marker also depends on the absence of somatic mutations in this region of DNA, which could occur and invalidate interpretation.

3) MYELOPROLIFERATIVE DISEASES

(A) GENERAL FEATURES

The myeloproliferative diseases (MPD's) are a heterogeneous group of disorders characterized by an abnormal accumulation of cells of the myeloid lineages, which typically show an ability to differentiate into functional, terminal cells (red cells in PV, granulocytes in CML and platelets in ET). The MPD's have several common features. There is extensive evidence indicating their origin in a pluripotent stem cell. This evidence, initially obtained in CML by the demonstration of the Ph^1 chromosome in multiple lineages (221-223), was also confirmed using G6PD isoenzyme analysis (224) and extended to the other diseases in this group. In every case studied, one isoenzyme was found in circulating erythrocytes, granulocytes, monocytes and platelets of female CML patients heterozygous for the G6PD polymorphism (225,226). Similarly, only one type of G6PD isoenzyme was found in hemopoietic cell lineages of patients with PV (227), ET (228), and myeloid metaplasia (229), whereas both isoenzymes were present in their skin fibroblasts. The clonal nature of CML has also been shown using RFLP and methylation analysis of HPRT gene (230).

Another common biological abnormality to all of the MPDs is the presence of erythroid colonies able to grow in semi-solid media without addition of erythropoietin (231). This is universally found and diagnostic of the abnormal clone in patients with PV (232) but is also common in CML (231) and occurs in at least some cases of ET (231). The fact that MPDs are clonal multipotential cell disorders is also suggested by the frequency with which these diseases share certain hematological abnormalities. Thrombocytosis is for example, such a common feature. Approximately half of CML patients present with or develop thrombocytosis during the course of their disease and this can herald the occurrence of a blastic phase (233).

Thrombocytosis also occurs frequently in PV and myeloid metaplasia and it is the cardinal feature of ET. Similarly, an increase of granulocyte and red cell counts may be found in all MPDs, as well as abnormalities of leukocyte alkaline phosphatase and serum vitamin B_{12} values. This latter is almost always increased in CML and may be related to the fact that the transport protein transcobalamine I is of granulocytic origin (234). Usually, the serum vitamin B_{12} value decreases towards normal values under the influence of therapy. The Ph^1 chromosome, which is the hallmark of CML, has been detected in some patients who initially presented with features more typical of ET or myeloid metaplasia (235,236). Transition forms of PV to CML (237) and to myeloid metaplasia (238) have also been reported. Finally some similarities exist between all chronic MPDs from a clinical standpoint. All MPDs, with the exception of the blastic crisis of CML, usually represent non-acute conditions.

(B) CHRONIC MYELOID LEUKEMIA (CML)

Clinical background. CML is a MPD characterized by a neoplastic proliferation and accumulation of myeloid cells as well as their progenitors. The peak incidence of CML is between ages 40 to 49. Epidemiological studies have shown that ionizing radiation and to a lesser degree, chemical carcinogens increase the incidence of CML (239). Patients usually present with elevated granulocyte counts, which may be asymptomatic for several months or even years. The most common physical finding is splenomegaly, which is present in > 98 % of patients. With the progression of the disease, patients develop symptoms related to the infiltration of all hemopoietic organs with cells of granulocytic lineage. Initially, regression of symptoms can be obtained by single agent chemotherapy, but none of the therapeutic modalities initiated prolongs survival (240). Bone marrow transplantation performed during this phase is currently thought to be the only possibility of cure (241). After a median period of 3 to 4 years, the disease undergoes progression to an acute leukemia regardless of prior responsiveness to chemotherapy (242). In the majority of cases (70 %) the blast cells in the

acute phase have the characteristics of myeloblasts (243) but promyelocytic (244) monoblastic (244) erythroblastic (245) megakaryoblastic (246) and mixed (247) transformations have also been described, highlighting the pluripotential origin of the chronic phase clone. In approximately 25 % of cases, the blasts in the acute phase show lymphoid B-lineage features as demonstrated by cell surface markers (TdT and CD10 antigen positivity) and more recently by Ig gene rearrangements (248). Occasional blast crises in which cells with T-cell characteristics have been found, have been reported (249) as well as cases with blasts exhibiting both myeloid and lymphoid features (250).

Chromosomal abnormalities. CML was the first human malignancy in which a consistent chromosomal abnormality was discovered (119). Initially the Ph^1 chromosome was thought to be the same as that involved in Down's syndrome (#21); however, with the introduction of banding techniques, it was later identified as #22 (251), shortened by a partial deletion of its long arm (252). In 1973, Rowley also demonstrated that the missing long arm of the Ph^1 chromosome was translocated to the distal end of the long arm of chromosome 9 (9q+) (253). In 1977, it was further shown that the translocation is balanced and no genetic material is lost (254). In 1978, Watt and Page demonstrated that the translocation is in fact reciprocal between chromosomes 9 and 22 (255). This translocation is now designated as t(9;22)(q34;q11 or q12) and is found in > 95 % of patients with Ph^1-positive CML (134). In approximately 5 % of cases, the deleted chromosome 22 segment attaches to another chromosome (complex translocation). Additional chromosomal abnormalities frequently occur with the progression of the disease to the blastic phase, suggesting a multistep pathogenesis at different clinical stages of CML (256). The most common additional abnormalities include second Ph^1, trisomy 8 and isochromosome 17q.

In approximately 8 % of patients initially categorized as CML, cytogenetic analysis is unable to demonstrate the Ph^1 chromosome (134). These patients typically have a more aggressive disease with poor response to therapy and a shorter median survival (approximately 1 year) (135). The morphological assessment of cells from these patients has led some authors

to conclude that many might in fact have another condition such as myelodysplasia, rather than CML (257). Molecular analysis revealed in approximately half of these patients, a genomic rearrangement typically seen in Ph^1-positive CML (256) as it will be detailed below.

Clonal analysis in CML. Evidence for the multipotential stem cell origin and clonal nature of CML has been discussed above. It seems likely that the cell in which the Ph^1 chromosome first appears is commonly a stem cell with B-lymphoid as well as myelopoietic potential. The question of whether this cell also has T-lymphoid potential is more controversial as there is no clearcut evidence for Ph^1 positive T-cells. Clonality studies have also been undertaken to look for evidence as to whether acquisition of the Ph^1 chromosome is a primary or a secondary event. Fialkow et al showed that both Ph^1-positive and Ph^1-negative EBV-transformed cell lines were obtained from a G6PD heterozygote patient with a Ph^1-positive CML. Because the Ph^1-negative lines were all of the same isoenzyme type as the Ph^1-positive cells and showed an increased frequency of cytogenetic abnormalities, these investigators suggested that the occurrence of Ph^1 chromosome may be a secondary event. Other evidence supporting such a multistep hypothesis, has been obtained by Lisker et al, who reported on 2 patients who converted to Ph^1-positive CML after an apparently initially Ph^1-negative phase (258). Three similar cases exist in the literature (182). Unfortunately, none to date have rigorous sequential cytogenetic, molecular and clonality data.

The clonal analysis in Ph^1-negative CML is limited to the study of a single case reported by Fialkow et al (259), demonstrating a single G6PD isoenzyme pattern in granulocytes, platelets and monocytes, consistent with the hypothesis that, this is also a clonal multipotential stem cell disorder.

Hemopoietic progenitors in CML. Clonogenic assays performed in a large number of patients with CML have shown that, although colonies obtained from these patients are morphologically undistinguishable from those obtained from normal controls, they originate from the same neoplastic clone, as demonstrated by G6PD analysis (225) and cytogenetics of individual colonies (260,261). When the number of progenitors obtained from the marrow of

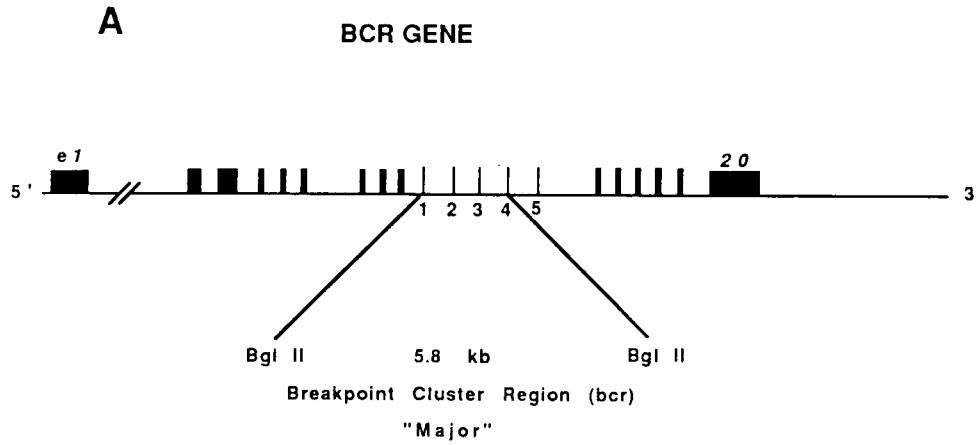
CML patients is compared to normal controls, these do not seem to be significantly changed (262) but this might be related to the fact that only relative progenitor numbers can be assessed in marrow samples (35) and peripheral blood progenitor assays might allow a quantitative evaluation of these values. These latter have shown that there is a considerable increase of all circulating hemopoietic progenitor numbers during the chronic phase, and this include erythroid (263) megakaryocytic (264) and pluripotent progenitors (265). This finding is consistent with the concept of an amplification of all progenitor compartments in CML (266) which is also suggested by cycling assays showing a persistent proliferative activity of all classes of blood and marrow progenitors (266) as opposed to normal controls in whom the most primitive blood and marrow progenitors have always been found to be quiescent (37). The reasons for the increased numbers of circulating progenitors in CML are not well understood. Changes in sialyltransferase activity at the surface of CML granulocytes (267) and decreased adhesive properties of CML progenitors as opposed to their normal counterparts (268) could reduce their binding ability to marrow microenvironment. Unique regulatory deficiencies are also likely to be involved to control lineage specific progenitors, as suggested by the fact that CML patients commonly have anemia despite the presence of considerably increased numbers of erythroid progenitors (263) which also frequently show Epo-independence in in vitro assays (263). Standard LTMC have not been found to be useful in studying regulation of Ph^1-positive CML progenitors, as it has been shown that these cells rapidly decline and disappear when such cultures are initiated with marrow cells (as discussed below). In an effort to maintain Ph^1-positive progenitors in vitro and to study their cycling characteristics, this system was modified by starting CML marrow or peripheral blood cultures on pre-established, irradiated feeder layers from normal bone marrow (269). Cycling experiments showed that in these conditions, Ph^1-positive progenitors were maintained in a continuously proliferative state for 1-2 months, whereas progenitors from normal controls were induced in and out of cycle with regular medium changes. This would suggest that CML progenitors are able to ignore in these

conditions, negative regulatory influences from normal mesenchymal cells and they remain continuously in cycle.

Persistence of normal progenitors in CML. In 1977, Smalley et al showed that cell cycle-specific chemotherapy could induce Ph^1-negative metaphases in the bone marrow of patients with Ph^1-positive CML (270). Other groups (271,272) later confirmed this finding using combination chemotherapy. However, the karyotypic conversion was of short duration. The question of normalcy of the Ph^1-negative progenitors in these patients was addressed by Singer and co-workers who showed in a patient heterozygous for G6PD, that 4 cycles of chemotherapy can induce non-clonal granulocytes and a significant proportion (76 %) of Ph^1-negative bone marrow metaphases (273). These reports strongly suggested the presence in vivo, of residual normal progenitors, which were undetectable by direct analysis of bone marrow metaphases (274) or cultured clonogenic cells (275). In 1983, Coulombel et al (276) demonstrated that the LTMC system allows preferential maintenance of Ph^1-negative progenitors in these cultures, whereas Ph^1-positive progenitors usually become undetectable within 4-6 weeks. These findings, initially reported in 4 patients, have now been extended to a large group of CML patients, and in many the "switch" to Ph^1-negativity has been found (266). The non-clonal nature of the emerging Ph^1-negative progenitors in these cultures was first demonstrated by Dube et al (277) in studies of a unique mosaic patient with Turner syndrome, in whom the Ph^1-clone, had arisen from a 45/XO cell. Subsequently the cells from another CML patient, who was heterozygous for G6PD, were also studied in LTMC and the non-clonal nature of some clonogenic cells emerging after 4 weeks of culture demonstrated (278). The mechanisms of the preferential disappearance of the Ph^1-positive progenitors in cultures initiated with CML marrow remain unknown at the present time. However, the selective maintenance of the Ph^1-negative progenitors in these cultures and their non-clonal nature has suggested that this could be applied as a "culture purging" method combined with autologous BMT. This approach has now been applied to 8 patients with Ph^1-positive CML in Vancouver.

Molecular changes in CML. In 1982, de Klein et al showed that the c-abl proto-oncogene, the human cellular counterpart of v-abl, is moved to the Ph^1-chromosome along with the rest of the translocated tip of chromosome # 9 (279). The breakpoints on the chromosome # 9 occur typically between the exon 1b and the exon 1a of the c-abl oncogene, these two exons being separated from each other by an extraordinarily large intron of >200 kb (280). In contrast to this breakpoint which can occur anywhere on the 5' region of c-abl, breakpoints on chromosome # 22 are found to be clustered in a small 5.8kb region, designated as the breakpoint cluster region or bcr (281). Further studies by Heisterkamp et al (282) demonstrated that the bcr is part of a large gene called BCR gene (Figure 9). The 5.8 kb region, which some authors propose to call the major bcr (M-bcr) occupies the central region of the BCR gene. The bcr contains 4 exons (b1 to b4) coding for two different species of mRNA of 4.5 and 6.7 kb. These are normally translated into a single protein product of 160 kd (283). The bcr breakpoint, which is unique to each CML clone, almost always occurs between b2-b3 or b3-b4 (284). As a consequence of the bcr-abl translocation, abl sequences from chromosome # 9 are fused distally, in a head-to-tail fashion, to the amputated bcr sequences from chromosome # 22, bcr being closer to the centromere. This large hybrid fusion gene is then transcribed into an abnormal hybrid bcr-abl mRNA of 8.5 kb (285). In this hybrid mRNA, the first 5' sequences are contributed by the bcr gene and the last 3' sequences by the abl gene from which the first two exons are always spliced out (284) (Figure 10). Thus, the most common fusions at the RNA level occur between b2-a2 and b3-a2. The novel transcript is translated into a hybrid protein product of 210 kd (p210), which shows increased tyrosine kinase activity (286), as opposed to the normal enzymatic activity associated with the normal, wild-type abl product of 145 kd (p145) (287).

One other consequence of the bcr-abl translocation is a change in the restriction enzyme sites located within or nearby bcr, in one of the chromosomes. This can be detected



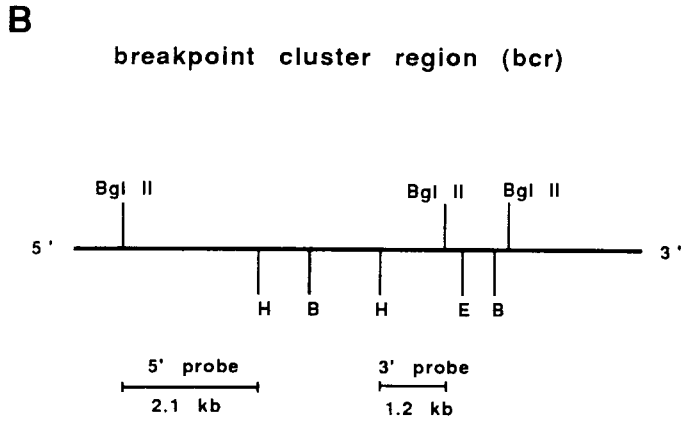


Figure 9.

Panel A. BCR gene located on Chromosome # 22.Black boxes and bars represent 20 known exons of the BCR gene. The first exon of the BCR gene is indicated by the symbol e1. Exons numbered from 1 to 5 are those of the "major" breakpoint cluster region (bcr) in CML.

Panel B. Restriction map of the breakpoint cluster region showing 5' and 3' probes used to detect rearrangements. The three most useful restriction enzyme sites for this purpose (in addition to Bgl II sites) are also indicated. B= BamH I; H= Hind III; E= EcoR I.

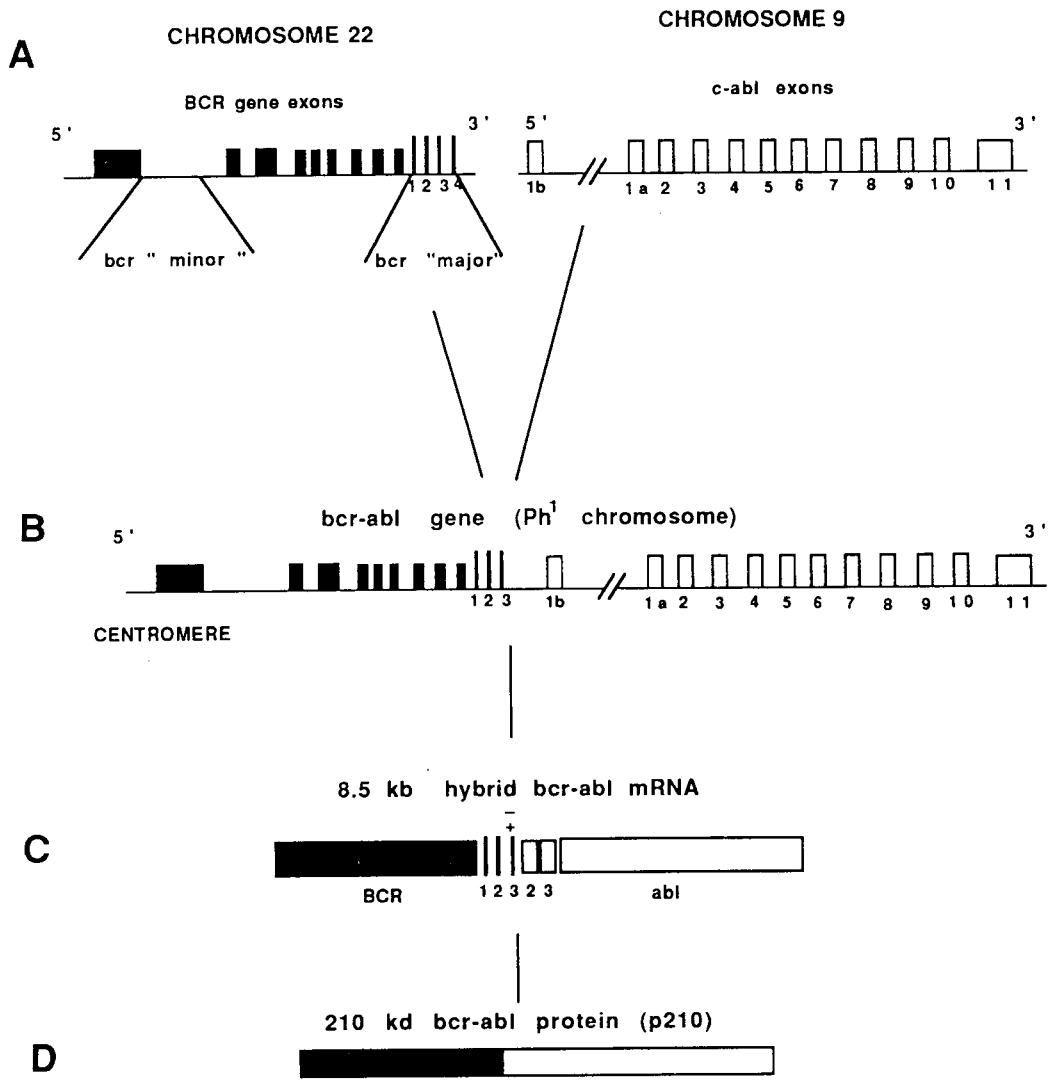


Figure 10.

Molecular events leading to the generation of bcr-abl gene and the abnormal p210 bcr-abl protein

A- bcr "major" indicates the breakpoint cluster region (bcr) on chromosome # 22. Breakpoints on chromosome 9 are higly variable, occurring in a large region of >200 kb, proximal or distal to exon 1b of the abl gene.

B- bcr-abl fusion gene with bcr (black boxes and bars) and abl (open boxes) sequences.

C- 8.5 kb hybrid mRNA in which sequences from exon # 3 of bcr might be present or absent according to the site of the breakpoint.

D- 210 kd protein product with increased tyrosine kinase activity.

by Southern blot analysis using a battery of restriction enzymes and probes that span the entire region. The modified configuration of the altered chromosome gives rise to a band of different size (the "rearranged", abnormal band) as compared to the normal band (the "germline" band). The germline band is of different size according to the restriction enzyme and probe used, but it remains identical in all individuals unless an RFLP affects it, and this usually will be unique to a single enzyme. The rearranged band, however, is unique to each patient and represents a specific clonal marker. Using the four most useful restriction enzymes (Bgl II, BamH I, Hind III and EcoR I) and probes spanning the entire bcr, a rearrangement is detectable in > 95 % of patients with Ph^1-positive CML (134). Two important pitfalls in the molecular analysis of bcr-abl rearrangement are the following. The first is that at least two restriction enzymes and/or a non-hematopoietic tissue DNA must be analysed to eliminate a possible RFLP (136,288). The second is the necessity to use both 3' and 5' probes, because of the occurrence of deletions of the 3' bcr sequences following the rearrangement, in 10-15 % of patients with CML (289). The use of a single 3' probe in these patients will give a false negative result.

Interestingly, a significant proportion of patients with Ph^1-negative CML show an otherwise typical bcr rearrangement leading to the same abnormal RNA transcript and protein product that is found in Ph^1-positive disease (135,136,290). The interstitial insertion of abl sequences to bcr sequences without reciprocal translocation (291) is thought to be the molecular mechanism involved in these cases. Relatively large series of Ph^1-negative CML patients are now available to show that the clinical course and survival patterns of these patients are similar to those with Ph^1-positive disease and the worse prognosis, classically associated with Ph^1-negative CML is probably true only if the bcr rearrangement is absent (292-294). The introduction of bcr rearrangement studies for the routine examination of CML patients can, like cytogenetic studies (295), allow assessment of responses to therapy, detection of residual disease and relapses (296). Molecular analyses have the great advantage that they can be performed on both dividing and non-dividing cells. The 3' location of the breakpoint

within the bcr, as opposed to 5' breakpoints, was reported to be associated with a more rapid progression to blast crisis (297) but this has not been confirmed by others (298,299).

More recently, the use of PCR technology to the study of CML has made it possible to detect extremely small numbers of cells carrying the bcr rearrangement (300,301). This technique, not applicable at the DNA level, due to the extreme variation of breakpoints and the large segments of DNA involved in the abl translocation, necessitates cDNA synthesis from the chimeric bcr-abl mRNA, followed by the amplification of this material detected by Southern blotting or ethidium bromide staining of gels. PCR analysis has been instrumental in detecting the presence of alternately spliced species of mRNA (302) and is being used to look for minimal residual disease in interferon-responsive patients (303) and patients treated with BMT (304-306). In this latter setting, the significance of detecting residual transcripts is unclear at the present time, as no concordant data has been obtained in different centres (307).

Ph^1-positive Acute Lymphoblastic Leukemia (ALL). The Ph^1-chromosome is found in leukemic lymphoblasts of 2-10 % of children who present with acute leukemia and in 15-20 % of adults who present with an otherwise typical ALL (120,308). These patients constitute a high risk group, as they have a high tumor burden, higher risk of CNS involvement and higher risks of remission failure or early relapse than cases without the Ph^1 chromosome (309-311). The significance of the Ph^1 chromosome in ALL and its relationship with the Ph^1 chromosome-positive CML has proven to be a complex problem (312). Some data strongly suggest that at least some patients with Ph^1-positive ALL have in fact experienced a clinically silent and hence undiagnosed chronic phase prior to the development of a lymphoid blast crisis. The persistence of Ph^1-positive bone marrow metaphases in chemotherapy-induced remissions (313) and rare cases of subsequent myeloid relapses after presentation with a Ph^1-positive ALL (312) support this view. On the other hand, in some patients with Ph^1-positive ALL, the Ph^1-chromosome becomes undetectable in the bone marrow metaphases during remission (310,313) unlike what is commonly seen after treatment of CML patients in lymphoid blast crisis where Ph^1-positivity usually persists (313). Other cytogenetic findings consistent with the possibility that

Ph^1-positive ALL may be a distinct entity is the absence of additional karyotypic abnormalities typically associated with either accelerated or blastic phase of CML (314). If de novo Ph^1-positive ALL exists as separate disease, it would be anticipated that the target for neoplastic transformation would be a B-lymphoid-restricted progenitor, thus making it analogous to Ph^1-negative ALL (315). Alternatively, Ph^1-positive ALL might be a disease that arises in a pluripotent stem cell but shows lymphoid-restricted expression of the neoplastic phenotype (315,316). In one case of a patient with Ph^1-positive ALL, cytogenetic analysis of erythroid colonies failed to reveal the Ph^1 chromosome (317). However, more commonly, myeloid involvement has been demonstrated by cytogenetic analysis of erythroid or granulopoietic colonies generated in vitro (316,317).

The discovery of the bcr-abl rearrangement in the generation of the Ph^1 chromosome has also been investigated in Ph^1-positive ALL and added a new dimension to these questions. Initial molecular studies using bcr probes showed that some patients with typical Ph^1-positive ALL display a rearrangement in the same region as Ph^1-positive CML patients, whereas others had no detectable rearrangements with these probes (318,319). In addition, in situ hybridization data demonstrated that in those patients without a detectable rearrangement, the 22q11 breakpoints were proximal to the 5' region of bcr and were located between this region and the more proximal Cλ locus (318). In 1987, 3 groups reported the existence of a novel abl-related protein of 185 to 190 kd (p190) which was associated with an abnormal transcript of 7-7.4 kb in some patients with a diagnosis of Ph^1-positive ALL (320-322). The same year, Fainstein et al (323) and Walker et al (324) demonstrated that the p190 is a chimeric translation product of a chimeric mRNA of 7kb, generated presumably by fusion of the first exon of BCR gene to abl exon #2 (323-325). Thus, in this gene product, only a small portion of the BCR gene is contributed, and the classic bcr is not involved. The p190 has an increased tyrosine kinase activity similar to p210 (323,324). The participation of the first exon of the BCR gene in the 7 kb abnormal transcript and the production of p190 indicated that in these cases, the rearrangement must have taken place in the first intron. This was first confirmed by

Hermans et al (326) who cloned several gene probes from the first intron of the BCR gene and then used these to demonstrate that a rearrangement takes place in this region (Figure 11). Very recently, 2 groups have cloned several regions of the first intron of the BCR gene and demonstrated another rearrangement cluster region in patients with Ph^1-positive,"bcr-negative" ALL (327,328). This region of approximately 16 to 20 kb, lies near ALU repeat sequences, close to an unusual deletion RFLP, which might render this portion of DNA prone to rearrangements (327). Initially thought to be specific for de novo Ph^1-positive ALL, the p190 has recently been demonstrated in Ph^1-positive AML with a breakpoint lying outside the bcr (329). Thus, the same molecular marker can be expressed in both lymphoid and myeloid lineages, indicating that p190 does not have a primary role in determining the phenotype of the neoplasia. One important question is whether the progression from the chronic phase to lymphoid blast crisis is accompanied by a transition of p210 synthesis to p190 synthesis, which theoretically can occur by alternative splicing (284). Initial evidence using PCR amplification of exon1-a2 and b2/b3-a2 junctions argues against this model,as cell lines obtained from lymphoid blast crisis cells do not have the "ALL" type, p190 product (330). The frequency distribution of DNA rearrangements using both bcr and first intron-BCR-probes, demonstrate that there are 2 types of Ph^1-positive ALL patients. Some patients exhibit a rearrangement pattern identical to that seen in CML and they are likely to produce p210. Others, have a rearrangement outside the bcr, most frequently in one of two clusters of the first intron of the BCR gene. These patients are more likely to produce p190. Correlation of these molecular findings with various phenotypic and other disease characteristics must await prospective studies involving larger groups of patients. However, the p210 and the p190 are highly specific to the Ph^1-positive leukemias and although their substrate of phosphorylation has not been identified, their transforming role has been shown by in vitro retroviral transfection experiments in both myeloid (331) and lymphoid (331-333) cell culture systems. More recently, a CML-like disease was induced in mice by transplantation of syngeneic bone marrow transfected with a p210-carrying retroviral construct (334).

