GENETIC INVESTIGATIONS OF HUMAN HEMOPOIESIS:
STUDIES OF CLONALITY AND GENE TRANSFER TO HEMOPOIETIC PROGENITORS

by

DONNA EILEEN HOGGE

B.Med.Sc., The University of Alberta, 1971
M.D., The University of Alberta, 1973

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

(Department of Pathology)

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May 1987
© Donna Hogge, 1987
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of Pathology

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date April 22, 1987
ABSTRACT

In most neoplasms malignant change occurs in a single cell which then proliferates. My purpose was to explore methods to study the cell that gives rise to hemopoietic cancer and to investigate the abnormalities at a molecular level.

Cytogenetic analysis of cells from individual hemopoietic colonies revealed that monosomy 7 syndrome, a hematologic disorder of childhood, arises in a primitive cell capable of differentiating down both myeloid and erythroid pathways.

Long-term bone marrow cultures (LTC) from patients with chronic myelogenous leukemia (CML) favor the growth of Philadelphia chromosome (Ph) negative progenitors which, although cytogenetically normal, could have been part of the malignant clone at a stage prior to the development of the Ph. LTC's were initiated with cells from 2 women with CML who were heterozygous for 2 electrophoretically distinct glucose-6-phosphate dehydrogenase (G6PD) enzyme variants. In one patient, 2/11 progenitors were Ph-negative after 4 to 6 weeks in LTC and 4/30 were nonclonal by G6PD enzyme analysis, i.e. the colonies expressed the enzyme not found in the malignant clone. In this case, karyotypically normal cells were truly normal.

Next, gene transfer to human hemopoietic cells was demonstrated using recombinant retrovirus carrying the selectable marker gene, neo\textsuperscript{R}. With the K562 human leukemic cell line as targets up to 60% of infected cells became G418 resistant (G418\textsuperscript{R}). Cloned populations of G418\textsuperscript{R} cells showed unique patterns of retroviral integration in K562 DNA. When the target cells were progenitors from normal marrow, CML blood or fetal liver, the highest frequencies of G418\textsuperscript{R} granulocyte-macrophage or large erythroid colonies was 16% and 5% respectively.
Experiments infecting bone marrow cells in LTC with neo\textsuperscript{R} virus produced up to 2% G418\textsuperscript{R} colonies after as long as 3 weeks in culture. Using v-src virus to infect LTC failed to perturb hemopoiesis, although infection of bone marrow-derived cells in these cultures was documented.

In summary:

1. Unique populations of hemopoietic progenitors can be identified in culture using several genetic markers including chromosomes, G6PD analysis or gene transfer.

2. The feasibility of retroviral-mediated gene transfer for use on human hemopoietic cells has been demonstrated.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Introduction</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>An Overview of Hemopoiesis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Markers of Clonality</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>Clonal Disorders of Hemopoiesis</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Oncogenes</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Gene Transfer</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Present Objectives</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>References</td>
<td>64</td>
</tr>
<tr>
<td>II</td>
<td>JUVENILE MONOSOMY 7 SYNDROME: EVIDENCE THAT THE DISEASE ORIGINATES IN A PLURIPOTENT HEMOPOIETIC STEM CELL</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Patients</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>79</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>84</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>87</td>
</tr>
<tr>
<td>III</td>
<td>HEMOPOIETIC PROGENITORS THAT ARE NOT PART OF THE MALIGNANT CLONE REVEALED IN LONG-TERM MARROW CULTURES FROM A G6PD HETEROZYGOTE WITH CHRONIC MYELOGENOUS LEUKEMIA</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>100</td>
</tr>
<tr>
<td>IV</td>
<td>GENE TRANSFER TO PRIMARY NORMAL AND MALIGNANT HUMAN HEMOPOIETIC PROGENITORS USING RECOMBINANT RETROVIRUSES</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Introduction</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>Materials and Methods</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td>Results</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>Discussion</td>
<td>128</td>
</tr>
<tr>
<td>TABLE</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>I</td>
<td>Classification of the Acute Nonlymphoblastic Leukemias</td>
<td>21</td>
</tr>
<tr>
<td>II</td>
<td>De Novo Acute Nonlymphocytic Leukemia in Adults with Specific Chromosome Defects</td>
<td>23</td>
</tr>
<tr>
<td>III</td>
<td>Oncogenes</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>The Proportion of Target Cells Showing Expression of the Transferred Gene Following Various Gene Transfer Techniques</td>
<td>38</td>
</tr>
<tr>
<td>V</td>
<td>Direct Cytogenetic Studies</td>
<td>77</td>
</tr>
<tr>
<td>VI</td>
<td>Bone Marrow Culture - Progenitor Numbers</td>
<td>80</td>
</tr>
<tr>
<td>VII</td>
<td>Peripheral Blood Progenitor Numbers From Patient 1</td>
<td>81</td>
</tr>
<tr>
<td>VIII</td>
<td>Bone Marrow Culture - Hemopoietic Colonies Cytogenetics</td>
<td>83</td>
</tr>
<tr>
<td>IX</td>
<td>Ratio of G6PD Isoenzyme Variants in Lysates of Peripheral Blood and Skin Fibroblast Cells</td>
<td>95</td>
</tr>
<tr>
<td>X</td>
<td>Hemopoietic Colony Cytogenetic and G6PD Analysis</td>
<td>96</td>
</tr>
<tr>
<td>XI</td>
<td>Titers of v-neo&lt;sup&gt;F&lt;/sup&gt; From Producer Cell Lines and pZipNeo</td>
<td>110</td>
</tr>
<tr>
<td>XII</td>
<td>Maximum Frequency of G418&lt;sup&gt;F&lt;/sup&gt; 1° Hemopoietic Colonies after Co-Cultivation with Various v-neo&lt;sup&gt;F&lt;/sup&gt; Producer Cells</td>
<td>119</td>
</tr>
<tr>
<td>XIII</td>
<td>Demonstration of v-neo&lt;sup&gt;F&lt;/sup&gt; Production in G418&lt;sup&gt;F&lt;/sup&gt; CFU-GM by Infectious Center Assay</td>
<td>122</td>
</tr>
<tr>
<td>XIV</td>
<td>Proportion of G418 Resistant Granulocyte-Macrophage Colonies from Nonadherent Cells in Human Long-Term Marrow Cultures Infected with Helper-Containing v-neo&lt;sup&gt;F&lt;/sup&gt;</td>
<td>137</td>
</tr>
<tr>
<td>XV</td>
<td>v-neo&lt;sup&gt;F&lt;/sup&gt; Titers in Medium From Infected Long-Term Cultures (LTC)</td>
<td>139</td>
</tr>
<tr>
<td>XVI</td>
<td>Infectious Center Assays: G418&lt;sup&gt;F&lt;/sup&gt; CFU-GM From v-neo&lt;sup&gt;F&lt;/sup&gt; Infected Long-Term Marrow Cultures</td>
<td>140</td>
</tr>
<tr>
<td>XVII</td>
<td>Transforming Viral Assays From v-src Infected Human Cells</td>
<td>143</td>
</tr>
<tr>
<td>FIGURE</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>--------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1.</td>
<td>Outline of Hemopoiesis as Defined by Colony Assays of Stem Cells and Committed Progenitors.</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Clonal Origin of Human Hematopoietic Cells in Chronic Myelogenous Leukemia.</td>
<td>12</td>
</tr>
<tr>
<td>3.</td>
<td>Chromosomal Rearrangement in CML.</td>
<td>18</td>
</tr>
<tr>
<td>4.</td>
<td>Glemsa-Banded Human Chromosome Map with 21 Oncogenes (Dot), 6 Cellular Genes (Triangle), and Breakpoints (Arrows) for Chromosomal Rearrangements Found in Cancer.</td>
<td>30</td>
</tr>
<tr>
<td>5.</td>
<td>Chromosomal Rearrangement in Burkitt's Lymphoma.</td>
<td>31</td>
</tr>
<tr>
<td>6.</td>
<td>Structure of Moloney Murine Leukemia Virus Retroviral Provirus DNA.</td>
<td>41</td>
</tr>
<tr>
<td>7.</td>
<td>Retroviral Life Cycle.</td>
<td>44</td>
</tr>
<tr>
<td>8.</td>
<td>Critical Steps in the Synthesis of Retroviral DNA.</td>
<td>46</td>
</tr>
<tr>
<td>9.</td>
<td>Construction of a Recombinant Retrovirus and Generation of Viral Stocks for Gene Transfer Experiments.</td>
<td>54</td>
</tr>
<tr>
<td>10.</td>
<td>Construction of a ( \Psi^- ) Amphotropic Retrovirus.</td>
<td>56</td>
</tr>
<tr>
<td>11.</td>
<td>Infection of Human Hemopoietic Cells with ( v\text{-neo}^R ).</td>
<td>106</td>
</tr>
<tr>
<td>12.</td>
<td>( G418^R ) K562 Colonies (% of Colonies Grown without ( G418 ) after Infection of ( 10^5 ) K562 Cells with Various Sources of ( v\text{-neo}^R ).</td>
<td>112</td>
</tr>
<tr>
<td>13.</td>
<td>( G418^R ) K562 Colonies (% of Colonies Grown without ( G418 ) after Infection of ( 10^5 ) Cells with ( v\text{-neo}^R ) from 3 ml Undiluted Supernatant or a 60 mm Dish of Confluent, Irradiated Viral Producer Cells of Various Types.</td>
<td>114</td>
</tr>
<tr>
<td>14.</td>
<td>Southern Blots of Total Cellular DNA Hybridized with a ( ^{32}p )-Labeled ( \text{neo}^R ) Specific Bam H1-Hind III Fragment from ( \text{pRSV-neo} ).</td>
<td>115</td>
</tr>
<tr>
<td>15.</td>
<td>Frequency (% of ( G418^R ) Primary Hemopoietic Colonies ( (G418^R ) Colonies/Total Colonies without ( G418 ) x 100) after 24 Hours Exposure of ( 5 \times 10^6 ) Cells to a 60 mm Dish of Irradiated, Confluent ( v\text{-neo}^R ) Producer Cell Lines at Various Viral Titers.</td>
<td>117</td>
</tr>
</tbody>
</table>
FIGURE 16. Frequency (%) of G418<sup>r</sup> Primary Hemopoietic Progenitors (G418<sup>r</sup> Colonies/Total Colonies without G418 x 100) after Infection of 5 x10<sup>6</sup> Cells by v-neo<sup>r</sup> from a 60 mm Dish of Irradiated Confluent Producer Cells or 5 ml Undiluted Supernatant of Various Types.

FIGURE 17. RNA Spot Blot of Total Cellular RNA from Pooled CML Granulocyte–Macrophage Colonies or K562 Cells Hybridized with a 32P-Labeled neo<sup>r</sup> Specific Bam HI–Hind III Fragment from pRSV-neo.

FIGURE 18. Total Nonadherent Cells per Long-Term Culture (Panel a) and Progenitor Numbers per 10<sup>5</sup> Nonadherent Cells (Panels b and c) in Cultures Initiated with Peripheral Blood Cells from a Patient with CML on Normal Bone Marrow Fibroblast Feeders.

FIGURE 19. Total Nonadherent Cells per Long-Term Bone Marrow Culture from a Patient with Myelodysplasia or Preleukemia.

FIGURE 20. Total Nonadherent Cells per Long-Term Bone Marrow Culture (Panel a) or Granulocyte/Macrophage Colony-Forming Cells (CFU-C) per 10<sup>5</sup> Nonadherent Cells (Panel b) from a Patient with Myelodysplasia or Preleukemia.
ACKNOWLEDGEMENTS

I wish to express my sincere appreciation:

To my supervisor, Dr. R.K. Humphries, and Dr. C.J. Eaves for their support and guidance throughout my research;

To members of my advisory Committee, Drs. P.A. Baird, A.C. Eaves, D.K. Kalousek and W.L. Dunn for their helpful suggestions;

To Drs. L. Coulombel and K. Shannon for scientific collaboration and help in obtaining patient samples suitable for these studies;

To Gloria Shaw, Marjorie Hutchison, Dianne Reid, Darlene Nipius and Sheryle Taylor for expert technical assistance;

To Susan Hayley and Michele Coulombe for secretarial assistance during preparation of the manuscript;

To the National Cancer Institute of Canada for financial support.
CHAPTER I

INTRODUCTION

1) AN OVERVIEW OF HEMOPOIESIS

The maintenance of hemopoiesis is felt to be the result of the behavior of primitive hemopoietic stem cells. These cells retain capacities for both self-renewal and differentiation along a pathway committed to the development of a particular lineage of blood cells. The property of self-renewal allows hemopoiesis to be maintained throughout the lifetime of an animal. This occurs although functional blood cells, including granulocytes, monocytes, red blood cells, platelets and most lymphocytes which are the ultimate products of stem cell commitment and differentiation, are relatively short-lived. Current evidence favours the existence of a hierarchy among hemopoietic progenitors along each committed pathway. Each successive level exhibits reduced proliferative and little or no self-renewal capacities as it differentiates toward functional blood cells. The regulation of this complex process of hemopoiesis and the precise factors which determine stem cell commitment vs self-renewal are subjects of intense interest and investigation but have not been completely elucidated. Major discoveries which have formed our current understanding of hemopoietic stem cell and growth factor physiology will be described in the following section.

Hemopoietic progenitors are extremely rare cell types even in blood-forming organs such as the bone marrow (less than one in one thousand nucleated cells). In addition, they cannot be identified morphologically.
These two factors have made it necessary to use indirect methods to study stem cell behavior. In 1961 Till and McCulloch (1) injected murine bone marrow cells into lethally irradiated syngeneic mice. Discrete nodules of hemopoietic cells formed on the recipient animal's spleen 8 to 10 days after injection. These nodules contained either pure populations or various combinations of erythroid, granulocytic, megakaryocytic or undifferentiated cells (2). Each of these nodules was shown to represent a clone derived from a single primitive cell by injecting cells with specific stable chromosomal abnormalities into irradiated, cytogenetically normal recipients. The abnormal karyotype was seen in more than 95% of metaphase cells in some colonies (3). If a suspension of cells derived from a single spleen colony is injected into an irradiated mouse new spleen colonies of approximately the same size are seen to form ten to fourteen days later (4). The formation of these secondary spleen colonies is evidence of the self-renewal capability of the cells from which the colonies are derived. (These cells were called CFU-S for spleen colony forming units). If cells from a spleen colony containing a pure population of cells, such as granulocytes or erythrocytes, were injected into an irradiated recipient colonies would form which contained several cell types (5,6). Thus, one marrow cell, CFU-S, is capable of forming a colony containing three myeloid cell types. This same cell, in addition to its ability to proliferate and differentiate, can undergo self-renewal.

Further studies into the stem cell hierarchy have provided evidence for the existence of a pluripotent stem cell capable of giving rise to lymphoid as well as myeloid cells (7). More recent work using unique sites of retroviral insertion into hemopoietic cell DNA as markers of clonality have shown that the same stem cell can repopulate both thymus and bone marrow. The proliferative capacity of these cells was considerable as a single clone was
often sufficient to repopulate an entire animal (8,9). In human beings, study of glucose 6 phosphate dehydrogenase (G6PD) heterozygotes with clonal disorders of hemopoiesis have also demonstrated a common cell of origin for lymphoid and myeloid cells (10).

In vitro colony assays have been developed which permit the growth and development of progenitors which give rise to all types of mature blood cells. Thus, rare primitive cells are recognized by their differentiated progeny. Cells are suspended in viscid (methylcellulose) or semisolid (agar) media which partially immobilizes the dividing cells so that the offspring of a single progenitor form a cluster or colony. The concentration of cells plated in each dish is chosen so that colonies are widely dispersed and can be recognized as separate entities and counted. The type of colony growth that is favoured by a particular assay depends on the addition of various stimulatory factors. Colony-forming units in culture (CFU-C) or granulocyte-macrophage colony forming units (CFU-GM) are descendants of CFU-S but are committed to granulocyte-macrophage lineages and have limited proliferative capacity (7). Erythroid burst forming units (BFU-E) are the primitive erythroid counterparts of CFU-C and appear to be closely related to CFU-S. BFU-E form large, hemoglobinized erythroid colonies composed of 3 or more clusters of cells in the presence of high concentrations of erythropoietin. Erythroid colony forming units (CFU-E) form small clusters of 8 to 32 erythrocytes and are located late in erythroid maturation (11). Megakaryocyte colony forming units (CFU-Meg) have been described which appear to be the megakaryocyte counterparts of CFU-C and BFU-E. In addition to progenitors of restricted lineage assays have been developed which identify pluripotent primitive cells, (CFU-GEMM) which give rise to colonies containing a mixed population of granulocytes, erythrocytes, monocytes and megakaryocytes (12).
In general, the more primitive progenitors give rise to larger colonies which require 2 to 3 weeks in culture and many cell divisions to produce terminally differentiated blood cell progeny. The more mature progenitors, e.g. CFU-E, develop within a week in vitro and after only a few cell divisions produce small colonies of mature cells (Figure 1).

The mechanism by which stem cells are regulated and the way in which choice between self-renewal and differentiation into one of the various types of committed progenitors is made has not been elucidated. However, at least three models have been proposed. The first, known as the stochastic model proposed by Till et al in 1964 (13) holds that differentiation along any lineage is a random event with a defined chance of occurring with each cell division. Two other models state that stem cell determination is dependent on either the specific microenvironment surrounding the cells (14) or soluble humoral factors (15). Studies on the self-renewal of hemopoietic stem cells during mixed colony formation in vitro suggest that the choice to self-replicate or not is determined by a mechanism that is at least partly random and intrinsic to the cell itself (16). Recent work has identified a unique type of colony consisting of primitive hemopoietic progenitors (17). The study of these blast cell colonies has provided further evidence for the stochastic model of self-renewal and stem cell differentiation. Paired daughter cells of unicellular origin from blast cell colonies have been shown to generate colonies of diverse multi and oligo lineage combinations. Each cell division of progenitors may yield progenitors with dissimilar lineage potentials. The loss of lineage potential does not appear to proceed in a defined sequence but is a random process.

Various substances have been found to be necessary to support the growth of hemopoietic cells in vitro and many of these may also be important in vivo. A number of serum components are necessary for the growth of mammalian
FIGURE 1. Outline of Hemopoiesis as Defined by Colony Assays of Stem Cells and Committed Progenitors.
cells in culture. These include trace elements and nutrients, various proteins and endocrine hormones such as insulin, thyroid hormones and steroids. Erythropoietin, another endocrine hormone, and certain non-endocrine growth factors are specific requirements for the growth of certain hemopoietic cells in vitro (18). The most convenient sources for enriched concentrations of these latter protein products are; a) medium conditioned by the presence of white blood cells that have been stimulated by agents such as phytohemagglutinin, or b) conditioned media from certain immortal cell lines. Leukocyte conditioned medium, in particular, contains a number of hemopoietic growth factors several of which have been purified to homogeneity and the genes encoding their protein sequence cloned. A brief review of factors felt to be important in regulating human hemopoiesis follows.

A. **Interleukin 3 (IL-3)**

Both the murine and the human IL-3 genes have been cloned and sequenced (19,20). The proteins are glycosylated and there is relatively little homology between the mouse and human DNA sequences. The activities of IL-3 on bone marrow cells in vitro include a permissive role for proliferation of early multipotential progenitors, increasing the number of cells capable of responding to erythropoietin, and promoting the growth and differentiation of granulocytes and macrophages and mast cells. Thus, IL-3 induces proliferation and differentiation of a relatively early stem cell that can differentiate to a variety of cell types.

B. **Granulocyte-Macrophage Stimulating Factor (GM-CSF)**

Granulocyte/macrophage colony formation in vitro requires the continual presence of a second glycoprotein produced by activated T cells and other cell
types, GM-CSF. Both the murine and human forms of this protein have been purified, cloned and sequenced (21,22). The two molecules show substantial sequence homology at the nucleotide (69%) and amino acid (54%) levels (23). In spite of this similarity, the murine and human GM-CSF's show no cross species activity in biological assays. The availability of recombinant human GM-CSF has allowed the identification of activities of this factor not previously appreciated (24). It stimulates the growth of BFU-E derived erythroid colonies and mixed lineage colonies derived from CFU-GEMM as well as granulocyte/macrophage colonies. Human GM-CSF also functions as a neutrophil activator as shown by its induction of granulocyte superoxide production and inhibition of neutrophil mobility (25).

C. **Granulocyte Colony Stimulating Factor (G-CSF)**

A third colony stimulating factor G-CSF stimulates the growth of neutrophil colonies and the terminal differentiation and function of both normal and malignant granulocytes in vitro. Like GM-CSF, G-CSF is able to support the growth and maturation of multilineage and erythroid colonies suggesting action at multiple levels of hemopoietic progenitor cell differentiation (26). The human factor shows species cross-reactivity as it will stimulate the growth of murine granulocyte colonies. Both the murine and human forms of this protein have been purified and the genes cloned (27,28).

D. **Macrophage Colony Stimulating Factor (M-CSF)**

A fourth factor, M-CSF or CSF-1, has also been highly purified and the gene for the human factor cloned (29). It stimulates the growth and differentiation, and function of monocytes and macrophages and shows species cross reactivity.
E. Erythropoietin

A fifth factor which is critical to in vitro hemopoietic cell development is erythropoietin. This is the only known hemopoietic growth factor which is a true endocrine hormone. It is produced by renal tubular cells in response to tissue hypoxia and like other hemopoietic factors is a glycoprotein (30). The murine and human genes for erythropoietin have also been cloned (31,32) and large quantities of recombinant hormone are available. The predominant action of erythropoietin is to induce terminal differentiation of late committed erythroid progenitors, notably CFU-E (30).

F. Interleukin 2 (IL-2)

Human IL-2 triggers the proliferation of both mouse and human activated T lymphocytes and may also stimulate B cells via specific receptors (33).

All of the preceding 6 hemopoietic factors appear to act initially by binding to high affinity cell surface receptors on responsive cells. The number of receptors is relatively low on normal cells (less than 1000/cell) and the factors have their maximum activity at extremely low (pM) concentrations. The molecular mechanisms by which any of these molecules induce changes in target cells after receptor binding is unknown.

The availability of large quantities of recombinant hemopoietic growth factors has allowed in vivo studies of their activity to be done. Recombinant erythropoietin has been shown to correct the anemia of chronic renal failure in human beings (34). Human GM-CSF and G-CSF cause a dramatic rise in granulocytes in cytopenic monkeys (35,36). GM-CSF causes a rise in platelets and reticulocytes as well. Thus, it appears that factors that have for some years been known to be essential for in vitro hemopoietic cell growth have important roles in vivo as well.
G. **Hemopoietic Microenvironment**

Much of the regulation of hemopoiesis in vivo probably occurs at a local level. This is suggested by the fact that hemopoiesis normally is confined to the bone marrow in adult humans and the bone marrow and spleen in adult rodents. It would seem likely that the specific composition of these organs allows hemopoiesis to occur and the substances necessary to support hemopoiesis are not present in other parts of the body. The S1/Sl mouse provides a model in which severe anemia and a decreased production in all lines of hemopoietic cells is the result of a defective hemopoietic microenvironment (37). Much work has been done to study various factors of potential importance in the local control of hemopoiesis. Recently cells of the type present within the bone marrow microenvironment have been shown to produce some of the growth factors to which hemopoietic progenitors respond. For example, activated macrophages will produce interleukin 1 (IL-1) which then stimulates fibroblasts and endothelial cells to make both GM and G-CSF (38). Other substances such as tumor necrosis factor will also induce hemopoietic growth factor production from mesenchymal cell types that are prominent within the bone marrow microenvironment (39). Other studies have investigated the potential role of the extracellular matrix and direct cellular interactions in regulating hemopoiesis. The long term bone marrow culture originally developed by Dexter (40) and modified for human studies by Greenberger (41) and others (42) allows one to investigate cellular and stromal-cellular interaction that take place in the hemopoietic microenvironment. In this system bone marrow cells in appropriate nutrient medium are placed in a tissue culture dish. Over a period of several weeks a layer of cells adherent to the culture dish forms which consists of fibroblasts, endothelial cells, adipocytes and a variety of hemopoietic cells
including the majority of primitive progenitors. The organization and composition of this adherent layer in many ways resembles the bone marrow microenvironment in vivo. In the medium above this adherent layer is a population of nonadherent hemopoietic cells which consists primarily of differentiated cells but also some progenitors. Cells from this nonadherent fraction can be sampled periodically and studied for various properties. Long term bone marrow cultures established with murine cells will maintain hemopoiesis for 6 months or longer. Cells harvested from the nonadherent fraction in these cultures are rich in hemopoietic progenitors including CFU-S. Human long term marrow cultures are less successful in that hemopoiesis, although maintained for at least 10 weeks, declines steadily as measured by both numbers of nonadherent cells and progenitor content within both the adherent and nonadherent fractions. The composition of the extracellular matrix in the adherent layer of long term cultures has been analyzed and shown to contain various types of collagen, fibronectin, laminin and proteoglycans as well as other substances (43). However, the role of any of these substances in supporting hemopoiesis is unclear. Similarly, the relative importance of nonhemopoietic cells in the marrow environment in hemopoietic regulation is unknown. In addition, the methods besides growth factor production by which these cells may modulate hemopoiesis are speculative at this time. Nevertheless, the long term marrow culture provides a convenient in vitro system in which to study various aspects of hemopoietic regulation.

Thus, normal hemopoiesis is a complex process which requires an intricate network of regulatory controls. A number of in vitro and in vivo model systems have been developed which have been used to elucidate most of the current knowledge in this area.
2) **MARKERS OF CLONALITY**

Normal hemopoiesis is polyclonal. That is, an organism contains many hemopoietic stem cells which through their proliferation contributes to the pool of differentiated blood cells. Although the ultimate proliferative potential of a single stem cell is unknown, it has been demonstrated that a single pluripotent cell is capable of at least temporarily repopulating the hemopoietic system of an irradiated mouse (8,9). Similar information is not available for human hemopoiesis. In contrast to the polyclonal nature of normal hemopoiesis, hemopoietic neoplasms are almost always clonal. The techniques which have been used to establish these facts will be discussed in the following section.

