MANIPULATION OF HAEMOPOIETIC STEM CELLS FOR CLINICAL USE

RODERICK JOHNSON

The Haematological Malignancy Diagnostic Service
Department of Haematology
The General Infirmary at Leeds
Great George Street
Leeds
LS1 3EX

ABSTRACT

Normal haemopoiesis derives from a bone marrow pool of pluripotent stem cells capable of self renewal and multilineage differentiation. Further developments in autografting and gene therapy will rely on a clearer definition and understanding of these primitive cells and an ability to manipulate them effectively. Phenotypic and functional differences between bone marrow and peripheral blood stem cells have been identified and exploited. Techniques for the mobilisation and harvesting of stem cells are established in clinical practice but consensus is lacking on the optimum approach for different diseases. In malignancy, there is great interest in removing contaminating tumour cells from harvests in the hope of reducing relapse rates. Stem cell selection using the CD34 antigen has been commonly used for such purging strategies. The concentration of stem cells produced by CD34 selection provides an ideal product for further manipulation by cell expansion and/or genetic modification. These techniques should facilitate the future engineering of more specific therapeutic cell products.

I have examined three specific aspects of the manipulation of haemopoietic stem cells with the aim of generating clinically useful therapeutic products. These were: a disease-specific mobilisation regimen in CML, clinical scale CD34 selection and transplantation in myeloma and genetic modification of stem cells to alter their function using PNH as a model disease.

A disease-specific mobilisation procedure was studied in patients with CML in an attempt to collect mainly normal progenitors for subsequent transplantation. This in vivo purging strategy relies on the differential mobilisation of Ph+ve/Ph-ve stem cells following myelosuppression. The Hydroxyurea/G-CSF regimen used was markedly less toxic than existing methods and the results compared favourably, with 28% of the harvests entirely Ph-ve and 56% showing a major response. Cytogenetic responses have been demonstrated post transplant in some patients.

Clinical scale ex vivo tumour purging was performed in myeloma patients by CD34 selection using an immunoaffinity column (Ceprate^{TM}). This procedure can effect a 3-4 log tumour reduction and also yields cells suitable for further laboratory

manipulation. I wished to confirm that the method was clinically acceptable. All but one patient achieved an adequate CD34+ve cell dose for transplant post selection and engraftment was normal, confirming the safety and efficacy of the approach. Genetic modification of peripheral blood stem cells was achieved using a retroviral construct (CD59-TM) capable of ameliorating the complement sensitivity in PNH. This was used successfully to transduce CFU-GM and BFU-E in peripheral blood and stem cell harvests from patients with haematological malignancy. Transduction efficiency was maximised by multiple cycles of infection in the presence of Il3, Il6 and SCF. A technique was developed for the simultaneous assessment of cell complement lysis and surface phenotype by flow cytometry to detect the presence and function of the novel construct. In PNH, the most primitive peripheral blood stem cells are of normal phenotype which has prompted suggestions from some authors that they might be collected and used for autologous transplantation. I have shown however, that G-CSF mobilises mainly cells of PNH phenotype in these patients. This, combined with the observation that the overall progenitor numbers in these hypoplastic patients are low suggests that the prospects for autografting or gene therapy are poor in this disease.

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ABBREVIATIONS

AChE	Acetylcholinesterase
AML	Acute Myeloid Leukaemia
β2M	Beta-2-microglobulin
BSA	Bovine Serum Albumin
BFU-E	Burst Forming Unit of Erythropoiesis
bp	base pairs
CD	Cluster of Differentiation
CFU-GM	Colony Forming Unit, Granulocyte/Macrophage
CML	Chronic Myeloid Leukaemia
CR	Complete Remission
DAPI	4,6-Diamino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
EBV	Epstein-Barr Virus
FBC	Full Blood Count
FACS	Flow Activated Cell Scanning/sorting
FCS	Foetal Calf Serum
FISH	Fluorescent in-situ Hybridisation
FITC	Fluoroscene Isothiocyanate
G-CSF	Granulocyte Colony Stimulating Factor
GPI	Glycosyl Phosphatidyl Inositol
HAT	Hypoxanthine,Aminopterin,Thymidine
4-HC	4-Hydroxyperoxycyclophosphamide
HDT	High Dose Therapy
HGPRT	Hypoxanthine-Guanine Phosphoribosyltransferase
HSC	Haemopoietic stem cell
IFN	Interferon
IgH	Immunoglobulin Heavy Chain gene
IL3	Interleukin 3
IL6	Interleukin 6
MAB	Monoclonal Antibody
MDS	Myelodysplastic syndrome
MFI	Mean Fluorescence intensity
MIL	Membrane Inhibitor of Reactive Lysis (CD59)
MM	Multiple Myeloma
MNC	Mononuclear Cell
MOI	Multiplicity of Infection
MoMLV	Moloney Murine Leukaemia Virus
MRC	Medical Research Council U.K.
MRD	Minimal Residual Disease
NAP	Neutrophil Alkaline Phosphatase

NEO	Neomycin resistance gene
NHL	Non Hodgkins Lymphoma
nt	nucleotide
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PBSCs	Peripheral Blood Stem Cells
PBSCH	Peripheral blood stem cell harvest
PBSCT	Peripheral Blood Stem Cell Transplantation
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
Ph-ve/+ve	Philadelphia Negative/Positive
PI	Propidium Iodide
PI-PLC	Phosphatidyl-Inositol Phospholipase C
PNH	Paroxysmal Nocturnal Haemoglobinuria
RT-PCR	Reverse Transcriptase PCR
SCF	Stem Cell Factor
SCID	Severe Combined Immune Deficiency
WBC	White Blood Cell

Chapter 1.

Introduction

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1.1   The Haemopoietic Stem Cell

Marrow production of mature blood cells throughout life is accomplished by the activity of a small pool of haemopoietic stem cells (HSC) which are defined by their ability to self renew or differentiate into any of the lymphohaemopoietic lineages (Gordon & Blackett, 1994; Scott & Gordon, 1995; Leitman & Read, 1996) (Figure 1.1). There has been intense debate regarding the nature of the HSC and in recent years other developments such as the increasing diversity of bone marrow transplantation strategies and research into gene therapy have quickened the pace of stem cell research owing to their dependence on an accurate understanding of the behaviour of these elusive but important cells. (Apperley & Williams, 1990a).

History

It had long been suggested that there were specialised cells capable of maintaining haemopoiesis but evidence for this did not begin to accrue until the late 1940s when it was shown that lead shielding of the spleen could save rodents exposed to lethal radiation (Jacobson *et al* 1949). This implied that protected HSCs in the spleen migrated to blood and marrow and repopulated the haemopoietic system. Further animal work supporting this theory was published over the next few years including cross-circulation experiments between irradiated and non-irradiated rats and demonstration of the radioprotective effects of marrow infusion in mice and guinea pigs (Ford *et al* 1956; Lorenz *et al* 1951).

Figure 1.1

Diagram showing a scheme for the development of mature, terminally differentiated cells of all lineages from a single putative pluripotent stem cell. This cell can renew itself by division or differentiate down any of the pathways shown.

In the 1960s, Till and co-workers used the spleen colony forming assay (CFU-S) to assay haemopoietic stem cells (Till & McCulloch, 1961; Till et al 1964). They showed that spleen irradiation after lethal irradiation led to the formation of haemopoietic tissue in the spleens in the form of these nodules which could be enumerated macroscopically, since colony spleen colonies tended to

Haemopoietic Stem and Progenitor Cells

pluripotent stem

CFU$_{GEMM}$

mixed myeloid progenitor

lymphoid progenitor

BFU$_E$

burst-forming unit (erythroid)

CFU$_E$ CFU$_{Meg}$

CFU$_{GM}$ CFU$_{Bas}$ CFU$_{Eo}$

thymus

erythroid   megakaryocyte
progenitor   progenitor

granulocyte/monocyte
progenitor

eosinophil
progenitor

B   T

red cells   platelets   monocytes   neutrophils   basophils   eosinophils   lymphocytes

colonies were derived from single myeloid progenitors or from closely co-mingled cells (Becker, Wu & McCulloch, 1965). However, more reductive/definitive assays measure in vitro colony formation (CFU and CFU in liquid or semi-solid media) of haemopoietic myeloid and progenitor cells to various specific growth agents (McCulloch & Sminovitch, 1964; Sminovitch & Sachar, 1977; Bradley & Metcalf, 1966) and various growth/progenitor specific (Metcalf et al 1967). Functional blood

In the 1960s, Till and co-workers used the spleen colony forming assay (CFU-S) in mice to elucidate these observations (Till & McCulloch, 1961; Till *et al* 1964). They showed that marrow infusions after lethal irradiation led to the formation of heterogeneous haemopoietic colonies in the spleens of these animals which could be harvested and re-transplanted into second hosts where more colonies would be formed (Siminovitch *et al* 1963). Analysis of these secondary colonies showed that all lymphoid and myeloid cell types were represented. (Wu *et al* 1967; Wu *et al* 1968). This work implied that the marrow contained cells capable of both multilineage differentiation and of self renewal and a model of the HSC and its behaviour was put forward which is still valid, if contentious (Till *et al* 1964). Over the last three decades since this work was first published, increasingly sophisticated methodology has been employed to further delineate, investigate and purify the putative HSC.

General features of the HSC

There is as yet no single accepted test by which the pluripotent HSC can be defined. The search for this elusive sub-population has involved a large number of techniques including: cell separation using physical properties such as density centrifugation and filtration (Goodman, 1960; Morrison, 1967), in-vivo radioprotection assays (Ploemacher & Brons, 1988), in-vivo colony formation (CFU-S)(Till & McCulloch, 1961), a variety of in vitro culture assays (see below), exposure to cell cycle specific cytotoxic agents (Harrison & Lerner, 1991; Hodgson & Bradley, 1979; Smith *et al* 1991) and immunophenotypic analysis (Terstappen *et al* 1991; Sutherland *et al*

1993; Baum *et al* 1992; Lansdorp *et al* 1990). These approaches have all helped to shed light on the biology and behaviour of HSCs but they may define different cell types and/or stages of maturation and no consensus has been reached as to the precise relevance of each assay or the stage of cell development that it concerns. Results from these areas of work have led to a generally agreed view that the early, pluripotent HSC resembles a small lymphocyte morphologically and is relatively quiescent in steady state haemopoiesis, the majority being in G_0 phase of the cell cycle at any one time (Lajtha, 1979; Gordon & Blackett, 1994; Morrison & Weissman, 1994). They can self renew or differentiate into any blood cell type and can protect animals from otherwise lethal doses of radiation by effecting long term marrow repopulation (Ford *et al* 1956; Lorenz *et al* 1951). They are resistant to the effects of cell cycle specific cytotoxic agents such as 5-Fluorouracil or 4-Hydroxyperoxycyclophosphamide (Harrison & Lerner, 1991; Hodgson & Bradley, 1979; Smith *et al* 1991) and to retroviral infection (Williams *et al* 1984a) and are capable of initiating long term marrow cultures in vitro (Dexter *et al* 1977; Gartner & Kaplan, 1980). By flow cytometry their surface phenotype can be shown to include: CD34+ve[bright] (Sutherland *et al* 1993), CD38-ve (Terstappen *et al* 1991), Thy-1+ve (Baum *et al* 1992), rhodamine-123[dull] (Lansdorp *et al* 1990), and CD45RO+ve (Lansdorp *et al* 1990) and to lack the expression of specific antigens associated with commitment to any of the haemopoietic lineages.

Stem cell kinetics

The HSC is capable of maintaining blood cell production throughout life in a healthy individual and also of repopulating the marrow following injury such as cytotoxic chemotherapy or radiation. There have been many theories concerning the behaviour of these cells and there is as yet no agreement. The controlling factors determining the cell's decision to renew, differentiate or proliferate have been envisaged as being stochastic (random), intrinsic, cell age related or extrinsic from cytokines or the marrow microenvironment. There is a wealth of evidence for all of these models and more than one may be important.

The first models of HSC kinetics were based on the concept of a biologically homogeneous population of HSC in which the decision to renew or differentiate was a stochastic event for each cell (Till *et al* 1964; Ogawa *et al* 1983; Gordon & Blackett, 1994) . This theory was supported by the results of murine CFU-S work (Till *et al* 1964) and was able to offer an explanation for the observed heterogeneity of spleen colonies by invoking a mechanism whereby HSCs would randomly undergo variable numbers of renewal divisions before differentiating. Some colonies would therefore be expected to contain many re-platable CFU-S from HSCs which had continued to self renew and others more differentiated progeny of varying lineages (Siminovitch *et al* 1963). Stochastic models allow for biologically identical HSCs to achieve short and long term haemopoiesis depending on the number of self-renewal divisions they undergo before differentiating (Gordon & Blackett, 1994; Gordon & Amos, 1994). Gordon et al have argued for this interpretation and have demonstrated just such in vitro heterogeneity in the maturity of progenitors derived

from single, primitive plastic adherent cells (Gordon, 1994; Gordon *et al* 1995; Gordon *et al* 1996). Kay suggested that the self renewal capacity of HSCs might be time related, diminishing with successive cell divisions and proposed the theory of clonal succession in which haemopoiesis is sustained largely by one lineage of HSC and its progeny until their proliferative capacity is exhausted (Kay, 1965). Others have suggested that multiple lineages sustain haemopoiesis and each cell's capacity for self renewal is an intrinsic, not stochastic feature (Metcalf & Moore, 1971; Rosendaal *et al* 1979). Models have been put forward where there is an envisaged continuum of renewal potentials intrinsic to each cell and dependant on the age of that cell (Helman *et al* 1978). Others have suggested a number of discreet maturation stages or subpopulations of HSCs with, within each, a relatively consistent and pre-determined potential for renewal (Micklem & Ogden, 1976; Morrison & Weissman, 1994). Morrison and Weissman have proposed such a model in which the HSC pool is subdivided into three phenotypically distinct subpopulations each with a different potential for self renewal and differentiation. In this deterministic model, the decision whereby a cell renews or differentiates is an intrinsic feature and not random or due to external influence. Here, there would only be a finite number of renewal divisions in each subpopulation and this is backed experimentally by the ability to predict the self renewal potential of HSCs by their phenotypic differences (Morrison & Weissman, 1994). The haemopoietic microenvironment and an increasing number of cytokines and their interaction with HSCs has been suggested to influence stem cell behaviour (Metcalf, 1989), while some argue that these factors only influence committed cells, the decision to self renew being intrinsic (Ogawa, 1993).

At present then, there are two major distinctions between workers in the field of stem cell biology. Firstly there is dispute as to whether the stem cell pool is homogeneous (Gordon & Blackett, 1994) or is separated into biologically distinct subpopulations (Morrison & Weissman, 1994; Morrison *et al* 1995) and secondly there is the debate over the mechanisms controlling HSC behaviour, be they stochastic (Ogawa *et al* 1983), intrinsic (deterministic) (Morrison *et al* 1995), age related (Micklem & Ogden, 1976) or external (VanZant & Goldwasser, 1979). It is of course entirely possible that a combination of these and other factors may act in consort either throughout stem cell life or at specific stages of haemopoiesis.

Gene marking studies in marrow reinfused after myeloablative treatment have provided insights into stem cell behaviour during and after haemopoietic reconstitution. It has been shown that in the early post transplant stages, haemopoiesis is characterised by rapid and erratic fluctuations in many stem cell clones whereas a small number of more stable lineages come to dominate in the long term. (Capel *et al* 1989; Capel *et al* 1990; Jordan & Lemischka, 1990). Stochastic models would suggest that this phenomenon is due to progressive clonal extinction as HSCs randomly differentiate or terminally mature. Deterministic or intrinsic theories would see this as a pre-programmed fate of cells belonging to different sub-populations responsible for long and short term haemopoiesis.

The experimental evidence underlying these various hypotheses is substantial but tends in the main to rely on work from three areas: Animal models (mainly mouse), non-physiological in vitro culture assays where the complex effects of growth factors and the interactions with the haemopoietic microenvironment cannot be reproduced and finally, extremes of human physiology in the setting of reconstitution following

bone marrow transplantation. All data from such studies must be cautiously interpreted. In view of this, it is perhaps not surprising that there are many models which have fundamental differences and for which there are equally vociferous proponents. The behaviour of the HSC is likely to remain even more contentious than its definition and description for some time to come. Despite these difficulties, the consideration of kinetic models is important as it has major implications for the outcome of transplantation strategies such as purging (Champlin, 1996; Johnson *et al* 1996b), stem cell expansion (Haylock *et al* 1992b; Brugger *et al* 1993) and the survival of therapeutic genetic material introduced into these cells (Apperley & Williams, 1990a; Brenner, 1996).

Haemopoietic stem cell assays

Following the demonstration of colonies in the spleen of the mouse and the protection of animals from the otherwise lethal effects of radiation by marrow infusion (Lorenz *et al* 1951; Ford *et al* 1956; Till & McCulloch, 1961) assay systems have been devised based on in vitro growth of cells under a range of different culture conditions. More recently, work on the surface immunophenotype of HSCs has allowed these cells to be identified and sorted. It has been possible to combine phenotyping and culture experiments in an attempt to more accurately define stages of stem cell development (Terstappen *et al* 1991).

In-vitro culture assays

It is relatively easy to create conditions in which colonies of committed progenitor cells will grow from samples of human blood or marrow. Such assay systems exist for erythroid (BFU-E), granulocyte-macrophage (CFU-GM) and megakaryocytic (CFU-Mk) progenitors. In addition to this there are a number of techniques for the growth of cell types that have some or most of the features of HSCs. These more primitive cell assays fall into two main types depending whether the system measures the cells directly or by the secondary production of more mature daughter cells (Gordon, 1993). Direct clonogenic assays of primitive stem cells include the CFU-GEMM (which gives rise to granulocytic, erythroid, monocytic or megakaryocytic lines and may have a limited in-vitro capacity for self renewal) (Gordon, 1993), blast colony forming cell assay (Bl-CFC) (Nakahata & Ogawa, 1982), cobblestone area forming cell assay (CAFC) (Breems et al 1994) and the high proliferative potential colony forming cell assay (HPP-CFC) (Bradley & Hodgson, 1979). The Bl-CFC described by Ogawa shows both secondary replating ability and multilineage differentiation. These cells are almost all out of cycle and together these features suggest that it is a measure of a very primitive cell. Similar blast colonies can be seen if human marrow cells are seeded onto pre-formed stromal layers and are probably similar to the cobblestone area forming cell (CAFC) described in this setting in mice (Breems et al 1994). Subsets of these stromal adherent Bl-CFC/CAFC have been described which develop rapidly (5 days) or more slowly (21 days), the latter having more primitive features (Tucker et al 1993). The HPP-CFC were originally described in murine culture where they are characterised by the

ability to form large colonies of macrophages, their resistance to 5-FU, multilineage potential and haemopoietic reconstitution in vivo (Bradley & Hodgson, 1979). Human counterparts for this cell type have been described (McNiece *et al* 1989). Phenotypic studies and replating experiments suggest that the CAFC and Bl-CFC are probably ancestral to HPP-CFC and CFU-GEMM but they all show primitive features and probably represent overlapping populations of closely related cells. All such assays share an important limitation as there is no technique that can reproduce the degree of self renewal that occurs in vivo. This could imply that these assays are failing to detect the most primitive HSCs or simply that physiological cell behaviour cannot be seen in these artificial conditions.

Assays that rely on measurements of daughter cells (secondary clonogenic potential) include long term bone marrow culture (LTBMC) (Dexter *et al* 1977; Gartner & Kaplan, 1980), the long term culture initiating cell assay (LTCIC) (Eaves *et al* 1992) and delta assays, in which subsets of primitive cells are selected by adherence properties (Dowding & Gordon, 1992; Gordon, 1994) or 4-HC treatment (Smith *et al* 1991) and then cultured, following which their production of CFU-GM can be assessed. LTBMC consists of the maintenance of haemopoiesis by primitive cells within or adherent to a layer of stroma. This is done either by innoculating marrow onto tissue culture plastic where a stromal layer will form or by a two stage process where the stromal layer is pre-formed and irradiated after which cells of interest can be added. The LTCIC assay is a quantitative version of this principle in which the supernatant CFU-GM numbers are measured at 5 weeks of culture. The 5 week delay ensures the death of CFU-GM and more committed HSCs that might have been present in the innoculum at the beginning of the culture. LTCICs have been

estimated at 1-2 per 10,000 marrow cells or 1-2% of CD34+ve cells (Sutherland *et al* 1990). Their numbers have been shown to correlate significantly with CAFCs (Reading *et al* 1994).

Delta assays of adherent cells also delineate cell types with features of HSCs. For these assays, the cells of interest are pre-selected by adherence to stroma or plastic, washed to remove non-adherent committed progenitors and then cultured for a week after which the production of CFU-GM in the supernatant is measured (Dowding & Gordon, 1992; Gordon, 1994). An alternative to adherence is to treat the cell source with 4-HC and culture as above (Smith *et al* 1991). Cells capable of giving rise to CFU-GM in these systems have been shown to have features of very primitive HSCs and probably overlap with the LTCIC and CAFC/Bl-CFC (Gordon, 1993; Gordon, 1994; Gordon *et al* 1996).

Assays based on surface immunophenotype

The CD34 antigen is widely used to identify stem cells. It was discovered using strategies to identify surface markers for primitive cells representing minor MNC subsets present in marrow but scarce in peripheral blood (Civin *et al* 1984). Cells bearing this antigen can be rapidly quantitated by flow cytometry after staining with appropriate MABs and this has been adopted as the simplest and quickest technique for stem cell assay in blood or marrow, particularly for clinical use (Trischmann *et al* 1993; Siena *et al* 1993) (see chapter 2, sections 2.26 and 2.28 for methodology). CD34 is a heavily glycosylated transmembrane glycophosphoprotein belonging to the sialomucin family of surface molecules. It is expressed on around 1.5% of

marrow MNCs but on less than 0.5% of peripheral blood cells (Civin *et al* 1984; Krause *et al* 1996b). Expression on other tissues is debated but it is certainly present on small vessel endothelial cells and embryonic fibroblasts (Fina *et al* 1990). Its predicted structure shows no strong overall homology to other proteins. The cytoplasmic region of the molecule shows a very high degree of conservation between human, canine and murine CD34 suggestive of an important functional role (Krause *et al* 1996b). There are no enzymatic motifs in the structure but parts of the extracellular portion show similarity to molecules with known functions in cell adhesion. Interestingly, early data from CD34 knock out mice shows that they survive normally and have normal blood counts and differentials (Krause *et al* 1996b). Such animals can respond appropriately to radiation induced marrow suppression but show reduced colony formation in vitro. At present then, the exact function of CD34 remains uncertain. Two possible functional roles have been suggested, namely cell-cell adhesion and inhibition of stem cell differentiation. It is hypothesised that stem cells may bind to marrow stroma by a mechanism involving an L-selectin like molecule and CD34 and that such interactions could be critical to their localisation within the haemopoietic microenvironment and thus have functional significance (Baumheter *et al* 1993; Baumhueter *et al* 1994). There is also evidence that terminal differentiation of myeloid cells requires downregulation of CD34 and it may thus have a role in the differentiation process both in normal and leukaemic stem cells (Fackler *et al* 1995).

CD34 selected cells are of blast or small lymphocyte morphology (Figure 4.1) and are enriched for colony forming cells of all types including the primitive LTCIC and Bl-CFCs (Srour *et al* 1991; Terstappen *et al* 1991; Gordon *et al* 1996). By contrast,

in culture, the CD34-ve fraction is depleted for these cells. The CD34+ve cell fraction contains precursors capable of short and long term haemopoietic reconstitution as evidenced by transplantation experiments in animals and man (Berenson *et al* 1988; Berenson *et al* 1991). Culture of the CD34+ve subset yields colonies of progenitor cells at different stages of maturation and this antigen seems to be expressed on all primitive precursors up to and including more mature progenitors such as CFU-GM or BFU-E. In view of this heterogeneity there has been much interest in phenotypically subdividing CD34+ve cells into functionally different compartments. It has been shown that the level of expression of CD34 varies with maturity, the CD34^{bright} cells being more primitive than the CD34^{dim} (Andrews *et al* 1989; Bernstein *et al* 1991). Other antigens have been used to further subdivide the CD34+ve fraction. The combination of CD38 negativity and Thy-1 positivity with lack of lineage specific antigens such as CD13 and CD33 is seen on the most primitive precursors. (Srour *et al* 1991; Terstappen *et al* 1991; Morrison & Weissman, 1994).

1.2   Autologous Stem Cells in Haematological Malignancy

Rationale for autologous transplantation

During this century, the treatment of haematological malignancy with cytotoxic agents has progressed from relatively non-toxic single agent approaches through increasingly complex and myelosuppressive combination chemotherapy regimens (De *et al* 1972) to myeloablative therapy with marrow rescue from allogeneic or autologous stem cell sources (Perry & Linch, 1996). The considerable increases in toxicity have been offset at least in part by improvements in supportive care and greater understanding of the problems of the neutropenic patient. Allogeneic transplantation offers the additional potential benefit of immunological tumour kill and is thus not simply a form of dose intensification (Nash & Storb, 1996; Marmont, 1993). This form of therapy is however only applicable to patients of a relatively young age for whom an adequately HLA-matched marrow donor can be found. Even where this is available there is a substantial treatment related morbidity and mortality and consequently only a small minority of patients at presentation will ultimately undergo and survive such a procedure. This has led to increasing interest in supporting high dose treatment with autologous stem cells, historically from the patients marrow but now increasingly with cells mobilised into the peripheral blood following chemotherapy and/or growth factor administration (To *et al* 1997). Stem cell support allows high doses of cytotoxic drugs to be administered, achieving increased tumour cell kill in sensitive malignancies, which might be expected to translate into improved disease control and long term survival.

This theory has been tested in a wide range of haematological malignancies. Initial small series are being increasingly supplemented by larger clinical studies. In certain areas, the adoption of HDT with stem cell support may confer a true survival advantage but despite a large body of publications, definitive evidence for this is still sparse in the literature and much work is still in progress. In the malignant lymphomas, only the PARMA study (Philip *et al* 1995) has so far been able to show a survival advantage in 109 patients with NHL studied over a 7 year period. In myeloma there is emerging evidence for prolonged survival with HDT (Attal *et al* 1996). In the acute leukaemias there are many trials of autologous transplantation but as yet no consensus as to its advantage and in CML there is no prospective randomised data although a recent analysis of the literature has suggested a plateau in the survival curve after autologous marrow or stem cell transplantation (McGlave *et al* 1994b).

The ability to collect autologous stem cells easily from patients has other potential applications apart from supporting high dose therapy. Such cells could be manipulated in vitro or in vivo with the aims of reducing tumour cell contamination before reinfusion (Johnson *et al* 1996b), expanding the stem cell and/or committed progenitor pool to enhance engraftment characteristics (Haylock *et al* 1992b) or introducing therapeutic genetic material into a primitive cell in which it might continue to be usefully expressed after transplantation (Brenner, 1996).

Sources of stem cells for autologous transplantation

Stem cells can be obtained directly from the bone marrow cavity by needle puncture or via the peripheral blood using an apheresis machine. Both sources have been used extensively and there is clear data to show that they achieve rapid and sustained haematopoietic reconstitution following myeloablative treatment (To *et al* 1997). Despite this, there are important quantitative and qualitative differences between marrow and blood stem cells. These differences have been responsible for dramatic changes in clinical practice with the percentage of autologous transplants using blood rather than marrow cells rising from 15% to 75% in the first four years of the 1990's according to the European Blood and Marrow Transplant Group (EBMT) (Gratwohl *et al* 1996). The use of PBSCs lagged behind marrow by around 15 years, only becoming routine in the mid 1980's (To *et al* 1997). Previously, while it had been appreciated that there were small numbers of stem cells in peripheral blood, their use for transplantation had not seemed practical. Richman et al first demonstrated that their numbers could be transiently increased by chemotherapy (Richman *et al* 1976) suggesting that blood might be an adequate source. This process, discussed below, is termed stem cell mobilisation. Mobilised stem cells could be collected by apheresis and were subsequently shown to provide both rapid and durable engraftment (Mahendra *et al* 1996; Vesole *et al* 1996a; To *et al* 1997). PBSC collections have been shown to contain adequate numbers of primitive stem cells and greater numbers of more mature progenitors which are considered responsible for the shorter period of neutropenia seen following PBSCT (Bender *et al* 1991; Craig *et al* 1993). It is now accepted that PBSCs provide more rapid

engraftment and this has become the most important reason for the decline in the use of marrow in such procedures (To *et al* 1992). Most of the data confirming this has been by historical comparison with marrow transplant results although randomised trials have been done (Beyer *et al* 1995). In addition to this benefit, their use is generally preferred because patients find it easier to undergo stem cell mobilisation rather than a marrow harvest under general anaesthetic and there is evidence that the degree of tumour cell contamination may be less than that in the marrow in certain diseases, although this may not necessarily translate into improved survival (Sharp *et al* 1995; Henry *et al* 1996). In some cases there is the advantage of mobilising cells in patients who have had pelvic disease or radiotherapy and would be difficult to harvest conventionally for practical reasons.

In summary then, it is possible to use either marrow or blood stem cells to support HDT but PBSCs have now largely replaced marrow as the preferred source for reasons of convenience, rate of engraftment and perhaps the degree of tumour contamination.

Mobilisation and collection of PBSCs

In the early 1970's it was appreciated that progenitor cells could be transiently mobilised into the peripheral blood following administration of endotoxin or adrenocorticotrophin and after strenuous exercise (Barrett *et al* 1978). By 1980 it had been demonstrated that large numbers of CFU-GMs could be collected from the peripheral blood by leucapheresis (Korbling *et al* 1980) and it was then feasible to combine these two observations and successfully collect adequate progenitors from

the blood for transplantation. The first mobilisation regimens used myelosuppressive chemotherapy alone (To *et al* 1984). Later it was found that the combination of growth factors and chemotherapy enhanced the progenitor yield and since then it has become standard practice to combine chemotherapy with G-CSF or GM-CSF (Schwartzberg *et al* 1992). GM-CSF was the first growth factor used in this setting but is now less common, mainly due to an inferior side effect profile.

The phenomenon of progenitor cell mobilisation remains poorly understood. It is believed that it involves a perturbation of the normal adhesive interactions between haemopoietic cells and the marrow stroma which in health localise blood production to the marrow cavity (Simmons *et al* 1994; Turner, 1994). Primitive haemopoietic cells express a wide range of cell adhesion molecules (CAMs). The ligands for many of these are found on marrow stromal cells (Long, 1992). There is evidence of changes in expression and/or function of some of these ligand pairs after exposure to a range of factors known to induce mobilisation and also during stem cell maturation. Examples include the release of progenitor cells into the circulation after functional blocking of the β-1-integrin VLA-4 (Papayannopoulou & Nakomoto, 1993) and the observation that c-kit expression (the SCF receptor) falls during maturation of CD34+ve cells and is very low in mobilised peripheral blood CD34+ve cells compared to their marrow counterparts (Mohle *et al* 1993). Despite this latter observation, it has been shown that G-CSF is far less efficient at mobilising stem cells in mice deficient in SCF or its receptor (Steel/WW strains) suggesting that mobilisation may involve a number of different interactions (Cynshi *et al* 1991). This concept is supported by observations that in vitro interference with adhesion molecule function produces rapid but short lived effects whereas the in

vivo kinetics of G-CSF mobilisation in patients shows a time lag, with CD34+ve cells appearing in the peripheral blood after several days. The implication of this is that G-CSF administration sets off a chain of events leading to release of stem cells from the marrow, the basis of which is as yet unknown. A more complete understanding of the mechanism may, in the future, allow more efficient mobilisation, perhaps with patients benefiting from reduced apheresis requirements. An additional possibility might be the construction of mobilisation regimens which specifically release the cells of interest while reducing tumour cell trafficking in which CAMs have also been shown to play a part (Dorudi & Hart, 1993). This could allow a form of in vivo cell purging at the point of stem cell collection with reduction in tumour cell contamination.

The dosage of G-CSF used in conjunction with chemotherapy is usually in the range of 3-6µg/kg/day and is generally started the day after chemotherapy although it may be possible to delay the growth factor until the fifth or even eighth day post chemo with a cost saving (Haynes *et al* 1995). Chemotherapy alone produces a variable increase in progenitors up to 50 fold and this appears to be roughly proportional to the degree of myelosuppression (Schwartzberg *et al* 1992). The addition of growth factors was shown in one large series to double the mononuclear cell yield and increase the CD34+ve numbers 4-6 fold (Schwartzberg, 1993).

There is a substantial body of literature concerning the optimum form of chemotherapy to use in a mobilisation regimen. The most commonly employed is cyclophosphamide at doses ranging from 1.5 to 7 grams per square metre. As well as its long pedigree as a mobilisation agent, this drug has the advantages of low toxicity to non-dividing stem cells, a reasonable side effect profile and activity against a wide

range of tumours (To *et al* 1997). Other agents alone or in combination have been used with success and in many clinical settings it is possible to collect stem cells following the chemotherapy that is being administered for disease control rather than perform a separate mobilisation procedure (Craig *et al* 1992). This latter approach is convenient but may suffer from variable progenitor yields from different regimens and requires considerable flexibility regarding leucapheresis services.

Mobilisation using only growth factors (without chemotherapy) is applicable to some situations and cells collected by leucapheresis following G-CSF alone are capable of rapid haemopoietic reconstitution (Sheridan *et al* 1992). This approach is indicated for collecting cells from normal donors for allogeneic stem cell transplantation where chemotherapy toxicity would be unacceptable. G-CSF at a dose of around 10µg/kg/day for 4 days is the commonest regimen and studies have suggested that escalation of the dose beyond 16µg/kg/day does not improve yield. The side effect profile of G-CSF is good with mild to moderate bone pain and flu-like symptoms being seen in around 30% of subjects and occasional transient increases in liver enzymes. Currently available products vary in terms of their glycosylation and some suggest that the glycosylated form may be superior (Hoglund *et al* 1995). Clinical studies have shown both to be effective.

As well as G or GM-CSF, a number of other factors and combinations have been investigated including: SCF, SCF+G-CSF, IL3, Flt3 ligand and MIP-1α. These have all been shown in animals or humans to mobilise to some degree and there is continuing interest in combinations of such factors and the use of sequential therapy (Andrews *et al* 1994; Brasel *et al* 1995). Since the simple use of G-CSF is established and adequate for most clinical settings, clinicians are seeking approaches

that would improve the mobilisation in those cases where marrow damage from heavy pre-treatment has rendered cell yields poor.

How many stem cells are required ?

In the clinical setting there are essentially three types of assay that are applied for the assessment of harvest quality: MNC counts, semi-solid progenitor colony assays, and enumeration of CD34+ve cells by flow cytometry. As described above, both culture and flow cytometry can be used to attempt to separate and quantify the more primitive subsets of stem cells which in theory might be most relevant to stable long-term marrow reconstitution. Despite much work in this area, these culture assays remain more complex, expensive and time consuming and in many instances do not yield results in time to be clinically useful (for example the 5-8 week lag for the LTCIC assay). In routine clinical practice, it has not yet been found useful to look beyond the relatively simple CFU-GM or CD34 assay for the prediction of good engraftment from a harvested product and some workers find the simple MNC count adequate. There have been many reports concerning the relationship between MNC, CD34 and CFU-GM cell dose reinfused and speed and quality of engraftment following high dose treatment. Two thresholds have been established although there is some disagreement on the exact levels chosen. The lower threshold seeks to define a number of CD34+ve cells or CFU-GMs below which rapid and sustained engraftment may not occur. This level is around 20×10^4 CFU-GM/kg or $1\text{-}2 \times 10^6$ CD34+ve cells/kg patient body weight (Bender *et al* 1992; Weaver *et al* 1995). It follows that these are minimum rather than desirable results of harvest. With greater

numbers there is a steady improvement in rate of engraftment but there appears to be an upper threshold beyond which there is no further benefit. This probably lies around 50×10^4 CFU-GM/kg or $5\text{-}8 \times 10^6$ CD34+ve cells/kg (Weaver *et al* 1995).

Similar assays have been used to determine the best time to commence apheresis after mobilisation. Regimens using chemotherapy alone used the peripheral white count and commenced apheresis when this rose above 1×10^9/l. Later approaches using combinations of chemotherapy and growth factors usually aimed for a higher count of $2\text{-}5 \times 10^9$/l. and more recently this has been improved by the rapid estimation of CD34 numbers in the peripheral blood with most centres starting apheresis when the CD34 count is above $10\text{-}20/\mu l$. After G-CSF alone it is customary to collect on the 5th or 6th day when the white count will often be greater than 30×10^9/l.

In our centre, we have been studying the relationship between engraftment and other more 'stem cell' specific markers by flow cytometry including Thy-1 and L-selectin (CD62L). There does appear to be a correlation with sustained engraftment using these markers but whether this will be clinically useful is as yet uncertain (Rawstron *et al* 1998).

1.3   Manipulation of Haemopoietic Stem Cells

Background

It is clear from a consideration of the uses of stem cells described above that different cell products have different properties and it would be logical to collect and process them in such a way that the final result best suits the particular clinical need. Cells can be manipulated in a variety of ways and it is likely that in the future the reinfused products will be better characterised and more specific than is currently possible. Methods of cell manipulation of current clinical interest include: selection, expansion and genetic modification. These approaches are not mutually exclusive. For example, it might be possible to select a particular population, genetically modify it and then expand it ex-vivo before putting it to clinical use. At present such technology has not advanced sufficiently to produce definite clinical benefit but there is promising work in all of these areas.

Cell selection

Selection is the process by which the products derived from marrow or mobilised peripheral blood are altered either to enhance the content of a desired cell type (positive selection) or reduce and if possible eliminate unwanted cells (negative selection).   There is no reason why these processes should not be combined to maximise the purity of a final product.   The major applications of these techniques are in purging harvests of contaminating tumour cells in malignancy (Shpall *et al*

1994), altering the proportions of immune reactive T cells in allogeneic transplantation and treatment of autoimmune disease (Russell *et al* 1996; Snowden *et al* 1997) and selecting pure populations of repopulating cells for further manipulation such as expansion or gene therapy (Alcorn & Holyoake, 1996; Brenner, 1996). A wide variety of selection methods have been investigated both in vivo and in vitro. In vivo, specific mobilisation techniques have been shown to allow collection of Ph-ve stem cells in patients with CML (Carella *et al* 1997) and I describe a clinical study of one such novel regimen in chapter 3 (Johnson *et al* 1996a). More commonly, cells are selected after collection. Examples of such in vitro methods have included: specific culture conditions favouring survival of normal cells in CML (Barnett *et al* 1993), exposure of collections to cytotoxic agents active against malignant cells in AML (Rowe & Liesveld, 1996), complement-mediated lysis of T-cells induced by the Campath antibody before allogeneic transplantation to reduce graft versus host disease (Butturini & Gale, 1988), removal of contaminating tumour cells by immune adsorption in lymphoproliferative disorders using MABs to lymphoid antigens (Gribben *et al* 1991) and selecting specifically for the stem cell fraction in collections using antibodies to CD34 (Berenson *et al* 1991). Cell selection using the CD34 antigen (which was described in section 1.1) has been the most widely studied of all methods as it is present on cells with long term repopulating ability and is absent on most solid tumours (Krause *et al* 1996b). It is therefore ideally suited for tumour purging strategies and for producing cell products which are starting points for expansion and genetic modification.

Berenson et al demonstrated in 1988 that CD34 cells selected using MAB 12.8 could restore full haemopoiesis in lethally irradiated baboons (Berenson *et al* 1988).

Since then, there have been a number of studies demonstrating the clinical applicability of the technique and the adequacy of short and long term engraftment using CD34 selected products (Berenson *et al* 1991; Schiller *et al* 1995). I describe one such study in chapter 4 (Johnson *et al* 1996b) and the methodology of selection in chapter 2. A major part of the interest in this method of selection has revolved around its ability to achieve a tumour purge in harvested cell products. Outside the haemopoietic system, CD34 is expressed only on endothelial cells and some fibroblasts (Krause *et al* 1996b). It is not generally detectable in solid tumours nor in most lymphoproliferative disorders. The recent explosion of interest in intensive HDT with stem cell reinfusion in these diseases has led to research looking at MRD, harvest tumour cell contamination and the origins of relapse after autografting. It has been shown that malignant cells frequently contaminate PBSCHs and that in some diseases there may even be a specific mobilisation of tumour cells by the chemotherapy/growth factor combination (Rill *et al* 1994; Owen *et al* 1996; Brugger *et al* 1994). The gene marking studies of Brenner and others has shown that malignant cells reinfused in autografts can contribute to relapse in some cases (Brenner *et al* 1994). If tumour cells are present in harvests and can contribute to relapse, there is a rationale for the use of CD34 selection. The efficiency of purging by this method has been investigated (Cagnoni & Shpall, 1996). Ideally, one would wish to achieve adequate CD34 yields with a high purity correlating with a maximum tumour reduction for all patients. In practice, while CD34 yields appear clinically adequate from most studies, the purity is inconsistent. Clonal cells are still detected in some products after selection and malignant contamination below the level of assay detection is possible and may be relevant. Where it has been

quantitated, the log reduction in tumour burden has been quoted at between 2.7 and 4.5 (Schiller *et al* 1995). It seems unlikely that CD34 selection alone will adequately purge products and whether the current technology will impact on disease free survival is unknown (To *et al* 1997; Cagnoni & Shpall, 1996). Studies of HDT in breast cancer and lymphoma have suggested that survival is improved in those patients who are autografted with successfully purged products (Fields *et al* 1996; Gribben *et al* 1991) but these may simply represent the patients with low disease burden and no randomised trial of autografting with unmanipulated versus CD34 selected stem cells has yet been reported. Tumour reduction may be improved by combining positive CD34 selection with negative tumour depletion steps using MABs to tumour-specific antigens. While this may improve the purging efficiency, loss of cells occurs at all stages reducing yield and potentially affecting engraftment and the procedure becomes more costly and labour intensive. It is too early to say whether such strategies will find a place in clinical practice.

An alternative approach to post harvest stem cell selection has been reported by Tricot et al who have used high speed cell sorting to isolate a population of CD34+ve, Thy-1+ve, lineage marker negative cells which are thought to be phenotypically close to the pluripotent stem cell. They have autografted myeloma patients using this technology with some initial success but report problems with delayed engraftment and increased opportunistic infections (Tricot *et al* 1995; Siegel *et al* 1997).

Cell expansion

The use of cell culture systems to investigate the complexities of haemopoiesis has been discussed in section 1.1. The ongoing refinement of culture systems and the elucidation of the actions of a multitude of soluble agents such as growth factors and interleukins has opened the door to the possibility of clinical scale ex vivo cell expansion (Alcorn & Holyoake, 1996). It is now proving possible to expand cell numbers many fold, harvest the result and reinfuse them into patients following chemotherapy. The safety and practicality of this approach has been well documented (Silver *et al* 1993; Brugger *et al* 1995a) but it remains to be seen whether long term repopulating cells are expanded (or indeed preserved) by these systems (Srour *et al* 1993). Haemopoietic reconstitution after truly myeloablative chemotherapy has not yet been consistently achieved in human subjects using solely ex vivo expanded cell products (Holyoake *et al* 1995).