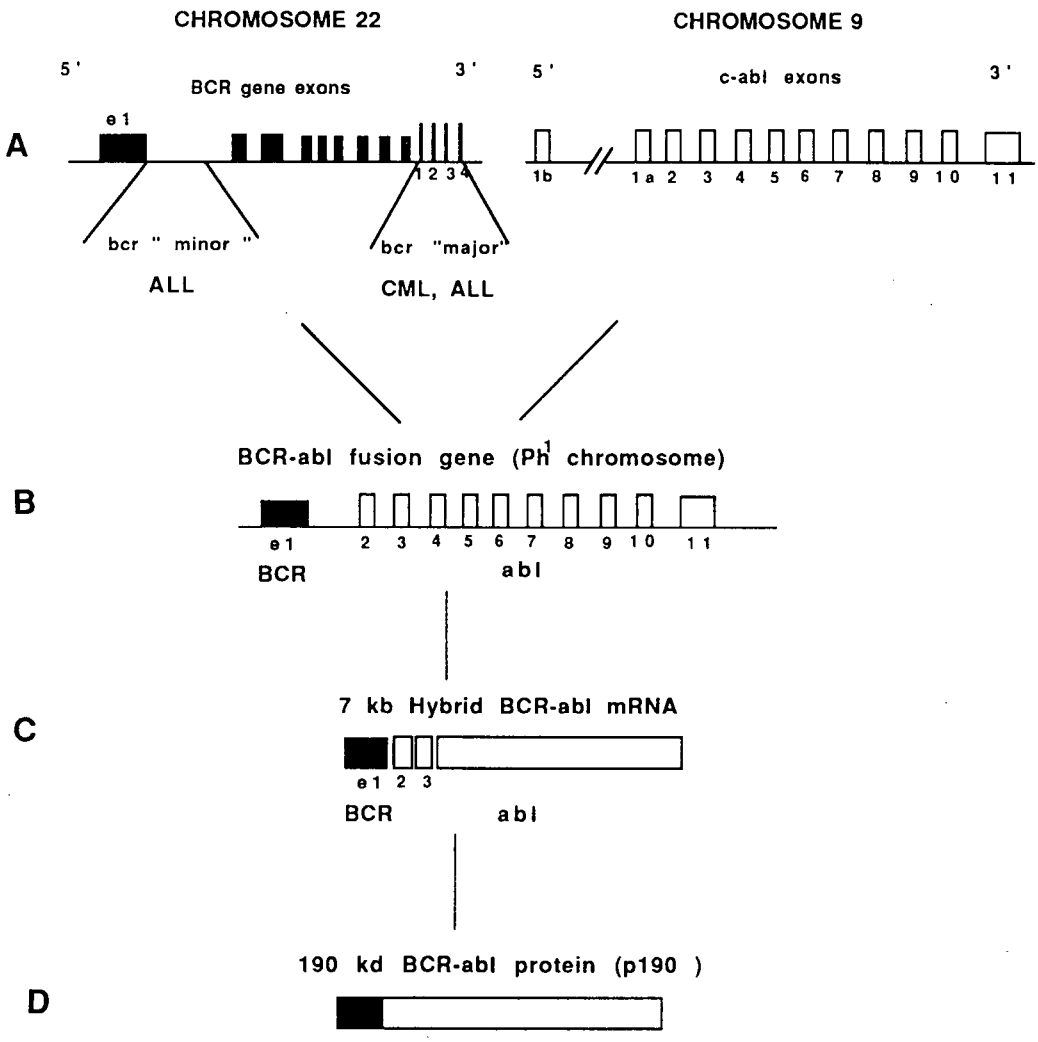


Figure 11.

Molecular rearrangement on the first intron of the BCR gene without involvement of the "major" bcr, leading to 190 kd BCR-abl protein (p190)

A- Black boxes and bars represent BCR exons and open boxes abl exons.

B- BCR-abl fusion gene to which only the first exon of the BCR (e1) contributes.

C- Schematic representation of 7 kb mRNA resulting from the splicing of e1 sequences to a2 (second exon of abl) sequences.

D- Chimeric 190 kd protein with enhanced tyrosine kinase activity.

(C)- POLYCYTHEMIA VERA (PV)

Clinical background. PV is a chronic MPD characterized clinically by an increased red blood cell synthesis, leading to an increased red cell mass. The mean age of onset is 60. Patients usually present with vascular accidents and other symptoms related to the increased red cell mass and splenomegaly. The disease has a chronic course under the influence of phlebotomies or alkylating agents, but more aggressive stages can occur, such as post-polycythemic myelofibrosis and transformation into acute leukemia, the latter being probably influenced by the previous treatment of the disease (335). Initial laboratory values show an increased hemoglobin and hematocrit, leukocytosis with commonly an absolute granulocytosis and a moderate thrombocytosis. Hyperplasia of all bone marrow elements is almost always found (336). The differential diagnosis between PV (which is a MPD) and secondary polycythemia usually relies on a search for a secondary cause of erythrocytosis, e.g. chronic hypoxemia or inappropriate erythropoietin production. Recently, the detection of erythropoietin-independent erythropoiesis in vitro has proven to be a reliable diagnostic marker of PV, as it will be detailed in the next sections. The Polycythemia Vera Study Group (PVSG) has established a number of criteria in order to generate an exclusive PV patient population for assessing various clinical results (337). These criteria are indicated in Table 3.

Chromosomal abnormalities. There are no specific chromosomal abnormalities known in PV. However, some chromosomal abnormalities are encountered non-randomly, in as many as 50 % of patients, especially in the advanced stages. The most commonly reported abnormalities are monosomy 5 or partial deletions of chromosome 5, along with trisomy 8 and 9 (338). Other non-random chromosomal abnormalities are partial deletions of #20 and trisomy 1q (339). There is no evidence that the presence of chromosomal abnormalities

TABLE 3. DIAGNOSTIC CRITERIA - POLYCYTHEMIA VERA

MAJOR CRITERIA

I. Red cell mass >36ml/kg (males) and >32ml/kg (females)

II. Normal arterial O_2 saturation (>92%)

III. Splenomegaly

MINOR CRITERIA

Thrombocytosis: Platelet count > 400 x $10^3/\mu l$

Leukocytosis > 12 x $10^3/\mu l$ (no fever or infection)

Leukocyte alkaline phosphatase activity > 100
(no fever or infection)

Serum Vitamin B_{12} > 900 pg/ml or
Vitamin B_{12}-binding capacity (unbound) > 2200pg/ml

PV diagnosis requires:
Criteria I + II + III or
Criteria I + II + any 2 minor criteria.

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at diagnosis is a poor prognostic sign in PV, as there is no difference in the progression of the disease between the patients with and without chromosomal abnormalities (340). However, in the case of a leukemic transformation, a karyotypic abnormality is almost always found (340).

Hemopoietic progenitors in PV. PV patients, compared to normal controls, do not show an increase of the marrow or blood erythroid progenitor numbers (341) except in patients with marrow fibrosis which is often associated with an increase of all types of progenitors in blood. Thymidine incorporation assays show that the majority of the BFU-E, CFU-E and some CFU-GM are usually in cycle, both in blood and bone marrow (342). The role of an abnormal response to Epo as an important factor in the pathophysiology of PV, was suggested by early studies of Krantz (343) and later confirmed in 1974 by Prchal and Axelrad (232). These authors were the first to report the growth of erythroid colonies in semi-solid media, without added

Epo, in 8 patients with PV. The ratio of the number of progenitors growing without Epo to those growing in the presence of Epo varied from 9 to 37; thus, not all progenitors were able to grow in low-Epo conditions. The authors speculated that an increased sensitivity of some PV progenitors to trace amounts of Epo present in the medium could be the cause of this phenomenon. In 1978, extension of the above observations and establishment of dose response curves to Epo in patients with PV clearly showed the presence of 2 types of erythroid progenitors: some progenitors grow with no added Epo (called Epo-independent) and others require the presence of Epo in the medium,(Epo-dependent), at concentrations required for the growth of normal progenitors (344). Epo-independent growth, was consistently found in all PV patients (341) and therefore, serves as a reliable marker of the neoplastic cell clone, although not all of the erythroid progeny within the clone will display this phenotypic abnormality (231). Epo-independence is thus useful in the differential diagnosis of PV from secondary polycythemia. It seems likely that the Epo-independent phenotype of the erythroid cells in PV is responsible for the overproduction of mature red cells even when this leads to a decrease in Epo levels in vivo. The genetic mechanisms that lead to an Epo-independent phenotype are unknown.

Clonal analysis in PV. Red cells, platelets, granulocytes, monocytes and macrophages are part of the same clone in PV, as demonstrated by G6PD isoenzyme markers (227). Evidence for the involvement of B-lymphoid cells has also come from experiments performed on EBV transformed B-cell lines, obtained from a G6PD heterozygous patient with PV (182). Although most mature blood cells are clonal even after the disease is brought under control by phlebotomy or chemotherapy (345), non-clonal hemopoietic progenitors can also be demonstrated (346). The in vitro detection of non-clonal progenitors in PV and their lack of detection in vivo, even in remission, is consistent with the expectation that normal erythropoiesis would be suppressed by the low Epo levels found in newly diagnosed PV patients. However, how other lineages are suppressed and the mechanisms by which clonal dominance is achieved within them is unknown.

The cycling behavior and Epo-independence of clonogenic progenitors from PV patients have also been investigated in the LTMC system. The proportion of Epo-independent erythroid progenitors obtained from the non-adherent (347) or the adherent (342) fractions of these cultures, was found to be constant after several weeks of LTMC, indicating clearly their maintenance in such a system, as opposed to the result obtained in CML marrow cultures (276). Thymidine- suicide experiments to assess the cycling control of progenitors in the adherent layer of such cultures, demonstrated that the primitive progenitors are continuously in cycle (342) in contrast to the alternating quiescent and activated populations seen in normal LTMC. This situation, analogous to the cycling behaviour of CML blood progenitors in the presence of normal marrow adherent layers (269) suggests that normal mechanisms of a negative regulation may be altered, ineffective, or overridden, in both PV and CML.

(D)- ESSENTIAL THROMBOCYTHEMIA (ET)

Clinical background. ET is a clonal myeloproliferative disorder characterized by megakaryocytic hyperplasia and an increased number of circulating platelets. Because thrombocytosis can occur in all of the MPDs, as well as in patients with iron deficiency (348), chronic inflammatory conditions (348) and neoplasia (348), the diagnosis of ET is usually made by excluding other causes of thrombocytosis. The most commonly used criteria for scientific purposes are those established by the PVSG (349) (see Table 4). However, since these criteria were established primarily to exclude false positives, they are not satisfactory for routine diagnostic or classification purposes, as some, perhaps many patients with ET will not be included.

TABLE 4. DIAGNOSTIC CRITERIA - ESSENTIAL THROMBOCYTHEMIA

I. Platelet count> 600 x 10^3/ μl

II. Hemoglobin< 13g/100ml or normal red cell mass
(males< 36ml/kg, females< 32ml/kg

III. Stainable iron in marrow or failure of iron therapy
(<1g/100 ml of hemoglobin rise after 1 month)

IV. No Philadelphia chromosome

V. Collagen fibrosis of marrow absent (A) or
<1/3 biopsy area without both splenomegaly and
leukoerythroblastic reaction (B)

VI. No known cause for reactive thrombocytosis

ET is also difficult to study because it is a rare disease, occurring mostly in the elderly, some of whom are probably never referred for study. No predisposing factors have been identified. The disease presents and progresses by thrombotic and/or hemorrhagic complications. The major difference with PV is the absence of an expanded red cell mass. Granulocytosis is found at similar level in both disorders at diagnosis (348). Therapy is similar to that for PV, (except for phlebotomy) and includes the use of alkylating agents and P^{32}, both of which have been shown to be potentially leukemogenic in these patients. The occurrence of acute leukemias in the absence of any treatment (350) and transition to other MPD's (351) have been reported. Recently, α-Interferon has been shown to be effective in some patients with ET (352).

Chromosomal abnormalities. No specific chromosomal abnormalities have been identified in patients with ET (120), although aneuploidy has been frequently reported. The

occurrence of the Ph^1-chromosome and bcr rearrangement in patients presenting with a typical ET has also been reported (235).

Clonal analysis in ET. In 1981, Fialkow et al (228) studied 3 G6PD heterozygotes patients with ET, and showed that in all, a single isoenzyme pattern was present in granulocytes, red cells and platelets, whereas both A and B enzymes were present in skin fibroblasts. In two of these patients, the analysis was performed post-cytoreductive chemotherapy (P^{32} and busulfan), indicating that this treatment did not induce a return to a non-clonal state. In 1982, Gaetani et al reported similar results in 2 patients with ET (353). The involvement of the lymphoid system was also suggested by studies of the G6PD pattern of extracts from lymphoid cell lines established from a patient with ET, which contained only a single isoenzyme, and the same one as was present in granulocytes and platelets (354). Thus, the multipotentiality of the abnormal stem cell clone is a similar feature between ET, CML and PV.

Hemopoietic progenitors. Epo-independent erythroid colony growth has been found in some patients with ET (355,356). In general progenitor numbers have not been found to be significantly elevated (262), although circulating progenitors, particularly CFU-Mk may be increased (357). The extent to which this may be related to fibrosis in these patients has, however, yet to be determined. Abnormal cycling of primitive marrow progenitors has been identified in some patients with ET (231).

4- THESIS OBJECTIVES

It is known that in normal human hemopoietic system, at any given time, many stem cells are actively contributing differentiated progeny into the circulating pool of mature blood cells. Conversely, there is data to support the hypothesis that hemopoietic malignancies, like many other cancers, represent clonal overgrowths of single transformed cells that have

acquired deregulated proliferative properties. What is not well established is, the extent to which these two behaviors are exclusive to or even indicative of these two biological conditions: i.e., does polyclonal hemopoiesis occur in some hemopoietic malignancies (and if so are these clones normal -Figure 12A) and can normal hemopoiesis be clonal (Figure 12B). The answers to these questions might have important implications both for our understanding of normal and neoplastic hemopoiesis and in terms of the design of future therapies. To explore these possibilities I chose to study two extreme situations, one where normal hemopoiesis is maximally stimulated (i.e., hemopoietic regeneration after transplantation of normal human marrow) and the other where neoplastic hemopoiesis is minimally different from normal (i.e., in the various myeloproliferative disorders).

The possibility that a single normal hemopoietic cell might have sufficient proliferative activity to repopulate the entire hemopoietic system of experimental animals has been demonstrated using retrovirally marked stem cell transplants in mice (discussed above). In humans retrovirally marked stem cell transplantation experiments are not yet permissible. I therefore chose to explore this question by application of the recently developed clonality analysis method based on examination of methylation-sensitive RFLP's, to cells generated after transplantation of normal bone marrow from HPRT and/or PGK heterozygous donors. The results of these studies are presented in Chapter IV.

However, in order to investigate the validity of this approach I first evaluated the frequency of heterozygous females (to ensure that sample acquisition would not be limiting). In addition, I examined the fidelity of the banding patterns expected for clonal and polyclonal populations using physically cloned cell lines that had also been transformed. The results of these control studies are described in Chapter III.

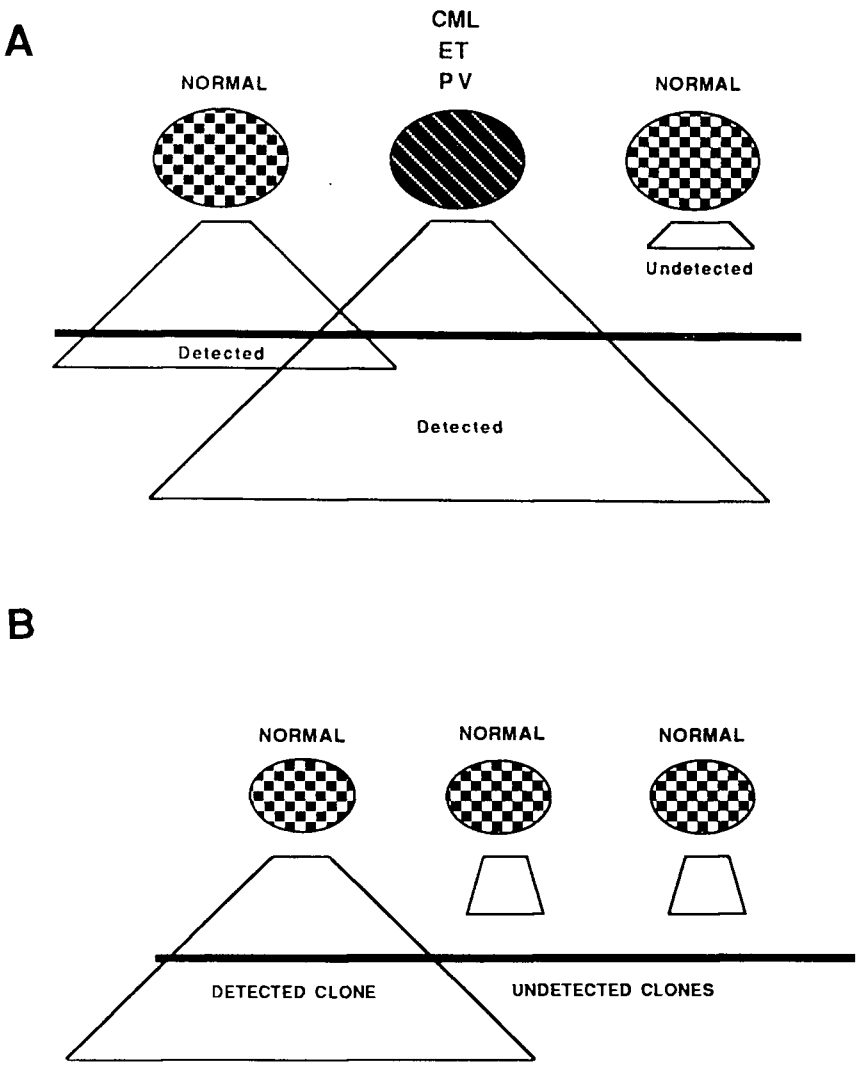

Figure 12A.
Polyclonal Hemopoiesis in Hemopoietic Malignancies

Figure 12B.
Clonal Expansion of Normal Hemopoietic Cells as an Extreme of Stem Cell Behavior

Normal hemopoietic cells are thought to be suppressed in many hemopoietic malignancies, including essential thrombocytosis. However, the question of whether non-clonal hemopoietic cells coexist with the abnormal transformed clone in any or most patients with ET has not been addressed. My next objective was therefore to identify likely ET patients who were also heterozygous at the HPRT or PGK loci and to correlate the clonality status of their granulocytes with other markers associated with neoplastic hemopoietic populations such as Epo-independent erythroid colony growth and/or abnormal progenitor cycling. From these studies I sought to determine whether polyclonal cells could be detected in the presence of cells with other markers of malignancy or alternatively, if clonality results and the presence of these markers would always be correlated. Such a demonstration has the obvious implication that modulation or activation of such non-clonal cells might then be considered as a possible therapeutic approach in this (or other) myeloproliferative disorders. The results of these studies are presented in Chapter V.

In addition to investigating ET, I also explored related questions in cell populations derived from patients with CML. The specific new area appropriate to address in the context of this disease was indicated by recent LTMC findings and a clinical trial just initiated in Vancouver to evaluate the feasibility of using LTMC cells from CML patients as autografts to enable potentially curative doses of chemo-radiotherapy to be given to the patient. It was therefore of interest to see if molecular techniques could be used to detect the disappearance of leukemic cells in the LTMC system. For this I used probes to detect DNA from cells carrying a rearranged BCR gene which is involved in the formation of Ph^1-chromosome. In addition I planned to compare this with changes in the clonality of the hemopoietic cells produced if suitable patients were available. I also planned to analyse the hemopoietic cells regenerated in vivo after transplantation of the cultured bone marrow with respect to the same two markers (bcr rearrangement and clonality status). The results of these efforts are presented in Chapter VI.

Another situation where I envisaged that polyclonal normal hemopoiesis and neoplastic cells might coexist in the same patient within the hemopoietic system was where the origin of the neoplastic clone might have occurred in a lineage-restricted derivative of the most primitive type of pluripotent stem cell. An example of a hematologic malignancy where this issue had not been resolved was Ph^1-positive ALL, still considered by some to represent an entity distinct from CML in lymphoid blast crisis. While in the latter the multipotential stem cell origin of the disease was well established (Figure 13), in the former there was not convincing data demonstrating a lymphoid-restricted origin. My goal was therefore to use both X-linked and bcr

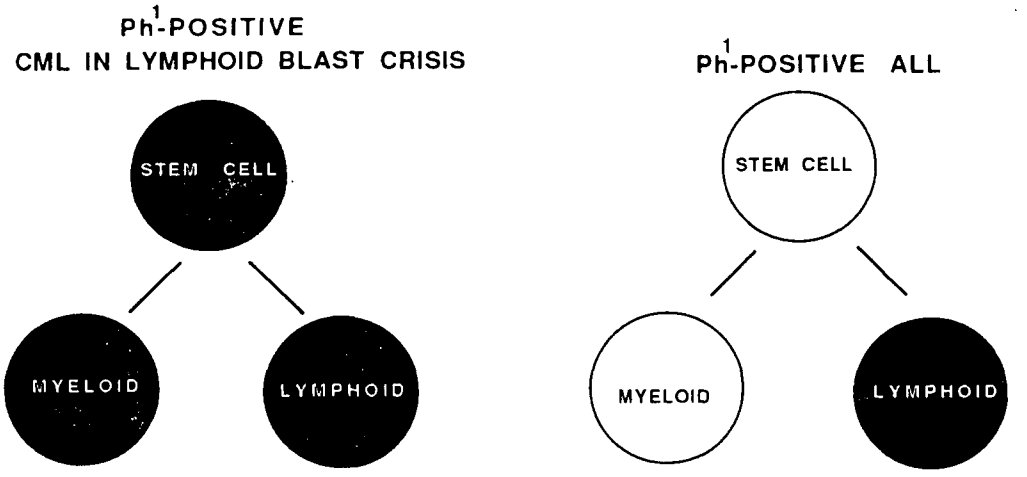

Figure 13.
Diagram showing blast crisis of CML in which there is evidence of pluripotent stem cell involvement (black circle) and Ph^1-positive ALL in which pluripotent stem cell may not be involved (open circle).

probes to determine the involvement of cell populations from patients presenting with a clinical diagnosis of Ph^1-positive ALL. The results of this work is presented in Chapter VII.

From these investigations, I hoped to obtain a better understanding of the limits of normal and malignant hemopoietic stem cell behavior.

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C H A P T E R II

MATERIALS AND METHODS

1- PATIENTS

Samples of cells from blood and marrow specimens were obtained as leftover material from specimens referred to the Stem Cell Assay Service of the Cancer Control Agency for routine clinical evaluation. This included ~150 patients with a differential diagnosis of CML, PV, ET, some form of myelodysplasia or acute leukemia. In addition ~78 samples were obtained with informed consent from normal volunteers or patients with non-hematologic malignancies (blood) and normal marrow transplant donors (marrow). In some cases, various cell populations in different specimens were cryopreserved in medium containing 20% FCS and 10% DMSO and then subsequently thawed rapidly at 37°C to maximize viability prior to use.

2- CELL SAMPLES

a. Blood.

(i) Purification of granulocytes: Heparinized sterile peripheral blood samples were separated by density gradient centrifugation on Ficoll-Hypaque (supplier) to give a light density (<1.070 g/cm^3) mononuclear fraction (PBMC) and a pure granulocyte fraction overlaying the pelleted cells. These two fractions were removed separately and the red cells contaminating the granulocyte fraction lysed by exposure to an NH_4Cl buffer (0.83% NH_4Cl and 0.1% NaHC03, pH=7) for a few minutes at 4°C. Cell suspensions were then centrifuged at 250g for 10 minutes and the pelleted cells washed twice in PBS prior to DNA extraction. Removal of an aliquot from

the granulocyte fraction for morphological assessment showed these suspensions to contain routinely >90% granulocytes in May-Gruwald Giemsa stained cytospin preparations.

(ii) T-cell isolation: When the mononuclear cell fraction contained adequate numbers of cells, a T-cell separation was performed using sheep red blood cell rosetting (1). SRBC were treated with AET (5-2-aminoethylisothiouronium bromide hydrobromide) which was prepared to a concentration of 0.14 M, pH=9, just prior to use. In a sterile 50-ml centrifuge tube, 16 ml of AET solution and 1 ml of washed, packed SRBCs were combined, gently mixed and incubated at 37^{o}C for 15 minutes. SRBC were then suspended in PBS, pelletted by centrifugation for 10 minutes at 300g and this was repeated once. From this stock solution, an AET-SRBC suspension of 1% was prepared just before use, by combining 4cc of FCS, 6cc of alpha medium and 0.1 ml of packed AET-SRBC. Light density cells obtained from the Ficoll-Hypaque separation were treated with heparin (Sigma Chemicals, 100 units/ml) and washed twice with alpha medium. After counting cells, these were diluted to 5 x 10^6 per ml. For rosetting, equal volumes of 1% AET-SRBC solution and cells were mixed in a 50 ml centrifuge tube and incubated at 37^{o}C for 5 minutes. Cells were then pelletted by centrifugation at 300g for 5 minutes and incubated on ice for 1 hr. The rosetted cells were then suspended, loaded onto Ficoll-Hypaque in 50 ml tubes (3 parts Ficoll-Hypaque and 2 parts rosetted cells) and spun for 30 minutes at 450g. Interface cells were then removed, washed, counted and plated in methylcellulose assays or used for DNA extraction (as described below). Pelletted cells were lysed with cold NH_4Cl-Tris buffer, washed and a second rosetting performed on an aliquot of 0.5 X 10^6 cells. Rosetted and non-rosetted cells were then counted and the efficiency of the procedure calculated. The proportion of rosette-positive cells after this T-cell separation procedure varied between 70% and 94%. Cells from the initial light density fraction that remained after the second Ficoll-Hypaque separation (i.e. were not pelletted), consisted primarily of monocytes and B-cells. In the text they are referred to operationally as the non-T cell fraction.

b. Bone Marrow (BM)

Marrow aspirates had been collected originally into 1 ml of sterile heparinized medium. Because of the limited number of cells in some samples, cells for use in the present studies were not always available. Cells were processed for DNA extraction directly after lysis of the red cells using NH_4Cl as for blood granulocytes. Whenever possible, an aliquot of the marrow sample was used to initiate fibroblast cultures by seeding 1-2 x 10^6 cells in 25 cm^2 flasks containing 8 ml of alpha-medium plus 20% FCS. Fibroblasts were grown to confluence and subcultured at 2-3 week intervals for several passages and then harvested and, if not used directly, stored at -20°C.

c. SV-40 Transformation of Marrow Fibroblasts

Marrow fibroblasts from 3 patients (Chapter 3) were transformed by a high titer SV-40 virus stock using a procedure previously described (2). Dense colonies obtained on the initial coverslip cultures were individually trypsinized using cloning wells and plated in alpha medium plus 20% FCS made viscid with 0.8% methylcellulose. Colonies of fibroblasts were only obtained from cultures containing SV-40 infected cells. Individual well-isolated clones were then removed with a pasteur pipette, transferred to fresh flask cultures containing liquid medium amplified prior to harvesting for DNA extraction.

3- LONG-TERM MARROW CULTURES (LTMC)

a. Regular LTMC

Two 2.5 x 10^7 nucleated marrow cells were used to start these cultures at 37°C in 60 mm x 15 mm Falcon tissue culture dishes, each containing 8 ml of long-term culture medium. This latter consisted of alpha medium supplemented with inositol (4mg/100ml), folic acid (1mg/100ml), glutamine (40mg/100ml), FCS (12.5%), horse serum (HS, 12.5%) 2

mercaptoethanol (2-ME, 10^{-4} M) and hydrocortisone sodium hemisuccinate (10^{-6} M). After 3 or 4 days, the cultures were switched to 33°C and then fed at weekly intervals with half medium changes and simultaneous removal of half of the nonadherent cells. Adherent cells were harvested following removal of all of the nonadherent cells and exposure of the remaining cells to trypsin. This allowed all of the remaining cells in the culture to be suspended and thus resulted in termination of the culture. Cells from both nonadherent and adherent fractions were suitable for use in colony assays.

b. LTMC for CML autografts

These were established and maintained for 10 days as described above for regular LTMC with the exception that the first half-medium changed on Day 7 was eliminated so that the possibility of infection due to handling of the cells or medium during the first 10 days was greatly reduced. At the end of the first 10 days, flasks containing cells destined for autografting were harvested and infused into the patient, with aliquots being retained for DNA analysis (see below) and colony assays. The number of cells infused to patients varied between 1.6×10^8 and 2×10^8/kg of body weight. A number of small replicate LTMC were also maintained for more prolonged periods of time to allow assessment of their clonogenic progenitor content and genotype. In the case of patient UPN#248, LTMC were also set up at a later time with a thawed aliquot of cryopreserved marrow removed from the same harvest used to initiate cultures for autografting. The recovery of viable cells (based on 1% nigrosin dye exclusion) after thawing these cells was 40% and 10^8 "viable" cells were then inoculated into each of eight $75cm^2$ flasks containing 30 ml of standard long-term culture medium (3). These were maintained for 4 weeks as regular LTMC's, at which time one culture was sacrificed and all of the cells harvested to allow colony assays and cytogenetic analysis of the colonies obtained (4) to be performed. At that time DNA was also extracted from the trypsinized adherent layer cells. After 4 weeks, all of the medium containing all of the non-adherent cells was then removed weekly and the cultures were each time refed with a modified long-term

medium (containing 30% FCS instead of 12.5% FCS; and 12.5% HS and 10% medium from one week old confluent 5637 cell cultures) (5). Removal of all nonadherent cells and replacement of all the old medium with an equal volume of this modified medium was continued weekly up to week 8 at which time all cells were harvested and the experiment terminated.