A. **Chromosomal Markers**

Radiation induced karyotypic abnormalities were used several decades ago to establish the clonal origin of spleen colonies in a host animal (3). The discovery of unique chromosomal changes in human malignancies was suggestive evidence of clonality in diseases such as CML (44). Finding the same abnormal chromosome in hemopoietic cells of various lineages was the first indication that neoplastic change in CML originated in a pluripotent hemopoietic cell (45) (Figure 2). The observation of karyotypic evolution in acute phase CML and acute nonlymphoblastic leukemia (ANLL) where new chromosomal changes were typically superimposed upon previously recognized abnormalities as the disease progressed was further evidence that the cytogenetic abnormalities represented unique clonal markers (46,47). Nevertheless, it was rightly argued by some investigators that such karyotypic alterations could be secondary changes occurring late in the evolution of a particular disease and in many cells. In such a case chromosomal abnormalities would not be reliable indicators of
FIGURE 2. Clonal Origin of Human Hematopoietic Cells in Chronic Myelogenous Leukemia.

The schema is based on the results of isozyme and chromosome studies. A plus sign indicates that the cell is definitely involved in the leukemic clone, a question mark indicates that it is not clear whether the cell is involved in the leukemic clone. BFU-E denotes erythrocyte burst-forming unit; CFU-E, erythrocyte colony-forming unit; CFU-C, colony forming unit in culture; CFU-EO, eosinophil colony-forming unit; and CFU-MEG, megakaryocyte colony-forming unit.
clonality. In addition, there are many conditions both benign and malignant in which analysis of clonality is important but cytogenetic markers are lacking. For these reasons it was important to develop other techniques for investigating clonality.

B. X-Chromosome Inactivation

According to the Lyon hypothesis (48) early in embryogenesis one X chromosome in each cell of a female organism becomes inactivated. The genes on the active X are the ones expressed in a given cell and all the progeny of that cell. If an organism is heterozygous for a particular X-linked trait some cells will express one phenotype for that trait and other cells the second phenotype. Because X chromosome inactivation occurs when the embryo is composed of only a few cells and because the determination of which X will be inactivated is random the ratio of cells which express each of the two phenotypes for a heterozygous trait is not always 50:50. In a minority of cases the ratio may be skewed towards one phenotype or another. The enzyme G6PD, which is a component of the hexose monophosphate shunt, is encoded on the X chromosome. The gene for this enzyme is highly polymorphic in the human population. Although well over 100 variants of G6PD have been discovered, most of them are extremely rare. Some variants results in a hemolytic anemia which is exacerbated by oxidative stress and others are clinically silent. A number of these isoenzymes can be distinguished by their electrophoretic mobility. These latter include G6PD-B which is the most common, or "normal" isoenzyme in human populations and isoenzyme G6PD-A which is a clinically silent variant found in one third of the black population in the United States and parts of Africa. Another isoenzyme found in one tenth of blacks is
G6PD-A\(^{-}\) which has the same electrophoretic mobility as G6PD-A but causes a hemolytic anemia in affected black males when they are exposed to oxidative stress such as sulfa or antimalarial drugs (49). One third of black women are heterozygous for G6PD-B and one of the A variants. Because of the phenomenon of X chromosome inactivation some of the cells in these women express only G6PD-B while some cells express only G6PD-A. Because normal hemopoiesis is polyclonal both enzymes are expressed in blood and bone marrow cells of normal heterozygous individuals. Similarly, both enzymes are seen in skin fibroblasts, or any other normal body tissue. In contrast, if a neoplasm arises in a single cell one would expect that all the cells in that tumor would express a single enzyme variant in G6PD heterozygotes while a neoplasm arising in many cells would express both isoenzymes (50).

G6PD analysis has been used to demonstrate that the large majority of human malignancies are clonal in origin (50). This includes most hemopoietic neoplasms some of which typically lack cytogenetic markers, e.g. polycythemia rubra vera (51). In addition, clonality has been demonstrated in some instances where malignancy was not otherwise apparent. For example, in several cases of ANLL where clinically normal hemopoiesis returned after chemotherapy the peripheral blood cells of the patients who were G6PD heterozygotes continued to express only 1 isoenzyme, the same one that had been expressed by the leukemic blasts (52). This finding suggests either that chemotherapy had merely returned the patient from frank leukemia to a pre-leukemic malignant state or that normal hemopoiesis had regenerated from a single pluripotent stem cell. G6PD analysis of hemopoietic cells in a heterozygous patient with sideroblastic anemia has been used to demonstrate the common origin of erythrocytes, granulocytes, macrophages, platelets and T and B lymphocytes from a single pluripotent cell (10). Thus, G6PD analysis
has provided considerable insight into clonality in both normal and malignant hemopoiesis. However, because this technique can only be used on the one third of black women who are heterozygous for electrophoretically distinct isoenzymes of G6PD it has limited applicability. Recent developments in molecular genetics now allow clonal analysis to be done on tissue from women who are heterozygous for other, more common markers on the X-chromosome.

C. HPRT, DNA Methylation and RFLPs

Genes on the inactive X chromosome in female cells are more heavily methylated than those on the active chromosome (53,54). Restriction endonucleases exist which will cleave demethylated but not methylated DNA at specific nucleotide sequences (55). Polymorphisms in nucleotide sequence exist throughout the human genome and result in restriction endonuclease digests of human DNA often producing fragments of different size from otherwise normal genes (56). For example, the gene for hypoxanthine phosphoribosyl transferase (HPRT) is found on the X-chromosome. A restriction enzyme fragment length polymorphism (RFLP) in the HPRT gene can be detected on 19% of X chromosomes. This allows distinction between the maternal and paternal gene in 19% of women. Further digestion of DNA with an enzyme which preferentially cleaves demethylated DNA will allow detection of which of the two gene copies is inactive. If DNA from a polyclonal population of cells is digested some of both the maternal and paternal X chromosomes will be inactive, heavily methylated at the HPRT locus and will not be cut by the second enzyme. The remainder of both copies of the gene will be digested. This means that on a Southern blot hybridized with a probe for the HPRT gene both the fragments from the single enzyme digest (representing methylated DNA resistant to the second enzyme) and new fragments generated by digestion of
demethylated DNA will be present. In contrast, in DNA from a monoclonal population of cells in which the same X chromosome is inactivated in every cell, the DNA fragment(s) generated by the active chromosome in the single digest will be lost completely in the double enzyme digest and new fragments generated while the fragment(s) from the single digest of the inactive chromosome will remain. Digests of tumor cell DNA from women heterozygous for the RFLP in the HPRT gene have been used to demonstrate clonality in a number of solid tumors (57) and ANLL (58). In addition these techniques have shown that mature granulocytes in some patients with ANLL in remission are clonal and may be part of the leukemic population (59).

The methods described above have extended the use of the phenomenon of X-chromosome inactivation in clonality studies to a larger proportion of the population than was possible with G6PD analysis. Further developments extending the use of RFLPs to other genes on the X chromosome will undoubtedly soon make it possible to study all women in this fashion.

D. "Marker Genes"—Retroviral Insertion

In the last several years it has become possible to insert foreign genes into mammalian chromosomes. The most efficient way to accomplish this and at present the only practical method for hemopoietic cells is by retroviral infection. Retroviral genomes become incorporated into cellular DNA as part of their life cycle. If the retrovirus carries an identifiable foreign gene this can be located in the host cell by Southern blotting. The site of retroviral integration within the host cell DNA appears to be random. However, once the virus is integrated within the cell DNA all subsequent daughters of that cell inherit the retroviral gene. The presence of the retroviral-linked marker gene at a specific site as indicated by restriction
enzyme digests and Southern blotting can be used to identify a monoclonal population of cells.

Retroviral-mediated gene transfer has been used in the analysis of hemopoietic reconstitution of lethally irradiated mice (8,9). In these experiments gene transfer techniques provide an opportunity to study the dynamics of hemopoiesis in detail that has previously been impossible. Many other experimental and clinical goals can be approached through the transfer of new genes into target cells. The technology and its present and future applications will be discussed in the final section of this introduction.

3) **CLONAL DISORDERS OF HEMOPOIESIS**

The fact that hemopoietic malignancies are clonal, that is, they originate in a single cell was first demonstrated in chronic myelogenous leukemia (CML). Cytogenetic analysis of bone marrow cells from patients with this disease revealed an abnormal chromosome 22 - the Philadelphia Chromosome (Ph) - which results from the translocation of genetic material between the long arms of chromosomes 9 and 22 (59) (Figure 3). Although CML is characterized primarily by increased members of peripheral blood granulocytes and their precursors other hemopoietic cells including erythroblasts, monocytes, eosinophils and B lymphocytes have been shown to have the Ph chromosome in this disease (60-63) (Figure 2). In addition, G6PD isoenzyme studies of various hemopoietic cells from patients with CML heterozygous for 2 electrophoretically distinct enzyme variants have shown that only one and the same isoenzyme is expressed in granulocytes, monocytes, red cells, platelets and some B lymphocytes (64,65). These two lines of evidence indicate that CML is a clonal neoplasm arising in a pluripotential hemopoietic progenitor. Other hematologic disorders, including ANLL, polycythemia vera, idiopathic
Chronic Myelogenous Leukemia

FIGURE 3. Chromosomal Rearrangement in CML.

abl and sis are the cellular protooncogenes c-abl and c-sis. bcr
is breakpoint cluster region. • marks chromosomal location of
these genes. + marks the site of the breakpoints in the Ph
translocation t(9;22)(q34;q11).
myelofibrosis, myelodysplasia and one case of aplastic anemia have been shown to be of clonal origin using these techniques (66-68). However, the cellular composition of the abnormal clone varies in each disorder and provides the basis for the clinical diagnosis. For example, granulocytes predominate in CML while an elevated red cell count characterizes polycythemia vera. Nevertheless, G6PD analysis reveals the presence of at least the three lineages of myelopoiesis in the abnormal clone of both disorders.

As a clonal hemopathy ANLL varies from the myeloproliferative disorders such as CML and polycythemia vera in two important ways. Using G6PD analysis Fialkov et al found that although some patients showed the usual pattern of trilineage myelopoietic differentiation in the abnormal clone; others showed granulopoietic but not erythroid maturation (69). These findings may indicate that ANLL may begin either in pluripotent stem cells or in progenitors committed to granulopoiesis. Alternatively, the transformation may occur in pluripotent stem cells which lose the capacity for erythropoiesis either at the time of transformation or during clonal evolution. The second distinctive feature of ANLL clones is the presence within them of a predominant population of blast cells showing little or no morphological evidence of differentiation.

Blast cells appear during the course of diseases other than ANLL. In particular, the terminal phase of CML is accompanied by proliferation of these undifferentiated cells. Thus, the abnormal clones in this and other hemopathies are genetically unstable and subject to progression. The nature of the precise genetic changes responsible for the initiation and progression of these or any other human malignancy are at present unknown. Nevertheless, it is believed that genetic abnormalities are central to malignant transformation. Some of the evidence for this belief is discussed below.
Theodor Boveri published a treatise on the origin of malignant tumors in 1914 in which he hypothesized that malignant cells have an abnormal chromosome constitution and that these abnormal chromosomes cause the malignant growth (70). Subsequent evaluation of metaphase cells from human tumors in the cytogenetic era before chromosome banding techniques were available revealed many abnormalities in cancer cells. However, these appeared to be extremely variable and were thought to most probably be random abnormalities of no pathogenetic importance. The Ph chromosome described by Nowell and Hungerford in 1960 in the leukemic cells of 2 patients with CML was the first consistent chromosomal abnormality described in human cancer (44). Subsequent analysis of many hundreds of patients with CML have revealed the Ph abnormality in at least 90% of patients with the disease (71,72). The application of chromosome banding techniques to metaphase cells from patients with CML have revealed that the Ph abnormality is actually a reciprocal translocation between the long arms chromosomes 9 and 22 and that the breakpoints in these chromosomes are quite specific: t(9;22)(q34;q11) (59) (Figure 3). Occasionally more complex translocations, involving, for example, a third chromosome are observed in CML cells but detailed analysis typically shows that the translocation of material from the long arm of chromosome 9 to the long arm of 22 still takes place (71,72).

Extensive surveys of chromosomes abnormalities in all the common hematological malignancies have now been accomplished (71). A variety of nonrandom changes have been described some of which appear to be associated with a particular type of clinical disorder. For example, ANLL may be subgrouped according to the French-American-British (FAB) classification into a number of categories (Table I) on the basis of morphological criteria (73). Up to 100% of patients with ANLL will have cytogenetic abnormalities in their
TABLE I
Classification of the Acute Nonlymphoblastic Leukemias

<table>
<thead>
<tr>
<th>Morphologic Types</th>
<th>Subtypes (FAB Designation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myeloid</td>
<td>AML</td>
</tr>
<tr>
<td></td>
<td>Myeloblastic without maturation (M1)</td>
</tr>
<tr>
<td></td>
<td>Myeloblastic with maturation (M2)</td>
</tr>
<tr>
<td></td>
<td>Hypergranular promyelocyte (M3)</td>
</tr>
<tr>
<td></td>
<td>Hypogranular promyelocyte (M3 variant)</td>
</tr>
<tr>
<td>Myelomonocytic</td>
<td>AMML (M4)</td>
</tr>
<tr>
<td>Monocytic</td>
<td>Poorly differentiated (M5a) AMoL</td>
</tr>
<tr>
<td></td>
<td>Differentiated (M5b) AMoL</td>
</tr>
<tr>
<td>Erythroid</td>
<td>AEL (M6)</td>
</tr>
<tr>
<td>Mast cell</td>
<td>Acute mast cell leukemia</td>
</tr>
<tr>
<td>Megakaryocyte</td>
<td>Acute megakaryocytic leukemia</td>
</tr>
</tbody>
</table>

Abbreviations: FAB, French-American-British; AML, acute myeloblastic leukemia; AMML, acute myelomonocytic leukemia; AMoL, acute monocytic leukemia; AEL, acute erythroleukemia.
high resolution banding techniques (74,75). When these cytogenetic changes are compared with the FAB category of the corresponding leukemic cells a number of striking correlations are observed. A t(15;17) (q22;q21) is virtually pathogenomic of acute promyelocytic leukemia, M3 (76). An 8;21 translocation is seen in myeloblastic leukemia with evidence of differentiation, M2 (77). Many other associations have been described (Table II) some of which appear to have prognostic importance, i.e. inv (17) in myelomonocytic leukemia M4 with dysplastic eosinophils, is associated with a good response to therapy and prolonged survival (78). Similar studies of malignant cells in acute lymphoblastic leukemia (ALL) (79) and nonHodgkin's lymphoma (80) have revealed characteristic cytogenetic abnormalities in various subgroups some of which appear to have prognostic importance. Among solid tumors detailed karyotypic information is less plentiful. However, it appears that most malignant tumors have an abnormal chromosome content (71).

The karyotype of malignant cells typically does not remain stable. A stepwise rearrangement of the abnormal karyotype is sometimes observed, often as the clinical illness enters a more aggressive phase. CML provides a convenient example. When this disease enters the phase of acute or blast transformation the karyotype of bone marrow cells shows changes in addition to the Ph is 80% of cases. The most common of these additional changes are an additional Ph, trisomy of chromosome 8, and an isochromosome of the long arm of chromosome 17 (46).

Thus, cytogenetic studies provided some of the first and most convincing examples of genetic abnormalities in cancer cells. However, because of the gross nature of these changes at the chromosomal level it has not been possible to determine which genes are altered either in their structure or
### TABLE II

De Novo Acute Nonlymphocytic Leukemia in Adults with Specific Chromosome Defects

<table>
<thead>
<tr>
<th>Frequency (%)</th>
<th>FAB Category</th>
<th>Chromosome Defects</th>
<th>Median Survival (month)</th>
<th>Median Age of Onset (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M1, M2, M4, M6</td>
<td>inv 3 or t(3;3)</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>1</td>
<td>M2</td>
<td>del 5q</td>
<td>Few cases</td>
<td>Few cases</td>
</tr>
<tr>
<td>3</td>
<td>M2, M1, M4, M5, M6</td>
<td>-7/del 7q</td>
<td>3</td>
<td>51</td>
</tr>
<tr>
<td>2</td>
<td>M2, M1, M4</td>
<td>t(6;9)</td>
<td>Few cases</td>
<td>34</td>
</tr>
<tr>
<td>5-20</td>
<td>M2</td>
<td>t(8;21)</td>
<td>14+</td>
<td>38</td>
</tr>
<tr>
<td>9</td>
<td>M2, M1, M4, M5, M6</td>
<td>+8</td>
<td>9</td>
<td>52</td>
</tr>
<tr>
<td>8</td>
<td>M1, M2</td>
<td>t(9;22)</td>
<td>Few cases</td>
<td>Few cases</td>
</tr>
<tr>
<td>9</td>
<td>M4, M5a, M2</td>
<td>t(V;11)</td>
<td>Few cases</td>
<td>34</td>
</tr>
<tr>
<td>6</td>
<td>M3</td>
<td>t(15;17)</td>
<td>19</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>M4, M2, M5b</td>
<td>inv 16</td>
<td>15+</td>
<td>49</td>
</tr>
<tr>
<td>14</td>
<td>M1, M2, M4, M5a, M6</td>
<td>Complex defects</td>
<td>2.5</td>
<td>60</td>
</tr>
</tbody>
</table>

Abbreviations: FAB, French-American-British; V, variable. From Reference 72.
expression and function by the documented abnormalities. This has also made it difficult to prove that any cytogenetic changes are of pathogenetic or primary importance in the onset of malignant transformation. Recent developments in molecular genetics and recombinant DNA technology have made analysis of structural chromosome rearrangements possible at the molecular level. The discovery of cellular genes homologous to the transforming genes of oncogenic retroviruses has identified a number of candidate genes on which to focus the search for tumor etiology.

4) **ONCOGENES**

It has been known for a number of years that certain RNA tumor viruses which cause tumors after short periods of latency in animals do so because they contain genetic sequences that encode for transforming genes or oncogenes. A major scientific discovery of this decade has been the identification of genes within normal cells that are highly homologous with these oncogenes (81). These genes have been highly conserved throughout evolution and thus are thought to encode proteins that are vital to some facet of the cellular life cycle. It appears that the retrovirus has incorporated a cellular gene into its genome as a consequence of its life cycle which requires that the virus integrate into host cellular DNA. The new gene becomes an oncogene or transforming gene in the virus as a result of mutation to the gene that occurs at the time of its transduction into the virus or during its subsequent reproductive cycles. Expression of the gene may also be altered because of the presence nearby of retroviral promoters and enhancer sequences. Although the precise function of most of these now more than 30 cellular oncogenes is unknown some general categories have begun to appear and certain genes have been assigned specific protein products (82) (Table III).
<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
<th>Protein (Location/Structure)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>abl</td>
<td>Abelson virus (v)</td>
<td>cytoplasm/protein kinase (PK)</td>
<td>?</td>
</tr>
<tr>
<td>bas</td>
<td>Murine sarcoma v</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Blym</td>
<td>B cell lymphoma</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>ELA</td>
<td>Adenovirus</td>
<td>nuclear protein</td>
<td>?</td>
</tr>
<tr>
<td>erb A</td>
<td>AEV</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>erb B</td>
<td>AEV</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>ets 1,2</td>
<td>ALV E26</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>fes/fps</td>
<td>Fujinami SV</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>fgr</td>
<td>PK</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>fms</td>
<td>Feline SV</td>
<td>PK</td>
<td>CSF-1 receptor</td>
</tr>
<tr>
<td>fos</td>
<td>FBJ osteosarcoma v</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>hst</td>
<td>Human stomach ca</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>int 1,2</td>
<td>M mammary tumor</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>jun</td>
<td>Avian SV</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>mas</td>
<td>Mouse sarcoma</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>met</td>
<td>Human osteosarcoma</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>mil</td>
<td>AV-MH2</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>mos</td>
<td>MSV-SD</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>myb</td>
<td>AMV</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>c-myc</td>
<td>AMV-MC29</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>L-myc</td>
<td>Lung tumors</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>N-myc</td>
<td>Neuroblastomas</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>P-myc</td>
<td></td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>R-myc</td>
<td>Rhabdomyosarcoma</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>neu</td>
<td>Neuroblastoma</td>
<td>membrane/PK</td>
<td>? receptor</td>
</tr>
<tr>
<td>N-ras</td>
<td>Harvey SV</td>
<td>GTP binding</td>
<td>? signal</td>
</tr>
<tr>
<td>K-ras</td>
<td>Kirsten SV</td>
<td>GTP binding</td>
<td>transduction</td>
</tr>
<tr>
<td>N-ras</td>
<td>Neuroblastoma</td>
<td>GTP binding</td>
<td>&quot;</td>
</tr>
<tr>
<td>raf</td>
<td>MSV 3611</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>rel</td>
<td>Turkey lymphoma v</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>ros</td>
<td>ASV UR2</td>
<td>PK</td>
<td>? receptor</td>
</tr>
<tr>
<td>sis</td>
<td>Simian SV</td>
<td>PK</td>
<td>PDGF-B</td>
</tr>
<tr>
<td>src</td>
<td>Rous SV</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>SV40-T</td>
<td>SV40 virus</td>
<td>nuclear</td>
<td>?</td>
</tr>
<tr>
<td>trk</td>
<td>Human colon ca</td>
<td>PK</td>
<td>?</td>
</tr>
<tr>
<td>yes</td>
<td>ASV-773</td>
<td>PK</td>
<td>?</td>
</tr>
</tbody>
</table>
A number of the cellular counterparts to viral oncogenes encode proteins that are localized to the nucleus, in some cases binding to DNA. These proteins may have some regulatory function in cell division. This category includes the c-myb and the myc gene family (82).

A second group of cellular oncogenes appear to encode growth factors or their receptors. C-sis encodes a protein highly homologous to the β chain of platelet-derived growth factor (PDGF) (83). c-erb-B is homologous to the epidermal growth factor receptor and v-fms to the M-CSF receptor (84,85). Other oncogenes have structures highly suggestive of the possibility that they, too may be growth factors or receptors, e.g. ros, neu (86,87). Sporn and Todaro have put forward a hypothesis that describes autocrine stimulation of malignant cells (88). According to this theory autocrine stimulation occurs when a cell begins to produce a growth factor for which it already possesses a receptor. In this way a cell becomes independent of the need for exogenous stimulatory factors. Examples of such behavior have been seen in cells transformed by V-sis containing retroviruses (89).

Finally, there are oncogenes that may function within the cytoplasm in signal transmission. For example, the ras gene family bears many structural similarities to the G proteins which are known to transduce signals from various cell-surface receptors to adenylcyclase (82).

Thus, the cellular oncogenes for which such information is available appear to function in the control of cell growth and division. It seems self-evident that perturbation of such functions could lead to malignant transformation.

After the discovery of cellular oncogenes in normal cells investigators began to look at malignant tissue for abnormalities involving these genes or their products. A number of theoretical changes that could cause a normal
gene to become a cancer gene were hypothesized and examples of all of these possibilities have been found in nature.

A. Gene Amplification

An increased number of copies of a gene could result in overproduction of an oncogene product. Members of the myc family of oncogenes have been shown to be amplified in the HL-60 cell line, (c-myc) (90) metastatic neuroblastoma (N-myc) (91) and small cell lung carcinoma (N-myc) (92). In some cases dozens of copies of the gene are found in tumor cell DNA and high levels of expression of the gene are found at the RNA level.

B. Point Mutations

It has been known for many years that a number of inherited human diseases are caused by a single nucleotide substitution in a critical gene, e.g. sickle cell anemia. It would not be surprising if similar changes in oncogenes were important for malignant transformation. Members of the ras family of oncogenes are often found to possess point mutations in tumor cell DNA. These nucleotide substitutions seem to occur at particular sites, e.g. codon 12, that are presumably important for carcinogenesis (93). N-ras and K-ras are genes that have been found to have point mutation in some samples of human leukemic cell DNA (94,95). These mutated genes have been identified by their ability to cause the formation of transformed foci of cells when leukemic cell DNA is transfected into murine fibroblast cell lines. This suggests, but certainly does not prove, that the mutated ras genes are important for producing or maintaining the malignant phenotype in the leukemic cells from which they originated.
C. **Gene Rearrangement**

Gross structural changes have been found to occur in oncogenes in many retroviruses. For example, although the v-erb-B oncogene is highly homologous to the EGF receptor gene it has been severely truncated in the extracellular or ligand-binding region. This is felt to result in a growth factor receptor product which is constitutively active in the absence of ligand (84).

D. **Altered Gene Expression**

It is possible that a potential oncogene could remain undamaged by a mutational event but that genetic rearrangements nearby could affect its expression in a significant way. The avian leukosis virus causes lymphomas in chickens although it does not contain an oncogene. However, detailed analysis of tumor cell DNA from lymphomas induced by this virus reveal that the virus has integrated near the c-myc oncogene in the cellular DNA. This results in over-expression of the c-myc gene product, a circumstance that may be important in tumorigenesis (96).

E. **Interaction of More Than One Oncogene**

A number of oncogenes which efficiently transform immortalized cell lines transform primary cells at extremely low frequency or not at all. However, when a second oncogene is introduced into the primary cell transformation occurs with high efficiency. This phenomenon of co-operation between two oncogenes in malignant transformation has been demonstrated most frequently for genes of the ras and myc families. The target cells have been various, including primary rat embryo fibroblasts and B lymphoblasts (97,98). The activation of two transforming genes in a single tumor is consistent with the observation that most de novo tumors in animals and man show prolonged latency
and increasing aggressiveness as the disease progresses. This suggests that multiple genetic events are necessary to cause most malignancies.