Theoretically, there are many applications for this technology (Alcorn & Holyoake, 1996). Stem cell expansion could shorten harvest procedures and allow useful products to be derived from inadequate collections in heavily pre-treated patients. The expansion of stem cells from a small PBSC collection might have a tumour purging effect as there is evidence that contaminating clonal cells do not grow in such systems (Brugger *et al* 1995b). It might also be possible to enlarge the stem cell content of cord blood samples allowing safe adult allo-transplants and to expand populations of genetically modified primitive cells as vehicles for gene therapy protocols. Large numbers of committed, lineage restricted cells could be produced

and infused after autografting to provide short term support pending engraftment and so reduce the period of severe neutropenia (Williams *et al* 1996).

Initially, studies concentrated on murine marrow and were aimed at delineating the factors that were most important for cell growth and differentiation in simple liquid culture systems. Combinations of IL3 and IL6 were the first to be used successfully and these remain important in most systems (Bodine *et al* 1989). Successive studies defined several other important agents, most notably: SCF, G-CSF or GM-CSF, IL1β and erythropoietin. These liquid culture systems yielded large increases in total cell numbers and, importantly, cell types associated with long term repopulating potential were also increased (Ploemacher *et al* 1993). Muench et al demonstrated that lethally irradiated mice could be rescued with allogeneic marrow cells that had been expanded ex vivo and that they retained long term donor derived haemopoiesis (Muench *et al* 1993). This and other murine studies suggested that ex vivo expansion might provide a cell source fitting all the criteria required for human transplantation.

A huge amount of resources have been targeted at clinical scale stem cell expansion in the last few years. It is important to note that for most such applications, purified CD34 cells have been the preferred cell source at input but that some workers have suggested that there may be advantages in using 'younger' stem cells derived from cord blood or foetal liver tissue (Traycoff *et al* 1995). These latter two sources may yield cells of greater ultimate proliferative capacity as suggested by their increased telomeric DNA (Vaziri *et al* 1994). Two basic approaches have been scaled up to a clinically practical level. These are: suspension cultures supplemented with cytokines and stromal-based cultures. The former are simply modifications of the

original liquid culture methods described above with optimised conditions. Cells are generally cultured for between 8 and 21 days in such systems with many fold increases in total numbers (Haylock *et al* 1992a). Progenitor cells also increase under these conditions but their numbers tend to fall off approaching 21 days presumably as more primitive cells are 'exhausted' through differentiation. A vital question concerning this approach is whether true repopulating cells are maintained or increased during the process. Data is conflicting but CD34 estimations and LTCIC assays suggest that this may in fact not occur (Williams *et al* 1996; Srour *et al* 1993). This is worrying and as yet there is virtually no clinical data available. The alternative approach is to use a stromal-based culture system. This is conceptually appealing as it seeks to mimic the interactions that occur in the marrow microenvironment. Liquid cultures by contrast require a better understanding of the soluble factors which stroma releases and cannot supply the potentially important cell-cell interactions. The hope is that 'physiological' stroma-based methods may achieve expansion of the most primitive cells which must be a major goal. The systems are, however, considerably more complex and expensive. There is now emerging evidence that such stromal cultures, scaled up in bioreactors with constant perfusion of medium and the necessary stimulating factors can lead to expansion of LTCIC (Koller *et al* 1995).

While such products have been successfully transfused with no toxicity into patients there is still no firm clinical evidence that current expansion techniques can produce adequate long term haemopoietic reconstitution at a clinical scale (Holyoake *et al* 1995).

The goal of research at present is to elucidate the factors and conditions required to reliably expand primitive stem cell numbers in a simple practical system. If this is achieved it could have major implications for transplantation and the delivery of gene therapy.

1.4   Cell Modification - Gene Therapy

Introduction

Gene therapy may be defined as the introduction of new genetic material into a cell in order to change that cell's phenotype. Since recombinant DNA technology became available it has been rapidly incorporated into medicine with a range of new diagnostic tools and therapeutic products. Although the application of the new genetic understanding to gene therapy appeared obvious, it has required a concerted and co-ordinated development in diverse areas of scientific and medical interest to reach clinical practice and the earliest phase one clinical studies are only now being reported (Boucher *et al* 1994; Grossman *et al* 1994; Malech *et al* 1995). This difficulty reflects the many pre-requisites of any effective gene therapy protocol which include: accurate molecular definition of a disease and choice of the genetic target, the construction of novel therapeutic genetic material and associated regulatory factors if required, a suitable vector with which to introduce this material and a mechanism for targeting this vector to the required tissue in which stable

integration and expression of the gene over a useful timescale and at an effective level will be required (Dunbar, 1996a; Flotte & Ferkol, 1997).

Applications

The range of human disease that could theoretically benefit from such an approach is wide and growing (Apperley & Williams, 1990b; Dunbar, 1996a). The first results of a clinical gene therapy protocol were reported in 1989. This was a gene marking experiment to investigate the fate of modified lymphocytes in malignant melanoma (Rosenberg *et al* 1990). More than 100 clinical trials in human gene transfer have been approved in the USA alone since then, some of which are reporting preliminary data (Flotte & Ferkol, 1997). The majority of these protocols are in malignant disease. These include: gene marking studies, attempts to improve tumour immune recognition ('tumour vaccine') by transducing tumour cells with activating cytokines such as IL2, the introduction of suicide genes to cancer cells such as HSV thymidine kinase and attempts to alter drug sensitivity by manipulating drug resistance gene mechanisms. Outside the field of malignancy, gene therapy can be applied to genetic disorders which result in low or absent levels of a protein or enzyme for example: Cystic Fibrosis, Chronic Granulomatous Disease, the Haemophilias, Gauchers disease, Fanconi's anaemia and SCID due to adenosine deaminase deficiency. Certain other acquired, infectious or multifactorial diseases have also been investigated such as AIDS, peripheral vascular disease and rheumatoid arthritis. The target tissue and choice of vector will depend on the disease and the chosen approach. Examples of such varied approaches include the re-implantation of ex-

vivo transduced hepatocytes to correct familial hypercholesterolaemia (Grossman *et al* 1994) and the in vivo transduction of lung epithelial cells by aerosolised vector in cystic fibrosis (Boucher *et al* 1994).

Haemopoietic stem cells as targets for gene therapy

Many gene transfer protocols have focused on the haemopoietic stem cell (Dunbar, 1996a). These are highly desirable targets due to their capacity for self renewal and multilineage differentiation. They can be easily collected from blood or marrow, genetically manipulated and reinfused into patients where their kinetic properties would in theory allow long term maintenance of a constantly proliferating cell source containing the new gene. In addition, as has already been discussed above, these cells might be purified and/or expanded ex vivo before or after genetic alteration to improve transduction efficiency and increase numbers for transplantation (To *et al* 1997; Holyoake & Alcorn, 1994; Alcorn & Holyoake, 1996). There has been a considerable amount of work on gene transfer to animal and human haematopoietic stem cells over the last decade. Some fundamental problems have complicated the research including the difficulty of isolating true long term repopulating cells for which there is no specific assay and the natural quiescence of these cells which renders them resistant to transduction by some vectors (Miller *et al* 1990). The same properties complicate selection and expansion of these cells with the result that the most desirable cell target for gene therapy has proved by its very nature the most inaccessible.

Vectors for gene transfer

A vector is a means to introduce novel DNA sequences into a target cell. They can be broadly divided into viral and non-viral and table 1.1 lists some of the vectors used in gene transfer both in vivo and in vitro. Currently, viral vectors remain the most efficient vehicles for DNA delivery. Key questions remain to be answered concerning the available viral agents. Firstly, do they have the ability to transduce the most primitive stem cells? Secondly, what design of vector and which conditions are optimal for maximising transduction efficiency? Thirdly, are the kinetics of primitive cells likely to be altered by the process of achieving efficient transduction such that they will lose some or all of their self-renewal capacity and be stimulated to differentiate? Finally, what implications will the answers to these questions have for the duration of stable transduction and long term gene expression in vivo? An idealised vector for stem cell transduction would be easily produced in high titre, free of helper (wild-type) virus, non-pathogenic to man, capable of packaging large DNA inserts, efficient and stable at integration, invisible to the host immune response and capable of transducing non-dividing cells. None of the currently available candidates fulfils all these criteria.

Retroviral vectors such as those based on the MoMLV have been the most widely studied. It is now over 13 years since it was first demonstrated that gene vectors based on murine leukaemia viruses were able to successfully incorporate specifically designed genetic material into murine haemopoietic stem cells with subsequent expression of the new gene in cells of both myeloid and lymphoid lineages (Williams *et al* 1984b).

Retroviral vector production based on the MoMLV is depicted in figure 1.2. A packaging cell is produced by transfection with a MoMLV whose packaging sequence (ψ) has been deleted such that no intact viral particles can be produced. When this cell line is transfected with the engineered retroviral construct, a producer cell line is created. The gag, pol and env genes of the modified virus are replaced by the DNA insert of choice but the ψ sequence is intact allowing the virus to utilise the gag, pol and env of the packaging cell to create infective viral particles (Miller, 1990). These are released into culture supernatant and can be collected and used for transduction. Details of methodology related to this process are described in chapter 2. Retroviral vectors enter target cells via specific cell receptors, following which their RNA undergoes reverse transcription using the reverse transcriptase enzyme from which the virus name is derived (Coffin, 1990). The cDNA so produced enters the nucleus upon mitosis and is stably integrated into host DNA. Since the vector has no gag, pol or env genes, the resulting replication products cannot form new infective viral particles and are thus termed replication defective. The target cell is capable of continuing production of the engineered gene without further infective risk to other host cells. Recombination events within producer lines could, however, lead to the production of wild-type virus and refinements of technique are required to safeguard the process (Markowitz *et al* 1988b). Retroviral vectors have the attractive property of stable nuclear integration (Coffin, 1990) and they have been the most studied system to date. They are however limited in the insert size they can accept (up to 7Kb) and are in general of fairly low titre (10^5-10^7 cfu/ml).

Figure 1.2

Diagram depicting the production of a retroviral producer cell line. The packaging line is created by transfection with an enfeebled MoMLV from which the Ψ sequence has been deleted. The lack of this sequence means that this cell line contains the genes encoding for the retroviral gag, pol and env proteins but cannot produce intact particles. A new retroviral construct is then created in which the gag, pol and env genes are excised and replaced by the desired DNA insert. This construct has a normal Ψ sequence. When the packaging cells are transfected with this, they are able to supply the gag, pol and env proteins for assembly by the Ψ sequence into intact, infective viral particles containing the new insert. This is now a producer cell line. The particles released can only infect one cell as they will not be able to produce their own gag/pol/env for production of new virus. The insert is however integrated into the genome and is available for transcription by the infected cell.
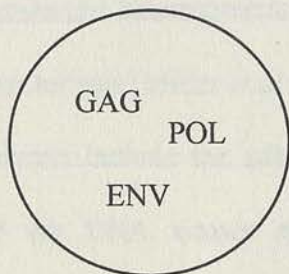
'ENFEEBLED' RETROVIRUS

5'LTR	Ψ	GAG	POL	ENV	3'LTR

Delete Ψ sequence

Transfection of a cell line

GAG
POL
ENV

PACKAGING CELL LINE

RETROVIRAL CONSTRUCT

5'LTR	Ψ	DNA INSERT	3'LTR

GAG/POL/ENV replaced
with chosen DNA insert

Transfection of the
packaging cell line

Ψ
GAG/POL/ENV
DNA INSERT
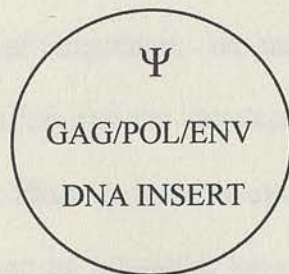
PRODUCER CELL LINE

Intact, infective viral perticles
containing DNA insert
but no GAG/POL/ENV
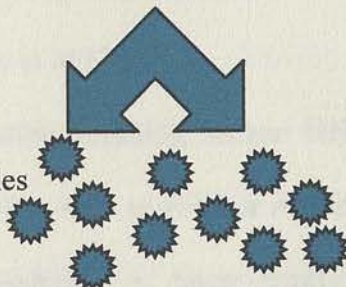
Target cell specificity can be altered by the use of vectors with different envelope glycoproteins, termed retroviral pseudotypes and there is evidence that efficiency of transduction is improved with certain strains, for example the gibbon-ape leukaemia virus (GALV) (Miller *et al* 1991). Perhaps most importantly, the physical barrier of the nuclear membrane appears an absolute one at present and it seems unlikely that truly quiescent cells such as pluripotent haemopoietic cells could be transduced using these viruses unless they enter mitosis (Miller *et al* 1990).

Other viral vectors of current interest include the adenoviruses (AV) and adeno-associated viruses (AAV). AV are DNA viruses responsible for mild upper respiratory illness in man and enter cells by receptor mediated endocytosis after which they induce lysosomal disruption and are then free to enter the nucleus (Horowitz, 1990). Replication defective AV vectors can be produced by deletion of viral genes (E1a, E1b and E3) and replacement with the relevant construct. They have the advantage of infecting non-dividing cells with great efficiency and being produced in high titre (Jaffe *et al* 1992). Despite this, there are problems with transient expression due to lack of integration, the induction of an inflammatory epithelial response in animal studies and the development of a strong immune response to repeated exposure. Phase 1 trials in cystic fibrosis where repeated aerosols of vector are administered by inhalation have confirmed these unwanted effects in human subjects (Boucher *et al* 1994).

AAV are non-pathogenic, replication defective, human DNA parvoviruses which require co-infection with helper virus such as HSV or AV for productive infection. In the absence of these, AAV establishes a latent infection in the host genome through site specific integration on chromosome 19 (the AAVS1 site) (Chatterjee *et*

al 1995). This integration is very stable and has been maintained in tissue culture for over 100 passages. The promise of a harmless, high titre vector which achieves stable site specific integration and can infect non-dividing cells has made this an appealing virus for study. Some initial hopes may be unrealistic however as the genetically engineered vectors do not appear to share the site specific integration of the wild type virus, increasing the possibility of insertional mutagenesis. It has also been found that transgene persistence after transduction may not always reflect stable integration (Flotte *et al* 1994; Russell *et al* 1994). Despite these doubts, they are currently an important area of study.

Efficiency of gene transfer into stem cells

The majority of available data concerns retroviral vectors. In animal stem cell transduction experiments, it has been possible to achieve expression of transgenes for at least 6-12 months, with the best results in murine models (Wilson *et al* 1990; Correll *et al* 1992). The efficiency of transfer has been improved by prior concentration of primitive cells using pre-treatment with cycle-specific cytotoxic agents and stimulation with cytokines (Bodine *et al* 1991). Under optimal conditions, with selected stem cells, growth factors and co-culture with viral producer cell lines, murine LTCICs have been successfully transduced, with up to 50% of daughter cells containing the marked gene (Fraser *et al* 1990). Similar experiments in larger animals have however been less successful. Although multilineage marking can be achieved, the proportions of marked cells are low at around 0.1-1% of marrow cells (Schuening *et al* 1991).

Table 1.1   Vectors for Gene Transfer

Non-Viral Vectors	Viral Vectors
Calcium phosphate precipitation (MLV)	Murine Leukaemia viruses (MLV)
Electroporation	Adenovirus
DMSO shock	Adeno-associated virus
Microinjection (single cell)	Herpes Simplex Virus
Liposome-mediated	MLV pseudotypes
Receptor-mediated transfer	

Human in vitro data has followed this work and high transduction efficiencies (often >50%) of CD34+ve selected cells from marrow, blood or cord blood have been achieved using progenitor cell assays as markers of success. Optimal transfer is again dependent on the addition of growth factors and/or stromal elements (Nolta & Kohn, 1990; Moore *et al* 1992). The most important question this raises is the extent to which we can infer from these assays that true repopulating cells are transduced. In addition to these surrogate assays, answers may come from long term gene marking studies in vivo. Trials have been reported showing persistence of marker genes in patients following marrow transplantation with ex vivo retrovirally transduced cells (Brenner, 1996; Dunbar *et al* 1995). Some patients remain PCR positive for the reporter gene in multiple lineages for over 18 months following the procedure but again, the proportion of marked cells is generally low. Clinical protocols attempting stem cell retroviral transduction and reinfusion using active gene constructs are underway, notably in SCID/ADA deficiency, Fanconi's anaemia and chronic granulomatous disease (CGD). There are preliminary reports of persistent detection of the therapeutic genes in vivo (Malech *et al* 1995).

The study of AAV gene transfer is less advanced than that of retroviruses. Despite this, there is evidence that lethally irradiated mice reconstituted with AAV transduced cells retain multilineage gene expression for >6 months, implying that primitive stem cells can be targeted (Podsakoff *et al* 1994). In vitro data is available for human stem cell transduction and suggests that AAV vectors are highly efficient at transferring genes to CD34+ve cells, cord blood and LTCIC (Zhou *et al* 1994; Goodman *et al* 1994). These and other vectors may offer considerable future promise.

In summary, the field of gene therapy is in its clinical infancy with only phase 1 data available and many trials underway. Viral vectors remain the most efficient methods of gene transfer but much is still unknown concerning the life cycle of these agents and their mechanisms of infection, integration, persistence and immunogenicity. The goal of long term, stable, controlled gene expression is still elusive but it seems increasingly likely that this will ultimately be achieved.

1.5   PNH: Stem Cells and Gene Therapy

PNH is caused by an acquired somatic mutation in a haemopoietic stem cell which leads to the co-existence of normal and 'PNH' haemopoiesis. It is therefore an interesting model disease in which to study normal and abnormal stem cell behaviour and the possibility of gene replacement as therapy.

History and clinical features of PNH

The molecular biology of this rare but fascinating disorder has recently been clarified (Miyata *et al* 1993; Rosse & Ware, 1995) but as a clinical entity it has puzzled physicians and scientists for two hundred years. The first probable description of the disease in the literature was an "Account of a singular periodic discharge of blood from the urethra" written in 1794 by a Scottish surgeon, Charles Stewart (Stewart,

1794). Several case reports in the nineteenth century probably describe cases of PNH but the first detailed description is credited to Paul Strübing in 1882 (Strubing, 1882). The rarity of PNH probably accounts, at least in part, for the delay between the first reference to a probable case and the recognition of the disorder as a distinct clinical entity. Strübing's description was of a 29 year old man with a six year history of the passage of brown-black urine intermittently in the mornings, always clearing by noon. His conclusions were detailed and astonishingly perceptive, suggesting as he did that there was intravascular haemolysis with persistent haemosiderinuria and that some of the patient's symptoms were due to thrombosis. He even concluded (correctly) that a red cell defect was to blame and that attacks were precipitated by sleep. Despite this insight, the work was largely ignored and when Marchiafava reported a case in Italy 29 years later it was regarded as a new entity (Marchiafava & Nazari, 1911). Marchiafava published a further case in 1928 and showed that both patients had haemosiderinuria as a clinical feature. He named this 'new' disease "chronic haemolytic anaemia with perpetual haemosiderinuria" (Marchiafava, 1928). Over the next 3 years, Micheli studied these cases and others from the literature and published his observations in 1931 in which PNH acquired the new title of "splenomegalic haemolytic anaemia with haemoglobinuria and haemosiderinuria, Marchiafava-Micheli type" from which the disease retains the eponym to this day (Michelli, 1931). The modern term PNH was in fact first coined shortly prior to Micheli's paper in a description of a case from the Netherlands by Enneking (Enneking, 1928).

The clinical features of PNH can be considered under two broad headings. Firstly there is the strong association with aplasia (first described in 1944 in a patient with

Fanconi's anaemia and PNH (Dacie & Gilpin, 1944)) and the possible link with subsequent clonal malignancy in the form of MDS or AML (Tichelli *et al* 1988) and secondly there are the classical features of haemolysis and thrombosis which led to the first descriptions of the syndrome described above (Socie *et al* 1996). Aplasia is probably always present to some degree but may range from the dominant clinical manifestation with anaemia, infections and haemorrhage to apparent normality of cell counts with only laboratory culture evidence of reduced marrow reserve. It is clear that PNH can co-exist with, antedate or post-date an episode of hypoplasia (Dacie & Lewis, 1961; Dacie & Lewis, 1972) and that the aetiology of the hypoplasia (idiopathic, viral or drug induced) is not specific to the development of PNH (De Planque *et al* 1989). Even in cases when there is insufficient evidence to diagnose AA, an isolated cytopenia (most commonly thrombocytopenia) is very common. Where MDS or AML occur they will usually supervene over any other features and frequently lead to the patient's death. It is uncertain whether PNH itself is truly pre-leukaemic. Cases of AML and MDS have been reported, some where the malignant cells have been of PNH phenotype and some where they were normal (Shichishima *et al* 1993; Stafford *et al* 1995). In view of this it might be more appropriate to take the view that patients with AA may go on to develop PNH or AML/MDS rather than to consider PNH as a separate, pre-leukaemic disorder.

Patients presenting with classical PNH are mostly young adults (median age 35 years) although cases at both extremes of life have been reported. There may be a slightly higher incidence in females. There is no reported racial variation (Sirchia & Lewis, 1973; Krautrachue *et al* 1978). Haemolysis is common and results in the classical episodes of haemoglobinuria that first drew attention to the disease. The

anaemia is related both to the degree of haemolysis and the extent of aplasia and ranges from a normal Hb level to severe recurrent anaemia requiring multiple, life-long transfusions (Hillmen *et al* 1995). Thrombosis is the other cardinal clinical feature. Panton, in 1924 was the first to describe this association and it has been confirmed in many series since (Panton *et al* 1924; Dacie & Lewis, 1972). Although it is largely venous, occasional reports of unusual arterial occlusions in patients of young age without vascular risk factors suggest that this is not exclusive. The commonest sites are the hepatic (Budd-Chiari syndrome), portal, intestinal, pulmonary and deep peripheral veins but at autopsy there have been studies showing thrombosis in many unusual sites such as veins of the pancreas, omentum, seminal vesicle and bladder (Dacie & Lewis, 1972; Peytremann *et al* 1972). Physical signs in PNH are non-specific and largely related to complications with the exception of modest splenomegaly which can be found in nearly half of patients. There is a spectrum of disease from acute haemolysis or thrombosis and little or no evidence of aplasia to patients with severe AA or other haematological disorders who are noted on investigation to have detectable PNH clones by laboratory testing only.

Hillmen et al have examined the records of 80 consecutive PNH patients registered at the Hammersmith hospital in London between 1940 and 1970 giving an insight into the long term clinical course of the disease (Hillmen *et al* 1995). In this paper, the median age at diagnosis was 42 years, with 84% of patients having episodes of frank haemoglobinuria as a chief symptom at some stage of their illness. A diagnosis of aplasia was made prior to the diagnosis of PNH in 29% and 5 of the patients who presented with frank haemolytic PNH subsequently developed aplasia. The median survival from diagnosis was 10 years which was substantially less than that of age

and sex matched controls. 58% of the 60 deaths to date in this cohort were directly attributable to PNH, with thrombosis being the commonest cause and Budd-Chiari syndrome the commonest fatal thrombotic event. In total, 39% of all patients suffered a thrombosis during follow up. Interestingly, spontaneous remission was noted in 12 cases in this series and none of the 80 patients developed MDS or AML.

Pathophysiology of PNH

Initial attempts to explain the clinical features understandably concentrated on the visible haemolytic component. With modern techniques it is now appreciated that individuals can have PNH cells detectable in the absence of typical clinical features and that there is a strong relationship with AA. Furthermore it is appreciated that normal and 'PNH' haemopoiesis co-exist in the marrow, the proportion varying from case to case and over time in individuals (Rosse, 1973). The underlying cause of PNH is an acquired genetic mutation (Miyata *et al* 1993). Most workers believe that a degree of marrow damage/failure is an essential pre-requisite for PNH to develop in the presence of this mutation and that clinical sequelae depend on the nature of the initiating insult and the proportion of the patient's haemopoiesis that is PNH (Bessler *et al* 1994b). An understanding of the pathophysiology of the disease requires knowledge of this genetic defect, the pressures that lead to the development of PNH following mutation, the biochemical consequences of the defect and the mechanisms by which this translates into the clinical phenotype.

Attempts to understand the basis of the haemolysis began in Rotterdam in 1911 when Hÿmans van den Bergh noted that PNH erythrocytes were sensitive to lysis in
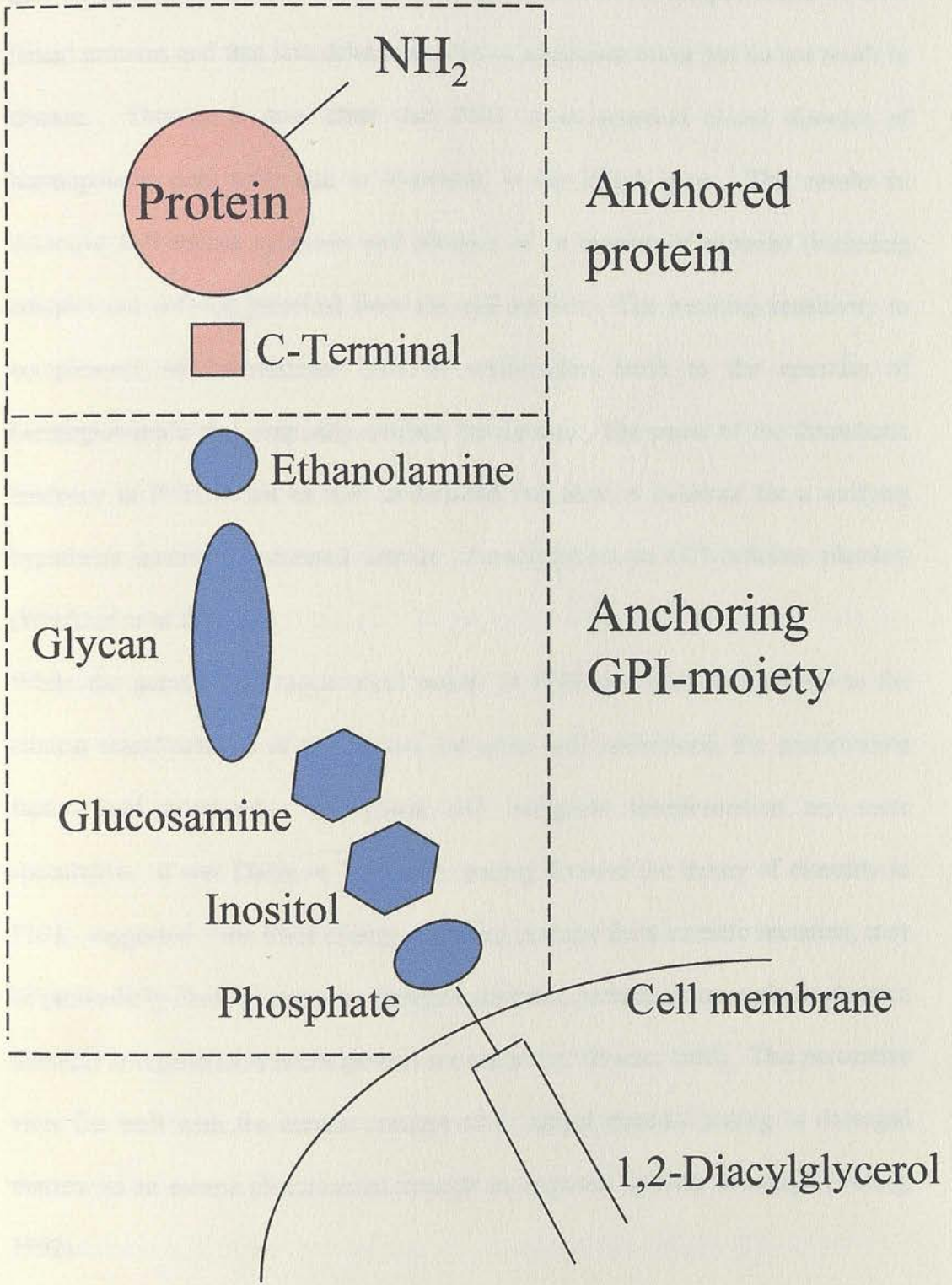
vitro when exposed to carbon dioxide (Hymans van den Bergh, 1911). By the 1930s, it had been shown that the erythrocyte lysis was caused by complement and was pH dependent with optimal lysis in acidified conditions (Dacie *et al* 1938). Despite a substantial body of laboratory work clarifying the haemolytic defect in PNH erythrocytes it was not until the late 1980s that a cellular mechanism could be hypothesised. Following earlier observations in the 1950s that some membrane bound proteins were absent from PNH cells, a diverse range of missing proteins was described (Rotoli *et al* 1993). Some of these (particularly DAF and MIRL) were involved in the regulation of complement activity at cell surfaces and it was presumed that their absence led to the erythrocyte sensitivity and subsequent haemoglobinuria. The initial belief that DAF (CD55) was the important molecule was proved incorrect when it was shown that individuals of the so called Inab red cell phenotype, who have an inherited DAF deficiency, had no clinical illness or in vitro red cell complement sensitivity (Telen & Green, 1989). MIRL (CD59) was the next candidate. This is an 18kD protein which inhibits formation of the complement membrane attack complex. When red cells were treated with blocking antibodies to CD59, they were indeed rendered complement sensitive. Further evidence for the significance of this molecule came in 1992 when a 22 year old man was described who had a homozygous deficiency of CD59 on all haemopoietic cells and suffered PNH-like symptoms with haemolysis and a cerebral thrombosis (Yamashina *et al* 1990). It seems likely that the haemolytic and thrombotic features of PNH are mediated by complement sensitivity and that CD59 deficiency is an important cause of this.

A major step in appreciating the underlying disease pathology came in 1986 when it was shown that all the proteins absent in PNH were attached to the membrane by a glycolipid (GPI) anchor (Davitz *et al* 1986). It was clear from this that the defect in PNH cells lay in the GPI structure, the details of which are depicted in Figure 1.3. Studies using labelled intermediates have demonstrated that GPI synthesis in PNH cells is almost absent due to failure of addition of N-acetylglucosamine to phosphatidyl-inositol early in the biosynthetic pathway, although the exact detail is as yet unclear (Hillmen *et al* 1993).

A gene whose cDNA is able to correct the GPI synthetic defect in all human cell lines transfected to date has now been discovered and cloned (Miyata *et al* 1993). This gene is situated on the short arm of the X chromosome, contains 6 exons and codes for a protein of 54kD in size which has homology to some plant glycosyl transferases. The gene has been named PIG-A, a term derived from its ability to restore the GPI synthetic defect in class A murine cell lines which belong to the same complementation class as lymphoblastoid cell lines derived from PNH patients (Bessler *et al* 1994a). Since the gene was cloned, several workers have sought mutations in PNH patients and it has become clear that this is a consistent finding in the disease. A recent report summarised the mutations found to date (Rosse & Ware, 1995). 84 mutations were found in 72 patients but the list is growing rapidly. The commonest are deletions or insertions which between them account for well over half. Most are small (often single nucleotide) but 2 large (>100 nt) deletions have been described. A number of patients have been described in whom there is more than one mutation in the gene (Rosse & Ware, 1995; Bessler *et al* 1994b).

Figure 1.3

Diagram of the GPI anchor. Proteins anchored through this structure are absent in PNH cells. The PNH defect appears to be at an early stage in the synthesis of the structure and involves failure of the addition of N-acetyl glucosamine to phosphatidyl inositol.

NH₂ → NH$_2$

Protein

C-Terminal

Anchored protein

Ethanolamine

Glycan

Anchoring GPI-moiety

Glucosamine

Inositol

Phosphate

Cell membrane

1,2-Diacylglycerol

Since all the mutations described to date abolish or severely impair GPI synthesis, it may be that the development of PNH requires an almost complete lack of GPI-linked proteins and that less deleterious PIG-A mutations occur but do not result in disease. Thus it is now clear that PNH is an acquired clonal disorder of haemopoietic stem cells due to mutations in the PIG-A gene. This results in defective GPI-anchor synthesis and absence of a number of proteins (including complement defence proteins) from the cell surface. The resulting sensitivity to complement and subsequent lysis of erythrocytes leads to the episodes of haemoglobinuria that originally defined the disease. The cause of the thrombotic tendency in PNH is not as well understood but there is evidence for a unifying hypothesis involving increased activity of complement on GPI-deficient platelets (Wiedmer *et al* 1993).

While the genetic and biochemical events in PNH and their relationship to the clinical manifestations of the disease are quite well understood, the precipitating factors and relationship to aplasia and malignant transformation are more speculative. It was Dacie in 1963 who, putting forward the theory of clonality in PNH, suggested "the PNH change, resulting perhaps from somatic mutation, may be particularly likely to occur in damaged marrows, perhaps where early or abortive attempts at regenerative haemopoiesis are occurring" (Dacie, 1963). This perceptive view fits well with the current concept of a clonal disorder arising in damaged marrow as an escape phenomenon through an acquired growth advantage (Young, 1992).

There is now considerable support for the idea that PNH clones proliferate in the presence of external selection pressure which favours expansion of GPI-deficient

cells. There are several lines of evidence for this: The strong relationship with marrow failure or disruption that has already been discussed is one. PNH haemopoiesis is clearly able to thrive in a marrow whose normal stem cell pool is severely diminished. Molecular evidence in the presence of more than one (sometimes several) independent PIG-A gene mutations in different PNH lines in the same patient implies that there is a selection pressure operating for GPI deficient cells (Bessler *et al* 1994b). Culture experiments do not suggest any intrinsic growth advantage for GPI deficient cells, indeed there is generally a reduction in the numbers of progenitor colonies in PNH blood and marrow (Rotoli & Luzzatto, 1982). Two 'dynamic' experiments are worthy of mention. Firstly, it has been shown that mononuclear cells from PNH patients can inhibit the growth of normal progenitors in culture but that this inhibition is removed if the normal cells are stripped of their GPI-linked proteins by PI-PLC treatment before culture (Rotoli *et al* 1995). This is direct evidence that a cell (probably a CD8+ve T cell) mediated suppression of the marrow may be operating in AA/PNH to which GPI deficient cells are partly resistant. Secondly, patients treated with the CAMPATH 1H antibody have been shown to develop GPI deficient T cell clones. This antibody causes lysis of CD52+ve cells and as this antigen is GPI linked there is a strong selection pressure for CD52-ve cells. The frequency with which this has been observed suggests that PIG-A mutations may be common in normal individuals and merely require suitable selection pressure for such a clone to be manifest. In our institution, we have been able to demonstrate that the PIG-A mutation in one such CAMPATH treated patient was present pre-treatment in stored blood by using an allele specific PCR (Rawstron *et al* 1997b).

In summary, PNH is an acquired stem cell disorder due to mutation of the PIG-A gene. The lack of GPI anchored proteins (particularly CD59) on PNH cells probably accounts for the clinical features of the disease and may well also provide a basis to understand the mechanism by which PNH haemopoiesis can develop in damaged or hypoplastic marrow.

Treatment options in PNH

Treatment can be directed at the disease process itself or the clinical manifestations. The former requires immunosuppression or bone marrow transplantation in a similar way that one would approach the treatment of severe AA (De Planque *et al* 1989). The success of this approach is itself supportive evidence for the theory of PNH pathogenesis described in the preceding section. As an alternative or additional mode of treatment, supportive therapy is available for some of the clinical manifestations including cytopenias and thrombosis.

Supportive therapy includes the use of folic acid, blood transfusions and iron chelation for the treatment of anaemia, anticoagulation for the prevention of thrombosis and perhaps the use of growth factors where cytopenia is severe (Ikeda *et al* 1993; Ninomiya *et al* 1993). There have been reports of improvements in all cell line counts and of the disappearance of PNH clones after administration of G-CSF and Erythropoietin but no controlled trials exist to quantitate this effect and distinguish it from the natural fluctuations of the disease and occasional spontaneous remissions that are seen (Balleari *et al* 1996; Bourantas, 1994). Whether growth factors impact on survival in PNH is unknown.

Immunosuppression can be effective in the management of PNH associated with severe aplasia just as it is in AA without a PNH clone (Stoppa *et al* 1996; van *et al* 1995). Combinations of anti-lymphocyte globulin, steroids and cyclosporin A are used. Steroids alone are used in the treatment of PNH by some clinicians but their effectiveness is in doubt and has never been proven in a randomised trial.

Allogeneic bone marrow transplantation is a more radical approach to treatment and the usual indication in PNH has been the coexistence of AA. Storb et al performed the first reported transplant in 1973. This patient achieved remission with a fully functioning marrow graft and no PNH clone (Storb *et al* 1973). Since then there have been a number of further cases of PNH with associated AA which have been transplanted (Kawahara *et al* 1992) but the high mortality and morbidity of the procedure makes it generally unsuitable for the treatment of patients with classical PNH who lack significant cytopenias.

Syngeneic transplantation for PNH has been reported by several workers either with or without conditioning chemotherapy (Graham *et al* 1996; Endo *et al* 1996). To date, 8 out of the 9 cases 'transplanted' without conditioning have relapsed with PNH despite initial responses. Those given chemotherapy appear to sustain their remissions. This observation is in keeping with the hypothesis previously stated that AA and subsequent PNH is caused by an autoimmune stimulus and that part of the successful treatment is to eradicate this either by immune suppression or myeloablation. In the unconditioned syngeneic cases the donated normal marrow was presumably subject to the same autoimmune destruction and the PNH clone again thrived under this selection pressure.
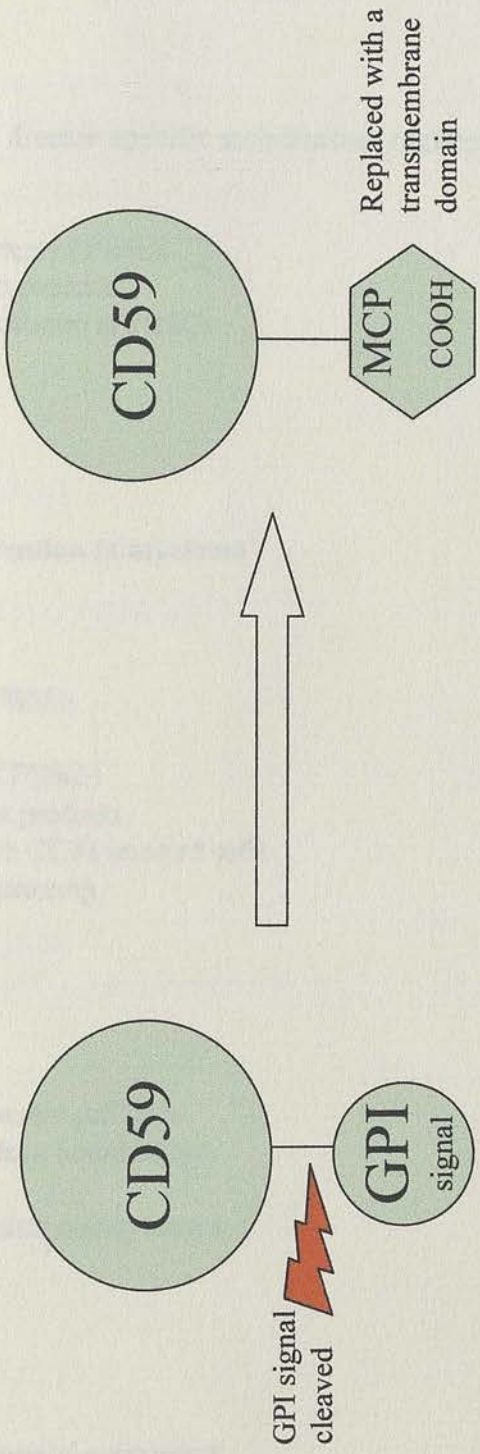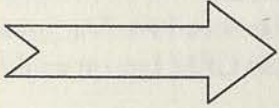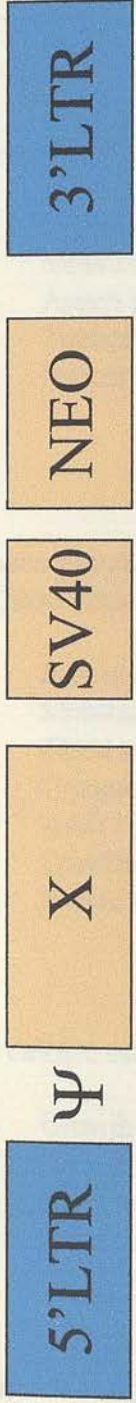
Potential targets for gene therapy in PNH

PNH is due to an abnormality in a single gene and is therefore a disease which could lend itself to genetic modification. While it might be thought that the obvious approach would be replacement of the mutated PIG-A, the experience with syngeneic transplantation in PNH described above suggests that this might not be advantageous. Recurrence after infusion of normal syngeneic cells shows that the aetiological process is still active and can destroy normal haemopoiesis. If this theory is correct then the introduction of genetically normal PIG-A constructs, even with conditioning chemotherapy, might simply lead to a situation where the modified PNH cells are as suppressed as the remaining marrow and patients would trade PNH for AA which is likely to be clinically more deleterious. A more elegant solution would be to target the GPI-linked proteins which are responsible for the selective growth advantage in PNH and modify the normal stem cells in this way. This would allow growth of normal marrow in the face of immune attack and in turn remove the advantage from the PNH stem cells which would no longer be competitive. This approach would require a precise understanding of the mechanism of selection pressure in PNH/AA which we do not as yet have and also presumes that the growth advantage and the sensitivity to complement reside in different GPI linked proteins. An alternative approach has been investigated by Rother et al where CD59 activity alone is restored to PNH cells using a retroviral vector containing a modified CD59 construct anchored to cell surfaces through a transmembrane domain rather than a GPI anchor (CD59-TM) (Rother *et al* 1994). Since CD59 is largely responsible for erythrocyte lysis and possibly also for the thrombotic tendency, the major clinical

features of classical PNH might be abrogated if this alternative CD59 construct was expressed on PNH stem cells. These cells would theoretically retain their selective growth advantage and avoid the AA that could ensue if PIG-A itself were corrected. The CD59-TM construct was generated by replacing the GPI anchor signal of CD59 with the carboxy terminus of membrane cofactor protein (MCP) amplified from HeLa cells. The amplified fragment contained a region known to comprise a transmembrane domain (TM). This CD59-TM was then further subcloned into the retroviral vector pLXSN which is displayed schematically in figure 1.4. This vector was introduced into the amphotrophic packaging cell line PA317. Viral supernatant from this cell line has been used by these workers to achieve stable expression of CD59 in murine 3T3 cells and human PNH B-lymphoblastoid cell lines. They have shown that the presence of this novel molecule on these cells increases their in vitro resistance to complement when compared to untransduced controls (Rother *et al* 1994). This work provides a rationale for attempts to transduce human PNH stem cells in an attempt to correct the clinical phenotype without inducing or worsening aplasia.