4- METHYLCELLULOSE ASSAYS

Cell suspensions to be assessed for their content of clonogenic hemopoietic progenitors were washed, diluted and then plated in methylcellulose cultures containing 30% FCS, 1% deionized BSA, 10^{-4} M 2-ME, and 10% human leukocyte-conditioned medium (6). Cultures to which Epo (7) was added contained a final concentration of Epo of 3 U/ml. Cultures to which no Epo was added contained <0.002 U/ml of Epo (8). Cells were plated in 35-mm petri dishes at a final concentration of 2×10^5 fresh marrow cells, 4×10^5 PBMC (or lower as required in some cases of CML), and 10^5 LTMC cells per 1.1 ml culture. Small erythroid colonies were scored 10-12 days later and large erythroid colonies, granulopoietic colonies and occasional mixed colonies were scored after 18-21 d according to standard criteria (6-8).

5- DNA EXTRACTION

a. Reagents.

SDS (Sodium Dodecyl Sulphate) (BRL) 20% w/v in water,filter sterilized.

Proteinase K. (BRL) 100 mg of proteinase K powder were dissolved in 10 mls of water and boiled 10 minutes. This stock solution was then aliquoted and stored at -20°C.

Phenol. Chromatography-grade crystalline phenol (BDH) was thawed at 65°C and then equilibrated with 0.5M Tris, pH 7.5. Simple shaking resulted in the formation of an interlayer after which the supernatant was then removed and discarded. After two Tris equilibrations, the

phenol was saturated by adding an equal volume of TNE buffer (10mM Tris pH 8.0, 150mM NaCl, 10mM EDTA), the mixture shaken briefly, and the aqueous upper layer was then removed. This equilibration was performed 3 times and the final stock phenol was then stored at -20°C in 50-ml tubes wrapped in aluminum foil.

Chloroform. (BDH) Mixed with isoamylalcohol to the ratio of 24:1 prior to use.

b. Procedure.

Cells were washed twice in PBS, suspended in TNE buffer (up to 2 ml for each 10^7 cells), and then 40µl of 20% SDS and 40 µl of proteinase K (10 mg/ml stock) added (per 10^7 cells). The sample was then incubated overnight at 37^oC. After the SDS-proteinase K digestions, the sample was ready for DNA extraction. The cell lysate was mixed with an equal volume of TNE-equilibrated phenol and gently shaken by hand until aqueous and organic phases were mixed. Two phases were separated by spinning on a bench top centrifuge at 300g for 10 minutes. The DNA containing aqueous top layer was removed using a large pipette avoiding the denatured protein at the interface. Following a second phenol extraction using the same volume of new phenol, the sample was extracted once with an equal volume of phenol-chloroform (1:1) and then twice with an equal volume of chloroform. The aqueous layer from the last chloroform extraction was then dialyzed against 100x volumes of 1xTE solution at 4^oC, with at least 3 changes. After dialysis, the DNA solution was collected and its concentration determined using UV spectrophotometry. A260-280 ratio was also determined to assess protein contamination. DNA samples were kept subsequently at 4^oC.

6- SOUTHERN ANALYSES

a. Restriction enzyme digestions

Restriction enzymes were purchased from Bethesda Research Laboratories (BRL, Gaithersburg, MD) and assayed using conditions recommended by the manufacturer. Routinely, 5 to 10 units of enzymes were used for 1 µg of DNA digested.

For HPRT analyses, 15-20 µg of DNA was first digested with BamH I for 2-4 hours at 37°C in the buffer provided by the manufacturer using a total reaction volume of 400 µl. The sample was then precipitated by adding 100 µl of 10 M ammonium acetate and 1000 µl of ice-cold ethanol, and incubated in dry ice for 20 minutes. After centrifugation at 12000g at 4°C for 20 minutes, the precipitated DNA pellet was washed with 70% ethanol, incubated 5 minutes in dry ice, and centrifuged again at 12000g for 5 minutes. The supernatant was carefully discarded without mobilization of the pellet which was dried in a vacuum centrifuge. When completely dry, the pellet was dissolved in 600 µl of Tris-EDTA buffer (3mM Tris and 0.2mM EDTA, pH=7.5) and incubated at 37°C for 30 minutes. This volume was then divided in 3 equivalent aliquots of 200 µl each. One aliquot (labeled a) was left undigested at 4°C. The two others (labeled b and c) were further digested with a 5-fold excess of either Hpa II or Hha I, respectively, in a reaction volume of 400 µl. They were then precipitated as above along with the sample a, dried and suspended in 16 µl of 1X TA buffer. After addition of 4 µl of tracking dye, the samples were vortexed, centrifuged briefly at 12000g incubated at 65°C for 5 minutes and loaded in 1% agarose gels.

For PGK analyses, a similar strategy was followed, using first digestion of DNA with EcoR I and Bgl II in order to bracket the differentially methylated region (9), followed by the Bgl I digestion. The sample was then divided in 2 aliquots; one was left undigested and the other further digested by Hpa II. In some cases, the BstX I polymorphism of the PGK gene was also used in conjunction with Pst I as described by Vogelstein et al (9). These samples were then electrophoresed in 1.5% agarose gels.

For other RFLP analyses required to document polymorphisms, a number of DNA samples from unrelated patients were digested with the most useful enzymes reported in the literature according to the recommendations of the manufacturer.

For bcr rearrangement studies, DNA samples were digested with BamH I, EcoR I, Bgl II and Hind III, which are the 4 most useful enzymes for detection of this rearrangement. The samples were then electrophoresed in 1% agarose gels.

b. Agarose gel electrophoresis

Reagents. Ultrapure agarose (BRL) was prepared w/vol in 1 X TA buffer by boiling and cooling to 50^{o}C prior to pouring into gel trays. Routinely, 200 ml of agarose was used in a gel tray of 14.5cm X 16.5 cm, resulting in a thickness of 5mm.

Procedure. DNA samples were carefully loaded into individual wells and electrophoresed at 2-4v/ cm of gel length for 12-16 hours (in practice overnight) until the first dye marker had progressed more than two-thirds of the way across the gel. The gel was then stained with ethidium bromide for 15-20 minutes, destained for 15-20 minutes in TA buffer and photographed under the UV light. The purpose of this was not only to detect incomplete digestions but also to determine if the samples subjected to methylation-sensitive enzymes were equivalently distributed after the initial digestion with BamH I, Bgl I and BstX I. In critical samples, the completeness of the digestion was assessed by running an aliquot of the reaction mixture on a diagnostic gel prior to the termination of the experiment. All critical samples were analysed at least twice under identical conditions. Molecular weights were determined using standard molecular weight markers (λ Hind III fragments) that could be readily seen in the polaroid photograph, allowing calculation of the molecular weights to be made.

c. Southern blotting

After photography, gels were generally depurinated for 10 minutes in 0.25 M HCl, except for PGK analyses. A piece of Zeta probe (Bio-Rad Laboratories, Mississauga, Ontario) was cut to the size of the gel and soaked in double-distilled and deionized water for 10 minutes. An alkaline transfer procedure (10) was then carried out in 0.4 M NaOH buffer, laying first the gel onto two pieces of 3MM Whatman filter paper. The water-saturated Zeta Probe was then placed onto the gel and care was taken to eliminate all air bubbles. Two wetted pieces of Whatman paper were layered on top of the membrane with removal of all air bubbles. A stack of paper towels was then placed on top of the 3MM filter paper. A glass plate with a small weight was placed last on top of the paper. The transfer was allowed to take place for at least 8-10 hours, or longer for high percentage gels. After transfer, the membrane was neutralized in 2 X SSC for 10 minutes, and either baked under vacuum at 80^{o}C for 30 minutes (if not hybridized immediately) or processed as for prehybridization.

d. Prehybridization and Hybridization.

(i) Reagents:

SSC Buffer, 20X (NaCl 3M, Na Citrate 0.3M, pH=7)

Formamide (BRL, Gaitersburg, MD) was deionized using methanol-washed Rexyn I-300 (Fisher Scientific Laboratories). 6 grams of resin per 100 cc of formamide was stirred at 4^{o}C in dark overnight. After filtration through glass wool, the OD at A270 was routinely < 0.100 and pH=7.0. Formamide was kept at -20°C and thawed immediately prior to use.

Dextran Sulfate (Sigma Chemical Co., St. Louis, Mo.) was prepared as stock solution of 50% in water, containing 0.2% of sodium azide and was stored at 4°C.

Blotto (Carnation Milk) was prepared as a 10% stock solution in water containing 0.2% sodium azide, stored at 4°C.

Salmon sperm DNA (Sigma Chemicals) was denaturated and sheared by boiling and then by being passed through an 18-gauge needle several times. A stock solution of 10 mg/ml in water was kept at 4°C.

(ii) Procedures:

Prehybridization Procedure- This was performed for 4-6 hours at 60°C in a 20 ml buffer (0.9 M NaCl, 0.09 M sodium citrate, 10% formamide, 1% SDS, 2mM EDTA, 1% nonfat dried milk, and 0.5 mg/ml denaturated salmon sperm DNA). Hybridization conditions were the same except for the inclusion of dextran sulfate to a final concentration of 10% and the increase of formamide concentration to 20%. After hybridization for 18-20 hours, filters were washed at 60°C every 30 minutes in 0.1% SDS, 0.1% SSC and 0.1% sodium pyrophosphate with 4 changes. A final wash at 68°C was necessary when the PGK probe was used, in order to eliminate cross-reaction with PGK-related genes (9). Similarly, a final wash at 65°C was often required to reduce background hybridization with the use of HPRT-p800 probe.

(iii) Probes:

Both HPRT probes (p600 cloned in the AccI site of pGEM4 plasmid and p800 cloned in the AccI/PstI site of pGEM4) and the PGK probe (pSPT phosphoglycerate kinase cloned in the BamHI/EcoRI site of pSP64) were generously provided by Dr.B.Vogelstein (John Hopkins University, Baltimore, MD). These fragments were isolated from the 1.7kb 5' HPRT probe originally cloned by Jolly et al (11). HPRT p600 contains a 600bp Hpa II subfragment and HPRT p800 contains an 800bp Hpa II-Pst I subfragment of pPB1.7 (9). bcr probes used included the 1.2 kb Hind III-Bgl II fragment from the 3' end of the bcr (probe-1, Oncogene Sciences, Mineola, NY) and a 2.1 kb Bgl II-Hind III fragment from the 5' region of the bcr (generously provided by Dr.D.Leibowitz). Six probes (human c-Ha-ras, pEFD64.2, pEKZ130, pYNZ86.1, pYNZ22, and pCMM12) for the detection of high frequency RFLP's on somatic chromosomes (12) were also purchased from the American Type Culture Collection and processed as summarized below.

To prepare plasmids, bacteria were transformed, amplified, lysed with 0.25 M lysozyme (10 mg/ml pH 8), and then centrifuged at 43700g for 35 minutes. Pellets were discarded, and cleared cell lysates loaded on CsCl-ethidium bromide gradients for ultracentrifugation at 160000g at 15°C for 18-24 hours. The sealed tubes were then examined under the UV light and the lower band corresponding to plasmid DNA was aspirated using a syringe and needle. The sample was dialyzed against 1xTE with 3 changes, then precipitated in 0.2 M ammonium acetate and 2 volumes of ice-cold ethanol. The concentration was determined using UV spectrophotometry and diagnostic gels with several restriction enzyme cuts were performed to verify both the quality of the plasmid preparation and the restriction cuts required for the isolation of the desired insert. Appropriate cuts were then performed to isolate subfragments of the plasmids.

Plasmids were then digested for 4-6 hours as appropriate to liberate desired inserts, precipitated with ethanol and electrophoresed in 1.5-1.8% agarose gels for 2-4 hours using preparative combs. After staining with ethidium bromide and photography under the UV light, the desired bands were cut out of the agarose gels using a sterile razor blade. The bands were placed in large dialysis bags, which had been previously prepared by boiling in 2% Na_2HCO_3 with 1 mM EDTA. 1 ml of 1 x TA buffer was added to the bags and dialysis clips placed after taking out all air bubbles. The tubes were then placed horizontally in an electrophoresis apparatus and completely immersed in 1 x TA buffer. A high voltage between 150-200 volts was then applied for 1 hour, at the end of which the polarity of the current was briefly (1-2 minutes) reversed, in order to remove DNA fragments from the bag walls. The solution was carefully taken out from the bag, 1 ml of 1 x TA buffer was added and the bag thoroughly massaged. This fraction was added to the initial recovery and centrifuged at 12000g for 10 minutes to remove gel fragments pelletting at the bottom of the tubes. The supernatants were then transferred to new microtubes, and extracted twice with an equal volume of chloroform. After ethanol precipitation in 0.2M ammonium acetate, DNA pellets were washed with 70% ethanol, dried under vacuum and suspended in 1 x TE buffer (10 mM Tris.Cl, 1 mM EDTA, pH

8). Diagnostic gels were then performed with serial dilutions of the fragment obtained, to check the quality of the insert and its concentration, and compared to the uncut plasmid control.

The probes were radiolabelled following the multiprime labelling (oligolabelling) procedure (13). Multiprime DNA labelling kits were purchased from Amersham Canada. 20-50 ng of DNA was mixed with 10µl of a Tris-H6l buffer pH 7.8, containing dATP, dGTP, dTTP and 2-ME. 5µl of a solution containing random hexanucleotides and BSA were then added and the reaction volume was brought up to 48µl by adding an appropriate volume of water and 5µl of high-specific activity [α-^{32}P]dCTP (3000 Ci/mmol). The DNA was denatured by boiling the reaction volume for 5 minutes and then cooled on ice for 2 minutes. The enzymatic reaction was then started by adding 2 µl of a solution containing the Klenow fragment of the DNA polymerase I at a concentration of 1 unit/µl. After incubation at room temperature for 2-6 hrs, unincorporated nucleotides were removed by passing the reaction volume through a column of G-50 Sephadex (Pharmacia). Using this procedure, the specific activity of the labelled products was routinely between 10^8 and 10^9 dpm/µgr of DNA. Probes were mixed with salmon sperm DNA, boiled for 10 minutes and added to hybridization buffers.

A procedure to remove repetitive sequences from the HPRT p800 probe was used because this probe contains repeated sequences that otherwise induce high backgrounds on the blots. Human DNA obtained from HL-60 cell lines was denatured by boiling in 0.4M NaOH, cooled on ice and neutralized to pH=7 by adding 3M Na Acetate pH=5. After dialysis against 1 x TE, DNA was precipitated and suspended in 1 x TE buffer to high concentration, between 1-2 mg/ml. Denatured salmon sperm DNA at a concentration of 10 mg/ml was also used in some reactions. Following essentially the original protocol (14), 100µl of the labelled HPRTp800 probe was mixed with 100 µl of 20 X SSC and 200µl of HL-60 DNA, boiled 10 minutes, incubated at 68^{o}C for 20-30 minutes and added to the hybridization bag along with the denatured salmon sperm DNA. If the latter was used in the preannealing reaction, it was omitted in the hybridization mixture.

(iv) Autoradiography

After washing, blots were exposed to Kodak XAR-5 radiographic films using intensifying screens for 13-96 hours at -70^{o}C. The films were screened using a Hoefer scanning densitometer.

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C H A P T E R III

EXPERIMENTAL VALIDATION OF CLONALITY ANALYSIS BY ASSESSMENT OF METHYLATION-SENSITIVE RFLPs ON THE X-CHROMOSOME

1. INTRODUCTION

My first objective was to evaluate the validity of the methodology to be used for assessment of the clonality status of patient samples (or primary cultures established from them). I thus undertook a series of "control" experiments to determine: (1) the consistency of the expected single band in Southern blots of DNA from male patients after hybridization with the p600 or p800 HPRT probes, or with the pSPT PGK probe using the protocol to be adopted for all subsequent studies and described in detail in Chapter II; (2) the proportion of informative females in British Columbia, i.e. the proportion of females who were heterozygous for the two X-linked polymorphisms required for clonality analysis; (3) the clonality of normal tissue samples from such female heterozygotes; and (4) the effect of Hpa II or Hha I digestion on the size of fragments obtained from DNA extracts of physically cloned neoplastic cell lines. The results of these studies are described below.

2. RESULTS

A. Frequency of Heterozygosity in Females and Hemizygosity in Males

Peripheral blood and/or bone marrow samples were obtained from patients with various non-hematological malignancies (total of 50), patients with a diagnosis of MPD, AML, myelodysplasia (total of 150), as well as from normal bone marrow donors and normal peripheral blood volunteers (total of 28). From these, DNA was extracted, digested and then analyzed by Southern blotting using both HPRT and PGK probes. Figure 14 shows two

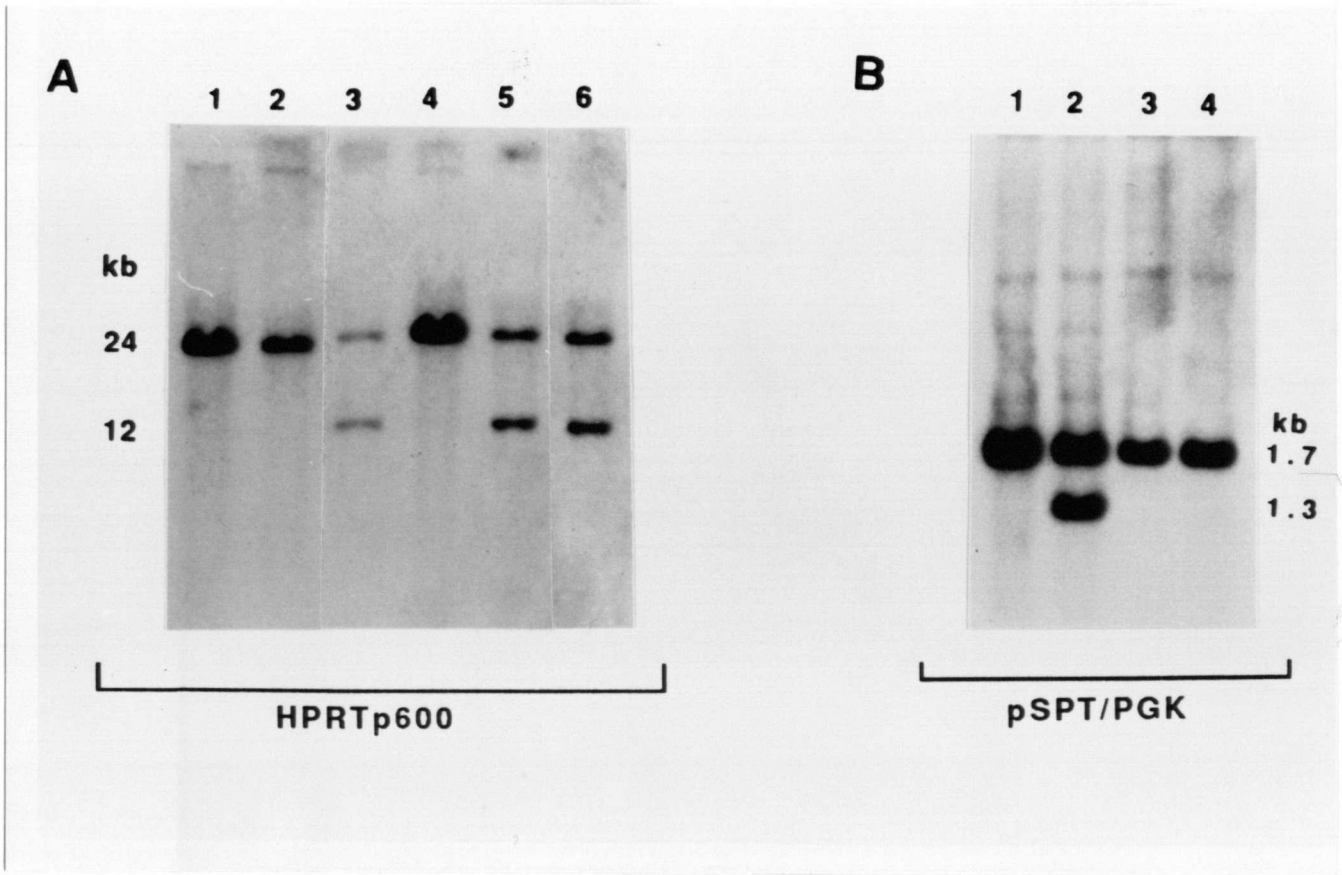


Figure 14

Two representative examples of screening blots to determine heterozygosity for BamH I or Bgl I polymorphism.
Panel **A**, Lane 1, shows a male BMT recipient without the polymorphic BamH I site at the HPRT locus. Two females also lacking this site (lanes 2, 4) and 3 females heterozygous for this site (lanes 3, 5, 6) are shown.
Panel **B**, DNA from 4 patients digested with EcoR I, Bgl II, Bgl I and probed with pSPT/PGK. Lane 1: Male BMT recipient. Lane 2: Heterozygous female. Lanes 3 and 4: two homozygous females

representative examples of screening blots to determine heterozygosity for HPRT/BamH I (panel A) or PGK/ Bgl I (panel B) polymorphisms. In two male BMT recipients analysed, an expected single band was found (panel A, lane 1 and panel B, lane 1). Three female patients were heterozygous for the BamH I polymorphism of the HPRT gene (panel A, lanes 3, 5, and 6) whereas two patients lacked the polymorphic site (panel A lanes 2 and 4). Figure 14, panel B shows one patient heterozygous for the Bgl I polymorphism (lane 2) and two other females lacking this site, therefore homozygous and not analysable by this technique (lanes 3 and 4). As summarized in Table 5, approximately half of women analysed (total of 206) in British Columbia are heterozygous for at least one of the two polymorphic sites studied as also found by Vogelstein et al in a similar survey (1). All male samples studied showed a hemizygous pattern as expected.

TABLE 5
PREVALENCE OF HPRT/PGK HETEROZYGOSITY

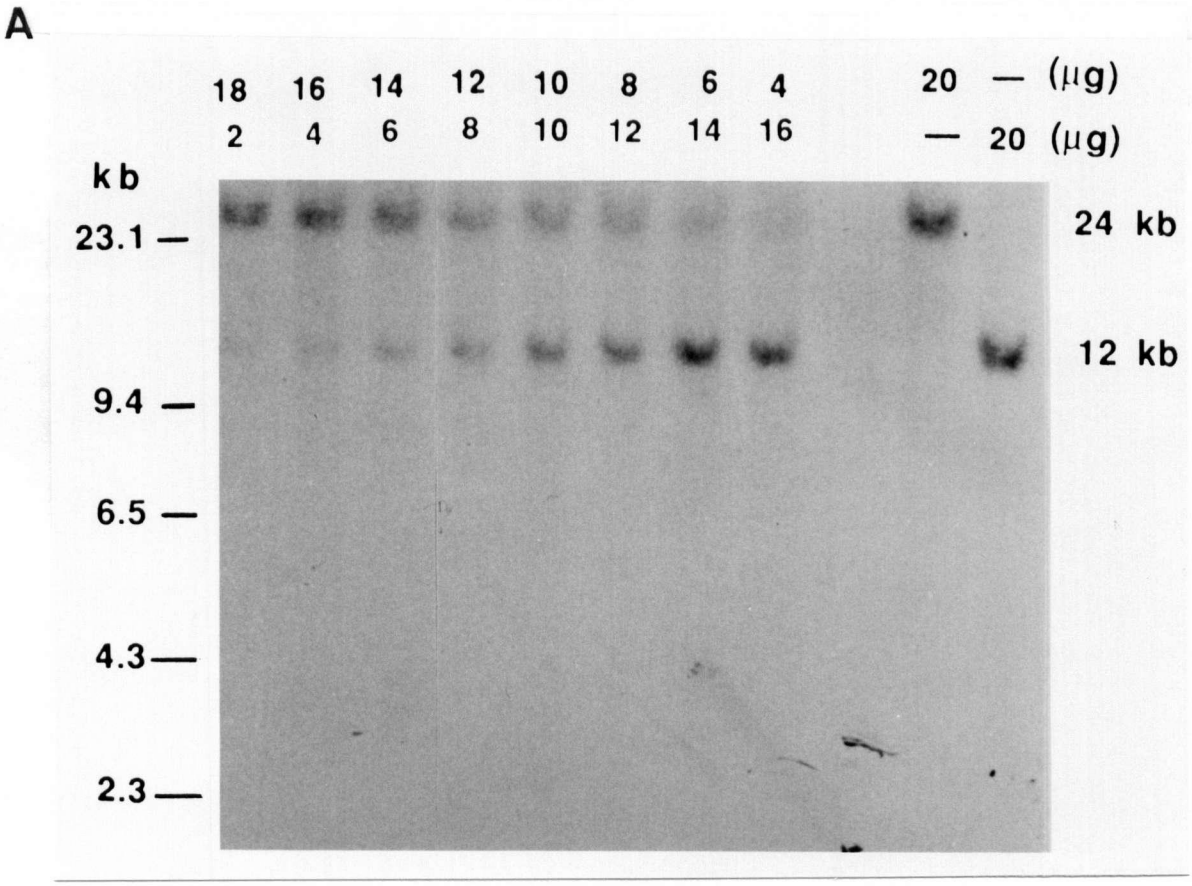
GENE	PATIENTS SCREENED	INFORMATIVE	HETEROZYGOSITY (%)
HPRT	206	51	25
PGK	206	48	23
HPRT/PGK (Double Heterozygotes)	206	7	3
TOTAL	206	106	51

B. Clonality of Normal Tissue Samples

Sensitivity- An estimate of the sensitivity of the method was necessary for an accurate evaluation of clonality patterns. Since the method involves the comparison of bands digested with methylation-sensitive enzymes to those not digested with methylation-sensitive enzymes,

the first important variable was the consistency of the intensity of large and small allelic fragments in lanes loaded with DNA that had been digested to reveal the polymorphic site but not with HpaII or HhaI. The intensity of these two fragments was expected to be equal, giving a value close to 0.5 if the signal intensity of one band (S_1) was compared to the combined signal intensity of both bands (S_1+S_2). Such measurements were performed on several autoradiographic films exposed between 1 and 4 days. The mean ratio obtained was 0.5 ± 0.045 (1SD), (n=20). Occasionally, the intensity of both bands was not similar, and ratios as high as 0.60 or as low as 0.43 respectively, were encountered. An explanation for this finding could be that the probe had repetitive sequences hybridizing more intensively to large alleles (as appeared to be the case in some patients where the PGK probe was used) or that the transfer of one fragment was less efficient than the other. In order to test this latter variable, experiments were performed with serially diluted amounts of restricted DNA to see if loading, transfer and hybridization efficiencies were in a linear range. Band intensities were measured by densitometry and integrated values plotted as a function of the amount of DNA loaded. Figure 15A shows such an experiment where increasing and decreasing amounts of DNA's were mixed from two male patients, one without the BamH I HPRT polymorphism (i.e. having a single 24 kb allele), the other possessing the polymorphic site (therefore having a single 12 kb allele). As can be seen in this Figure, the 12 kb allele was readily demonstrated after 13 hours of exposure and a linear relationship was found between the amounts of DNA loaded and the resulting densitometric intensities (Figure 15B). The relationship between the amount of DNA loaded on and the intensity of the 24 kb allele was also linear.

Unequal Xpat/Xmat inactivation ratio- One important variable related to the methylation patterns of both alleles is the distribution of the methylated and unmethylated fragments between the maternal (Xmat) and paternal (Xpat) chromosomes. In polyclonal cell populations, an equal distribution of these fragments between both alleles and hence an equal



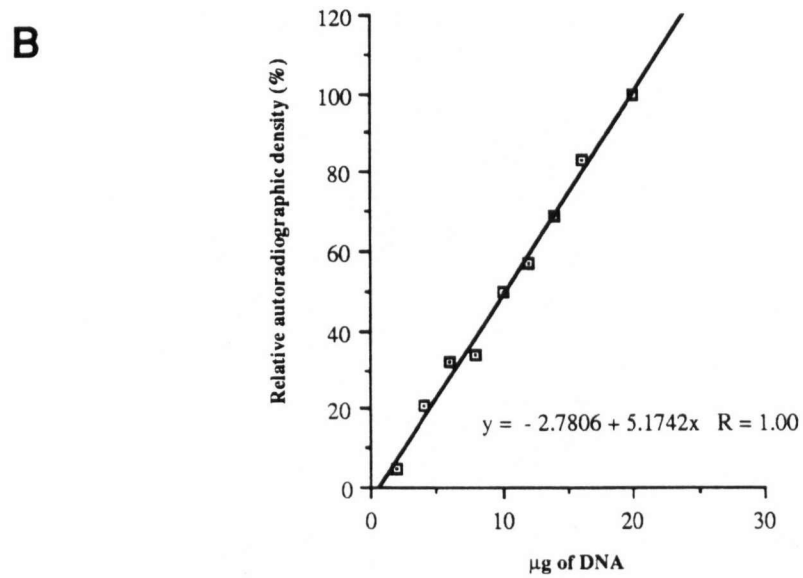


Figure 15

Panel **A**- Mixing experiment of DNAs from two male patients, one having a BamH I polymorphic site at the HPRT locus (12 kb band), and the other lacking this site (24 kb band).
Panel **B**- Relationship between the amounts of DNA loaded in each well and relative autoradiographic densities for 12 kb band.

reducation of the intensities of both bands are expected. However, unequal Xmat/Xpat inactivation ratios would skew patterns of distribution of unmethylated and methylated X-chromosomal DNA. The evaluation of somatic, non-hemopoietic DNA is thus an essential control in any case where a skewed distribution is found in order to determine whether this can be used to infer clonality. Figure 16 shows a representative example of such a difference between the methylation patterns of DNA obtained from marrow fibroblasts and blast cells from the same patient with a diagnosis of Ph^1-negative ALL, suggesting that the pattern exhibited by the DNA from the blast cell fraction was consistent with a true clonal overgrowth. An unequal Xpat/Xmat inactivation ratio was found to be a rare event and has been identified only in one normal bone marrow donor at the HPRT locus which resulted in a skewed distribution of methylation patterns between the two alleles. This pattern was not identified at the PGK locus for which this patient was also informative.