F. Oncogene Involvement in Chromosomal Rearrangements

There are now almost 40 different oncogenes identified either by the investigation of transforming retroviruses or the analysis of tumor cell DNA in various transfection assays. Undoubtedly, many oncogenes remain to be discovered. It is a formidable task to study human tumors for mutations in all these potential transforming genes so investigators have looked for clues from other areas of genetic research on cancer cells. Chromosomal analysis of human malignant cells has provided many valuable insights into which of these many oncogenes might be perturbed in a given tumor (71). The cellular oncogenes have in most cases been mapped to specific regions on the human chromosomes (Figure 4). By comparing the location of the oncogenes to the breakpoints involved in translocations seen in human malignancies it has been possible to predict that certain genes would move from one chromosome to another as a consequence of the translocation. By cloning the regions of DNA around these chromosomal breakpoints and studying this DNA for gene rearrangements it has been possible to demonstrate structural or functional alterations in both oncogenes and other cellular genes in several tumors.

i) Burkitt’s Lymphoma

Characteristic cytogenetic abnormalities can be identified in more than 80% of these tumors. In the majority of cases the karyotype of malignant cells shows a translocation between the long arms of chromosomes 8 and 14. However, in some cases chromosomes 8 and 2 or 8 and 22 are involved (99). The breakpoint on chromosome 8 is at the site where the c-myc gene has been
FIGURE 4. Giemsa-banded human chromosome map with 21 oncogenes (dot), 6 cellular genes (triangle), and breakpoints (arrows) for chromosomal rearrangements found in cancer. (Reference 72).
 Burkitt's Lymphoma
translocation 8; 14 = c-myc; Ig rearrangement

FIGURE 5. Chromosomal Rearrangement in Burkitt's Lymphoma.
mapped. On chromosome 14 the immunoglobulin heavy chain gene is at the breakpoint whereas on chromosomes 2 and 22 it is the \( \lambda \) and K light chain immunoglobulin gene respectively that is at the breakpoint (Figure 5). Cloning of the breakpoint in the 8;14 translocation has revealed that the c-myc gene has been translocated into the Ig heavy chain gene locus near the Ig enhancer region. It has been shown that this translocated c-myc gene is expressed in the tumor cells while the normal gene is not (100). In some Burkitt's Lymphoma tumor samples c-myc expression is unusually high. Thus, it is likely that the chromosomal translocation seen in Burkitt's lymphoma, a B-cell malignancy, allows a potential transforming gene, c-myc, to be regulated by the Ig enhancer which normally functions at high levels in B cells. The abnormal regulation of c-myc in these cells may be critical to their malignant transformation.

ii) CML

In this disorder the t(9;22) which is seen in more than 90% of patients results in the translocation of the c-abl oncogene from chromosome 9 into a gene on chromosome 22 known as bcr (breakpoint cluster region) (Figure 3). This rearrangement causes abnormal splicing of the two gene products so that a fusion bcr-abl RNA transcript is produced (101). This RNA is translated into an abnormal protein with altered enzymatic activity, i.e. the normal c-abl product, has little or no detectable tyrosine protein kinase activity whereas the bcr-abl fusion protein has a very high and promiscuous level of this activity (102,103). Thus, the Ph translocation results in a new rearranged or hybrid gene which ultimately yields a new and abnormal protein. The abnormal bcr-abl RNA species has been seen in cells from patients with CML who appeared
cytogenetically normal (Ph negative CML) (104) and in CML patients with complex 3-way translocations in the karyotype of their malignant cells (105). Thus, this rearrangement of the c-abl oncogene appears to be highly characteristic and perhaps diagnostic of CML.

Cytogenetic abnormalities have been used as clues to begin investigation of hereditary carcinomas at the molecular level. Hereditary retinoblastoma is often accompanied by deletion or rearrangements in the long arm of chromosome 13 in tumor cell metaphases (106). Southern blotting analysis of tumor cell DNA hybridized with probes to genetic sequences which map to this region of chromosome 13 have revealed the following facts. It appears that children may inherit a mutation on one chromosome 13 and that tumors arise when a somatic mutation occurs at the same site on the previously normal chromosome 13. These mutations may be visible as chromosomal abnormalities or may require more subtle molecular analysis (107). Recently the putative "retinoblastoma" gene has been cloned and it remains to be seen what characteristics it may have in common with previously described oncogenes (108).

When it was discovered that the cellular homologues of several viral oncogenes were growth factors or their receptors it became natural to suppose that other known growth factor or receptor genes might act as oncogenes. As previously mentioned, a number of the hemopoietic growth factor gene have been cloned and recombinant factors produced. Using such reagents it has been shown that a significant minority of leukemic blast cells from patients with ANLL both secrete and respond to GM-CSF (109). In a human leukemic cell line the GM-CSF gene has been shown to be rearranged by Southern blotting (110). Therefore, at least some leukemic cells seem to be responding to autocrine stimulation and a hemopoietic growth factor gene which has not been identified as an oncogene in the usual sense may be playing an important role in malignant transformation.
5) **GENE TRANSFER**

When studying established human malignancies the investigator is looking at the result of both genetic and nongenetic events which give rise to a very complicated array of abnormalities. It is difficult to determine which changes are of primary importance and which are secondary to the neoplastic process. A more directed or simplified approach to studying malignant transformation might be to use normal cells as a target for introduction of suspect cancer genes and study the results. Similarly, the genetic control of many normal cell functions is poorly understood and could potentially be dissected in this way. The use of transferred genes to mark and identify certain cell populations has been discussed in the previous section.

A clinical area in which gene transfer may have applicability is in the therapy of inherited human disorders in which a molecular genetic defect has been identified. In some cases the organ system in which the disease is primarily manifest would be accessible to genetic manipulation, e.g. the bone marrow in various hemoglobinopathies. However, because globin synthesis and gene regulation are unusually complicated and not completely understood, other diseases are likely to be the first candidates for gene therapy. Those disorders which are caused by a missing enzyme or protein whose level does not need to be regulated precisely are more approachable. Three diseases which are undergoing active investigation at present include the Lesch-Nyhan syndrome caused by the absence of hypoxanthine-guanine phosphoribosyl transferase (HPRT) and the two immune deficiency disorders caused by lack of purine nucleoside phosphorylase (PNP) or adenosine deaminase (ADA). The introduction of the normal gene for these factors into patient bone marrow cells may result in the production of only a fraction of the normal enzyme level but this may be sufficient to reverse many of the clinical
abnormalities. On the other hand, a mild overproduction of these enzymes should not be harmful to the cells producing them.

The above are but a few of the instances in which an efficient means of transporting specific genetic sequences into selected target cells have potential usefulness. Recent advances, to be discussed in this paper, make such manipulations possible now or in the near future.

A. History of Gene Transfer - (112, 113)

Transfection experiments began several decades ago with the addition of metaphase chromosomes to cell suspensions. The chromosomes were taken up rather inefficiently by the cells and gave rise to unstable variants at low frequency. Intact chromosomes rarely survive this procedure and the recipient cell usually gains a fragment of a donor chromosome which is unstable because it lacks a centromere. Only rarely do stable lines arise after integration of donor material into a recipient chromosome (112).

Purified DNA in suspension will enter eukaryotic cells but with such low efficiency that the successful events cannot be effectively studied. A great technical advance was made with the discovery that when DNA is precipitated with calcium phosphate (CaPO₄) before adding it to cells growing in a monolayer, many more transformed foci appear than when CaPO₄ is absent. Although it appears that the cells actually phagocytize the DNA-CA⁺⁺ granules, the mechanisms of preferential uptake of CaPO₄ precipitated DNA by cells is unclear. Several stages can be identified in the transformation process. First, the CaPO₄-DNA precipitate enters the cytoplasm of all or nearly all of the cells exposed. However, only 1-5% of cells will show the presence of DNA complexes in the nucleus. These cells will show transient expression of the exogenous genes they have taken up. The frequency of this transient
expression is 10 to 100 fold higher than the frequency of stable gene expression which requires integration of the foreign DNA into a host chromosome. Even when DNA is injected directly into the nucleus, although 50 to 100% of cells show transient gene expression, only 1 of 500-1000 show a stable genetic change (113). The competence of the cells to undergo this permanent change is the result of a number of factors, many of which are not understood. It appears that cells in the early phase of DNA synthesis in the cell cycle are the most likely to be transformed, perhaps because enzymes involved in DNA metabolism such as DNA polymerase, are most active at this time (114). The DNA stably introduced into eukaryotic cells by CaPO\textsubscript{4} co-precipitation has been found to be integrated into a single random site in a host chromosome usually in a large concatamer of many units linked together and containing up to 1000 kilobases. Although virtually any cloned DNA segment can be introduced into adherent tissue culture cells using this technique, the efficiency of gene transfer is never greater than 1:10\textsuperscript{3} even in the most competent cells. The technique also has a relative, though not absolute, requirement for the targets to be adherent cells rather than floating in suspension. This limits the applicability of the technique to a relatively select group of cell types.

Recently certain groups of investigators have had success using the technique of protoplast fusion for gene transfer (115,116). Protoplasts are derived by treating bacteria containing the plasmid DNA of interest with lysozyme. The protoplasts are then fused to cells in the presence of polyethylene glycol. Reported efficiencies of stable gene transfer range around 3 x 10\textsuperscript{-3}. Although this appears better than that achieved with CaPO\textsubscript{4}-DNA precipitates, the success comes from a very small number of laboratories. In the hands of most investigators, protoplast fusion is very
toxic to many cell types, once again limiting the applicability of the technique.

A third technique of DNA mediated gene transfer uses electroporation (117). Cells are incubated in suspension with the DNA of interest and then subjected to an electric current of 2000-4000 volts which opens cellular membrane pores and allows the DNA to enter the cells. The efficiency of transferring selectable genes into lymphocytes or fibroblast cell lines has been reported at up to $3 \times 10^{-4}$. With this technique the number of copies of the genes transferred is lower (1 to 15 per cell) than is usually seen with traditional CaPO$_4$-DNA precipitates which may be an advantage for some experiments. The range of cell types successfully transfected with technique may also be broader than with CaPO$_4$ precipitates, although reports of successful electroporation are still relatively scarce in the literature.

With any of the three gene transfer techniques discussed so far; CaPO$_4$-DNA precipitates, protoplast fusion or electroporation, the frequency of gene transfer is still quite low - too low to be reliably successful when the target cell of interest is rare in the total population, e.g. the hemopoietic stem cell in the bone marrow (Table IV).

DNA can be injected directly into the cell nucleus with an efficiency of stable transformation of about $1 \times 10^{-3}$. The technical demands of this microinjection procedure are considerable, making it unsuitable for the usual gene transfer experiments. However, it has been discovered that, when DNA is injected into the pronucleus of a fertilized zygote, the frequency of stable integration and expression is vastly increased over that achieved by injecting genes into somatic cell nuclei (118,119). After a successful microinjection, from 1 to 25 copies of the gene are located at a single chromosomal site. In the order of 5% of successfully injected zygotes result in viable offspring.
<table>
<thead>
<tr>
<th>Technique</th>
<th>Transient Gene Expression</th>
<th>Efficiency of Stable Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaPO₄-DNA precipitate</td>
<td>1-5%</td>
<td>1-10 x 10⁻⁴</td>
</tr>
<tr>
<td>Protoplast fusion</td>
<td></td>
<td>3 x 10⁻³</td>
</tr>
<tr>
<td>Electroporation</td>
<td></td>
<td>3 x 10⁻⁴</td>
</tr>
<tr>
<td>Microinjection</td>
<td>60-70%</td>
<td>5%</td>
</tr>
<tr>
<td>Retrovirus</td>
<td></td>
<td>~100%</td>
</tr>
</tbody>
</table>
when placed in a pseudopregnant mother. The foreign DNA in these mice is then in the germ line, and thus will be present in all cells of the new organism and transmitted to offspring as Mendelian traits. The creation of such "transgenic" animals has been used to study tissue specific regulation of the immunoglobulin and insulin genes among others and to cure β thalassemia in a murine model (120). The limitations of the microinjection technique include its considerable technical demands and its successful use primarily only in germ line cells.

B. Viral Vectors

Efforts to harness the infective cycle of viruses for gene transfer have involved many classes of viruses. DNA viruses have shown promise for gene transfer. Recombinant adeno or SV40 viruses (121), DNA packaged pseudo SV40 (122) or polyoma virions (123) and recombinant parvovirus (124) all have been used with some degree of success. A conditionally nonreplicating adenoviral vector has been developed that will efficiently infect animal and human cells with only one or a few copies of the recombinant virus integrated into the host genome (121). However, evidence of stable gene expression in primary cells has not been forthcoming. Another DNA virus, the bovine papilloma virus, which replicates extrachromosomally may be useful for maintaining genes in cells in an unintegrated state (125), but experiments to show its usefulness in targets such as hemopoietic cells, have yet to be reported.

SV40 is of interest because of its broad range of species and tissue infectivity, its well-characterized genome and its general lack of pathogenicity. A recombinant SV40 virus has been used to carry the chloramphenicol acetyltransferase (CAT) gene into hemopoietic cell lines and primary bone marrow cells and achieve transient gene expression. However,
assays designed to demonstrate stable gene transfer and expression with similar vectors were unsuccessful in primary progenitors (126).

A method of gene transfer that shows great promise at present is the use of retroviral vectors (111,127). Genes can be transferred as a consequence of the normal life cycle of these viruses. The genes to be transported can be those selected by the investigator and inserted into the appropriate vector using standard recombinant DNA technology. The efficiency of gene transfer should approach 100% and the procedure is nontoxic to eukaryotic cells. A relatively small number of gene copies will integrate in host DNA, in most cases only one.

Because of these features, retroviruses appear to have the most immediate promise for gene transfer. In order to optimize their use, it is necessary to understand details of their structure and life cycle which will be discussed in the following sections.

C. Retroviral Structure (128,129)

During infection by retroviruses, double-stranded viral DNA is generated from an RNA template and covalently joined to host chromosomes. The RNA genomes of all retroviruses contain 3 coding regions that participate in replication (Figure 6). The gag region codes for viral core proteins, pol for the enzyme reverse transcriptase (RNA-directed DNA polymerase), and env encoding sequences for synthesis of envelope glycoproteins. Each end of the virus RNA contains a repeated sequence R and segments characteristic of the 3' (U3) or 5' (U5) end of the virus. The U3 and U5 regions contain sequences necessary for retroviral integration into host DNA, initiation of viral transcription of the viral genome, polyadenylation of RNA transcripts and a promoter which enhances transcription. A 150 bp sequence 5' to the gag region
FIGURE 6. Structure of Moloney Murine Leukemia Virus Retroviral Provirus DNA.

Abbreviations: E, enhancer; P, promoter; I, initiation (Cap) site for viral RNA synthesis; r-, replication initiation site for minus DNA strand; D, donor splice site; Ψ packaging sequence; A, major acceptor splice site; r+, replication initiation site for plus DNA strand; T, terminal (poly-A addition) site for viral RNA synthesis; LTR, long terminal repeat; U3, R, and U5 are portions of the LTR; gag, viral core proteins; pol RNA-dependent DNA polymerase (reverse transcriptase); and env, envelope proteins.
has also been identified which is essential for packaging of viral components into completed viral particles. Within each viral core are 2 identical subunits of RNA genome, as described above, each 5 to 9 Kb in length. The reason for this genetic redundancy is unknown and unique among animal viruses to the RNA tumor viruses.

Although the above represents the essential components of a replication competent retrovirus, in the course of its life cycle a virus may be altered by mutational events that occur within the host cell genome or recombinational events among viruses themselves. Examples would include the endogenous retroviruses present in many animal species, most of which have lost the ability to replicate except under special circumstances, or the acutely transforming retroviruses which appear to have acquired a cellular "oncogene" at some points in their history.

D. Host Range of Retroviruses (130)

One feature of retroviruses which makes them attractive for gene transfer is their wide host range. There are viruses which will infect most potential eukaryotic hosts and most functional tissues. However, each virus does have a specific host requirement which is determined by several factors.

Most importantly, there are receptor restrictions which are determined by the viral env sequence. Although the virus adsorbs nonspecifically to the cell surface by a process which can be enhanced by polycations such a polybrene which decrease cell surface charge, the virus must bind to a specific receptor if it is to enter the cell and replicate. Most cells possess 1 to 5 x 10^5 such receptors on their surface. While these structures presumably do not exist solely for the convenience of the retrovirus, their "nonviral" or usual function is unknown. In vivo studies have shown some
tissue specificity exists for retroviral receptors. For example, recombinant thymic lymphoma viruses react with receptors that are restricted to a subset of thymocytes. Retroviruses have been classified according to their receptor specificity. C-type murine retroviruses viruses are categorized as follows:

i) ecotropic - replicating only in mouse cells.

ii) xenotropic - replicating poorly or not at all in most mouse cells, but infecting a wide range of other species including rat, mink, human and quail.

iii) amphotropic - replicating in both mouse and other mammalian cells, including human.

Each of the above types of virus recognizes a unique cell surface receptor. There also exists a category of virus known as polytropic from which the env product will recognize more than one receptor, for example, both the ecotropic and the xenotropic receptor. Recombinant viruses have been constructed in which an ecotropic virus has its env sequence replaced by a xenotropic or amphotropic env. When this is done, the new virus acquires the host range of the virus from which the new env came. Such manipulations allow the infectivity of a given virus to be manipulated at will.

How the interaction between virus and cell receptor facilitates viral replication is not known, although likely candidates include aiding viral penetration of the cell or viral uncoating.

E. Retroviral Replication (128,129)

Once the virus enters the cell it begins to reproduce itself (Figure 7). The key event in retroviral replication occurs when the viral enzyme reverse transcriptase converts single-stranded viral RNA into double-stranded DNA.
Binding and infection

Adsorption of virus to the cell and binding to a cell surface receptor is followed by the entry of single-stranded RNA genome into the cytoplasm. There it is reverse transcribed into a double stranded complementary DNA molecule. This DNA as a circular molecule is transported to the nucleus where it integrates into a host chromosome. The integrated provirus serves as a template for synthesis of the subgenomic mRNA's encoding the gag, pol and env proteins as well as full-length transcripts of the viral genome. These components are then packaged and shed from the cell as infectious virus. (From Reference 126).

FIGURE 7. Retroviral Life Cycle.
The viral genome consists of dimers of 2 identical strands of RNA joined at the 5' ends and with a complex secondary structure. During the course of replication, a portion of the viral RNA is duplicated to form the identical LTRs (long terminal repeats) found at the 5' and 3' end of proviral DNA. The LTR unit represents a fusion of sequences from the 3' end of viral RNA (U3), the R (repeated) sequence and sequences from the 5' end of viral RNA (U5) in the order 5'-U3-R-U5-3' and is terminated by short sequences forming inverted and often imperfect repeats. On comparing the structure of viral RNA and DNA, it becomes clear that DNA synthesis requires the transfer of DNA strand twice between templates during reverse transcription (Figure 8).

Linear duplex viral DNA appears in the cell cytoplasm within a few hours of infection. From there it must migrate to the nucleus where a second unintegrated form of viral DNA is formed; covalently closed circular DNA containing 1 or 2 copies of the LTR. It is now known that the formation of the circular DNA species with the two LTRs is necessary for proviral integration. The junction between the two LTRs in the circular DNA forms a so-called "att" site which is necessary for integration (131).

Although we do not understand the enzymatic mechanisms involved, retroviral integration into host DNA is a highly ordered process influenced by sequences within the viral LTR. A relatively small number (1-20) copies of proviral DNA are integrated per cell. This may be a small percentage of the viral DNA actually synthesized in the cell. The site of integration appears to be random in that no specific host nucleotide sequence has been identified that favors it nor has restriction enzyme mapping located gross genetic sequences that are likely to surround the site of viral DNA entry. In contrast, the viral nucleotides which join to cellular DNA are rigidly determined. In all published cases, exactly 2 base pairs are missing from the 5' end of the 5' LTR and from the 3' end of the 3' LTR.
FIGURE 8. Critical Steps in the Synthesis of Retroviral DNA.

The priming events for (-) and (+) stands of viral DNA (A and C) and the two transfers of nascent strands between templates (B and D), pictured on an expanded scale, lead to production of a linear duplex with two copies of the LTR unit (E and F; reduced scale). RNA is shown as wavy lines, DNA as straight lines with arrows denoting direction of synthesis; the 5' end of (-) strand DNA is indicated by a filled circle, the 5' end of (+) strand DNA by an open circle. (-)PB is the transfer RNA primer binding site, (+)P is the putative (+) strand primer sequence. The short vertical lines designate the boundaries of U5, R, and U3 in all panels. (A) A nascent (-) strand copy of R-U5 at the extreme 5' end of its template. (B) After removal of the 5' end of the primary template, the nascent (-) strand has base-paired with the R sequence at the 3' terminus of the same or a companion RNA subunit and is extended along its secondary template of viral RNA. (C) Synthesis of (+) strand DNA commences at a priming site at the boundary of U3, and (+) strand is extended through a portion of the transfer RNA sequence originally bound to viral RNA; the (-) strand concurrently elongates toward the 5' end of viral RNA, into or beyond the (-)PB site. (D) The nascent (-) strand in (C) has base-paired with the (+) strand, with complementary sequences from the (-)PB region. The (+) strand is then extended on its second template, (-) strand DNA, and the (-) strand is extended by displacement synthesis along its third template, (+) strand DNA. (E) The events in (D) are shown at a reduced scale to indicate the use of either one RNA subunit (left) or two (right) during DNA synthesis. (F) Complete extension of both (+) and (-) strands has produced a linear duplex terminated with LTR's. (From Reference 127).
Once the provirus has successfully integrated it must be expressed to complete the viral life cycle. Host cell factors are responsible for production of viral RNA, maintenance of the DNA template and synthesis and processing of viral proteins. The viral LTR contains sequences which initiate transcription and a promoter which is found in the U3 region. Sequences are also found in the LTR for polyadenylation and termination of the RNA transcripts. Many molecules of viral RNA are produced per cell. Some viral or RNA molecules code for the entire genome while others code only for 3' segments. The RNA molecules may be cleaved into individual protein coding sequences before protein translation or the whole RNA molecule may be processed and the protein product cleaved into components later. 0.1% to 1% of total cellular RNA in a productively infected cell is viral-specific. Among mRNA molecules 5 to 10% may be of viral origin, several thousand copies per cell.

Viral proteins are synthesized on cellular ribosomes, modified by glycosylation and/or phosphorylation, then cleaved and assembled along with the RNA genome, into viral particles. The viruses then exit the cell by budding from plasma membranes and mature upon release (Figure 7).

There are many ways in which the retrovirus and the host cell can affect each other's behavior. After viral penetration some of the first instances are seen at the time of proviral integration. Retroviruses may act as mutagens if they integrate within a cellular gene and destroy its function, e.g. the transformed phenotype of some Rous sarcoma virus (RSV) has been reverted to normal by superinfection of the cells with a nontransforming murine leukemia virus which was shown to have integrated within the RSV genome (128). The 3' viral LTR may act as a promoter for a cellular gene that lies nearby. An example of the latter would be the avian leukosis virus which
acquires transforming ability when it integrates near the cellular "myc" gene (96). Other acutely transforming retroviruses appear to have been created by recombination between the provirus and cellular DNA allowing the virus to acquire a cellular sequence, an oncogene which is responsible for the transforming activity of the virus (132). There are now at least 30 examples of this phenomenon. A related theoretical, but as yet undemonstrated phenomenon, would be the transposition of a cellular gene from one location to another within the genome much as transposable elements move genetic material in lower organisms. Finally, the virus itself may be altered after the process of integration. As components of cell chromosomes, the provirus is subject to the same mutational risks and regulatory influences as host DNA. If anything, provirions appear to be more labile than host chromosomes. Studies of cloned viral transformed lines have allowed the discovery of deletions, point mutation, duplications of coding sequences, nonsense mutations and insertional mutations within proviral DNA and affecting viral expression.

During transcription, host cell factors also operate to modify the efficiency of viral RNA production (129). The factors which cause the same provirus to produce different amounts of RNA in different cells are poorly understood but may include the nature of flanking cellular DNA sequences (cis factors), the methylation state of proviral DNA, and the host chromosome structure in the region of viral integration which could affect the 2° structure of proviral DNA. Regardless of the reasons for variations in viral RNA expression, there are now many instances where it has been shown to vary by several logs (128). For example:

i) strains of the Rous sarcoma virus which will successfully infect mammalian, as well as avian cells, show 2-3 logs less viral RNA in the mammalian targets.
ii) different virally-infected clones of the same cell type containing the same type of viral DNA will show differences in the number of viral RNA molecules produced per cell from 0 to several thousands.

iii) different proviruses of the same type within the same cell can show 1 to 2 log differences in expression.

iv) over time, a single provirus within a cell may show changes in viral expression as shown by the spontaneous reversion to normal from a transformed phenotype of many virally transformed cell lines. This occurs without any observable change in the proviral genome and is caused by presumed epigenetic mechanisms.

v) transacting factors, such as hormones, may also interact with the provirus, e.g. steroids interact with sequence in the U3 region of the mouse mammary tumor virus LTR increasing viral transcription and leading to the malignant transformation of cells caused by the virus.

The general condition of the infected cells also affects the level of viral expression. In particular, cells actively in cycle, particularly in the DNA synthesis phase, support the synthesis of viral DNA whereas noncycling cells do not. Cellular DNA synthesis is necessary for proviral integration and viral replication and is more efficient in cycling cells. In many cells, one round of mitosis after the acute viral infection may be necessary to initiate virus production. Terminally differentiated cells are not able to produce viral DNA, at least partly because they are no longer able to divide themselves. Although it is clear that the viral life cycle proceeds most efficiently in cycling cellular hosts, the requirement may not be absolute in all circumstances. It has been shown that infected cells which have been maintained in a stationary state by serum starvation and which are not
producing virus will begin to show viral replication when the cells are refed serum and enter cell cycle.

In spite of the many factors, both known and unknown as outlined above, that will affect the success of gene transfer mediated by retroviral vectors, the concept is still more encouraging for many types of work than any other technique available at present. The potential advantages of retroviruses include (111):

i) An efficiency of cell infection approaching 100%. This means that not only a large number of cells receive foreign genes but that the occasional rare target in a large population of less interesting cells, for example the hemopoietic stem cell among differentiating bone marrow cells, may be infected at high frequency.

ii) Retroviral DNA generally integrates into host cell chromosomes as a single copy. This is in contrast to other methods of gene transfer where the DNA usually exists in the recipient cell as a concatamer of as many as several hundred copies integrated at a single site. In some situations this may be important for gene function or expression.

iii) The structure of the integrated proviral DNA is maintained in most infected cells. Although recombination events do occur among retroviruses, this is a relatively rare event compared to the possibility of deletion or rearrangement of the transferred DNA in chemical or physical techniques.

iv) Retroviral infection is nontoxic to the target cells. Again exceptions occur (e.g. Human immunodeficiency virus appears toxic to many lymphocytes) but there are available many retroviral vectors that do not harm the infected cells or alter their growth in any measurable way.
v) There are available a large number of potential retroviral vectors with many different host and tissue type ranges such that there should be virtually no limit to the target cell employed.

vi) The retrovirus provides its own promoter for gene expression in its LTR. This should allow many foreign genes to be expressed in the host cell without further manipulation. For example, the neomycin resistance gene (neo<sup>r</sup>) can be expressed in mouse bone marrow cells from the retroviral LTR (8,133). There also exist retroviruses with enhancers that can be manipulated by chemicals or hormones, such as the mouse mammary tumor virus. Use of vectors based on such a virus would allow flexibility and some control over how the host cell expressed the transferred genes. The successful expression of genes carried by retroviruses is an important contrast to genes introduced by physical or chemical techniques where "normal" expression is the exception rather than the rule. To correct this problem, the exogenous DNA is often linked to a foreign promoter before use in experiments involving techniques such as microinjection (119). However, only a handful of known genomic promoters have been successful in this situation, e.g. metallothionein, immunoglobulin, transferrin, elastase. Many more such promoters fail to function after DNA-mediated gene transfer perhaps due to methylation or other unknown mechanisms. Thus, the ability of the retrovirus to enhance its own expression may prove to be one of its most attractive features.