Figure 1.4

Diagram showing the basic structure of the LXSN retroviral construct. The virus name is an acronym: L=LTR (long terminal repeat), X=engineered DNA insert of choice, S=SV40 promoter sequence, N=Neomycin phosphotransferase resistance cassette. Transcription of the insert is driven from the 5'LTR while the SV40 promoter drives the Neomycin gene. The virus was packaged in PA317 cells which supplied the gag/pol/env genes for particle assembly. In this case, the insert 'X' is a form of CD59 which has been modified by excision of the GPI anchor signal and replacement by a transmembrane (TM) domain from MCP (membrane cofactor protein). This modification of CD59 allows it to attach to PNH cells which lack GPI anchors (see text).

5'LTR | Ψ | X | SV40 | NEO | 3'LTR

CD59

GPI signal
GPI signal cleaved

CD59

MCP
COOH

Replaced with a transmembrane domain

Chapter 2

Methods

In-vivo PBSC purging in CML by a disease-specific mobilisation regimen

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2.2   Mobilisation collection and storage of PBSCs
2.3   Assays performed on the harvest products
2.4   Transplant conditioning and reinfusion of PBSCs
2.5   Patient follow up

Ex-vivo purging and stem cell purification in myeloma by CD34 selection

2.6   Patients
2.7   Mobilisation and collection of PBSCs
2.8   CD34 selection procedure
2.9   Cryopreservation and storage of PBSCs
2.10 Assays performed on the harvest products
2.11 Conditioning and reinfusion with CD34 selected cells
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Cell culture methods

2.13 Growth and maintenance of adherent cell lines
2.14 Growth and maintenance of cells in liquid culture
2.15 Cryopreservation of cell lines
2.16 Semi solid haemopoietic progenitor colony assays

Using retroviral producer cells

2.17 Production and collection of retroviral supernatant
2.18 Assaying potency of retroviral supernatant

Retroviral gene transfer

Assessing transduction efficiency

Flow Cytometry

Mobilisation of PNH patients with G-CSF

In-vivo PBSC purging in CML by a disease-specific mobilisation regimen

2.1   Patients

Eighteen patients with Ph+ve CML were mobilised using a regimen of high dose hydroxyurea and G-CSF in an attempt to collect mainly Ph-ve PBSCs (Johnson *et al* 1996a). To date, seven of these patients have been transplanted with cells harvested in this way (Johnson & Smith, 1997). Details of these patients including disease status and previous therapy are described in chapter 3.

2.2   Mobilisation, collection and storage of PBSCs

Newly diagnosed patients underwent a mononuclear cell collection on a single day as an autologous backup for future transplant procedures. Following this, their condition was initially stabilised with hydroxyurea.   For mobilisation, patients initially received 6 grams of hydroxyurea orally for 7 days but doses varied as experience was gained (see chapter 3). Overall, the average dose was 3.5 grams/m^2 daily for 7 days rounded up to the nearest gram. It was given entirely as an outpatient in all cases. The day after hydroxyurea finished, G-CSF (Filgrastim, Amgen UK Ltd, Cambridge, UK) was commenced at a dose of 300µg/day subcutaneously. This was continued until the last day of harvesting. Prophylactic antibiotics were not given. In order to accurately define the first day of harvesting we combined two parameters: a rise in peripheral white cell count above at least $1 \times 10^9/l$. and the appearance in the peripheral blood of CD34+ve

cells rising above our threshold of detection (>0.05% of viable mononuclear cells). When these criteria were met, apheresis commenced using the mononuclear cell program on a Baxter CS3000+ (Baxter Healthcare Ltd., Berkshire, UK), 12 litres of blood being processed daily. Between 2 and 4 aphereses were required to achieve the target counts of >$2x10^8$/kg MNC and at least $1x10^6$/kg (preferably $2x10^6$/kg) CD34+ve cells. Venous access was via bilateral antecubital vein cannulation or, in those with poor access, a double lumen femoral line. Those with an indwelling line were admitted while those with easy venous access were harvested on an outpatient basis. The product of each days apheresis was cryopreserved in 10% final concentration DMSO in a controlled rate freezer and then stored under liquid nitrogen.

2.3   Assays performed on the harvest products

Samples from the products were taken for assessment of harvest quality and detection of contaminating cells from the CML clone. Harvest quality was assessed by MNC counts (section 2.10), CFU-GM assay (section 2.16) and CD34 quantitation (section 2.10). Contaminating clonal cells were sought by cytogenetic analysis for the Ph chromosome, RT-PCR for the BCR-ABL transcript and FISH studies for the t(9;22) translocation. Cytogenetic and FISH analysis was performed by Helen Dickinson and Sue Ricketts in the cytogenetics department at St James's Hospital, Leeds, U.K. and RT-PCR by Paul Evans from the molecular pathology department at the General Infirmary, Leeds, UK.

For cytogenetic analysis, samples from each leucapheresis were incubated in culture medium (RPMI+glutamine+FCS) for 48 hours, the last 17 hours of this in the presence of 0.2µg/ml colcemid. Cells were then washed, incubated with 0.075M KCL for 15mins

and fixed with a 3:1 methanol:acetic acid mixture. G-banding was performed on the cells prepared in this way and as many intact and assessable metaphases as were produced were examined microscopically for the presence of the Philadelphia chromosome. FISH Studies were carried out using the mBCR-ABL (minor breakpoint) translocation DNA probe (from Appligene-oncor Ltd., Watford, UK) following the manufacturers instructions with minor modifications. Slides were viewed with a triple bandpass filter which allows simultaneous viewing of the FITC (green), Rhodamine (red) signals and the DAPI (blue) counterstain. A combined red and green signal (often appearing yellow) indicates the translocation. Interphase nuclei and any metaphases seen were scored from each sample. A minimum of 50 cells were assessed for all the samples in this series. Overlapping or damaged nuclei were excluded as were any with high levels of background fluorescence. Marrow slides from CML patients at presentation were used as positive controls. Samples from normal marrows and patients with Ph-ve acute leukaemias were used as negative controls and produced a background false positive rate of 0-4% resulting from random juxtaposition of the signals. The presence of a fusion transcript from the abnormal BCR-ABL gene was detected using a nested RT-PCR technique. A 0.5ml sample of the leucapheresis product from each days harvest was obtained. This contained a mean of 0.8×10^8 cells (range $0.45 - 1.2 \times 10^8$). RNA was then extracted from this using a commercial preparation based on the acid guanidinium phenol chloroform method (Trizol^{TM}, Life Technologies ltd., Paisley, Scotland) to give a final volume of 65µl. 20µl of this was reverse transcribed using random hexamers as primers and MoMLV reverse transcriptase giving a final volume of 40µl. These steps were controlled by a single round PCR for the ABL gene to demonstrate that adequate quality cDNA had been produced. Nested PCR was performed on 1µl of the cDNA

using p210 specific primers and the products were visualised on a 2% agarose gel by ethidium bromide staining. The B3A2 product yielded a 458 bp band and the B2A2 a 383 bp band. Controls included 1/100 dilutions of known positive B2A2 and B3A2 cDNAs, two known negative cDNAs and RT and PCR blanks. The sensitivity of the test in our hands is of the order of 1 positive cell in 10^5 negatives.

2.4 Transplant conditioning and reinfusion of PBSCs

Transplant conditioning was either with busulphan alone (16mg/kg orally divided over 4 days) or busulphan (same dose) and cyclophosphamide (120mg/kg intravenously divided over 2 days following the busulphan) depending on the centre involved. Appropriate hydration, MESNA, antiemetics and anticonvulsants were given concurrently according to regional transplant protocol. The cryopreserved PBSCs were thawed at the bedside directly from liquid nitrogen in a sterilised 37^0C waterbath and each bag was infused within 15 minutes of thawing via a central line.

2.5 Patient follow up

All patients were commenced on α Interferon therapy as soon as peripheral blood counts allowed. They remained on this indefinitely if it was tolerated. Patients were regularly followed up and examined clinically and by laboratory methods for signs of disease and evidence of Ph+ve haemopoiesis. Bone marrow examination was performed at intervals to assess the %Ph+ve cells in the marrow.

Ex-vivo purging and stem cell purification in myeloma by CD34 selection

2.6   Patients

PBSCs from eight patients with multiple myeloma were CD34 selected, one of them undergoing two procedures. Six of these patients were autotransplanted with these selected cells, one of them twice (Johnson *et al* 1996b). Details of the patients' disease status, history and therapy are described in chapter 4.

2.7   Mobilisation and collection of PBSCs

PBSCs were mobilised with a combination of Cyclophosphamide and G-CSF (Filgrastim, Amgen UK Ltd., Cambridge, UK). Two schedules were used depending on the hospital the patients attended. Five patients (WW, BD, PC, JL, GL, in table 4.1) received Cyclophosphamide at a dose of 2 grams/m^2 by 1 hour intravenous infusion followed by 300µg/day of G-CSF from day +1. The other three patients (KK, IB, PH, table 4.1) received 1.5 grams/m^2 and 960µg/day G-CSF. Appropriate hydration, MESNA and antiemetics were given in all cases to minimise side effects from the cyclophosphamide. PBSCs were harvested at a mean of 8.5 days post cyclophosphamide when the absolute peripheral WBC was rising through 5x10^9/l. Patients were harvested on 3 successive days with the first 2 days products pooled for CD34 selection and the third days separately frozen as a backup. The cells were collected using the mononuclear cell program on a Baxter CS3000+ cell separator (Baxter Healthcare Ltd., Berkshire,

U.K.). Venous access was either via peripheral veins or (in most cases) a double lumen femoral catheter inserted for the purpose under local anaesthetic. Twelve litres of blood was processed daily.

2.8   CD34 selection procedure

This was performed using the Ceprate™ immunoaffinity system (Supplied by Cellpro Ltd, Wezembeek-Ottem, Belgium). The first days harvest was kept overnight at 4^0C and pooled with the second the following day. The cells are then washed, counted and incubated with a biotinylated anti CD34 antibody (from Cellpro). They are then washed again and passed through the Ceprate™ avidin column which has been primed with saline and human albumin solution. This and subsequent steps are integrated and semi automated under computer control. The column is then flushed to remove unbound cells and this waste is collected and assayed (section 2.10). The CD34+ve cells which have been retained in the column are released by gentle agitation using an integral magnetic stir bar and flushed into a collection bag in a final volume of around 100ml.

2.9   Cryopreservation and storage of PBSCs

The CD34 selected cells were volume reduced and frozen in a controlled rate freezer in 10% (final concentration) DMSO and human albumin solution and stored under liquid nitrogen. The day 3 backup harvest was cryopreserved in the same manner but without any form of manipulation.

2.10   Assays performed on the harvest products

These were performed on samples taken before and after processing on the column and from the column waste. Product quality was assessed by measurements of CD34+ve cells, MNC counts, CFU-GM assays (section 2.16) and in some cases the P-delta assay (section 2.16). Tumour cell contamination was measured by IgH PCR. MNC counts were calculated from the total nucleated cell count on a Coulter automated blood cell counter with an adjustment by manual differential to exclude neutrophils and nucleated red cells. The CD34 quantitation was performed by Andy Rawstron using a modification of the Baxter method (provided by K. Unverzagt, Baxter Healthcare Ltd., Newbury, Berkshire, UK) on a Becton Dickinson FACScan^{TM} (Becton Dickinson, Cowley, Oxford, UK). One million cells are labelled with CD34-PE and CD45-FITC then resuspended in FACSFlow^{TM} with 50µl of PI. Cells are then analysed on the FACScan using LYSIS II software. A live gate is set on viable leucocytes (CD45+ve, PI-ve) and 50-200 thousand events are collected. CD34+ve events are defined by their CD34 Vs. CD45 and FSC Vs. SSC characteristics. CFU-GMs were assayed as described in section 2.16 and expressed as colonies of >50 cells at day 14 of growth x10^4/kg body weight. P-delta assays were performed on two patients according to the method described in section 2.16.

IgH PCR was performed by Dr Roger Owen on all but one patient (PH) who did not amplify. DNA was prepared by proteinase K digestion, phenol-chloroform extraction and cold ethanol precipitation. The rearrangements were detected using CDR3 and CDR1 fluorescent PCR techniques (Owen et al 1996). Both utilise a consensus JH primer which is labelled with a green fluorochrome. The CDR3 PCR utilises a second consensus primer from the framework 3 region of the gene. All samples were analysed

using this technique. If the result was polyclonal or amplification unsatisfactory the CDR1 PCR was used. This requires 6 reactions each using a family specific VH primer from the framework 1 region in addition to the fluorescently labelled JH primer. A schematic of the method is shown in figure 2.1. The PCR products were analysed using a DNA sequencer (Applied Biosystems 373A) whose computer software converts the fluorescent gel image for each sample into an 'electrophoretogram' which consists of a variable number of peaks whose height corresponds to the fluorescence intensity on the gel. A polyclonal pattern appears as a number of peaks arranged in a normal distribution while clonal rearrangements appear as distinct peaks either alone or within a polyclonal background (see figure 2.2). Size standards are loaded into each gel and this enables product sizing to within 1 base pair. By diluting clonal B cells into normal peripheral blood MNCs we have shown that the CDR3 PCR has a sensitivity of 10^{-3}-10^{-4} and the CDR1 PCR a sensitivity of at least 10^{-4}.

Figure 2.1

Schematic of the IgH PCR. The heavy chain variable (VH), diversity (D) and joining (JH) regions are depicted, consisting of three complementarity determining regions (CDR 1-3). Framework 1,2,3 and 4 (Fr 1-4) are relatively constant areas of the sequence suitable for the design of PCR primers. All reactions utilise the same JH consensus primer (JH con), depicted by the arrow below Fr4. All samples were first tested using this primer and a Fr3 primer (the arrow below Fr3). If they failed to amplify with this, primers from the Fr1 region were used. This latter PCR needed 6 primers for the 6 VH1 families. These are shown by the arrows below Fr1. Around 65% amplify with the Fr3 PCR and this can be increased to over 80% using the 6 Fr1 primers.

VH

Fr1	CDR1	Fr2	CDR2	Fr3	N	N D	N	JH
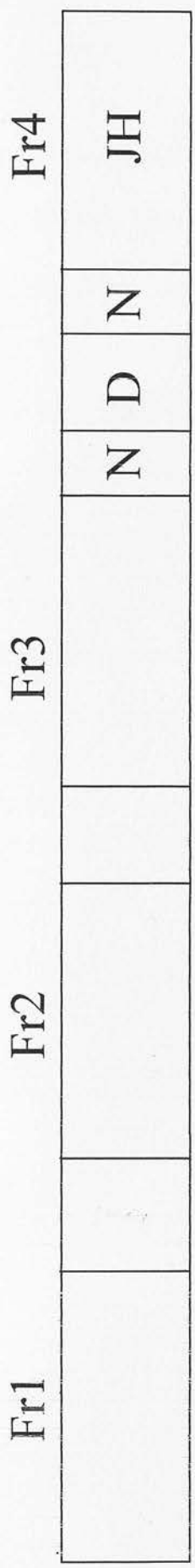
CDR3
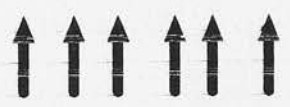
Fr1 (VH1 - 6)　　　　　　　　Fr3　　　　　　　　JH con

Figure 2.2

Examples of electrophoretogram read-outs of the fluorescent IgH PCR on the applied biosystems gene scanner (see text, section 2.10). The x-axis shows the product size in base pairs and the y-axis is an expression of fluorescence intensity.

A. Polyclonal fingerprint from normal B cells in the peripheral blood. There is a normal distribution of product sizes (peaks are 3 bp apart).

B. A single 126 bp clonal band is demonstrated with suppression of all normal polyclonal B cell populations. This could be the picture in blood or marrow from a presentation ALL sample or myeloma with a large plasma cell burden.

C. A 96 bp clonal band is clearly seen on a background of normal polyclonal B cells. This could represent molecular MRD in a remission sample in ALL or be seen in myeloma with low disease bulk pre or post treatment.
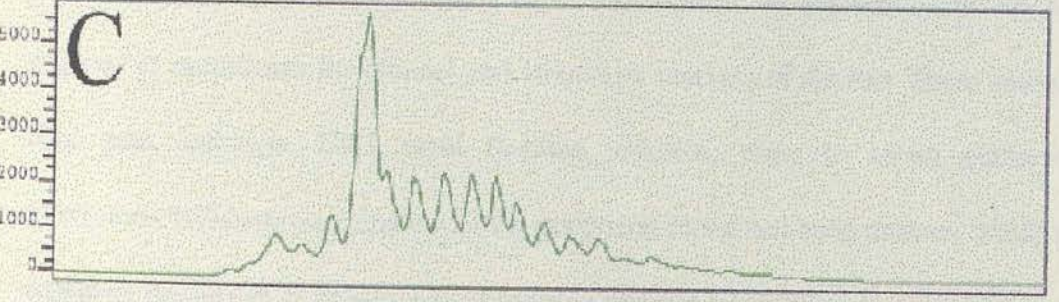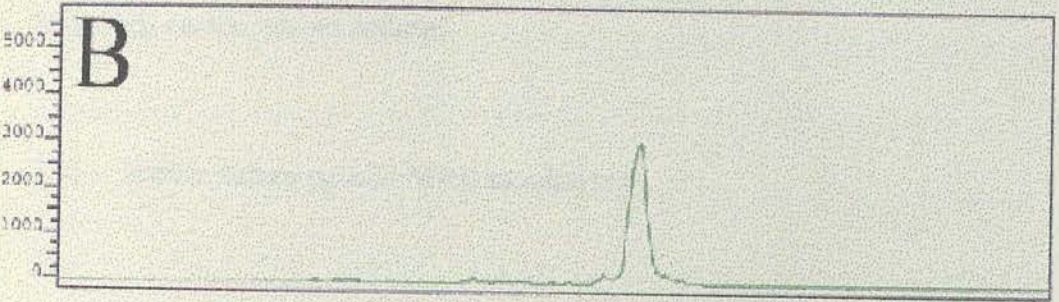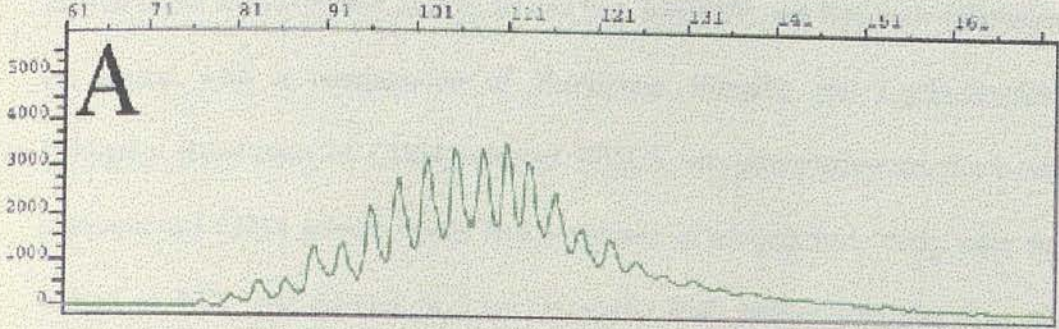
2.11 Conditioning and reinfusion with CD34 selected cells

All patients were conditioned for transplant with melphalan alone at a dose of $200mg/m^2$ intravenously with appropriate hydration and monitoring. The cells were returned at least 24 hours following this. Patient IB had two autografts, the first with melphalan and the second with a combination of Busulphan 16mg/kg and Cyclophosphamide 120mg/kg. Reinfusion of CD34 selected PBSCs was straightforward. Each vial of cryopreserved CD34 selected cells had a volume of around 9ml. Vials were rapidly thawed at 37^0C and diluted in 20ml of sterile PBS before administration via an indwelling central venous catheter.

2.12 Patient follow up and MRD monitoring

Following discharge from the ward after engraftment, all patients were regularly assessed as outpatients for clinical and laboratory markers of disease. These included: bone pain, infection, FBC, renal function, calcium, urine or serum paraprotein estimations, β2M and examination of the peripheral blood and bone marrow. MRD was sought by the IgH PCR on serial bone marrow samples taken at approximately 3, 6 and 12 months post transplant.

Cell culture methods

2.13 Growth and maintenance of adherent cell lines

Adherent cell lines were maintained in DMEM supplemented with 5% FCS both from Life Technologies Ltd., Paisley, Scotland. Media were supplemented with penicillin and streptomycin (pen/strep) from a 100x stock solution containing 1 gram of streptomycin and 1 Megaunit of Penicillin per 100ml. For routine use cells were grown to near confluence in vented $25cm^2$ tissue culture flasks covered with 5ml of DMEM+5%FCS at 37^0C in a humidified incubator with 5% CO_2. When approaching confluence, the medium was pipetted off and after washing with sterile 0.9% saline, 2ml of Trypsin-EDTA (Life Technologies Ltd.) was added and the flask placed in the incubator for 5 minutes. It was then agitated to achieve a single cell suspension and 0.1-0.3ml (depending on the rate of cell growth) of the trypsin/cell suspension was added to 5ml fresh DMEM+5%FCS in a new flask. The cell lines in use were split in this way every 3-4 days and rarely required interim feeding. There were four adherent cell lines used:

i)   NIH 3T3 murine fibroblasts.

ii)   GP+envAm12. This amphotrophic packaging cell line (Markowitz *et al* 1988a) produced an MFG based retroviral construct (MFG-LacZ) which contains a nuclear localising beta-galactosidase gene allowing identification of successfully transduced cells by a colour enzyme reaction described in section 2.18. This cell line has no selectable marker and the viral titre (around 10^4-10^5 cfu/ml on NIH 3T3 cells) tends to fall with repeated passaging. Consequently it was frozen down and fresh aliquots used

approximately every 3 months following confirmation of viral titre (section 2.18). This cell line was kindly donated by Dr Paulam Patel of St James's Hospital, Leeds, UK.

iii)   VSN2 retrovirus in a fibroblast packaging cell line. This VSN2 producer line was selectable in Dulbecco's HAT medium (Life Technologies Ltd., Paisley, Scotland) on the basis of HGPRT activity. The Hypoxanthine and Thymidine in this selection medium supply pre-formed purines and pyrimidines for cell DNA synthesis by salvage pathways dependent on HGPRT. This rescues them from the toxicity of the Aminopterin in the medium which inhibits de novo nucleoside synthesis. The cells were grown to around 60% confluence in DMEM+5%FCS. The medium was then removed and after washing, 5ml HAT+5%FCS was added and the cells returned to the incubator. The flask was fed with fresh HAT+5%FCS every 4 days. After 14 days the cells were washed and the HAT replaced by DMEM+5%FCS. When they were near confluent in this fresh medium they were split as described above. Cells were passaged twice in normal medium before harvesting viral supernatant (section 2.17).

iv)   PA317 amphotrophic packaging cells (murine fibroblast). Two producer lines based on these cells were used. Both had been created by transfection with the pLXSN retrovirus. LXSN consists of an LTR-driven (L) construct (X) and an SV40 promoter-driven (S) neomycin resistance cassette (N) (Miller & Rosman, 1989). They thus have a marker (Neo) that can be PCRed or selected by exposure to the antibiotic G418 (see section 2.24). One producer line contained only the retrovirus with no additional DNA sequence and the other contained a novel CD59 (MIRL) molecule which had been modified by the addition of a transmembrane protein domain (CD59-TM) and was thus not reliant on GPI linkage for cellular attachment (Rother et al 1994).

These lines were kindly donated by Dr Russell Rother of Alexion Pharmaceuticals, Newhaven , Connecticut, USA.

2.14 Growth and maintenance of cells in liquid culture

These lines were carried in RPMI 1640 medium supplemented with 10% FCS (Life Technologies Ltd., Paisley, Scotland). The cultures were held in $80cm^2$ vented tissue culture flasks in a 37^0C, 5% CO_2, humidified incubator. Cultures were fed with fresh medium by demi-depletion approximately every 3-4 days. The lines used were kindly donated by Dr Peter Hillmen. They were EBV transformed B lymphoblastoid cell lines derived from patients with PNH. PNH and normal B cell lines from several such patients were used.

2.15 Cryopreservation of cell lines

For storage purposes, cells were frozen and kept under liquid nitrogen. The adherent cell lines were frozen by taking one $25cm^2$ flask at around 75% confluence and trypsinising as described above (section 2.13). The single cell suspension achieved was washed in DMEM/FCS then resuspended in 2.75ml DMEM with 50% FCS. An equal volume of 20% DMSO diluted in DMEM was slowly added to this after both solutions had been cooled on ice to 4^0C giving a final concentration of 10% DMSO. Still on ice, this 5.5ml volume was aliquotted into 3x 1.8ml cryotubes which were transferred to a -90^0C freezer in a small polystyrene container and allowed to freeze before transfer to a liquid nitrogen store. Lines in liquid culture were frozen one day after feeding. They were spun

down and resuspended and diluted in RPMI+50% FCS to a cell concentration of around 5×10^6/cells/ml. Equal volumes of 20% DMSO were added on ice at 4^0C as for the adherent lines described above and frozen in the same way. All cell lines were recovered from frozen in the same manner. A 1.8ml cryotube containing the cells of interest was removed from liquid nitrogen and thawed in a 37^0C water bath. As soon as it defrosted it was transferred to a sterile hood and 10ml of warm medium was slowly added over 3 minutes. They were washed twice in the same medium and finally resuspended in 5ml in a 25cm^2 flask and transferred to an incubator.

2.16   Semi-solid haemopoietic progenitor colony assays

Assays were performed for: CFU-GM, BFU-E and the P-delta assay for more primitive progenitors (Gordon, 1994). The MNC plated out in these assays came from three sources: Density centrifugation of bone marrow obtained by needle aspiration, density centrifugation of peripheral blood obtained by venepuncture and apheresis samples obtained from a cell separator procedure. The latter was obtained using the mononuclear cell program on a Baxter CS3000+ apheresis machine (Baxter Healthcare Ltd, Newbury, Berkshire, U.K.). An aliquot of this MNC product was washed twice in MEMα medium (Life Technologies ltd.) supplemented with 15% FCS (MEMα+15%FCS) and re-suspended in the same medium at 1×10^6 MNC/ml for use in the assays described below. Density centrifugation was performed by layering blood or marrow onto an equal volume of lymphoprep^{TM} (Nycomed Pharma, Oslo) and spinning at 1800rpm for 20-35mins depending on the sample volume. The turbid MNC layer produced was carefully

pipetted off and washed and diluted in the same manner as the apheresis product described above. CFU-GM and BFU-E assays were adapted to use methylcellulose rather than agar as a supporting matrix so that colonies could be more readily picked (aspirated) from the plates for analysis. All FCS used in the assays was batch tested and shown to support colony growth. Methylcellulose (2% solution=4000 centipoises, from Sigma-Aldrich company, Poole, Dorset, UK) was dissolved in MEMα to a concentration of 2.4% (1.8grams in 75ml) in an autoclaved conical flask containing a magnetic stir bar. This was agitated with the stirrer for 2-4 days in the dark at 4^0C. When a clear solution was achieved it was aliquotted and stored in light tight vessels at -20^0C. This can be thawed and re-frozen repeatedly. Growth factor supplementation for the CFU-GM assays was provided by 5637 (bladder carcinoma cell line) conditioned medium kindly donated by Debbie Clark, St James's Hospital, Leeds, UK. In the BFU-E assay, erythropoietin (EPO) came from Terry Fox labs, Vancouver, Canada and 35% bovine Serum albumin (BSA) from Sigma-Aldrich company, Poole, UK.

CFU-GM:

MEMα medium	0.75ml
FCS	1.0ml
5637	0.5ml
MNC (10^6/ml)	0.5ml
methylcellulose	2.25ml (0.9% final conc.)
	=5ml in total

BFU-E:

MEMα medium	0.5ml
FCS	1.5ml
BSA (35%)	130µl
EPO	10 units
2-Mercaptoethanol	10µl (50mM)
MNC (10^6/ml)	0.5ml
Methylcellulose	2.25ml (0.9% final conc.)
	=5ml total

In a sterile cabinet, the above reagents are added to a universal container and slowly mixed together using a syringe and 16 gauge needle. When the methylcellulose mixture is of an even consistency it is aliquotted out in 1ml amounts (10^5 MNC/dish) into four 35mm covered petri dishes. These are gently rotated to spread the mixture evenly on the bottom and then placed in a humidified container within a 37^0C, 5%CO_2 incubator. Colony growth is scored at day 10-14 by direct vision under an inverting microscope counting >50 cell aggregates as colonies for CFU-GMs and identifying clusters of erythroid bursts for the BFU-E assay. The result is expressed as the average number of colonies or bursts per 10^5 MNC plated (i.e. per dish).

P-Delta assay: (method courtesy of Debbie Clarke, St James's Hospital, Leeds, UK)

Preparation of MP+α medium:

MEMα medium is supplemented with 10% CFU-GM tested FCS, 10% Horse serum, methylprednisolone (MP, final conc. $2x10^{-6}M$) and 2mM L-Glutamine. The MP is diluted from a 40mg vial (Pharmacia and Upjohn Ltd., Milton Keynes, UK) in 1ml water. Add 0.25ml of this to 12.25ml medium and store in 0.5ml aliquots at -20^0C. This is finally diluted 1:1000 into the medium.

This assay is thought to assess the presence of progenitor cells more primitive than CFU-GM or BFU-E (Gordon *et al* 1996). 10^7 MNC prepared as described above are incubated for 2 hours in 10ml MEMα+15%FCS in a $25cm^2$ tissue culture flask at 37^0C/5%CO_2 to allow adherence of primitive cells to the plastic. The medium is then removed and the flask is washed three times with MEMα before replacing it with 9ml MP+α and 1ml 5637 and placing back in the incubator for 7 days. The medium is then drawn off, spun for 4mins @1500rpm, washed and resuspend in MEMα+15%FCS and this is plated in a standard CFU-GM assay as described above. The result is expressed as the total number of CFU-GM colonies derived from the original 10^7 cells adhered.

Using retroviral producer cell lines

2.17 Production and collection of retroviral supernatant

Adherent Producer cell lines were grown to near confluence in 80cm² tissue culture flasks under the conditions described above. They were then washed and 16ml of DMEM+5%FCS was added to each flask. This was left for 24 hours after which time the medium was aspirated and passed through a 0.45μm filter to remove any cell debris. The filtered supernatant from all flasks was pooled and aliquots taken to assess the number of viral particles per ml. The supernatant was divided into volumes suitable for single use and stored at -90°C.

2.18 Assaying titre of retroviral supernatant.

Supernatants containing retroviruses with the selectable neomycin resistance cassette (from VSN2 and LXSN-based lines) were used to transduce NIH 3T3 cells which were then subjected to Geneticin sulphate (G418, Life Technologies, Paisley, Scotland, UK) selection at a concentration of 400μg/ml. Transduction was performed by plating 3T3s in 25cm² tissue culture flasks containing a presumed number of viral particles based on a titre of 10⁵ CFUs/ml suggested by the supplier. Three dilutions of virus were used giving an expected 10, 100 and 1000 CFUs/flask at that titre. The medium was supplemented with 6μg/ml of polybrene to improve viral attachment. After 8 hours incubation, the viral supernatant was removed and G418 was added and replaced every 3 days. Surviving G418 resistant colonies were scored under an inverting microscope at 10

days. An alternative and more rapid way to assess the quality of batches of supernatant from the CD59-TM producing virus and to cross check for contaminating producer cells is by flow cytometry. CD59 is not normally present on murine 3T3 cells which allows distinction of transduced from non-transduced cells by staining with this MAB. Cells were transduced as already described and then trypsinised, washed and resuspended in FACSFlow^{TM} (Becton Dickinson, Cowley, Oxford,UK). They were transferred to a 96 well microtitre plate and stained with 5µl of appropriately titred CD59-PE conjugated MAB. After 15 minutes room temperature incubation, the cells were washed and analysed in a Becton Dickinson FACScan^{TM}. The proportion of transduced cells can be rapidly displayed as the CD59 positive peak on histogram analysis using the machine's lysis II software. Non-transduced 3T3 cells and 3T3s transduced with the LXSN virus alone were assayed in parallel as controls (see chapter 5 and figure 5.2).

MFG virus supernatant containing a beta-galactosidase gene was assayed using the same transduction method on 3T3s followed by staining of the transduced cells using X-GAL. Successfully transduced cells expressing the enzyme stain blue following exposure to X-GAL and can be read and counted in the same way as G418 resistant colonies under an inverting microscope (see figure 5.1).

X-GAL is prepared as a stock solution of 40mg/ml in dimethylformamide which can be frozen at -20^{0}C. 2.5ml of this is added to 100ml of buffer giving a 1mg/ml working solution which should be filtered (0.2µm pore) to remove crystal deposit prior to use. The solution is light sensitive and should be kept in the dark. The buffer is made up as follows:

Phosphate buffered saline	100ml
Sodium deoxycholate	10mg
NP40(detergent)	200μl (0.02%)
Magnesium Chloride	0.0406g
Potassium Ferricyanide	0.1646g (0.05M)
Potassium Ferrocyanide	0.2112g (0.05M)

(All reagents from Sigma-Aldrich company, Poole, UK). Cells to be stained are fixed with 0.05% Glutaraldehyde for 20 mins then washed. X-GAL is then added for 2-6 hours. The cells are finally washed again and read under the microscope.

Retroviral Gene Transfer

2.19   Retroviral gene transfer into NIH 3T3 cells.

This was done using supernatants containing VSN 2, MFG-LacZ, LXSN and CD59-TM retroviruses. This allowed confirmation that the producer cell lines described in section 2.13 were functioning and also allowed quantitation of viral titre as described in section 2.18. Integration and expression of the constructs in 3T3 cells were assessed in a number of ways: flow cytometry (CD59-TM expression, section 2.25), PCR and G418 selection for those with a neomycin resistance cassette (sections 2.21 and 2.18) and in situ staining with X-GAL for beta-galactosidase (section 2.18). The method of gene transfer has been briefly described in section 2.18 in relation to the techniques for assessing viral titre. 3T3 cells are plated at low density in 25cm^2 tissue culture flasks

with 5ml DMEM+5%FCS and allowed to establish themselves overnight in an incubator. The medium is then removed and replaced with the viral supernatant of interest with polybrene at a concentration of 6µg/ml. Fresh supernatant can be added repeatedly if desired to improve the MOI if required. Each time supernatant is added it is left on for 6-8 hours. After this, the cells are washed and allowed to grow overnight. They can then be processed in a manner appropriate to the assays described above. Cells for FACS (section 2.18) or PCR (section 2.21) are trypsinised off and washed prior to assay while those for X-GAL staining or G418 selection (section 2.18) are washed and left in situ.

2.20   Retroviral gene transfer into human PBSCs

This was attempted with the supernatants containing VSN 2, MFG-LacZ, LXSN and CD59-TM retroviruses. A number of improvements and alterations were made over time to optimise the transduction efficiency and these are described in the relevant results sections. The optimum method achieved was as follows: PBSCs were obtained either by venesection or by apheresis on a Baxter CS3000+ machine (Baxter Healthcare Ltd, Newbury, Berkshire, UK). PBSCs were separated from venesected whole blood by density centrifugation as described in section 2.16, while the apheresis samples required no further processing. Venesected cells came from patients with haematological malignancies, patients with PNH or normal volunteers while all apheresis samples were from haematology patients preparing for PBSCT. These latter patients had received chemotherapy and G-CSF (Filgrastim, Amgen UK) to mobilise stem cells into the peripheral blood. Cells were washed twice in saline before resuspension in

DMEM+5%FCS. 6×10^6 MNC were placed in a 25cm^2 flask with 8ml of DMEM+5%FCS supplemented with growth factors (SCF donated by Amgen UK Ltd., IL3 donated by Sandoz Pharmaceuticals, Camberley, Surrey, UK and IL6 donated by Roz Banks, St James's Hospital, Leeds, UK, all at a concentration of 50ng/ml).

The flask was incubated for 48hrs and then the cells and medium were transferred into 2 universal containers and centrifuged. The medium was tipped off and the cells resuspended in 8ml viral supernatant with 2ml fresh DMEM+5%FCS, the same growth factors as above and 6µg/ml of polybrene. This 10ml volume was placed in a flask and after 6hrs incubation it was again centrifuged and the process repeated with fresh viral supernatant and supplements as above. The supernatants used had viral titres of around $1-5 \times 10^5$ cfu/ml which, with the 2 exposures described may achieve an MOI of approximately one. Pre-incubation with a cocktail of growth factors is intended to encourage cell division and therefore rates of transduction as retroviruses will only infect dividing cells. Following exposure to the virus, the cells are washed and set up in semi-solid colony assays as described in section 2.16.

Assessing transduction efficiency

2.21   PCR for the neomycin phosphotransferase gene.

Single round PCR was shown to be insufficiently sensitive for most applications therefore a nested technique was used. The 2 sets of primers were synthesised in-house by Fraser Lewis, the sequences being as follows:

External primers

Forward   5' TCC ATC ATG GCT GAT GCA ATG CGG C 3'

Reverse   5' GAT AGA AGG CGA TGC GCT GCG AAT CG 3'

Internal primers

Forward   5' AAG CGA AAC ATC GCA TCG AG 3'

Reverse   5' ATA TTC GGC AAG CAG GCA TC 3'

Primers were stored at a concentration of 50pmol/μl in ammonia which was dried off in a vacuum centrifuge for 20-30mins when required. 1ml of a master PCR mix sufficient for 40x 25μl tests for each round was made as follows and frozen at -20^{0}C:

> 20μl of both primers   (25pmol/test)
>
> 5μl of dNTP's (200x)
>
> 100μl of 10x Tac buffer
>
> 10μl of 100mM MgCl
>
> Distilled H$_2$0 up to 1ml

24μl of this mix was added to the target in each test microcentrifuge tube. Tac polymerase (Supertac, 5units/μl, from HT Biotech, Cambridge, UK) was added to the mix before it was aliquotted to the test tubes to give 1 unit of Tac per test. Finally, all tubes were covered with 2 drops of mineral oil. Positive (1μl diluted VSN2 DNA) and negative controls and PCR blanks were set up at the same time. All tubes were

transferred to a pre-heated (95^{0}C) block on a programmable thermal controller (PTC-100^{TM} , Genetic Research Instrumentation Ltd., Dunmow, Essex, UK). The program used was: 30 cycles, Temperatures 55-72-95^{0}C with one minute at each stage and a 15 minute final extension with a magnesium concentration of 1 mM. This produces a 420 bp fragment from the positive control. For the second round, 1μl of each first round product was transferred to a fresh tube and the process was repeated using exactly the same conditions with the internal primer mix. This yields a final 190 base pair fragment. The results are visualised and photographed under ultraviolet light after running on a 2% agarose gel. This gel is made by dissolving 2.4g agarose in 120ml TAE buffer in a conical flask heated to boiling in a microwave. Ethidium bromide (15μl of 10mg/ml) is added when hand hot and the liquid poured into a carrier with well markers and allowed to set. 10μl of the PCR products are placed into the wells after mixing with loading buffer and the gel is run along with standard 100bp ladders at 130 volts for 1 hour.

2.22   Harvesting colonies from semi-solid medium.

CFU-GMs or BFU-Es were carefully aspirated from the methylcellulose under direct vision using an inverting microscope. This is a fiddly and highly operator-dependent technique. The age and quality of the colony plates had a significant impact on the success of the procedure and it was best to pick colonies early at around 12 days. Older plates tend to dry and were difficult to aspirate. A fine plastic micro-pipette tip was fixed to a standard yellow tip on a 10μl pipette. This was carefully positioned under the microscope into the centre of a colony and the pipette released, drawing some or all of the cells into the tip. The micropipette tip was then detached and fixed to a standard

yellow tip on a 200µl pipette pre-loaded with PBS+1%BSA and flushed out into a microcentrifuge tube. The cells were either processed for FACS analysis (section 2.27), or PCR (section 2.21).

2.23 Assessing retroviral transduction of colonies using X-GAL

X-GAL has been described in section 2.18. Colonies grown from cells transduced using MFG-LacZ supernatant were visualised after 12 days growth by the direct addition to the petri dishes of 1ml of X-GAL which was allowed to diffuse into the methylcellulose for 6 hours before observing the colour change under the inverting microscope.

2.24 Assessing retroviral transduction of colonies using G418

Colonies grown from cells transduced with the retroviruses containing the neomycin resistance gene (VSN2, LXSN, CD59-TM) could be selected using G418 (section 2.18). Following transduction with retroviral supernatant the cells are split. One aliquot is kept in G418 containing medium (400µg/ml) for 24 hours then plated out for CFU-GMs or BFU-Es using methylcellulose (section 2.16) to which G418 has been added at the same concentration. The other aliquot is the control with no G418. The difference in growth between the two gives an approximate estimate of the proportion of cells successfully transduced. Parallel plates of non-transduced cells are set up with and without G418 to ensure the assay works and that G418 at these concentrations fully inhibits growth of normal cells.

2.25 Assessing retroviral transduction by Flow Cytometry

The Flow Cytometric methods used are described in detail in the next sections (2.26-2.31). Two of these techniques were used for assessing transduction efficiency: Firstly, CD59 expression on 3T3 cells was used as a marker of successful transduction by the CD59-TM vector as described in sections 2.18 and 2.19 (figure 5.2). Secondly, as CD59-TM (unlike native CD59) is not cleaved by the enzyme PI-PLC, normal human cells were transduced with CD59-TM supernatant and then treated with PI-PLC (section 2.31) before FACS analysis. Native CD59 (which is GPI linked) is entirely removed by this enzyme and the remaining fluorescence is due to the CD59-TM and is a measure of the number of cells transduced.

Flow Cytometry

2.26 Basic Flow Cytometry methods.

Stem cell quantitation was performed on a FACSort™. All other assays were done on a FACScan™ (both manufactured by Becton Dickinson Ltd, Cowley, Oxford, UK). Two colour compensation settings were adjusted using the manufacturers CALIBrite™ flow cytometer beads and the software appropriate to each machine (AUTOComp on the FACScan and FACSComp on the FACSort). Three colour work required additional settings which were done manually according to the manufacturers recommendations. Results were analysed using LYSYS II software on the FACScan™ and CELLQuest on

the FACSort^{TM}. The MABs used were either commercially supplied or manufactured in-house. Table 2.1 shows a list of MABs, the clone from which they were derived and the source. All MABs used were pre-conjugated with FITC or PE. Cells for analysis were prepared by several methods depending on the source and the nature of the assay. Separation of MNCs from peripheral blood or bone marrow by density centrifugation has already been described in section 2.16. Whole blood lysis was performed by adding 20ml of 0.8% ammonium chloride (NH4Cl) to 2.4ml of peripheral blood in a universal container and incubating for 5 mins at 37^0C with intermittent mixing. This was then spun down at 2000rpm for 5mins and the pellet resuspended in 25ml of FACSFlow^{TM} and re-spun. Cells were washed twice in this way before being finally resuspended in the same solution and counted on an automated blood analyser (Sysmex K 1000, from Sysmex Ltd., Milton-Keynes, UK). Adherent cell lines were trypsinised off tissue culture plastic (section 2.13), washed twice and resuspended and counted in the same way. Liquid cell cultures and apheresis samples were simply aspirated, spun down, washed and counted as above. When dealing with small numbers of cells, 1% BSA was added to the FACSFlow^{TM} to minimise losses. Routine antibody staining was performed in round bottomed 96 well microtitre plates. The MABs used were titred such that 10µl of MAB would adequately stain up to a million cells. For staining, up to 10^6 cells were placed in a microtitre well and 10µl of each MAB was added. Plates were then agitated on a plateshaker to ensure adequate mixing and incubated at room temperature for 20 minutes. Cells were then washed twice manually in FACSFlow^{TM} and resuspended and transferred to Kahn tubes ready for analysis. Cells were not fixed but if not processed immediately were kept at 4^0C.