Detection of non-clonal DNA- Contamination of clonal cell populations with sufficient polyclonal cells to prevent detection of the neoplastic clone is another important variable that confounds the use of methylation analysis of X-linked RFLP's to detect persisting clonal cells. The limits of detecting clonal DNA in the presence of non-clonal cells was quantitatively evaluated in experiments where DNA with an established polyclonal pattern was mixed in decreasing amounts with DNA with a known monoclonal pattern. Such mixing experiments showed that non-clonal HPRT and PGK alleles would be detected if present as greater than 1% to 3% of the sample analysed.

Criteria for clonal patterns- Because of the above considerations and technical aspects related to loading, transfer and hybridization variabilities, I used clonality criteria suggested by Vogelstein et al (1) throughout this thesis. According to these criteria, a sample is called monoclonal if the intensity of one allele is reduced more than 85% and the other less than 40%

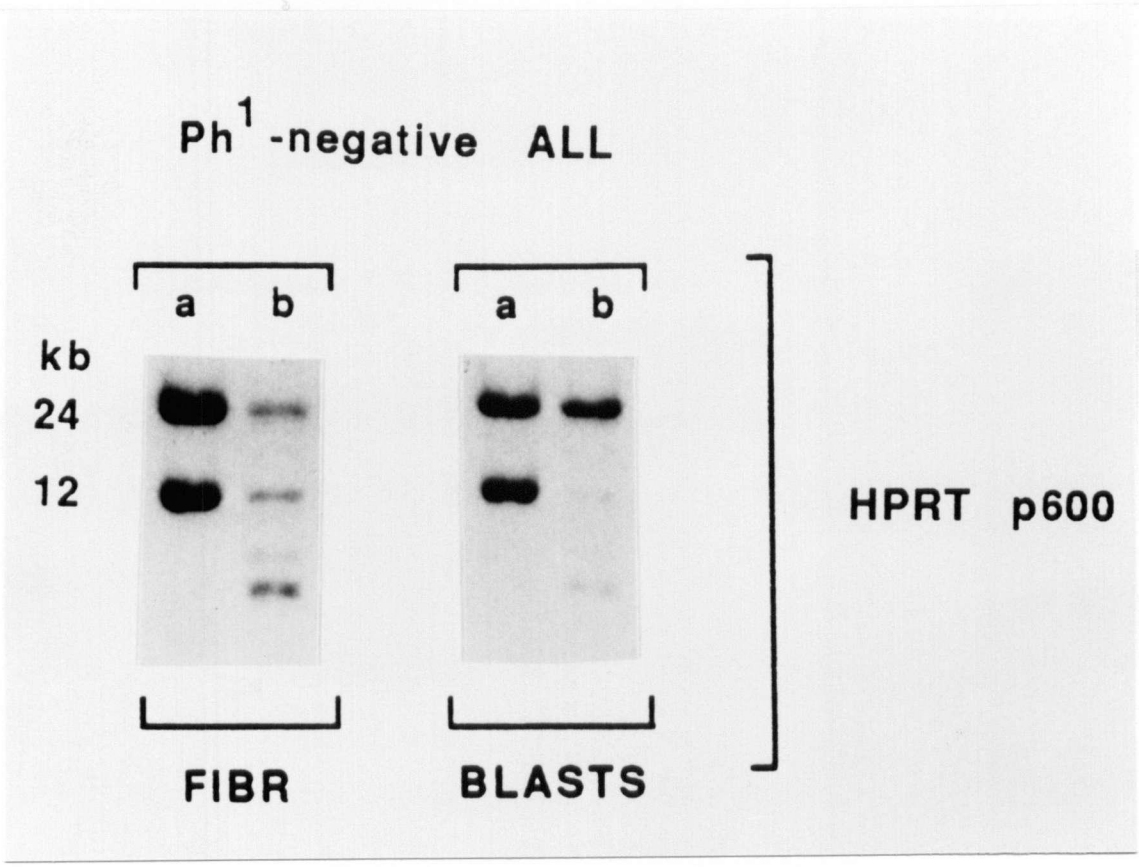


Figure 16

Marrow fibroblasts and blast cell DNA obtained during the terminal relapse in a patient with Ph^1-negative ALL. A typical polyclonal pattern is seen in the fibroblast DNA (left), whereas a monoclonal pattern is found in blast cell fraction (right). In lanes marked a: DNA was digested with BamH I. In lanes marked b: Half of the sample was additionally digested with Hpa II.

after digestion with Hpa II or Hha I. It is called polyclonal if the intensities of both bands are decreased by 30 to 70%. In cases where none of these criteria are met, the sample is called oligoclonal, in the absence of a proven unequal Xpat/Xmat inactivation ratio. In all normal controls analysed, a polyclonal pattern was demonstrated using both Hpa II and Hha I enzymes. DNA from one individual displayed a methylation pattern suggestive of a skewed Xpat/Xmat inactivation ratio as discussed above. The assessment of clonality was slightly complicated by the presence of two types of inactive alleles as determined by the methylation status of Hpa II sites at HPRT locus. In clonal cell populations arising from a type II inactive alleles, the p800 probe can not distinguish between polyclonality and monoclonality because it hybridizes to both inactive and active alleles. The use of p600 in these cases is required in order to demonstrate clustering of methylated or unmethylated fragments to one allele, consistent with monoclonality. Conversely, in clonal cell populations arising from a type I inactive allele, p600 probe fails to show any significant hybridization to large size fragments whereas p800 can hybridize to active fragments clustered either to Xmat or Xpat, suggesting monoclonality. Figure 17 illustrates two examples of cell populations with predominantly type II (Panel A) or type I (Panel B) inactive alleles. These results highlight the importance of using both probes and both Hpa II and Hha I enzymes to explore clonal patterns.

C. Analysis of Physically Cloned SV-40 Transformed Cell Lines

To further validate clonality analysis based on the interpretation of methylation patterns of HPRT and PGK RFLP's I examined known clonal populations, i.e. cell populations that had been physically cloned from single cells in vitro. Several available and well established cell lines originally derived from female patients and which could be readily cloned were therefore tested for heterozygosity, but none proved to be informative for the required PGK and HPRT polymorphisms. As part of another project, a number of SV-40 transformed, physically cloned cell lines were being established in the Terry Fox Laboratory from marrow fibroblasts of various patients (2). I determined that 3 of these patients were informative for PGK or HPRT

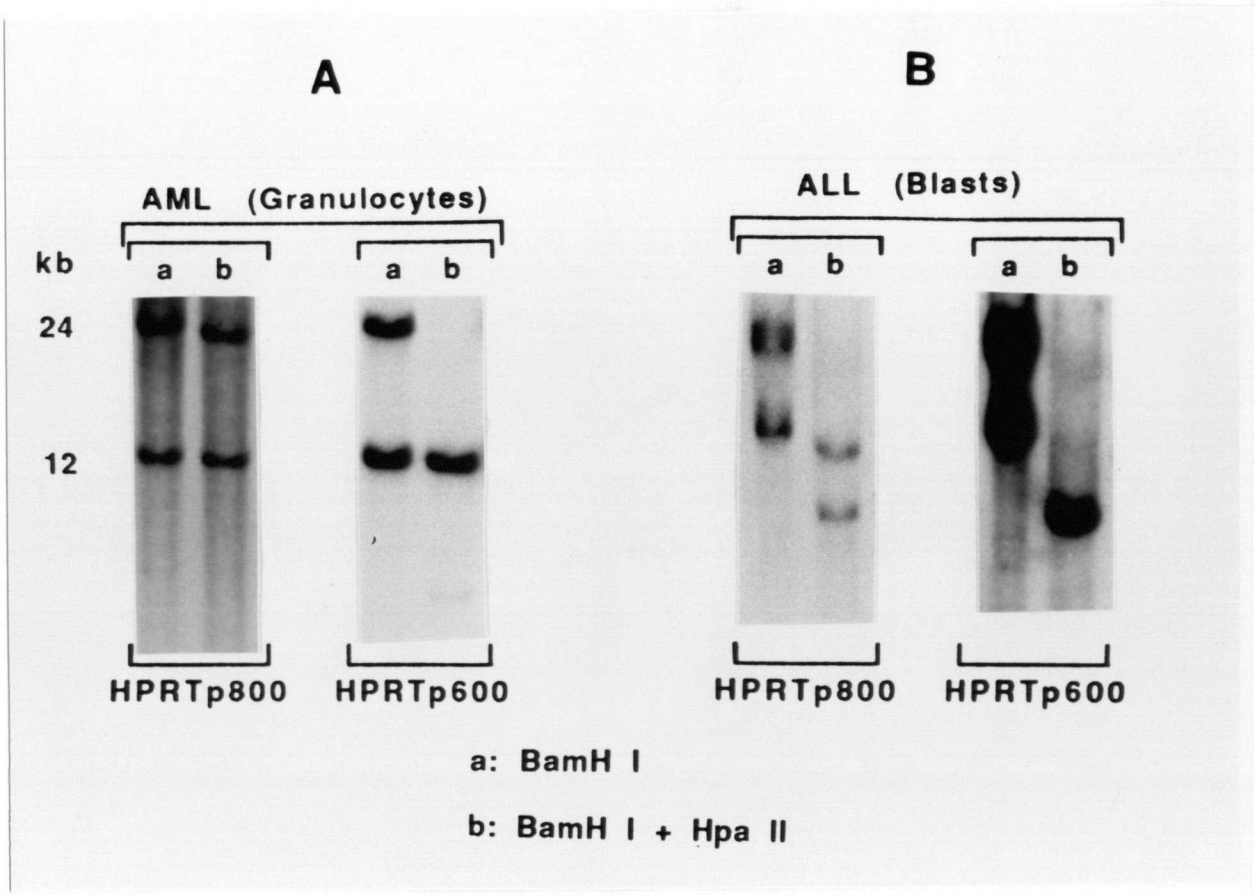


Figure 17

A- Example of a leukemic cell population with predominantly Type II inactive alleles. DNA was digested with BamH I (lanes a) or BamH I + Hpa II (lanes b). Blots were successively hybridized to HPRTp800 and HPRTp600 probes. The apparent resistance to Hpa II digestion when HPRTp800 is used, suggests that cells had predominantly Type II alleles. In the example shown here, HPRTp600 demonstrates a typical monoclonal pattern.

B- Example of a leukemic cell population with predominantly Type I inactive alleles. HPRTp800 is able to demonstrate a monoclonal pattern in these cases.

TABLE 6
CLONAL ANALYSIS OF SV-40 TRANSFORMED MARROW FIBROBLASTS

PATIENT	CLONES	HPRT 600	HPRT 800	PGK
D.P.	S.V.	MONOCLONAL		
B.H.	BH1	MONOCLONAL	N.D.	
	BH3	MONOCLONAL	MONOCLONAL	MONOCLONAL
	BH4	MONOCLONAL	MONOCLONAL	OLIGOCLONAL
	BH5	MONOCLONAL	MONOCLONAL	OLIGOCLONAL
	BH12	MONOCLONAL	N.D.	
	BH15	MONOCLONAL	N.D.	
A.U.	U43.1			POLYCLONAL
	U9.1			MONOCLONAL

N.D.= Not Determined

polymorphisms and lines derived from their marrow fibroblasts were therefore suitable for the analysis required.

Table 6 summarizes the results for 9 separately cloned cell lines derived from three different heterozygous patients. One patient was heterozygous for HPRT, one for PGK and the third patient for both. One cloned line established from patient D.P was found to have only one X chromosome after transformation, thus precluding its use for the clonality analysis. In another cloned line, however, both X chromosomes were present, and following Hpa II and Hha I digestion, DNA from this line showed a pattern consistent with a clonal population in contrast to that obtained with DNA from the original primary marrow fibroblast culture (Table 6). Six independently isolated lines were obtained from the patient BH who was heterozygous for both PGK and HPRT. Figure 18 shows the patterns obtained on three of these as representative examples. Digestion of DNA from each of these with BamH I and Hpa II followed by hybridization with the small p600 probe resulted in a disappearance of both the 12 kb and the 24 kb bands. This is the predicted result for a clonal cell population with type I inactive allele.

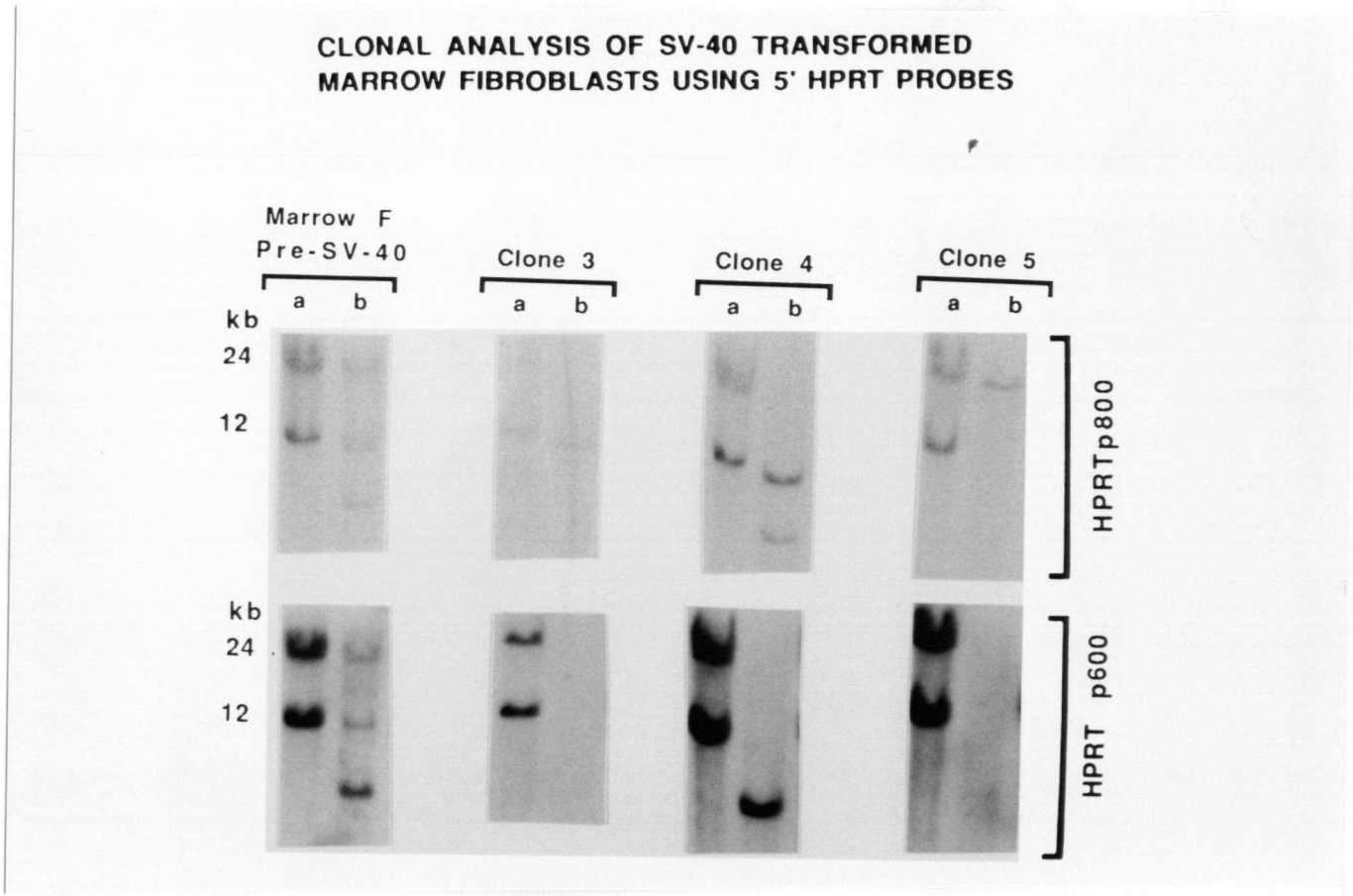


Figure 18

Clonal analysis of SV-40 transformed marrow fibroblasts from a patient informative for HPRT polymorphism. Marrow fibroblasts prior to SV-40 transformation (Marrow F-Pre SV-40) and three clones were analysed first with HPRTp800 probe (top filters) and then with HPRTp600 probe (bottom filters). DNA was digested with BamH I (lanes a) or with BamH I + Hpa II (lanes b). Pre-SV-40 sample is polyclonal with both probes whereas a monoclonal pattern is demonstrated with HPRTp800 in all three clones, suggesting that SV-40 induced transformation occurred in each of 3 clones in a cell with Type I inactive allele.

However, when the same blot was hybridized to the p800 probe, one allele only became detectable again as predicted for such a clonal population. The results with the p800 probe also showed the random nature of the SV40 transforming event in different cells in the original infected population by the fact that different bands were retained in different clones. For example, in clone 5 (Figure 18), the polymorphic site was on the inactive X-chromosome, whereas in clone 4 this site was on the active allele (which is detected by the p800 probe but only slightly shortened by the cleavage at the Hpa II site #1). As this patients was also heterozygous for the Bg1 I/PGK polymorphism, her fibroblast clones were also analysed using this probe. A clonal pattern with >85% reduction of the intensity of one allele was present in all 3 clones (data not shown). In cell lines established from the third patient heterozygous for the Bgl I polymorphism of the PGK gene, a monoclonal pattern was found in one clone (U9.1) whereas a typical polyclonal pattern was present in the other clone (U43.1). The significance of this is uncertain and is discussed below.

3- DISCUSSION

There are two important considerations that must be included in interpretation of methylation-sensitive RFLP data. One is that two or more clones with the same methylation pattern can not be distinguished from a single clone. The use of the binomial distribution, however, allows an estimate to be made of this likelihood. The second pertains to the sensitivity of the method. Thus a pattern indicative of clonality will occur only if the clone sufficiently outnumbers other cells present and can also not exclude the persistent presence of small numbers of other (polyclonal) cells. The results that I have obtained with both patient samples and SV-40 transformed, physically cloned cells lines provide strong support for the applicability of methylation-sensitive RFLP analysis to identify both normal and neoplastic clonal populations in humans.

This approach is particularly attractive because of the high prevalence of heterozygous females in the general population for HPRT and PGK polymorphisms. In the B.C. female

population that I have analyzed, this prevalence was approximately 50%, confirming data from the literature. In the future, it may be expected that this will approach 100%, with the discovery of new polymorphic sites in the X-chromosome.

The use of the physically cloned SV-40 cell lines has also shown that methylation patterns are relatively well maintained even after transformation, although in one line, isolated using the same cloning procedure, a non-clonal pattern was found. The significance of this is not clear; one possibility might be that abnormalities of the PGK gene methylation can occur in vitro, as has been shown to occur at the globin locus during serial passages of human fibroblasts (3). However, data obtained from immortal mouse cell lines indicates a more stable methylation patterns in these lines compared to that observed in normal fibroblast cultures from mice, hamsters and humans (4). Thus, consistent with data reported in immortal cell lines, in most clones that I analysed, methylation patterns behaved exactly as predicted, so that if deregulation of methylation does occur, it may be a relatively uncommon event. Another possibility is, of course, that the population was not truly clonal due to an uncontrolled technical failure during the cloning procedure. The presence of a single second cell with a different inactive X-chromosome would have been sufficient to cause a polyclonal pattern. This highlights the fact that this type of analysis is more reliable as a mean to detect monoclonal cell populations than to rule out the persistence of a dominant clone in the presence of other polyclonal cells.

One interesting phenomenon was the loss of one X-chromosome in an SV-40 transformed clone. Loss of one X-chromosome has been reported in human malignancies, although this occurs relatively rarely (5). Whether the X-chromosome loss seen here preceded or followed SV-40 transformation is unknown. Even if it occurred after, the possible role of the SV40 transformation events are also not known.

Clonality analysis using X-linked DNA polymorphisms appears to be a powerful tool to study human cell populations. I have shown that this analysis can be performed routinely in 50% of females and that transformation per se does not appear to cause major deviations in the

inherited fidelity of methylation patterns on which interpretation of the Southern data to infer clonality status is based.

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CHAPTER IV

ANALYSIS OF HEMOPOIETIC CLONALITY AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION

1- INTRODUCTION

Allogeneic bone marrow transplantation is now a well established therapeutic modality for the treatment of a variety of conditions, particularly acute myeloid and lymphoid leukemia (AML and ALL), and chronic myeloid leukemia (CML)(1). Usually, successful hemopoietic reconstitution is obtained as a result of the proliferation of cells in the marrow graft, although examples of partial and/or transient engraftment have been documented (2,3). The development of methods to characterize DNA, based on analysis of restriction fragment length polymorphisms (RFLP's), has greatly expanded our ability to distinguish cells from different individuals, even when they are closely related and of the same sex. Application of this methodology to assess the extent of donor and host derived cells in the blood and marrow of allogeneic bone marrow transplant recipients has helped to document chimerism (4,5), to provide early evidence of graft failure (6), and to identify the origin of recurrent leukemia or a secondary lymphoproliferative malignancy that may appear in the transplant patient (7).

In mice transplanted with syngeneic marrow, there is longstanding evidence indicating that the regenerated marrow and lymphoid elements often derive from the same pluripotential stem cell population (8,9). More recent experiments with retrovirally-marked murine marrow cells have shown that repopulation of the entire blood-forming system of a mouse can be achieved by the proliferative activity of a single, putatively normal, hemopoietic stem cell

(10-12). The demonstration of clonal populations of hemopoietic cells in humans has, in the past, relied primarily on analysis of glucose-6-phosphate dehydrogenase (G6PD) isoenzyme expression. This type of analysis has revealed clonal hemopoiesis in female heterozygotes with a variety of disorders including AML, CML and other myeloproliferative diseases (13). However, this approach is of limited applicability because of the very low frequency of informative (heterozygous) G6PD females in the general population. I thus applied the clonality analysis method developed by Vogelstein et al (14, 15) to cell populations regenerated in recipients of bone marrow transplants from HPRT and/or PGK heterozygous donors. The results obtained in a series of 12 patients where this method could be used to assess the frequency and origin of clonal hemopoiesis after allogeneic marrow transplantation are described in this Chapter.

2- RESULTS

Marrow Donors

Patient selection for this study was based solely on the identification of an informative female donor for recipients sequentially entered into the adult allogeneic bone marrow transplant program in Vancouver, irrespective of the type or stage of the disease of the recipient. Twelve donor-recipient pairs were accumulated in this way. As shown in Table 7, seven of the donors were heterozygous at the HPRT locus, yielding both a 12kb band and a 24kb band in Southern blots of their Bam HI digested marrow or blood granulocyte DNA after probing with the p600 HPRT fragment. Donors #2 and 8 were also informative at the PGK locus, showing a 1.3 kb band as well as the more prevalent 1.7 kb band. Donors #3, 7, 9, 10 and 11 were informative only at the PGK locus. Additional digestion of the donor DNA samples with HpaII or HhaI showed a methylation pattern consistent with polyclonality criteria (i.e. equal distribution of active and inactive alleles between the paternal and maternal alleles) in every case except in DNA from donor #8 which showed a skewed pattern of X-inactivation

Table 7. Genotype Data for the Patients Studied.

	Donor				Recipient			
Pair No.	Age[a] (yr)	Sex	HPRT Genotype[b]	PGK Genotype[b]	Age[a] (yr)	Sex	HPRT Genotype[b]	PGK Genotype[b]
1	53	F	24/12	1.7/1.7	47	M	24/-	1.7/-
2	65	F	24/12	1.7/1.3	36	M	24/-	1.7/-
3	17	F	24/24	1.7/1.3	19	M	24/-	1.7/-
4	29	F	24/12	1.7/1.7	32	M	Not Done	
5	15	F	24/12	1.7/1.7	18	F	24/12	1.7/1.7
6	49	F	24/12	1.7/1.7	44	F	24/12	1.7/1.7
7	42	F	24/24	1.7/1.3	50	F	24/24	1.7/1.3
8	23	F	24/12	1.7/1.3	21	M	Not Done	
9	21	F	24/24	1.7/1.3	19	M	Not Done	
10	42	F	24/24	1.7/1.3	45	M	Not Done	
11	43	F	24/24	1.7/1.3	37	F	24/24	1.7/1.3
12	24	F	24/12	1.7/1.7	25	F	24/24	1.7/1.7

[a] Age at BMT
[b] Alleles defined by banding pattern of digested DNA on Southern analyses.

of the HPRT loci. This pattern was not found at the PGK locus of the same patient.

Recipients

All patients were treated at the Leukemia/Bone Marrow Transplant Unit of the Vancouver General Hospital between January 1987 and February 1988. Initial diagnoses and disease status at the time of transplantation are given in Table 8. Recipients were treated with a conditioning regimen consisting of either cyclophosphamide 120 mg/kg and busulphan 16 mg/kg (patients, #2, 7, 9, 10, 11 and 12), cyclophosphamide 150 mg/kg, etoposide 1.8 g/m^2, and total body irradiation (1200cGy) (patients #1, 3, 5, 6 and 8), or cyclophosphamide 7.2 g/m^2, etoposide 2.4 g/m^2, and BCNU 0.6 g/m^2 (patient #4). The allogeneic marrow transplant was given on Day 0. The number of nucleated cells infused ranged from 2.0 to 9.9 x 10^8 cells/kg body weight.

Seven of the 12 recipients were male and the clonality status of their hemopoietic cells before transplantation could therefore not be investigated. For one of the female recipients (#12), a pre-BMT sample was not available. Her HPRT and PGK genotype was subsequently established by analysis of DNA extracted from fibroblast monolayers generated from a marrow aspirate obtained 9 months after transplantation (Table 7). She turned out to be homozygous at both loci and hence clonality studies of her hemopoietic cells would not have been possible. Of the remaining four female recipients, three were heterozygous for either the HPRT allele (patient #5), or the PGK allele (patients #7 and #11). As indicated in Table 8, patient #11 was found to have monoclonal circulating granulocytes 2-3 weeks prior to transplantation. This finding is not surprising, as she had a myelodysplastic syndrome with recurring blasts. The production of mature granulocytes within the neoplastic clone in similar patients has been documented before (13,16). On the other hand, the presence of a monoclonal pattern in the pre-transplant granulocyte and marrow DNA from patient #5 (data not shown) was not

Table 8. Summary of Clonality Results on Hemopoietic Cell Populations from BMT Recipients.

Recipient No.	Pre-BMT			Post-BMT			
	Clinical Status	Clonality		Clinical Outcome	Clonality Results		
		Sample	Result		Sample	Origin	Result
1	Ph[1] CML in 1st chronic phase	Not done		CCR (D+530)	D+28 G	Donor	Polyclonal
					" MC	"	"
					D+111 G	"	"
					" BM	"	"
					D+140 G	"	"
					" T	"	"
					" non-T	"	"
2	AML in 1st remission	Not done		CCR (D+503)	D+28 G	Donor	Polyclonal
					" T	"	"
					" non-T	"	"
					" BM	"	"
					D+90 G	"	"
					" T	"	"
					" non-T	"	"
3	Ph[1] CML in accel. phase	Not done		Died (D+182) from aspergillosis	D+33 G	Donor	Polyclonal
					D+54 G	"	"
					D+159G	"	"
					" T	"	"
4	Refractory Hodgkin's	Not done		Died from leukemia (D+267)	D+37 G	Donor	Polyclonal
					" T	"	"
					" non-T	"	"
					D+103G	"	"
5	ALL (L2) in remission	D-13 G	Monoclonal	Died (D+267) from GVHD	D+30 G	Donor	Polyclonal
		BM	"		D+85 G	"	"
					" T	Not Determined	"
					D+152G	Donor	"
					" T	Not Determined	"
					" non-T	"	"
6	Ph[1] CML in 2nd chronic phase	D-9 G	Monoclonal	Died (D+70) from GVHD	D+28G	Not Determined	Polyclonal
7	Ph[1] CML in 1st chronic phase	D-9 G	Monoclonal	CCR (D+294)	D+28G	Not Determined	Polyclonal
					" BM	"	"
					D+45G	"	"

....con't

Table 8. Summary of Clonality Results....(con't)

Recipient No.	Pre-BMT			Post-BMT			
	Clinical Status	Clonality		Clinical Outcome	Clonality Results		
		Sample	Result		Sample	Origin	Result
8	Lymphoblastic lymphoma	Not done		CCR (D+263)	D+28G " MC " BM	Donor " "	Polyclonal " "
9	AML in 1st remission	Not done		CCR (D+132)	D+33G	Donor	Polyclonal
10	Ph^1 CML in 1st chronic phase	Not done		CCR (D+159)	D+28G " BM D+61G D+75G	Donor " " "	Polyclonal " " "
11	RAEB in relapse	D-22 G	Monoclonal	Died (D+51) from GVHD, no leukemia	D+29 MC	Donor	Monoclonal
12	AML in relapse	Not done		Died (D+315) from leukemia	D+36 G D+267G " T	Donor " "	Oligoclonal " "

CCR: Clinical complete remission; D+ : Days after transplantation; D- : Days before transplantation; G: Granulocytes; BM: Bone marrow; MC: Mononuclear cells; RAEB: Refractory anemia with excess of blasts; AML: Acute myeloblastic leukemia; GVHD: Graft-versus-host disease; CML: Chronic myeloid leukemia.

anticipated. This patient was diagnosed as a Ph^1-negative adult ALL 9 months before being transplanted and at the time of the transplant was considered to be in complete remission.