There are a number of theoretical and real disadvantages to retroviral gene delivery systems (111).
i) There is an intrinsic limit to the size of the DNA which can be inserted into these vectors. The Moloney murine leukemia virus (MoMuLV) must not be larger than 9 to 12 kb in order to be packaged. Two to 3 Kb are necessary for essential function leaving 6 to 9 Kb available for inserts.

ii) The DNA to be transferred must be a cloned fragment while whole cellular DNA can be used in physical techniques such as calcium phosphate transfection.

iii) The use of traditional retroviral vectors results in a productive infection of target cells which can spread to other cells beyond the intention of the initial experiment. This problem appears to have been overcome by newer constructed vectors to be described below.

iv) Retroviruses appear to have a strong propensity for deleting sequences during virus replication. For example, the murine sarcoma virus (MSV) DHFR-NEO vector which produces neo<sup>r</sup> expression in mice has lost a portion of its DHFR gene during production of the viral particles (134). Cell replication also appears to be necessary for viral integration. This would make it impossible to infect nondividing cells such as brain cells.

v) The randomness of retroviral integration may prove to be a disadvantage. It would be important for some experiments to direct the vector to a specific chromosomal site. At present this appears to be a formidable task in mammalian cells.

vi) Although the retroviral LTR appears to function in most cell lines as an efficient promoter in primary cells, particularly those which are primitive or undifferentiated such as found in embryos and
perhaps hemopoietic stem cells, it appears to operate less well (8,9,135). This may mean that exogenous promoters will have to be linked to the gene to be transformed by the retrovirus in order to achieve adequate gene expression.

F. Construction of Retroviral Vectors (111,127) (Figure 9)

The proviral DNA for the desired retroviral vector, commonly either the Moloney murine leukemia virus (MoMuLV) or murine sarcoma virus (MSV), is inserted into a convenient bacterial plasmid to make it possible to produce it in large quantities. The viral structural genes can then be replaced with the exogenous genes of choice by standard recombinant DNA techniques. This vector is now incompetent to replicate because it lacks the ability to produce a full complement of viral proteins. The vector construct is used to transfect cells such as NIH-3T3 cells by calcium phosphate co-precipitation. If such cells already contain packaging or helper virus proviral DNA, appropriate viral proteins are available to package the recombinant virus. The recombinant virus is produced and buds off the cells into the medium. Either the medium or the viral producer cells can be used to infect target cells. However, if an intact helper virus such as the Moloney murine leukemia virus (MoMuLV) is used, helper virus as well as recombinant virus is produced and the successfully infected target cells themselves now become infectious.

The continuous production of virus, both helper and recombinant, by infected target cells will be a disadvantage for many experiments including any attempt at gene transfer to human beings. Two groups of investigators have developed retroviral packaging mutants that produce a defective helper retrovirus (136,137). A site 5' of the retroviral gag gene termed the Ψ region has been found necessary for viral packaging in both the Rous sarcoma
PRODUCTION OF RETROVIRUSES FOR GENE TRANSFER

Step 1: Construct Recombinant Virus in a Plasmid

Step 2: Produce Virus


Step 1: Viral regulatory sequences present primarily in the long terminal repeat (LTR) of the retroviral genome and the gene of interest to be transferred (neo<sup>R</sup>) are inserted into a bacterial plasmid vector to allow large quantities of the recombinant molecule to be generated in bacterial hosts.

Step 2: Plasmid DNA is transfected into a packaging cell line and integrates into the host cell DNA. The recombinant proviral DNA provides the template for viral RNA synthesis. The packaging cell line contains viral DNA sequences that code for viral structural proteins and allow the recombinant viral DNA to be packaged and bud from the cells. Illustrated is a "helper defective" packaging cell in which the helper proviral DNA lacks sequences necessary for helper virus replication (see text). Only recombinant "neo<sup>R</sup>" virus is produced by this cell.
virus and MoMuLV (Figure 10). A deletion of 350 bp fragment encompassing this region between the 5' LTR and the gag codon results in a recombinant virus which will provide viral proteins for another defective recombinant virus coding for exogenous genes without replicating itself. The proviral DNA of the Y deficient vector is inserted into NIH-3T3 cells. Proviral DNA containing the exogenous gene of interest is inserted into the same cells by calcium phosphate transfection. Viral proteins produced by the Y deficient provirus are used to successfully package the other recombinant virus, which contains the intact Y region, and which then buds off the cells and can be used to infect target cells. The infecting virus cannot replicate in the target because it lacks genes coding for viral proteins. The virus thus acts as a gene delivery system rather than an infectious agent.

Unfortunately, in practice when such Y-helper viruses are used sufficient recombination occurs between helper and recombinant viral genomes such that helper virus is generated with unacceptably high frequency in many cases. To overcome this problem further deletions have been made in the helper virus genome primarily in the LTRs. This strategy appears to make recombination events and the generation of helper virus highly unlikely if not impossible (138).

It would be important to develop packaging defective viruses of broader host range than the MoMuLV for experiments using human cells. Two groups of investigators have accomplished this by replacing the env gene in the Y deficient MoMuLV with the env from an amphotropic retrovirus (137,139) (Figure 10).

There is concern, particularly among those hoping to use retroviruses for gene therapy in humans, that the viral LTRs may act as abnormal promoters for cellular genes with potential deleterious results for the target cells.
CONSTRUCTION OF AN AMPHOTROPIC PACKAGING-DEFECTIVE VIRUS

Ecotropic MoMLV

Amphotropic Virus

Amphotropic MoMLV

Amphotropic Packaging-Defective Virus

FIGURE 10. Construction of a $\psi^-$ Amphotropic Retrovirus.

Abbreviations: MoMLV, Moloney murine leukemia virus; LTR, long terminal repeat; SD, splice donor site for generation of env mRNA; $\psi$, packaging signal; gag, viral core proteins; pol, reverse transcriptase; SA, splice acceptor for generation of env mRNA; env, envelope proteins. The env region of the MoMLV is replaced with the env of the amphotropic virus to produce an amphotropic MoMLV. The packaging signal is then deleted to give an amphotropic virus that will provide proteins to package a recombinant virus but cannot package itself.
Situations exist in animal systems where, for example, the avian leukosis virus causes increased expression of the cellular myc gene with resulting malignant transformation of the infected cells (96). For this reason some investigators are developing retroviral vectors with "crippled" LTRs which enable the virus to integrate into host DNA, but which cannot promote or enhance gene expression (140). With these vectors it will be necessary to provided an exogenous promoter and enhancer sequence to see expression of the proviral DNA.

G. Studies on Retroviral-Mediated Gene Transfer to Hemopoietic Cells

i) Murine Studies

Several investigators have now shown that it is possible to introduce foreign genes into murine bone marrow stem cells and committed progenitors using recombinant retroviruses. The first report of the successful use of retroviruses to transfer genes into hemopoietic cells demonstrated G418 resistant granulocyte-macrophage colony formation after exposure of mouse bone marrow cells to virus containing the gene for neomycin resistance (neo<sup>R</sup>) (133). Subsequently, several groups have reported high efficiencies of gene transfer to more primitive hemopoietic cells including CFU-S (8,9,134,141). Successfully infected cells have also reconstituted W/W<sup>y</sup> mice or normal irradiated recipients (8,9). Gene transfer efficiencies to CFU-S approaching 100% have been obtained using high titer virus (141,142). Various factors may be important to allow such high efficiencies. These include, co-cultivation of target with viral producer cells, pre-treatment of mice with 5 fluorouracil and the addition of growth factors such as IL-3 during the infection period. These latter two manipulations presumably act by increasing the proportion of target cells in the active part of the cell cycle.
As mentioned in a previous section, retroviral marking of hemopoietic stem cells has been used to analyze patterns of bone marrow repopulation and progenitor differentiation in vivo. The site of retroviral integration in target cell DNA is random and provides a unique molecular marker for that cell and its progeny. Infected bone marrow cells have been injected into lethally-irradiated recipients. In some cases the pattern of retroviral integration into spleen, bone marrow and thymus DNA from reconstituted mice indicates that a single clone can sometimes repopulate all three tissues (8,9). Thus, a pluripotent stem cell of both myeloid and lymphoid potential has been infected and this cell has an enormous proliferative capacity. In the same experiment progenitors of more restricted potential were identified that populated only the thymus or the bone marrow. Long term studies have been performed on such reconstituted animals and successive waves of proliferation have been identified from progenitors of different clonality (142). That is, a clone that has repopulated bone marrow may disappear and be replaced by cells of different clonality only to reappear at a later time. The fact that very few stem cell clones may be active at a given time in murine transplant recipients and the evidence for clonal succession of these cells may be relevant to human marrow transplantation and hemopoiesis.

In contrast to the above studies where gene transfer was clearly highly efficient experiments designed to show expression of the foreign gene in primary hemopoietic cells have been much less successful. Most published results have used vectors in which promoter function for the transferred gene would be provided by the retroviral LTR or SV40 viral sequences. Progenitors derived from CFU-S known to carry the neo<sup>R</sup> have given rise to colonies only 10% of which show G418 resistance (8,9). Similar difficulties have been encountered with expression of the human adenosine deaminase (hADA) gene
(143). These vectors which result in poor expression from CFU-S appear to function well in established cell lines or more differentiated progenitors such as CFU-GM. Mechanisms may be active in primitive pluripotent cells which result in inactivation of certain promoters such as that found in the retroviral LTR. Vectors which rely on different promoters for gene expression are currently being tested in a number of laboratories. Preliminary evidence indicates that the herpes simplex thymidine kinase promoter or myeloproliferative sarcoma virus may allow improved gene expression in primitive hemopoietic cells (135,144).

Preliminary studies exploring the potential role of genes such as oncogenes or growth factors in malignant transformation of hemopoietic cells have been conducted by a number of workers using retroviral-mediated gene transfer to murine cells. Lang et al (145) infected a growth factor-dependent hemopoietic cell line with a virus containing the mGM-CSF gene. Infected cells not only lost their dependence on exogenous growth factor but became leukemogenic when injected into irradiated mice suggesting that escape from "normal" growth control in these hemopoietic cells was related to their malignant potential. In somewhat analogous studies Witte et al (98) infected long term cultures of murine cells designed to favor the growth of B cells with viruses containing viral oncogenes; H-ras, v-myc, or both in combination. Growth of primitive B cells was greatly enhanced in the cultures infected with both viruses and the infected B cells caused B-cell lymphomas in mice. In this study it appears that the transferred genes were carcinogenic for murine B cells.

In addition to the intrinsic interest of the studies themselves, results such as those described above provide a basis for the belief that the technique of retroviral-mediated gene transfer is a useful tool for dissection of the genetic events involved in malignant transformation.
ii) Human Studies

Retroviruses have also been used for gene transfer to human cells. Although the available data is less extensive than for murine targets it has been possible to demonstrate corrections of hypoxanthine phosphoribosyl transferase (HPRT) deficiency in human fibroblasts (146) and B lymphoblasts (147) after infection with an HPRT-containing virus. Similar results have been reported for experiments in which a virus containing the hADA gene was used to infect human ADA deficient B and T lymphocytes (143,148). Evidence of sustained retroviral infection in human long term bone marrow cultures with virus containing oncogenes or the human HPRT gene has also been reported (149,150). However, quantitative studies of the level of gene transfer or expression in hemopoietic progenitors in these cultures was not determined. Hock and Miller have recently published more precise data demonstrating retroviral-mediated gene transfer to primary human marrow progenitors using retroviruses carrying the neo$^r$ or mutant dihydrofolate reductase gene (151).

In summary, gene transfer via retroviruses is not only feasible but very efficient using murine hemopoietic targets. The available data on human hemopoietic cells is very limited but also indicates the feasibility of the technique. Problems of obtaining high levels of expression of the transferred gene appear to be considerable when primitive primary cells such as hemopoietic stem cells are the targets. Nevertheless, retroviral-mediated gene transfer is an exciting technique for use both in the genetic labelling of cells and in the study of gene regulation.

6) PRESENT OBJECTIVES

Malignancies of the hemopoietic system present clinically as the disordered production of blood cells. Although the malignant cells may
differentiate along several lineages, in most, if not all, of these diseases, the essential malignant event(s) occurs in a single cell which then proliferates, i.e. hemopoietic malignancies are clonal in origin.

The hypothesis behind my research is that genetic change is fundamental to the development of malignancy. The purpose of this research was to explore methods to study not only the cell in which the changes occur that give rise to hemopoietic cancer but also to investigate the specific abnormalities at a molecular level.

In the first phase of my research, I used cytogenetic analysis of cells from individual hemopoietic colonies of various lineages to demonstrate that, in a myelodysplastic disorder of childhood associated with monosomy of chromosome 7, the malignancy arose in a pluripotent stem cell capable of differentiation down both myeloid and erythroid pathways. Although this is a relatively unusual pediatric disorder, it is clinically and cytogenetically very similar to the increasingly common myelodysplasias of adulthood. Both diseases typically terminate in refractory acute leukemia. Thus, it is likely that myelodysplastic disorders originate in a very primitive hemopoietic progenitor which initially retains some of its ability to differentiate into functional cells. However with time, progressive changes occur and a part of the clone loses this capacity and is seen as leukemic blasts.

In the second part of my research, I took a different approach to study the clonal evolution of malignant cells. In this case, using the disorder CML and G6PD isoenzyme analysis, I investigated the possibility that there might be cytogenetically normal hemopoietic progenitors that would, nevertheless, be part of the malignant clone. Long-term bone marrow cultures from patients with CML are known to favour the growth of Ph-negative progenitors. Such cultures were initiated with cells from 2 women who were heterozygous for 2
G6PD enzyme variants. In one of these patients a proportion of hemopoietic progenitors was found to be Ph-negative after 4 to 6 weeks in long-term culture. A similar proportion of progenitors were found to be non-clonal by G6PD enzyme analysis, i.e. some colonies expressed the enzyme not found in the malignant clone. In this case karyotypically normal cells appear to be truly normal and a Ph-negative clonal population has not been identified. Although it is clearly possible to identify the G6PD enzyme produced by individual hemopoietic colonies, the technique is limited in its applicability to the 1/3 of black women who are heterozygous for two electrophoretically distinct enzyme types. Experimental approaches which would be valid for a larger proportion of clinical samples are necessary before such studies can be carried out in a more comprehensive fashion.

The third phase of my research involved developing the technique of retroviral-mediated gene transfer to move foreign genes into human hemopoietic progenitors. The purpose of this was two-fold. Firstly, marking the DNA of hemopoietic progenitors with a foreign gene would allow them and their progeny to be followed through the processes of proliferation and differentiation. Various clonal populations of marked cells could be identified by the unique site of integration of the retroviral genome into cellular DNA. Secondly, genes of potential interest in the study of hemopoiesis, e.g. growth factors or oncogenes, could be transferred into target cells to allow the study of gene function or regulation. I have demonstrated the feasibility of retroviral-mediated gene transfer to human hemopoietic cells using recombinant virus carrying the selectable marker gene, neo\(^r\), which confer resistance to the neomycin analogue G418 on successfully infected cells.

These experiments established the feasibility of retroviral-mediated gene transfer to human hemopoietic cells. Further experiments using bone marrow
cells in long-term culture as a target and the neoF virus as infecting agent were performed with the hope of showing successful and stable gene transfer over a period of several months. These were also preliminary experiments to those in which we plan to use viruses containing genes more pertinent to the study of hemopoietic regulation, e.g. growth factors or oncogenes.

In summary, the experiments described in this thesis will show that:

i) Hemopoietic progenitors can be studied in culture using several genetic markers. Cytogenetic analysis can be used in patients with a hemopoietic malignancy characterized by an abnormal karyotype. Where chromosomal markers are lacking other techniques such as G6PD isoenzyme analysis or gene transfer may provide ways to identify unique populations of cells.

ii) Retroviral-mediated gene transfer may provide ways to study the regulation of normal and malignant hemopoiesis at the molecular level using in vitro model system such as long-term bone marrow culture.
REFERENCES


38. Kaushansky K, Broudy VC, Lin N, Adamson JW. Granulocyte/macrophage (CM) and granulocyte (G) colony-stimulating factors (CSFs) are synthesized by candidate cells of the marrow microenvironment in response to interleukin-1 (IL-1). Blood 68 (Suppl 1) 167a, 1986.


CHAPTER II

JUVENILE MONOSOMY 7 SYNDROME: EVIDENCE THAT THE DISEASE ORIGINATES IN A PLURIPOTENT HEMOPOIETIC STEM CELL

1) INTRODUCTION

The association of chronic myeloproliferative disorders or refractory cytopenias with a bone marrow karyotype showing monosomy of chromosome 7 is well-documented in the pediatric literature (1-6). The bone marrow is typically hypercellular with disordered maturation in one or more cell lines. Although this disease may present as a typical myelodysplastic disorder (5,6) it often has features more commonly associated with the myeloproliferative syndromes, such as hepatosplenomegaly and an elevated white blood cell count with a left-shifted differential and eosinophilia (1,3,4). The presenting features may vary somewhat, but the ultimate prognosis is predictably poor and the majority of patients enter a terminal phase of refractory acute nonlymphoblastic leukemia (ANLL) within a few years.

The cell in which malignant change originates in this disease has not been elucidated. Some patients show only leukocytosis and left-shifted peripheral blood granulocytes in association with marrow myeloid hyperplasia, suggesting an origin in cells committed to the granulocyte-macrophage pathway. In other children dyserythropoiesis, reticulocytopenia and severe anemia have been observed during the preleukemic period. This could reflect involvement of cells of the erythroid lineage but might also be explained by secondary effects. Direct investigation of the differentiation potential of the
malignant cells in children with bone marrow monosomy 7 and myelodysplasia was made possible in this study by the combined use of hemopoietic progenitor assays and hemopoietic colony cytogenetics.

2) PATIENTS
   A. Case 1
      An 18 month old boy presented with bruising, hepatosplenomegaly and abnormal blood counts. His Hb was 8.9 gm/dl, platelets 36,000/μl, wbc 49,000/μl (neutrophils 42%, metamyelocytes 4%, myelocytes 2%, promyelocytes 0.5%, monocytes 10%, blasts 3%). His bone marrow was hypercellular with reduced megakaryocytes and an abnormal karyotype including cells showing monosomy of chromosome 7 (See Results and Table V). The patient was treated with multiple chemotherapeutic agents without benefit. He developed progressive organomegaly, pancytopenia and an increasing proportion of myeloid blasts in marrow and blood. A diagnosis of ANLL was made 17 months after presentation and the patient died 3 months later.

   B. Case 2
      A 5 month old boy presented with hepatosplenomegaly, anemia and thrombocytopenia. His Hb was 4.9 gm/dl, platelets 39,000/μl, wbc 10,400/μl with 14% circulating nucleated red blood cells but no other blasts. The bone marrow was hypercellular with an M:E ratio of 1:3, dyserythropoiesis and reduced megakaryocytes. Bone marrow cytogenetic analysis showed the presence of cells with monosomy 7 (See Results and Table V). The patient was initially treated with corticosteroids. He underwent splenectomy at age 8 months for refractory thrombocytopenia. Allogeneic bone marrow transplantation was performed at age 9 1/2 months after conditioning with cyclophosphamide,
### TABLE V
Direct Cytogenetic Studies

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tissue Studied</th>
<th>Time of Sample</th>
<th>Karyotype</th>
<th>Number of Analyzed Metaphases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>blood lymphocyte</td>
<td>at diagnosis</td>
<td>46,XY</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>bone marrow</td>
<td>at diagnosis</td>
<td>46,XY/45,XY,-7/47,XY,+8</td>
<td>9/8/4</td>
</tr>
<tr>
<td></td>
<td>bone marrow</td>
<td>17 months post-diagnosis - onset of ANLL</td>
<td>45,XY,-7</td>
<td>25</td>
</tr>
<tr>
<td>2</td>
<td>bone marrow</td>
<td>at diagnosis</td>
<td>46,XY/45,XY,-7</td>
<td>NA*</td>
</tr>
<tr>
<td></td>
<td>blood lymphocyte</td>
<td>3 months post-diagnosis</td>
<td>46,XY</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>splenic aspirate</td>
<td>3 months post-diagnosis</td>
<td>45,XY,-7</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>bone marrow</td>
<td>1 month post marrow transplant</td>
<td>46,XY/45,XY,-7</td>
<td>98/3</td>
</tr>
<tr>
<td></td>
<td>bone marrow</td>
<td>4 months post transplant</td>
<td>45,XY,-7/46,XY</td>
<td>20/3</td>
</tr>
</tbody>
</table>

* NA = not available.
busulfan and cytosine arabinoside. The donor marrow was from the patient's mother and had been depleted of alloreactive T lymphocytes with soy bean lectin agglutination and sheep erythrocyte rosetting (7). Although he became hematologically normal, bone marrow cytogenetics following transplantation showed autologous reconstitution. The patient remains alive at age 10 months with increasing marrow dysfunction and at the time of writing had just received a second marrow allograft.

3) MATERIALS AND METHODS

A. Hemopoietic Colony Assays

Erythropoietic (CFU-E and BFU-E) and granulopoietic (CFU-GM) progenitors from bone marrow buffy coat or Ficoll-Hypaque-separated peripheral blood mononuclear cells were assayed in methylcellulose and scored as previously described (8,9, Appendix IV). A final concentration of 10% leukocyte conditioned medium was added to all cultures. Small and large erythroid colony growth was evaluated in cultures both with and without human urinary erythropoietin (3 U/ml, 1000 U/mg, and <0.002 U/ml, respectively). Cells were plated at several concentrations to prevent developing colonies from overlapping one another. Control values were determined using similar reagents and criteria as reported previously (9).

B. Cytogenetic Studies

Bone marrow was processed for direct cytogenetic analysis by standard techniques (10) and cells harvested both immediately and after 24 hours in liquid culture. To study the karyotype of individual hemopoietic colonies large, well-isolated erythroid or granulocyte/macrophage colonies were plucked and processed individually as previously described (11, Appendix V). A
minimum of 2 G-banded metaphases were analyzed per colony. Pools of smaller colonies of the same lineage were also analyzed. Karyotypes were established by Giemsa banding (12).

4) RESULTS

A. Progenitor Assays

Hemopoietic progenitor assays were performed on bone marrow cells from both patients several times during their clinical course. Peripheral blood cells from patient 1 were also analyzed. Representative colony counts from marrow samples obtained shortly after diagnosis are shown in Table VI. Qualitative abnormalities in colony growth were striking in both patients' cultures although the actual yield of erythroid colonies per $10^5$ marrow cells did not vary significantly from normal. In both cases, 5 to 10% of CFU-E and BFU-E often generated visually detectable hemoglobinized colonies in the absence of erythropoietin, and even those colonies forming in the presence of normally optimal concentrations of erythropoietin showed poor development and maturation. Granulopoietic colonies were increased in frequency in patient 1's marrow cultures. Some of these became macroscopic, containing several thousand cells. When such colonies were individually plucked and cell morphology examined on Wright-Giemsa stained cytocentrifuge preparations, the majority of cells appeared to be monocytes or macrophages. CFU-GM in patient 2 cultures were normal or reduced in frequency and generated only small colonies.

Peripheral blood from patient 1 was consistently found to contain elevated numbers of progenitors of all classes (Table VII). The frequency of CFU-E, BFU-E and CFU-GM in blood gradually increased throughout the patient's course so that by 12 months after diagnosis the proportion of progenitors in
TABLE VI
Bone Marrow Culture – Progenitor Numbers*

<table>
<thead>
<tr>
<th>Patient</th>
<th>CFU-E</th>
<th>BFU-E</th>
<th>CFU-GM</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
<td>125</td>
<td>808</td>
<td>- erythroid colonies small, poorly hemoglobinized, 5-7% erythropoietin independent - many very large macrophage colonies</td>
</tr>
<tr>
<td>2</td>
<td>48</td>
<td>224</td>
<td>180</td>
<td>- erythroid colonies small, poorly hemoglobinized, 7-10% erythropoietin independent - repeat culture showed no normal granulocyte/macrophage colonies</td>
</tr>
</tbody>
</table>

Normal Range
(99%) (10-299) (4-336) (7-156)

* Colonies per $10^5$ marrow buffy coat cells.
**TABLE VII**

Peripheral Blood Progenitor Numbers
From Patient 1*

<table>
<thead>
<tr>
<th>Time Since Diagnosis (months)</th>
<th>CFU-E</th>
<th>BFU-E</th>
<th>% Abnormal**</th>
<th>CFU-GM</th>
<th>Blood wbc/μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>0</td>
<td>752</td>
<td>0</td>
<td>4317</td>
<td>8000</td>
</tr>
<tr>
<td>9</td>
<td>1830</td>
<td>18800</td>
<td>36</td>
<td>166375</td>
<td>21000</td>
</tr>
<tr>
<td>12</td>
<td>22856</td>
<td>68572</td>
<td>0</td>
<td>348572</td>
<td>26300</td>
</tr>
<tr>
<td>Normal Range (99%)</td>
<td>(1-345)</td>
<td>(21-841)</td>
<td>0</td>
<td>(2-290)</td>
<td>(6000-17500)</td>
</tr>
</tbody>
</table>

* Colonies per ml of blood.

** % Erythroid colonies grown without added erythropoietin.
the blood was almost 2% of the total wbc count. These exhibited the same spectrum of qualitative abnormalities of erythroid and granulopoietic colony growth as were seen in the marrow cultures.

B. Cytogenetic Studies

Direct cytogenetic studies of bone marrow cells initially showed more than one karyotype in both patients: 45,XY,-7/47,XY,+8/46,XY in patient 1 and 45,XY,-7/46,XY in patient 2 (Table V). In patient 1, the proportion of bone marrow cells with the 45,XY,-7 karyotype subsequently increased, reaching 100% in several samples in which at least 25 metaphases were analyzed by 17 months after diagnosis. This coincided with clinical progression to acute leukemia. In patient 2, the 45,XY,-7 karyotype was present in all 50 metaphases from spleen cells obtained 3 months after diagnosis. Wright-Giemsa stain of the splenic aspirate processed for cytogenetics revealed predominantly erythroid cells, some of which showed dyserythropoiesis. After bone marrow transplantation the patient showed cytogenetic evidence of autologous recovery with 3 of 101 cells containing the 45,XY,-7 karyotype and the proportion of metaphases with this karyotype subsequently increased (Table V).