Table 2.1 Monoclonal Antibodies used for Flow Cytometry

Specificity	Clone	Source
IgG_1	PAP7	ATCC, Maryland, USA
IgG_{2a}	W6/32HK	ATCC, Maryland,USA
CD3	OKT3	ATCC, Maryland, USA
CD13	WM15	Dr K. Bradstock, Westmead Hospital, Australia
CD15	C3D-1 (1)	DAKO Ltd.
CD16	3G8	Prof. Unkeless, Mount Sinai Hospital, New York
CD33	WM54	Dr K. Bradstock, Westmead Hospital, Australia
CD34	BIRMA K3	Dorothy McDonald, Transfusion service, Birmingham, U.K.
CD38	OKT10	ATCC, Maryland, USA
CD45	9.4	ATCC, Maryland, USA
CD55/DAF	143-30	Cymbus Biosciences Ltd.
CD59/MIRL	MEM 43	Cymbus Biosciences Ltd.
Thy1	F15.42	Immunotech Ltd., Marseille, France
Glycophorin A	R10+R18	Ludwig Institute for Cancer Research, London, UK

2.27   FACS analysis of semi-solid colonies.

This was done for whole plates (35mm petri dishes) of CFU-GM/BFU-E or for individual picked colonies. Whole plates (volume = 1ml of methylcellulose+reagents) were dissolved in 20ml of FACSFlow^{TM}+1%BSA at around 12 days of growth. This solution was gently mixed for 30 mins. to allow the methylcellulose to fully dissolve and then centrifuged at 2000rpm for 5 mins. before tipping off the supernatant, resuspending the cell pellet and washing twice more in the same solution. Cells were then transferred to microtitre wells for staining as described in section 2.26. It was possible to harvest cells from individual colonies and analyse these by FACS after MAB staining. Colonies are grown and picked as described in sections 2.16 and 2.22. Once an individual colony has been transferred to a microcentrifuge tube, it is gently vortexed and then allowed to stand for 10 minutes to allow the methylcellulose to fully dissolve. The tube is then centrifuged for 2mins @2000rpm in a microcentrifuge. From this point on the tubes must be very gently handled to avoid loss of cells as there may only be a few hundred to a thousand present in each. The supernatant is carefully aspirated using an orange needle attached to a suction pump. A little liquid is left to avoid disturbing the cells. The cells are resuspended and 5µl of each MAB of interest is added. This is mixed by pipetting and incubated at room temperature for 30 mins. The cells are then washed twice with 200µl of FACSFlow^{TM}+1%BSA using the same careful technique for centrifuging and aspirating. After the second wash, 200µl FACSFlow^{TM} is added and the cells mixed by pipetting and transferred to a Kahn tube. The microcentrifuge tube is washed out with a further 200µl FACSFlow^{TM} and this is also transferred to the Kahn which can then be analysed on the FACScan^{TM}.

2.28 Stem cell phenotyping.

CD34 positivity was taken to define stem cells at all stages of development. CD34 quantitation to assess the quality of PBSC harvests in relation to the stem cell mobilisation and selection procedures described in chapters 3 and 4 has already been discussed in section 2.10. Further CD34 subset analysis was undertaken in some cases, particularly in relation to the work on PNH stem cells described in chapter 7. This more extensive phenotyping work was performed by Andy Rawstron at the Leeds General Infirmary, Leeds, UK.

Leucocytes were prepared either by whole blood lysis (section 2.26) or by density gradient centrifugation (section 2.16). For antibody staining, 2-3 million cells were incubated in microtitre plates for 20 minutes at room temperature with $10\mu l$ of each pre-titred antibody conjugate per million cells, then washed twice in FACSFlow^{TM}. Cells were analysed on a Becton Dickinson FACSort^{TM} with CELLQuest^{TM} software. The antibodies used were (see table 2.1): CD34-PE, CD45-FITC, CD38-Cy5, IgG1 control-PE/Cy5, IgG2a control- PE/Cy5, and CD59-FITC. CD59-FITC was supplied by Cymbus Biosciences U.K. Ltd. All other conjugates were prepared in-house by Dr Richard Jones.

For CD34 quantitation, cells were incubated with CD34-PE and CD45-FITC, washed, and resuspended in $150\mu l$ FACSFlow^{TM} with PI. A 50,000 event file was collected. Regions were set around the viable leucocytes (CD45+ve,PI-ve) and progenitor cells (CD34+ve,SSC^{lo}), the latter being a wide gate including some contaminating events. A further 200 - 500,000 events which satisfied both these regions were then acquired. A 'tight' CD34 region was then set around the

progenitor cells using their CD34 *Vs* CD45 characteristics. FSC, SSC and PI of these gated cells were assessed to ensure that no apoptotic cells or debris had been included. CD34+ve cells were calculated as a percentage of viable leucocytes. An equivalent number of leucocytes stained with IgG1/2a control-PE, CD45-FITC and PI were acquired, and the percentage of control events were deducted to give a final CD34+ve percentage. The absolute numbers of circulating progenitors was then calculated from the total nucleated cell count. The main steps in this process are shown in Figure 2.3.

For CD34 subset analysis, 50,000 events were acquired and a wide region was set around the progenitor cells (CD34+ve,SSC^{lo}). A 200 - 500,000 event file was then collected using this gate. In order to allow simultaneous analysis of CD38 and CD59 expression by progenitor cells, only CD34 and light scatter characteristics were available for definition of progenitor cells. To analyse sufficient CD34+ve cells, large numbers of leucocytes had to be acquired, resulting in increased contamination of the CD34+ve region. To overcome this problem, regions were used from the CD34 quantitation assay to improve definition. In each case, the CD34 vs SSC and FSC vs SSC regions were assessed to determine whether they alone could identify progenitor cells with >95% accuracy, as defined by their combined CD34, CD45, PI, and light scatter characteristics. These regions were then applied to allow analysis of CD38 *vs* CD59, and controls.

Figure 2.3

FACS dot plots showing the main steps in CD34 quantitation as described in the text, section 2.28. The bottom left plot shows a 50,000 event file of CD45 vs PI. The R1 gate excludes non-viable (PI+ve) cells. The top left plot shows gate R2 which contains events from R1 which are CD34+ve and CD45^{low}. In the right hand plot, the forward and side scatter characteristics of the R1 and R2 gated cells are applied allowing a tighter gate to be placed around the stem cell population.
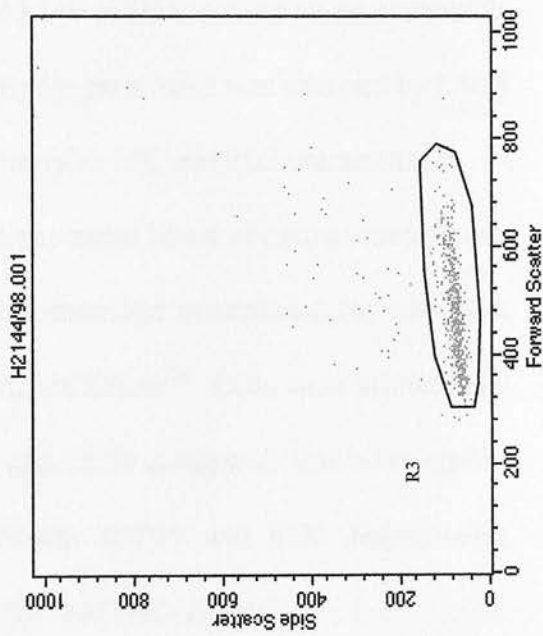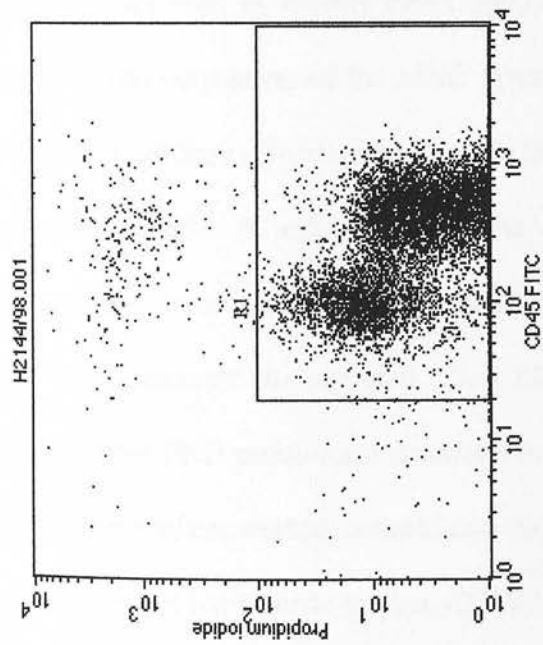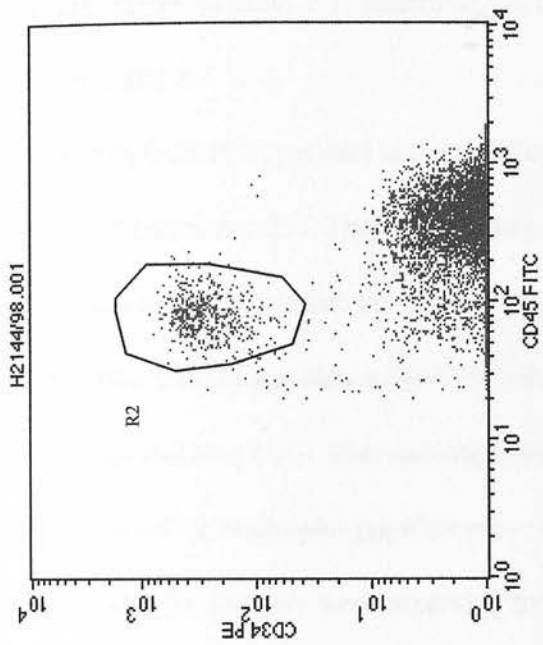
2.29   Phenotyping in PNH.

Cell preparation and FACS methods are described in sections 2.16 and 2.26. The MABs used are shown in table 2.1. Examples of these analyses are described and shown in chapters 6 and 7.

Neutrophils from PNH patients and normal controls were prepared for analysis by whole blood lysis (section 2.26). They were stained with pre-conjugated MABs reactive with GPI linked antigens present on normal neutrophils but absent on PNH cells (CD16, CD59 and/or CD55) and also a non-GPI linked MAB (CD15) present on all neutrophils as a control (section 2.26). The staining intensity for each MAB was analysed by FACS after gating on the neutrophil population by virtue of its FSC and SSC characteristics.

Lymphocytes for analysis were separated from peripheral blood of normal controls and patients with PNH by density centrifugation as described in section 2.16, with final washing and suspension of the MNC layer in FACSFlow™. Cells were stained with MABs against the GPI-linked antigens CD55 and CD59 along with antibodies against non-GPI linked B and T lymphocyte antigens (CD19 and CD3 respectively). Lymphocytes were identified by characteristic FSC and SSC profiles.

PNH cell lines were also analysed. These EBV transformed B lymphoblastoid cell lines derived from PNH patients are described in section 2.14. Aliquots of cells from liquid culture were taken, washed, counted and suspended in FACSFlow™. They were stained with the pan-B lymphocyte marker, CD19 (non-GPI linked) for identification and with CD55 and CD59 in the same way as described above for peripheral blood lymphocytes.

2.30   Assaying complement mediated cell lysis by FACS.

The relative sensitivity of different cell populations to complement mediated lysis was assessed by FACS using the property of retention or loss of different fluorescent dyes after exposure to a sensitising antibody and fresh human serum (as a source of complement). This allowed concurrent MAB staining to identify cell subtypes. Assays were performed using two different fluorescent dyes, Calcein-AM ester (Molecular Probes Inc., Oregon, USA) and Propidium Iodide (PI). The PI was eventually preferred as it was possible to adjust the FACScan^{TM} compensation settings so that this fluoresced in channel FL2 or FL3 allowing potential use of 2 other fluorochromes for cell identification. The Calcein-AM fluoresces in channel FL1 only prohibiting the use of FITC-conjugated MABs.

The assay was initially developed to lyse peripheral blood lymphocytes and the EBV transformed B-cell lines described in section 2.14. For the Calcein-AM based assay, cells were prepared, washed and placed in microtitre wells at 1×10^6 cells/well (see sections 2.16 and 2.26). They were loaded with calcein and sensitised by incubating for 15 minutes at room temperature with 5µl of appropriately titred Calcein-AM and 20µl of Anti HLA W6/32 (clone = W6/32HL, source = ECACC, Porton Down, Wiltshire, UK). After washing, the cells were incubated with complement for 30 minutes at 37^0C. The source of complement was human serum which was collected and immediately frozen in aliquots at -90^0C. 50µl aliquots of an appropriate dilution of this serum was added to each test well. The optimum dilution of serum for the assay was defined as that at which there was the greatest disparity in lysis between normal and PNH cells both in peripheral blood lymphocytes and in the B-cell lines. This was determined by using serial dilutions

of serum (in FACSFlow™) and proved to be 35% (see chapter 6). Controls using serum

heat inactivated at 56°C for 45 minutes (to remove complement), FACSFlow™ alone

and without any sensitising HLA antibody were set up in parallel to confirm that no non-

specific lysis occurred during the assay and that all test cells took up and retained

Calcein-AM in the absence of complement. After further washing, the cells were finally

stained with appropriate MABs (e.g. CD59 to distinguish normal from PNH cells) and

analysed by FACS. It was important to add CD59 or CD55 after the lysis stage as these

antibodies proved to be partly blocking and inhibited the inactivation of complement by

these molecules on normal cells. Lymphocytes were identified by their FSC and SSC +/-

additional MAB staining. Non-lysed cells retain the same intensity of fluorescence for

Calcein-AM and the same FSC and SSC. Cells whose membranes have been

compromised by complement have a lower staining intensity and reduced FSC and SSC

allowing easy identification. Examples of this analysis are shown in chapter 6.

The assay was subsequently modified to use PI allowing 3 colour analysis. This allowed

simultaneous staining of a lineage specific antigen (e.g. CD19 on B-cell lines) and a GPI

linked antigen (e.g.CD59) along with PI to assess the degree of lysis. The method was

identical to that described above except Calcein is not used and the PI is added to the

Kahn tubes after MAB staining and washing, 5mins. before analysis on the FACScan™.

In contrast to Calcein, PI is taken up by cells with damaged membranes therefore the

lysed cells are identified by PI positivity and reduced FSC and SSC.

Both the Calcein-AM and PI based assays were applicable to cells derived from semi-

solid colony assays after recovery of these cells from methylcellulose culture as

described in sections 2.16, 2.22 and 2.26. The assay conditions used were identical to

those described above for lymphocytes. MABs to glycophorin-A and CD13+33 were used to discriminate BFU-Es and CFU-GMs respectively.

2.31   Using PI-PLC.

Recombinant PI-PLC from *bacillus Thuringiensis* (Oxford Glycosystems, Abingdon, UK) was used. This is an enzyme which cleaves the GPI anchor, effectively producing PNH cells by a chemical means. It was used to cleave GPI linked structures from intact cells. The CD59-TM construct can be differentiated from native CD59 by virtue of its resistance to PI-PLC cleavage. For use, PI-PLC was diluted in FACSFlow™ at a concentration of 1 unit/ml. Cells were treated with the enzyme in microtitre wells (up to 1×10^6 per well). 100µl of PI-PLC (1 unit/ml) was added to each well and incubated at 37^0C for 60 minutes along with appropriate controls. This procedure could be combined with antibody staining and/or a complement lysis assay in the same wells.

Mobilisation of PNH patients with G-CSF

2.32   PNH patients

Four patients were investigated. All were under regular review with an established diagnosis of PNH. Informed consent for the procedure was obtained. The clinical details and baseline haematological parameters for these patients are described in detail in chapter 7.

2.33   Baseline investigations prior to G-CSF mobilisation.

Before administration of G-CSF, written informed consent was obtained from all patients. Blood was taken for the following assessments:

Haematology: FBC, differential WBC, blood film, reticulocyte count.

NAP score: This was performed by Sheila O'Connor in our laboratory. NAP stock substrate was prepared by dissolving 30mg of napthol AS phosphate in 0.5ml dimethyl formamide and adding 100ml 0.2M tris buffer at pH 9.1. The NAP stain was prepared by adding 2mg fast blue BB to 10ml of this stock substrate. Blood films from patients and controls were fixed in buffered cold formol acetate for 30 seconds then rinsed. The slides are overlain with the NAP stain for 20 minutes, rinsed and counterstained with aqueous neutral red for 1 minute. NAP positivity is indicated by the presence of blue/black granulation in the neutrophil cytoplasm which is conventionally scored from 0-4. The NAP score for a sample is the sum of the scores of 100 neutrophils assessed by light microscopy. The normal range in our laboratory is 40-100.

Biochemistry: A standard biochemical profile including urea, electrolytes, creatinine, liver function tests, calcium, protein and albumin estimations.

FACS phenotyping: The patients neutrophils, red cells and lymphocytes were typed with appropriate MABs (table 2.1) to GPI linked antigens to assess the size of the PNH clone in each cell line according to the methods described in sections 2.26 and 2.29. The absolute numbers and proportions of stem cells in the peripheral blood of these patients was measured using the CD34 antigen and stem cell subsets were investigated as described in section 2.28.

Progenitor colony assays: CFU-GM, BFU-E and P-delta assays were set up from all patients peripheral blood as described in section 2.16.

Complement sensitivity: A PI-based lysis assay was performed by FACS on all patients' separated peripheral blood lymphocytes as described in section 2.30.

2.34 G-CSF mobilisation Regimen in PNH.

After the above baseline investigations had been performed, patients were given G-CSF (Filgrastim, Amgen U.K. Ltd.) in an attempt to mobilise PBSCs. G-CSF was given at a dose of 10μg/Kg/day by subcutaneous injection for 4 consecutive days. This was administered as an outpatient with daily monitoring. Patients were assessed clinically by a haematology specialist nurse and a member of haematology medical staff each day. Phenotypic analysis of neutrophils and CD34 quantitations were carried out daily by FACS. CD34 subset analysis was performed on steady state blood and on day 5 (one day post G-CSF) as described in sections 2.28, 2.29 and 2.33.

2.35 Collection and processing of mobilised peripheral blood.

The day after G-CSF treatment was completed (day 5), 350ml of peripheral blood was removed via an ante-cubital vein using a sterile venesection pack containing Acid citrate dextrose anticoagulant (Baxter healthcare Ltd., Berkshire, UK). Subsequent work was carried out in a sterile safety cabinet to avoid contamination of the cells prior to culture. The MNCs were separated from the blood by density centrifugation (section 2.16) in 50ml Falcon™ centrifuge tubes with a spin of 35 minutes at 1800rpm. The MNCs were

pooled, washed and assayed. Excess MNCs were frozen in aliquots in 10%DMSO at -

90°C and transferred to liquid nitrogen (see section 2.15). The same assays that were

performed at baseline (section 2.33) were done on day 5. In addition, separated MNCs,

hopefully enriched for CD34+ve cells, were exposed to CD59-TM retroviral supernatant

as described in section 2.22 in order to assess the possibility of transduction of PNH

stem cells with this construct.

Chapter 3.

In-Vivo PBSC Purging in CML by a Disease-Specific Mobilisation Regimen

3.1   Introduction
3.2   Patient details
3.3   Mobilisation of PBSCs in CML with Hydroxyurea
3.4   Harvest quality
3.5   Contaminating clonal cells in the harvest products
3.6   Engraftment following reinfusion of PBSCs mobilised with Hydroxyurea
3.7   Patient follow up
3.8   Discussion

3.1   Introduction

CML is a clonal myeloproliferative disease which clinically progresses from

an initial chronic phase through an accelerating phase and finally terminates in an

inevitable blast transformation which results in the patients' death. The disease is

characterised by the presence of a definitive molecular event resulting in the

formation of a fusion gene (BCR-ABL) which codes for an abnormal tyrosine kinase

which has an ill-defined transforming activity on myelopoiesis (Gordon & Goldman,

1996). This abnormality is generally detected cytogenetically by the presence of the

Philadelphia (Ph) chromosome but can also be assayed by FISH showing the t(9;22)

translocation or by PCR for the BCR-ABL fusion mRNA (Cross *et al* 1993). At

diagnosis, most patients have entirely Ph+ve metaphases in their marrow and blood.

Conventional drug therapy has been largely unable to alter this even in those whose disease is well controlled. This led to the view that haemopoiesis was entirely replaced by stem cells bearing the Ph abnormality in CML. This view has now been revised and it is appreciated that there is residual normal haemopoiesis in patients' marrows and that, as discussed below, certain laboratory and clinical manoeuvres can exploit this therapeutically (Gordon & Goldman, 1996; Goldman & O'Brien, 1993).

Treatments considered to confer a survival advantage in CML are all associated with reduction, suppression or apparent elimination of Ph+ve cells (Giralt *et al* 1995). To date, the only widely accepted approaches which can achieve this are allogeneic marrow transplantation and IFN therapy (Allan *et al* 1995; Goldman *et al* 1988). Despite these promising approaches, much remains to be done in management as allografting is available to only a small minority of patients and IFN is not a cure. This has contributed to increasing interest in autologous marrow and stem cell transplantation in CML over recent years (Goldman, 1994). The use of ABMT in this disease grew from observations that some patients were rendered wholly or partially Ph-ve following accidental busulphan induced hypoplasia. Subsequently, similar findings were demonstrated after deliberate administration of AML-type chemotherapy regimens (Sharp *et al* 1979). This led to the use of ABMT initially for patients in blast crisis but increasingly in chronic phase (O'Brien & Goldman, 1994). Although there has been no large prospective randomised trial comparing survival between autografted and conventionally treated patients, over 200 transplants have now been reported in the literature (O'Brien & Goldman, 1994; McGlave *et al* 1994a). It seems clear that those transplanted in chronic phase do better than those in accelerated phase who in turn do better than those in blast crisis. Interestingly, at least half of those in chronic phase

achieve partial or complete cytogenetic responses. These cases generally relapse over time but may occasionally be durable. A recent analysis of the results shows a plateau in the survival curve following ABMT which has never been present in conventionally treated patients (McGlave *et al* 1994a). Retrospective comparisons must be viewed with caution but the suggestion is of a survival advantage for autografting with either marrow or peripheral blood stem cell support.

If Ph negativity is a treatment goal and it is accepted as a possibility that clonal cells in harvest products can contribute to relapse in some malignancies (Brenner, 1996) then there may be a rationale for purging strategies in CML. Attention is now being directed to the manipulation and purging of such stem cell products in an attempt to reduce the number of Ph+ve cells reinfused (O'Brien & Goldman, 1995). If harvested CML marrow is placed in long term culture the Ph+ve cells tend to decline and an increase in the proportion of Ph-ve cells may be seen. Such cultures have been used as the source of stem cell support for CML autografts and despite some problems with engraftment a significant number of cases have been successfully transplanted with a reduction in Ph positivity, sometimes to zero (Barnett *et al* 1993). Other attempts to purge Ph+ve cells ex-vivo have included the use of in vitro IFN or chemotherapeutic agents and incubation with antisense oligonucleotides. There have been a number of small studies addressing the feasibility of combining one or more of these approaches to minimise the Ph+ve clone. Despite some encouraging preliminary results, it is impossible to say whether this will ultimately translate into clinical benefit.

Since 1989, Carella et al in Genoa have been pioneering a technique for collecting 'in vivo purged' stem cells (Carella *et al* 1991). This relies on the differential recovery of Ph+ve and Ph-ve peripheral blood stem cells following myelosuppression. Variations of

this method have been used by a number of groups and it has been established that such cells can be collected in most CML patients and used for autografting (Johnson & Smith, 1997; Carella *et al* 1997). As with conventional autografting, there are patients many months out from such procedures whose bone marrows remain Ph-ve. The chemotherapy regimens used by most groups have proved toxic, with severe myelosuppression and a mortality of at least 5%. In this chapter, I describe a technique for the mobilisation of predominantly Ph-ve PBSCs using a less toxic oral regimen of Hydroxyurea and G-CSF. We have mobilised PBSCs from 18 patients in this way and 7 of them have proceeded to PBSCT using only these cells (Johnson *et al* 1996a; Pratt *et al* 1998). The details of the patients, the mobilisation and harvest data, success of Ph-ve cell collection and outcome of transplantation are described and a comparison made with other regimens currently employed regarding toxicity and efficacy.

3.2   Patient details

Eighteen patients were studied. All had proven typical Ph+ve CML. Median age was 48 years with a range of 29-59. Median disease duration was 12 months (range 2-48). One patient (ES) was in blast crisis at the time of mobilisation and one (GJ) was in second chronic phase. The remaining 16 were all in first chronic phase. The type and number of previous modalities of treatment varied. Nine of the patients had received only Hydroxyurea and 6 had received Hydroxyurea and α-interferon. Three had received other cytotoxic agents in addition. Patient details, disease status, duration and treatment are shown in table 3.1.

Table 3.1   **Clinical Details of the CML Patients pre-Mobilisation**

	Age	Sex	Disease Status	Duration(months)	Previous Therapy
GJ	36	F	CP2	36	HU, DAT 3+8
ES	48	F	BC	48	HU only
AL	33	M	CP	8	HU only
CM	35	M	CP	30	HU, IFNα
RK	46	M	CP	15	HU, IFNα
CH	39	F	CP	2	HU only
SR	39	F	CP	4	HU only
AP	55	M	CP	10	HU, IFNα
CB	49	M	CP	12	HU, IFNα
MD	36	F	CP	36	HU, IFNα, Busulphan
DJ	56	F	CP	6	HU only
JG	29	F	CP	36	HU, IFNα, Busulphan
JW	59	F	CP	2	HU only
PS	50	M	CP	7	HU only
RD	55	M	CP	4	HU only
EP	59	F	CP	3.5	HU only
BM	49	F	CP	19	HU, IFNα
JT	53	M	CP	30	HU, IFNα

(CP=Chronic Phase, BC=Blast Crisis, HU=Hydroxyurea, IFNα=alpha-Interferon, DAT=Combination Chemotherapy)

3.3   Mobilisation of PBSCs in CML with Hydroxyurea

PBSCs were mobilised using a combination of high dose oral Hydroxyurea and G-CSF (Filgrastim). The procedure is summarised in chapter 2 section 2.2.

Hydroxyurea dosage: The first case was a young female patient (GJ) whose transformation of CML had reverted to a 2nd chronic phase following intensive chemotherapy. Assuming her marrow reserve to be poor following this treatment, she was given a fairly low dose (3g daily for 7 days) of Hydroxyurea followed by G-CSF (300µg daily). The WBC nadir was $1.1 \times 10^9/l$. and she underwent 4 aphereses, 14 days post Hydroxyurea, commencing when the WBC rose to $1 \times 10^9/l$. Despite this being a poor risk case, adequate cell numbers were obtained, all of which were Ph-ve. The doses that were used for each of the 18 patients are shown in table 3.2 along with the duration and depth of WBC nadir.

Initially, patients were given 6g of Hydroxyurea daily for at least seven days with further doses beyond this if the WBC had not fallen below the normal range. However, the WBC continues to fall after the end of treatment so subsequently Hydroxyurea was given for a fixed period of seven days. Two of the patients received higher doses (8g for 10 days (RK) and 6g for 10 days (AL)). They suffered no increased side effects and were successfully harvested. In 3 cases, the initial Hydroxyurea dose failed to achieve a satisfactory nadir (WBC remained 2.2-$3.6 \times 10^9/l$.) and the procedure was abandoned before apheresis. Not surprisingly, these patients experienced no toxic effects. All 3 of these mobilisations were repeated using either the same or a slightly increased dose. They were all successfully harvested on the second occasion. The data suggests that Hydroxyurea produces a useful nadir in the majority of cases if given at a dose of

$3.5g/m^2$ daily for 7 days. This probably errs on the side of caution and some patients may require more. In these cases, apheresis should not be attempted and repeat procedures are easily performed. This is preferable to using a higher initial dose which, for some patients, might produce unnecessary and unwanted side effects.

Kinetics of Mobilisation: Most patients show a slow reduction in WBC starting after 4 to 5 days of Hydroxyurea and continuing for 2 to 4 days beyond it. Nadir is reached around day+3. A significant number of patients show a transient rise in the WBC shortly after nadir is reached but this falls again within a day or two. This is presumably a response of residual neutrophils to G-CSF. At this time there are no detectable CD34+ve cells in the peripheral blood which allows differentiation of this transient rise from true marrow recovery. Attempts to harvest 2 patients at this time were made initially but no stem cells were collected and successful harvests were subsequently achieved from them 9 to 11 days later. As shown in table 3.2, the duration of leucopenia following Hydroxyurea proved quite consistent (mean 13.8 days, range 6-18) and the mean depth of WBC nadir was $0.8x10^9/l$. Only one patient differed significantly from the rest of the group in their recovery time. This patient was in accelerated phase and recovered after only 6 days. The WBC tends to recover slowly over several days at the end of nadir. Because of this, the optimum time to commence apheresis can be difficult to judge. In our experience, the most useful guide is to combine the observation of a rising daily WBC with the detection of increasing numbers of CD34+ve cells in the peripheral blood. Reliance on only an arbitrary rise in WBC may mean that apheresis is started too early and large numbers of fruitless daily procedures may be performed.

Table 3.2 Hydroxyurea Dose and Kinetics of Mobilisation

Patient	Grams/Days of HU	Nadir WBC	Days to Harvest
GJ	3g/7	1.1	14
ES	6g/7	0.5	6
AL	6g/10	0.5	14
CM	6g/7	1.6	14
RK	8g/10	0.5	15
CH	6g/7	0.6	16
SR	6g/7	0.4	15
AP	6g/7	0.8	18
CB	8g/6	0.5	15
MD	8g/7	0.6	10
DJ	6g/6	1.0	15
JG	4g/6	0.6	16
JW	5.5g/7	0.2	13
PS	7g/7	1.2	13
RD	7g/7	0.9	13
EP	6g/7	1.4	12
BM	8g/7	0.5	16
JT	7g/7	ND	14
MEAN	-----	0.8	14

(HU=Hydroxyurea, Days to Harvest=Days post HU until first leucapheresis)

Toxicity: The side effect profile of the procedure can be considered in two parts: inevitable haematological toxicity and specific effects of high dose Hydroxyurea. The haematological toxicity is predictable from the nadir achieved. One third of cases were treated entirely as outpatients with no episode of neutropenic fever. The others were admitted with fever which responded to standard broad spectrum intravenous antibiotics in all but one case. This one patient went on to receive amphotericin B although fungal infection was not documented. All patients recovered fully from the mobilisation procedure and spent a median of 9 days in hospital. Twelve of the 18 patients required an average of 2 units of blood and 4 units of platelets, the remaining 6 needing no blood product support. Side effects directly related to Hydroxyurea were skin rash and mild oral mucositis affecting about half of the patients. Nausea or vomiting was not a feature in any of the patients. One patient reported some thinning of their hair but no significant alopecia was seen.

3.4   Harvest quality

Table 3.3 shows details of the measures of harvest quality for each patient. A mean of 3.4 aphereses was required to achieve adequate cell numbers (range 1-6). Mean values and ranges for harvested MNC, CD34+ve cells and CFU-GMs were respectively: 4.3×10^8/kg (range 1.2-9.8), 3.0×10^6/kg (range 0.2-6.7) and 64.4×10^4/kg (range 2.3-170). Only three of the 18 patients (17%) mobilised less than a million CD34+ve cells per kg, which we would normally consider a relative contraindication to transplant.

Table 3.3   **Harvest Quality Assays**

Patient	MNC $\times 10^8$/kg	CD34's $\times 10^6$/kg	CFU-GM $\times 10^4$/kg
GJ	3.6	1.6	80
ES	1.9	2.0	113
AL	2.6	6.6	67
CM	5.9	1.4	74
RK	3.6	6.7	ND
CH	5.2	0.75	121
SR	4.1	6.2	170
AP	3.5	3.0	ND
CB	3.0	0.3	71
MD	2.8	5.4	38
DJ	7.7	2.4	ND
JG	8.1	2.0	ND
JW	1.2	3.3	19.2
PS	4.5	0.2	2.8
RD	2.2	2.3	14.5
EP	4.8	2.0	2.3
BM	9.8	4.4	ND
JT	2.7	3.4	ND
MEAN	**4.3**	**3.0**	**64.4**

3.5   Contaminating clonal cells in the harvest products

Assessment of clonal contamination by cytogenetics, FISH and PCR was made in all the apheresis products as described in chapter 2 section 2.3. Table 3.4 shows the results of these assays for each individual patient where the data is available. There is no available data on two of the patients as cytogenetics failed for technical reasons and the centre where the harvesting was carried out did not perform FISH. Fourteen patients are evaluable by conventional cytogenetics. All had 96-100% Ph+ve metaphases in their marrow prior to mobilisation. Of these, 4 (28.5%) had a complete response and a further 4 (28.5%) a major response (defined as >50% reduction in Ph+ve metaphases). One patient had a 46% response and the remaining 5 showed little or no response. FISH was a useful supplement to cytogenetics. It demonstrated that even in those harvests that were negative by cytogenetics there were 1-19% contaminating clonal cells by FISH. In addition it gave information on two further patients both of whom appeared to have had major responses but in whom conventional cytogenetics failed for technical reasons. Overall, over half (56%) of the patients showed a complete (28%) or major (28%) cytogenetic response to mobilisation with Hydroxyurea using this regimen.

3.6   Engraftment Following Reinfusion of PBSCs Mobilised with Hydroxyurea

Seven of the patients have been transplanted to date using only the PBSCs mobilised following Hydroxyurea/G-CSF. Two of the patients (ES and AL) were transplanted after transformation to blast crisis. The remaining 5 were in chronic phase. Conditioning chemotherapy and the techniques of reinfusion are described in chapter 2 section 2.4.

Engraftment of neutrophils to $>0.5 \times 10^9/l$. occurred at a median of 17 days and platelets $>20 \times 10^9/l$. at 21 days. One patient failed to make a clinical and haematological recovery from the procedure and died of veno-occlusive disease (VOD) and engraftment failure 3 months after return of PBSCs despite the infusion of additional backup buffy coat cells. The other 6 patients all left hospital within 33 days without any clinical problems beyond those that would be considered routine following a high dose procedure. Individual figures for engraftment are shown in table 3.5.

3.7   Patient follow up

Three of the seven transplanted patients have died. The causes of death were as follows: Patient AP died from transplant related toxicity (VOD) as described above. Patient AL relapsed with blastic disease. He had been transplanted in blast crisis, achieved a second chronic phase but relapsed again five months post PBSCT and was given a second autograft using buffy coat cells collected at diagnosis. He did not enter a second remission following this procedure and died from his disease eight months from the original procedure. Patient ES also died of disease relapse following transplantation in blast crisis but did achieve a 27 month second chronic phase with excellent quality of life which would be considered an impressive outcome in this setting. The remaining four patients are alive and well with follow up from 16 to 28 months. All are in chronic phase, three maintained deliberately on IFN and one requiring Hydroxyurea to control their counts. Disease status has been monitored by cytogenetics. All patients had 100% Ph+ve bone marrows prior to transplant. The Ph status of the stem cells reinfused in each case is shown in tables 3.4 and 3.6. All patients were evaluated at 3 months

following the procedure by bone marrow cytogenetics. Two of them showed a major response (CM and MD) with 18% and 34% Ph+ve metaphases in their marrows respectively. Both are showing an increase in the Philadelphia clone with time. At 14 months post autograft (the last assessment) the figures have risen to 86% and 50% positive respectively. Table 3.6 shows the follow up and outcome data along with cytogenetic response for all seven transplants.

Table 3.4   **Assessment of Harvests by Cytogenetics, FISH and PCR**

Patient	No.Ph+ve Metaphases /Total No.assessed	%Ph+ve metaphases	%Cells FISH+ve for t(9;22)	PCR for BCR/ABL
GJ	0/12	0	ND	ND
ES	1/42	2.4	9	+ve
AL	2/7	28.6	16	+ve
CM	Failed	Failed	10	+ve
RK	0/28	0	6	+ve
CH	10/31	32.2	13	+ve
SR	0/45	0	1	+ve
AP	41/50	82	49	ND
CB	2/11	18.2	12	+ve
MD	0/7	0	19	+ve
DJ	62/67	92	40	+ve
JG	46/49	94	37	+ve
JW	39/70	56	ND	ND
PS	Failed	Failed	ND	ND
RD	30/31	97	ND	ND
EP	Failed	Failed	ND	ND
BM	66/67	99	ND	ND
JT	Failed	Failed	32	ND

Table 3.5   Engraftment Data for the CML Transplants

Patient	Stage at transplant	N>0.5	N>1.0	Plt>20	Plt>50
ES	BC	22	75	157	210
AL	BC	15	29	13	17
CM	CP	17	23	23	110
MD	CP	17	17	38	41
AP	CP	33	NR	21	NR
JT	CP	16	20	11	14
RK	CP	15	15	13	33

(N=Neutrophils x10^9/l., Plts=Platelets x10^9/l.)

Table 3.6

Post Transplant Follow Up

	%Ph+ve Harvest	%Ph+ve BM Pre	%Ph+ve BM post	Status now/ months post
ES	2.4	100	100	Died of disease relapse/27
AL	28.6	100	100	Died of disease relapse/8
CM	10	100	18	CP on IFN, 86%Ph+ve/14
MD	0	100	34	CP on IFN, 50%Ph+ve/14
AP	82	100	100	Died of VOD/3
JT	32	100	100	CP on HU, 100% Ph+ve/18
RK	0	100	100	CP on IFN, 100%Ph+ve/26

(Pre=pre transplant, Post=3 months post transplant, BM=bone marrow, IFN=Interferon, HU=Hydroxyurea, VOD=veno-occlusive disease, CP=chronic phase)

3.8   Discussion

CML is an inevitably lethal malignancy curable only by allogeneic bone marrow transplantation. Pooled data from over 200 autografts in this disease (McGlave *et al* 1994a) suggests that this procedure may extend life if performed in chronic phase, although few if any are likely to be cured.   Gene marking experiments implicate contaminating tumour cells in autologous harvests in the relapse process in some diseases (Brenner, 1996). These observations allow us to hypothesise that purging the graft in CML before reinfusion might have clinical benefits. The differential recovery of normal and Ph+ve cells in the peripheral blood of these patients following myelosuppression has allowed collection of cytogenetically normal CD34+ve cells for autografting (Carella *et al* 1991). This in vivo purging technique has been investigated using intensive chemotherapy priming but there has been substantial morbidity and some mortality associated with the procedure (Chalmers *et al* 1994; Carella *et al* 1997). In this chapter, I have described the collection of such cells using a less toxic regimen (Johnson & Smith, 1997). Eighteen patients with Ph+ve CML were mobilised using the Hydroxyurea/G-CSF regimen and 7 have been autografted. (Johnson *et al* 1996a; Pratt *et al* 1998). One patient in blast crisis (ES) achieved an adequate and entirely Ph-ve collection and remained well for 2 years post transplant. By contrast, some patients mobilise no negative cells even shortly after diagnosis. It is felt that the success of these procedures varies inversely with disease duration and prior treatment (Carella *et al* 1997) but this cohort is too small to draw conclusions for this particular regimen.

The use of Hydroxyurea requires attention to dosage as the regimen is designed to produce a transient and short lived WBC nadir as opposed to intensive intravenous

regimens which accept profound myelotoxicity in all cases. The optimal starting dose from this data was $3.5g/m^2$ Hydroxyurea daily for seven days followed by G-CSF at 300μg daily thereafter until the last day of harvesting. This produced a mean WBC nadir of $0.8x10^9/l$ (range 0.2-1.6). with a mean duration of 14 days. There were no prolonged cytopenias. This dose was inadequate for 3 out of the 18 patients who were not apheresed but re-mobilised after a short interval. The occasional need to repeat the schedule seems acceptable by contrast to the marked myelotoxicity of intravenous regimens. Hospital stay was a median of 9 days and one third of cases were treated as outpatients. Hospitalisation is almost universal for intravenous regimens with a median stay of 29 days and a 5% procedural death rate (Carella *et al* 1993; Chalmers *et al* 1994).

Harvest quality from the Hydroxyurea regimen was adequate in most cases. A mean of 3.4 (range 1-6) aphereses was required, giving mean values and ranges for harvested MNC, CD34+ve cells and CFU-GMs of: $4.3x10^8/kg$ (range 1.2-9.8), $3.0x10^6/kg$ (range 0.2-6.7) and $64.4x10^4/kg$ (range 2.3-170). Only 3 of the 18 patients (17%) mobilised less than 10^6 CD34+ve cells/kg, which we would normally consider a relative contraindication to transplant.

The purging efficacy of this regimen appears very similar to that of other published data, although numbers are small and this should be viewed with caution. Overall there have been about 200 mobilisations reported using an Idarubicin and Cytarabine based regimen and around one third of the collections have been entirely Ph-ve with half demonstrating a major (>50% Ph+ve reduction) response (Carella *et al* 1997). We found almost the same with 28% Ph-ve and 56% major response.

Results from the 7 transplanted patients are interesting. Engraftment and procedural toxicity appear comparable to standard autografting in CML with one death from VOD at 3 months and all other patients recovering normally. Two other patients have died, both from disease relapse, both having been transplanted in blast crisis. One of these (ES) survived over 2 years from PBSCT with a good quality of life. The remaining 4 patients are alive in chronic phase. Two of these achieved major cytogenetic responses but these are slowly being lost over time. The transplant results from other more intensive regimens are similarly variable. Several authors have reported small cohorts of between 5 and 16 patients with small proportions of these cases becoming Ph-ve post procedure (Talpaz et al 1993; Chalmers et al 1994). Some patients have remained in cytogenetic remission approaching 3 years (Carella et al 1994) but long term follow up is needed.

In summary, Ph-ve PBSCs can be collected and successfully reinfused in CML patients, particularly during the early stages of their disease. The optimum method remains to be determined but the severe toxicity of some of the more aggressive intravenous regimens may not be justified without proof of increased efficacy. Whether any form of autotransplantation will improve survival in CML is as yet unknown and whether any patients can be cured by such procedures remains doubtful at present.

This form of in vivo stem cell purging has proved practical at a clinical level and could in theory be combined with other forms of purging ex vivo and become part of the management of CML for those without an allogeneic option.

Chapter 4.