A peripheral blood sample was obtained from all patients approximately one month after transplantation and, except for patients #6, 8, 9, and 11, later samples were also analysed (Table 8). All post-transplant cell samples from patients #1-10 were consistently polyclonal, including DNA extracted from purified granulocyte and T-cell populations (Figure 19). The donor origin of the cells in patients #1-4, and 8-10 was deduced from the same RFLP analysis because the recipients were males and hence assumed hemizygous for HPRT and PGK. Analysis of all of their post-transplant samples showed the presence of a donor-specific RFLP band. This was confirmed in patients #2, and 3 using c-Ha-ras and pYNZ22 probes respectively for highly polymorphic loci (pYNZ22 is an anonymous probe obtained from ATCC and described in Ref. 17). Analysis of post-transplant samples from patient #5 with the PYNZ22 probe similarly showed donor origin.

Results of analysis of DNA from blood cells of the other two recipients (#11 and 12) was in marked contrast to the obvious polyclonal patterns seen in post-transplant hemopoietic cell samples from patients #1-10. Patient #11 was a female patient with a refractory anemia with excess of blasts, in relapse at the time of transplantation with marrow from her sister. Only a single sample of mononuclear cells (<1.077 gm/cm^3) was obtained 29 days after transplantation. At that time, the white cell count was 11300 per microliter with 2060 monocytes and 330 lymphocytes (with a similar differential in the preceding and subsequent weeks). Analysis of this DNA showed a monoclonal pattern (Figure 20a). The origin of the cells could not be determined by this analysis, because both sisters were heterozygous for the BglI polymorphism in the PGK gene. However, a difference between their DNA was detected using a BamHI polymorphism based on the variable number of tandem repeats flanking the c-Ha-ras gene (17). As shown in Figure 20b, neither of the alleles for this polymorphism were shared between the recipient #11 and her donor, and in the day +29 sample no evidence of recipient

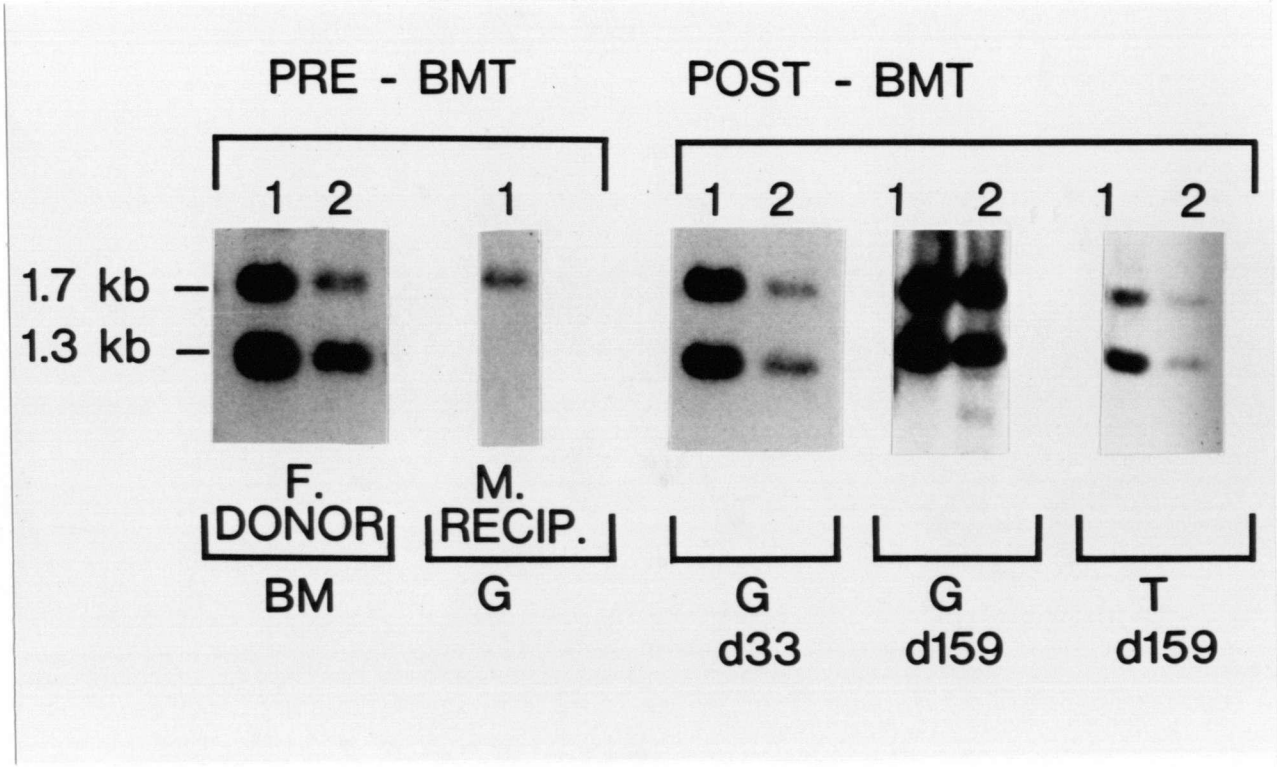

Figure 19

Polyclonal pattern of hemopoietic reconstitution in patient #3 shown by Southern blot analysis of the BglI polymorphism of the PGK gene. Lanes marked 1: DNA was digested with BglII, EcoRI and BglI to reveal the two PGK alleles in the female donor (F) and a single PGK allele in the male recipient (M). In lanes 2: Half of each DNA was further digested with HpaII for methylation analysis and then lanes 1 and 2 were compared. BM: bone marrow, G: granulocytes, T: T-cells, d= days post-transplant.

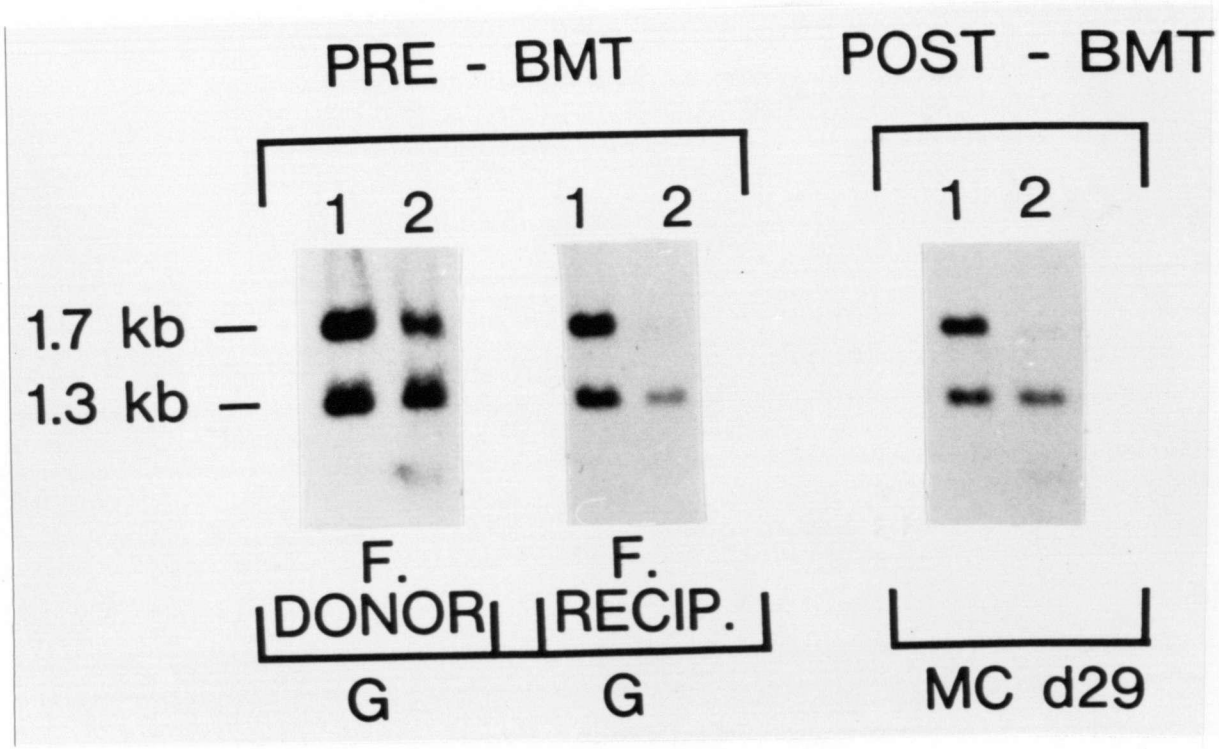

Figure 20a

Monoclonal pattern of hemopoietic reconstitution in mononuclear cells of patient #11, shown with methylation analysis of the BglI polymorphism of PGK gene. Lanes marked 1: DNA was digested with BglII, EcoRI and BglI and Lanes marked 2: DNA digested additionally with HpaII as explained in Figure 19. Both donor and recipient were heterozygous for Bgl I polymorphism; therefore undistinguishable with the use of this RFLP alone. G: Granulocytes, MC: Mononuclear cells, d= days post-transplant.

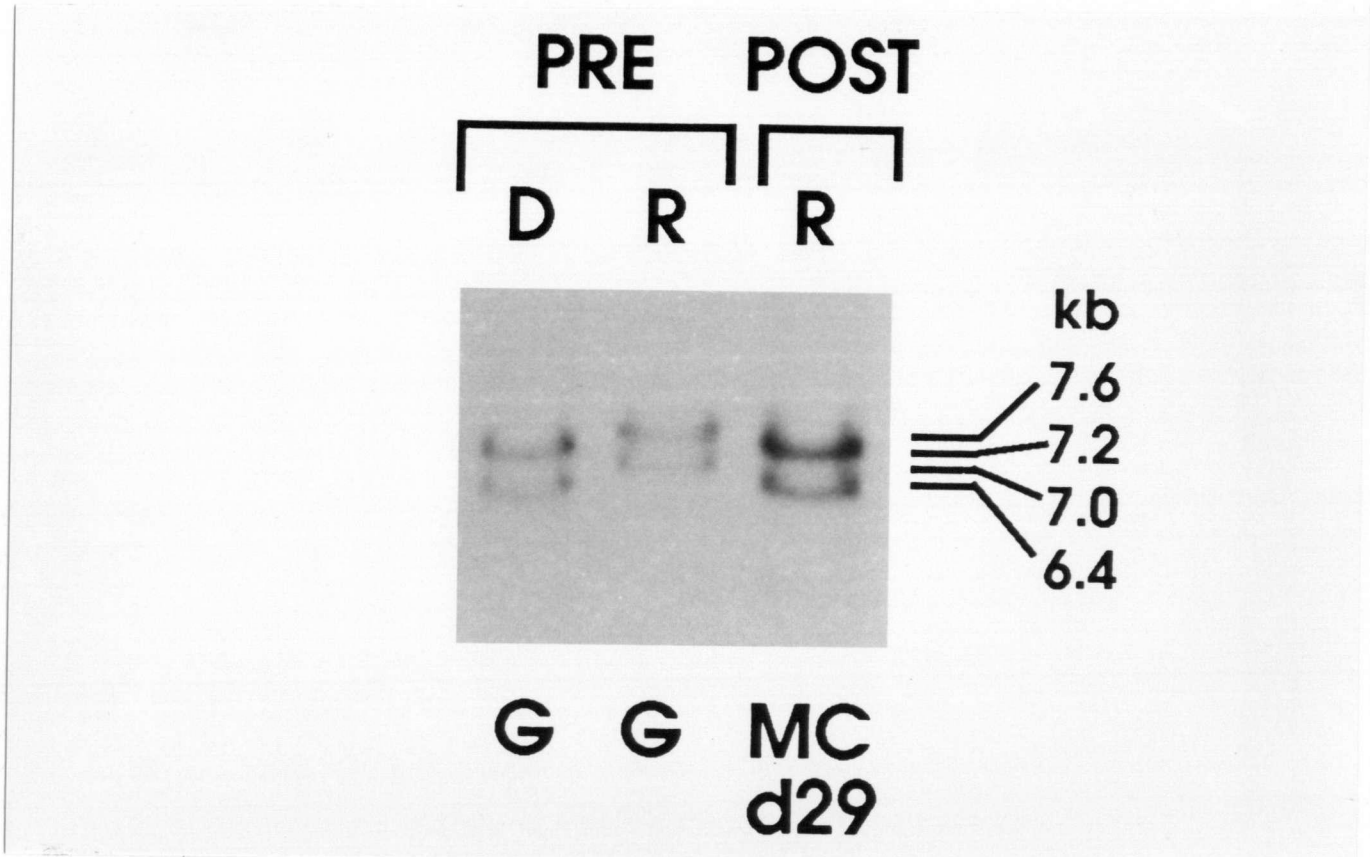

Figure 20b

Southern blot analysis demonstrating donor origin of the MC cells in patient #11 at 29 days post-transplant. DNA was digested with BamHI, electrophoresed, transferred to a nylon membrane and hybridized to a 6.4kb insert containing the human c-Ha-ras gene. Pre-transplant DNA samples show 7.2kb and 6.4kb alleles in the donor and 7.6 and 7kb alleles in the recipient. In the post-transplant sample, only donor-type alleles are present. In order to rule out the possibility that the DNA in the middle lane had migrated aberrantly, the same blot was also hybridized to a 3' bcr probe. This showed a single band of 3.3 kb at the same position in all three lanes (data not shown). D: Donor, R: Recipient, G: Granulocyte, MC: Mononuclear cells.

DNA was detected. Patient #11 died of severe GVHD 51 days after transplantation with no circulating blasts and no evidence of recurrent leukemia. Post-mortem examination of hemopoietic organs also showed no leukemic infiltrates.

Patient #12 was a female AML patient, also in relapse at the time of transplantation. She was homozygous at both the HPRT and PGK loci. DNA from granulocytes obtained 36 days after transplantation showed a preferential cleavage of the 24 kb HPRT allele after HpaII digestion (Figure 21). This suggested the presence at that time of a dominant myeloid clone, although densitometric measurements (90% reduction of the 24kb allele versus 50% reduction of the 12kb allele, compared to 79% vs 88% reduction respectively, in the pretransplant donor sample) did not meet the strict criteria for monoclonality. Another sample was not obtained from this patient until 267 days after transplantation. Southern analysis of DNA from granulocytes and T-cells purified from the blood obtained at this later time (80% T-cells in the rosetted fraction) showed in both cases the same persistent selective reduction of the 24 kb allele band following HpaII digestion (Figure 21) . The donor origin of the cells was indicated by the presence of the donor-specific 12 kb band.

3- DISCUSSION

The results obtained in this study show that analysis of methylation-sensitive X-linked RFLP's may be used to assess the clonality status, and sometimes the origin, of different hemopoietic lineages regenerated in patients transplanted with allogeneic marrow from heterozygous donors. Most of the patients in the unselected series described here (i.e. 10 of 12) yielded results indicating engraftment and long-term maintenance of hemopoiesis by multiple donor-derived stem cell clones. Similar findings were recently reported by Nash et al (18). For the other 2 patients, application of the same type of RFLP analysis to various blood cell populations obtained after transplantation revealed a digestion pattern suggestive of a dominant clone. In one of these 2 patients where the evidence for clonality data was clearest,

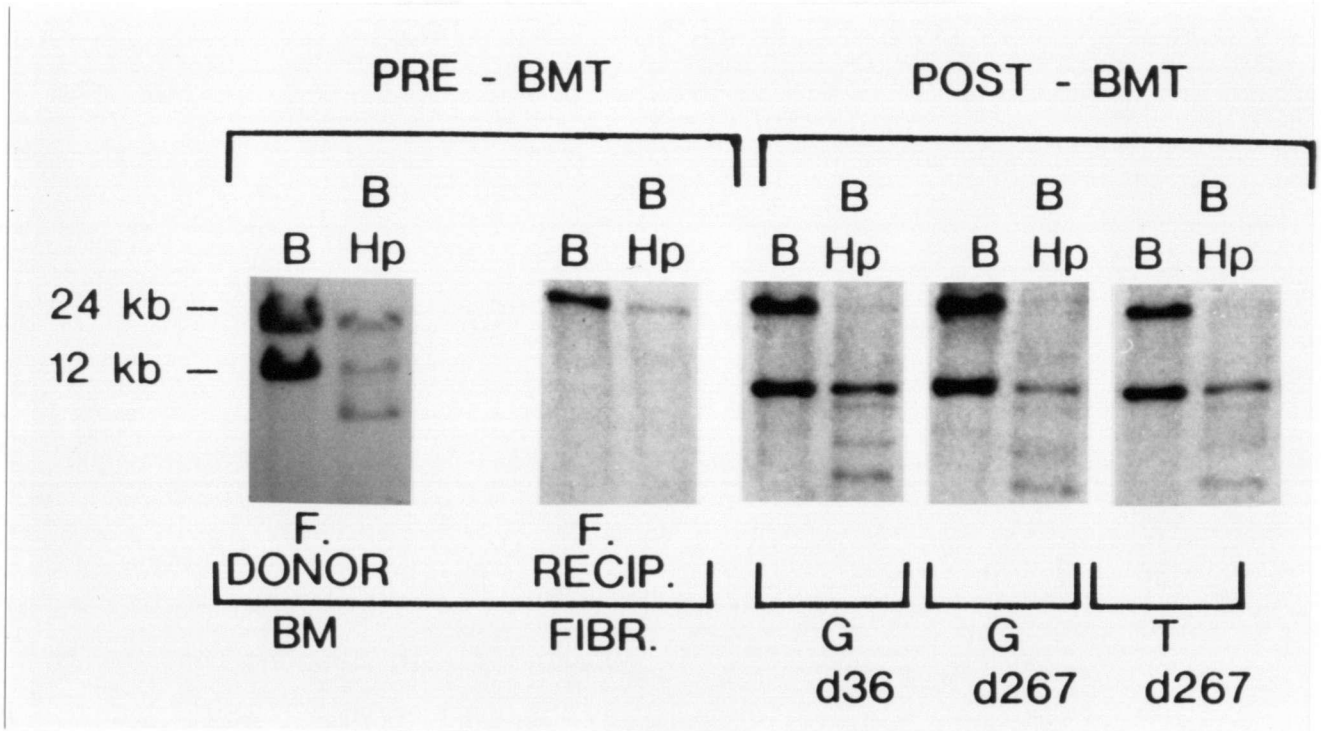

Figure 21

Dominant clonal pattern of hemopoietic reconstitution shown using the BamHI polymorphism of the HPRT gene in patient #12. Both donor and recipients are females but the recipient DNA lacks the polymorphic site, and has a single 24kb band after probing with the p600 probe. The donor is heterozygous for the BamHI polymorphism. Lanes marked B : DNA digested with BamHI alone. Lanes marked B/Hp: DNA additionally digested with HpaII. G: Granulocytes, T: T lymphocytes, Fibr: Fibroblasts, H: HpaII. Bands smaller than 12kb in BamHI plus HpaII lanes are HpaII cleavage products.

the use of a non-X-linked, RFLP probe showed that the blood cells analysed were donor-derived and hence presumably normal. This patient died 3 weeks later of severe GVHD. The possibility of a clonal expansion of alloactivated T-cells related to her GVHD in her day+ 29 sample is unlikely, due to the small contribution of the lymphocyte DNA to the mononuclear cell fraction. In the second patient, the data were consistent with the presence of additional minor clones, although the digestion pattern after the use of methylation-sensitive enzymes still appeared abnormally skewed suggesting one predominant clone. This pattern was found to persist for more than 6 months at which time it was demonstrated separately in the DNA isolated from the T cells as well as from the granulocytes. The same data showed that both of these cell populations were derived from the marrow transplant.

The most likely explanation for these findings is that normal adult human marrow contains a population of primitive pluripotent hemopoietic stem cells that possess enormous proliferative capacity, sufficient to repopulate and maintain both T lymphoid and myeloid compartments for many months. Analysis of methylation sensitive RFLP's cannot distinguish between single and multiple clones with the same active X-chromosome. However, the likelihood that patients #11(and possibly patient #12) had been repopulated with more than 2 clones identical in this regard is low, and the chances that more than 4 such clones were involved is <0.05 (assuming an equal probability of either allele being inactivated as suggested by analysis of the donor DNA and application of the binomial distribution). Thus it is reasonable to conclude that hemopoiesis regenerated after allogeneic bone marrow transplantation can be due to the activity of a single or, at most, a small number of stem cells.

The concept that undifferentiated hemopoietic stem cells with lymphopoietic as well as myelopoietic potential are present in normal adult human marrow is not new; however evidence for this has been limited. In a large number of patients with various neoplastic hemopoietic disorders, analysis of the clonal organization of hemopoiesis has revealed single clones encompassing multiple lineages of mature blood cells in every case examined (13,16). However, it is possible that the proliferative or differentiative potential of the cells maintaining

such abnormal clones may not accurately reflect the normal potentiality of the first cell to acquire a proliferative advantage. Lineage infidelity or promiscuity is a well recognized feature of some neoplastic hemopoietic cells (19,20), including populations that appear otherwise normal (21). The present studies circumvent this issue, since the hemopoietic cells in patient #11 were derived from the normal marrow transplanted.

The ability of one or very limited numbers of stem cells to dominate the hemopoietic system after allogeneic transplantation raises questions about the significance of clonal hemopoiesis in other situations. The recent demonstration of clonal remissions in AML (13,21), or in ALL (patient #5), could reflect the proliferation in these individuals of cells at an early stage in the development of the malignant clone. Alternatively, such remissions could be due to the regenerative activity of a single normal stem cell amongst the reduced numbers surviving the therapy used for remission induction. Such a possibility is suggested by results of chemotherapy-induced stem cell depletion experiments in a cat model (22). Recent evidence also indicates that oligo- or monoclonal hemopoiesis can be observed during hemopoietic recovery after intensive chemotherapy in patients with breast cancer (23). Clearly, a larger series of patients needs to be studied to evaluate the relative importance of these possibilities.

The present results extend to the human setting of clinical marrow transplantation the possibility of using very small numbers of hemopoietic stem cells to rescue patients from marrow ablation, defective marrow function, and constitutional problems amenable to gene therapy. A first direct test of this possibility in mice has recently been published (24). In these experiments, transplantation of less than 50 purified marrow stem cells gave detectable progeny in lethally irradiated recipients several weeks later. Similar results were obtained using a different stem cell purification strategy (25). Development of analogous procedures applicable to human hemopoietic stem cell populations is less advanced. This is due, in part, to the lack of a method for directly quantitating such cells in vitro. Current assays for hemopoietic colony-forming cells of either mouse or human origin, including those capable of multi-lineage colony formation, do not appear to be suitable (25-27). Progress in stem cell

purification in the murine system has been possible because transplantation, although cumbersome, can be used as the basis of an assay. Initiation of sustained hemopoiesis in long-term marrow cultures may offer a possible alternative for human cells (28).

In summary, these observations suggest that a clonal cell population which dominates the entire hemopoietic system, can occur in recipients of allogeneic bone marrow transplants without evidence of an intrinsic neoplastic process. Further long-term studies are required to document the frequency of this phenomenon, and whether it has any prognostic significance.

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CHAPTER V

CLONAL CHARACTERISTICS OF HEMOPOIETIC CELLS AND THEIR ASSOCIATION WITH OTHER BIOLOGICAL ABNORMALITIES IN PATIENTS WITH THROMBOCYTOSIS

1-INTRODUCTION

Thrombocytosis is a common feature of all MPDs including CML, PV and Myelofibrosis. In addition to being clonal disorders of the pluripotent hematopoietic stem cell (1), the neoplastic cells in these diseases also share other abnormalities, such as an ability of some terminally differentiating erythroid progenitors to bypass the normal Epo requirement (2) and a deregulation of cycling control of the most primitive neoplastic progenitors, demonstrable by thymidine suicide assays (3). However, it is unlikely that all patients with thrombocytosis have an underlying MPD as there are other causes of an increased platelet count. Patients of this latter type are most readily distinguished when they do not show any of the clinical, hematological or cytogenetic findings typical of CML, PV or myelofibrosis (4). Thus, for example, occasional examples of inflammatory or non-hematologic neoplastic conditions have been associated with thrombocytosis (4). In cases where no evidence of other conditions are found, the term "essential" thrombocythemia (ET) has been used to infer a subcategory of MPD, although the inadequacy of this diagnosis is well recognized. The Polycythemia Vera Study Group (PVSG) established a number of criteria (5) to exclude potential ET patients from being included with other MPD categories in attempting to develop "homogeneous" patient groups for analysis of treatment results. Accordingly, the PVSG criteria for diagnosing ET are not satisfactory as they are not based on any specific biological abnormality inherent to the disorder.

One of the most convincing lines of evidence for the existence of ET as a distinct entity within the MPD group has been the demonstration of clonal hemopoiesis involving all lineages in a patient with this diagnosis (6). However, both because of the low incidence of ET and the low prevalence of G6PD heterozygotes in the general population, this has not been a practical approach.

Previous studies in the Terry Fox Laboratory showed that hemopoietic progenitors from a number of patients with clinical features suggestive of ET showed the same abnormal Epo-independence and unregulated cycling typical of neoplastic hemopoietic cells from patients with PV or CML (7). In this chapter, I describe studies which were undertaken to assess the clonality status of granulocytes from a number of patients with thrombocytosis who were also heterozygotes at the PGK and/or HPRT loci. The results of these studies have then been compared with simultaneously but independently performed Epo-independence and progenitor cycling analyses where data was available. The two major questions that these studies address are as follows: (1) Can PGK and/or HPRT analysis detect monoclonal hemopoiesis in the absence of other markers of neoplastic hemopoiesis (i e. Epo-independent growth and/or unregulated cycling)? and (2) When Epo-independence and/or abnormal cycling is detectable, is the neoplastic clone always sufficiently dominant to be detected as monoclonal or, alternatively, can PGK and/or HPRT analyses detect polyclonal hemopoiesis in the presence of an abnormal neoplastic clone ?

2-RESULTS

Tables 9 and 10 summarize the clinical and hematological characteristics of the 17 patients included in this study. The median age was 61 with a range of 32 to 82 years. The only selection criteria applied were that patients be PGK and/or HPRT heterozygotes with a platelet count >500x10^3/μl and did not fit a diagnosis of CML (presence of Ph^1 chromosome or

TABLE 9- CLINICAL AND HEMATOLOGICAL CHARACTERISTICS OF 17 PATIENTS WITH THROMBOCYTOSIS

PATIENT NO.	AGE AT DIAGNOSIS	BLOOD AT DIAGNOSIS			MARROW DIAGNOSIS[a]	CYTOGENETICS
		Hb g/100ml	Platelets $x10^3/\mu l$	WBC $x10^3/\mu l$		
1. E.T.	68	12.1	1,572	11.8	MPD, consistent with ET	46, XX (N)
2. A.R.	73	14.5	1,158	17.	MPD, consistent with ET	N/A
3. B.D.	54	11.6	609	8.4	Not consistent with MPD	46, XX (N)
4. O.T.	59	13.9	514	4.4	MPD, consistent with ET	46, XX (N)
5. M.M	75	15	870	8.1	Not consistent with MPD	46, XX (N)
6. W.M.	63	11.0	708	6.6	Not consistent with MPD, Iron deficiency	46, XX (N)
7. L.G.	33	14.5	640	6.1	Non-diagnostic, Early MPD?	46, XX (N)
8. A.M.	61	11.1	871	9.2	MPD, consistent with ET	N/A
9. L.L.	39	13.7	632	4.5	N/A	N/A
10. A.A.	69	7.6	826	9.	Not consistent with MPD, Iron deficiency	N/A
11. H.N.	82	11.4	1,544	17.6	MPD consistent with ET	N/A
12. F.R.	63	9.9	736	14.5	Not consistent with MPD	46, XX (N)
13. M.F.	61	13.8	830	10.2	MPD, consistent with ET	46, XX (N)
14. W.B.	52	8.5	1,200	12.9	N/A	N/A
15. D.A.	30	12.4	1,100	6.7	MPD, consistent with ET	N/A
16. F.R.	72	14.3	1,071	11.1	MPD, consistent with ET	46, XX (N)
17. W.H.	32	14.4	940	11.6	MPD, Consistent with ET	46, XX (N)

[a]Obtained independently from referring pathologist
N=Normal, N/A=Not Available
MPD: myeloproliferative disorder; ET: Essential thrombocythemia.