Table VIII shows the results of cytogenetic analyses of hemopoietic colonies. The majority of individual and pooled erythroid colonies from patient 1's cultures showed the 45,XY,-7 karyotype. One BFU-E was 47,XY,+8. No cytogenetically normal BFU-E were detected. CFU-GM showed a similar picture, some belonging to the -7 clone, some to the +8 clone and some showing a normal karyotype. In patient 2's cultures the majority of erythroid colonies, individual and pooled, were 45,XY,-7, even at times when metaphases from direct marrow samples were largely normal. Attempts to obtain cytogenetic data from the small granulocyte/macrophage colonies produced in patient 2's assays were unsuccessful.
### TABLE VIII

**Bone Marrow Culture - Hemopoietic Colony Cytogenetics**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Karyotype</th>
<th>BFU-E</th>
<th>CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45,XY,-7</td>
<td>9 (7)*</td>
<td>3 (12)</td>
</tr>
<tr>
<td></td>
<td>47,XY,+8</td>
<td>1</td>
<td>3 (20)</td>
</tr>
<tr>
<td></td>
<td>46,XY</td>
<td>0</td>
<td>1 (9)</td>
</tr>
<tr>
<td>2</td>
<td>45,XY,-7</td>
<td>22 (48)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>46,XY</td>
<td>4 (16)</td>
<td></td>
</tr>
</tbody>
</table>

* Number of individual colonies (number of metaphases from pools of cells from similar colonies).
5) DISCUSSION

Preleukemic disorders are uncommon in children. However, they have many similarities to the increasingly prevalent forms of adult myelodysplasia. In particular, karyotypic abnormalities are frequent in the adult disorder and deletions of 7q or monosomy 7 are among the most common cytogenetic changes observed (13,14). These changes confer a particularly poor prognosis as a large proportion of patients with them ultimately develop refractory ANLL. Myelodysplasia and abnormalities of chromosome 7 often follow treatment of a primary malignancy with cytotoxic chemotherapy, notably alkylating agents, and these patients, too, fare badly (15-17). In children with monosomy 7 and marrow dysfunction, whether the presentation has features more consistent with a myelodysplastic or myeloproliferative nature, the prognosis is similarly poor (1-6). Refractory cytopenias develop, become progressively severe and typically evolve into ANLL which is unresponsive to conventional anti-leukemic therapy.

The fact that hemopoietic malignancies may arise in a pluripotent hemopoietic progenitor was first demonstrated in chronic myelogenous leukemia (CML) by cytogenetic and glucose 6 phosphate dehydrogenase (G6PD) isoenzyme studies (18,19). In this disorder the malignant stem cell gives rise to differentiated progeny that function as relatively normal blood cells. However, the eventual emergence of the blast phase of CML in more than 80% of patients is reminiscent of the evolution of myelodysplasia to ANLL. G6PD analysis has also been used to demonstrate involvement of erythrocytes, platelets, granulocytes and lymphocytes in the malignant clone of two adult cases of myelodysplasia (20,21). Recently a single case of therapy-linked myelodysplasia and monosomy 7 in a child has been described with karyotypic changes in both BFU-E and CFU-GM (17) providing further evidence for pluripotent progenitor involvement in preleukemic states.
In this report erythroid progenitors that are cytogenetically marked and differentiate abnormally have been detected in two children with dyshemopoiesis associated with monosomy of chromosome 7. In one case morphologic and karyotypic changes in colonies derived from granulopoietic progenitors were also demonstrable. Erythropoietin-independent erythroid colony growth was prominent in both patients' cultures. Although this phenomenon was first described in polycythemia vera (22), it has subsequently been revealed in other myeloproliferative disorders (23) and erythroleukemias (24), and appears to be specifically associated with clonal disorders of hemopoiesis. The large increase in progenitor content of the peripheral blood in patient 1 is reminiscent of similar changes typical of CML and other adult myeloproliferative disorders (25). Various growth abnormalities have been described in cultures of hemopoietic cells from patients with myelodysplasia. They include, reduced granulocytic and erythroid colony formation (26-29) and increased small cluster formation (27-29). Our second patient's cultures showed a decreased CFU-GM content in later cultures similar to what was described in the above studies. Thus, both patients' progenitors exhibited abnormal growth in culture consistent with the malignant nature of their hemopoietic cells. Monosomy 7 was demonstrated in many of the cytogenetically analyzed erythroid and granulopoietic colonies verifying the proliferation of neoplastic hemopoietic progenitors in these cultures. Malignant change in this disease must therefore involve primitive hemopoietic cells capable of erythroid and, in at least some cases, myeloid differentiation.

Studies of blood and bone marrow cells from patients with de novo acute leukemia also suggest that some of the cells within the malignant clone retain the ability to differentiate along one or more lineages under certain conditions both in vitro and in vivo (30-32). Thus, hemopoietic progenitors
and, in particular, the pluripotent stem cell appear to be common targets for events that give rise to a variety of malignant disorders including myelodysplasia.
REFERENCES


HEMOPOIETIC PROGENITORS THAT ARE NOT PART OF THE MALIGNANT CLONE
REVEALED IN LONG-TERM MARROW CULTURE FROM A G6PD HETEROZYGOTE
WITH CHRONIC MYELOGENOUS LEUKEMIA

1) INTRODUCTION

Chronic myelogenous leukemia (CML) is a clonal neoplasm in which malignant transformation occurs in a primitive hemopoietic cell with the potential to differentiate along both myeloid and lymphoid lineages (1,2). In most patients 100% of bone marrow metaphases will show a cytogenetic marker, the Philadelphia chromosome (Ph) (3,4). When marrow or blood cells from CML patients are cultured in semi-solid media usually all of the erythroid, granulocytic and multi-lineage colonies generated are also Ph-positive, and these findings are not altered after conventional chemotherapy (5,6). However, studies of patients treated with high dose chemotherapy (7-9) or α interferon (10) have demonstrated the reappearance of Ph-negative, and in two cases, nonclonal hemopoiesis. From quantitative studies of progenitor numbers and genotypes in CML patients, it has been shown that failure to detect Ph-negative cells in untreated or less aggressively managed patients is due to two factors: dilution of primitive Ph-negative progenitor cells by early expansion at the stem cell level of the Ph-positive clone as well as accompanying suppression of Ph-negative progenitor cell differentiation (6,11). More recent studies using the long-term marrow culture system have shown that Ph-negative progenitors can often be revealed in cultures derived
from recently diagnosed patients, including cases where these karyotypically normal cells were not initially demonstrable (11-13). Although the Ph-negative progenitors appearing in long-term CML marrow cultures are karyotypically and phenotypically normal, it is not certain that they represent truly normal cells. An alternative possibility is that they may belong to the neoplastic clone but derive from a cell at a stage prior to development of the Ph translocation (2). In a previous study of a patient mosaic for the karyotypes 46,XX and 46,X with CML, it was shown that the Ph-negative hemopoietic progenitors detected after long-term culture of marrow were not part of the malignant clone (14). In the present study I have extended this observation to a second CML patient using glucose-6-phosphate dehydrogenase (G6PD) analysis to distinguish clonal and normal populations.

2) MATERIALS AND METHODS

Twelve black women with CML were identified and skin biopsies used as a source of fibroblasts for G6PD analysis. Six of these patients were found to be heterozygous for two electrophoretically distinguishable G6PD isoenzymes. Of the six patients; one was lost to follow-up, two had marrow aspirates of insufficient quantity for establishing long-term cultures because of previous therapy or myelofibrosis and a fourth patient refused marrow aspiration. The remaining 2 patients are the cases in this report.

A. Case Reports

Patient 1. Patient 1 was a 45 year-old woman from West Africa. The diagnosis of CML was made in March, 1985 when she was discovered to have massive splenomegaly and an abnormal peripheral blood picture (white cells 490,000/mm³ with 30% immature myeloid forms including promyelocytes,
myelocytes and metamyelocytes, 3% basophils, hemoglobin 5.4 gm/dl, platelets 800,000/mm³). At that time cytogenetic analysis showed the standard Ph translocation: t(9;22) (q34;q11) in all 30 marrow metaphases examined. The patient was treated with oral hydroxyurea for 10 days before a second bone marrow aspiration was performed, a portion of which was used for the studies reported here. At this time her white blood cell count was 10,500/mm³, hemoglobin 7.8 gm/dl, platelets 500,000/mm³.

Patient 2. Patient 2 was an 11-year old girl from Guadeloupe who presented in August, 1983 with a peripheral blood white cell count of 105,000/mm³ and a platelet count of 1,000,000/mm³. She was observed without treatment until August, 1984. Physical examination at that time showed hepatosplenomegaly and her peripheral blood white cell count was 185,000/mm³ with 38% neutrophils, 3% eosinophils, 1% basophils, 25% myelocytes, 19% metamyelocytes, 8% promyelocytes, 4% blasts, 2% lymphocytes. Her hemoglobin was 10 gm/dl and her platelet count was greater than 1,000,000/mm³. A bone marrow examination revealed myeloid hyperplasia at all levels of maturation and increased megakaryocytes. All 25 marrow metaphase cells examined showed the standard Ph translocation: t(9;22)(q34;q11). She was then treated with hydroxyurea and allopurinol intermittently until November 1984 when the marrow sample used for the studies reported here was taken. At this time the peripheral blood white cell count was 58,300/mm³.

B. Cultures

Skin samples were obtained by punch biopsy and placed in sterile medium. Bone marrow aspirate and blood cells were collected in heparin. All samples were placed on ice from the time of collection in Paris until arrival in Vancouver 18 to 24 hours later.
Skin samples were minced with scissors and allowed to adhere to tissue culture dishes before flooding the dishes with α medium plus 20% fetal calf serum. After three to four weeks confluent fibroblasts were harvested by trypsinization for G6PD analysis.

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque separation.

Bone marrow buffy coat cells were used to initiate long-term cultures as previously described (12, Appendix III). An aliquot was plated in methylcellulose cultures for assessment of erythroid and granulocytic colony-forming cell numbers, karyotype and G6PD analysis. Long-term cultures were maintained by weekly removal of half of the growth medium and nonadherent cells and replacement of half of the growth medium. After 4 to 6 weeks adherent layers were suspended using collagenase and the cells plated in methylcellulose at $10^5$ cells/ml for assessment of the number and types of progenitors present (15, Appendix IV).

C. G6PD Analysis

Lysates were made from cells pelleted by centrifugation by repeated freezing on dry ice and thawing. Samples were then applied to cellulose acetate membranes. Individual colonies were plucked from methylcellulose directly onto cellulose acetate membranes before freeze/thawing. Samples were then subjected to electrophoresis in Supre-Heme buffer pH 8.4 (Helena Laboratories, Beaumont, Texas) for 40 minutes at 375 volts. The membranes were stained immediately with G6PD isoenzyme reagent (Helena Laboratories) and the position and relative intensity of the enzyme bands estimated visually and compared to established standards.
D. Cytogenetic Studies

Cytogenetic analysis was performed on individual colonies and pools of colonies of the same lineage plucked from methylcellulose assays as previously described (16, Appendix V). Karyotypes were established after Giemsa banding (17).

3) RESULTS

G6PD analysis of skin fibroblasts (Table IX) showed the patient to be heterozygous for enzyme variants A and B at a ratio of 50:50. Whole blood lysates from patient 1 were 100% G6PD-B. Blood from patient 2 was separated into red and white cell fractions and lysates of both were found to be 100% G6PD-A.

Cytogenetic analysis of colonies removed individually from methylcellulose assays of the initial marrow sample showed all 10 colonies examined from patient 1 and all 12 colonies from patient 2 to be Ph-positive (Table X). G6PD analysis of colonies from the same assays showed 26/26 colonies from patient 1 assays to be G6PD-B and 22/22 colonies from patient 2 assays to be G6PD-A. Similar results were obtained for colonies produced in simultaneous assays of peripheral blood progenitors (data not shown).

For both patients the number of granulopoietic progenitors present in the nonadherent fraction of long-term marrow cultures was consistently low from the second week of culture onward, and no erythroid progenitors were detectable in the non-adherent fractions after this time. Adherent layers were harvested after 4 weeks for patient 1's cultures, and after 4 and 6 weeks of culture for patient 2. The majority of the granulocyte colony progenitors present at these times were in the adherent layer, as has been found previously (12,13) and hence cytogenetic and G6PD data were obtained only from these assays. An occasional erythroid colony was produced in assays of the 4
<table>
<thead>
<tr>
<th>Patient</th>
<th>Blood</th>
<th>Fibroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A : B</td>
<td>A : B</td>
</tr>
<tr>
<td>1</td>
<td>rbc* 0 : 100</td>
<td>50 : 50</td>
</tr>
<tr>
<td></td>
<td>(whole blood)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>rbc 100 : 0</td>
<td>50 : 50</td>
</tr>
<tr>
<td></td>
<td>wbc† 100 : 0</td>
<td></td>
</tr>
</tbody>
</table>

* rbc - red blood cells
† wbc - white blood cells
TABLE X
Hemopoietic Colony Cytogenetic and G6PD Analysis

<table>
<thead>
<tr>
<th>Patient</th>
<th>Initial Marrow</th>
<th>Long Term Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Karyotype</td>
<td>G6PD</td>
</tr>
<tr>
<td>1</td>
<td>BFU-E*</td>
<td>6 - Ph§</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - N¶</td>
</tr>
<tr>
<td></td>
<td>CPU-C†</td>
<td>4 - Ph</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - N</td>
</tr>
<tr>
<td></td>
<td>Pooled-C‡</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>BFU-E</td>
<td>10 - Ph</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - N</td>
</tr>
<tr>
<td></td>
<td>CPU-C</td>
<td>2 - Ph</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0 - N</td>
</tr>
</tbody>
</table>

* BFU-E = large erythroid colonies.
† CPU-C = granulocyte/macrophage colonies.
‡ Pooled-C = a pool of many small granulocyte colonies.
§ Ph = 46,XX,t(9;22)(q34;q11).
¶ N = 46,XX.
week adherent layer cells from patient 1's cultures. No erythroid colonies were detected in the assays of the 4 and 6 week adherent layer cells of patient 2's cultures.

From the assays of patient 1's long-term cultures, 3 large erythroid colonies, 9 large granulocytic colonies and a pool of many smaller granulocytic colonies were examined cytogenetically. All were found to contain only Ph-positive metaphases (Table X).

From the assays of patient 2's long-term cultures, 11 individual granulocytic colonies were examined cytogenetically. Nine were Ph-positive. Two were Ph-negative. Another 30 individual granulocytic colonies were examined for their G6PD isoenzyme type. Twenty-six contained G6PD variant A, the isoenzyme associated with the malignant CML clone. Four contained G6PD variant B.

4) DISCUSSION

Previous studies have shown that in most cases, Ph-positive hemopoiesis is not maintained in long-term cultures initiated with marrow cells from patients with Ph-positive CML. In contrast, chromosomally normal progenitors, even if initially undetectable, appear to be maintained with kinetics similar to those typical of long-term cultures initiated with normal marrow (12,14). However, whether or not Ph-negative progenitors are detected in long-term CML cultures depends on the extent of dilution of the Ph-negative stem cell population. If the content of Ph-negative stem cells in the initial marrow inoculum is sufficiently small, then it is unlikely that their progeny would be detected in spite of a rapid decline in Ph-positive cells. Examples of such a pattern have been observed previously and are likely explanations for the absence of detectable Ph-negative progenitors in cultures established with
marrow from patient 1, and the relatively low numbers of Ph-negative progenitors detected in cultures established with marrow from patient 2. Both patients had high white blood cell counts at the time of diagnosis, and hence probably also had a greatly expanded pool of Ph-positive progenitors (11). Ph-negative progenitors are also less commonly detected in long-term cultures initiated with cells from treated patients, and again the present findings are consistent with that observation.

Nevertheless G6PD analysis clearly showed that at least some of the progenitors from the patient 2's cultures were not part of the malignant clone as they expressed the G6PD enzyme variant not found in the CML cells. These data confirm previous findings with a patient mosaic for karyotypes 46,XX and 46,X with CML. Ph-negative progenitors detected in 4 to 6 week old long-term marrow cultures were demonstrated to not be part of the CML clone by cytogenetic analysis in this case (14). These results do not eliminate the possibility of a Ph-negative step in the pathogenesis of CML, nor even that clonal Ph-negative cells were present in low numbers in the cultures examined here. However, at least for the 2 patients thus far examined, both the 46,XX/46,X mosaic and patient 2 in the present study, the data suggest that the majority, if not all, Ph-negative cells are not part of the malignant clone.

Convincing evidence of a cytogenetically normal but clonal population in G6PD heterozygotes will depend on analyses of patients or cultures where nonclonal elements are either absent or suppressed. Such a situation has been recently demonstrated in a patient with acute myelogenous leukemia (AML) in remission (18). It has been shown long-term culture may favor the maintenance of phenotypically normal progenitors in AML as well as CML (19). It would be of interest to determine if these apparently normal progenitors selected for
long-term culture are clonal and possibly "premalignant" or truly normal in diseases such as AML.

The unequivocal demonstration of normal hemopoietic precursors in patients with CML and the enrichment for these progenitors in long-term marrow cultures offers possibilities that may be exploited clinically. Selective enrichment of normal progenitors in CML marrow by in vitro treatment with 4 hydroxycyclophosphamide has also recently been reported (20). Further development of in vitro techniques, either separately or in combination, may eventually provide a method of harvesting normal autologous hemopoietic stem cells for bone marrow transplantation in situations where an allogeneic transplant is not possible.
REFERENCES


CHAPTER IV

GENE TRANSFER TO PRIMARY NORMAL AND MALIGNANT HUMAN HEMOPOIETIC PROGENITORS USING RECOMBINANT RETROVIRUSES

1) INTRODUCTION

The delivery of exogenous genetic material into various target cells is important for many experimental and clinical goals. Recombinant retroviruses provide an attractive vehicle for gene transfer for a number of reasons. These include the high efficiency with which they are able to enter cells and integrate into host DNA, their wide target cell range and their lack of toxicity (1). Mammalian bone marrow is a convenient source of primitive cells with high proliferative and self-renewal capacity on which to test the feasibility of using a gene transfer technique to primary cells. A number of workers have already shown that it is possible to introduce a variety of foreign genes into the bone marrow stem cells of mice using recombinant retroviruses (2-7). These authors have shown a high efficiency of gene transfer and the transferred gene has remained stably integrated through serial marrow transplants. Although some expression of the transferred genes has been demonstrated, difficulties in obtaining satisfactory levels of the products of certain genes such as human adenosine deaminase have been encountered (7). In addition, the long-term stability of gene expression has been questioned (5). Nevertheless, it is clear that retroviruses are capable of delivering functional genes into murine hemopoietic progenitors.
Retroviruses have also been used for gene transfer to human cells, although the available data is less extensive than for murine targets (7-12). The enzyme deficiency in hypoxanthine phosphoribosyl transferase (HPRT) negative human fibroblasts and B lymphoblasts has been corrected by infection with recombinant virus containing the HPRT gene (8,9). Similar results have been reported for experiments in which a virus containing the human adenosine deaminase (ADA) gene was used to infect human ADA deficient B lymphocytes (7). Some data exists for cells of the hemopoietic system from experiments in which long-term human marrow cultures were infected with virus containing oncogenes or the human HPRT gene (10,11). Viral replication in the hemopoietic cells in these cultures was documented but the extent to which the gene of interest had been successfully transferred and expressed by primitive blood cell progenitors was not studied. Recently, more direct quantitative data has been published by Hock and Miller (12) demonstrating retroviral-mediated gene transfer to primary human marrow progenitors with recombinant viruses carrying the neo^R or the DHFR^* gene.

The present study was undertaken to investigate and identify variables that may influence the frequency of successful gene transfer to human hemopoietic progenitors. A number of the available viral packaging lines and retroviral vectors have been evaluated using a spectrum of target cell types including an established cell line as well as fresh progenitors of both normal and leukemic origin.

2) MATERIALS AND METHODS

A. Cells and Culture Conditions

Cell lines were cultured in Dulbecco's modified Eagle medium with high glucose (4.5 g/l) and 10% heat inactivated calf serum (for WAM or W2 cells) or
10% fetal calf serum for all other cell types in 5% CO₂ atmosphere at 37°C. The amphotropic retrovirus packaging lines, $Y_A$, PA12 and PA317, and the ecotropic packaging line, $Y_2$, have been previously described (13-16).

Primary human cells were obtained either from consenting adults (CML peripheral blood or normal bone marrow) or from second trimester abortions (fetal liver cells) after approval of the Clinical Screening Committee for Research Involving Human Subjects of the University of British Columbia. Blood and bone marrow cells were passed over a percoll density gradient (density 1.063) and light density cells collected and used in subsequent experiments. Fetal liver was minced with scissors, and incubated for 3 hours at 37°C in α medium with 20% fetal calf serum and collagenase 1 mg/ml (Sigma Chemical Co., St. Louis, MO). The cells were then passed through a succession of needles of decreasing gauge, washed and used for the experiments described.

Primary human cells were grown in α medium supplemented with 20% fetal calf serum and 10% agar-stimulated leukocyte conditioned medium while in suspension culture and in methylcellulose assays as previously described (17, Appendix IV) for assessment of hemopoietic progenitors.

B. Virus Production and Assay

The general strategy for generating high titer retroviral producer cell lines was as outlined by Miller et al (18). The retrovirus packaging line, $Y_2$, was plated at $5 \times 10^5$ cells per 60 mm dish on day 1. On day 2, 10 μg of viral plasmid DNA (pSVXZipNeo or pN2) (17,19) was transfected into the cells by calcium phosphate co-precipitation. These plasmids are both MoMuLV-based vectors which code for the gene for neomycin phosphotransferase ($neo^r$). After 24 hours, the medium was changed and on day 3 the medium which contained neo$^r$ virus (v-neo$^r$) produced by the $Y_2$ cells was removed, centrifuged at 3000 rpm x
5 minutes to remove cells and debris and aliquots used to infect the amphotropic packaging lines. YAM, PA12 or PA317 cells had been plated at $10^5$ cells per 60 mm tissue culture dish the previous day. They were incubated with medium from the transfected Y2 cells containing 8 µg/ml polybrene for 2 hours at 37°C. Then fresh medium was added followed, in 48 hours, by trypsinization, dilution 1:10 and selection for v-neo<sup>r</sup>-producing clones in medium containing the neomycin analogue G418 at 1 mg/ml (Gibco Laboratories, Chagrin Falls, Ohio). G418 was dissolved in distilled water and added to growth medium to achieve the desired final concentration in total mg/ml (the effective drug concentration was approximately 50% of that value for the 2 lots of G418 used). Colonies were isolated by cloning rings, expanded and examined for v-neo<sup>r</sup> titer on 3T3 cells and for amphotropic helper virus using the S<sup>+</sup>L<sup>-</sup> assay (15). The sarcoma virus-containing non-producer (S<sup>+</sup>L<sup>-</sup>) cells were cat CCC-81 cells and the background cells on which the release of transforming virus was scored were rat NRK cells. For amphotropic helper virus assay, $10^4$ CCC-81 cells were plated per 60 mm dish on day 1. On day 2 the medium was aspirated and replaced with test medium containing virus and 4 µg/ml polybrene. On day 3 the medium was replaced with 4 ml medium containing 2 x $10^5$ NRK cells per dish. 4 to 5 days later, foci resulting from rescue of the sarcoma virus present in the CCC-81 cells were counted. Ecotropic viruses do not score in this assay because CCC-81 cat cells are resistant to infection by ecotropic viruses. Ecotropic helper virus was assayed by secondary v-neo<sup>r</sup> titers on 3T3 cells. That is, medium was removed from the original 3T3 viral assays and reassayed on new 3T3 cells for v-neo<sup>r</sup>.

C. **Viral Infection**

The general protocol for viral infection is illustrated in Figure 11.
INFECTION OF HUMAN HEMPOIETIC CELLS WITH V-NEO\textsuperscript{r}

\begin{align*}
\text{LTR} &-\text{neo}^\text{r}-\text{LTR} \quad \text{pN2 or pZip neo} \\
+ &\quad \text{retroviral packaging cell line} \\
\quad &\quad (\psi AM, PA12 or PA317) \\
\downarrow &\quad \text{X-rays} \\
\quad &\quad \text{viral producer lines} \\
&\quad + \\
\downarrow &\quad \text{K562} \\
&\quad \text{or} \\
&\quad \text{normal marrow} \\
&\quad \text{or} \\
&\quad \text{fetal liver} \\
&\quad \text{or} \\
&\quad \text{CML blood} \\
\downarrow &\quad 24\text{h at }37^\circ\text{C} \\
\downarrow &\quad \text{suspension culture} \\
\quad &\quad \times 48\text{h} \\
\downarrow &\quad \text{methylcellulose assay} \\
+ &\quad \text{G418 2mg/ml}
\end{align*}

FIGURE 11. Infection of Human Hemopoietic Cells with \(v\)-neo\textsuperscript{r}.

Viral producer lines were produced by transfecting \(\text{neo}^\text{r}\) plasmid DNA into viral packaging lines. Irradiated producer cells were used to infect human target cells (K562, normal marrow, fetal liver or CML blood cells). Target cells were incubated on viral producer cells for 24 hr (in some experiments the infection was done with viral culture supernates for 2 hr at 37\(^{\circ}\)C) and then placed in suspension culture for 48 hr to allow integration and expression of the viral genome. Target cells were then placed in methylcellulose assay with or without G418 2 mg/ml and colonies scored 14 to 18 days later.
The K562 human leukemic cell line or primary cells were infected with v-neore by either co-cultivation with amphotropic viral producer cells which had received 1500R irradiation or incubation in supernate from viral producer cells with 8 μg/ml polybrene for various periods of time. After the infection period they were maintained in suspension culture for 24 to 48 hours before plating in methylcellulose assay with or without G418. Control cultures that were not exposed to virus were grown in suspension culture and plated with or without G418 at the same time as the infected cells. Colonies were scored after plating in methylcellulose on day 5 to 7 for K562 and day 10-14 for granulocyte macrophage colonies (from CFU-GM) and day 18-21 for large erythroid colonies (from BFU-E) for primary progenitor assays. Colonies were not scored unless they contained at least 30 cells and, in the case of BFU-E, had at least 3 clusters and were clearly hemoglobinized.