Ex-Vivo Purging and Stem Cell Purification in Myeloma by CD34 Selection

4.1   Introduction
4.2   Patient details
4.3   CD34 selection on the Ceprate™ column
4.4   Engraftment following CD34 selected PBSCT
4.5   Patient follow up and monitoring of MRD
4.6   Discussion

4.1   Introduction

Myeloma   is a malignant haematological disease characterised by the progressive accumulation of clonal plasma cells in the marrow and sometimes at other sites (Boccadoro & Pileri, 1995). These cells usually produce a monoclonal immunoglobulin (paraprotein) detectable in serum and/or urine. In advanced disease, these findings are associated with a range of serious clinical manifestations including bone destruction, hypercalcaemia, marrow failure, infections and renal impairment. These complications cause considerable morbidity and the disease itself is generally fatal with a median survival from diagnosis of around 30-36 months (Barlogie *et al* 1989). Conventional chemotherapy for the disease has involved the use of alkylating agents and steroids, most commonly melphalan and prednisolone. Other drugs have been added including anthracyclines and vinca alkaloids and a variety of regimens based on combinations of these agents has been investigated with varying results. There is

no international consensus on the interpretation of these trials, with some strongly favouring a particular combination and others unconvinced that there is any superiority to oral melphalan and prednisolone (Gregory *et al* 1992; Bergsagel, 1995). This conventional chemotherapy achieves disease response in around 50% of cases but perhaps only 5% achieve CR (if this is strictly defined) and only 5% will be alive 10 years from diagnosis (Kyle, 1983). The introduction of infusional chemotherapy regimens and intensification of treatment with high doses of melphalan with or without stem cell support appears to have improved disease response with up to 50% CR rates in some series (Vesole *et al* 1996b). Importantly, there is now evidence that this more aggressive approach can extend survival (Attal *et al* 1996). In the light of this evidence, high dose therapy (HDT) supported by stem cell reinfusion is becoming more or less standard treatment for myeloma although many centres rightly restrict this to the context of large scale randomised trials.

Despite the enthusiasm that this dose intensification has generated in the clinical community, the excellent responses that can be achieved appear not to be durable (Vesole *et al* 1996b; Harousseau *et al* 1995). Current HDT is therefore unlikely to cure the disease and for this reason, further modifications of the procedures are being evaluated.

One such modification to autografting in myeloma is CD34 selection of stem cell products. Theoretical aspects of CD34 selection are discussed in chapter 1 and the methodology used for the work described here is outlined in chapter 2. The generation of small volume, highly purified stem cell products by this technology could have benefits in myeloma and other diseases either by acting as a starting point for therapeutic gene modification or through tumour purging. In myeloma, CD34

selection should result in a product greatly depleted of tumour cells, provided that they do not express the CD34 antigen. Conventional wisdom has assumed the clonal plasma cell, which is CD34-ve, to be the cell of origin of myeloma (Rawstron *et al* 1997a). In recent years however, there has been much debate regarding the identity of the proliferating cell pool in this disease and this is as yet unresolved. The plasma cells in myeloma can be shown by PCR techniques to be clonal by demonstrating patient specific rearrangements of the immunoglobulin heavy chain gene (IgH) (Owen *et al* 1996). It has been shown by ASO-PCR that B-lymphocytes with the same IgH rearrangement as the myeloma plasma cells circulate in small numbers in patient peripheral blood (Billadeau *et al* 1992). It has been suggested that these clonal B cells are the proliferating cells in myeloma (Pilarski & Belch, 1994). Further work has demonstrated, in the same patients, the presence of the myeloma-specific IgH rearrangement in association with different heavy chain constant genes (Billadeau *et al* 1993). This implies that the myeloma clone may arise from a pre-switch B cell but whether this is the 'tumour stem cell' or a non-malignant stage of myeloma development requiring further tumorigenic events is unclear. It has therefore been suggested that there could be a primitive CD34 expressing pool of cells which are clonally relevant in myeloma although phenotypic work suggests this is not the case (Vescio *et al* 1994).

In addition to the potential purging effects, CD34 selection in myeloma might lend itself to the investigation of gene therapy for the disease. As already discussed above, myeloma is chemosensitive and demonstrates a dose response to melphalan and other alkylating agents (Barlogie *et al* 1997). Drug resistance genes have been demonstrated to play a part in the ultimate failure of therapy (Sonneveld, 1995). The

effectiveness of chemotherapy might be enhanced by genetic modification of tumour cells to sensitise them or of normal stem cells to protect them and allow dose escalation. The genetic modification of normal CD34 cells in myeloma to secrete substances implicated in myeloma growth or development such as inhibitors to IL6 is also conceivable (Hata *et al* 1993) but as yet none of these approaches is viable without further advances in gene therapy techniques and clearer understanding of myeloma cell biology. The isolation of stem cells and their concentration in a small volume is advantageous to current strategies for gene therapy as it improves the MOI in viral transduction protocols as discussed in chapter 1 (Bregni *et al* 1992). CD34 selection is one way of achieving this at clinical scale and it is thus important to investigate the clinical effects of such manipulation on engraftment post transplantation and to demonstrate its feasibility for a wide range of clinical applications.

In this chapter, I describe eight patients with myeloma who underwent CD34 selection using the Ceprate^{TM} avidin-biotin immunoaffinity column. Six of these went on to have PBSCT with the selected cells. The intention was to establish how applicable the technique was to clinical scale transplantation. The cell products were investigated for their putative stem cell content at all stages of processing and for the presence of contaminating cells related to the myeloma clone. I wished to establish by laboratory and clinical monitoring whether this approach could yield adequate cells for PBSCT with acceptable short and long term engraftment capability and if there was a significant reduction in tumour cell contamination in the selected products (Johnson *et al* 1996b).

4.2 Patient Details

Eight patients with myeloma were CD34 selected following mobilisation of PBSCs as described in section 2.7. One of them (PH) went through 2 procedures due to poor cell yields. The median patient age was 53 years (range 46 to 61). All but one of the patients (GL) were male. All patients received cytotoxic treatment until maximum response before undergoing mobilisation. Six of the eight patients had received only one modality of treatment (5-8 courses of combination chemotherapy consisting of Cyclophosphamide, Vincristine, Adriamycin and Methylprednisolone-CVAMP as per the UK MRC Myeloma VII trial protocol) before harvesting, while the other two had been heavily pre-treated, one with ABCM (Adriamycin, BCNU (carmustine), Cyclophosphamide and Melphalan as per the UK MRC Myeloma VII trial protocol), α-interferon and CVAMP and the other with CVAMP followed by an unselected PBSCT. All patients had myeloma as defined by the UK MRC trial criteria. The median disease duration prior to transplant was 13 months with a range of 8 to 52. Table 4.1 shows the age, disease subtype, previous treatment and disease status at mobilisation for each of the 8 patients. We defined a partial remission (PR) according to the criteria used in the UK MRC Myeloma VII trial. This requires a 50% reduction in paraprotein (or 50% reduction in marrow plasma cell infiltrate in the case with light chain myeloma). We further defined a 'good PR' as >75% reduction in paraprotein/plasma cell infiltrate with <15% plasma cells in the marrow. At the time of the procedure, as shown in Table 4.1, five patients were in a 'good PR', two were in a PR and one was in relapse. None of the patients was in complete remission (CR).

4.3   CD34 selection on the Ceprate™ column

Nine CD34 selection procedures were performed on mobilised PBSCs from the eight

patients (PH being processed on 2 separate occasions). The selection process on the

Ceprate™ column is described in section 2.8.   A mean of $45x10^9$ (range 17-122) MNC

were processed on the column for each patient. The pre-column product contained a

mean of 1.2% CD34+ve cells (range 0.1-2.3). The post column products contained a

mean of $2.4x10^8$ MNC an average of 49% of which were CD34+ve (range 18.4-98%).

Despite wide differences in the cell numbers processed and the percentage of these that

were CD34+ve the yield of CD34+ve cells was relatively constant with a mean of 30.1%

(range 24-37.8). There was no correlation between yield and the percentage CD34+ve

cells in the harvest pre-processing. Figure 4.1 shows a photomicrograph of CD34

selected cells, most of which resemble myeloblasts or small lymphocytes. Table 4.2

shows a breakdown of the absolute numbers of cells processed for each patient at each

stage of the processing and of the CD34+ve proportions and yields before and after

selection. On the left of the table, the first 3 columns show the absolute cell numbers

put onto the column (top), retrieved from the column after CD34 selection (bottom) and

lost in the column waste (waste). On the right of the table, the CD34+ve percentages are

shown at the top (pre-processing) and the bottom (final product post processing. The

figures on the extreme right of the table show the %yields of CD34+ve cells in the final

selected product.

Figure 4.1

Photomicrograph of CD34 selected cells (post column/final product) stained with MGG. Most of the cells have the morphology of small lymphoid cells or larger blasts. One contaminating neutrophil is seen.

Table 4.1   Clinical details of patients being CD34 selected

Patient	Age	Diagnosis/Date	Previous Therapy	Status at Mobilisation
WW	53	IgG MM, Feb/94	7x CVAMP	Good PR
KK	56	IgM MM, Nov/94	8x CVAMP	Good PR
BD	56	IgG MM, July/94	6x CVAMP	Good PR
PC	46	IgG MM, Sept/93	5x CVAMP	Good PR
IB	48	IgD MM, Feb/94	5x CVAMP	Good PR
JL	48	BJ(k)MM,Oct/91	8xABCM,αIFN, then 6xCVAMP	PR (20% plasma cells)
LG	53	IgG MM, Sept/93	6x CVAMP	PR (15% plasma cells)
PH	61	IgA MM, 1992	6x CVAMP then unselected PBSCT	Relapsing post PBSCT

(BJ=Bence Jones, PR=Partial Remission (see text section 4.2), CVAMP/ABCM/IFN - see text section 4.2)

Table 4.2

Summary of Ceprate™ Processing Figures (see text)

PATIENT	Total cells processed x10⁹ Top	x10⁸ Bottom	x10⁹ Waste	%CD34 cells at top of column	%CD34 cells in final product	%CD34 cell yield in final product
WW	27.2	2.9	13	1.2	42.6	37.8
KK	76.4	2.43	66.6	0.8	86.3	34.3
BD	31.1	2.0	31.0	0.6	30.9	33.1
PC	17.1	2.16	1.42	ND	60	ND
IB	122	6.5	74.1	2.3	90.7	21
JL	22.1	2.07	21.3	2.0	63.4	29.7
GL	39	1.71	36.1	ND	98	ND
PH 1	56.1	1.3	30.4	ND	47	ND
PH 2	17	0.23	16.9	0.1	18.4	24.8
MEAN	45	2.4	32.3	1.2	49	30.1

CFU-GM assays were performed on 5 of the patients and showed an increase in numbers in the final, selected product with very few colonies in the waste (77.5×10^4/kg vs. 10×10^4/kg). P-Delta assays, which may equate to the LTCIC (Gordon, 1994) were performed in two patients and showed a similar concentration of progenitors in the product. These results lend support to the assumption that selecting for CD34+ve cells also selects the primitive precursors capable of long term marrow reconstitution.

4.4 Engraftment following CD34 selected PBSCT

Six patients were transplanted with a median of 2.0×10^6/kg CD34+ve cells (range 1.5-9.4). Two patients were not transplanted, one due to rapid disease progression (BD) and the other because of an inadequate cell collection (PH). This latter patient had undergone a previous unselected PBSCT. In the 6 patients transplanted, engraftment of neutrophils to $>0.5 \times 10^9$/l. took a mean of 16 days and platelets $>20 \times 10^9$/l. took a mean of 17 days. This is similar to other reported experience with this selection method (Schiller et al 1995) and to our own experience in non-selected stem cell transplants in pre-treated myeloma patients. All six patients had unremarkable clinical courses and were discharged home well and self caring. One patient (IB) who had presented with predominantly tissue disease of IgD type relapsed within a month of the procedure with further skin lesions and underwent a second transplant with 1.6×10^6 CD34+ve cells/kg (stored from the original selection procedure). This patient achieved rapid neutrophil recovery but never engrafted platelets and died two months later of massive relapse of their disease. Table 4.3 summarises the engraftment data.

Table 4.3   **Engraftment Data for the CD34 Selected Patients**

	CD34+ cells reinfused x10^6/Kg	Days until:			
		N>0.5	N>1.0	Plts>20	Plts>50
WW	1.6	12	ND	12	12
KK	2.9	15	26	26	54
PC	1.5	17	19	14	30
IB 1	7.8	14	17	13	15
IB 2	1.6	13	21	13	never
JL	2.0	27	35	25	36
GL	2.7	13	14	15	37

(N=Neutrophil count x10^9/l., Plts=Platelet count x10^9/l.)

4.5 Patient follow up and monitoring of MRD

Seven of the 8 patients had detectable IgH rearrangements in their marrows at presentation. The same rearrangement was present in two of these cases in the leucapheresis product. Both of these products were successfully purged by the column and all final products were PCR negative. In addition to this, the polyclonal PCR electrophoretogram pattern seen in the pre-column products was absent from all final products indicating a substantial reduction in normal B cells following CD34 selection. There were 2 early deaths in the 6 transplanted patients. Patient IB relapsed within one month and underwent a second selected PBSCT but died 2 months after the procedure with massive disease relapse. Patient KK died 4 months post transplant with progressive neurological signs and CT scan evidence suggesting progressive multifocal leucoencephalopathy. There was no evidence of disease progression at the time. PCR data post transplant is unavailable for these two cases. The remaining 4 patients were monitored for MRD by PCR and conventional methods. Post PBSCT, two achieved CR with PCR negativity (patients PC and WW), one was in conventional CR but PCR positive (JL) and one achieved only a PCR positive PR (GL). This latter patient remained stable for some time but eventually progressed and died two years post PBSCT. The three who achieved CR remain alive 4 years post transplant but only one is still in CR. This patient (PC) is well and persistently PCR negative.

In summary, of 6 transplanted patients, 2 died early, 2 achieved CR and are alive but have needed further treatment, 1 achieved stable PR and died after 2 years and 1 achieved CR, remains alive and well at 4 years without treatment and has no evidence of disease down to the molecular level.

4.6   Discussion

This chapter describes the laboratory and clinical outcome of nine PBSCH CD34 selection procedures in eight patients with myeloma, six of whom have been transplanted with a   follow up to the present day of 4 years for those surviving. This cohort serves to demonstrate the clinical utility of the selection procedure.

The absolute number of CD34+ve cells harvested   ranged from 1.9 to $28x10^6$. This heterogeneity in mobilisation is common in our experience but we have not been able to relate it to any specific factor in this cohort.   One surprising feature is the relatively constant yield of CD34+ve cells (range 21-37.8%) despite wide variation in both the CD34 percentage and absolute numbers loaded onto the column.   CD34 purity in the final product ranged from 18% to 90% (mean 49%). The relationship of purity to tumour cell contamination may be important as a product of low CD34 purity may increase the likelihood of tumour cell contamination. Our PCR analysis does not show any increased detection of clonal cells in the products that were less pure but numbers are small and contamination below the level of detection by this assay may be relevant.

Molecular analysis of IgH rearrangements in this study demonstrates that CD34 selection can reduce the level of contaminating B cells to below that at which they can be detected by PCR. Despite this, the two patients whose harvests were PCR positive before selection remained positive post transplant in their marrows even though their reinfused stem cells had been apparently purged by the procedure. This highlights the point that eradication of disease in the body by adequate conditioning is an essential part of treatment. If the body burden of disease is incurable by intensive HDT then there is little point in manipulating the reinfusion product by CD34 selection in an attempt to

purge it of tumour. Myeloma may not currently be an ideal disease for CD34 selection if this is envisaged as a purely tumour purging strategy to improve survival.

Short and long term engraftment after selected PBSCT appeared normal in this series with all the transplanted patients recovering and further problems being associated with disease relapse/progression rather than graft failure. CFU-GM's and p-delta cells were monitored as an indicator of engraftment potential and an increase in their numbers was seen in the final product. Although reassuring this can only be regarded as a very indirect indicator of stem cell numbers. The most important (and only direct) indicator of harvest quality is the ability to restore and maintain marrow function after reinfusion and this has been successfully achieved. Despite the difficulties of directly monitoring long term repopulating potential we feel that taken together this data supports the view that 'true' stem cells are contained within the CD34 fraction and are efficiently collected in a viable state by the Cellpro system and this finding has been consistent in other series (Schiller et al 1995). It is possible to conclude from this work and that of others that CD34 selection by this methodology is practical on a clinical scale and provides effective haemopoietic support for high dose therapy.

While this chapter has described a cohort of patients with myeloma, this has been done mainly to demonstrate the applicability of the process and does not imply that this is the only or indeed a suitable area for this technology. As discussed in chapter 1, the ability to select CD34 cells can be regarded as an initial stage in the further purification and/or expansion of stem cells ex vivo (Alcorn & Holyoake, 1996). It can achieve a degree of tumour cell purging which may or may not ultimately improve survival in some diseases (Schiller et al 1995; Brugger et al 1994) and it may produce cell products which are well suited to gene therapy applications (Bregni et al 1992).

Chapter 5.

Gene Transfer into Haemopoietic Stem Cells

5.1   Introduction
5.2   Assessing the potency of retroviral supernatants
5.3   Assessing transduction efficiency
5.4   Gene transfer into haemopoietic cells
5.5   Discussion

5.1   Introduction

One of the therapeutic possibilities arising from an ability to collect and manipulate primitive stem cells is the introduction of novel DNA into these cells with the intention of reinfusing them into patients to alter a disease phenotype (Apperley & Williams, 1990b).   Stem cells are unique in their ability to both proliferate and self renew (Gordon & Blackett, 1994) and these attributes offer the possibility of long term survival and expression of any new genetic material which they may have been used to deliver. This has made them an attractive target for the rapidly increasing number of gene therapy protocols (Dunbar, 1996a).

There are many potential applications for stem cell modification by gene therapy, as has been discussed in chapter 1. Mechanisms of relapse in malignant disease are being clarified by gene marking experiments (Brenner *et al* 1995) and various

approaches to cancer treatment are under investigation including: tumour vaccine strategies, introduction of suicide genes and engineered expression of drug resistance cassettes in normal stem cells as chemoprotectants. Outside the field of malignancy there is great interest in the restoration of normal protein function in monogenetic disorders such as Cystic Fibrosis or the Haemophilias (Boucher *et al* 1994) and models for the treatment of diseases as diverse as AIDS and peripheral vascular disease are being developed.

Chapter 1 describes a model system for therapeutic gene transfer into haemopoietic progenitors in PNH. This involves the use of a novel construct containing a form of CD59 modified to bind to cell membranes through a non-GPI linked mechanism (CD59-TM). This would confer complement resistance to PNH cells (Rother *et al* 1994). In order to further investigate the potential of this approach, experiments were undertaken to demonstrate efficient gene transfer to human haemopoietic progenitor cells using retroviral vectors including one containing CD59-TM. This chapter describes the experimental approach and results of this gene transfer protocol using human stem cells derived from G-CSF mobilised and steady-state blood.

5.2   Assessing the potency of retroviral supernatants

In order to validate the supernatants from all the producer cell lines used in these experiments, assays were performed to assess the titre (expressed as colony forming units (cfu) per ml of supernatant) of infectious viral particles produced. Details of the

methods are described in chapter 2, section 2.18. Viral supernatant was collected, batched and frozen at -90^0C and the titre of each new batch was measured.

Titre of the MFG-LacZ construct using X-GAL

10^4 NIH-3T3 cells were plated in wells and allowed to grow for 24 hours. Serial dilutions of MFG-LacZ supernatant were added (from neat to 1 in 10^5) with polybrene (6µg/ml) and incubated for 6 hours before being replaced with fresh medium and cultured for a further 24 hours. Plates were then stained with X-GAL as described in section 2.18. The lowest dilution of supernatant that still produced positive results was taken as the titre. The titre was assessed on two separate batches of supernatant and also using supernatant supplied frozen from the same source as the cell line (Dr Paulam Patel, St. James's Hospital, Leeds). The titre was no better than 10^4 cfu/ml on any batch despite X-GAL staining of the producer cell line showing that almost all cells expressed the construct (Figure 5.1).

Titre of VSN 2 and LXSN-based supernatants using G418

The VSN 2 and LXSN based retroviral constructs all contained a neomycin resistance cassette allowing survival of successfully transduced cells in the antibiotic G418. An estimate of titre was made by infecting 3T3 cells with serial concentrations of these supernatants and then placing the cells under G418 selection pressure. Surviving colonies of successfully transduced 3T3s were visible at around 10-12 days. The method is described in detail in section 2.18. VSN 2 and LXSN were assessed in this way. The LXSN-based CD59-TM construct was initially also assayed by this method but thereafter it was validated by a more rapid FACS based

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assay described in the next section. Results indicated that the VSN 2 had a titre of around 10^5 cfu/ml and this could be raised to nearly 10^6 after selection in HAT medium (see section 2.13). LXSN was shown to have a titre of between 10^5 and 3.5×10^5 in different batches and the initial CD59-TM titre was 2×10^5 cfu/ml.

FACS - based assessment of CD59-TM supernatant potency

Since NIH 3T3 cells do not normally express the CD59 surface protein, successful transduction can be visualised by MAB staining for this antigen on a flow cytometer. The method is described in section 2.18. The first batch of CD59-TM supernatant was assayed using G418 selection giving a titre of 2×10^5 cfu/ml. An aliquot of the same batch was used to infect 3T3s which were then analysed by FACS. This gave a %positivity for CD59 transduced cells in the 3T3s of 20.4% (see figure 5.2). Subsequent batches were assessed by this rapid method giving results between 18 and 28% on frozen batches but up to 31% in fresh supernatant.

Effects of storage on CD59-TM supernatant

The rapid FACS assay allowed investigation of the effects of storage and freezing on the potency of the CD59-TM supernatant. Figure 5.3 is a series of representative results showing that batches retain about 65% of their potency after freezing. If thawed and re-frozen this falls to around 30% and if left for 24 hours at room temperature or at 4^0C almost all ability to successfully transduce 3T3s is lost. Wherever possible, fresh supernatant was used in these gene transfer experiments to maximise efficiency and it is clearly vital to avoid delays in using the supernatant after thawing.

Figure 5.1

Photomicrograph of the fibroblast producer cell line for the MFG-LacZ viral construct. The producer cells have been treated with X-GAL which stains cells blue if they contain the LacZ sequence, coding for a nuclear localised-beta-galactosidase. As shown, all the producer cells are stained positively indicating expression of the construct. See section 2.18 for methodology.

Figure 5.2

FACS histograms showing retroviral transduction of NIH-3T3 fibroblasts. The left hand plots are unstained cells after exposure to viral supernatant and the right hand plots are the same cells stained with a MAB to CD59 (FITC). The top plots were using LXSN vector with no CD59 construct, the middle plots are LXSN containing the wild-type CD59-GPI linked molecule and the bottom plots are LXSN containing the modified transmembrane CD59, CD59-TM. As shown, only the CD59-TM leads to expression as the GPI-linked form is not bound to this mouse fibroblast line. This FACS technique was a rapid method of validating serial batches of the CD59-TM supernatant and checking for contamination in the others.

21:LXSN001\FL1-H\Gamma-1 Control FITC
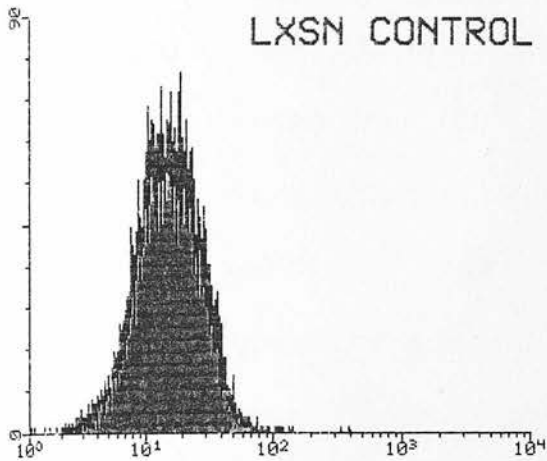
LXSN CONTROL

21:LXSN002\FL1-H\CD59 FITC

LXSN CD59
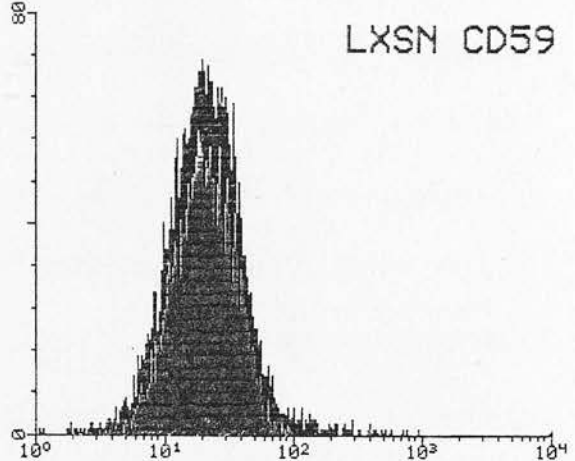
21:GPI001\FL1-H\Gamma-1 Control FITC

GPI CONTROL

21:GPI002\FL1-H\CD59 FITC

GPI CD59

M1

21:TM001\FL1-H\Gamma-1 Control FITC

TM CONTROL

21:TM002\FL1-H\CD59 FITC

TM CD59

20.4% POSITIVE
M1
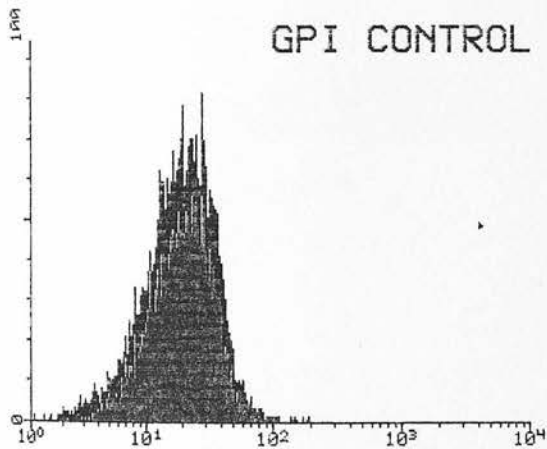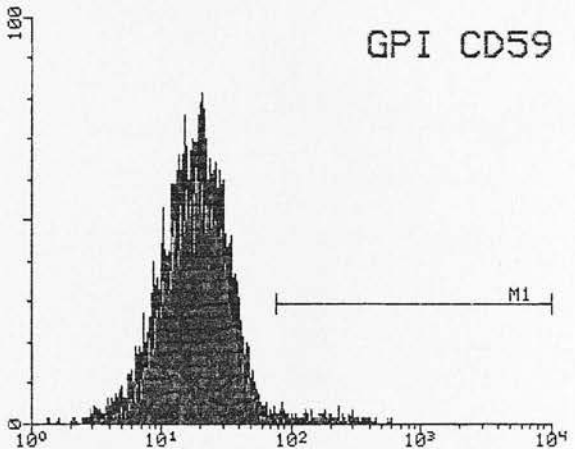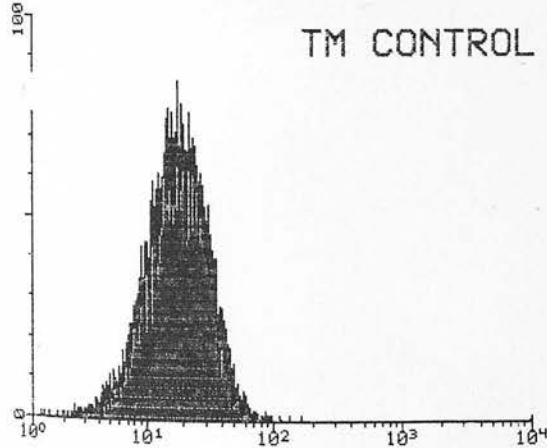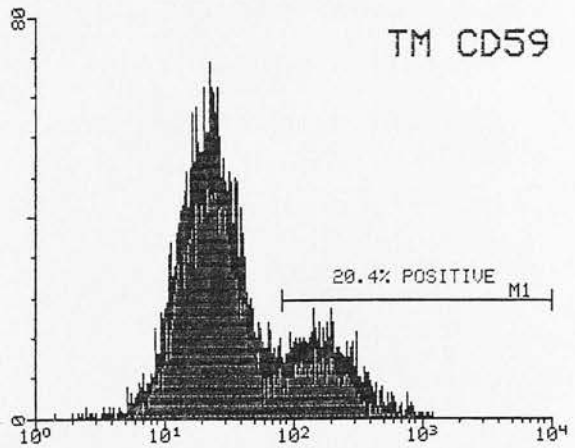
Figure 5.3

FACS histograms showing the effects of storage and freeze/thawing on the potency of the CD59-TM supernatant based on its ability to transduce NIH-3T3 cells. The top plot shows that these cells are uniformly negative before exposure to supernatant. The remaining plots show the percentage transduction efficiency of different batches and the effects of freezing and being left to stand. While the supernatant tolerates one freeze thaw cycle fairly well (batch 1 frozen), further freezing or being left to stand overnight causes serious deterioration in efficacy. (RT = room temperature).
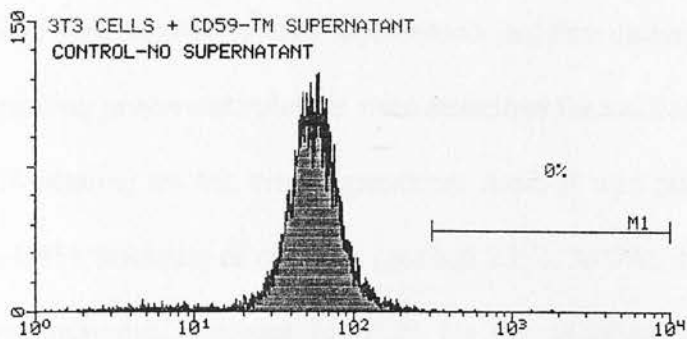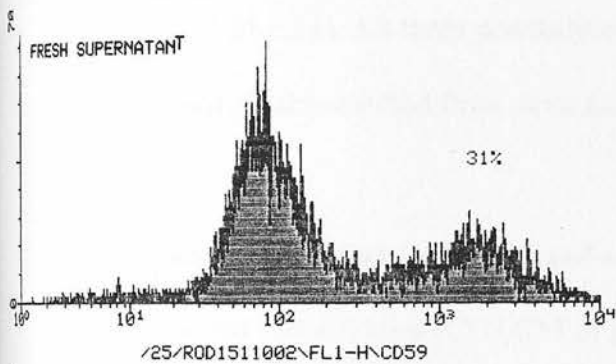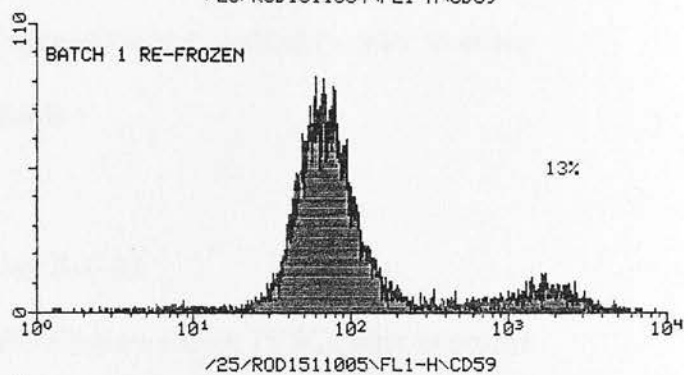
3T3 CELLS + CD59-TM SUPERNATANT
CONTROL-NO SUPERNATANT

0%

M1

/25/ROD1511001\FL1-H\CD59

FRESH SUPERNATANT

31%

/25/ROD1511002\FL1-H\CD59

BATCH 1 FROZEN

22%

/25/ROD1511003\FL1-H\CD59

BATCH 2 FROZEN

22%

/25/ROD1511004\FL1-H\CD59

BATCH 1 RE-FROZEN

13%

/25/ROD1511005\FL1-H\CD59

BATCH 1 LEFT AT 4 DEGREES FOR 24 HOURS

4%

/25/ROD1511006\FL1-H\CD59

BATCH 1 LEFT AT RT FOR 24 HOURS

2%
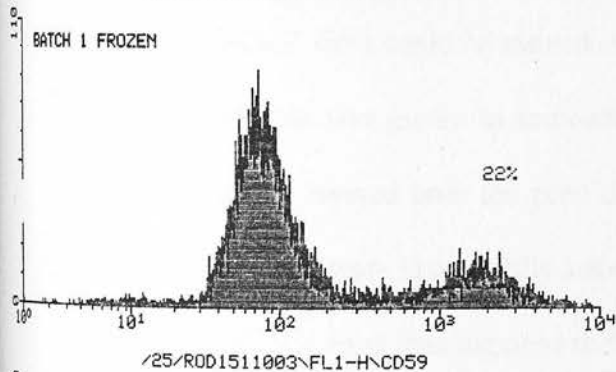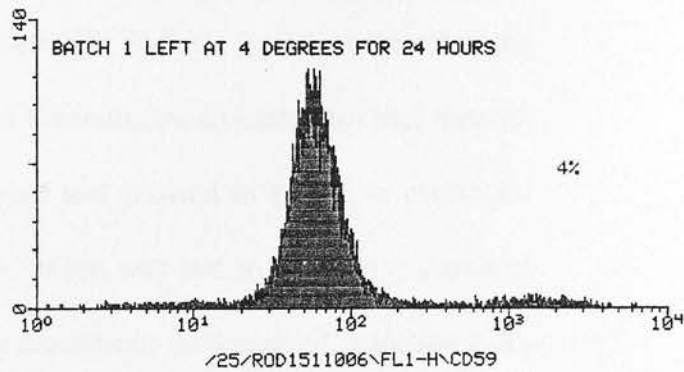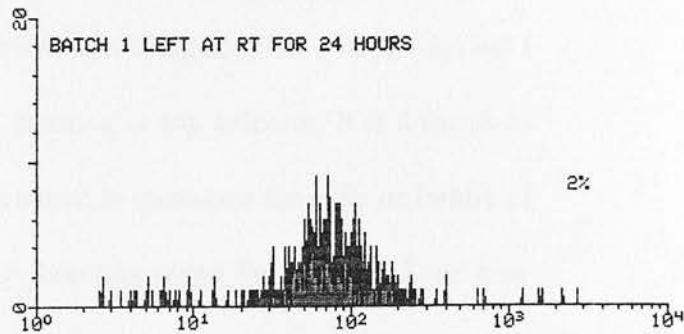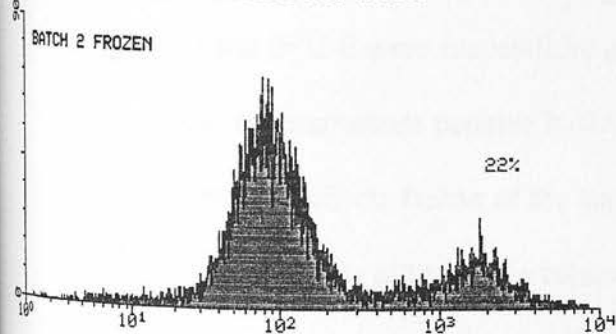
5.3   Assessing transduction efficiency

Human stem cells were exposed to viral supernatants and then cultured in semi-solid medium. The resulting progenitor colonies were examined for evidence of successful transduction. Depending on the viral supernatant used, it was possible to assess transduction by G418 selection of colonies (section 2.24), X-GAL staining (section 2.23) or picking individual colonies for PCR for the neomycin resistance gene (sections 2.21,2.22). All these possibilities were initially examined in order to arrive at the most reliable method from those available.

Assessing transduction by MFG-LacZ using X-GAL

It was hoped that colonies (CFU-GM or BFU-E) grown from PBSCs after exposure to the MFG-LacZ virus could be stained in situ with X-GAL. In order to test this, the producer cell line was grown in semi-solid medium (methylcellulose) and then X-GAL was gently layered onto the petri dishes and allowed to diffuse in overnight. The producer cells were successfully stained in this way and an example is shown in figure 5.4. PBSCs were then exposed to the supernatant as described in section 2.20. CFU-GM and BFU-E were successfully grown from these cells on 3 occasions but I was unable to demonstrate positive X-GAL staining in any colonies. It is difficult to say whether this reflects failure of the supernatant to transduce the cells or failure of the staining procedure although the relatively low titre noted for the MFG-LacZ may suggest the former. As a consequence of this, future experiments employed other viral constructs.

Figure 5.4

Photomicrograph of the fibroblast producer cell line for the MFG-LacZ construct grown in semi-solid culture medium (methylcellulose). X-GAL has been gently layered onto the medium and allowed to diffuse in. The cells are successfully stained blue by the process due to the presence of the construct coding for a beta-galactosidase.

Assessing transduction by G418 selection

Initially, normal colonies were grown in the presence of varying concentrations of G418 along with controls. Concentrations above 400µg/ml of G418 had significant effects on colony growth although the effects were highly variable. Concentrations at or above 800µg/ml generally abolished growth. Pre-incubation of the cells for 24 hours in 400µg/ml G418 containing medium before plating in the semi-solid assays proved more consistent at abolishing colony growth when the cells were subsequently plated with 400µg/ml G418 in the methylcellulose. Following on from these dose finding experiments, PBSCs were exposed to viral supernatants containing a neomycin resistance gene. The intention was to use the difference in survival of transduced colonies with and without G418 selection as a quantitative measure of transduction efficiency. Appropriate controls were always set up in parallel. Unfortunately, the results of the selection and the intrinsic variation in colony growth between plates were never sufficiently reliable to be used in a quantitative manner for assessment and this technique was abandoned in favour of the more laborious but more accurate process of picking individual colonies to PCR for the neomycin gene as described in the next section.

Assessing transduction by NEO PCR

The most reliable method for demonstrating transduction of colonies was found to be PCR. The method is described in sections 2.21 and 2.22. After exposure to retroviral supernatant, cells were set up in semi solid assays and the resulting colonies were individually picked and transferred to a PCR tube. The PCR mix was added directly

to this target. Appropriate controls were run in parallel. These included non-transduced colonies, PCR blanks and VSN 2 DNA as a positive control.

The initial attempts with a single round PCR showed no positive signals in any colonies despite a working positive control. Further experiments were set up where the colonies were split into two PCR tubes, one for NEO PCR and the other for a β-globin PCR already working in the department (courtesy of Dr Paul Evans). As shown in figure 5.5, the β-globin PCR worked for all colonies demonstrating that an adequate cell target had been picked and transferred to each tube. Viral producer cells were then PCRed (using around 500 cells/tube as a target- a similar quantity to a picked colony). Only occasional weak signals for NEO were obtained even in these cells, implying that the single round PCR was insufficiently sensitive. Internal primers were designed and synthesised for a nested PCR for NEO. Figure 5.6 shows results using this method compared to the single round PCR. The nested PCR gave reliable results for the detection of the NEO cassette. This nested technique was subsequently shown to work consistently on transduced colonies as described in the next section.

PCRing a range of cell numbers from producer cell lines suggested that the NEO PCR worked best with a target of 50 to 800 cells under these conditions. Fewer than 50 cells were unreliably detected and more than 500-800 cells also began to produce negative signals. This range in fact corresponds well to the numbers of cells generally obtained from picking colonies as described above. Since the PCR could pick up 50 cells or less there could be a problem with small numbers of contaminating transduced cells in a culture plate which could be accidentally aspirated along with a negative colony. This problem would be greatest in plates

with large numbers of colonies where significant degrees of overlap were likely. Figure 5.7 shows an experiment where positive signals for NEO were generated from apparently cell free aspirates of methylcellulose (deliberately picked as controls) in a plate in which there were large numbers of colonies. These false positives proved only to be a problem in plates where there was heavy growth.

5.4   Gene transfer into haemopoietic cells

Details of the method for retroviral gene transfer are described in section 2.20. Experiments were done to assess transduction rates in CFU-GM and BFU-E derived from human PBSCHs and peripheral blood. All these samples were taken from patients with haematological malignancies in the PBSCT program in our department. The samples were taken after mobilisation with Cyclophosphamide and G-CSF. Initially some transductions were performed without pre-incubating the cells with the growth factor combination described in section 2.20. A few colonies were successfully transduced using these simple conditions but the rates shown in tables 5.1 and 5.2 were only achievable with the protocol described.

Tables 5.1 and 5.2 summarise the results from the gene transfer experiments performed. Table 5.1 relates to CFU-GM and BFU-E colonies grown from 6 different patients' PBSCHs (samples 1-6). The numbers of colonies individually assayed by PCR and the number and proportion of these which were positive for the NEO reporter gene are shown. Table 5.2 shows the same data for colonies grown from the peripheral blood of patients post G-CSF.

The median transduction rate of colonies with the LXSN virus was 36% (range 35-50) and with the CD59-TM was also 36% (range 17-63.5). The median rate for both PBSCHs and peripheral blood cells was the same at 36% (ranges: PBSCHs 25-63.5, blood 17-50). Median rate for CFU-GMs was 33% (range 17-58) and for BFU-Es was 43% (range 33-63.5). Overall then, both the CD59-TM and LXSN supernatants produced useful and broadly similar rates of transduction. There is insufficient data to compare infection of CFU-GM and BFU-E but they are both transduced well using this protocol. Equally there appears in this limited data to be no difference between the ease of transduction of cells in harvest products or in the peripheral blood. Photographs of PCR gels showing examples of transduced CFU-GM and BFU-E are shown in figures 5.8 and 5.9.

Figure 5.5

Photograph of a PCR gel for the β-globin gene. This single round PCR produces a 350bp fragment if the target is present. The PCR tests have been performed on individual CFU-GM colonies picked from semi-solid medium. Lanes 1 to 9 are:

1   100bp ladder

2   positive control

3   CFU-GM

4   CFU-GM

5   CFU-GM

6   CFU-GM

7   CFU-GM

8   Blank

9   100bp ladder

As shown, the process of picking colonies always resulted in sufficient DNA target for this PCR.