TABLE 10- IN VITRO COLONY GROWTH, PROGENITOR CYCLING AND CLONAL CHARACTERISTICS OF 17 PATIENTS WITH THROMBOCYTOSIS

PATIENT #	Epo-Independent Erythroid-Colonies[a]		Cycling[a] (Blood)	Clonality of Granulocytes	
	Blood	Marrow		PGK	HPRT
1. E.T.	No	No	No	---	Monoclonal
2. A.R.	Yes	Yes	---	---	Monoclonal
3. B.D.	No	No	---	Polyclonal	---
4. O.T.	Yes	Yes	---	Monoclonal	---
5. M.M	Yes	Yes	Yes	Polyclonal	---
6. W.M.	No	No	No	---	Polyclonal
7. L.G.	Yes	Yes	Yes	Oligoclonal	---
8. A.M.	Yes	No	---	Polyclonal	---
9. L.L.	No	---	---	Polyclonal	---
10. A.A.	No	No	No-	Polyclonal	Polyclonal
11. H.N.	Yes	Yes	---	Monoclonal	---
12. F.R.	No	No	---	Polyclonal	---
13. M.F.	Yes	No	No[b]	Polyclonal[b]	---
14. W.B.	No*	---	---	Polyclonal	---
15. D.A.	No*	No	No	Polyclonal	---
16. F.R.	Yes	Yes	Yes	Monoclonal	---
17. W.H.	Yes	Yes	Yes	---	Monoclonal
Normal Individuals	No	No	No	Polyclonal	Polyclonal

[a]Data available by permission from Drs. A. Eaves, C. Eaves and J. Cashman; Epo-independence and cycling assessed as described previously (2,3) yes for Epo-independence when $\geq$ 1% CFU-E and/or BFU-E showed Epo-independent growth in vitro (no for <1%); yes for cycling when all blood progenitors or marrow progenitors of high proliferative potential (3) showed >25% kill in Thymidine suicide assay (no for cycling when these same progenitors showed <10% kill)

[b]Oligoclonal on an earlier blood specimen at which time circulating progenitors showed Epo-independence

bcr-rearrangement), PV (PVSG criteria) or Myelofibrosis. These latter diagnoses could be eliminated in most of the patients. Because the goal was to study all patients with thrombocytosis, all heterozygous patients likely to have reactive as well as primary causes of their elevated platelet counts were included.

A- Monoclonal hemopoiesis in the absence of Epo-independence or abnormal progenitor cycling. In one patient (Pt #1) clonality analysis of purified granulocytes showed a typical monoclonal pattern using the BamH I polymorphism of the HPRT gene. This patient presented with a very high platelet count, ($1,572x10^3/\mu l$), a normal WBC and Hb, and a normal karyotype. Peripheral blood and marrow progenitor assays performed (on two different occasions for the blood) did not demonstrate the formation of any Epo-independent erythroid colonies. Thymidine suicide assays of peripheral blood progenitors revealed these to be quiescent, as is typical of normal individuals (8). The monoclonal pattern was demonstrated with both p600 and p800 HPRT probes and with both Hpa II and Hha I enzymes on the same two peripheral blood samples used for progenitor studies and indicated origin of the clone in a cell with an inactive allele mainly of Type II.

B-Correlations between clonality, Epo-independence and abnormal cycling. Table 11 shows the results obtained when clonality data was correlated with in vitro growth patterns of peripheral blood and/or bone marrow progenitors. The majority of patients whose granulocytes appeared to be polyclonal also did not show detectable Epo-dependent erythroid colony formation in vitro, indicating absence of a neoplastic clone by both methods. Similarly, the majority of patients whose granulocytes appeared to be oligo- or monoclonal, were also found to have Epo-independent erythroid progenitors. In contrast, 3 patients had detectable numbers of Epo-independent erythroid progenitors although the granulocytes were clearly polyclonal.

TABLE 11

ASSOCIATION OF CLONAL HEMOPOIESIS AND EPO-INDEPENDENCE*

CLONALITY OF GRANULOCYTES	EPO-INDEPENDENT GROWTH SEEN	EPO-INDEPENDENT GROWTH NOT SEEN	TOTAL
POLYCLONAL	3	7	10
MONO- OR OLIGOCLONAL	6**	1	7
TOTAL	9	8	17

* Values shown are the number of patients showing both characteristics indicated.
** 1 patient oligoclonal, 1 patient transiently polyclonal (see Tables 10 & 14).

In 9 patients, a repeat blood and/or bone marrow sample was obtained in the absence of any cytotoxic chemotherapy and a thymidine-suicide assay of the hemopoietic progenitors was performed. Table 12 shows a comparison of the results of these measurements with the clonality data. (Table 13 shows an example of the actual data obtained on one of the more interesting cases, described in detail below.) Of the 9 patients, 4 were found to have clonal circulating granulocytes. In 3 of these, (pt # 7, 17 and 16) an abnormal pattern of progenitor turnover (high thymidine suicide values) was also seen. (The other patient (Pt.#1) who showed no evidence of increased progenitor turnover or Epo-independent growth was noted above.) Amongst the 5 patients where granulocytes appear to be polyclonal, 4 showed no evidence of altered progenitor turnover, but 1 did (patient #5). In patient #5, evidence of polyclonal granulocytes was obtained on each of two samples taken 21 months apart. In patient #13, an oligoclonal pattern was found when the granulocytes were first evaluated (August, 1988) but a polyclonal pattern was found in the sample obtained 10 months later (June, 1989). Interestingly, this patient showed evidence of Epo-independence only in her peripheral blood progenitors, but not in her marrow progenitors.

TABLE 12

ASSOCIATION OF CLONAL HEMOPOIESIS AND ABNORMAL (DEREGULATED) HEMOPOIETIC PROGENITOR CYCLING*

CLONALITY OF GRANULOCYTES	PROGENITORS CYCLING	PROGENITORS NON-CYCLING	TOTAL
MONO- OR OLIGOCLONAL	3	1	4
POLYCLONAL	1	4	5
TOTAL	4	5	9

*Values shown are the number of patients showing both characteristics indicated.

In addition to these two cases suggesting the presence of normal, polyclonal hemopoiesis together with cells showing neoplastic markers, I had the opportunity to study another such patient where transient resolution of her disease was associated with a normal pregnancy (see below).

C- Transient polyclonal hemopoiesis associated with pregnancy-induced remission.

This patient was a 32 year old woman who was first seen by her physician in October 1985 for non-specific bowel symptoms. The WBC at that time was 11.6 x 10^3/µl; the hemoglobin (Hb), 14.4 gm/100 ml; and the platelet count, 940 x 10^3/µl. The peripheral blood smear showed occasional giant platelets. The bone marrow aspirate biopsy was consistent with a diagnosis of MPD. The LAP score was normal, as was the karyotype of all 27 marrow metaphases examined. The red cell mass was normal (30 ml/kg of body weight). Although not clinically palpable, the spleen was enlarged on nuclear scan. The patient received no treatment and remained asymptomatic apart from her colonic dysmobility. Seven months later, she became pregnant and her platelet count then returned spontaneously to normal values (Figure 22).

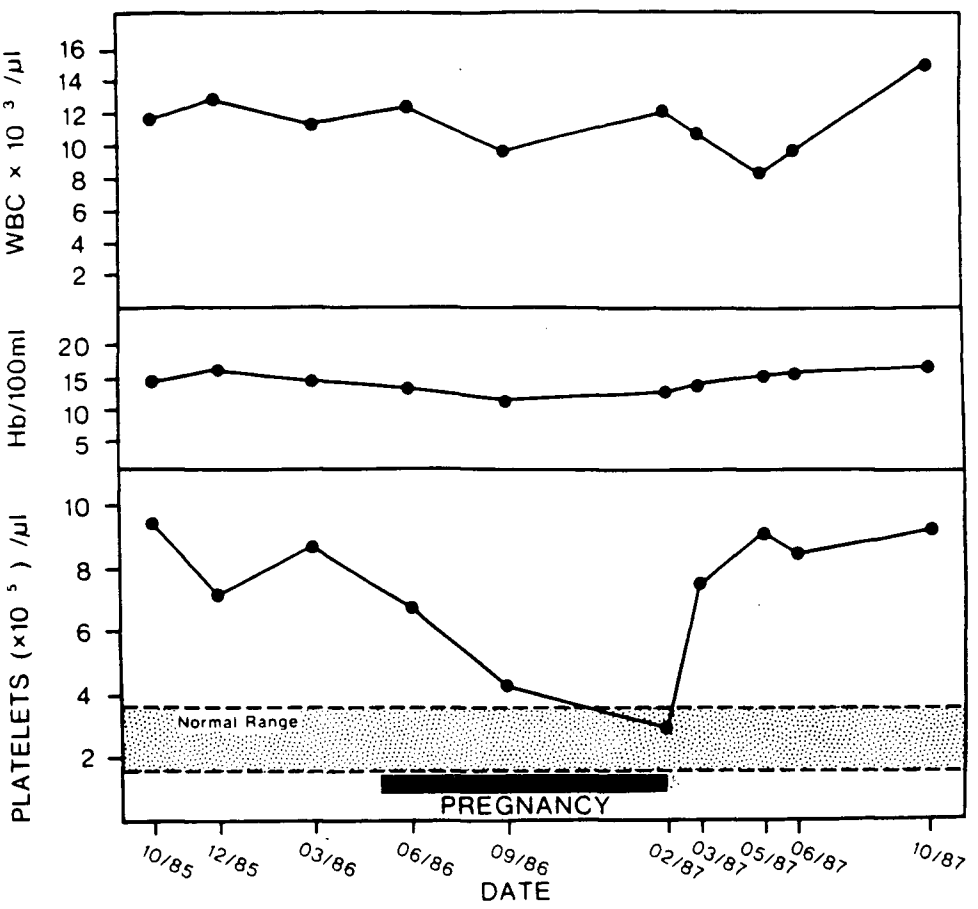

Figure 22

Changes in the peripheral blood counts of patient # 17 studied from the time of her diagnosis. Shaded area indicates normal range of platelet counts. Hb: Hemoglobin; WBC: White blood cells.

Following the birth of a normal child, her platelet count rose to 903 x $10^3/\mu l$. As can be seen in Figure 22, the temporal association between the start and end of her 9 month pregnancy and a corresponding decline and resurgent rise in the platelet count is clearly evident. Since she received no treatment during the entire period, the change in her platelet counts was most likely related to physiologic parameters associated with her pregnancy.

Two bone marrow samples and 4 peripheral blood samples were obtained for assessment of clonogenic progenitor numbers and Epo-independence. As detailed in Table 13, at no time did there appear to be a significant increase in the concentration of any erythroid progenitor type assessed, although her circulating granulopoietic progenitors were consistently at the high end of the normal range. However, on every occasion some readily scored Epo-independent CFU-E and BFU-E were detected. Of particular note was the detection of Epo-independent CFU-E and BFU-E in her September 1986 blood sample which was obtained in the middle of her pregnancy at a time when her platelet count had spontaneously decreased to 426 x $10^3/\mu l$.

Table 14 summarizes the results of the thymidine suicide studies performed on her peripheral blood and marrow progenitors obtained 3 and 4 months postpartum, respectively, at a time when her platelet count had again risen to >800 x $10^3/\mu l$. The data shown in Table 14 are limited to those progenitor categories which in normal individuals appear quiescent (8). In every instance there was an abnormal increase in progenitor cycling in the corresponding progenitor cell types from this patient.

Clonality analysis was performed on sequential samples, using the Bam H1 polymorphism of the HPRT gene (Figure 23a). At mid-pregnancy (September 1986), her granulocyte DNA showed an equal sensitivity of the 24 kb and 12 kb HPRT fragments to HpaII digestion (lane 2) indicating that the active alleles were equally distributed, as expected from a polyclonal cell population. Similarly, 4 months postpartum, DNA obtained from fibroblast cultures showed a polyclonal pattern (Fig. 23b, lanes 1 and 2). In contrast, analysis of post-pregnancy peripheral blood granulocyte DNA (3 months postpartum) revealed a complete

Table 13. Assessment of the Total and Epo-Independent Progenitor Concentrations in Blood and Marrow Samples Obtained from Patient #17[a]

Date	Status relative to period of pregnancy	Platelet count $x10^3/\mu l$	Specimen	CFU-E			BFU-E			CFU-GM
				+Epo	-Epo	(% of +Epo)	+Epo	-Epo	(% of +Epo)	
10/1985	Before	940	Blood	88	19	(22%)	490	49	(10%)	228
			Marrow	69	46	(67%)	96	28	(29%)	87
09/1986	During	426	Blood	89	188	(100%)	648	201	(31%)	347
05/1987	After	903	Blood	25	14	(56%)	283	141	(50%)	85
06/1987	After	847	Blood	64	13	(21%)	747	97	(13%)	352
			Marrow	25	8	(32%)	38	6	(17%)	38
Normal values[b]			Blood	1-345	0		21-841	0		2-290
			Marrow	19-598	0		7-671	0		13-312

[a]Values for blood are expressed on a per ml basis. Values for marrow are per 2 x 10^5 buffy coat cells. Data courtesy of A Eaves.
[b]Normal values show the range defined by $\pm$ 3 S.D. (2).

Table 14. Progenitor ^{3}H-Thymidine Suicide Values[a] in Blood and Marrow Samples from Patient #17

Specimen	Progenitor	% kill[b] Patient		% kill[b] Normal[c]
Blood	CFU-E	49	(48/95)	1.1 ± 2.6
Blood	BFU-E	42	(648/1118)	0 ± 1.2
Blood	CFU-GM	62	(171/450)	0 ± 1.9
Bone Marrow	Primitive BFU-E (>16 clusters)	36	(9/14)	3.6 ± 1.7
	(>8 clusters)	41	(29/49)	13.6 ± 2.1

a Ascertained 3 (blood) and 4 (marrow) months post-partum.

b Calculated from assays of a total of 2.4 x 10^6 blood cells, or 1.2 x 10^6 marrow cells, plated in 6 replicate 1.1 ml cultures per treatment group. Values in brackets represent the actual total number of colonies scored in all 6 dishes with/without ^{3}H-thymidine. Data courtesy of J. Cashman and A. Eaves

c Values are the mean ± 1 SEM from 9 normal blood donors and for 20 marrow samples obtained either from donors of marrow transplants or from untreated patients undergoing hematologic assessment whose marrows showed no disease involvement (8).

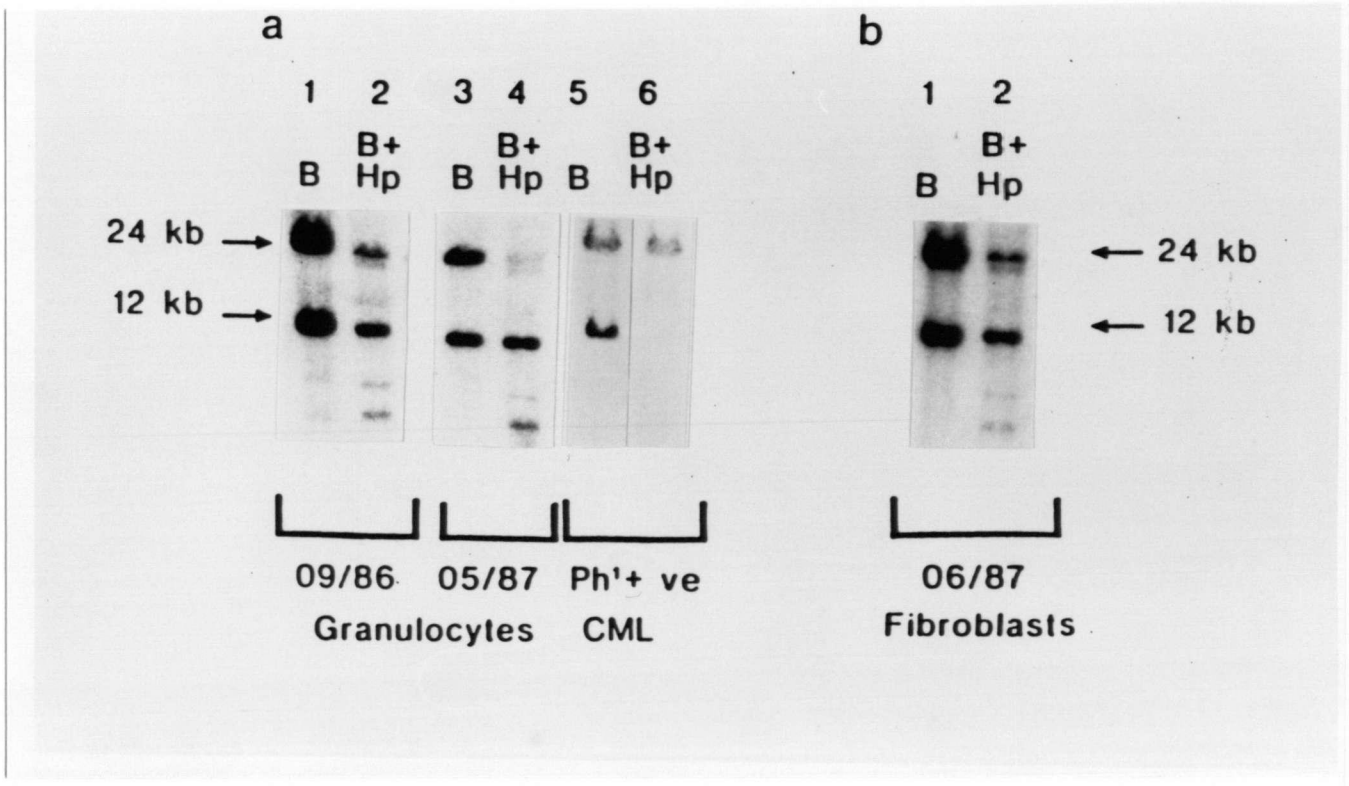

Figure 23

a- Southern blot analyses of granulocyte DNA obtained from patient # 17 during pregnancy (09/86) and 3 months post-partum (05/87) showing a polyclonal pattern during the pregnancy-associated clinical remission (lanes 1,2) whereas a monoclonal pattern is demonstrated in the post-partum sample (lanes 3,4). A Ph^1-positive CML patient is shown as an example of a typical monoclonal pattern (lanes 5,6).

b- Southern analysis of DNA from cultured marrow fibroblasts obtained from the 06/87 marrow aspirate (lanes 1,2), showing a polyclonal pattern.

disappearance of the 24 kb band, without a significant change in the intensity of the 12 kb band, indicative of a monoclonal cell population (lanes 3 and 4). This pattern is analogous to that exhibited by the granulocyte DNA of a female patient with CML who was studied simultaneously as a control using the same approach (lanes 5 and 6). The only difference in the pattern shown in this latter case is the persistence of the 24 kb band and disappearance of the 12 kb band after HpaII digestion indicating the origin of the CML clone in a cell with an active 12 kb allele.

3- DISCUSSION

Patients presenting with an increased platelet count and no evidence of an increased red cell mass, no Ph^1 chromosome and no myelofibrosis often represent a diagnostic challenge. In the absence of an identified secondary cause of thrombocytosis, these patients are considered as likely to have a MPD referred to as ET, a disease believed to be biologically distinct from the other MPD's, with a chronic course and marked with thromboembolic accidents. However, very little is known about the biology of this disorder, although G6PD studies in one patient demonstrated that the granulocytes, red cells and platelets were clonal (6).

The major goal of this study was therefore to determine the clonal characteristics of hemopoietic cells obtained from a number of patients with increased platelet counts (excluding obvious PV's, CML's or patients with myelofibrosis, but otherwise unselected). These findings could then be examined in relation to two other biological abnormalities characteristic of MPD's in general; i.e. Epo-independence and abnormal cycling of primitive hemopoietic progenitors. In general, a strong association was found between all three parameters; i.e. when circulating granulocytes were clonal the other two abnormalities were also usually demonstrable. However, one exception to this was noted, and in this case the marrow biopsy report suggested

the presence of a MPD. It should be noted that Epo-independence, although consistently seen in PV (3), is not always seen in CML (9), and hence it is possible that it might be variably expressed in another MPD.

Results were somewhat more discordant in patients in whom PGK/HPRT analyses indicated the granulocytes to be polyclonal. Of 10 such patients, 7 showed no evidence of other progenitor abnormalities, but 3 did show Epo-independent growth. This highlights an important limitation of clonality analyses. In cell populations in which a large but not necessarily overwhelmingly dominant clone coexists with normal clones, the abnormal clone will not be detected by PGK/HPRT analyses even though its presence may be readily identified by other tests, such as those that detect the presence of Epo-independent erythroid progenitors. The likelihood of such an explanation in these 3 cases is strengthened by the chance opportunity to follow a patient during the spontaneous resolution of her disease during a normal pregnancy and subsequent rapid return of clonal hemopoiesis post-partum.

The present studies constitute the first documentation of persisting normal hemopoietic cells in patients with ET, thus extending to this disease findings that have been previously reported for patients with PV (10) and CML (11). The transient decrease of clonal cells during pregnancy seen in one of the patients studied indicates that some changes associated with pregnancy, either hormonal or immunological may selectively induce the suppression of the neoplastic clone. Recently, a clinical remission apparently associated with pregnancy has also been reported in 3 patients with ET (12). It is interesting to note that alpha interferon has been found to be effective in reducing platelet counts in patients with ET (13), and that interferon-like substances are known to be produced by the placenta (14).

From a practical point of view, the results presented here suggest that clonality analysis using RFLP of X-linked genes may be useful additional laboratory procedure to facilitate the distinction of ET from secondary thrombocytosis.

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CHAPTER VI

CLONALITY ANALYSIS OF Ph^1-NEGATIVE CELLS REVEALED IN LONG-TERM MARROW CULTURE AND AFTER TRANSPLANTATION OF CULTURED MARROW IN CML

1-INTRODUCTION

The Philadelphia (Ph^1) chromosome is the hallmark of chronic myeloid leukemia (CML), a clonal myeloproliferative malignancy which is believed to originate in a pluripotent hemopoietic cell (1). Recently, molecular studies have identified a small region on chromosome 22, called the breakpoint cluster region (bcr) which is broken and rejoined to the c-abl protooncogene, normally located at the distal end of the long arm of chromosome 9 (2,3,4). This results in the formation of the hybrid bcr-abl gene and the synthesis of an abnormal gene product with tyrosine kinase activity (5,6) analogous to that exhibited by the oncogenic murine v-abl gene product (7). Using appropriate DNA probes, it is now routinely possible to detect the presence of non-dividing Ph^1-positive cells using Southern or PCR techniques (8,9) thereby increasing accessibility of the neoplastic clone to analysis beyond those amenable to cytogenetic studies (which are restricted to proliferating cell populations). Molecular studies have thus facilitated assessment of treatment response in CML in a variety of settings, in particular to demonstrate persistence of residual disease after suppression of the neoplastic clone by interferon (10) or intensive therapy supported by allogeneic bone marrow transplantation (11). Such studies have also made it possible to analyze various purified subpopulations of hemopoietic cells and these

have helped to establish a lack of correlation between the particular site of the breakpoint within the BCR gene and the involvement of lymphoid and/or myeloid cells in the neoplastic clone (12).

This Chapter describes the results of molecular studies of the hemopoietic stem cell population present in 10 day "culture-purged" CML marrow autografts (13) based on bcr and clonality analyses of DNA extracted from their differentiated progeny generated either after further maintenance in vitro, or in vivo following transplantation of the cultured autografts.

2-RESULTS

A- Patients

Table 15 summarizes the clinical details of the three CML patients studied. All had Ph[1]-positive CML and were in a 1st, 2nd, or 3rd chronic phase at the time of autografting.

Table 15. Clinical and Hematological Characteristics of 3 CML Patients Transplanted with Autologous Marrow Maintained in Culture

Patient (UPN)[1]	Age[2] (years)	Sex	Pre-BMT Status (% Ph[1]-positive cells)[3]	Outcome
208	44	F	CML in 1st CP (100%)	Alive on α-interferon, clinically normal 26 months after autografting
232	53	F	CML in 3rd CP (90%)	Died of recurrent blast phase disease on day 125
248	41	F	CML in 2nd CP (84%)	Died of therapy-related toxicity on day 28

[1] Unique patient number
[2] At the time of autografting
[3] As determined from direct marrow preparations

B- Analysis of pre-transplant samples

Southern analyses of BamH I digested DNA from pre-transplant samples of marrow and blood cells showed a bcr rearrangement (Figures 24, patient 208; Figure 25, patient 232; Figure 27A and B, patient 248). In patient 248, a LTMC experiment was performed as outlined in Figure 26. The presence of a polymorphic Bgl I site in one of her X-linked phosphoglycerate kinase (PGK) genes, made it possible to undertake clonality studies (14). Southern analysis of DNA from pre-transplant blood and marrow samples from this patient after sequential digestion with EcoRI, Bgl II, Bgl I, and then Hpa II, demonstrated a pattern typical of a monoclonal cell population (Figure 28). Neither of the other 2 patients was suitable for clonality studies because of homozygosity at both the hypoxanthine phosphoribosyl transferase (HPRT) and PGK loci (data not shown).

C- Analysis of cultured marrow cells.

Cytogenetic studies of erythroid and granulopoietic colonies produced by progenitors harvested from the long-term marrow cultures established from all three patients indicated that after 4 - 6 weeks Ph^1-positive clonogenic cells had declined to very low levels, but that Ph^1-negative clonogenic cells were readily detectable. Since cytogenetic analyses tend to be biased towards the selection of larger colonies containing at least 500 immature cells (in order to ensure the presence of some metaphase cells), it was of interest to investigate the pattern of population changes demonstrated by a less selective, but nevertheless quantitative technique that could be applied to all hemopoietic cells in the culture, e.g. by Southern analysis of extracted DNA. Since I determined that one of the patients (UPN 248) was heterozygous for Bgl I polymorphism at the PGK locus, cultures from this individual offered the opportunity to assess changes in bcr-positivity and clonality simultaneously. However, prior to initiating such studies, it was important to establish a procedure that would yield exclusively hemopoietic

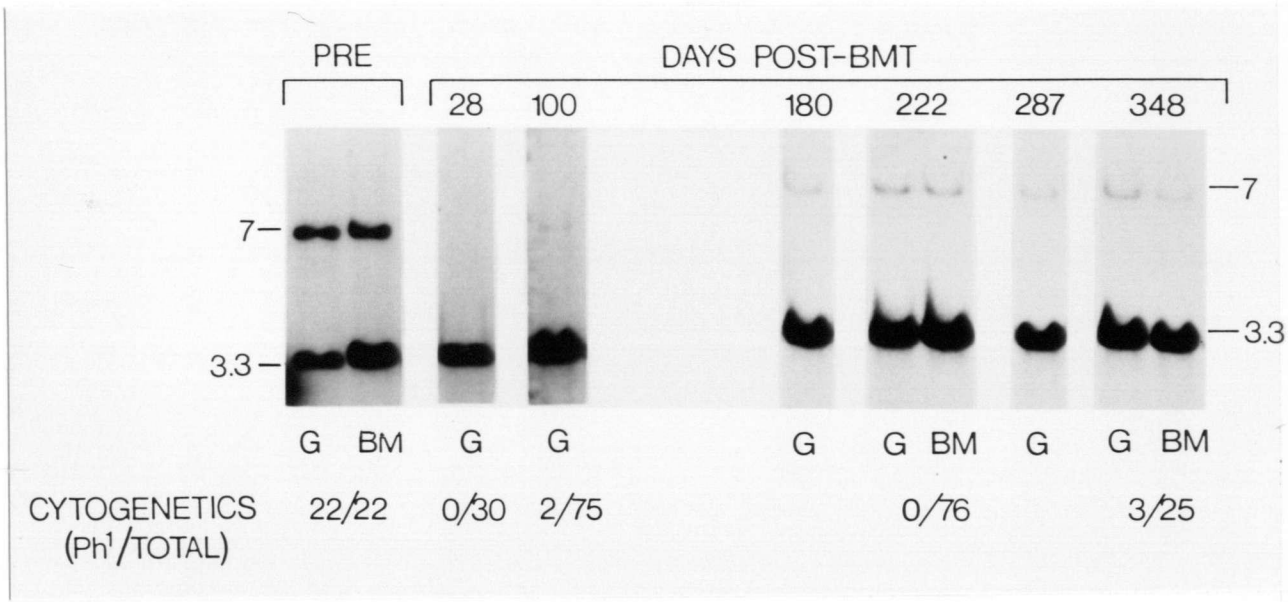

Figure 24.