The infectious center assay was done by plucking individual G418 resistant (G418r) granulocyte-macrophage colonies from methylcellulose assay and placing the dispersed cells from one colony in a 2 cm² tissue culture well containing 10⁶ NIH-3T3 cells in medium with 8 μg/ml polybrene. After overnight incubation, the medium was replaced with fresh medium containing G418 1 mg/ml. Seven days later the assay was scored for the presence of G418r 3T3 cells.

Cell and viral manipulations and cultures were performed under Level C containment following Medical Research Council of Canada guidelines for handling retroviruses and human samples.

D. DNA and RNA Studies

High molecular weight DNA was harvested from expanded clones of K562 cells and pooled hemopoietic colonies by cell lysis with 0.2% SDS and
proteinase K digestion overnight at 37°C followed by phenol and chloroform extractions. Southern blotting of K562 and primary hemopoietic colony DNA to nitrocellulose filters was performed by digestion of 10 μg of total cellular DNA with the appropriate restriction endonuclease following the manufacturer's recommendations. After agarose gel electrophoresis the DNA was denatured by soaking the gel in 0.5 M NaOH, 1.5 M NaCl 2 x 30 minutes followed by 1 M NaCl, 0.5 M TrisHCl pH 7.4 2 x 30 minutes to neutralize. DNA was transferred to nitrocellulose by Southern blotting with 6 x SSC (0.9 M NaCl, 0.09 M Na₃ citrate) overnight and the filters baked in a vacuum oven for 2 hours at 80°C (20).

In order to detect neo\textsuperscript{F} sequences, a 2.3 kb neo\textsuperscript{F} specific Bam HI, Hind III fragment isolated from RSV-neo plasmid DNA was \textsuperscript{32}P-labeled by nick translation (Bethesda Research Laboratories, Gaithersberg, MD, kit) for use as a probe. Filters were prehybridized, hybridized in 3 x SSC, 4 x Denhardt's solution (.08% Ficoll, .08% BSA, .08% polyvinylpyrolidine) and sonicated, denatured salmon sperm DNA 100 μg/ml at 68°C for 8 hours and hybridized under the same conditions with 3.3 x 10\textsuperscript{6} cpm/ml of, heat-denatured \textsuperscript{32}P-labelled probe (specific activity 5 x 10\textsuperscript{7} cpm/μg) for 20 hours. Hybridized filters were washed for 30 minutes each time in 0.1 x SSC, 0.1% SDS, 0.1% sodium pyrophosphate at 55°C x 3 and 65°C x 1.

Total cellular RNA was harvested by the method of Meinkoth and Wahl (21) from K562 or from primary hemopoietic colonies that had been individually plucked and pooled from methylcellulose assays. 1-5 x 10\textsuperscript{6} cells were resuspended in 45 μl ice cold 10 mM Tris-HCl (pH 7.0), 1 mM EDTA, with 10 mM vanadyl nucleoside as a ribonuclease inhibitor. Cells were lysed by addition of 5 μl 5% Nonidet P40, incubation on ice 5 minutes, addition of a further 5 μl 5% Nonidet P40 and a further 5 minutes on ice. The mixture was spun at
15,000 g for 2.5 minutes to remove nuclei and then extracted once with phenol and once with chloroform. 50 μl of supernatant was added to 30 μl 20 x SSC (3 M NaCl, 0.3 M Na₃ citrate, pH 7.0) and 20 μl 37% formaldehyde. The mixture was incubated at 60°C for 15 minutes to denature RNA. Serial dilutions were performed in 15 x SSC and the samples applied to nitrocellulose filters through a spot blot vacuum manifold (Schleicher and Schuell, Inc., Keen, NH). The filters were dried and then baked in a vacuum oven at 80°C for 1 hour. The spot blot filters were prehybridized and hybridized at 42°C in 50% deionized formamide, 5 x Denhardt's solution (0.1% Ficoll, 0.1% BSA, 0.1% polyvinylpyrolidone), 0.1% SDS, 100 μg/ml heat-denatured salmon sperm DNA and 5 x SSC (0.75 M NaCl, 0.075 M Na₃ citrate, pH 7.0). Prehybridization was done for 8 hours followed by hybridization overnight with 2 x 10⁶ cpm/ml of ³²P-labeled, nick translated probe as for Southern blots. Spot blot filters were washed at room temperature, 4 x 15 minutes in 2 x SSC, 0.1% SDS and 2 x 5 minutes in 0.1 x SSC, 0.1% SDS.

Filters were auto-radiographed at -70°C with the use of intensifying screens (20).

3. RESULTS

A. Viral Producer Cells

Many attempts were made to obtain the highest possible v-neo⁵ titer from various combinations of packaging lines and either the ZipNeo or the N2 vector (Table XI). A large number of clones were tested for both recombinant V-neo⁵ titers and helper virus. The neo⁵ titer from these clones varied over a range of 2 logs. For the ecotropic packaging lines, Y2, the highest V-neo⁵ titer amongst 40 clones was 7 x 10⁵ cfu/ml using the ZipNeo plasmid. At least half of the Y2 clones were positive for helper virus as judged by secondary
TABLE XI
Titers of v-neoR From Producer Cell Lines and pZipNeo

<table>
<thead>
<tr>
<th>Type</th>
<th># Clones Tested</th>
<th>$\bar{x}$ Titer (cfu/ml)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Psi$2 ecotropic +/-helper</td>
<td>40</td>
<td>$1.3 \times 10^5$</td>
<td>$&lt;1 \times 10^3 - 7 \times 10^5$</td>
</tr>
<tr>
<td>PA12 amphotropic + helper</td>
<td>21</td>
<td>$3.1 \times 10^4$</td>
<td>$&lt;1 \times 10^3 - 3 \times 10^5$</td>
</tr>
<tr>
<td>YAM amphotropic + helper - helper</td>
<td>29</td>
<td>$2 \times 10^3$</td>
<td>$&lt;1 \times 10^2 - 3 \times 10^4$</td>
</tr>
</tbody>
</table>

Titers of v-neoR From pN2

<table>
<thead>
<tr>
<th>Type</th>
<th>Viral Titer (cfu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA12 amphotropic + helper</td>
<td>$4 \times 10^6$</td>
</tr>
<tr>
<td>PA317 amphotropic - helper</td>
<td>$5 \times 10^5$</td>
</tr>
</tbody>
</table>
infection of 3T3 cells by supernatant from primary viral assays. For the combination of YAM cells and pZipNeo, the highest titer of helper-free virus obtained after screening 61 clones was $3 \times 10^4$ cfu/ml. With PA12 and pZipNeo, several of the 21 clones studied produced approximately $10^5$ cfu/ml but these were found to produce helper virus as well. With the combination of PA12 and the N2 vector, higher titers of v-neo$^\varepsilon$ ($4 \times 10^6$ cfu/ml) were obtained, but these also contained helper virus. The highest titers of helper-free v-neo$^\varepsilon$ ($5 \times 10^5$ cfu/ml) were obtained from the PA317 packaging cell line containing the N2 proviral DNA.

B. K562 Experiments

To optimize conditions for high frequency retroviral infection of human hemopoietic progenitors, we used K562 cells as a convenient model for experiments in which a number of variables were explored. Figure 12a shows the frequencies of G418$^\varepsilon$ K562 colonies obtained after exposure of $10^5$ K562 cells to various numbers of irradiated viral producer cells of different types plated in a 60 mm dish. The G418 concentration of 1 mg/ml completely inhibited all colony growth in control plates. The transformation efficiency increased as the number of producer cells increased from $10^2$ to $10^5$. Beyond this level, the producer cells began to approach confluence and the frequency appears to plateau at 1% for PA12/ZipNeo, 40% for PA317/N2 and 60% for PA12/N2. The higher the titer of v-neo$^\varepsilon$ produced by the cells, the higher was the frequency of G418$^\varepsilon$ K562 transformants obtained.

Figure 12b is a graph of similar data for infection of $10^5$ K562 cells after incubation with 3 ml of various dilutions of supernatant from cultures of v-neo$^\varepsilon$ producer cells. For the PA12/ZipNeo clone producing virus at $1 \times 10^5$ cfu/ml, there was a clear correlation between dilution of viral
FIGURE 12. G418^R K562 colonies (% of colonies grown without G418) after infection of 10^5 K562 cells with various sources of v-neo^R:

- PA12/N2 cell line with titer 4 x 10^6 cfu/ml, + helper virus;
- PA317/N2 cell line with titer 5 x 10^5 cfu/ml, helper-free;
- PA12/ZipNeo cell line with titer 1 x 10^5 cfu/ml, + helper virus;

a) co-cultivation for 24 hours with various numbers of irradiated v-neo^R producer cells.
b) incubation x 2 hours with 3 ml of v-neo^R containing supernatant at various dilutions.
supernatant and frequency of G418\(^{r}\) K562 cells, to a maximum of 0.25\%. For higher titer virus the frequency appeared to plateau at 1:10 dilutions and declined somewhat with undiluted supernatant. Maximum G418\(^{r}\) K562 cell frequencies were 20\% for PA317/N2 supernatant and 60\% for PA12/N2 supernatant.

Figure 13 shows the frequency of G418\(^{r}\) K562 colonies obtained by infection with undiluted v-neo\(^{r}\)-containing medium or co-cultivation with confluent cells of various types. The cell lines selected were those producing the highest v-neo\(^{r}\) titer for the various packaging line/neo\(^{r}\) plasmid combinations. At viral titers \(\leq 10^5\) cfu/ml the maximum frequency of G418\(^{r}\) K562 cells was approximately 2\% after co-cultivation with producer cells. Frequencies obtained after infection with supernatant from the same cell lines was at least 4-fold lower than that obtained by co-cultivation. At the highest titer, \(4 \times 10^6\) cfu/ml, the difference in efficiency between co-cultivation and supernatant disappeared.

A number of other variables that were investigated did not appear to affect the frequency of K562 cell transformation to G418\(^{r}\). These included extending the duration of co-cultivation from 6 to 48 hours, or extending the duration of exposure to supernatant from 2 to 48 hours.

The neo\(^{r}\) gene was demonstrated in G418\(^{r}\) K562 cell DNA by expanding individual colonies in liquid culture and extracting cellular DNA for Southern blots. Figure 14a demonstrates the 4 kb neo\(^{r}\) specific Xbal fragment found in 4 G418\(^{r}\) K562 colonies but not found in control K562 DNA. Figure 14b shows DNA from the same 4 G418\(^{r}\) K562 colonies and 2 uninfected control K562 colonies cut with enzymes Bam H1 (lanes 1-6) or EcoR1 (lanes 7-12) which cut only once within the neo\(^{r}\) proviral DNA. The neo\(^{r}\) specific fragments demonstrated in the G418\(^{r}\) colonies (lane 1-4 and 7-10) are single and unique to each colony indicating one random site of integration for the provirus in the cellular DNA from each K562 colony.
FIGURE 13. G418$^R$ K562 colonies (% of colonies grown without G418) after infection of $10^5$ cells with v-neo$^R$ from 3 ml undiluted supernatant or a 60 mm dish of confluent, irradiated viral producer cells of various types.

- supernatant  
- co-cultivation
FIGURE 14. Southern blots of total cellular DNA hybridized with a $^{32}$P-labeled neo$^R$ specific Bam HI-Hind III fragment from pRSV-neo.

a) K562 DNA from cloned G418$^R$ cells infected with YAM/ZipNeo viral stocks (lanes 1-4) or uninfected (lane 5) and digested with XbaI.

* 4kb neo$^R$ specific fragment in infected cells.

b) DNA from the same G418$^R$ K562 clones as in (a) (lanes 1-4 and 7-10) and uninfected clones (lanes 5, 6, 11, 12) digested with Bam HI (lanes 1-6), or EcoRI (lanes 7-12). These enzymes cut once within proviral DNA and once within cellular DNA to generate a single neo$^R$ specific fragment of unique size in each of the G418$^R$ clones.

c) DNA from pooled primary hemopoietic colonies either infected with PA12/N2 viral stocks (lanes 2, 3, 5) or uninfected (lanes 1, 6, 7) and digested with EcoRI. The expected 1.5 kb neo$^R$ specific fragment is seen in lane 2 (cells infected with viral supernatant but not selected in G418), lane 3 (G418$^R$ cells after infection with supernatant), and lane 5 (cells infected by co-cultivation with producer cells but not selected in G418). Lanes 4 shows no neo$^R$ specific hybridization from cells exposed to supernatant from PA317/N2 producer cells but not selected in G418.
C. Infections of Primary Hemopoietic Progenitors

A total of 4 normal marrow, 6 fetal liver and 6 CML blood samples were infected with v-neoRF from a variety of packaging cell line/neorF plasmid combinations. The infection procedure did not appear to be toxic to the target cells as shown by a lack of any effect on cell recovery or plating efficiency. (Values for infected cells were 80 to 100% of control values). A G418 concentration of 2 mg/ml (effective drug concentration 1 mg/ml) completely inhibited all colony growth in assays of uninfected primary progenitors. No difference in G418 sensitivity between the various target cell sources or between progenitors of different lineages (i.e. BFU-E and CFU-GM) could be demonstrated.

Figure 15a shows the frequency of G418RF CFU-GM seen after infection by v-neoRF producer cells at various viral titers. Each point represents a different experiment combining target cells of a certain type with a specific viral producer cell line. Although there was a correlation between viral titer and frequency of G418RF, considerable variation in transformation efficiency at any given titer was also found; for example, from 2.4 to 15.7% G418RF CFU-GM at a titer of 4x10^6 cfu/ml. There was also a low (0.045 to 0.4%), but reproducible incidence of G418RF colonies after infection with cells producing viral titers as low as 10^3 cfu/ml.

Figure 15b shows similar data for BFU-E. Infections with cells producing a v-neoRF titer of 1 x 10^3 cfu/ml, yielded a frequency of large G418RF erythroid colonies of less than 1% while frequencies up to 5.6% were seen after infections done with cell lines producing higher titers. However, the correlation of G418RF frequency with titer was less clear and the variation in G418RF frequency at any given titer was similar to that seen for CFU-GM. From these data (Figures 15a & 15b), it appears that BFU-E may be less readily
FIGURE 15. Frequency (%) of G418\textsuperscript{r} primary hemopoietic colonies (G418\textsuperscript{r} colonies/total colonies without G418 x 100) after 24 hours exposure of 5 x 10\textsuperscript{6} cells to a 60 mm dish of irradiated, confluent v-neo\textsuperscript{r} producer cell lines at various viral titers.

- normal bone marrow,  O CML blood,  ▲ fetal liver

a) granulocyte, macrophage colonies; CFU-GM
b) large erythroid colonies; BFU-E

Correlation (r) of log\textsubscript{10} frequency G418\textsuperscript{r} colonies vs. log\textsubscript{10} viral titer is 0.71 (p<.001) for figure a and 0.64 (p<.01) for figure b.
transformed by v-neo\textsuperscript{R} than CFU-GM. Although this may be true, it is possible that the apparent difference is the result of experimental variables which particularly affect erythroid colony growth. For example, it was found that in general, G418\textsuperscript{R} erythroid colonies were not as large or as red as those obtained from infected BFU-E plated in the absence of G418. It is possible that impurities in the G418 affect the growth of erythroid colonies containing the neo\textsuperscript{R} gene. Alternatively, the neo\textsuperscript{R} gene may be expressed at a lower level in erythroid cells as they differentiate. Therefore, it is likely that the frequencies recorded here for G418\textsuperscript{R} BFU-E represent minimum estimates.

The maximum frequencies of G418\textsuperscript{R} progenitors from these experiments are summarized in Table XII. The highest frequency was usually obtained after infection with the cell line producing the highest viral titer, PA12/N2 cells producing v-neo\textsuperscript{R} at 4 x 10\textsuperscript{6} cfu/ml in addition to helper virus.

The ability of viral supernatant, as compared to co-cultivation with producer cells of various types, to transform primary progenitors to G418\textsuperscript{R} is compared in Figure 16. At a titer of 6 x 10\textsuperscript{3} cfu/ml produced by YAM/ZipNeo cells, supernatant was unable to produce a significant incidence of G418\textsuperscript{R} colonies while co-cultivation yielded a frequency of approximately 0.1%. At titers of 5 x 10\textsuperscript{5} or 4 x 10\textsuperscript{6} cfu/ml, supernatant was effective at transforming both BFU-E and CFU-GM. However, the frequency was still 2 to 50 fold higher using co-cultivation. Nevertheless, the frequency of G418\textsuperscript{R} colonies was higher after infection with medium at high titers (10.6\% for CFU-GM at 4 x 10\textsuperscript{6} cfu/ml) than with co-cultivation at low titers (0.13\% CFU-GM at 6 x 10\textsuperscript{3} cfu/ml).

The frequencies of transformation for primary progenitors were not as high as those obtained for K562 cells. To try and increase the efficiency, target cells were allowed to remain on viral producer cells for as long as 7 days without any increase in the efficiency of transformation.
### TABLE XII

**Maximum Frequency of G418<sup>F</sup> 1<sup>o</sup> Hemopoietic Colonies after Co-Cultivation with Various v-neo<sup>F</sup> Producer Cells**

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Viral Producer Cells</th>
<th>v-neo&lt;sup&gt;F&lt;/sup&gt; Titer (cfu/ml)</th>
<th>G418&lt;sup&gt;F&lt;/sup&gt; Colonies/Colonies Without G418 (%)</th>
<th>CFU-GM</th>
<th>BFU-E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+ Virus</td>
<td>Control**</td>
</tr>
<tr>
<td>Normal Marrow</td>
<td>YAM/ZipNeo</td>
<td>3 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>26/2775 (0.94)</td>
<td>0/2970</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PA317/N2</td>
<td>5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>78/1860 (4.2)</td>
<td>0/630</td>
<td>85/3930 (2.2)</td>
</tr>
<tr>
<td></td>
<td>PA12/N2*</td>
<td>4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>127/1800 (7.1)</td>
<td>0/630</td>
<td>101/4260 (2.4)</td>
</tr>
<tr>
<td>Fetal Liver</td>
<td>YAM/ZipNeo</td>
<td>3 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>24/2960 (0.81)</td>
<td>0/3996</td>
<td>54/3333 (1.6)</td>
</tr>
<tr>
<td></td>
<td>PA317/N2</td>
<td>5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2/158 (1.3)</td>
<td>0/150</td>
<td>1/105 (1.0)</td>
</tr>
<tr>
<td></td>
<td>PA12/N2*</td>
<td>4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>13/414 (3.1)</td>
<td>0/150</td>
<td>2/690 (0.31)</td>
</tr>
<tr>
<td>CML Blood</td>
<td>YAM/ZipNeo</td>
<td>6 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>48/9900 (0.48)</td>
<td>0/6600</td>
<td>18/14320 (0.13)</td>
</tr>
<tr>
<td></td>
<td>PA317/N2</td>
<td>5 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>66/3120 (2.1)</td>
<td>0/3888</td>
<td>194/3480 (5.6)</td>
</tr>
<tr>
<td></td>
<td>PA12/N2*</td>
<td>4 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>656/4170 (15.7)</td>
<td>0/16920</td>
<td>1672/31650 (5.3)</td>
</tr>
</tbody>
</table>

* + Helper virus

** Control = Cells from the same sample plated in methylcellulose + G418 without exposure to v-neo<sup>F</sup> producer cells.
FIGURE 16. Frequency (%) of G418R primary hematopoietic progenitors (G418R colonies/total colonies without G418 x 100) after infection of 5 x 10^6 cells by v-neoR from a 60 mm dish of irradiated confluent producer cells or 5 ml undiluted supernatant of various types. All infections were done with CML progenitors except at titer of 3 x 10^4 cfu/ml where the target was fetal liver cells.

<table>
<thead>
<tr>
<th>cell lines/plasmid</th>
<th>viral titer (cfu/ml)</th>
<th>frequency (%) G418R progenitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA 317/N2</td>
<td>5 x 10^5</td>
<td>1</td>
</tr>
<tr>
<td>PA 12/N2</td>
<td>4 x 10^6 (+helper)</td>
<td>10</td>
</tr>
<tr>
<td>PA 317/N2</td>
<td>3 x 10^4</td>
<td>0.1</td>
</tr>
<tr>
<td>PA 12/N2</td>
<td>6 x 10^3</td>
<td>0.01</td>
</tr>
<tr>
<td>^AM/Zip neo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>^AM/Zip neo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BFU-E</td>
<td>super-natant</td>
<td>confluent producer cells</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>super-natant</td>
<td>confluent producer cells</td>
</tr>
</tbody>
</table>
The ability of G418<sup>r</sup> CFU-GM that had been infected with helper-containing v-neo<sup>r</sup> to infect 3T3 cells was tested by infectious center assay. In two experiments, 5 of 22 and 24 of 24 CFU-GM transformed 3T3 cells to G418 resistance (Table XIII). The production of G418<sup>r</sup> 3T3 in this assay requires that the infecting cells be producing v-neo<sup>r</sup>. To do so they, themselves, must have been infected by both v-neo<sup>r</sup> and helper virus, which may occur relatively infrequently. This would account for less than 100% of G418<sup>v</sup> CFU-GM being positive in the infectious center assay.

Figure 14c is a Southern blot of DNA harvested from pooled hemopoietic colonies grown from CML blood cells that were either uninfected or exposed to viral stocks containing v-neo<sup>r</sup>. After digestion with EcoR1 the neo<sup>r</sup> specific probe identified the expected 1.5 kb neo<sup>r</sup> specific fragment in G418<sup>r</sup> cells (lane 3). A less intense signal is seen in lane 2 where the same cells were exposed to medium containing v-neo<sup>r</sup> but not selected in G418 indicating that some of the cells did not contain the neo<sup>r</sup> gene, (8% of the CFU-GM in this sample were G418<sup>r</sup>). In lane 5 the DNA from cells exposed to viral producer cells but not selected in G418 shows a strong signal. In colony assays 15.7% of the CFU-GM in this sample were G418<sup>r</sup>. The strength of the hybridization signal in lane 5 as compared to lane 3 where an equivalent amount of DNA was hybridized indicates that at least 15.7% of the cells in this sample contained the neo<sup>r</sup> gene. DNA from cells from the same sample that were not exposed to virus, in lanes 1, 6 and 7 show no signal.

Expression of the neo<sup>r</sup> gene by v-neo<sup>r</sup> infected, pooled primary progenitors or K562 cells, was shown by RNA spot blot (Figure 17). Total cellular RNA was hybridized with a neo<sup>r</sup> specific probe. Uninfected, control cells show no evidence of neo<sup>r</sup> hybridization, while the same number of infected cells show a strong signal. The signal is strongest for cells that
TABLE XIII
Demonstration of v-neo<sup>R</sup> Production in G418<sup>R</sup> CFU-GM by Infectious Center Assay

<table>
<thead>
<tr>
<th>Experiment</th>
<th>+ ve CFU-GM/Total Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5/22</td>
</tr>
<tr>
<td>2</td>
<td>24/24</td>
</tr>
</tbody>
</table>
FIGURE 17. RNA spot blot of total cellular RNA from pooled CML granulocyte-macrophage colonies or K562 cells hybridized with a $^{32}$P-labeled neo$^+$ specific Bam HI-Hind III fragment from pRsv-neo. Control cells were not exposed to v-neo$^+$ or G418. Cells exposed to either PA12/N2 or PA317/N2 v-neo$^+$ producer cells were either selected in G418 (b) or unselected (a) in the case of CFU-GM. Insufficient cells were available to allow samples for the PA317/N2, $4 \times 10^5$ cells column. Therefore, these two spots are blank. All infected K562 cells were selected in G418. pZipNeo DNA serves as a positive control in the top lane (2 and 0.5 pg DNA from left to right).
were selected in G418 (lanes b, CFU-GM and all the infected K562 cells) but is also seen in cells that were infected but not selected (lanes a, CFU-GM).

3) DISCUSSION

These data demonstrate efficient transfer and expression of the neo<sup>F</sup> gene to both the K562 human leukemic cell line and a variety of normal and malignant primary human hemopoietic progenitors using recombinant retroviruses.

A number of variables were investigated to try to achieve the highest possible efficiencies of gene transfer. Co-cultivation with viral producer cells rather than infection with viral supernatant and use of high titer virus were both identified as important parameters, but viral titer clearly had the greatest influence on the frequency of transformation to G418 resistance. Considerable effort was devoted to studying viral producer cells generated by various packaging line/neo<sup>F</sup>-containing plasmid combinations. The choice of these variables clearly had a major impact on the titer of both v-neo<sup>F</sup> and helper virus. We were unable to generate high titer v-neo<sup>F</sup> from the PA12 packaging line that was not associated with helper virus. The use of the pN2 vector rather than ZipNeo raised the v-neo<sup>F</sup> titer but did not eliminate the problem of helper virus. Although the PA12 line has been constructed to generate helper-free recombinant virus, it appears that sufficient genetic recombination occurs between the defective packaging and recombinant neo<sup>F</sup> viral sequences to frequently generate significant helper virus titers with the vector used in this study. Helper-free v-neo<sup>F</sup> generated by the YAM packaging line containing pZipNeo DNA, was always of relatively low titer (<10<sup>5</sup> cfu/ml). The newer generation of retroviral packaging lines, such as the PA317 developed by Miller and Buttimore (16), may solve the problem of obtaining high titer recombinant virus without associated helper.
Although both ZipNeo and N2 contain the same neo\textsuperscript{r} gene under the control of the promoter in a Moloney leukemia virus LTR the two vectors are not identical in structure (6,20). Although the differences appear subtle they seem to affect the viral titers obtained from producer cells and might also cause different levels of expression of the transferred foreign gene in infected hemopoietic cells. For this reason we elected to test virus derived from both the N2 and ZipNeo vectors on our target cells. Although our data are not definitive, some of our results (e.g. Figure 13 where K562 were the target cells and a 5 fold increase in viral titer lead to a 20 fold increase in frequency of G418\textsuperscript{r} colonies) may be explained if neo\textsuperscript{r} was expressed more efficiently in cells infected with virus derived from N2 rather than ZipNeo.

In experiments done with helper-free virus, from a producer line derived from PA317 cells and the N2 plasmid, we were successful in demonstrating gene transfer at levels only slightly lower than those achieved with helper containing virus of significantly higher v-neo\textsuperscript{r} titer. No obvious effect of helper virus on the frequency of gene transfer was detected.