350bp→
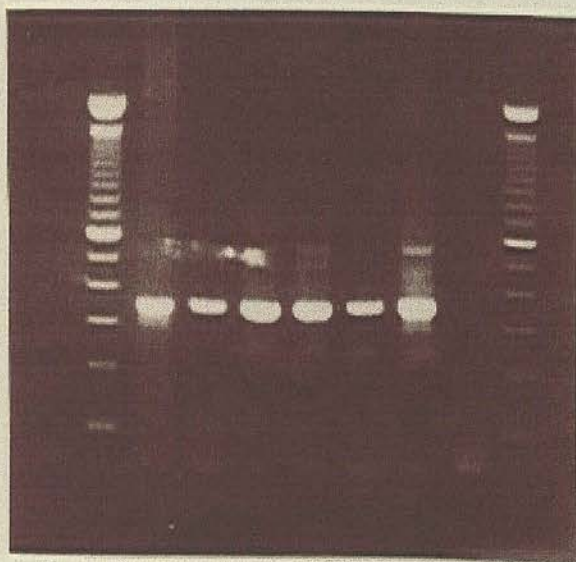
1 2 3 4 5 6 7 8 9

Figure 5.6

Photograph of a PCR gel. This shows single-round and nested PCR for the neomycin resistance cassette on VSN2 retroviral producer cells. The lanes are labelled as follows:

L   100bp ladder

+   positive control (VSN2 DNA)

B   PCR blank

1   VSN2 cells (nested PCR)

2   VSN2 cells (nested PCR)

3   VSN2 cells (nested PCR)

4   VSN2 cells (nested PCR)

5   VSN2 cells (single round PCR)

6   VSN2 cells (single round PCR)

7   VSN2 cells (single round PCR)

8   VSN2 cells (single round PCR)

The first 7 lanes (from left to right) are nested and the rest are single round. As shown, the controls are all correct with a 190bp fragment from the nested tests and a 430bp fragment in the single round. None of the single round cell samples (5-8) is positive while all 4 of the nested ones are, 1 and 2 being strongly positive and 3 and 4 weakly so.
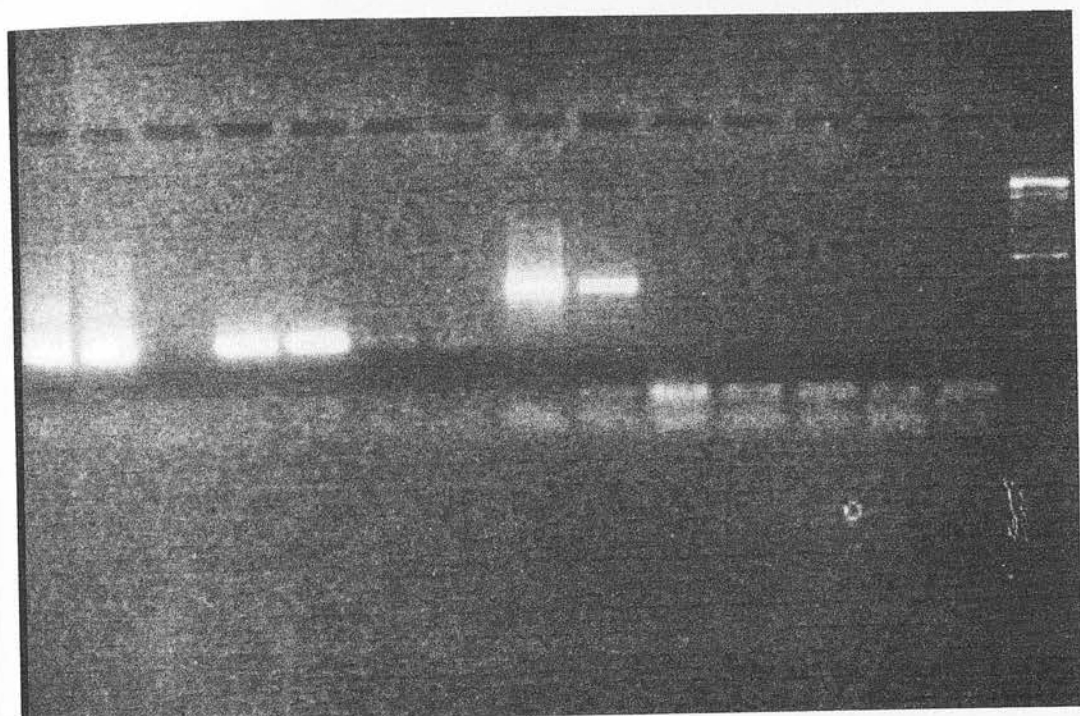
+ + B 1 2 3 4 + + B 5 6 7 8 L

Figure 5.7

Photograph of a PCR gel showing tests from samples taken from a 35mm petri dish containing CFU-GM colonies in semi-solid medium. All the samples are 'blank' in otherwords there was no visible colony in the plate area the medium sample was harvested from. Three of the samples are positive (1,2,3) for the 190bp fragment amplified by the nested NEO PCR. This demonstrates the possibility of false positives arising from contaminating cells in the plate when there are large numbers of CFU-GM present. (L = 100bp ladder).
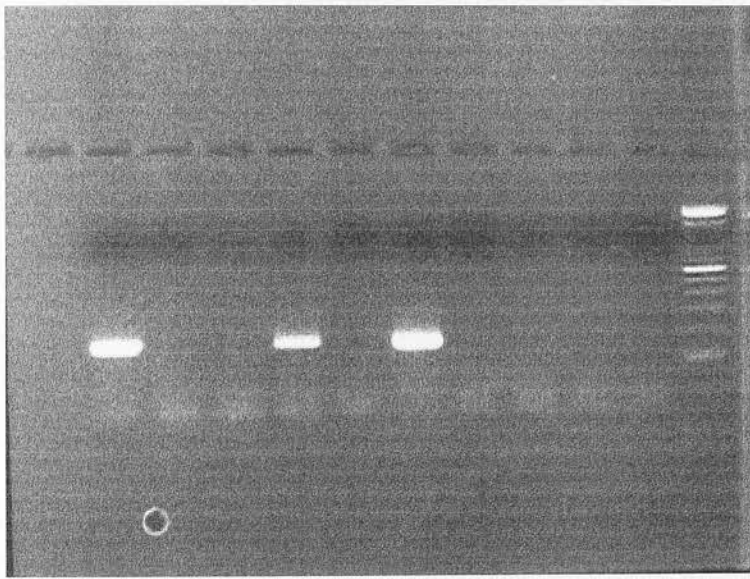
←190bp

1   2   3   L

Figure 5.8

Photographs of PCR gels for the NEO resistance gene which generates a 190bp fragment when positive. All samples tested are individual CFU-GMs, grown from a stem cell harvest and picked from the semi-solid medium (methylcellulose) using a fine pipette. The controls are colonies not exposed to any retroviral supernatant. None of these is positive for the NEO cassette. The top photo shows 10 samples from colonies grown from cells exposed to the LXSN retrovirus. 5 of these are positive indicating 50% successful transduction. The bottom photo shows the same experiment but using the retroviral construct containing the CD59-TM insert. 3 of these are positive indicating 33% successful transduction. Results are shown in table 5.1, sample 5. (L = 100bp ladder, B = PCR blank, + = positive control of VSN2 DNA).
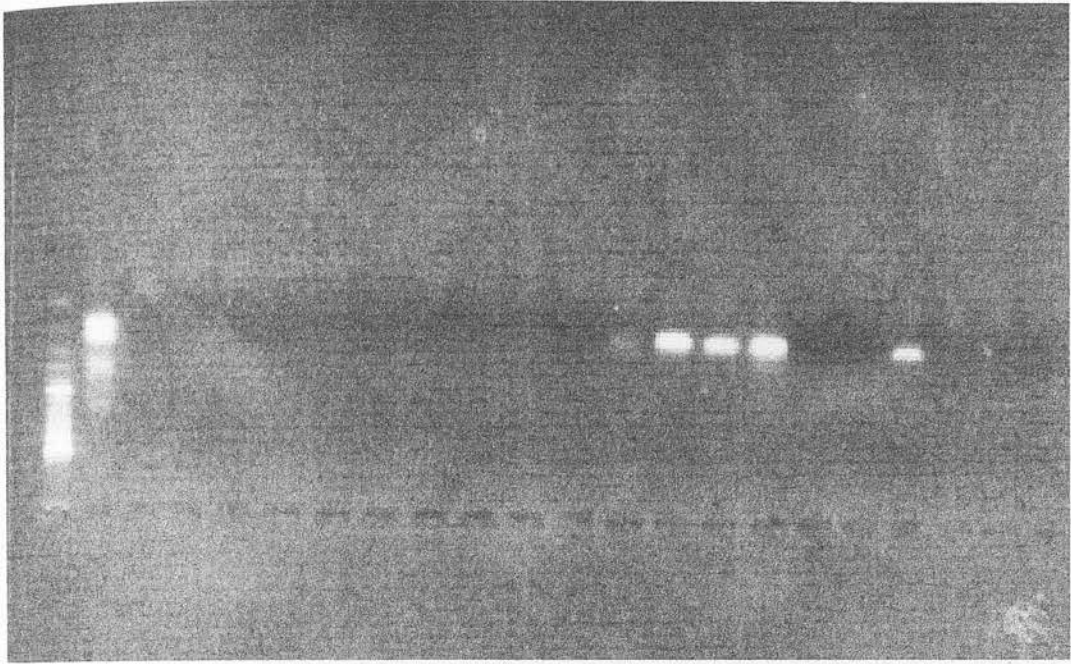
L +   10 Controls   10 CFUs + LXSN

L B   10 Controls   10 CFUs + CD59-TM

Figure 5.9

Photographs of PCR gels for the NEO resistance gene which generates a 190bp fragment when positive. All samples tested are individual colonies (CFU-GM/BFU-E), grown from peripheral blood and picked from the semi-solid medium (methylcellulose) using a fine pipette. The controls (C) are colonies not exposed to any retroviral supernatant. None of these is positive for the NEO cassette. All other samples shown have been exposed to retroviral supernatant containing the CD59-TM insert as well as the NEO cassette. The top half of the gel shows 12 BFU-Es so treated (table 5.2 sample 1) with 6 (50%) positive indicating successful transduction. The lower half of the gel shows 12 CFU-GMs treated in the same way with 2 (17.5%) positive. (L = 100bp ladder, B = PCR blank, + = positive control of VSN2 DNA).

L C C   12 BFU-Es + CD59-TM
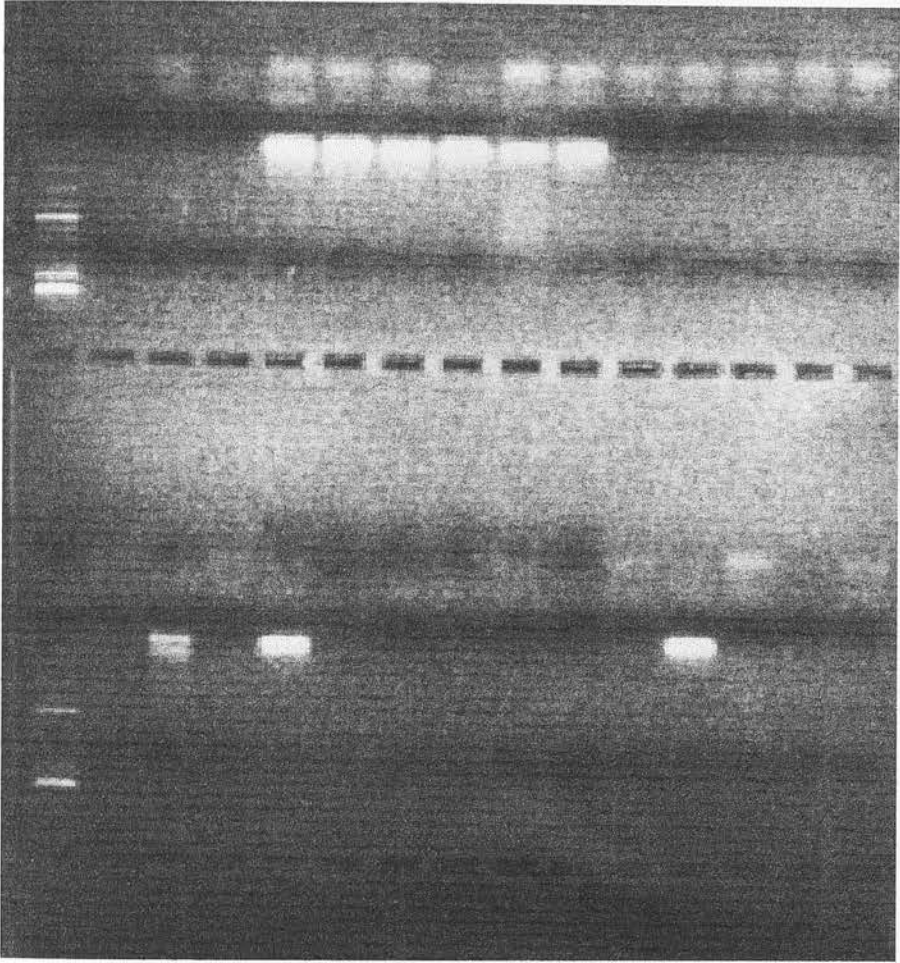
L B +   12 CFU-GMs + CD59-TM

Table 5.1   **PCR for NEO on Colonies from PBSCHs Following Gene Transfer**

	Colony Type	Virus	No. PCRed	No.+ve	%+ve
Sample 1	CFU-GM	CD59-TM	12	7	58
	BFU-E	CD59-TM	11	7	63.5
Sample 2	CFU-GM	CD59-TM	44	11	25
	BFU-E	CD59-TM	21	9	43
Sample 3	CFU-GM	LXSN	14	5	36
	BFU-E	LXSN	12	4	33
Sample 4	CFU-GM	CD59-TM	50	15	30
Sample 5	CFU-GM	CD59-TM	10	3	30
	CFU-GM	LXSN	10	5	50
Sample 6	CFU-GM	CD59-TM	14	4	29
	BFU-E	CD59-TM	12	5	42

Table 5.2   **PCR for NEO on Colonies from PB Following Gene Transfer**

	Colony Type	Virus	No. PCRed	No.+ve	%+ve
Sample 1	CFU-GM	CD59-TM	12	2	17
	BFU-E	CD59-TM	12	6	50
Sample 2	CFU-GM	CD59-TM	11	4	36
Sample 3	CFU-GM	CD59-TM	14	5	36

5.5   Discussion

One method by which stem cells may be manipulated for clinical use is by their genetic modification - gene therapy. In order to impact on disease phenotype, stable transduction of sufficient numbers of primitive cells would have to be achieved and this requires efficient methods for gene transfer. In order to establish a reliable protocol for the transduction of human peripheral blood stem cells, different retroviral vectors were investigated and attempts made to optimise the conditions for efficient transfer. The most appropriate assay technique for assessing transduction efficiency in human progenitor cells in this system was chosen.

Conditions for viral producer cell culture and methods for the harvest of viral particles were employed which achieved titres of between 10^4 and 10^6 cfu/ml. The MOI required for the transfer protocol was considered too low at 10^4 and the MFG-LacZ construct which could not be concentrated beyond this, was not used in the final method. The LXSN based vectors including that containing the CD59-TM sequence of potential interest in future studies in $\overline{\text{PNH}}$ was consistently produced at titres around 10^5 cfu/ml which was satisfactory.

Assays for transduction efficiency were performed by histochemical staining, FACS-based MAB staining and PCR techniques. Using the CD59-TM construct, it was convenient to use FACS analysis in experiments with non-human cells as they did not normally express this antigen. When working with human cells, PCR was used to detect the neomycin reporter gene in the LXSN-based vectors. It proved possible to reliably detect this gene in cell products and semi-solid human progenitor colonies

by adjusting the PCR conditions appropriately, allowing accurate assessment of transduction efficiency of progenitor cells.

The most efficient gene transfer protocol involved multiple exposures to virus in the presence of growth factors (IL3, IL6, SCF) after a pre-incubation step with the same cytokines. When this was applied to mobilised and steady-state human peripheral blood cells, it proved possible to consistently transduce CFU-GM and BFU-E using the LXSN-based retroviral constructs. Rates of transduction were up to 63% of progenitors (median 36%) with no clear differences between CFU-GM/BFU-E or between G-CSF mobilised or steady-state blood. This level of gene transfer and the consistency with which it was achieved in these relatively primitive cells would suggest that such approaches might be applicable to gene therapy research. The CD59-TM vector was efficiently transferred to human progenitors making it possible to use PNH as a model disease in which to investigate this form of stem cell manipulation.

Chapter 6.

Assessing the Outcome of CD59-TM Gene Transfer by Flow Cytometry.

6.1   Introduction
6.2   Development of the FACS-based complement lysis assay
6.3   Applying the assay to cell lines
6.4   Applying the assay to peripheral blood
6.5   FACS analysis and lysis of semi-solid colonies
6.6   Discussion

6.1   Introduction

Complement sensitivity is a clinical and laboratory hallmark of PNH and is though to be mainly due to lack of the GPI-linked molecule, CD59 (Yamashina *et al* 1990) (see Chapter 1, section 1.5). Abolishing this sensitivity could abrogate the resulting haemolysis and possibly the thrombotic risk in the disease (Rosse & Ware, 1995). Restoration of CD59 activity in PNH cells could achieve this. In chapter 5, I have described a human gene transfer protocol and its potential applicability to stem cells derived from PNH patients. This would involve the transfer of a novel CD59 construct anchored by a trans-membrane (TM) domain. It proved possible to transduce human progenitor cells with the novel CD59-TM construct with integration indicated by PCR for a marker gene. Expression of the new molecule following transduction was demonstrated in murine fibroblasts by flow cytometry

(these cells do not normally display this antigen). Having achieved integration and expression, the next logical step would be to demonstrate function, i.e. show that PNH cells were protected from complement by the presence of CD59-TM and to compare their complement sensitivity to that of normal cells and untransduced PNH cells. This required the development of an assay to measure the degree of functional correction following gene transfer in order to fully document the outcome of this gene therapy protocol. The conventional diagnostic test (Ham-Dacie) for PNH relies on complement sensitivity but is applicable only to erythrocytes, while the cells of interest for gene transfer in this setting would be mononuclear progenitors. It was therefore necessary to devise a means for assessing complement lysis which was applicable to the cell type of interest.

This chapter describes the development of a flow cytometry method for this quantitation in different cell types as a means of investigating the degree of restoration of CD59 activity by the novel CD59-TM construct proposed for use in PNH gene transfer experiments.

6.2   Development of the FACS-based complement lysis assay

Principle

Cells whose membranes have been compromised by complement can be visualised by FACS using two simple properties. Firstly they shrink and show reduced FSC and SSC and secondly they become permeable to certain fluorescent dyes. Calcein-AM (section 2.30) fluoresces in the FL1 channel (FITC) and cells pre-loaded with this dye lose it progressively following exposure to increasing amounts of complement.

Cells which have been permeabilised by complement will also take up PI. This latter dye has the advantage that it can fluoresce in channels FL2 or FL3 on the FACScan™ allowing simultaneous staining with MABs conjugated to FITC or PE for more accurate cell identification.

For the assay, cells are sensitised with anti-HLA W6/32 and then exposed to human complement along with appropriate controls (no serum and serum heat inactivated at 56^0C for 30mins. to remove complement). Calcein is added along with the HLA W6/32 or PI is added shortly before processing on the FACScan™. The detailed methodology is described in section 2.30. Several factors required investigation or optimising before the assay could be applied:

Complement concentration in the assay

The source of complement for these assays was human serum which was obtained by venepuncture, rapidly separated and frozen in suitable aliquots at -90^0C for repeated use, ensuring that a standard amount of complement from the same source was used in all experiments. A standard volume of varying concentrations of this serum was added to test cells in microtitre wells in order to establish the optimum amount of complement for the assay (see methods, section 2.30). Normal peripheral blood MNCs begin to lyse significantly at a concentration of 35% serum. The greatest differential in lysis between normal and complement-sensitive PNH cells (using cell lines from PNH patients) was also seen at this percentage. Stronger concentrations of serum caused lysis in a high proportion of normal and PNH cells which reduced the differential effect. 35% serum was therefore chosen as the optimum discriminating concentration in this system. Figures 6.1 and 6.2 illustrate typical examples. Figure

6.1 shows lysis in B-lymphoblastoid cell lines from PNH patient, demonstrating changes in FSC and SSC after exposure to complement. It also illustrates the differential complement sensitivity between these PNH and normal cells. Figure 6.2 shows lymphocytes from 3 normal volunteer peripheral blood samples with lysis beginning consistently at a 35% serum concentration.

Calcein or PI to indicate lysis?

These proved to be equally effective at indicating the degree of lysis. There were several reasons why PI was ultimately preferred: It was quicker and easier to use, being added at the end of the procedure just before FACS analysis. PI is also cheaper and easier to store than calcein. In addition to these practical issues, PI will fluoresce in the FL3 channel of the FACScan™ after appropriate adjustment of the compensation settings and this allowed routine use of FITC and PE conjugated MABs along with PI. Calcein utilises the FITC channel. Use of PI thus allowed identification of cell types by one MAB (e.g. B lymphoblastoid cell lines using CD19-PE), discrimination between PNH and normal cells using CD59-FITC and assessment of lysis in these cells using PI. Figure 6.3 shows a series of FACS histograms comparing calcein and PI as indicators of lysis in a normal (non-PNH) B lymphoblastoid cell line lysed as described above with increasing concentrations of serum. Both indicators show lysis beginning at 35% serum with no difference between the two.

Blocking effects of CD59/CD55

In order to investigate whether the MABs (CD59 and/or CD55) used in these experiments interfered with the function of their antigens and reduced the ability of cells to handle complement, lysis assays were performed on cell lines pre and post

labelled with the antibodies. Normal (non-PNH) cells could theoretically be rendered PNH-like if these MABs were blocking and their resistance to complement would be reduced. Figure 6.4 shows an example of this with the normal cell line showing less resistance to complement if pre-labelled with CD59 and CD55. Since these antibodies did appear to be at least partly blocking, subsequent experiments were always carried out by staining with MABs after the lysis step.

Quantifying lysis in PNH and normal cells

Cells that are lysed in this system suffer two possible fates: either they are totally disrupted and appear in the low FSC/SSC debris in the bottom left quadrant of a FACS plot or they will be compromised and become PI+ve/calcein^{low} with reduced FSC/SSC as described above. To quantify lysis in a mixture of PNH and normal cells, it was found preferable to express the result as the proportion of PNH cells remaining in the viable non-lysed PI-ve gate as this change in proportion takes into account those cells that are compromised and those that are disrupted. If only compromised, PI+ve cells are measured, the disrupted cells (many of which will be PNH) are excluded from the result. Figure 6.5 shows this method for a mixture of PNH and normal B lymphoblastoid cells. The cell population is gated loosely using FSC/SSC characteristics and a gate is then set round the PI-ve (viable) cells. This is then re-gated on FSC and SSC and this final population of cells with appropriate FSC and SSC which are viable (PI-ve) are separated using CD59 into normal and PNH. In this way the proportion of normal:PNH cells that remain viable after exposure to increasing concentrations of serum can be measured. The figure shows the proportion of PNH cells falling from 37% to 15% after lysis, indicating their increased susceptibility to complement.

Figure 6.1

FACS dot plots. The cell population analysed here is a mixture of normal and PNH phenotype B-lymphoblastoid cell lines derived from a patient with PNH (courtesy of Dr P Hillmen, Leeds Infirmary).

In the left hand column, the top 2 plots show the change in the FSC/SSC characteristics of the cells on exposure to complement. The main population with high FSC seen in the upper plot is shifted to the left as the cells are permeabilised and shrink on exposure to 50% human serum (second plot down). The third plot down shows the use of PI to gate out dead cells in the mixture before exposure to complement. Only the gated area at the lower right of the plot is viable (PI-ve). The lower plot shows the differentiation of PNH from normal cells using CD59 performed on the viable cells. The percentage of normal/PNH cells can be easily calculated from this.

The remaining 8 sequential plots show these cells with their respective proportions after exposure to increasing amounts of complement. As demonstrated, the proportion of normal cells rises steeply as serum concentration increases and the sensitive PNH cells are lysed, with optimum discrimination at 35%. At higher concentrations the lysis of all cells is considerable, reducing overall numbers for analysis and lowering the degree of discrimination (see text).

/25/ROD2509009
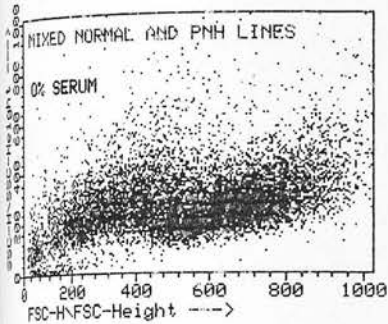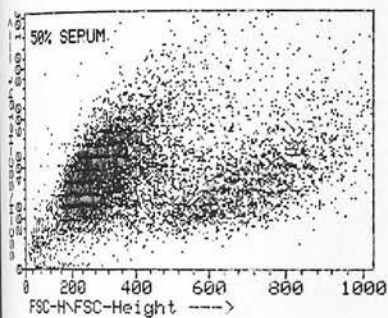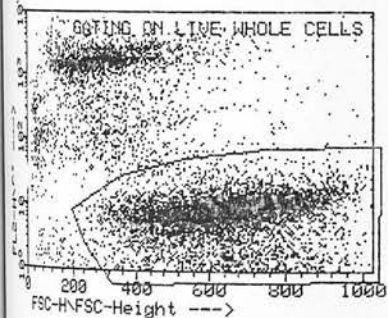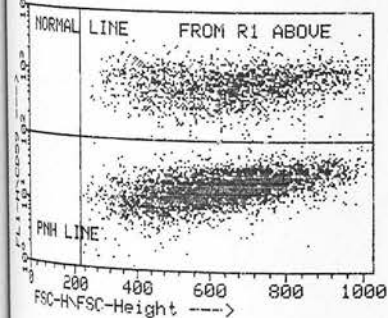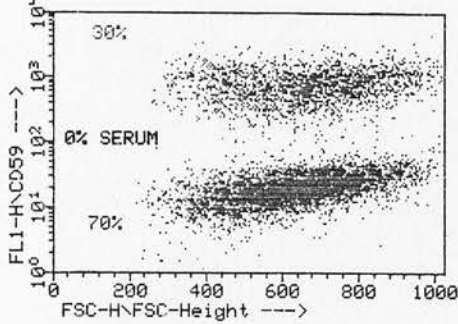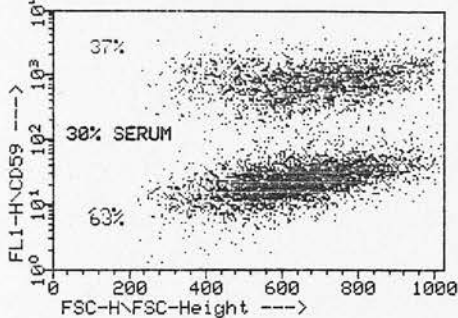
MIXED NORMAL AND PNH LINES

0% SERUM

FSC-H\FSC-Height --->

/25/ROD2509009

30%

FL1-H\CD59 --->

0% SERUM

70%

FSC-H\FSC-Height --->

/25/ROD2509013

50%

FL1-H\CD59 --->

45% SERUM

50%

FSC-H\FSC-Height --->

/25/ROD2509014

50% SEPUM

FSC-H\FSC-Height --->

/25/ROD2509010

37%

FL1-H\CD59 --->

30% SERUM

63%

FSC-H\FSC-Height --->

/25/ROD2509014

45%

FL1-H\CD59 --->

50% SERUM

55%

FSC-H\FSC-Height --->

/25/ROD2509009

GGTING ON LIVE WHOLE CELLS

FSC-H\FSC-Height --->

/25/ROD2509011

58%

FL1-H\CD59 --->

35% SERUM

42%

FSC-H\FSC-Height --->

/25/ROD2509015

36%

FL1-H\CD59 --->

75% SERUM

64%

FSC-H\FSC-Height --->

/25/ROD2509009

NORMAL LINE   FROM R1 ABOVE

PNH LINE

FSC-H\FSC-Height --->

/25/ROD2509012

54%

FL1-H\CD59 --->

40% SERUM

46%

FSC-H\FSC-Height --->

/25/ROD2509016

34%

FL1-H\CD59 --->

100% SERUM

66%

FSC-H\FSC-Height --->
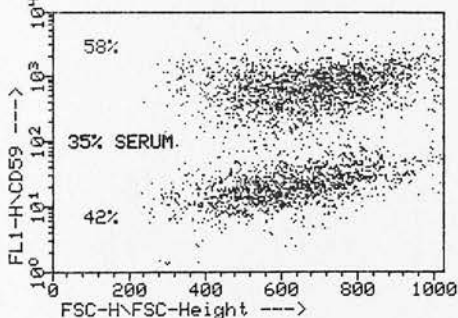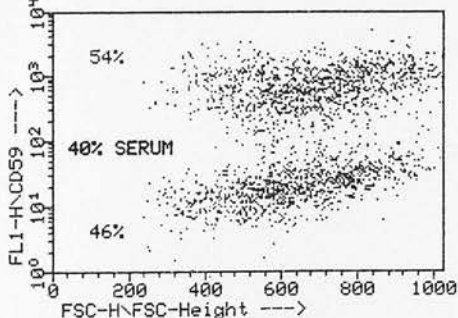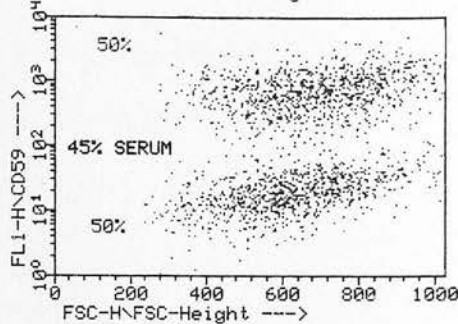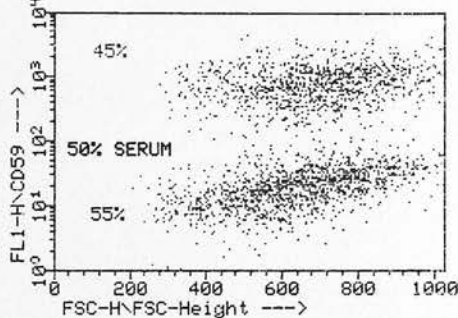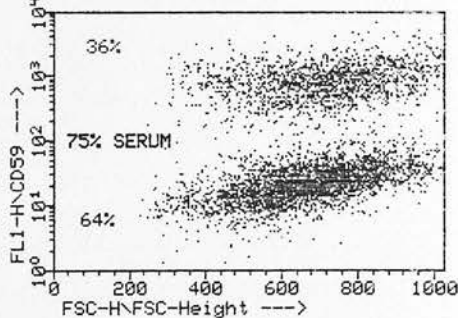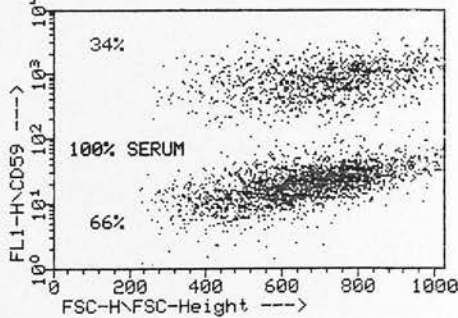
Figure 6.2

FACS histograms showing lysis of normal peripheral blood lymphocytes by complement with PI as an indicator dye. Each of the 3 columns shows one sample. The increasing percentages of human serum added are indicated on the plots reading downwards. The percentage of lysed (PI+ve) cells at each concentration is seen as a peak to the right of the histogram. This increases consistently at 35% serum in all cases.

Sample 1

#11:/25/ROD2007001\FL2-H\PI

0% SERUM

5.6% |———— M1 ————|

#11:/25/ROD2007002\FL2-H\PI

30% SESRUM

2.5%

#11:/25/ROD2007003\FL2-H\PI

35% SERUM

18.5%

#11:/25/ROD2007004\FL2-H\PI

40% SERUM

38%

Sample 2

#11:/25/ROD2007005\FL2-H\PI

4.5%

#11:/25/ROD2007006\FL2-H\PI

3.2%

#11:/25/ROD2007007\FL2-H\PI

12.4%

#11:/25/ROD2007008\FL2-H\PI

28.5%

Sample 3

#11:/25/ROD2007009\FL2-H\PI

3.7%

#11:/25/ROD2007010\FL2-H\PI

2.2%

#11:/25/ROD2007011\FL2-H\PI

19.7%

#11:/25/ROD2007012\FL2-H\PI

32.2%
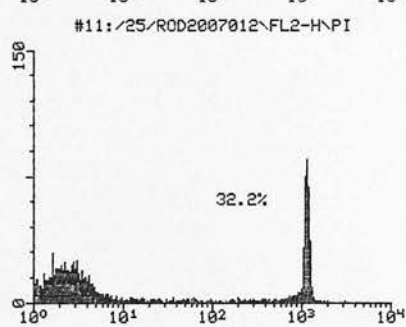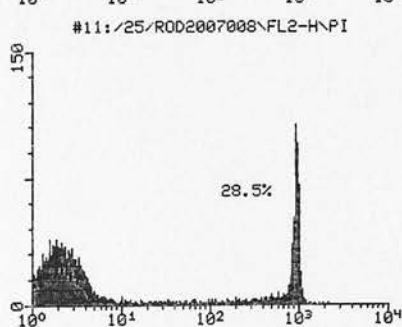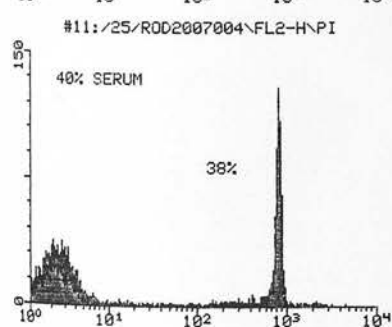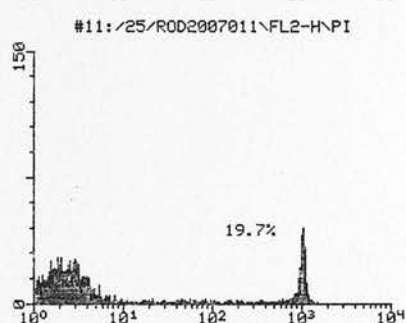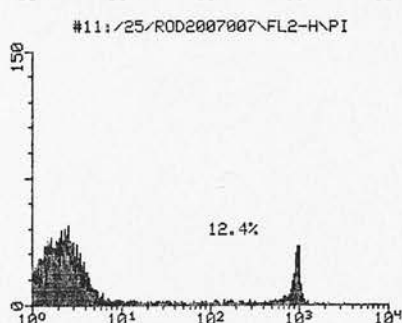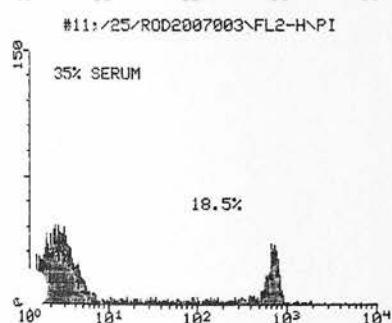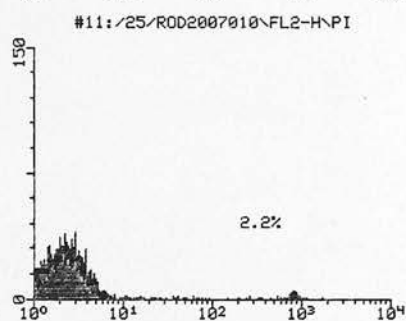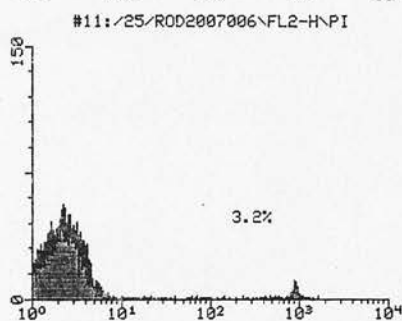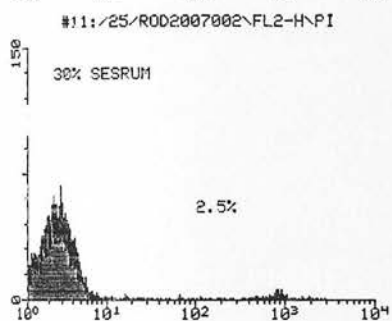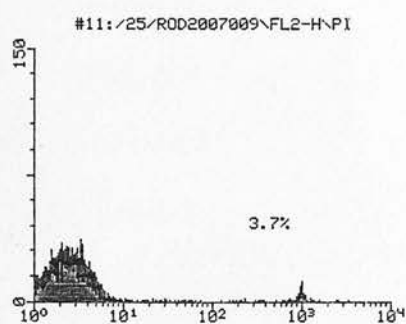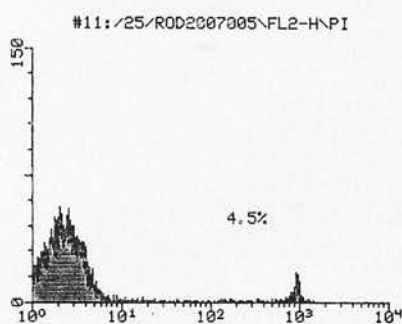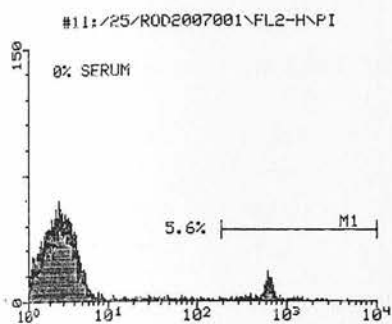
Figure 6.3

FACS histograms showing lysis of a normal (non-PNH) B cell line comparing the use of Calcein-AM and PI as dye indicators of cell lysis.

The upper half of the figure shows 6 plots using calcein and increasing concentrations of serum. The right hand histogram peak gets progressively smaller and the left hand peak increases as cells are lysed, lose their calcein and shift left in their fluorescence.

The lower half of the figure shows the same conditions exactly but with PI as the indicator. Here, lysed cells take up PI so that the right hand (PI+ve) peak increases with lysis.

The two dyes are equally useful as indicators as they both show lysis beginning at 35% serum concentration.

#11:/13/ROD1406010\FL1-H\CALCEIN

NORMAL LINE
CALCEIN

0% SERUM

98%

M1

#11:/13/ROD1406012\FL1-H\CALCEIN

25% SERUM

88%

#11:/13/ROD1406013\FL1-H\CALCEIN

30% SERUM

88%

#11:/13/ROD1406014\FL1-H\CALCEIN

35% SERUM

83%

#11:/13/ROD1406015\FL1-H\CALCEIN

40% SERUM

69%
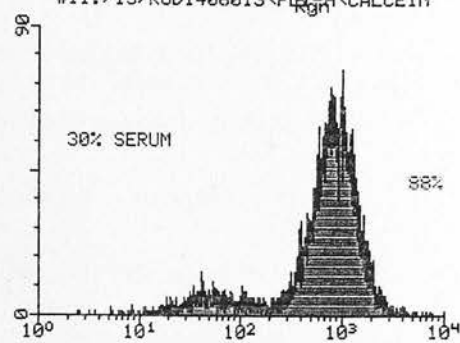
#11:/13/ROD1406016\FL1-H\CALCEIN

45% SERUM

63%

#11:/13/ROD1406010\FL2-H\PI

NORMAL LINE
PI

0% SERUM

6%

M1

#11:/13/ROD1406012\FL2-H\PI

25% SERUM

8%
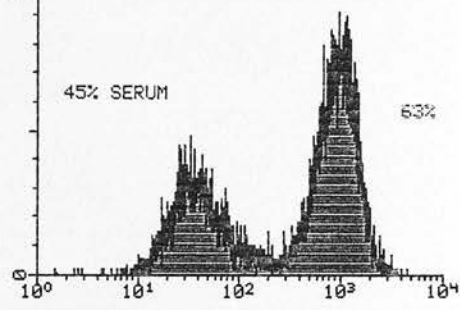
#11:/13/ROD1406013\FL2-H\PI

30% SERUM

7%

#11:/13/ROD1406014\FL2-H\PI

35% SERUM

12%

#11:/13/ROD1406015\FL2-H\PI

40% SERUM

25%
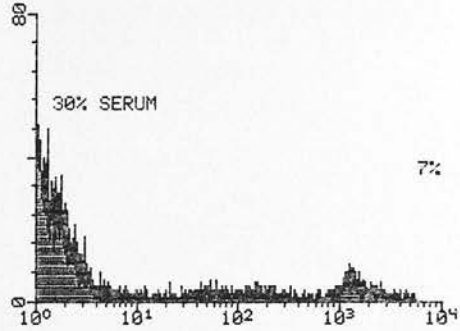
#11:/13/ROD1406016\FL2-H\PI

45% SERUM

33%
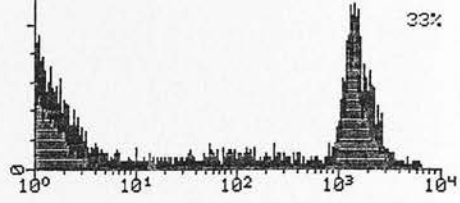
Figure 6.4

These FACS histograms show lysis of non-PNH, B-cell lines. The left hand column shows the experiment with pre-labelling by the MABs CD55 and CD59. The degree of lysis is higher than in the post labelled cells shown in the right hand column. The antibodies are functionally blocking these complement regulating molecules and converting normal cells to those with a 'PNH' phenotype with regard to their complement sensitivity.