Southern analysis of pre- and post-transplant DNA samples in patient UPN 208. 20 μg of DNA was digested with BamH1 and hybridized to a 1.2-kb 3' bcr probe. Pre-and post-transplant samples are from the different blots, but the same abnormal 7-kb rearranged band is detected in day+ 100 sample and in the following samples shown, until d + 348. Because of the slightly less amount of DNA loaded of day 28 sample, a repeat gel with 25 μg of DNA loaded also failed to show a rearranged band (data not shown). Cytogenetic data refers to analysis of direct bone marrow metaphases obtained at indicated dates after transplantation. (e.g. 22/22 : 22 metaphases out of 22 are Ph^1-positive). G: Granulocyte BM: Bone Marrow

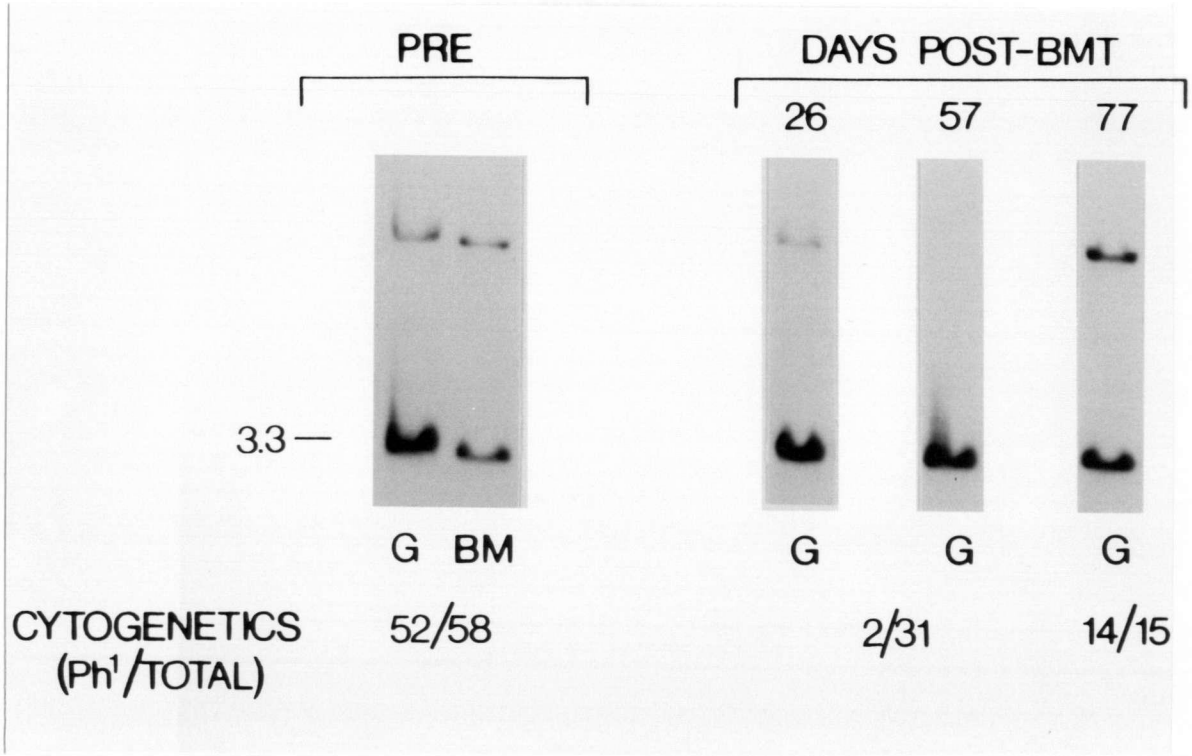

Figure 25.

bcr rearrangement analysis in pre- and post-transplant DNA samples obtained from patient UPN 232. Samples were digested with BamH I and hybridized to the 1.2 kb 3' bcr probe, resulting in a 3.3 kb normal "germline" fragment and an abnormal "rearranged" band. After a transient reduction, the amount of bcr-rearranged fragments is significantly increased at the terminal relapse at day +77. Cytogenetic data are as described in Figure 24. G: Granulocytes; BM: Bone marrow.

cells and in numbers sufficient for sequential Southern analyses.

In established (> 4 week-old) long-term cultures initiated with normal marrow at least half and usually more than 80% of all the cells present are part of the adherent layer, and of these, a large proportion are of non-hemopoietic origin (15,16). In analogous cultures established from CML marrow, the adherent layer, even if less well developed, nevertheless remains the majority population and contains a significant population of stromal cells. (17). On the other hand, the non-adherent fraction, although smaller, consists almost exclusively of terminally differentiating granulopoietic cells which are continuously released as a result of the proliferative activity of more primitive hemopoietic cells that remain in the adherent layer (15,18). Thus, to maximize detection of persisting bcr-positive cells in the culture, I explored strategies for enhancing the production and release of hemopoietic cells into the non-adherent fraction. Of a number of procedures tried, best results were obtained when I simply switched the feeding procedure after four weeks to a complete (rather than a half) medium change and altered its composition to more closely resemble that used to stimulate terminal granulopoiesis, e.g. by granulopoietic progenitors plated in semi-solid assays containing a source of granulopoietic stimulating factors. Preliminary experiments in our laboratory established that cultures fed in this way showed an enhanced release of granulocytes into the non-adherent fraction without a significant effect on the long-term hemopoietic activity of the culture (19). To ensure that the non-adherent cells obtained after initiation of this modified feeding procedure could be used to provide sequential samples of the preceding activity of the hemopoietic population remaining in the adherent layer, all non-adherent cells were removed each week for analysis. Figure 26 shows schematically the LTMC experiment performed. Using this method, 5-10 μg of DNA were routinely obtained per 30 ml culture (each initiated with 10^8 cryopreserved cells).

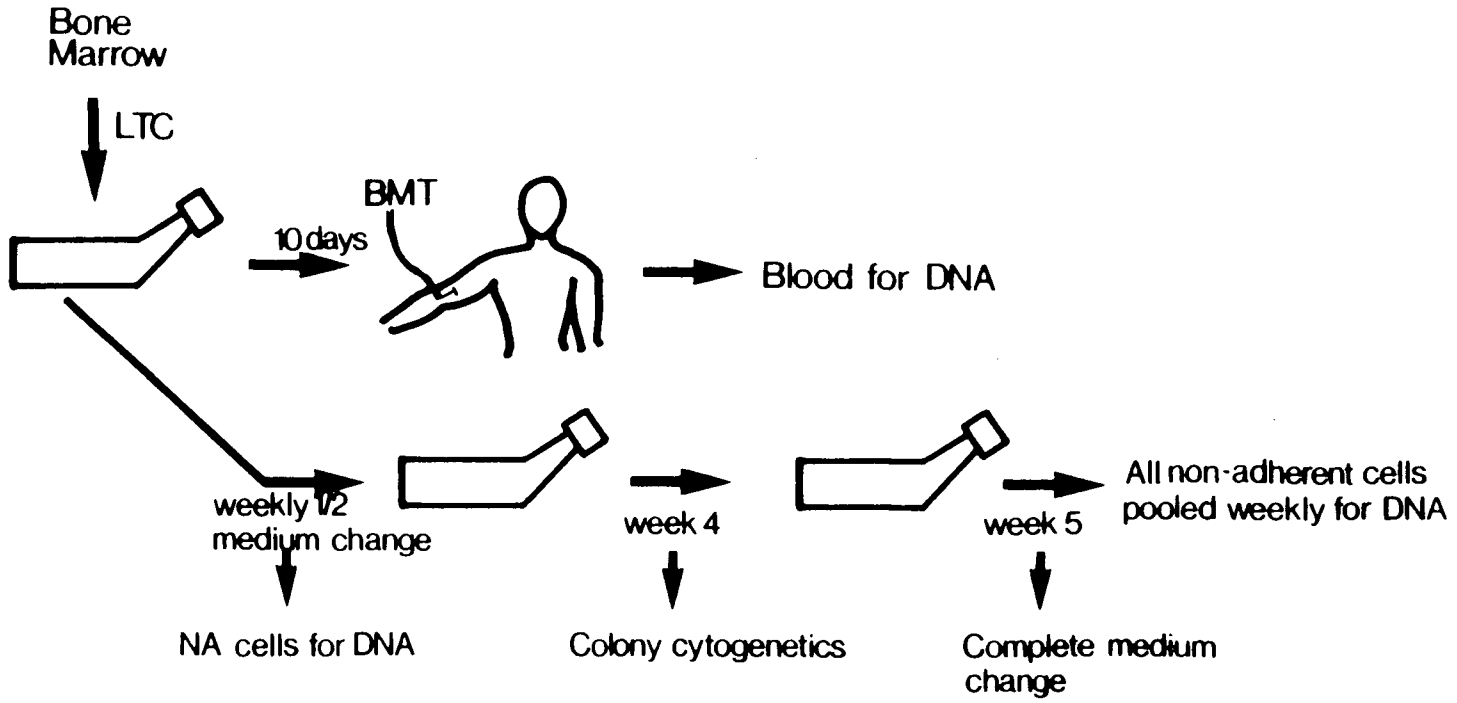

Figure 26.

Schematic representation of long-term marrow culture experiment in patient UPN 248. Bone marrow cells used for autologous transplantation were cultured in 8 large flasks containing standard long-term culture medium and half of non-adherent cells collected weekly for DNA analysis. Starting week 5, a complete medium change was performed using a modified culture medium (containing 30% fetal calf serum, 12.5% horse serum and 10% medium from one week-old 5637 bladder carcinoma cell cultures) and all non-adherent cells were collected and pooled for DNA analysis. NA: Non-adherent; LTC: Long-term culture.

Figures 27 and 28 show the results of both bcr and clonality (PGK) analyses of marrow cells from patient UPN 248 before and after varying periods of time in culture. At the end of the first week in culture, cells showing the bcr rearrangement typical of this patient's clone were still prevalent (Figure 27A). DNA extracted from these cells also showed a monoclonal pattern of methylated fragments (data not shown). The same results were also obtained on the original cultures that had been initiated with fresh marrow cells and harvested and infused after a continuous period of 10 days in culture. Analysis of the non-adherent population obtained after longer periods of culture showed a decline in bcr-positive cells which fell below the limit of detectability (~ 2% as established in parallel dilution experiments) by 5 weeks and remained undetectable thereafter (Figure 27A). bcr-positive cells were also not detectable in the adherent layer after 5 and 8 weeks (data not shown). Clonality studies showed a concomitant emergence of a second PGK band in Southern blots of HpaII digested samples of her non-adherent cells indicating a shift to polyclonal hemopoiesis in her cultures within 5 weeks (Figure 28) and this pattern continued for all subsequent samples.

D- Analysis of post-transplant samples

Results for each patient are shown respectively in Figures 24, 25, 27 and 28 and are summarized in Table 16. Twenty-eight days after the autograft when patient UPN 208 had largely regenerated her hemopoietic system and the peripheral granulocyte count had already recovered to 2.2 X 10^9/L, these did not include detectable (i.e. > 2%) bcr-positive cells (Figure 24). However, a subsequent sample taken ~ 3 months after autografting showed the reappearance of a minor (~ 5%) bcr-positive population which contained the same rearrangement seen prior to autografting and which remained detectable at about the same low level for another 8 months. This patient was then started on treatment with α-interferon (α-IFN) and subsequently, samples from her became bcr-negative (day+ 543 to day+ 718 post-BMT, see Table 16). Cytogenetic results of direct marrow preparations or colonies

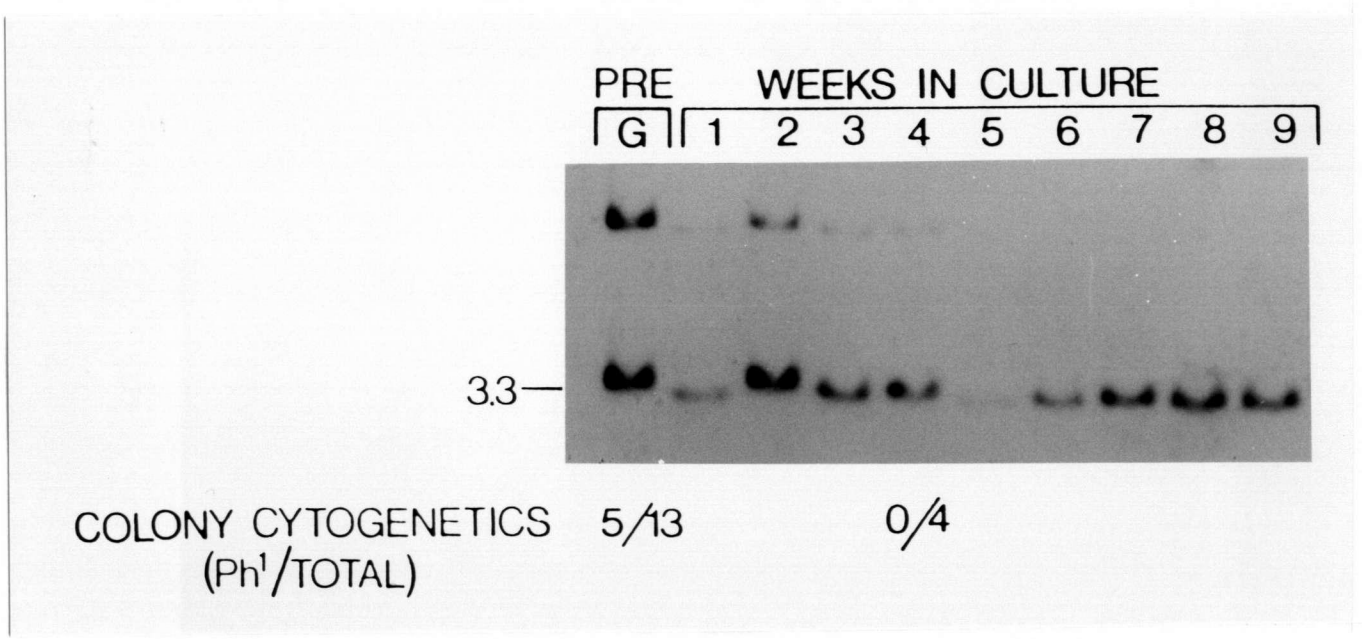

Figure 27A.

Analysis of bcr arrangement in non-adherent cells collected weekly from long-term marrow culture, compared to the pre-transplant granulocyte DNA in patient UPN 248. DNA was digested with BamH I and hybridized to the 1.2 kb 3' bcr probe. Pre-transplant granulocyte sample shows a typical bcr rearrangement which declines gradually and becomes undetectable after 5 weeks. Adherent cells collected by trypsinization after 5 weeks (1 flask) and 8 weeks (7 flasks) also did not show detectable bcr rearrangement. Cytogenetic data are from analysis of in vitro colony assays (number of Ph^1-positive colonies/total number of colonies).

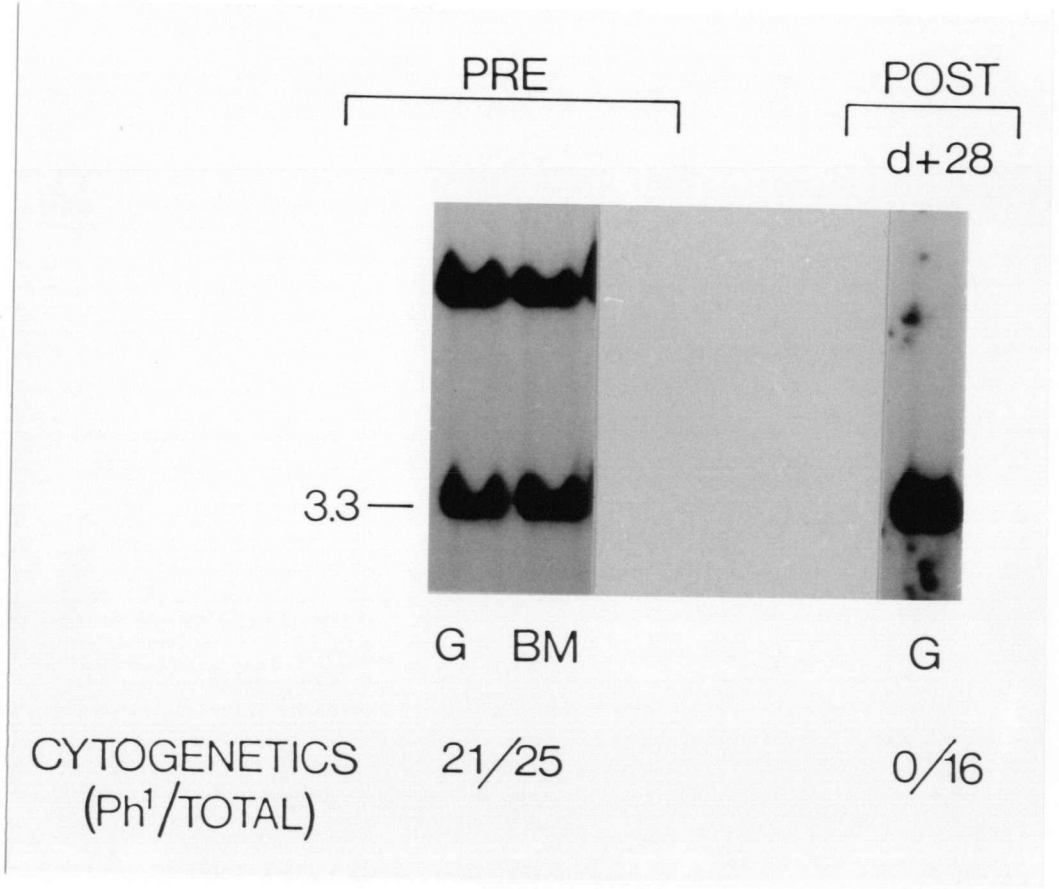

Figure 27B.

bcr analysis of day+28 post-transplant granulocyte DNA sample in patient UPN 248, showing no detectable bcr rearrangement after transplantation. Cytogenetic data are from analysis of direct bone marrow metaphases as described in Figure 24. G: Granulocytes; BM: Bone marrow.

derived from either marrow or peripheral blood progenitors gave the same picture, although given the size of the bcr-positive population that transiently reappeared 3 months after transplantation, it was not always possible to obtain sufficient metaphases to detect its presence (Figure 24).

In patient UPN 232, hemopoietic recovery was slower; nevertheless by day 26 after autografting the granulocyte count was at 0.6 X 10^9/L. As shown in Figure 25, Southern analysis of granulocytes obtained at this time showed that the original bcr-positive clone had been largely, but not completely, replaced by bcr-negative cells. This was confirmed by cytogenetic studies of direct metaphases in a marrow sample obtained 3 weeks later (Day 46) and by repeat Southern analysis of the peripheral blood granulocytes sampled on Day 57. By Day 77, a marked increase in the abnormal bcr band had become apparent in a Southern blot of DNA from the granulocytes in her circulation and a marrow sample obtained 5 days later was heavily infiltrated with lymphoblasts and a high proportion of proliferating cells with a duplicated Ph^1 chromosome. No further follow-up on this patient was possible and she succumbed to this third and final recurrence of blast phase disease.

The last patient (UPN 248) also showed good hemopoietic recovery after autografting but could only be studied for a limited period because she developed overwhelming therapy-related toxicity. Only 2 samples of circulating granulocytes were available for DNA studies and these were obtained 24 and 28 days after autografting, at which time her peripheral granulocyte count was 1.2 X 10^9/L. In neither of these preparations were bcr-positive cells detected (Figure 27B and Table 16). Since clonality studies (by analysis of the methylation status of the PGK gene) were also possible in this patient, both post-transplant granulocyte DNA preparations were also evaluated in this way. As illustrated in Figure 28, the typical monoclonal pattern exhibited by this patient's pre-transplant marrow and granulocytes was replaced in the post-transplant samples by a pattern indicative of a polyclonal population.

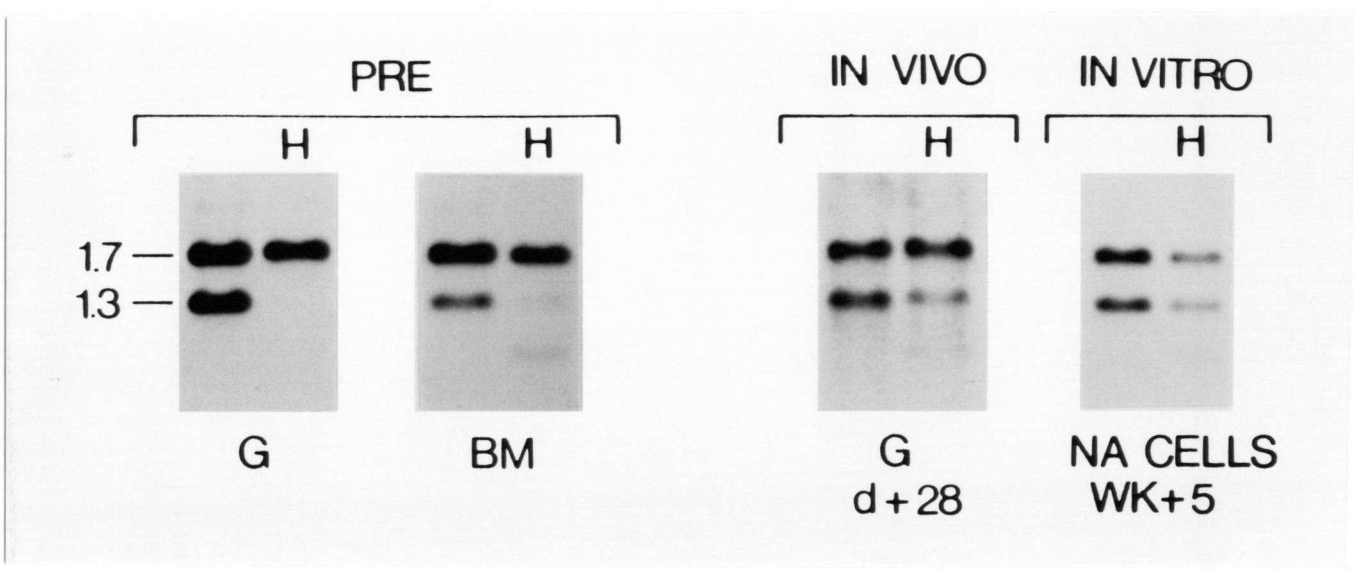

Figure 28.

Clonality analysis using Bgl I polymorphism of the PGK gene in DNA samples obtained from patient UPN 248. DNA samples were digested with EcoR I, Bgl II and Bgl I without or with Hpa II (lanes marked H). Methylation analysis of pre-transplant granulocyte (G) and bone marrow (BM) samples revealed a monoclonal pattern of methylation. In contrast, d+28 post-transplant granulocyte DNA sample and week + 5 non-adherent DNA sample from long-term marrow cultures revealed a methylation pattern consistent with reemergence of non-clonal cells, as indicated by the appearance of the 1.3-kb allele. G: Granulocyte; BM: Bone marrow; NA: non-adherent.

Table 16. Summary of bcr Rearrangements in 3 Patients Transplanted With Autologous Marrow Maintained in Culture

Patient (UPN)	Date of BMT	Pre BMT Samples			Post BMT Samples		
		Date	Tissue[1]	bcr[2]	Days Post BMT	Tissue[1]	bcr Status[2]
208	12/11/87	5/87	BM	+	18	G, LDC	-
		9/87	Gr	+	28	G, T, non-T	-
					100	G	± (weak)
					100	T, non-T	-
					120	G	±
					180	G	±
					222	G, BM	±
					287	G	±
					348	G, BM	±
					427	G	±
					453	G	±
					502	G	±
					543	G	-
					574	G	-
					614	G	-
					644	G	-
					718	G	-
232	26/2/88	9/87	G, LDC	+	26	G	+
		2/88	BM	+	57	G	± (weak)
		2/88	G	+	77	G	++ (strong)
248	6/5/88	12/87	G	+	24	G	-
		4/88	G, BM	+	28	G	-
		5/88	BM autograft	+			

[1] BM: bone marrow, G: granulocytes, LDC: peripheral blood light density (<1.077g/cm^3) cells, T: T-lymphocytes, non-T: T-lymphocyte depleted LDC, BM autograft: cells harvested from 10 day old BM cultures and used as autograft.

[2] bcr status = presence (+) or absence (-) of DNA showing a rearranged bcr gene.

3-DISCUSSION

The primary purpose of this study was to use molecular techniques to evaluate clonality of normal and neoplastic hemopoiesis both in vitro, using LTMC system, and in vivo, studying cell populations obtained from patients transplanted with an autologous cultured marrow. A major advantage of molecular techniques is their applicability to non-dividing as well as dividing cell populations. For the present analyses, it was possible to obtain relatively pure suspensions of maturing granulocytes and hence, assign genotypes to their precursors. In all three case that I analysed, both in vitro and in vivo, bcr data confirmed or extended results that had been obtained by cytogenetic analyses. In addition, in the one patient where clonality studies could also be undertaken, reappearance of bcr-negative hemopoiesis was paralleled by re-establishment of polyclonal hemopoiesis both in vitro and in vivo. The number of patients whose Ph^1-negative hemopoietic cells appearing after several weeks in culture could be subjected to clonality analysis, has been limited so far to two patients. The patient 248 analysed in this chapter represents a third case. Interestingly in each, the marker used to determine clonality has been different. The first such patient studied was a cytogenetic (Turner's syndrome) mosaic (20), the second a G6PD heterozygote (21), and the third, described here, heterozygous for a Bgl I polymorphism in the X-linked PGK gene. Nevertheless, in all three of these cases, the hemopoietic cells present after several weeks in culture were polyclonal indicating the presence of functional normal precursors amongst the cells in the initial suspension used to set up the cultures. The in vivo data on the patient that I studied also showed a temporal association between regeneration of bcr-negative and polyclonal hemopoiesis after autografting of cultured marrow. Given the rapidity of hematologic recovery in all three patients, it seems most likely that this was due to the proliferation and differentiation of a very primitive, genetically normal (Ph^1/bcr-negative, but polyclonal) population that was initially present in the fresh marrow and able to survive for 10 days in culture. These findings are important because they highlight the possibility that some CML

patients might be cured by intensive therapy and transplantation of autologous "culture-purged" marrow. Perhaps of even broader interest is the related implication that conditions are already available to allow preservation of the reconstituting function of a standard human bone marrow harvest for at least 10 days in culture. Such knowledge may be critical to optimizing the design of future therapies involving transplantation of marrow that is to be manipulated or activated in vitro.

In this study, I have also confirmed that a simple modification to the CML long-term marrow culture procedure may additionally stimulate granulopoietic progenitors residing in the adherent layer to increase their output of non-adherent granulocytes. This finding has both practical and theoretical implications of considerable interest. For example, in the present study sufficient non-adherent cells could be harvested each week from long-term CML marrow cultures that contained < 10% of the average number of clonogenic cells found in normal long-term marrow cultures (13) to allow sequential Southern analyses to be performed. Thus, it was possible to monitor the non-adherent cell population by molecular techniques and infer what changes had occurred previously in the more primitive population of hemopoietic cells from which the non-adherent cells are ultimately derived (22,18). These primitive hemopoietic cells remain throughout the life of the culture as a minor component of the adherent layer (22) and therefore cannot be genotyped directly. The ability to obtain sequential cohorts of their progeny as relatively homogeneous populations of non-adherent cells and in amplified numbers using the procedure described here may also provide a useful approach to evaluating primitive hemopoietic cell populations in the adherent layer in other experimental settings.

From a mechanistic point of view, the ability to enhance the output of terminally differentiating granulopoietic cells in long-term marrow cultures indicates that standard culture conditions do not maximally stimulate these cells. It appears that the provision of certain exogenous growth factors either alone or perhaps in an appropriate combination (as exemplified by 5637 cell conditioned medium) (23) can increase the number of mature cells obtained. Further experiments will be required to determine which factors have this potential,

whether their effects are mediated directly or indirectly, and at what level of hemopoietic cell differentiation.

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C H A P T E R VII

MOLECULAR ANALYSIS OF CLONALITY IN PH^1-POSITIVE ACUTE LYMPHOBLASTIC LEUKEMIA (ALL)

1- INTRODUCTION

Philadelphia chromosome (Ph^1)-positive ALL was first recognized in 1970 (1). Since that time the relationship of this disease to chronic myeloid leukemia (CML) and the role of the translocation in the development and progression of malignant hemopoietic clones has been the subject of intense speculation and investigation. In some patients presenting with Ph^1 chromosome-positive ALL, inclusion of myeloid cells within the neoplastic clone appears likely as evidenced by the persistence of large numbers of Ph^1-positive cells in the marrow even after the blast population is reduced by chemotherapy. Such cases are generally viewed as examples of patients with CML whose chronic phase escapes diagnosis and who are thus clinically recognized first in a lymphoid blast crisis (2). In other patients, clinical remission may be associated with the disappearance of Ph^1-positive marrow metaphases, and the extent of lineage involvement in the original disease is more difficult to establish.

Studies of the breakpoint on chromosome 22 have revealed a heterogeneous pattern in patients presenting with Ph^1-positive ALL (3-5). In some, the rearrangement occurs in the same 5.8 kb region ("major" bcr) of the BCR gene (6) that is involved in most cases of CML. In others, the breakpoint may occur 5' of this region but still within BCR (7). According to the site

of the breakpoint, two different c-abl proteins may be produced, a 210-kD bcr-abl fusion protein typical of CML cells,(8,9) and a 190-kD c-abl protein seen in some cases of Ph^1-positive ALL (10-12). This has raised the question as to whether these molecular differences might correlate with the cell type initially transformed (5,11).

In a recent study of 5 patients that presented with Ph^1-positive ALL, extensive cytogenetic analyses of direct marrow preparations and individually removed erythoid, granulopoietic and mixed erythroid-granulopoietic colonies derived from marrow or peripheral blood progenitors suggested variability in the involvement of myeloid cells in different patients (13). Additional material was available from 3 of these patients for molecular studies of clonality (13,14) and bcr rearrangement. The results of these studies provide additional evidence that one of these patients was a case of lymphoid-restricted Ph^1-positive ALL. Further, they show that the breakpoint on chromosome 22 in this patient's blasts occurred within the same region of bcr that was involved in the the two cases of multi-lineage Ph^1-positive ALL studied and in 8 cases of CML also analyzed.