In addition to normal bone marrow, we evaluated two alternative sources of primary human progenitors for retroviral infections. These were fetal liver and CML blood. In both cases, a high proportion of the primitive progenitors in these tissues are in the active part of the cell cycle as compared to their counterparts in normal marrow which are largely quiescent (22,23). Cell cycling status has been suggested as an important variable affecting the success of retroviral infection and integration into host DNA (24). Although some of our highest frequencies of G418\textsuperscript{r} colonies were seen in the CML blood cultures, there was sufficient variability from experiment to experiment that no overall difference was evident. However, there was clearly no dramatic improvement in the frequency of G418\textsuperscript{r} progenitors in experiments
using either of the primary human targets that were presumably cycling optimally.

Although encouraging, the frequency of gene transfer demonstrated to primary progenitors by these data is still much lower than the theoretical 100% that should be obtainable with retroviruses (1). Our frequencies are reasonably close to those reported by Hock and Miller for gene transfer to primary normal human hemopoietic progenitors from bone marrow (12), but are much lower than the 75 to 100% frequency reported by Eglitis et al for murine CFU-S (6). However, it is notable that the latter authors and others have found the level of expression of G418 resistance or neo\(^r\) gene product to be highly variable among different CFU-S containing the neo\(^r\) gene (4,5,6). The frequencies of G418 resistance in murine CFU-GM infected and selected in vitro have been approximately 10 to 30% (4,5) which is also lower than one might have predicted and consistent with our results on human progenitors.

A large number of techniques exist for transporting genetic material into cells. A number of these have been applied to primary hemopoietic progenitors including calcium phosphate co-precipitation and electroporation (25-27). Although initial results using the former technique to confer methotrexate resistance on murine bone marrow cells appeared promising, similar results have not been forthcoming for other genes or for human cells. Electroporation is a relatively new technique that has not been widely applied to gene transfer into primary cells. Initial results with human bone marrow progenitors demonstrate low levels of transfer and expression of the xanthine-guanine phosphoribosyl transferase gene in CFU-GM (27). The full potential of this technique remains to be explored. At the present time the bulk of evidence would indicate that the highest efficiencies of gene transfer are those mediated by retroviruses. Where high frequencies of transformation are
necessary and appropriate vectors are available, these viruses appear to be the current method of choice for transporting genes into cells.

The knowledge gained from the present studies should facilitate the successful use of the technique of retroviral-mediated gene transfer to study genetic factors important in normal and malignant hemopoiesis. In addition, there are a number of inherited human hematologic disorders that are known to have a molecular genetic etiology. Some of these may ultimately be curable by gene replacement therapy, possibly with retroviruses providing the means of transporting the normal gene into primitive hemopoietic progenitors (28).
REFERENCES


27. Toneguzzo F, Keating A. Stable expression of selectable genes introduced into human hematopoietic stem cells by electric field mediated DNA transfer. Proc Natl Acad Sci USA (in press).

1) **INTRODUCTION**

The long-term bone marrow culture system as developed by Dexter et al (1) for murine cells and modified by Greenberger (2) and others (3) for human cells provides an opportunity to study hemopoiesis in vitro over a period of several months. The cellular interactions and regulation that takes place in these cultures appear to mimic in many ways events that occur in vivo in the bone marrow microenvironment. One application of human long-term marrow cultures to the study of hemopoiesis is as a system in which to investigate techniques in vitro although their use in vivo is not yet possible. For example, retroviral-mediated gene transfer to hemopoietic progenitors has potential for use in the therapy of inherited genetic disorders (4). However, prior to in vivo experiments in humans, the use of these viral vectors require extensive testing, not only in animals, but in in vitro human systems. Such systems should assess the frequency and stability of gene transfer and the levels of expression of the transferred gene. The long-term marrow culture provides the opportunity to assess virally-infected hemopoietic cells over time for these features. In addition, experiments designed to study the importance of a particular gene in normal or abnormal hemopoiesis can be performed using retroviral-mediated gene transfer to hemopoietic cells in long-term cultures. Candidate genes include the various oncogenes.
A number of investigators have infected murine and human long-term marrow cultures with acutely transforming retroviruses containing oncogenes (5-8). Among the most interesting results are those reported by Dexter et al in which virus containing the v-src oncogene was used to infect murine cultures (7). Infected cultures showed increased progenitor numbers in the non-adherent cell fraction and spleen colony forming cells (CFU-S) from these cultures showed increased ability to serially repopulate the hemopoietic system of irradiated recipient animals. A number of immortal, non-leukemogenic factor dependent cell lines were derived from these cultures (9). Although it is not clear what role the v-src oncogene is playing in the etiology of these phenomena, these events are clearly of interest to those investigating the regulation of hemopoiesis particularly if they can be demonstrated to occur in human systems.

Retroviral-mediated transfer of the neo\(^r\) gene has recently been demonstrated to human hemopoietic cells in short-term methylcellulose assays (10). The experiments described in this paper describe attempts to extend that experience by using human hemopoietic cells in long-term culture as a target for transfer of the neo\(^r\) gene. Experiments in which retrovirus containing the v-src oncogene were used as the infecting vector are also described.

2) MATERIALS AND METHODS
A. Cells and Culture Conditions

Cell lines were cultured in Dulbecco's modified Eagle medium with high glucose (4.5 g/l) and 10% heat inactivated calf serum (for \(\Psi 2\) cells) or 10% fetal calf serum for all other cell types in 5% CO\(_2\) atmosphere at 37°C. The amphotropic retrovirus packaging line, PA12, the ecotropic packaging line, \(\Psi 2\),
and the 2-1-292 cell line producing amphotropic v-src-containing retrovirus have been previously described (11,12,13).

Primary human cells were obtained either from consenting adults (CML peripheral blood, preleukemic bone marrow, or skin fibroblasts) or from second trimester abortions (fetal liver cells) after approval of the Clinical Screening Committee for Research Involving Human Subjects of the University of British Columbia. Blood and some bone marrow cells were passed over a Percoll density gradient (density 1.063) and light density cells collected and used in subsequent experiments. Fetal liver was minced with scissors, and incubated for 3 hours at 37°C in α medium with 20% fetal calf serum and collagenase 1 mg/ml (Sigma Chemical Co., St. Louis, MO). The cells were then passed through a succession of needles of decreasing gauge, washed and used for the experiments described. Skin samples were obtained by 3 mm punch biopsy, minced with scissors and allowed to adhere to plastic dishes. After growth in α medium with 20% fetal calf serum for 3 weeks, fibroblasts were trypsinized and split into several dishes for the experiments described below.

Human long-term marrow cultures and methylcellulose assays for hemopoietic progenitors were established with marrow buffy coat cells as previously described (14,15, Appendices III, IV). Reconstituted long-term cultures were established using bone marrow fibroblast feeders and peripheral blood or marrow Percoll-separated light density cells. The bone marrow fibroblast feeders were produced by placing marrow buffy coat cells into tissue culture dishes in α medium with 20% fetal calf serum. After 3 weeks, with weekly total medium changes, a confluent adherent layer comprised primarily of spindle-shaped fibroblast-like cells was present. This was trypsinized and split into several dishes before use. Confluent bone marrow fibroblast feeders in 60 mm tissue culture dishes were inoculated with
approximately $2 \times 10^7$ light density marrow or blood cells in 8 ml long-term culture medium to create a reconstituted long-term culture.

**B. Virus Production and Assay**

The general strategy for generating high titer retroviral producer cell lines was as outlined by Miller et al (16). The retrovirus packaging line Y2 was plated at $5 \times 10^5$ cells per 60 mm dish on day 1. On day 2, 10 µg of viral plasmid DNA (pN2) (17) was transfected into the cells by calcium phosphate co-precipitation. This plasmid is a MoMuLV-based vector which codes for the gene for neomycin phosphotransferase (neo<sup>R</sup>). After 24 hours, the medium was changed and on day 3 the medium which contained neo<sup>R</sup> virus (v-neo<sup>R</sup>) produced by the Y2 cells was removed, centrifuged at 3000 rpm x 5 minutes to remove cells and debris and aliquots used to infect the amphotropic packaging line. PA12 cells had been plated at $10^5$ cells per 60 mm tissue culture dish the previous day. They were incubated with medium from the transfected Y2 cells containing 8 µg/ml polybrene for 2 hours at 37°C. Then fresh medium was added, followed in 48 hours by trypsinization, dilution 1:10 and selection for v-neo<sup>R</sup>-producing clones in medium containing the neomycin analogue G418 at 1 mg/ml (Gibco Laboratories, Chagrin Falls, OH). G418 was dissolved in distilled water and added to growth medium to achieve the desired final concentration in total mg/ml (the effective drug concentration was approximately 50% of that value for the 2 lots of G418 used). Colonies were isolated by cloning rings, expanded and examined for v-neo<sup>R</sup> titer on 3T3 cells and for amphotropic helper virus using the S<sup>+</sup>L<sup>-</sup> assay (16).

The 2-1-292 cell line producing amphotropic v-src-containing virus was a gift from Dr. S. Anderson. The titer of this virus was approximately $5 \times 10^5$ ffu/ml of medium conditioned by producer cells assayed for the ability to induce transformed foci on 3T3 cells.
The infectious center assay was done by plucking individual G418 resistant (G418\textsuperscript{r}) granulocyte-macrophage colonies from methylcellulose assay and placing the dispersed cells from one colony in a 2 cm\textsuperscript{2} tissue culture well containing 10\textsuperscript{4} NIH-3T3 cells in medium with 8 \mu g/ml polybrene. After overnight incubation, the medium was replaced with fresh medium containing G418 1 mg/ml. Seven days later, the assay was scored for the presence of G418\textsuperscript{r} 3T3 cells. Medium from long-term cultures was assayed for the presence of v-neo\textsuperscript{r} or v-src on 3T3 cells.

C. **Viral Infection**

Cells were infected with v-neo\textsuperscript{r} by either co-cultivation overnight with amphototropic viral producer cells which had received 1500R irradiation or incubation in supernate from viral producer cells with 8 \mu g/ml polybrene for 2 hours. After the infection period, cells were washed off the feeders or pelleted from the supernate. An aliquot of the infected or control cells were withheld from long-term culture and assessed in short-term methylcellulose assays with or without G418 at 2 mg/ml. (This concentration of G418 completely inhibited colony growth in uninfected cultures.) The remaining cells were resuspended in long-term culture medium and placed in tissue culture dishes, for marrow buffy coat cells, or on pre-established bone marrow feeders for light density blood or marrow mononuclear cells. Non-adherent or adherent cells were removed from the long-term cultures at various intervals and plated in methylcellulose assay with or without G418. Control cultures were not exposed to virus but were grown in suspension culture in medium with 8 \mu g/ml polybrene during the infection period and placed in long-term culture and methylcellulose assays at the same time as the infected cells. Colonies were scored after plating in methylcellulose on day 10-14 for granulocyte
macrophage colonies (from CFU-GM) and day 18-21 for large erythroid colonies (from BFU-E) for primary progenitor assays. Colonies were not scored unless they contained at least 30 cells and, in the case of BFU-E, had at least 3 clusters and were clearly hemoglobinized.

Cells were infected with v-src containing viral supernatant in the same manner as the v-neo<sup>F</sup> infections. Bone marrow fibroblasts which had been infected with and were producing v-src virus were used to infect light density hemopoietic cells in several experiments in which they were used as the permanent feeder or adherent layer for long-term cultures.

Cell and viral manipulations and cultures were performed under Level C containment following Medical Research Council of Canada guidelines for handling retroviruses and human samples.

3) RESULTS

A. v-neo<sup>F</sup> in Long-Term Culture

Three normal bone marrow samples were infected with virus from an amphotropic retroviral producer cell line producing v-neo<sup>F</sup> at 4 x 10<sup>6</sup> cfu/ml with helper virus. Two samples were infected as marrow buffy coats with cell-free viral supernatant. The third sample was processed to recover light density cells which were infected by co-cultivation with irradiated viral producer cells for 24 hours and then placed on pre-established bone marrow fibroblast feeder layers. Neither infection protocol appeared to be toxic to the target cells as the number of non-adherent and adherent cells and the number of progenitors in the infected long-term cultures was the same as in the uninfected, control cultures.
Table XIV shows the proportion of G418<sup>r</sup> granulocyte-macrophage colonies that were recovered from infected long-term cultures after various periods of time in culture. Bone marrow cells infected by v-neo<sup>r</sup>-containing supernate had a low but definite incidence of G418<sup>r</sup> colonies. The frequency of G418<sup>r</sup> colonies remained roughly stable over time. When light density bone marrow cells were infected by co-cultivation with viral producer cells, the frequency of G418<sup>r</sup> colonies was higher and also appeared to remain stable in culture, although the period of observation was short.

Since the virus used to do these infections contained helper virus, it was expected that a productive viral infection would be produced in the target cells. Table XV shows the v-neo<sup>r</sup> titers present in the medium of infected long-term cultures at various time points.

Individual G418<sup>r</sup> granulocyte-macrophage colonies were plucked from methylcellulose assays of the first two experiments and assayed for the production of v-neo<sup>r</sup> in infectious centre assays. Table XVI shows the results of these assays and indicates that at least some of these colonies had been infected by both v-neo<sup>r</sup> and helper virus. Some G418<sup>r</sup> colonies may have been infected by v-neo<sup>r</sup> without helper which would account for the negative colonies in these experiments.

B. v-src in Long-Term Culture

The above experiments indicate that it is possible to infect human hemopoietic progenitor in long-term culture with recombinant retrovirus and maintain a constant, albeit low, level of transformed cells. Based on this information and data from our own, and other laboratories on the ability of v-src containing retroviruses to perturb hemopoiesis in murine long-term cultures (7,9,18), a series of experiments were performed to assess the effects of this virus on human hemopoiesis.
**TABLE XIV**

Proportion of G418 Resistant Granulocyte-Macrophage Colonies From Nonadherent Cells in Human Long-Term Marrow Cultures Infected with Helper-Containing v-neor

<table>
<thead>
<tr>
<th>Marrow Sample</th>
<th>Source of Virus</th>
<th>Time of Analysis (day of culture)</th>
<th>Infected #G418&lt;sup&gt;+&lt;/sup&gt; CFU-GM</th>
<th>Control #G418&lt;sup&gt;+&lt;/sup&gt; CFU-GM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total #CFU-GM (%)</td>
<td>Total # CFU-GM</td>
</tr>
<tr>
<td>1</td>
<td>PA12/N2</td>
<td>1</td>
<td>13/5037 (0.25)</td>
<td>0/4425</td>
</tr>
<tr>
<td></td>
<td>Supernate</td>
<td>10</td>
<td>9/12900 (0.07)</td>
<td>0/1847</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>12/4150 (0.3)</td>
<td>0/1990</td>
</tr>
<tr>
<td>2</td>
<td>PA12/N2</td>
<td>1</td>
<td>2/6063 (0.03)</td>
<td>0/3780</td>
</tr>
<tr>
<td></td>
<td>Supernate</td>
<td>10</td>
<td>8/15248 (0.05)</td>
<td>0/6563</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21</td>
<td>9/6300 (0.14)</td>
<td>0/3450</td>
</tr>
<tr>
<td>3</td>
<td>PA12/N2</td>
<td>1</td>
<td>21/1124 (1.9)</td>
<td>2/2568</td>
</tr>
<tr>
<td></td>
<td>co-cultivation</td>
<td>10</td>
<td>18/1340 (1.3)</td>
<td>0/960</td>
</tr>
</tbody>
</table>
**TABLE XV**

`v-neo<sup>r</sup>` Titers in Medium From Infected Long-Term Cultures (LTC)*

<table>
<thead>
<tr>
<th>Marrow Sample</th>
<th>Time of Analysis (day of culture)</th>
<th>Titer of <code>v-neo&lt;sup&gt;r&lt;/sup&gt;</code> (cfu/ml of LTC medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>$4 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>$2.3 \times 10^3$</td>
</tr>
</tbody>
</table>

* Infections on day 0 were done with viral supernatant (marrow samples 1 or 2) or viral producer cells (sample 3) with `v-neo<sup>r</sup>` titer of $4 \times 10^6$ cfu/ml.
### TABLE XVI
Infectious Center Assays:

G418<sup>r</sup> CFU-GM From v-neo<sup>r</sup> Infected
Long-Term Marrow Cultures

<table>
<thead>
<tr>
<th>Marrow Sample</th>
<th>Time of Analysis (day)</th>
<th>v-neo&lt;sup&gt;r&lt;/sup&gt; Positive CFU-GM Total CFU-GM Tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>5/11</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>7/10</td>
</tr>
</tbody>
</table>
To assess the ability of the amphotropic v-src virus to infect primary human cells and to create human virus-producing feeders for subsequent experiments, human skin and bone marrow fibroblasts were infected with virus-containing medium. The cells gradually changed morphology over the two to four weeks following infection, acquiring large nuclei with prominent nucleoli, prominent cytoplasmic vacuolization, multiple cytoplasmic projections, and increased cell size. In place of an organized unicellular sheet of spindle-shaped cells, as was seen in uninfected fibroblast cultures, disorganized swirls and piled heaps of cells were seen in src-infected cultures. Approximately four weeks after infection, clumps of cells began to peel off the tissue culture dishes and float in the medium of infected fibroblast cultures. Assays for transforming virus from the medium in these cultures is shown in Table XVII. Both skin and marrow fibroblasts produced large amounts of v-src after many weeks in culture. However, in spite of this and the cells' morphological alteration, there was no major change in the growth rate of the infected fibroblasts and all cultures eventually senesced after passage in culture for up to six months.

The hemopoietic cells infected with v-src included fetal liver (1 case), peripheral blood or marrow from patients with chronic myelogenous leukemia (CML) (3 cases), and bone marrow from patients with myelodysplasia or preleukemia (5 cases). Fetal liver and CML samples were chosen because they provide a convenient source of large numbers of progenitors which behave relatively normally in culture, i.e. form morphologically distinct hemopoietic colonies of various lineages in methylcellulose assay and proliferate in long-term culture (14,19). In addition, a large proportion of progenitors in fetal liver and CML samples are in the active part of the cell cycle (20,21). This is in contrast to normal primitive progenitors that are largely quiescent
The efficiency of retroviral infection is felt to be influenced by the cycling status of the target cells such that viral integration and reproduction is more efficient in cycling cells (23).

The fetal liver sample was split into three portions. One was infected with v-src containing supernate, one was infected by continuous exposure in culture to a v-src producing bone marrow fibroblast feeder, and the third portion served as an uninfected control. The number of progenitors in these cultures dropped to undetectable levels within 4 weeks of long-term culture initiation. No difference was noted between the control and either of the infected samples. However, the adherent "fibroblast" layer of fetal cells in the infected cultures became morphologically abnormal and quickly began to peel off the tissue culture dish and float as balls of cells in the medium. The viral titer assayed in medium from these infected fetal liver cultures is shown on Table XVII and was maintained throughout the life of the cultures.

The CML samples consisted of one marrow sample which was infected with v-src-containing supernatant and 3 samples of CML blood light density cells (samples were obtained twice from one patient). Some of the blood cells were infected with viral supernatant and then placed on pre-established bone marrow feeder layers. The remainder of the cells were either infected by co-cultivation with v-src producing bone marrow feeder cells throughout the duration of the long-term culture or were not infected and placed on bone marrow feeders to serve as controls. Table XVII shows the viral titer recovered from medium in these cultures after various periods of time. Significant titers were present after more than 4 months in culture. Figure 18a shows the non-adherent cell counts from long-term cultures of two blood samples from one CML patient. There is no significant difference between infected and control cultures in either sample. Figure 18b and 18c
<table>
<thead>
<tr>
<th>Sample</th>
<th>Week of Culture</th>
<th>Titer (ffu/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Human Fibroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin</td>
<td>8+</td>
<td>$1.5 \times 10^4$</td>
</tr>
<tr>
<td>Marrow</td>
<td>8+</td>
<td>$1.7 \times 10^3$</td>
</tr>
<tr>
<td>B. Long-Term Cultures</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal Liver</td>
<td>2</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>$&gt;10^3$</td>
</tr>
<tr>
<td>CML-1</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>CML-2</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>CML-3(a)</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>56</td>
</tr>
<tr>
<td>Preleukemia-1</td>
<td>5</td>
<td>15</td>
</tr>
</tbody>
</table>
FIGURE 18. Total nonadherent cells per long term culture (panel a) and progenitor numbers per $10^5$ nonadherent cells (panels b and c) in cultures initiated with peripheral blood cells from a patient with CML on normal bone marrow fibroblast feeders. Some of the cultures were infected with amphotropic v-src-containing retrovirus. Pt. 3a and 3b represent two samples from the same patient.

- $\cdots\cdots$ pt3b-infected
- $\square\square\square$ pt3b-control
- $\diamond\diamond\diamond$ pt3a-infected
- $\square\square\square$ pt3a-control
show the number of large erythroid and granulocyte-macrophage colonies grown from non-adherent cells in these cultures. Again there is no significant difference between colony numbers in the infected or control cultures.

Infection by co-cultivation, rather than infectious supernatant, did not alter these findings. Similar results were obtained with the other two CML samples.

A final series of experiments were done using bone marrow samples from 5 patients with myelodysplastic or preleukemic disorders. These patients all had refractory cytopenias in two or more cell lines in the absence of bone marrow blasts. Previous work in our own laboratory and others have shown that hemopoietic progenitors from such patients fail to form either quantitatively or qualitatively normal colonies in vitro. Both erythroid and granulocyte-macrophage colony numbers are reduced and the formation of small clusters of undifferentiated cells is often noted (24-26). Having failed to show perturbation of hemopoiesis in human long-term cultures established with relatively normal cells, infection of myelodysplastic cells was undertaken to see if the expected abnormalities in in vitro hemopoiesis could be modified in any way. Table XVII shows an example of one preleukemic culture in which we were able to maintain viral titers in culture for a reasonable period of time. Figures 19 and 20a show that the number of non-adherent cells in preleukemic long-term cultures did not change whether or not the bone marrow cells were infected with v-src. The same results were obtained with two additional samples. Similarly in Figure 20b, which illustrates granulocyte-macrophage colonies in one of the preleukemic cultures, infection with v-src did not change the number (or appearance) of the colonies. The remaining 4 preleukemic samples did not produce enough scoreable hemopoietic colonies to allow such an analysis to be done.
FIGURE 19. Total nonadherent cells per long-term bone marrow culture from a patient with myelodysplasia or preleukemia.

♦ ♦ ♦ v-src-containing virus infected
□ ■ ■ uninfected control
FIGURE 20. Total nonadherent cells per long-term bone marrow culture (panel a) or granulocyte/macrophage colony-forming cells (CFU-C) per $10^5$ nonadherent cells (panel b) from a patient with myelodysplasia or preleukemia.

- ◆ ◆ ◆ v-src-containing virus infected
- □ □ □ uninfected control
Attempts to derive immortal cell lines independent of the need for fibroblast feeders from the non-adherent cells in the v-src infected CML or preleukemic long-term cultures were unsuccessful. Similarly, replating experiments, in which hemopoietic colonies grown from v-src infected cells were plucked, dispersed and replated in methylcellulose assay, failed to show any increase in replating potential over similar colonies plucked from control, uninfected cultures.

4) DISCUSSION

The data presented in this chapter indicate that human hemopoietic progenitors that are subsequently maintained in long-term culture can be successfully used as targets for retroviral-mediated gene transfer. Previous workers have exposed human long-term marrow cultures to a number of different retroviruses. Using a virus containing the human HPRT gene, one group has been able to demonstrate both viral replication and successful infection of progenitors (33). With viruses containing 'ras' oncogenes, retroviral replication was also demonstrated in long-term culture. However, quantitative data on the frequency of gene transfer and expression was not presented in the above studies. Table XIV shows the proportion of G418^r progenitors isolated at various times in long-term culture in the current experiments. Although the frequencies of G418^r colonies in these experiments using neo^r virus in supernatant were low, they were reproducible and could be improved somewhat by using co-cultivation with viral producer cells as the infection technique. Data from murine systems have shown frequencies of gene transfer as high as 100% to CFU-S (27,28). However, the frequency of gene expression, as measured by the ability of these cells to form G418^r hemopoietic colonies, was much lower (10-20%) (29-30). This difficulty with expression of the transferred
gene has also been seen with the human adenosine deaminase gene among others in primary hemopoietic cells (31). With the data available from the current studies, it is not possible to assess whether difficulties with gene transfer or gene expression was the primary problem leading to low numbers of G418^{r} colonies. Most likely both factors are contributing. Future experiments to address this problem will include the use of retroviral vectors in which expression of the neo^{r} gene is driven by a promoter other than the viral LTR. For example, the herpes virus thymidine kinase promoter is felt to function in primitive targets, such as embryonal carcinoma cells, and perhaps hemopoietic progenitors more effectively than the retroviral promoter (32).

We had hoped to use the long-term marrow culture system to study the stability of retroviral mediated gene transfer over several months in vitro. However, the current experiments were done with virus containing helper virus as well as the neo^{r} recombinant virus. Therefore many cells infected at the beginning of the long-term culture become infectious viral producers themselves, making it impossible to tell whether G418^{r} colonies were the result of a primary or secondary infection. The use of a helper-free recombinant virus would overcome this problem. However, our best helper free recombinant neo^{r} virus has a 10-fold lower titer than the helper-containing virus (4 x 10^5 cfu/ml and 4 x 10^6 cfu/ml respectively). The use of this helper free virus resulted in even lower, unworkable frequencies of G418 resistance (data not shown).

The low frequency of G418^{r} colonies also made it impossible to continue the experiments for periods longer than 3 weeks. The number of cells in human long-term cultures drops progressively with time in culture. A large number of cells was necessary for plating in these experiments in order to recover a measurable number of G418^{r} colonies. These cell numbers were not available
after 3 weeks. Nevertheless the co-cultivation experiment where the frequency of G418^R colonies was almost 2% and the availability of new viral vectors gives hope that the problems of low frequency will soon be overcome.

The demonstration of successful transfer of the neo^R gene to progenitors in long-term culture and our previous success with progenitors in short-term methylcellulose assay made it seem feasible to attempt the transfer of other retrovirally encoded genes to human hemopoietic progenitors. The v-src gene was selected because of the general interest in elucidating the role of known transforming oncogenes in human malignancies and the data from murine long-term cultures suggesting that the v-src containing virus perturbs hemopoiesis in that system. Dexter et al have shown increased colony numbers, increased self-renewal, and the generation of immortal, multipotent, factor-dependent cell lines from v-src infected murine cultures (7,9). The role of v-src in these changes is unclear as the viral gene is not present in the DNA of the cell lines (34). Nevertheless, the effects described are real and reproducible in other laboratories (18).