#11:/13/ROD1506005\FL3-H\PI
NORMAL LINE PRE LABELLED WITH CD55/59
25% SERUM
12%
M1
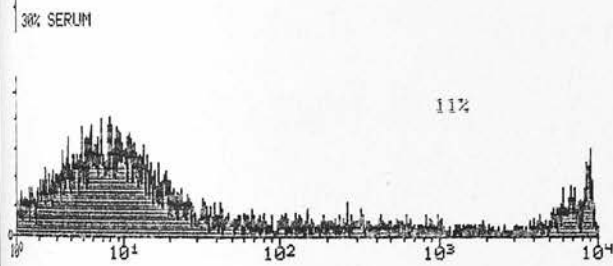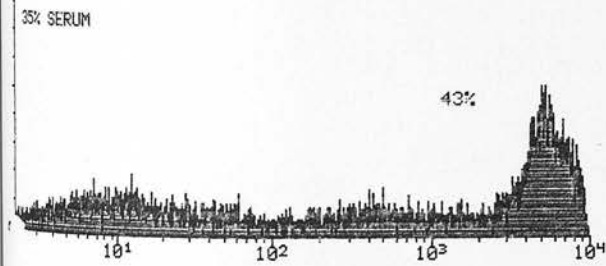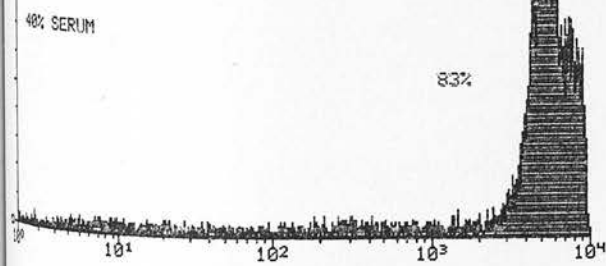
#11:/13/ROD1506009\FL3-H\PI   Rgn
NORMAL LINE POST LABELLED WITH CD 55/59
25% SERUM
9%

#11:/13/ROD1506006\FL3-H\PI
30% SERUM
11%

#11:/13/ROD1506010\FL3-H\PI
30% SERUM
12%

#11:/13/ROD1506007\FL3-H\PI
35% SERUM
43%

#11:/13/ROD1506011\FL3-H\PI
35% SERUM
22%
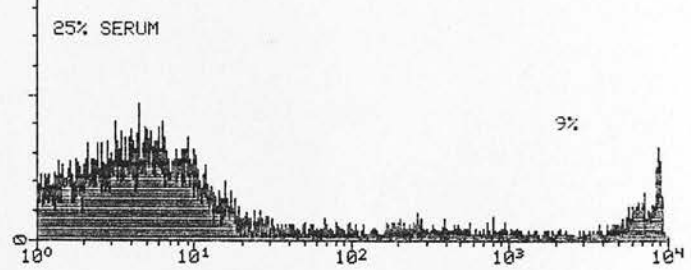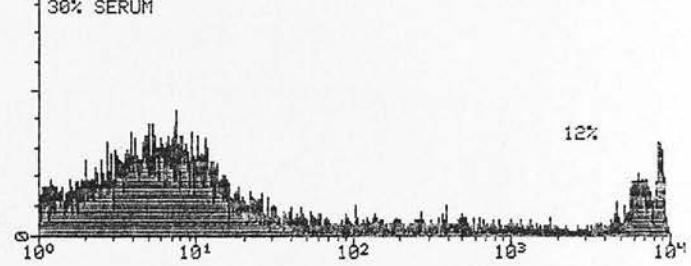
#11:/13/ROD1506008\FL3-H\PI
40% SERUM
83%
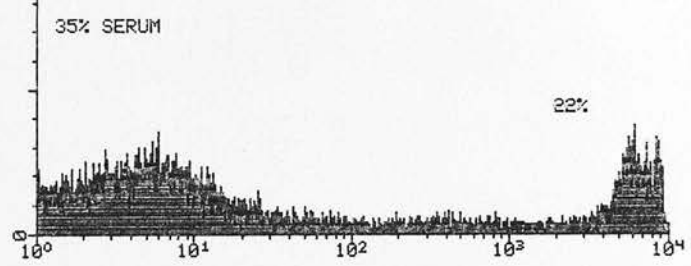
#11:/13/ROD1506012\FL3-H\PI
40% SERUM
66%
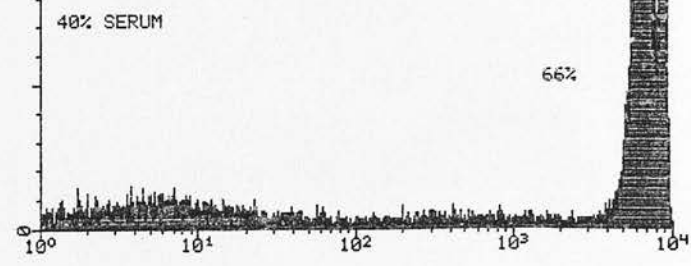
Figure 6.5

FACS plots/histograms showing the differential lysis sensitivity of B cell lines of normal and PNH phenotype. The left hand column shows the gating method. The mixed cell population is loosely gated by FSC/SSC characteristics (top) then non-viable PI+ve cells are excluded (middle). Finally, a tighter gate is applied to these viable cells before histogram analysis.

The right hand column shows the proportions of PNH:Normal cells remaining after exposure to two controls of no serum and heat inactivated serum (top and middle) and then 35% complement. As shown, the cell numbers fall with lysis after complement. The remaining cells are largely normal (right hand peak, CD59+ve) as most PNH cells have been destroyed.

#11:/25/ROD0911001
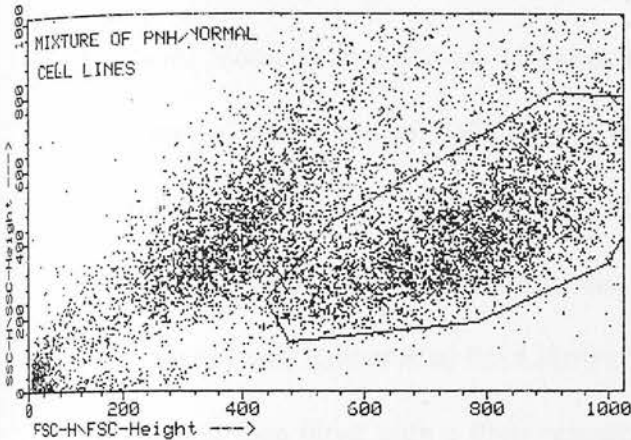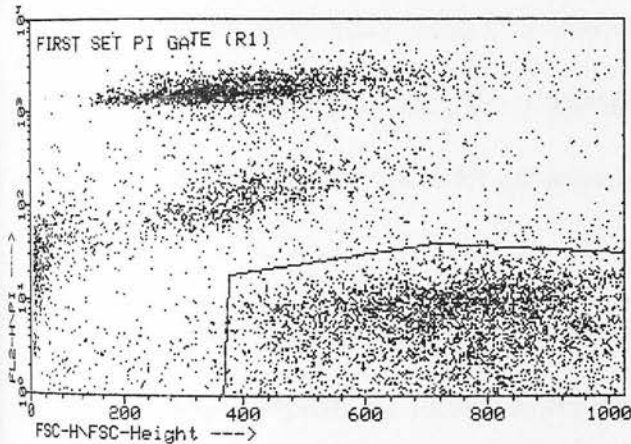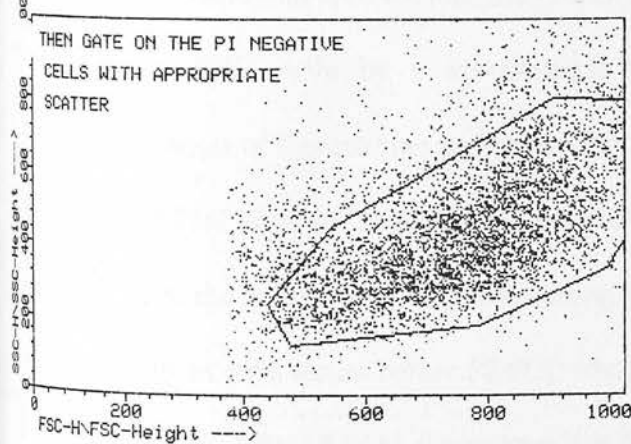
MIXTURE OF PNH/NORMAL
CELL LINES

#11:/25/ROD0911001

FIRST SET PI GATE (R1)

#11:/25/ROD0911001

THEN GATE ON THE PI NEGATIVE
CELLS WITH APPROPRIATE
SCATTER

#11:/25/ROD0911001\FL1-H\CD59

FINALLY, CD59 HIST ON GATE R1 + R2

NO SERUM

37%   63%

#11:/25/ROD0911002\FL1-H\CD59

35% HEAT INACTIVATED SERUM

38%   62%

#11:/25/ROD0911003\FL1-H\CD59

35% FRESH HUMAN SERUM

15%   85%
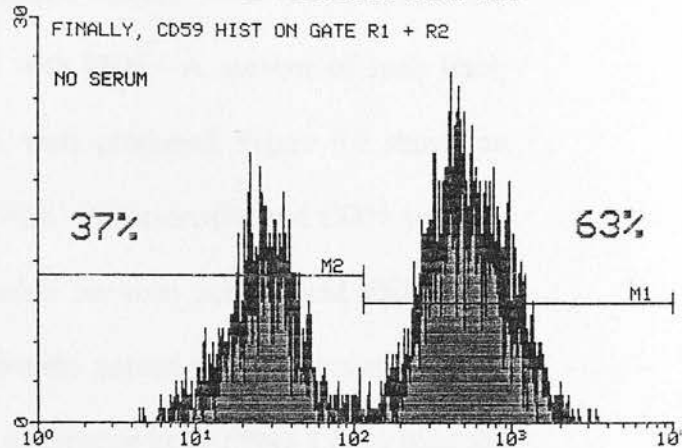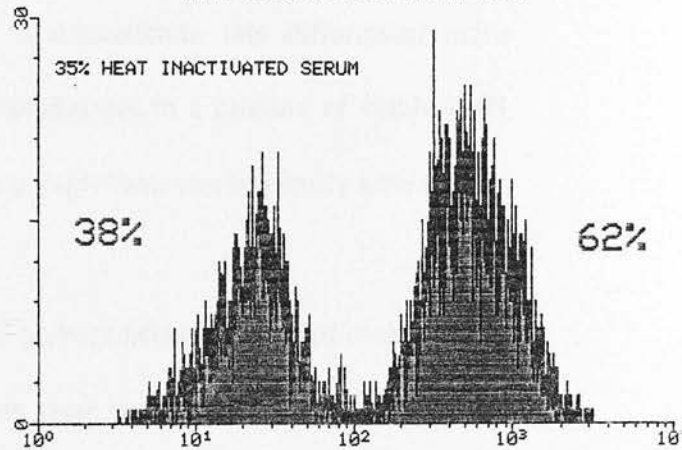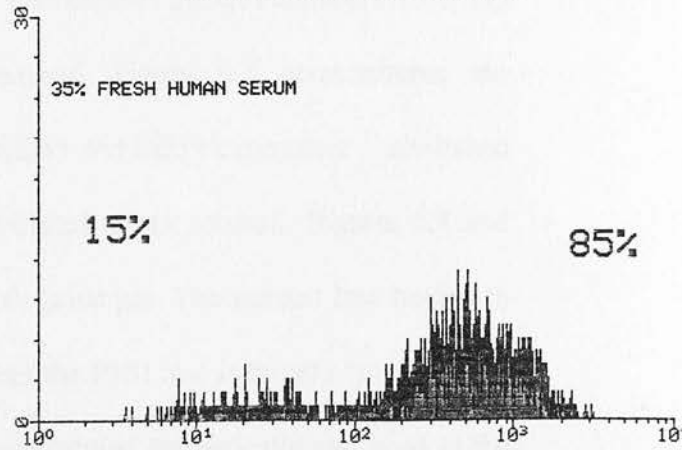
6.3   Applying the assay to cell lines

The cell lines used were produced by Dr Peter Hillmen and were kindly donated. These were EBV transformed human B-lymphoblastoid lines derived from the peripheral blood B-lymphocytes of patients with PNH. A number of such lines, some normal and some of PNH phenotype, were produced. Figure 6.6 shows an example of a normal and PNH line with FSC/SSC characteristics and CD59 staining. This antibody gives the clearest discrimination between normal and PNH cells. Using these lines, Rother et al have shown that the normal cells are less sensitive to complement than those with a PNH phenotype (Rother *et al* 1994). I have used the FACS based lysis assay described above to demonstrate this differential more clearly. Using these cell lines, the proportional changes in a mixture of viable PNH and normal cells before and after exposure to complement can be clearly seen (figure 6.5).
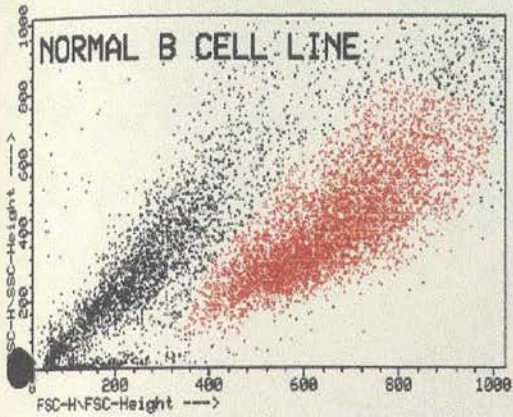
In order to show the importance of CD59 and perhaps other GPI-linked molecules in inactivating complement, further experiments were performed on the lines before and after treatment with the enzyme PI-PLC. This cleaves the GPI anchor, effectively creating PNH cells by a biochemical method. Figure 6.7 demonstrates the effectiveness of this enzyme and shows that CD55 and CD59 expression is abolished after PI-PLC with no change in the non GPI-linked CD19 control. Figures 6.8 and 6.9 show the results of a lysis assay using this principle. The normal line begins to lyse only at 40% serum before PI-PLC whereas the PNH line is largely lysed at 35% (figure 6.8). After PI-PLC, the normal line has changed dramatically and lyses at the same point and to a similar degree to the PNH line (figure 6.9).

Figure 6.6

FACS plots and histogram showing the CD59 staining characteristics of a normal and a PNH cell line. The red dots/peak show CD59+ve normal cells and the green dots/peak show the CD59 deficient PNH cells. All cells are CD19+ve B cells as shown on the y-axis of the dot plots in the centre of the figure. The CD59 GPI-linked antibody is particularly good at discriminating clearly between the populations in PNH.
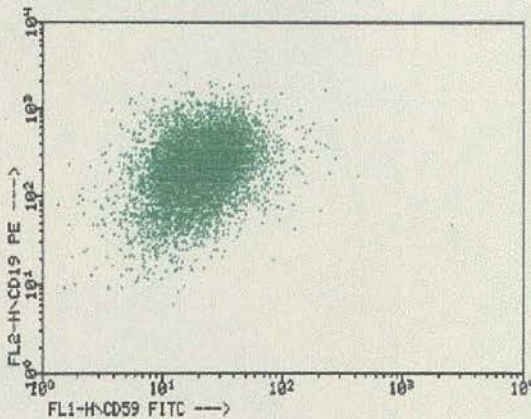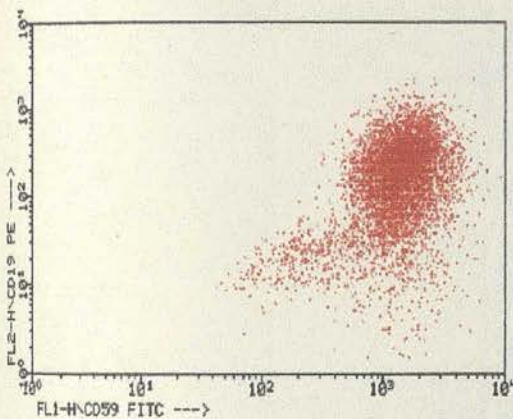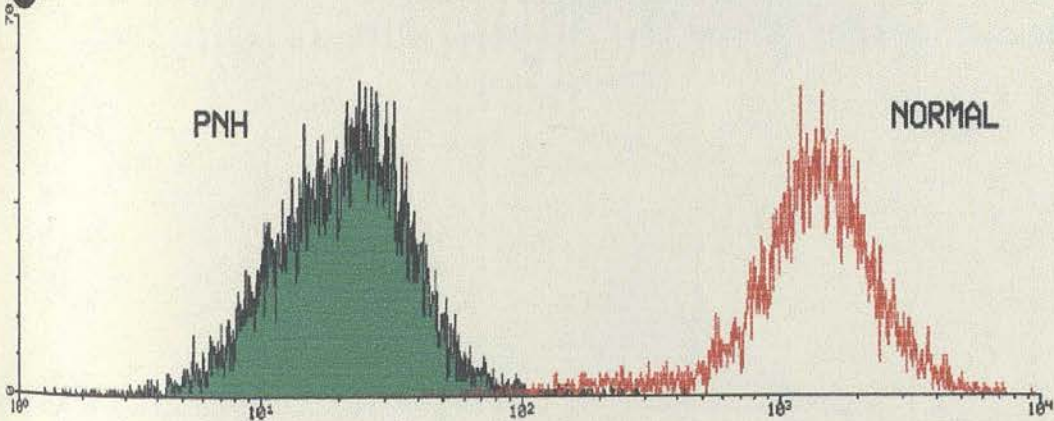
#11:/11/ROD001

NORMAL B CELL LINE

#11:/11/ROD002

PNH B CELL LINE

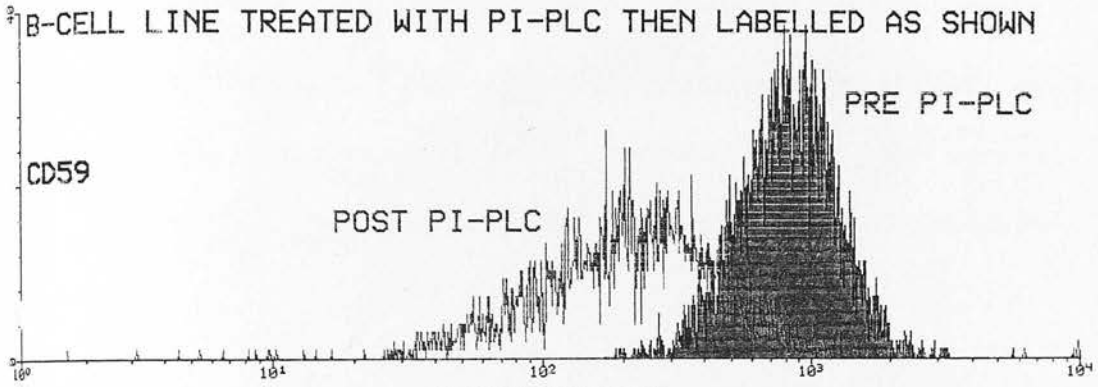#11:/11/ROD001
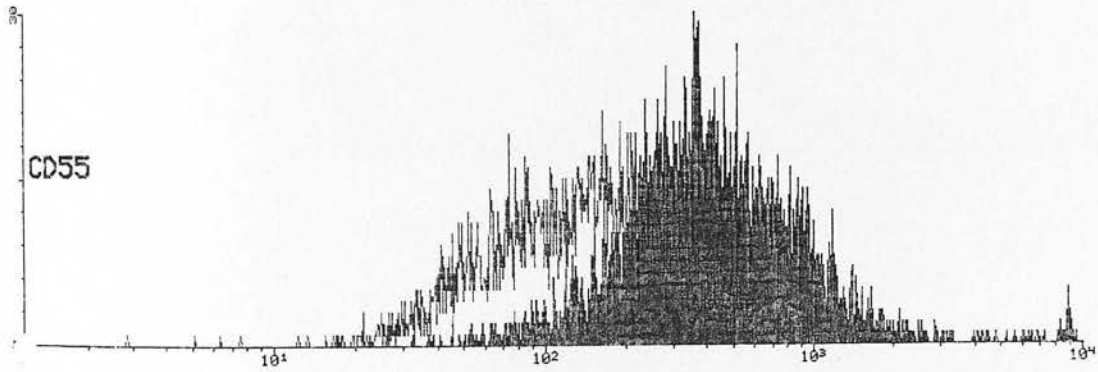
#11:/11/ROD002

#11:/11/ROD002\FL1-H\CD59 FITC

PNH
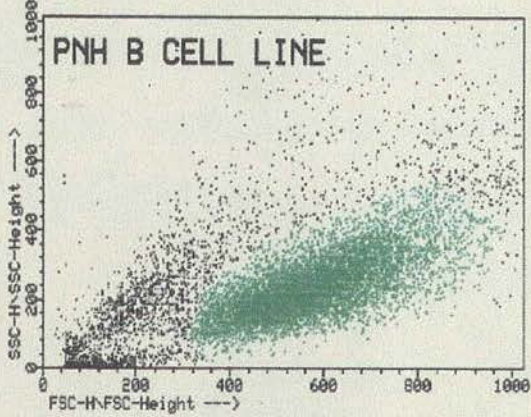
NORMAL

Figure 6.7

FACS histograms showing a normal B cell line stained for 3 MABs (CD59, CD55 and CD19) before and after treatment with PI-PLC. This enzyme cleaves the GPI anchor and thus removes CD59 and CD55 as shown in the top 2 plots. The non-GPI linked CD19 B cell antigen is not affected by this enzyme and acts as a useful control as seen in the bottom plot.
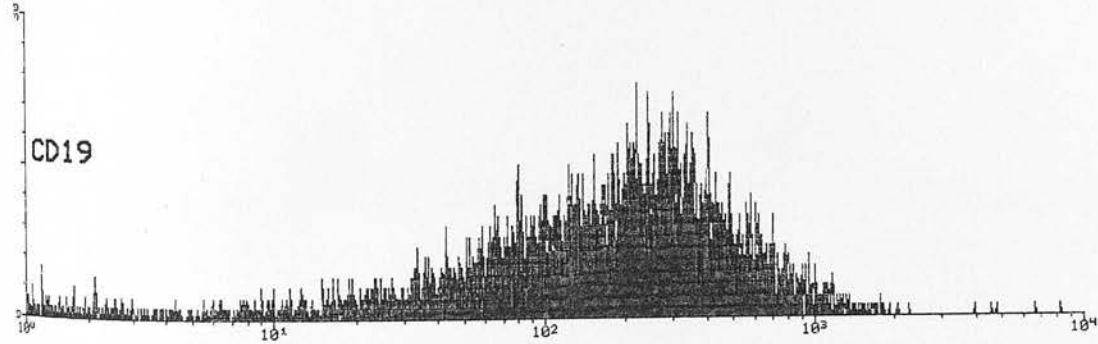
/25/ROD0406001\FL1-H\CD59

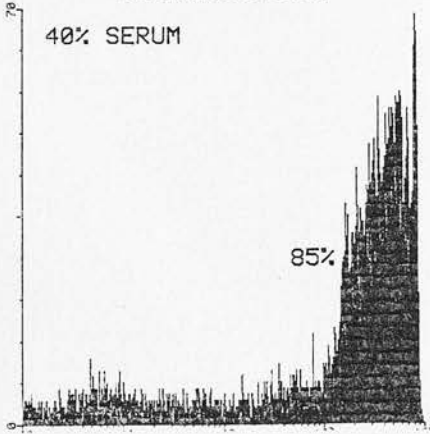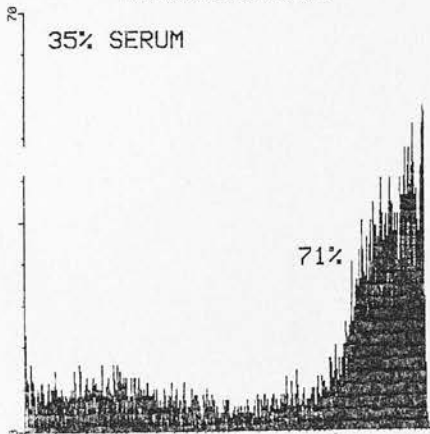B-CELL LINE TREATED WITH PI-PLC THEN LABELLED AS SHOWN

CD59

PRE PI-PLC

POST PI-PLC

/25/ROD0406001\FL2-H\CD55

CD55

/25/ROD0406001\FL3-H\CD19

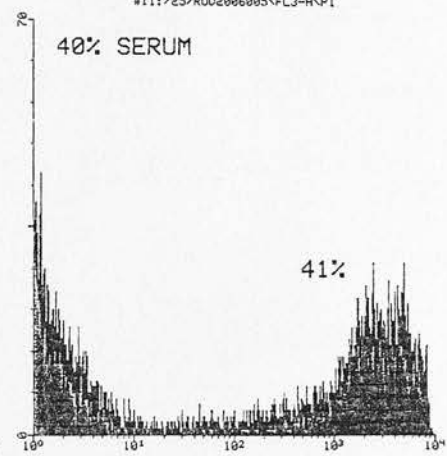CD19

Figure 6.8

FACS histograms showing a complement lysis assay in normal (top 4 plots) and PNH (bottom 4 plots) B-cell lines with no PI-PLC treatment. HI-serum is heat inactivated to remove complement and act as a control. The increasing amounts of serum shown lead to lysis as indicated by the increasing percentage of cells that take up PI (right hand histogram peak). As can be seen, the PNH cells are more sensitive and lyse at a lower serum concentration (35%). The normal cells can be made complement sensitive by PI-PLC treatment as demonstrated in figure 6.9 (see text).

NORMAL LINE + HI SERUM
NO PIPLC

25%

25% SERUM

23%

35% SERUM

25%

40% SERUM

41%

PNH LINE + HI SERUM
NO PIPLC

25%

25% SERUM

18%

35% SERUM

71%

40% SERUM

85%

#11:/25/ROD2006004\FL3-H\PI
#11:/25/ROD2006005\FL3-H\PI
#11:/25/ROD2006009\FL3-H\PI
#11:/25/ROD2006010\FL3-H\PI
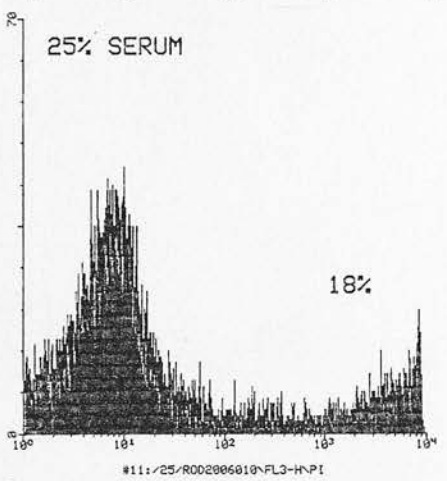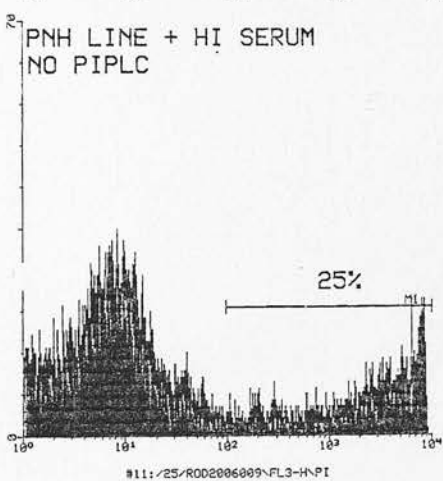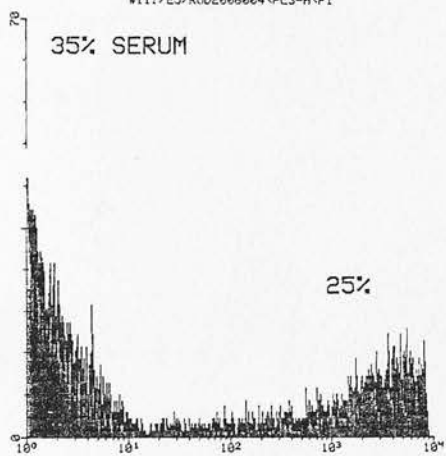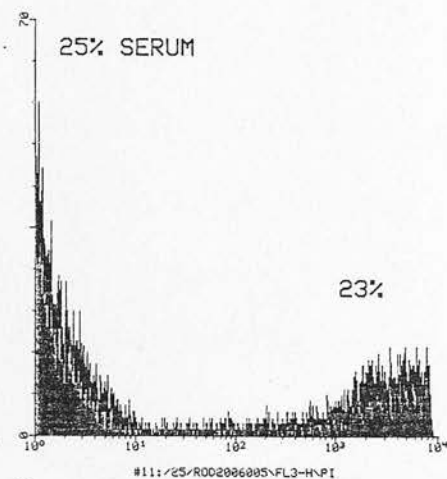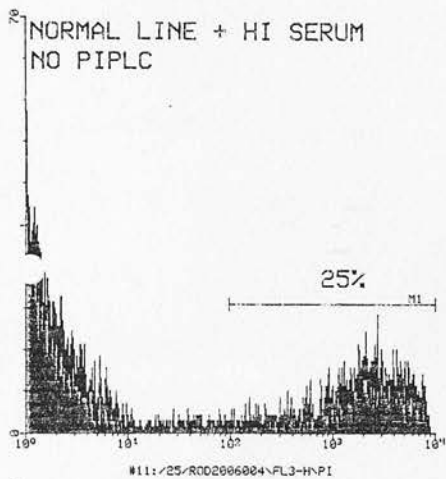
Figure 6.9

FACS histograms showing a complement lysis assay in normal (top 4 plots) and PNH (bottom 4 plots) B-cell lines after PI-PLC treatment. HI-serum is heat inactivated to remove complement and act as a control. The increasing amounts of serum shown lead to lysis as indicated by the increasing percentage of cells that take up PI (right hand histogram peak). By contrast with non-PI-PLC treated cells (figure 6.8), the normal cell line is now as complement sensitive as the PNH line because it has lost all GPI-linked regulatory molecules (mainly CD59). Both lines begin to lyse significantly at 35% serum concentration now.

NORMAL LINE + HI SERUM + PIPLC — 25% — M1 — #11:/25/ROD2006014\FL3-H\PI

25% SERUM — 22% — #11:/25/ROD2006015\FL3-H\PI

35% SERUM — 52%

40% SERUM — 62%

PNH LINE + HI SERUM + PIPLC — 23% — M1 — #11:/25/ROD2006019\FL3-H\PI

25% SERUM — 18% — #11:/25/ROD2006020\FL3-H\PI

35% SERUM — 60%

40% SERUM — 54%
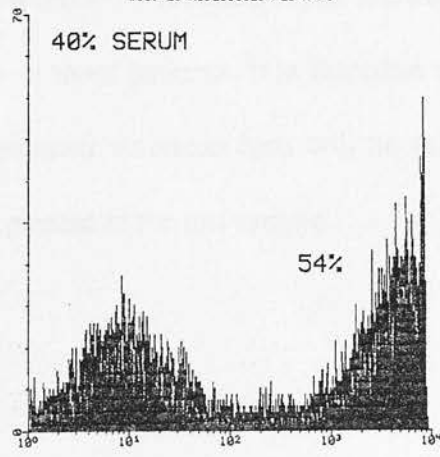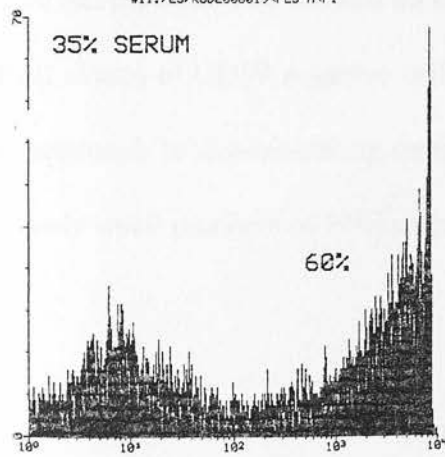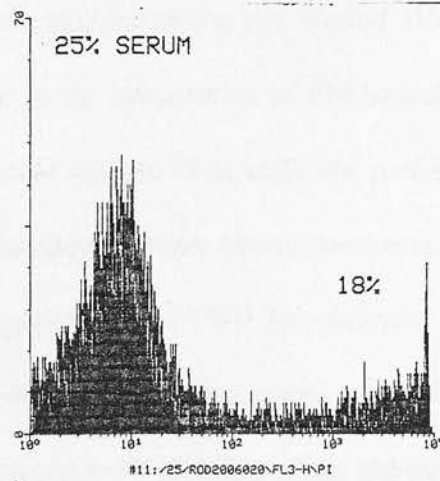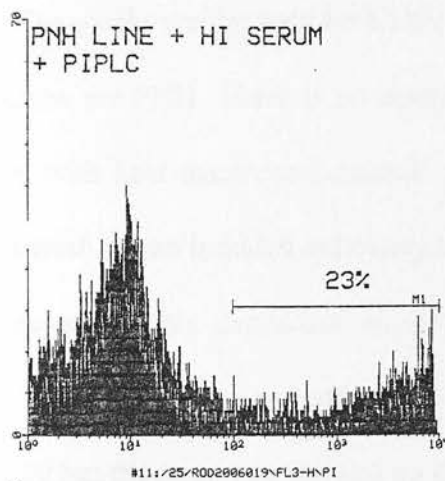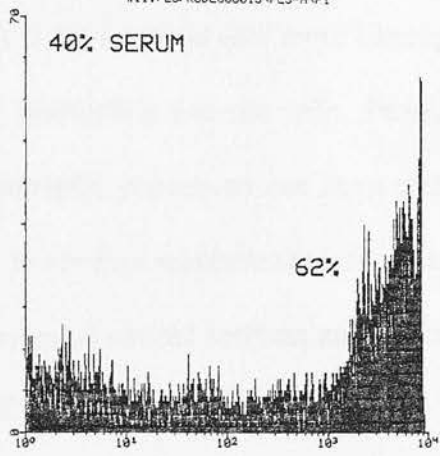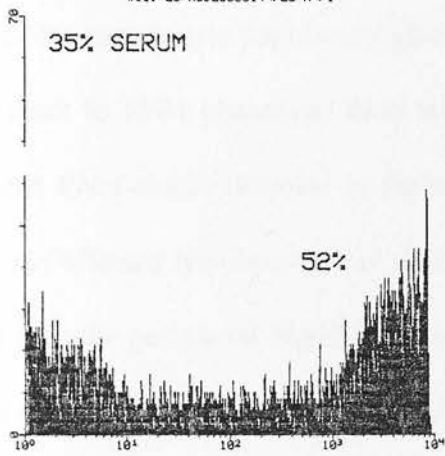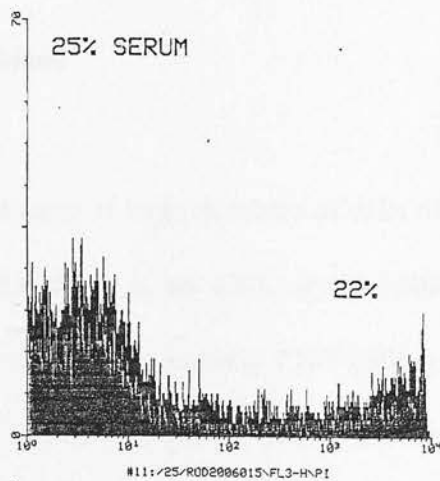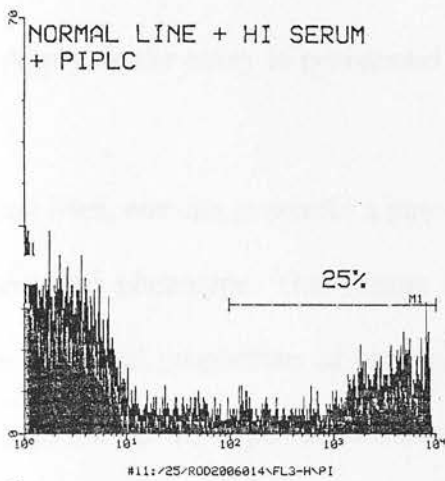
6.4   Applying the assay to peripheral blood

Using cell lines, one can guarantee a pure source of large numbers of cells of clearly pre-determined phenotype. This means that there is no difficulty in achieving a mixture of known proportions of entirely normal and entirely PNH cells in a test. This would not be the expected situation in PNH peripheral blood, particularly in regard to the lymphocyte population which is often a great deal more heterogeneous with respect to PNH phenotype than the neutrophils and red cells. Patients with substantial PNH clones detected in the neutrophil population can have quite small numbers of affected lymphocytes. In order to see how sensitive the lysis assay was, I applied it to the peripheral blood lymphocytes of normal subjects and of four PNH patients. Figure 6.10 shows the results of an assay on lymphocytes from a PNH patient. Two peaks can be seen for CD59 staining indicating that around 41% of the lymphocytes are PNH. There is no change in the proportions of PNH:normal after treatment with heat inactivated control serum but the PNH cells are preferentially lost after fresh serum is added indicating that they are more complement sensitive.

The assay was highly dependent on the population of PNH lymphocytes in each patient. Two of the four patients tested showed a positive result as displayed in figure 6.10 but the other two showed no detectable PNH cells by lysis although there were small clones of CD59 negative cells in these patients. It is therefore unlikely that this approach to demonstrating complement mediated lysis will be reliable if there are only small numbers of PNH cells present in the test sample.

Figure 6.10

These FACS plots and histograms show the differential complement sensitivity of normal and PNH peripheral blood lymphocytes from one of our patients. The left hand column shows the gating which is done in the same manner as described in figure 6.5. The right hand column shows 3 histograms: the top and middle ones are controls with no serum and heat-inactivated serum respectively, while the bottom one shows the effect of adding 35% fresh serum. As can be seen, the lymphocytes in this patient are about 40% PNH (CD59-ve,left hand peak) and 60% normal phenotype (CD59+ve,right hand peak). The bottom plot shows how the PNH lymphocytes are preferentially lysed by complement leaving only 31% PNH cells compared to 69% normal after serum exposure

/25/ROD3110007
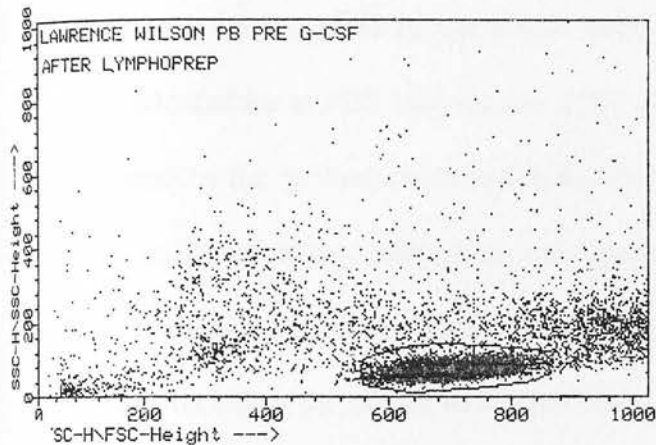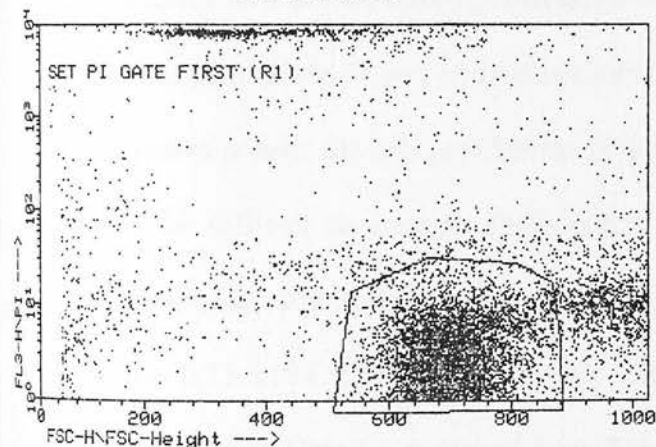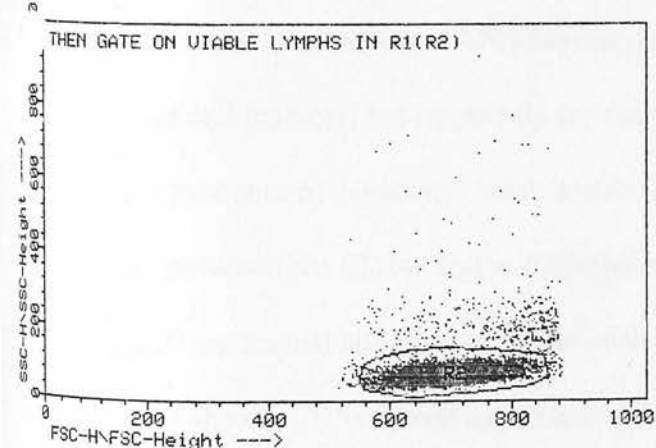
LAWRENCE WILSON PB PRE G-CSF
AFTER LYMPHOPREP

/25/ROD3110007

SET PI GATE FIRST (R1)

/25/ROD3110007

THEN GATE ON VIABLE LYMPHS IN R1(R2)

/25/ROD3110002\FL1-H\CD59   Rgn
FINALLY LOOK AT HIST OF CD59 IN THE GATE R1 + R2

NO SERUM ADDED

41%   59%

M1

/25/ROD3110007\FL1-H\CD59

35% HEAT INACTIVATED SERUM

43%   57%

M1

/25/ROD3110009\FL1-H\CD59

35% FRESH HUMAN SERUM

31%   69%

M1
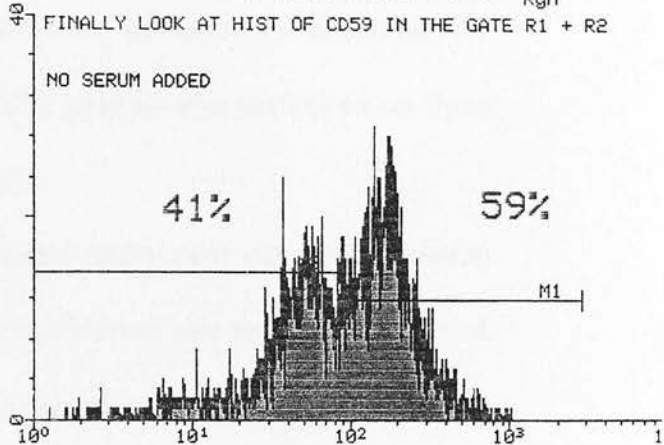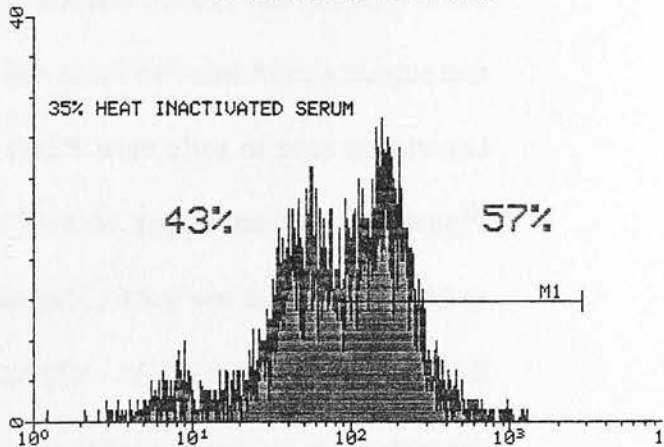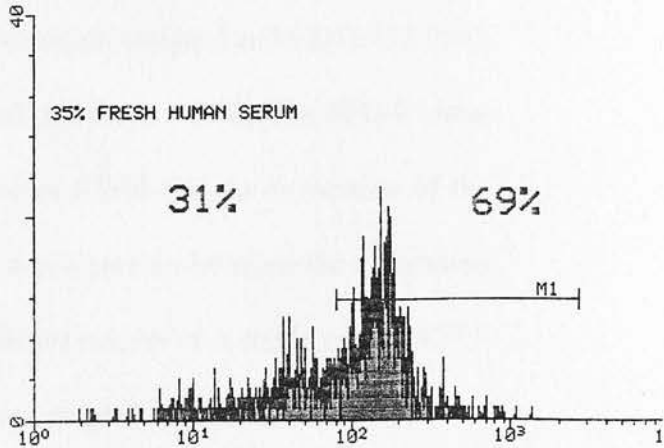
6.5   FACS analysis and lysis of semi-solid colonies

Semi-solid colonies (CFU-GM and BFU-E) were grown from peripheral blood samples or PBSCHs as described in section 2.16. They could be individually harvested from a plate or the whole plate could be harvested by dissolving the methylcellulose in PBS (see section 2.22). FACS analysis was performed on these colonies by the methods described in section 2.27.

The results of colony harvesting were inconsistent and a clear cut cell population was not visualised on the FACS for all samples. This was due to a combination of factors including the aspiration of the colony (a manual and very operator dependent technique), how dry the methylcellulose had become and the size and viability of the colonies themselves. Where there were only a few small colonies from a sample that had grown poorly, the cell populations on the FACS were often of poor quality and could be difficult to separate from debris. CFU-GM appear on the FACScan™ scatter plot as large cells of low to intermediate SSC. They are larger than BFU-E. Figures 6.11 and 6.12 show respectively scatter plots of 3 CFU-GM and 3 BFU-E single colonies. Gate R1 is placed around the populations which are quite discreet. The CFU-GM stain well for CD45 (leucocyte common antigen) and CD13+33 (both myeloid cell markers) but negatively for the red cell marker GPA. The BFU-E show GPA (glycophorin) positivity, weak expression of CD34 and no expression of the monocytoid antigen CD14. Figure 6.13 shows a comparison between the expression of CD59 on normal and PNH cell lines with the expression on a single normal CFU-GM. As shown, CD59 is well expressed on these progenitor colonies.