2- RESULTS

a- Patients. Table 17 summarizes clinical and cytogenetic characteristics of 3 patients analysed. All 3 Ph^1-positive ALL patients in presented with a typical ALL picture with 50-98% lymphoblasts in the marrow that showed features consistent with a B-lineage phenotype (patient #1, $CALLA^+$, DR^+; patient #2, $CALLA^+$, TdT^+, $B4^+$; patient #3, TdT^+, $CALLA^+$, DR^+, $B4^+$, $L12^+$, $CytIgM^+$), and all were treated as ALL's. Direct marrow metaphase preparations obtained at diagnosis showed the Ph^1 translocation in all 3 patients. Following remission induction the proportion of normal metaphases increased initially in all cases. However, with time Ph^1-positive metaphases became more prevalent in the marrow of ALL patients #1 and #2, and aspirate and biopsies were judged as consistent with an underlying diagnosis of CML.

Table 17. Clinical and Cytogenetic Data on the 3 Ph^1-Positive ALL Patients Studied*

Patient No.	Age/Sex	Date	Disease Stage	% Ph^1-Positive Cells+ Direct BM Preparations	Myeloid Colonies (Erythroid, Granulocytic and Multi-Lineage)
1	45/M	9/86	Diagnosis	27% (8/30)	22% (20/93)
		11/86	Remission	24% (6/25)	49% (25/51)
		2/87	Remission	68% (17/25)	
2	49/M	1/87	Diagnosis	100% (43/43)	100% (10/10)
		3/87	Remission	92% (23/25)	87 (26/30)
		6/87	Remission	100% (10/10)	100% (13/13)
3	23/F	9/86	Diagnosis	88% (22/25)	0% (0/52)
		10/86	Remission	0% (0/25)	0% (0/53)
		4/87	Relapse	3% (1/30)	0% (0/13)
		5/87	Relapse	60% (6/10)	0% (0/18)

* Blasts from all patients were typed as CALLA-positive and lymphoid morphology, and in the case of patient #3 were further shown to be positive for cytoplasmic IgM.

+Number of Ph^1-positive metaphases/total number of metaphases examined is shown in brackets.

Cytogenetic analysis of metaphases from cultured myeloid colonies (granulocytic, erythroid and mixed granulocyte/erythroid) revealed the presence of the Ph^1 translocation in a readily detectable proportion of myeloid progenitors from both ALL patients #1 and #2 (22-49% and 87-100%, respectively). In contrast, Ph^1-positive metaphases were not seen in direct preparations of marrow from ALL patient #3 except at diagnosis and later at the time of her terminal relapse, and were at no time detectable in her myeloid colonies (a total of 136 metaphases examined from colonies cultured at presentation, in remission and at the time of terminal relapse).

b- Clonality analysis using BamH I/ HPRT polymorphism in patient 3.

Evidence indicating a restricted distribution of malignant cells in ALL patient #3 to the B-lineage was provided by taking advantage of a BamH I restriction fragment length polymorphism in one of her X-linked HPRT genes (Fig 29A). BamH I digestion of DNA from purified blasts and granulocytes obtained simultaneously at the time of terminal relapse and subsequent Southern blot analysis revealed the two characteristic 24 kb and 12 kb bands corresponding to the two HPRT alleles (Fig. 29B, lanes 1, 4 and 7). Further cleavage of the granulocyte DNA with either Hpa II or Hha I (lanes 5 and 6 respectively) resulted in reduction, but not elimination, of the intensities of hybridization in both bands, indicative of active and inactive copies of both HPRT alleles in the original DNA, as expected for a polyclonal cell population. Analysis of normal control DNA from her marrow fibroblasts similarly revealed a pattern typical of a polyclonal population after Hha I digestion (Fig. 29B, lane 8). In contrast, combined digestion with BamH I and Hha I of DNA isolated from her blast cells completely eliminated the 24 kb allele, demonstrating their monoclonal origin (lane 3). Similarly, combined digestion of her blast cell DNA with BamH I and Hpa II resulted in the disappearance of both the 24 kb and 12 kb alleles, (lane 2) a pattern commonly seen when the small p600 HPRT probe is used to analyze monoclonal populations (14).

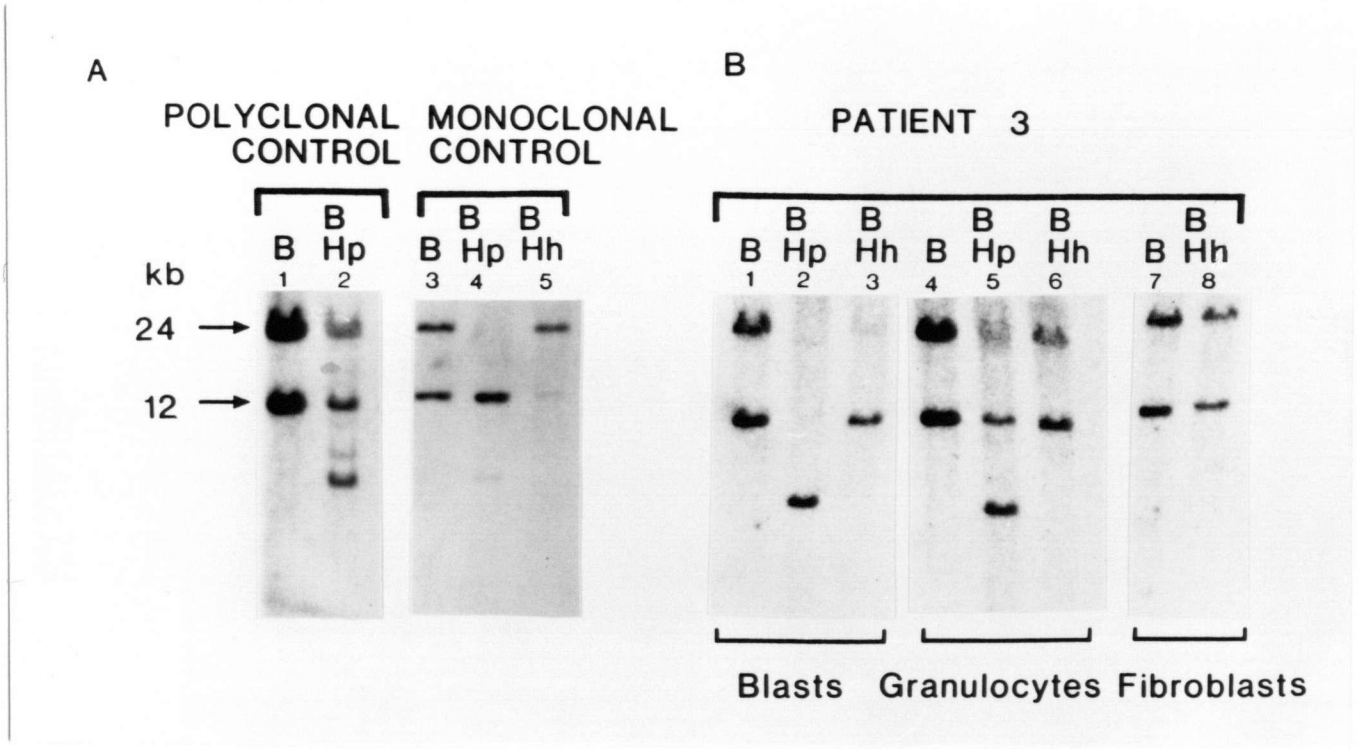

Figure 29

A.Clonality studies of bone marrow cells from two informative patients using BamH I polymorphism of the HPRT gene. Lanes 1 and 2, polyclonal pattern (acute leukemia in remission); lanes 3-5, monoclonal pattern (patient with a myeloproliferative disorder). B.Southern blot analysis of cells from patient #3 who was heterozygous for a BamH I polymorphism in the HPRT gene. DNA samples were digested with restriction enzymes and hybridized to HPRTp600 probe. B: BamH I; Hp: Hpa II; Hh: Hha I. A typical monoclonal pattern is revealed in the blast cell fraction using Hha I digestion. The disappearance of both bands in Hpa II digested sample (lane 2) suggests that clonal amplification originates from a cell with a type I inactive allele. A polyclonal pattern is found in both granulocyte and fibroblast DNA.

c- Analysis of bcr rearrangements. To investigate the type of bcr rearrangement present in the Ph^1-positive cells of the 3 patients, I performed BamH I, and Bgl II, EcoR I and Hind III digests of DNA samples from their granulocytes (ALL patients #1, #2, and #3), blast cells (ALL patient #3) and fibroblasts (ALL patient #3) (Fig. 30). Control DNA was obtained from marrow cells from a normal allogeneic transplant donor, as well as from marrow or blood cells from 8 patients with typical Ph^1-positive CML. Southern blot analysis using the 1.2 kb genomic bcr probe from the 3' portion of the 5.8 kb region of bcr typically involved in CML (3-5) showed a rearrangement in bcr for all 8 CML patients studied, but none in the normal transplant marrow sample (data not shown). bcr rearrangements were similarly detected in BamH I or Bgl II digested granulocyte DNA from ALL patients #1 and #2 using this probe (Fig.30A, lanes 1, 2 5 and 6). In contrast, this probe did not reveal any rearranged bands in either the polyclonal granulocytes or the monoclonal blasts from ALL patient #3 after digestion of the DNA's with BamH I, BglII, EcoR I and Hind III (Fig. 30A, lanes 3, 4, 7, and 8) (EcoR I and Hind III data not shown). However, a second 2.1 kb probe (17) from the 5' portion of the same 5.8 kb region of bcr clearly demonstrated a rearrangement in the blast cells of ALL patient #3 but not in her simultaneously obtained granulocytes (Fig. 30B, lanes 9-12). The pattern of rearrangement with EcoR I, Bgl II and BamH I, but not Hind III, is consistent with a translocation point in the middle (zone 2) (16) of bcr and deletion of sequences 3' to the breakpoint.

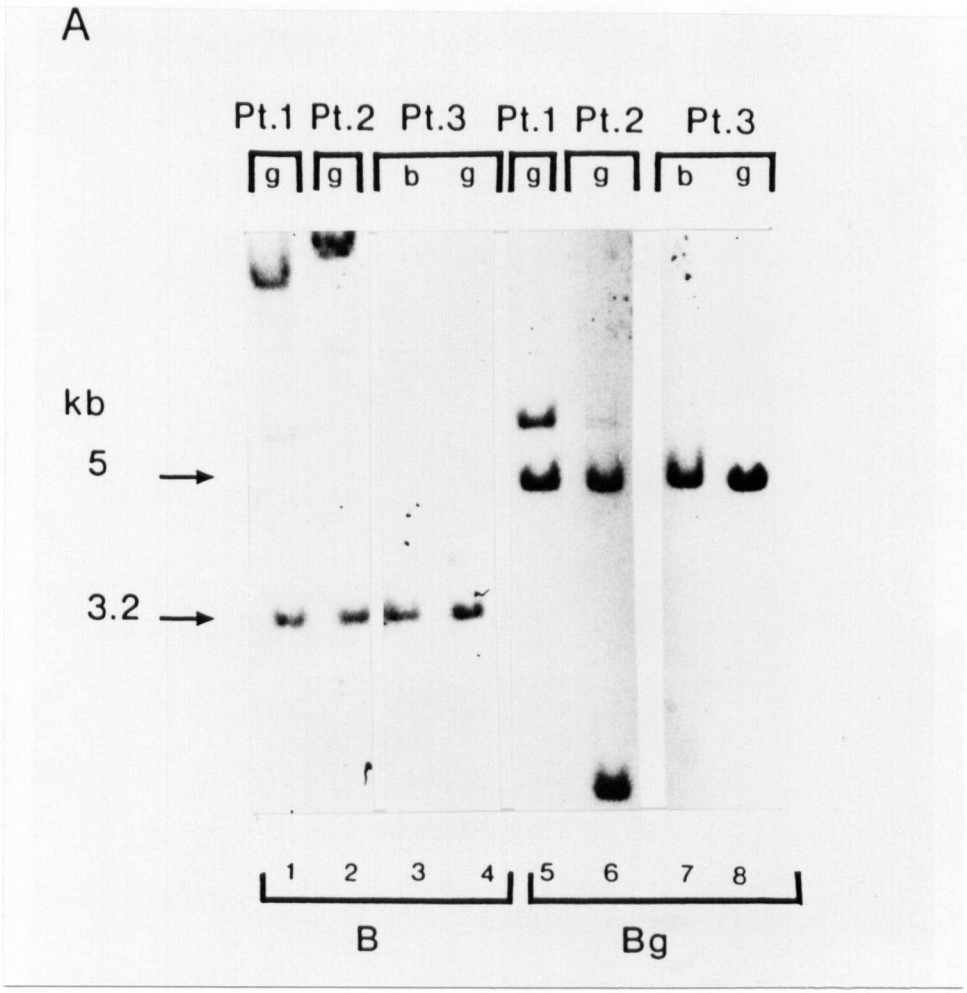

Figure 30A

Southern blot analysis of bcr rearrangement in cells from the 3 Ph^1-positive ALL patients using a 3' bcr probe.Lanes 1 and 5, granulocyte DNA from ALL patient #1; lanes 2 and 6, granulocyte DNA from ALL patient #2; lanes 3 and 7, blast cell DNA from ALL patient #3; lanes 4 and 8, granulocyte DNA from ALL patient #3. B, BamH I; Bg, BglII. Additional non-germ line bands are apparent for patients #1 and #2 with both enzymes.

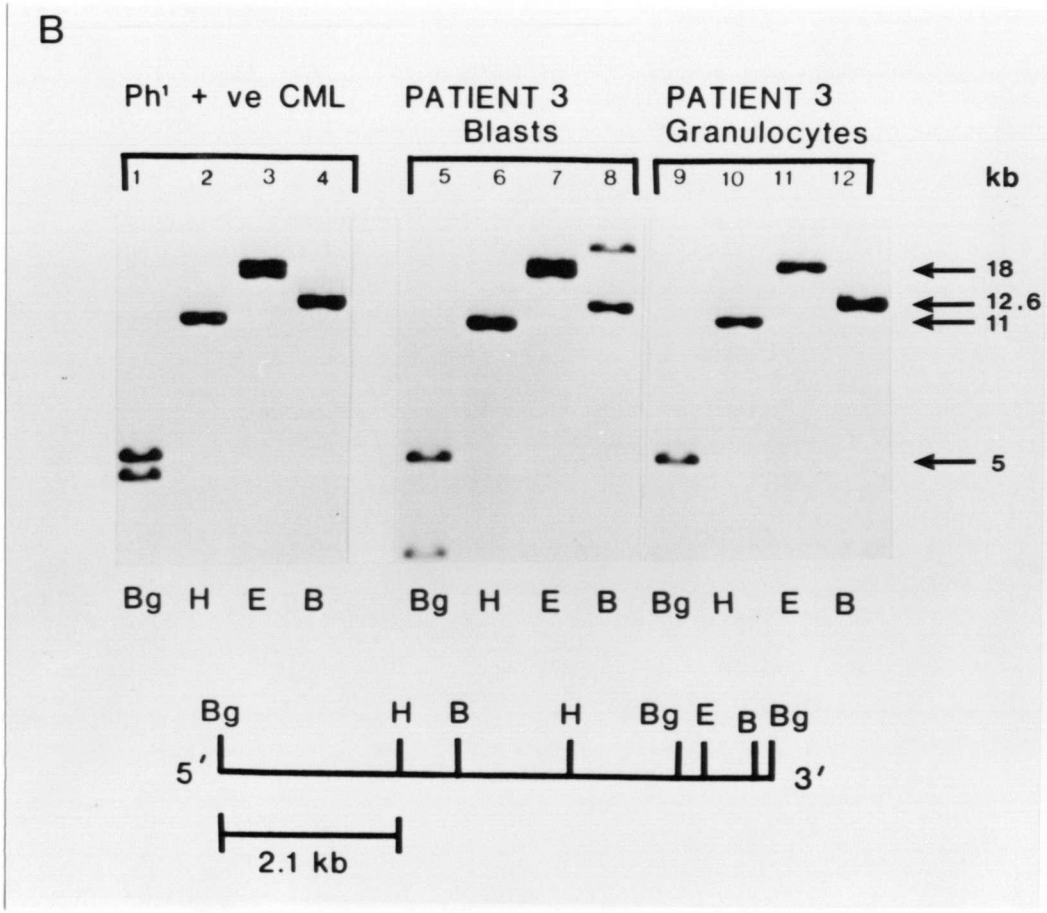

Figure 30B

Southern blot analysis of bcr rearrangements in DNA from ALL patient #3, using a 5' bcr probe. Lanes 1-4, granulocyte DNA from a Ph^1-positive CML patient; lanes 5-8, blast cell DNA from ALL patient #3; lanes 9-12, granulocyte DNA from ALL patient #3. Bg, BglII; H, HindIII; E, EcoR I; B, BamH I. Additional bands indicative of rearrangement are present for the CML sample with BglII and EcoR I and for patient #3 blasts with Bgl II, EcoR I and BamH I. The additional bands on EcoR I digestion are near but resolvable from the germ line band on the original X-ray.

3- DISCUSSION

Thus, the leukemic cells from all 3 ALL patients showed a rearrangement within the same 5.8 kb region of bcr usually associated with CML. For the first two patients, the cytogenetic and clinical follow-up findings also suggested that these two cases might have had a disease very similar to CML, characterized by a clone that originated in a stem cell with extensive myeloid as well as lymphopoietic potential. In contrast, ALL patient #3 showed no clinical evidence of pre-existing CML, and neither clonal nor Ph^1-positive myeloid cells were ever detectable, by both HPRT (Fig. 29) and bcr (Fig. 30B) analysis of her granulocytes and by cytogenetic analysis of her myeloid progenitors, even at the time when her leukemic clone was expanding uncontrollably 8 months post-diagnosis. It therefore seems likely that in this patient the leukemic clone was confined to the B lymphoid lineage and arose in a cell already restricted to this differentiation pathway. Since her leukemic blasts showed a bcr rearrangement similar to that seen in CML clones of multi-lineage stem cell origin, the findings reported here do not substantiate the prediction (5,11) that there is an association between the region of bcr that is rearranged and the cell type in which the rearrangement occurs. A bcr rearrangement restricted to a clonal lymphoblast fraction (as assessed by Ig rearrangement) without involvement of the concomittantly purified myeloid cells has also been reported in a patient with Ph^1-positive ALL (17). This case similar to the patient #3 analysed in this study illustrates the fact that a similar a rearrangement of the bcr undistinguishable to that found in Ph^1-positive CML can be associated with a lymphoid phenotype of blast cells. Interestingly, a lack of specificity has also very recently been found for Ph^1-positive multi-lineage disease (18-19) which in some cases may be characterized by a breakpoint outside of the 5.8 kb region of bcr resulting in the production of a 190-kD protein rather than the 210-kD bcr-abl fusion protein typical of CML (8,9). Phenotypically, the blasts of patients presenting with Ph^1-positive ALL and those seen in CML patients undergoing lymphoid blast crisis are also indistinguishable (2). Thus, the findings reported in this Chapter raise interesting questions

about the significance of the two different bcr-abl fusion proteins described in Ph^1-positive ALL and serve to emphasize the lack of tissue specificity of many transforming molecular genetic changes.

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C H A P T E R VIII

SUMMARY AND CONCLUSIONS

Hemopoietic stem cells have the remarkable capacity to maintain hemopoiesis throughout life by both their differentiation and their self-renewal properties. However, very little is known about the normal functional limits of these cells and thus it has been difficult to define changes from the norm that could be used as biological markers of neoplastic transformation. In mice, the use of genetically marked stem cells in transplantation experiments has shown that reconstitution of all the hemopoietic cells in all myeloid and lymphoid tissues in a reconstituted animal, can occur as a result of the proliferative activity of a single stem cell. Similarly, experimental leukemia can be induced by a variety of treatments and then subsequent changes at the molecular, cellular and tissue level monitored as the disease evolves. In humans, transplantation of hemopoietic cells that have been purposely modified in the laboratory has not yet been approved and systems for following the early phases of evolution of leukemic cell populations are practically non-existent.Thus, to address these questions in the context of the human hemopoietic system, alternative approaches are required. The work described in this thesis illustrates the type of new information in this regard that could be obtained using the method developed by Vogelstein et al (1) to distinguish monoclonal and polyclonal cell populations in samples from heterozygous females.

Following preliminary control studies to establish the feasibility and validity of this technique (Chapter III), I began to investigate the clonal characteristics of normal human hemopoietic cells regenerated in recipients of allogeneic bone marrow transplants. This involved screening sequential bone marrow donors for heterozygosity at the HPRT and/or PGK

loci to identify those recipients from whom to collect samples for clonality analysis during and after their hematologic recovery. In addition, donor (or recipient) origin of hemopoietic cells regenerating after transplantation was determined using RFLP's (including non-X-linked RFLP's where donor and recipient shared the same HPRT or PGK loci). The majority of the patients analysed (10 of 12) showed evidence of polyclonal hemopoietic recovery. However, in two patients, hemopoietic cells obtained after transplantation were donor-derived and mono-or oligoclonal. In one of these patients, the same oligoclonal pattern was seen in the circulating granulocytes for at least 6 months suggesting the persistence of a single dominant clone throughout that period. At the later time, T-cell involvement in the same dominant clone was documented. These findings support the concept that normal human adult marrow contains cells that have both lymphoid and myeloid differentiation potential, as has been previously inferred from similar analyses of neoplastic clones (2,3).

These findings have important pathophysiological implications, for example in the interpretation of clonality data in intensively treated, non-transplanted patients. Clonal remissions have been reported in ~25% of patients with acute myeloid leukemia (AML) (Table 18). Although in some of these patients chemotherapy might have returned the patient to a preleukemic state (e.g. as can occur in CML following suppression of blast phase disease),clonal remissions that have been observed in AML could also be due to the reactivation of a single residual normal stem cell. Although not conclusive, persisting prolonged survivals in some patients (>8 years) could be interpreted as providing support for this view. On the other hand, in the small subset of clonal remissions reported to date in AML, the emerging clonal cells have always shown the same G6PD phenotype or HPRT/PGK genotype as the original leukemic cells from the same patient. This therefore raises the alternative possibility of preleukemic mutational events that predispose a cell to clonal overgrowth with little effect on other biologic functions. In the future, more comprehensive longitudinal analyses are obviously required in larger groups of patients to determine the significance of these alternative possibilities.

TABLE 18: CLONAL REMISSIONS[a] IN ACUTE MYELOBLASTIC LEUKEMIA

Clonal Remissions/ Total Studied	FAB (# of patients)	Age	Longest Duration	Subs. Relapse
5/13	M4(1) M2(4)	2 1/2-65	NR	3/5
3/13	M4(2) M5(1)	42-50	>3 years	NR
2/8	NR	NR	>8 years	NR

NR: Not Reported
FAB: French-American-British Classification
[a] From Reference (4)

The second important implication of normal but clonal hemopoiesis pertains to gene therapy, which could theoretically be applied to certain diseases in the near future. My data suggest that transplantation of a small number of very primitive cells to which the desired gene has been transferred, may be sufficient to restore a deficient product and/or functional compartment. Moreover, if larger numbers of stem cells are transplanted and stem cell recruitment is low, then close to 100% gene transfer efficiencies must be obtainable for gene therapy to be effective. Finally, clonality analysis may be also useful in evaluating the role of growth factor treatments of transplants or transplant recipients on stem cell utilization.

The second goal of my work was to determine if normal, non-clonal cells could be detected in hemopoietic neoplasia. The demonstration of such cells is important because the development of therapeutic strategies that rely on their stimulation or modulation will be rationale only if they are present in these diseases. I first tried to determine the occurrence of such cells in ET. Et has been the least well studied of all the MPDs, both because of its rarity, and the previous lack of a widely applicable method to undertake clonality studies. In the

majority of the patients with a diagnosis of MPD consistent with ET, analysis of granulocyte DNA showed a typical monoclonal pattern. In contrast, in 3 patients with this diagnosis and evidence of circulating neoplastic cells (as indicated by Epo-independent erythroid progenitors and in some cases, their abnormal cycling characteristics), I found evidence of polyclonal hemopoiesis. This indicates that normal stem cells can persist in ET and even differentiate to generate detectable progeny in the presence of the abnormal clone. In one of these patients, the non-clonal cells were detected only transiently during a clinical remission apparently associated with a normal pregnancy. After the delivery, the patient has promptly relapsed with increase of her platelets to pregestation levels. At the same time her hemopoiesis became monoclonal. The study of this case suggests that as yet unidentified factors associated with pregnancy may be beneficial to the control of MPD. Recently, other cases of clinical remissions associated with pregnancy in ET patients have been reported (5). One possibility is that an interferon-like substance that has been shown to be present in bovine placenta (6) could be released during pregnancy in humans. The effectiveness of α-interferon in ET has also been demonstrated in recent clinical trials (7). During the course of these studies it has also become clear that clonality analysis using X-linked DNA polymorphisms can usefully complement the available diagnostic markers in ET and make the important distinction between the diagnosis of ET and that of a secondary or reactive thrombocytosis.

Concommitantly with the above studies, I pursued my goal of identifying non-clonal, non-neoplastic cells in CML, another clonal hemopoietic malignancy of pluripotent stem cell origin. This disease is universally fatal after a median duration of 3 years and no significant advances in therapy have been made for several decades except for the recent introduction of allogeneic bone marrow transplants in selected patients. In our laboratory, it has been shown that when marrow cells from CML patients are placed in long-term culture, Ph^1-positive cells disappear after 4-5 weeks whereas Ph^1-negative hemopoietic progenitors are maintained. In two cases previously studied, clonality analysis using a chromosomal marker in one case (8) and the G6PD polymorphisms in the other (9) demonstrated the non-clonal nature of some

cells emerging in LTMC. With the combined use of the X-linked clonality method and bcr analysis, I showed that the Ph^1-negative cells emerging in a long-term culture established from a PGK heterozygous CML patient were both non-clonal and bcr-negative. In addition, I had the opportunity to study these markers in cells regenerated in vivo in 3 CML patients after transplantation of their autologous marrow maintained for 10 days in culture. In the unique patient heterozygous for a PGK polymorphism, I showed that the regenerated granulocytes 28 days after transplantation had no detectable bcr rearrangement and were polyclonal, in contrast to pre-transplant granulocytes and bone marrow cells which were monoclonal and showed a typical bcr rearrangement. Two other patients,transplanted using the same procedure, also showed hematologic recoveries with predominantly bcr-negative cells. These findings are important because they show that non-clonal and therefore presumably normal cells with long-term repopulating ability exist in CML and they can be maintained unharmed for at least 10 days in LTMC. Their modulation in these conditions associated with some other biological modulations in the future, might represent a new and perhaps a curative approach for this disease.

My goal in analysing Ph^1-positive ALL was to determine if another extreme aspect of hemopoietic stem cell behavior could be documented in human leukemia by the demonstration of concomitant polyclonality and monoclonality within the hemopoietic system in the same patient. The Ph^1 chromosome, the hallmark of CML, has been documented in ~25% of cases of adult ALL and 8-10% of cases of childhood ALL. Clinically, this disorder, has been in some cases undistinguishable from a CML in lymphoid blast crisis with an undetected chronic phase whereas in others the clinical and hematological findings have been sufficiently different as to suggest a distinct disease entity arising in a lymphoid lineage-restricted rather than a pluripotent stem cell. This differential diagnosis has obvious pathophysiological and therapeutic implications. To investigate whether a B-lineage restricted stem cell involvement might be present in some patients with Ph^1-positive ALL, I used both X-linked RFLP and bcr probes to study cell populations in 3 patients with this diagnosis. In 2 male patients initially

diagnosed as Ph^1-positive ALL but later reclassified on clinical grounds as CML in lymphoid blast crisis, a classic bcr rearrangement was present in the circulating granulocytes. However, in one female patient with Ph^1-positive ALL who was heterozygous for the BamH I polymorphism of the HPRT gene, I found that granulocytes were polyclonal and bcr-negative, whereas concommitantly purified blast cells at the terminal relapse phase were both monoclonal and bcr-positive. These findings suggest that the target cell for neoplastic transformation in this disorder might be a lymphoid restricted stem cell. If this were the case then, the classic bcr rearrangement found in the majority of Ph^1-positive CML patients could not be assumed to occur only in pluripotent stem cells. Recently, a Ph^1-positive ALL patient with a clonal Ig rearrangement and bcr rearrangement present only in the blast cells (but not in the concommitantly purified granulocytes) has also been described (10). The use of PCR technology to detect specific leukemic transcript junctions will certainly contribute in the future to readdress the important questions of lineage restriction and target cell for transformation in Ph^1-positive ALL.

In summary, studies that I have presented in this thesis show that:

1- In humans, normal hemopoiesis can be reconstituted by the proliferative activity of a single or a very small number of primitive stem cells.

2- Conversely, normal stem cells persist in patients with both ET and CML. In ET they may actively produce detectable progeny even when the neoplastic clone is dominant. In CML they are selectively reactivated in LTMC and they can be transplanted as functionally active cells in vivo.

3- Ph^1-positive ALL and Ph^1-positive CML in lymphoid blast crisis can be different entities, although this may be relatively rare.

In conclusion, my studies have identified a large spectrum of activity levels for normal and malignant hemopoietic stem cell clones. These various proliferative activities should in the near future be amenable to experimental manipulation for therapeutic purposes in human hemopoietic neoplasia.

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