The literature describing infection of human long-term cultures with retroviruses containing oncogenes is relatively sparse (8). However, Greenberger et al were able to demonstrate retroviral infection of hemopoietic cells in human long-term culture with Kirsten or Harvey ras-containing retroviruses. The level of transferred gene expression in these hemopoietic cells is unclear from the data presented. Nevertheless, the only perturbation in hemopoiesis noted in the infected cultures was the generation of an increased number of Ebstein-Barr virus (EBV) transformed B lymphocytes in the infected cultures as compared to controls. In the experiments presented in this chapter, we were similarly unable to demonstrate any reproducible effect on human hemopoiesis in long-term culture by viruses containing the v-src
oncogene. This occurred in spite of v-src viral titers that persisted in culture for many weeks. The absence of effect may have been due to technical factors related to low levels of infection and gene transfer to hemopoietic progenitors in the long-term cultures or to the relatively short period of observation in the human as compared to murine system. Alternatively, there are intrinsic differences between murine and human cells that may allow the behaviour of the former to be more easily perturbed by the v-src oncogene. Finally, the v-src gene alone may be unable to transform hemopoietic cells and the effects seen in murine cultures may be due to the indirect effects on hemopoietic cells of infected stromal cells. The morphological abnormalities that were noted in the v-src infected human fibroblasts in this report are reminiscent of changes described in infected murine marrow fibroblasts (7). Thus the microenvironments in the infected human, as well as murine long-term cultures, were abnormal. It is not possible to determine from available data if these changes were quantitatively or qualitatively similar in the two systems. The Moloney leukemia virus which was the helper virus used in the murine experiments does not contain an oncogene. Nevertheless, it may have contributed in undetermined ways to the observed effects on hemopoiesis in murine cultures. The amphotropic helper virus used in the human infectious is a non-pathogenic murine virus which may be incapable of potentiating the "src" effect.

There is now a considerable body of experimental data indicating that more than one oncogene may be necessary to transform primary cells. Weinberg et al first demonstrated that rat embryo fibroblasts could be transformed by the ras and myc oncogenes together but not by either gene alone (35). Similar data now exists for other systems, including murine B lymphoblasts in long-term culture (36). Thus a second gene may be necessary to complete the transformation of v-src infected hemopoietic cells.
Although these experiments have failed to demonstrate a biological effect for a retrovirally transferred oncogene on human hemopoiesis in long-term culture, they have shown that it is possible to maintain retroviral infection in primary human cells for significant periods of time. The experiments using the neo\textsuperscript{R}-containing virus have shown that some hemopoietic progenitors in these cultures are successfully infected and express the transferred gene. Future work to enhance the efficiency of infection and gene expression with new retroviral vectors should allow long-term marrow cultures to be used as convenient and useful targets for gene transfer experiments.
REFERENCES


SUMMARY AND CONCLUSIONS

1) SUMMARY

Hemopoiesis is the result of the activities of primitive pluripotent cells, hemopoietic stem cells. In normal organisms, many of these cells exist and, through the processes of proliferation and differentiation, contribute to the pool of mature blood cells. The result is that normal hemopoiesis is polyclonal. Because these cells are able to reproduce themselves, i.e. self-renew, hemopoiesis continues throughout the life-span of the animal.

Hemopoietic malignancies arise most commonly in a single cell. The clonality of these disorders has been convincingly demonstrated using cytogenetic and G6PD isoenzyme analysis of malignant cells. The prototype disease for such studies was chronic myelogenous leukemia (CML) in which a pluripotent hemopoietic cell has been shown to be the target for malignant transformation (1,2). While similar data is available for a number of other neoplasms, notably other myeloproliferative disorders and some cases of acute nonlymphoblastic leukemia (ANLL), such analysis is limited to diseases which have cytogenetic markers and the one third of black women who are heterozygous for two electrophoretically distinct variants of G6PD. Recent developments in the field of molecular genetics, including restriction fragment length polymorphism (RFLP) (3) analysis, and the use of marker genes such as those encoded by retroviruses (4,5), will make detailed analysis of clonality more widely applicable to scientific research.
The regulation of hemopoiesis is a complex process involving various protein growth factors and cellular interactions within the bone marrow microenvironment. The precise mechanism of action of these factors, their level of interaction and control, and their relative importance are not presently understood for normal hemopoiesis. How such regulation is perturbed in hemopoietic neoplasms is even more obscure. However, a large body of evidence indicates that genetic change is fundamental to the development of malignancy. Such change has been demonstrated at the cytogenetic or chromosomal level for most human cancers (6). In the last few years, there has been an exponential rise in the number of molecular genetic abnormalities that have been found in human tumor cells, some of which appear chromosomally normal. Many of these molecular abnormalities involve cellular oncogenes, the discovery of which has allowed investigators to focus the search for critical alterations in cancer cells on a relatively small number of candidate genes (7). Impressive though, the results to date are, there are undoubtedly many "oncogenes" waiting to be discovered and the technical advances of molecular biology will continue to offer new approaches to the study of carcinogenesis.

One such approach is the technique of gene transfer using recombinant retroviral vectors. Genes of potential importance in the etiology of a particular tumor could be inserted into the appropriate vector allowing the transfer of this gene into a selected target cell population. Subsequent analysis of infected cells may reveal specific changes in their behavior or physical or chemical properties that can be attributed to the presence of the new gene.

In the present studies, several of the techniques alluded to above have been used to analyze clonality in human malignant cells. In addition, the technique of retroviral mediated gene transfer was developed for use on human hemopoietic cells.
A. Cytogenetic Analysis of Hemopoietic Colonies in Patients with Juvenile Monosomy 7 Syndrome

Using a technique that allows cytogenetic analysis of individual hemopoietic colonies plucked from methylcellulose assay, it was possible to show that erythroid progenitors and, in one case, myeloid progenitors were involved in the neoplastic clone of two patients with juvenile monosomy 7 syndrome. Thus, this myelodysplastic or proliferative disorder of childhood must originate, at least in some cases, in a primitive, multipotent hemopoietic cell. This disorder bears many clinical and cytogenetic similarities to the more common myelodysplasias seen in adults. In both adults and children with monosomy of chromosome 7 in bone marrow cells, the initial cytopenic or myeloproliferative phase of the disease typically evolves into frank ANLL within several years of diagnosis. De novo ANLL has been shown by G6PD analysis to often involve a pluripotent hemopoietic cell, particularly if the disease has a smouldering or gradual onset in an elderly patient (8).

Thus, primitive pluripotent hemopoietic progenitors appear to be common targets for neoplastic transformation in hematologic malignancy. This is true in spite of the fact that the phenotype of the disease varies considerably, i.e. myeloproliferative, dysplastic or acute leukemia. The precise nature of the genetic change that occurs in these multipotent malignant cells is, of course, of great interest and the subject of intense investigation in many centers.
B. Identification of Hemopoietic Progenitors that Are Not Part of the Malignant Clone in Long-Term Cultures from a G6PD Heterozygote with CML

In this study G6PD isoenzyme analysis was combined with hemopoietic colony cytogenetics to analyze the clonality of progenitors in long-term marrow cultures from patients with CML. Although 100% of bone marrow metaphase cells in patients with chronic phase CML typically show the Philadelphia chromosome abnormality (Ph) (1), studies in which patients have received aggressive cytotoxic chemotherapy or α interferon have revealed the presence of Ph negative hemopoietic cells in vivo (9,10). Similarly, Ph-negative hemopoietic colonies have been recovered from long-term culture established with CML marrow cells (11). The polyclonality of the Ph-negative cells in vivo has been demonstrated by G6PD analysis in one patient (9).

However, it has been suggested, and some data presented to support the idea, that at least some cytogenetically normal marrow and blood cells in CML are part of the neoplastic clone (12). That is, the Ph chromosome is a secondary finding that develops relatively late in the course of the disease. The long-term marrow culture provides a system in which one could potentially demonstrate this phenomenon of Ph-negative clonality. It is possible to obtain cytogenetically normal progenitors from CML long-term cultures on a relatively routine basis and to study these colonies for their G6PD isoenzyme type in the appropriate G6PD heterozygous patient.

Although cells from one dozen black women with CML were screened in the current study, only two were found to be suitable for these experiments. In cultures from one of these patients, it was possible to demonstrate hemopoietic colonies derived from cells in long-term culture that were not part of the malignant clone. Although this does not exclude the possibility that a cytogenetically normal clonal population of cells also exists, it does
show that at least some of the Ph-negative cells are truly normal. This finding may have practical importance for clinicians planning to use in vitro techniques such as the long-term culture to purge patient bone marrow of malignant cells prior to autologous marrow transplantation (13).

C. retroviral-Mediated Gene Transfer to Human Hemopoietic Cells

The technique of retroviral-mediated gene transfer was developed for use in human cells as a tool which would allow analysis of clonality in cultured cells as well as functional studies into the genetic regulation of normal and malignant hemopoiesis.

Short-Term Assays. Using recombinant virus containing the neoF gene, it was possible to demonstrate efficient gene transfer to normal and malignant hemopoietic cells, both established cell lines and primary progenitors in methylcellulose assay. Expression of the transferred gene was shown by RNA spot blot and G418 resistance of hemopoietic colonies formed by the infected cells. The clonality of isolated G418 resistant, virally-infected K562 cell lines was verified by Southern blotting with demonstration of a unique site of retroviral integration in the cellular DNA of each clone. A number of variables were optimized to increase the frequency of G418 resistant colonies and successful gene transfer was obtained with neoF virus both with and without associated helper virus. Although these experiments were generally successful, the highest frequency of gene transfer to primary progenitors obtained, as assessed by the proportion of G418 resistant granulocyte-macrophage colonies, was 15.7%. While this figure is encouraging, it is much less than the 60% frequency obtained with K562 cells or the 100% frequency reported by others for murine CFU-S (14,15). The reasons for this discrepancy could not be precisely determined in the current study but probably include:
(i) inadequate expression of the transferred gene from the promoter in the retroviral LTR, (ii) inefficient gene transfer due to factors such as the cycling status of target cells.

Long-Term Cultures. Having shown that retroviral-mediated gene transfer to primary hemopoietic progenitors was a feasible technique, the next phase of the research focused on the study of virally-infected progenitors maintained in long-term culture. The purpose of these experiments was to ultimately use the long-term culture as a system in which to investigate the stability of gene transfer and expression over time. Secondly, preliminary functional studies were planned using virus containing the v-src oncogene.

(i) Experiments using the neo^r virus were successful in demonstrating G418 resistant hemopoietic colonies after periods of up to three weeks in culture. However, the low frequency of G418 resistant granulocyte-macrophage colonies in these experiments (less than 2%) made definitive studies on the stability of the transferred gene using helper-free virus for longer periods of time in culture prohibitive.

(ii) Infections of various hemopoietic target cell populations were carried out using a recombinant v-src-containing retrovirus pseudo-typed with an amphotropic helper. Progenitors from fetal liver, CML blood or marrow, and myelodysplastic marrow were infected and followed in long-term culture for periods up to 4 1/2 months. Although no perturbations in the quantitative or qualitative aspects of hemopoiesis measured in the cultures were observed, it was possible to show successful infection by the v-src virus of primary human cells and their long-term maintenance in these cultures.

The above experiments represent preliminary experience with the technique of retroviral-mediated gene transfer. Several different viral vectors were
used and a variety of human cell types, including established leukemic cell lines, primary normal and malignant progenitors, and bone marrow and skin fibroblasts were used as targets. Two different culture systems, short-term methylcellulose assay and long-term cultures, were used to study the results of various infections. Although not all of these experiments were completely successful, the overall results are sufficiently encouraging to allow one to proceed in the expectation that the modification of existing techniques and the development of new ones will make future progress possible. Already, the availability of new retroviral vectors providing different promoters than that present in the retroviral LTR, make it likely that the level of gene expression following successful gene transfer will soon be routinely much higher than was achieved in the experiments described here (16). Vectors containing other genes of interest such as hemopoietic growth factors are being constructed. These will be important reagents for future experiments designed to explore the importance of various molecules in normal and malignant hemopoiesis.

2) CONCLUSIONS

The studies described here have used several established techniques to extend our knowledge of the clonality of hemopoietic progenitors in human malignancies. In addition to their theoretical interest, these findings may have importance for clinicians searching for new therapeutic modalities for hemopoietic neoplasms.

The technique of retroviral-mediated gene transfer has been adapted for use in human hemopoietic progenitors. Although much remains to be learned about this technique and its application, the experience described provides a good foundation for future work. The technique clearly has exciting potential
for use as a tool in basic and clinical research. The use of gene transfer to dissect the factors regulating various cell processes and mark cells for studies of various clonal populations is in its infancy but has already yielded important biological information. The era of gene therapy for inherited disorders with molecular genetic defects is close at hand (17) and may ultimately be made possible by techniques such as those described in this work.
REFERENCES


APPENDIX I

LIST OF ABBREVIATIONS

ADA  adenosine deaminase
AEL  acute erythroleukemia
ALL  acute lymphoblastic leukemia
AML  acute myelogenous leukemia
AMML  acute myelomonocytic leukemia
AMoL  acute monocytic leukemia
ANLL  acute nonlymphoblastic leukemia
bcr  breakpoint cluster region
BFU-E  burst forming unit-erythroid
bp  base pair
BSA  bovine serum albumen
Ca++  calcium
CAT  chloramphenicol acetyltransferase
cfu  colony forming unit
CFU-C  colony forming unit in culture
CFU-E  colony forming unit, erythroid
CFU-GEMM  mixed colony forming unit, granulocyte, erythroid, macrophage, megakaryocyte
CFU-GM  granulocyte macrophage colony forming unit
CFU-M  macrophage colony forming unit
CFU-Meg  megakaryocyte colony forming unit
CFU-S  spleen colony forming unit
cm  centimeter
CML  chronic myelogenous leukemia
CO₂ carbon dioxide

cpm counts per minute

CSF-1 colony stimulating factor 1

DHFR dihydrofolate reductase

dl deciliter

DNA deoxyribonucleic acid

EBV Epstein Barr virus

EGF epidermal growth factor

env gene encoding retroviral envelope glycoproteins

ep erythropoietin

FAB French-American-British

FCS fetal calf serum

ffu focus forming units

G418 geneticin

G6PD glucose 6 phosphate dehydrogenase

gag gene encoding retroviral coat proteins

G-CSF granulocyte colony stimulating factor

gm gram

GM-CSF granulocyte-macrophage colony-stimulating factor

GTP guanidine triphosphate

HBSS Hanks balanced salt solution

HPRT hypoxanthine phosphoribosyl transferase

HS horse serum

IgH immunoglobulin heavy chain

IgK immunoglobulin kappa light chain

Igλ immunoglobulin lambda light chain

IL-1,2,3 interleukin 1,2,3

Kb kilobase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTC</td>
<td>long term culture</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>M-CSF</td>
<td>macrophage colony stimulating factor</td>
</tr>
<tr>
<td>M:E</td>
<td>myeloid:erythroid</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mm</td>
<td>millimeter</td>
</tr>
<tr>
<td>MoMuLV</td>
<td>Moloney murine leukemia virus</td>
</tr>
<tr>
<td>MSV</td>
<td>murine sarcoma virus</td>
</tr>
<tr>
<td>MT</td>
<td>metallothionein</td>
</tr>
<tr>
<td>neo&lt;sup&gt;r&lt;/sup&gt;</td>
<td>neomycin resistance or neomycin phosphotransferase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia chromosome</td>
</tr>
<tr>
<td>PK</td>
<td>protein kinase</td>
</tr>
<tr>
<td>pM</td>
<td>picomolar</td>
</tr>
<tr>
<td>PNP</td>
<td>purine nucleoside phosphorylase</td>
</tr>
<tr>
<td>pol</td>
<td>retroviral gene for reverse transcriptase</td>
</tr>
<tr>
<td>R</td>
<td>rad</td>
</tr>
<tr>
<td>rbc</td>
<td>red blood cell</td>
</tr>
<tr>
<td>RFLP</td>
<td>restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RSV</td>
<td>Rouse sarcoma virus</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SV40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TCR.A</td>
<td>T cell receptor A chain</td>
</tr>
<tr>
<td>TCR.B</td>
<td>T cell receptor B chain</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>μg</td>
<td>microgram</td>
</tr>
<tr>
<td>V-neo&lt;sup&gt;R&lt;/sup&gt;</td>
<td>retrovirus encoding the gene for neomycin resistance</td>
</tr>
<tr>
<td>V-src</td>
<td>viral src oncogene from the Rous sarcoma virus</td>
</tr>
<tr>
<td>wbc</td>
<td>white blood cell</td>
</tr>
</tbody>
</table>
APPENDIX II

SELECTED CYTOGENETIC NOMENCLATURE

The cytogenetic nomenclature used throughout this work was the International System for Human Cytogenetic Nomenclature (1978).

In the construction of the karyotype the autosomes are numbered from 1 to 22 as nearly as possible in descending order of length. The sex chromosomes are referred to as X and Y. The symbols p and q are used to designate, respectively, the short and long arms of each chromosome.

A) Normal Karyotypes.

Normal human karyotypes are designated as follows:

46,XX Normal female.
46,XY Normal male.

B) Numerical Chromosome Aberrations.

The + and - signs are placed before the appropriate symbol to indicate additional or missing whole chromosomes. e.g.,

47,XX,+8 47 chromosomes, XX sex chromosomes, an additional chromosome #8.
45,XY,-7 45 chromosomes, XY sex chromosomes, a missing chromosome #7.

C) Chromosome Mosaics.

The chromosome constitution of the different cell lines in a mosaic are listed in order of the predominant clones.

45,X/46,XX A chromosome mosaic with two cell lines, the major one with 45 chromosomes and a single X, the other with a normal female karyotype.
(a) Reciprocal translocations are designated as follows:

46,XY,t(9;22) Breakage and reunion have occurred in chromosomes #9 and #22.

46,XY,t(9q;22q) Breakage and reunion have occurred in the long arm of chromosome #9 and the long arm of chromosome #22.

46,XY,t(9;22)(q34;q11) Breakage and reunion have occurred at bands q34 in chromosome #9 and q11 in chromosome #22.

46,XY,Ph Abbreviated nomenclature for a normal male karyotype with a standard Philadelphia chromosome resulting from a reciprocal t(9;22)(q34;q11).

(b) Deletions of part of a chromosome which shortens the length of one of its arms are designated as follows:

46,XX,del(5)(q13q31) Deletion of part of the long arm of chromosome 5 between bands 13 and 31 in an otherwise normal female karyotype.

(c) Inversions in which the region of a chromosome around the centromere becomes inverted relative to the distal portions of the long and short arms are designated as follows:

46XX,inv(16)(p13q22) Inversion of one chromosome 16 between bands p13 and q22 in an otherwise normal female karyotype.
Reference

APPENDIX III

METHOD FOR ESTABLISHMENT AND MAINTENANCE
OF HUMAN LONG TERM CULTURES

(Modified from Reference 1)

Cells. All marrow specimens were collected in sterile tubes containing 800 units of preservative free heparin in 1 ml of θ-medium. A buffy coat preparation of the marrow was made by centrifugation at 200 g x 41 and harvesting the upper layer of cells. Long-term cultures were initiated by placing 2 x 107 nucleated marrow buffy coat cells in 8 ml of growth medium in a 60 mm x 15 mm tissue culture dish (Falcon).

Growth Medium. θ-medium supplemented with extra glutamine (400 mg/l), inositol (40 mg/l), and folic acid (10 mg/l) was supplemented with horse serum (HS, 12.5%, Flow Laboratories), fetal calf serum (FCS, 12.5%, Flow Laboratories), 2-β-mercaptoethanol (10⁻⁴M), and hydrocortisone sodium succinate (10⁻⁶M). Mercaptoethanol was made up to 10⁻⁴M from a frozen stock solution of 10⁻²M, and hydrocortisone from a 10⁻⁴M solution kept at 4°C and made up fresh every week. Solutions of both of these reagents were made up in θ-medium. Both horse and fetal calf sera were selected initially for their ability to support hematopoiesis in culture: the horse serum in mouse CFU-C assays, and the fetal calf serum in mouse and human CFU-E and BFU-E assays.

Initiation of Long Term Cultures. Dishes were incubated during the first 3-4 days at either 37°C and then transferred to 33°C, in all cases in a humidified atmosphere of 5% CO₂ in air. At day 3 or 4 a total change of the growth medium was performed before the dishes were transferred to 33°C where they remained through the duration of the culture.

Maintenance. At weekly intervals, the half growth medium was replaced with fresh medium and half of the non-adherent cells were removed with the
spent medium. These cells were used for nucleated cell counts, and for assessment of the progenitor content by cloning aliquots in standard methylcellulose cultures.

**Enzymatic Detachment of the Adherent Layer.** Bacterial collagenase type I (200 units/mg) protein, Sigma Chemicals) was dissolved in calcium and magnesium-free Hanks balanced salt solution (HBSS-Ca-Mg) to a final concentration of 0.13% and the solution sterilized by passage through a 0.22μm millipore filter. This solution was kept at 4°C and used within 48 hours. Just before use, FCS was added to give a final concentration of 20% FCS and 0.10% collagenase. To detach adherent cells, non-adherent cells and all the growth medium were first removed from the culture dish. Cultures were then vigorously washed twice with fresh HBSS-Ca-Mg, and the additional detached cells then added to the non-adherent suspension. Ten ml of 20% FCS in 0.10% collagenase solution were then pipetted onto the adherent layer and the cultures incubated undisturbed for 3 hours at 37°C in an atmosphere of 5% CO₂ in air. At the end of the incubation period, many cells were completely detached and most of the remaining adherent cells could be readily resuspended by gentle but sustained pipetting.

Adherent cells were centrifuged at 180g for 10 min at room temperature, and then washed twice in 2% FCS in HBSS-Ca-Mg. After each centrifugation, the cell pellet was carefully resuspended to minimize the formation of clumps. Adherent cells were then assayed for progenitor content in standard methylcellulose assay.

**Reference**

APPENDIX IV

METHOD FOR HEMOPOIETIC COLONY ASSAYS IN METHYLCELLULOSE CULTURE

(Modified from Reference 1)

Erythropoietic (CFU-E and BFU-E) and granulopoietic (CFU-GM) progenitors were assayed in 0.8% methylcellulose in α-medium or Iscove's medium, supplemented with 30% FCS, 1% deionized bovine serum albumen (BSA) buffered with bicarbonate (1 ml of 7% bicarbonate for 40 ml of 10% BSA), 10^{-4}M 2-β-mercaptoethanol and 200 mM L-Glutamine. 3 units per ml of human erythropoietin (Ep, purified in the Terry Fox Laboratory to a specific activity of at least 100 units per mg of protein), was added to assays to assess both erythroid and granulopoietic progenitors. 10% of phytohemagglutinin (PHA)-stimulated human leukocyte conditioned medium was added to all assay dishes. PHA-conditioned medium was prepared by incubating blood buffy coat leukocytes (separated in 0.1% methylcellulose) at a concentration of 1 x 10^6 cells per ml with 0.5% PHA (Gibco, M-type), in Iscove's or α-medium with 10% FCS. Incubation was performed at 37°C during 7 days, and at the end of the incubation period, the medium was collected, spun at 2000 rpm to remove cells and cell debris, and then stored in frozen aliquots. Erythropoietin and conditioned medium preparations were both calibrated against standard preparations to ensure equivalence of activity in different batches.

Cells were plated in methylcellulose assays at a final concentration of 0.5 to 2 x 10^5 cells per 1.1 ml of culture. Buffy coat marrow cells were plated at 2 x 10^5 cells per 1.1 ml; Ficoll hypaque separated blood cells at 4 x 10^5 per 1.1 ml. Non-adherent cells from long-term cultures were usually
plated at $0.5 \times 10^5$ cells per 1.1 ml during the first 2-3 weeks, because of the increased concentration of CFU-GM in the non-adherent fraction during that period, and then subsequently at $1 \times 10^5$ cells per 1.1 ml. After 6-8 weeks, the number of cells recovered in the non-adherent fraction was often reduced so that as few as 1 to $2 \times 10^4$ cells was all that could be plated. Adherent cells were plated at $1 \times 10^5$ or $0.5 \times 10^5$ cells per 1.1 ml. As the adherent cell suspension was a mixture of some hemopoietic cells and many non-hemopoietic cells (including fibroblasts), it was found that it was important to select assay petri dishes that strongly prevented any spreading of the adherent cells during the 3 week period required for hemopoietic colony growth. If this was not done, fibroblast proliferation was extensive and inhibition of erythroid, and to a lesser extent of granulocytic, colony growth resulted.

Each assay was set up in duplicate or quadruplicate and scored 2 times to obtain reliable counts of both small and large erythroid colonies using the following criteria: isolated single or paired clusters of CFU-E derived erythroid cells were counted at day 7 or 8 after plating. Bursts containing 3-8 clusters were scored at day 12-14, and bursts containing more than 8 clusters after 16-18 days. However, in most instances, BFU-E numbers have been added together and presented as a single value.

Colonies of granulocytes and macrophages (from CFU-GM) containing more than 20 cells were scored at day 12-14, sometimes in the same assay dishes as used for the CFU-E and BFU-E estimate but more often in assays without added erythropoietin.

Reference

APPENDIX V

METHOD FOR CYTOGENETIC ANALYSIS OF METAPHASES FROM CELLS IN INDIVIDUAL HEMOPOIETIC COLONIES

(Modified from Reference 1)

Hemopoietic colonies were plucked from methylcellulose assays with a finely drawn out glass pipet. Each set of assay cultures was monitored at frequent intervals to select the optimal time for plucking i.e., when large colonies had matured to the point where they could be identified with confidence as erythroid, granulocytic or mixed, but colony growth had not yet stopped. This usually occurred after 9-12 days for BFU-E and up to 15 days for CFU-C. As a result, different types of colonies were usually harvested at different times in different assay dishes. One hour prior to harvesting, 0.1 ml of Colcemid (1 ug/ml), was evenly distributed over the methylcellulose surface with a 26 gauge needle (7-8 drops). Selected colonies were then plucked, and dispersed in a small volume of 0.075 M KCl in a round-bottomed well of microtiter plate (20 ul/microwell). After 15-20 minutes in hypotonic KCl, colonies were transferred one by one onto polylysine-coated slides and allowed to settle for 10 minutes. The cells were then fixed by dropping a mixture of 3:1 methanol:acetic acid onto the slide and blotting excess moisture from the edges of the slide with a cotton swab. The slides were dried and stained for Giemsa banding. Only colonies in which at least 2 metaphases could be analyzed were accepted.

Reference