Having established this method, I attempted to grow colonies from the peripheral blood of patients with PNH and analyse them on the FACS. These patients were mildly to moderately hypoplastic and as expected showed poor colony growth in culture making such experiments difficult. The size of the colonies that typically grew was less than ideal to visualise clearly on the FACS except in a few cases. Figure 6.14 shows the scatter plots for 6 single CFU-GM from one of these patients and below them it shows the CD59 expression of the colonies. Five of them are evaluable and of these, one appears to show a mixed population, one is negative and the other 3 are positive for CD59. The relatively small numbers of colonies that were successfully grown, picked and stained from PNH patient blood showed this pattern, with most of the colonies appearing to show a normal phenotype. Others have demonstrated the presence of dual populations of normal and PNH BFU-Es in such patients (Rotoli *et al* 1984)

It proved impossible to apply the lysis assay to the cell samples harvested from single colonies for practical reasons. The assay requires too many washes and incubations to handle such small cell numbers. It was possible however to demonstrate that CFU-GM could be lysed under the same conditions as lymphocytes by pooling all the cells from a whole methylcellulose dish. Figure 6.15 shows an example of a dish of colonies harvested and stained for a variety of positive and control antibodies. Figure 6.16 shows a similar sample subjected to lysis on the FACScan[TM]. As the concentration of serum rises beyond 30%, the cells lose their calcein as they are lysed in an almost identical pattern to that of lymphocytes.

Figure 6.11

FACS plots showing 3 individual CFU-GM colonies picked from methylcellulose. The left hand column shows the FSC/SSC characteristics of the cells in the colony and they are gated in region R1. The right hand column shows staining of the gated cells for: top plot=CD34+ve,CD13+33+ve, middle plot=CD45+ve,GPA(glycophorin A)-ve, bottom plot=non-stained control. These CFU-GM therefore stain as expected for primitive and myeloid-specific markers.
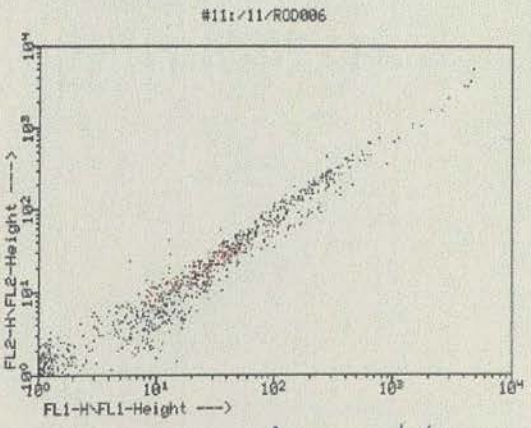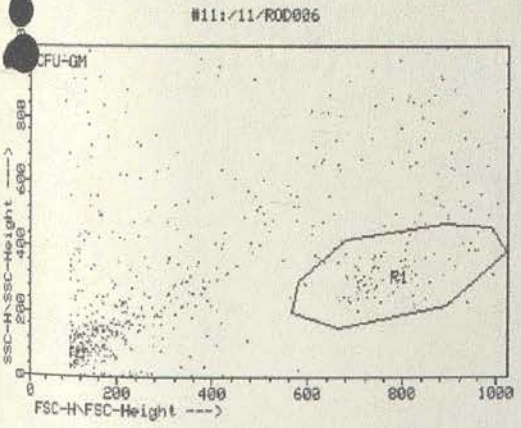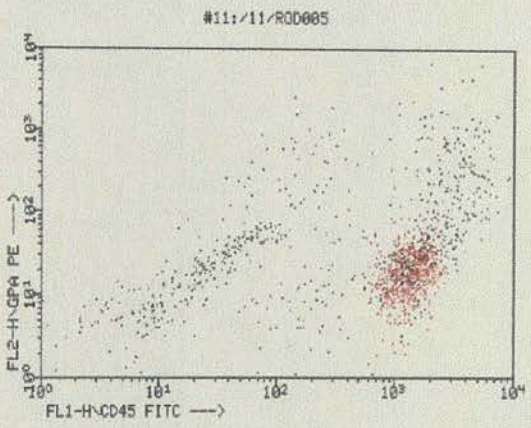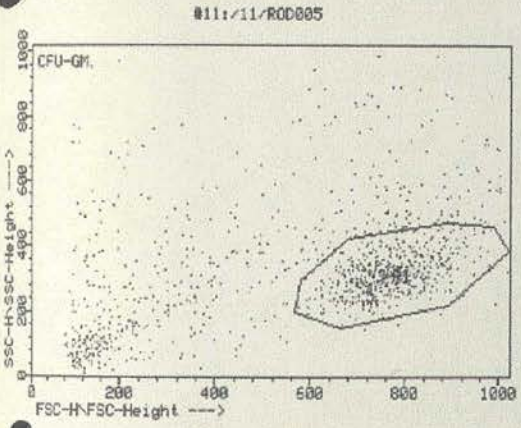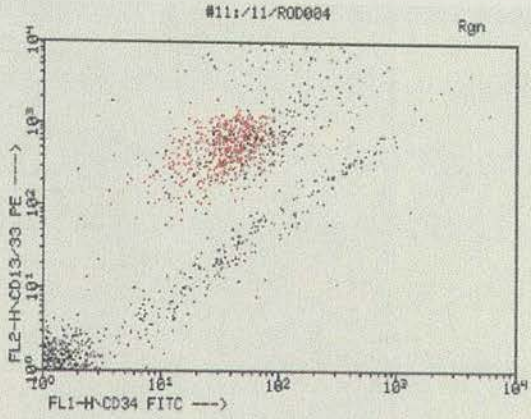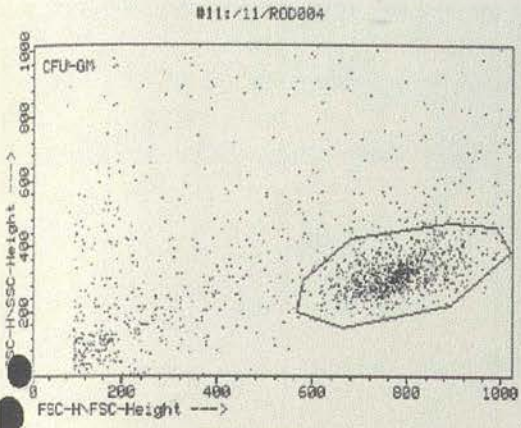
Figure 6.12

FACS plots showing 3 individual BFU-E colonies picked from methylcellulose. The left hand column shows the FSC/SSC characteristics of the cells in the colony and they are gated in region R1. The right hand column shows staining of the gated cells for: top plot=strong+ve Glycophorin(GPA), middle and lower plots=negative controls for CD14 (monocytic marker) and autofluorescence.

#11:/11/ROD001

BFU-E

#11:/11/ROD001   Rgn

#11:/11/ROD002

BFU-E

#11:/11/ROD002

#11:/11/ROD003

BFU-E

#11:/11/ROD003
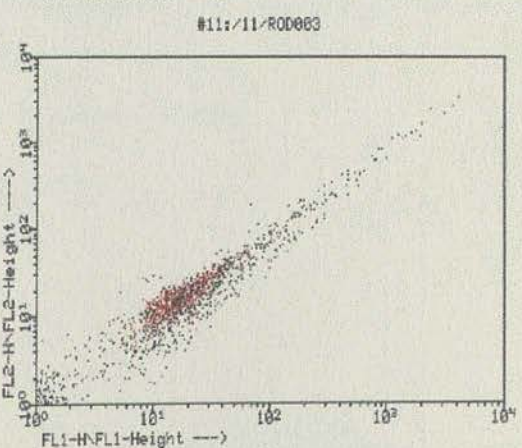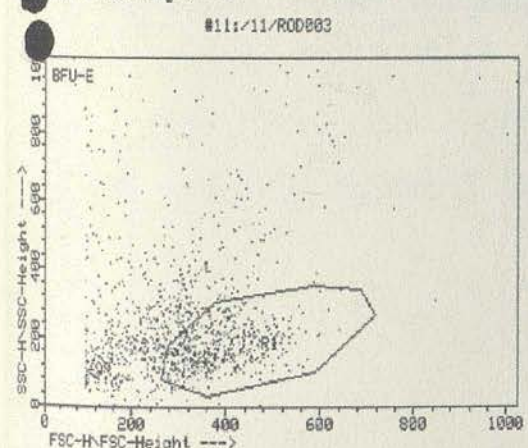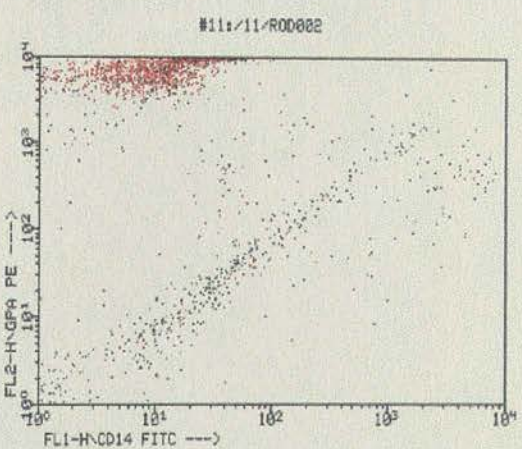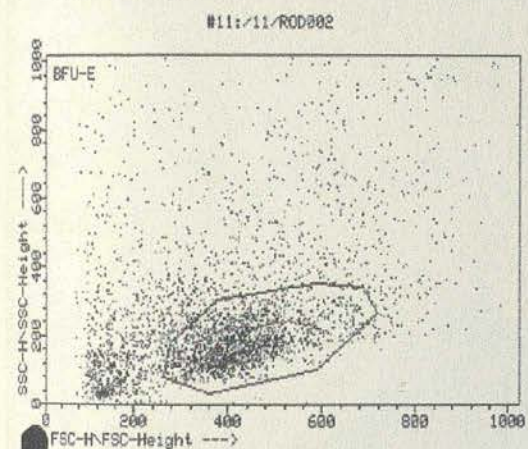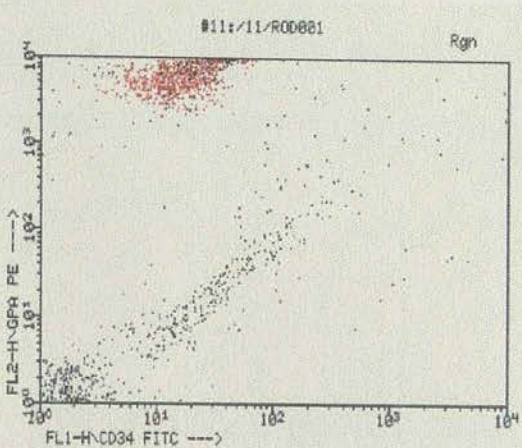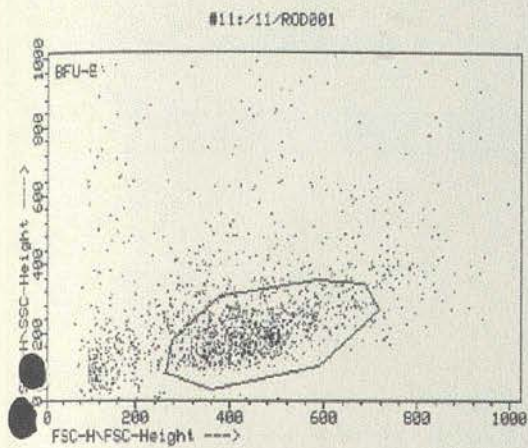
Figure 6.13

FACS histograms comparing the CD59 expression on PNH and normal B-cell lines with the expression on a single CFU-GM harvested from methylcellulose. The PNH line is negative and the normal line and the CFU-GM are positive for this antigen.

NORMAL CELL LINE   CD59

PNH CELL LINE

SINGLE NORMAL CFU-GM
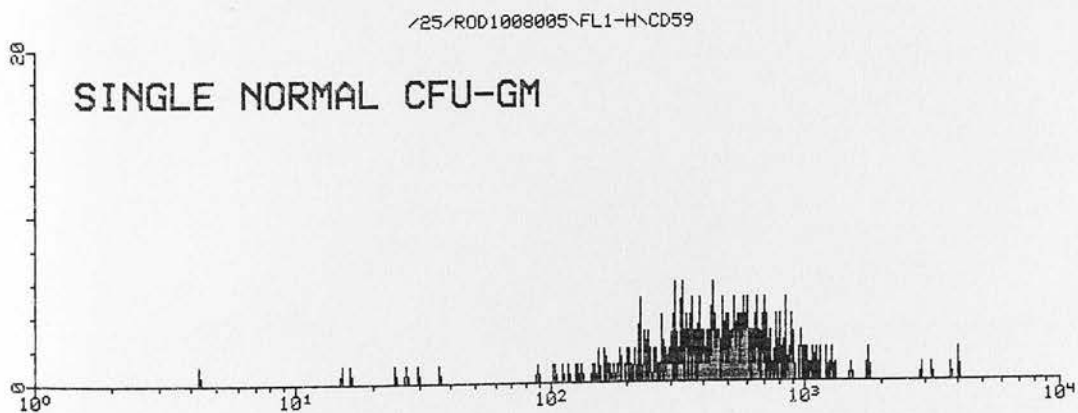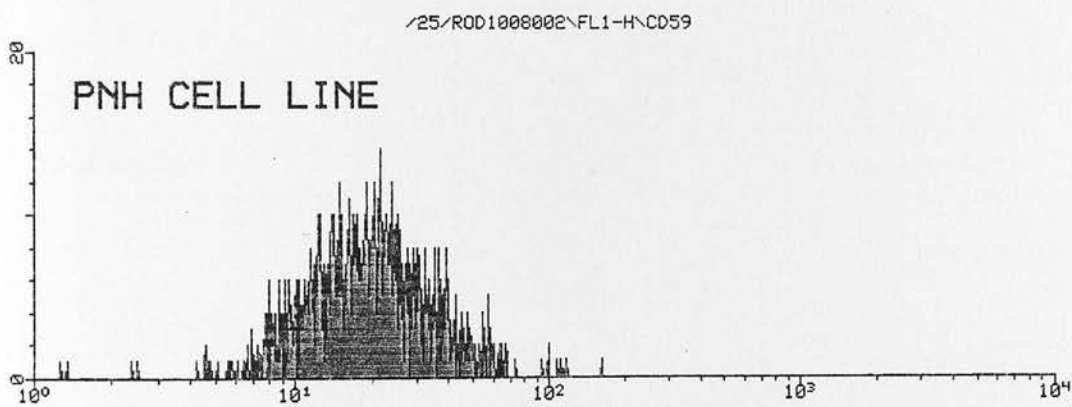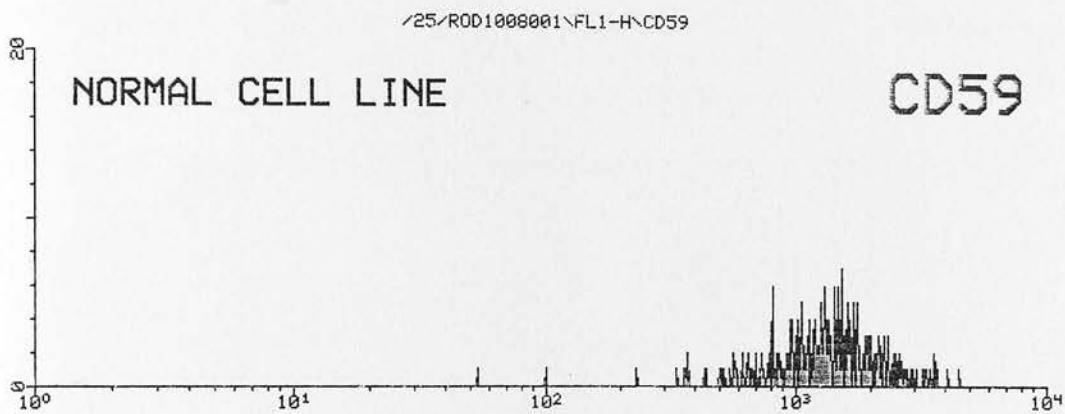
Figure 6.14

FACS plots and histograms showing CD59 staining on 6 individual CFU-GMs picked from methylcellulose. These colonies have been grown from the peripheral blood of a patient whose neutrophils are >90% PNH in phenotype. Of the 5 evaluable results shown, 4 are normal and one has a mixture of normal and PNH expression of CD59 (see text).

#11:/25/ROD2002001

SSC-H\SSC-Height --->
6. SINGLE CFU-GM FROM PB OF D.T.
R1
200   400   600   800   1000
FSC-H\FSC-Height --->

#11:/25/ROD2002002

SSC-H\SSC-Height --->
R1
0   200   400   600   800   1000
FSC-H\FSC-Height --->

#11:/25/ROD2002003

SSC-H\SSC-Height --->
R1
0   200   400   600   800   1000
FSC-H\FSC-Height --->

#11:/25/ROD2002004

SSC-H\SSC-Height --->
R1
0   200   400   600   800   1000
FSC-H\FSC-Height --->

#11:/25/ROD2002005

SSC-H\SSC-Height --->
0   200   400   600   800   1000
FSC-H\FSC-Height --->

#11:/25/ROD2002006

SSC-H\SSC-Height --->
R1
0   200   400   600   800   1000
FSC-H\FSC-Height --->

#11:/25/ROD2002001\FL2-H\CD59-PE
NEG
10^0 10^1 10^2 10^3 10^4

#11:/25/ROD2002002\FL2-H\CD59-PE
??
10^0 10^1 10^2 10^3 10^4

#11:/25/ROD2002003\FL2-H\CD59-PE
POS
10^0 10^1 10^2 10^3 10^4

#11:/25/ROD2002004\FL2-H\CD59-PE
POS
10^0 10^1 10^2 10^3 10^4

#11:/25/ROD2002005\FL2-H\CD59-PE
POS/NEG
10^0 10^1 10^2 10^3 10^4

#11:/25/ROD2002006\FL2-H\CD59-PE
POS
10^0 10^1 10^2 10^3 10^4

Figure 6.15

FACS plot and histogram showing the scatter characteristics and MAB staining pattern of CFU-GM grown from a stem cell harvest product from an individual with myeloma. All CFU-GMs in the dish have been pooled to obtain a larger cell sample for analysis. As shown, they stain for CD45,CD13+33,CD59 and CD55 but are negative for the control antibody Glycophorin-A (present on red cells and their precursors).

/25/ROD1309001

CFU-GM FROM A NORMAL
PBSCH. 1 DISH DISSOLVED
AND LABELLED

/25/ROD1309001\FL1-H\CD59

GLYCOPHORIN-A

CD13/33

CD45

CD59

CD55
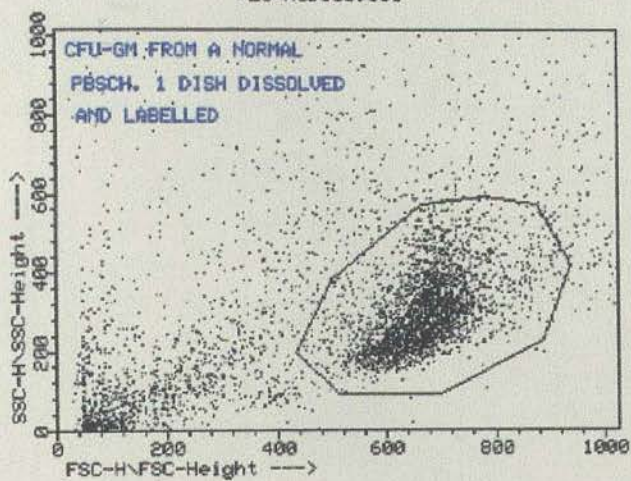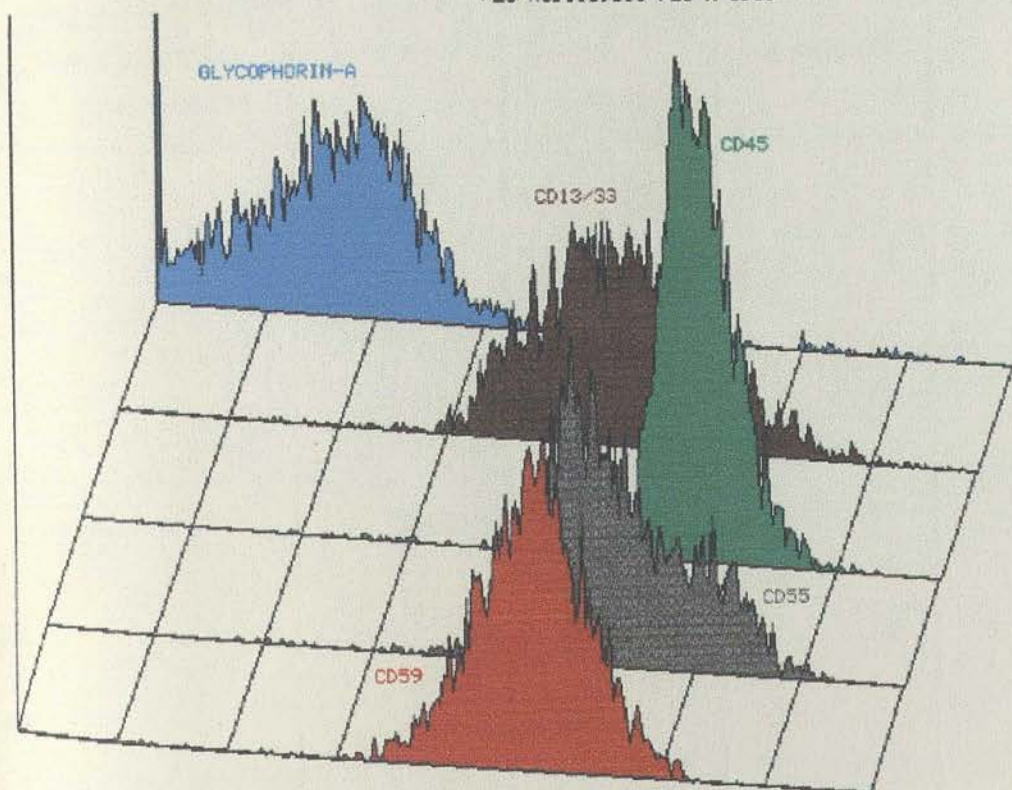
Figure 6.16

These plots show pooled CFU-GM from a normal individual exposed to increasing concentrations of complement. As shown, the percentage of calcein^{low}, CD13+33+ve cells (which are CFU-GM which have been permeabilised by complement) rises from 14% to 40% as the serum concentration increases (see text).
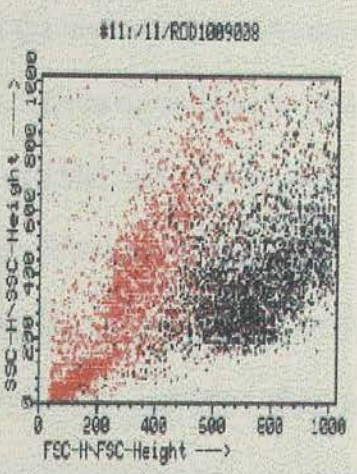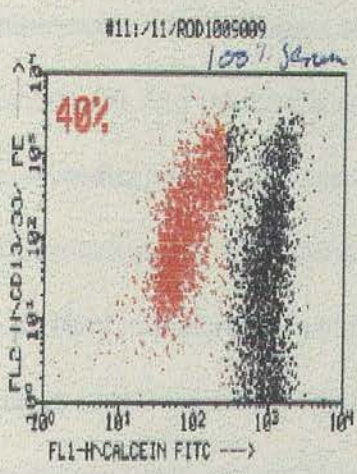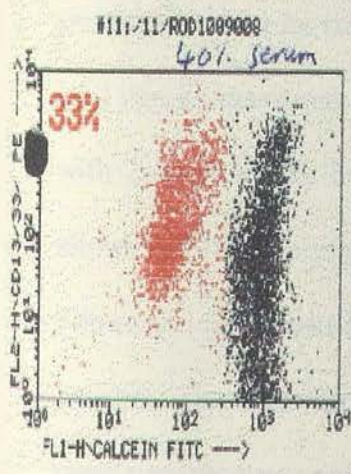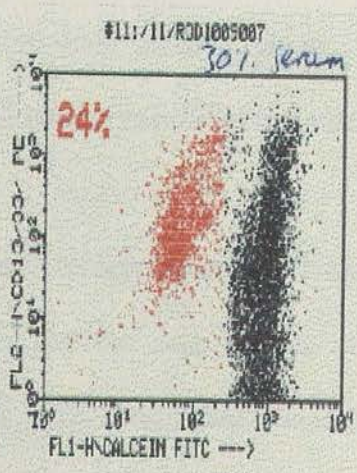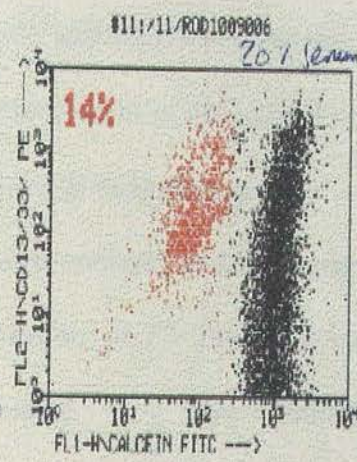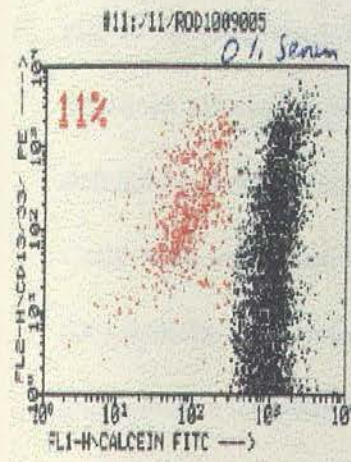
6.6   Discussion

In this chapter, I have described the results of a flow cytometry based assay for the demonstration of complement-mediated cell lysis. The assay is applicable to different cell types and because the degree of lysis is measured by fluorescence, it allows simultaneous cell identification using one or more labelled MABs. This approach allows the measurement of differential cell lysis in mixed cell populations defined by their surface antigen expression.

Cells were sensitised with antibody and then exposed to concentrations of human complement. A combination of FSC/SSC changes and the gain or loss of an appropriate autofluorescent vital dye defined cells that had undergone permeabilisation by complement. PI was chosen as the most suitable indicator dye as it can be measured in channel 3 of the FACScan™ allowing cells to be labelled with 2 other MABs for identification. In the case of PNH cells for example this allows PNH and normal stem cells to be defined using CD59 and CD34 staining.

The assay was applied to EBV transformed lymphoblastoid cell lines from PNH patients. The differential complement sensitivity of normal and PNH lines from these patients was easily demonstrated and could be measured in mixtures of these cells assayed simultaneously and separated by their CD59 staining characteristics. This principle has been used by Rother et al to demonstrate that retroviral transduction of these PNH lines with CD59-TM restores CD59 expression and function as judged by an increase in their resistance to complement lysis. Although this increase is significant, it does not fully equate with the level of function in non-PNH cells (Rother *et al* 1994).   As further evidence for the importance of CD59

activity (and perhaps other GPI-linked molecules) in inactivating complement, I have shown that biochemical removal of these GPI-linked structures using the enzyme PI-PLC renders normal cell lines as sensitive to complement lysis as their PNH counterparts. These experiments provide strong support for the central role of CD59 in the pathogenesis of some of the clinical features in PNH. They suggest that there is a rationale for attempting CD59-TM gene transfer into human PNH stem cells.

The results with this assay on human peripheral blood lymphocytes, as opposed to lymphoblastoid cell lines, served to underline the requirement for a reasonable number of test cells in order to achieve a clear result. The proportion of lymphocytes that are affected in PNH patients is often very small even when the clone is large as judged by neutrophil and/or red cell phenotype. I found that the small numbers of PNH lymphocytes in some samples limited the assay in PNH peripheral blood and only 2 of the 4 patients tested could be shown to have a clearly positive complement sensitivity test by flow cytometry. Neutrophils were not sensitive to lysis in this system and red cells do not take up PI, so the assay is not suitable for routine diagnosis of PNH in the manner of a Ham's lysis test.

In order to demonstrate functional correction of CD59 activity in PNH progenitors, it would be necessary to apply the assay to CFU-GM and BFU-E colonies. This was done by harvesting these cells from semi-solid growth medium (methylcellulose). It was demonstrated that these progenitor colonies are sensitive to lysis in this system. Two significant problems were encountered in attempting to grow colonies from PNH patient blood and assay them. Firstly there are technical problems as these hypoplastic patients have reduced blood progenitors and showed poor colony growth and secondly, the majority of progenitors appeared to be of normal phenotype unlike

the peripheral blood neutrophils. This phenomenon has been noted by others during immunophenotyping experiments on PNH blood and appears most marked in the most primitive precursors (Prince *et al* 1995; Musto *et al* 1997). This implies that collection of PNH stem cells from blood for gene transfer experiments would be difficult. I have proceeded to investigate the stem cell subsets in PNH patient blood before and after the administration of G-CSF in order to elucidate this unexpected observation (Johnson *et al* 1998) and to establish the practicality of attempting such a gene transfer experiment. The results are discussed in chapter 7.

Chapter 7.

PBSC Mobilisation in PNH:
a Prelude to Treatment by Autografting or Gene Transfer?

7.1   Introduction
7.2   Patient details and effects of mobilisation
7.3   Changes in haematological parameters on G-CSF
7.4   Phenotypic changes in peripheral blood neutrophils on G-CSF
7.5   Progenitor mobilisation
7.6   Stem cell subset analysis before and after G-CSF
7.7   Discussion: prospects for autografting and gene therapy

7.1   Introduction

PNH is an acquired haemolytic anaemia resulting from a somatic mutation in a

haemopoietic stem cell.   The disorder is discussed in detail in chapter 1.   The co-

existence of normal and abnormal stem cell populations in these patients, the links with

other stem cell disorders (AA/AML/MDS) and the ease with which PNH cells can be

identified (using flow cytometry) has made this an attractive disease model for stem cell

research.

Two speculative approaches to the treatment of PNH are autografting and gene therapy.

These would rely on a detailed understanding of the stem cell biology in this disease and

require satisfactory means of collection, phenotypic assay and manipulation of PBSCs in

these unusual patients. Important questions include: what are the effects of G-CSF in

PNH? Are there differences in the behaviour of normal and PNH stem cells? Can

normal and/or PNH stem cells be collected for clinical use and can methods of

mobilisation select for the normal cells thus effecting an in vivo purge as has been discussed in chapter 4 for CML?

The effects of G-CSF on normal individuals and those with a variety of haematological disorders have been studied extensively (Stroncek *et al* 1996; To *et al* 1997). It has been used in both congenital and acquired marrow failure states and has an established track record in ameliorating neutropenia in these conditions both in short and long term studies (Dale *et al* 1993). In a manner analogous to this, G-CSF has been used in PNH patients suffering aplasia-related infective problems and the suggestion is that they may derive benefit (Ninomiya *et al* 1993). Interestingly, these workers reported that G-CSF induced phenotypic changes in PNH cells suggesting that this drug might alter the biology of the disease. Other groups have looked at stem cell phenotype in the peripheral blood of PNH patients (not on G-CSF) and found that the majority of the most primitive PBSCs appear to be of normal phenotype despite the opposite situation in the bone marrow (Prince *et al* 1995). This has led to tentative suggestions that normal PBSCs could be collected and used for autologous transplantation (Musto *et al* 1997; Prince *et al* 1995). Clearly these observations, which are contrary to expectations, require further investigation by combining the study of primitive and mature cell types in PNH patients intentionally mobilised using G-CSF. This chapter describes the first such clinical investigation (Johnson *et al* 1998). I sought through this work to clarify the existing contradictory data surrounding G-CSF in PNH, collect PNH stem cells for laboratory manipulation in a gene therapy protocol (see chapters 5 and 6) and investigate the practicality of PBSC collection for autologous transplantation in this disease.

The clinical consequences and changes in haematological parameters during G-CSF therapy in these patients are described, along with experiments designed to explain the apparent phenotypic changes in PNH neutrophils referred to above. Progenitor cell mobilisation was examined by assays of CD34 cells and CFU-GM/BFU-E and compared to that of normal individuals. Finally, the phenotype (PNH vs normal) of subsets of the CD34 fraction was studied in order to establish whether the most primitive progenitors are more or less affected and whether normal stem cells could be recruited into the peripheral blood. All experimental detail is described in chapter 2 sections 2.28 to 2.35.

7.2   Patient details and effects of mobilisation

Four patients underwent stem cell mobilisation using G-CSF alone at a dose of 10μg/Kg/day for four consecutive days. All patients had been previously diagnosed in our department and had classical haemolytic PNH. Diagnostic criteria included a positive Ham-Dacie acid lysis test and the demonstration on patient red cells and neutrophils of a population of CD59/CD55 deficient cells by flow cytometry. One patient (DL) was on treatment for Aplastic Anaemia with Cyclosporin A (their original presenting diagnosis). All patients were prophylactically warfarinised to prevent thrombosis. Baseline clinical details and typical haematological values for the patients are shown in table 7.1 along with the size of their PNH clone, measured by flow cytometry on peripheral blood neutrophils (median 87% PNH, range 50-97). All four patients completed the mobilisation regimen. A bone marrow aspirate was taken post G-CSF in one case (DT). One patient (ST) developed a chest infection after treatment

precipitating a haemolytic crisis. The others had no ill effects apart from mild bone pain related to the growth factor.

7.3 Changes in haematological parameters on G-CSF

Haematological monitoring included the FBC, NAP score and reticulocyte count. The WBC rose in all patients on G-CSF from a baseline mean of 3.6×10^9/l. to a peak mean of 15.3×10^9/l. (Figure 7.1). Although this is a definite rise it is substantially less than that seen in normal volunteers on G-CSF whose absolute neutrophils count alone reaches mean peak levels of around 28×10^9/l. on similar regimens (Stroncek *et al* 1996). This presumably reflects the underlying hypoplasia in these patients. Interestingly, the NAP score (NAP being GPI-linked) rose in all patients during G-CSF treatment. Our normal range is 30-120. The mean NAP score on day 1 was 41 while the mean on day 5 was 162. Three out of the four patients showed a steady increase which only began to fall again after discontinuing G-CSF while one patient's score rose initially but began to fall back towards baseline before G-CSF finished (Figure 7.2). Platelet counts did not change. Haemoglobin and reticulocyte counts for the four patients are shown respectively in figures 7.3 and 7.4. One patient (DL) was transfused at the end of G-CSF (day 5) but this had been planned as this patient was due their regular transfusion at this time in any case. As previously mentioned, one patient (ST) did have a haemolytic crisis that started 2 days post G-CSF. This patient normally suffers bouts of haemolysis and it is impossible to say whether the procedure contributed.

Table 7.1   Baseline Details for the Four PNH Patients

Patient	Age/sex	diagnosed	Hb	WBC	Plts	Transfused	%PNH Neuts.
DL	43/M	1988	9.5	3.0	74	2-4 weekly	96
DT	53/M	1986	9.9	4.0	209	6 weekly	95
LW	30/M	1991	12	3.7	155	None	50
ST	65/F	1990	10	4.0	280	Occasionally	79

(Hb=Typical pre-transfusion haemoglobin in g/dl, WBC=total white blood count x10^9/l., Plts=Platelet count x10^9/l.,

Neuts=Neutrophils M=Male, F=Female, Age is in years.)

Figure 7.1

Graph showing the changes in WBC in the peripheral blood of the 4 PNH patients

over 8 consecutive days. The count on day 1 is pre-G-CSF (i.e. baseline) and this

drug was administered daily from days 1 to 4 inclusive. The rise in WBC during

G-CSF is shown as is the return to baseline levels 4 days after it was discontinued.

WBCx109/l.

G-CSFgiven on days 1-4 inclusive

DL
DT
LW
ST
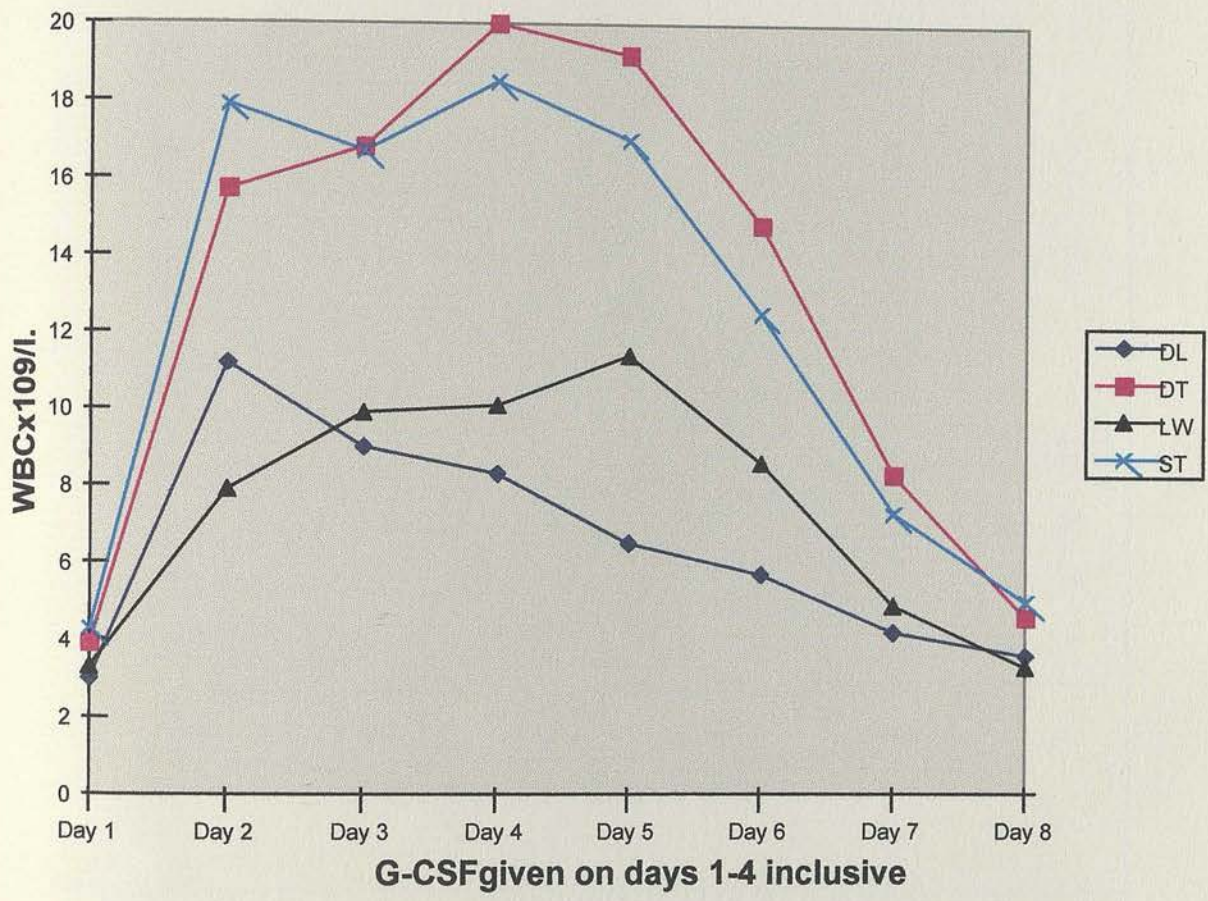
Figure 7.2

Graph showing the NAP scores in the 4 PNH patients during treatment with G-CSF. The scores rise dramatically in all cases (normal range in our laboratory is 30-120). While this phenomenon is well described in normal individuals on G-CSF, it has not been described in PNH where the NAP is classically low due to its GPI-linkage. See text for discussion.

Y-axis: NAP score

X-axis: Day 1, Day 2, Day 3, Day 4, Day 5

G-CSF given on days 1 to 4

Legend: DL, DT, LW, ST
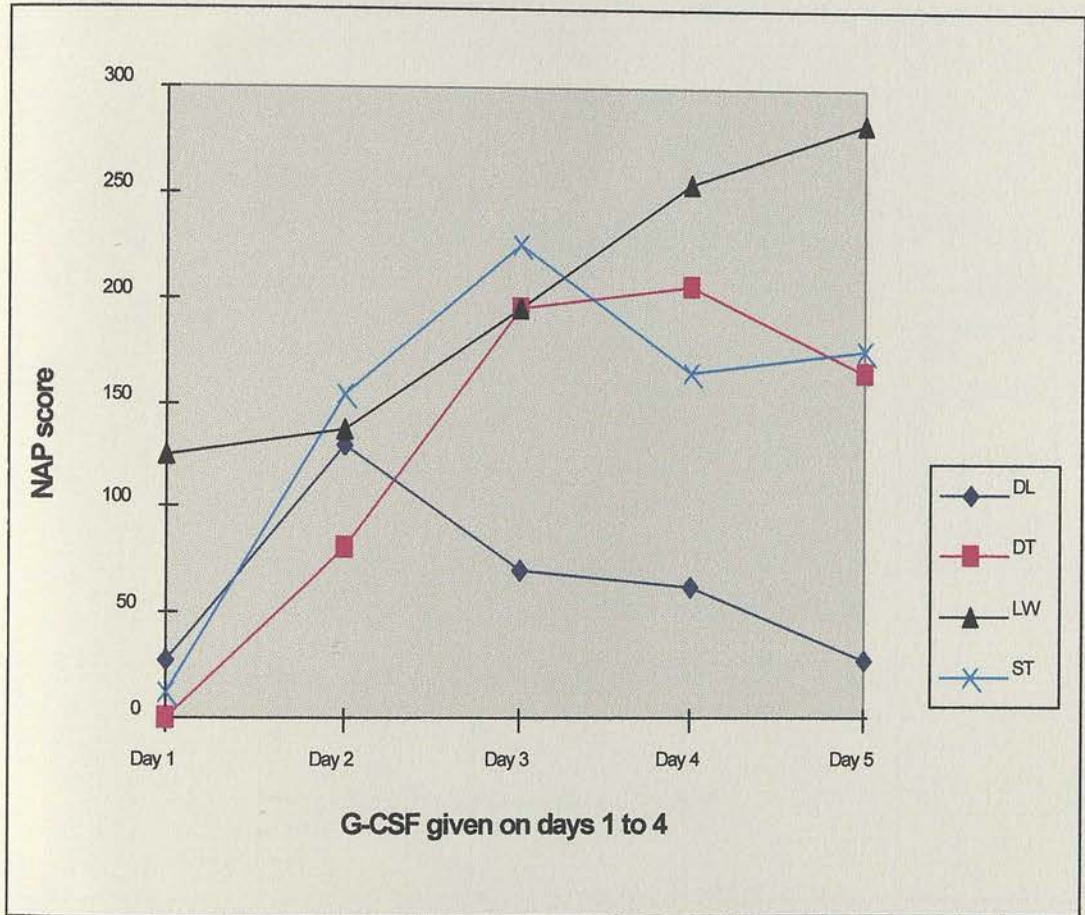
Figure 7.3

Graph showing the changes in Hb levels during G-CSF treatment in the 4 PNH patients.
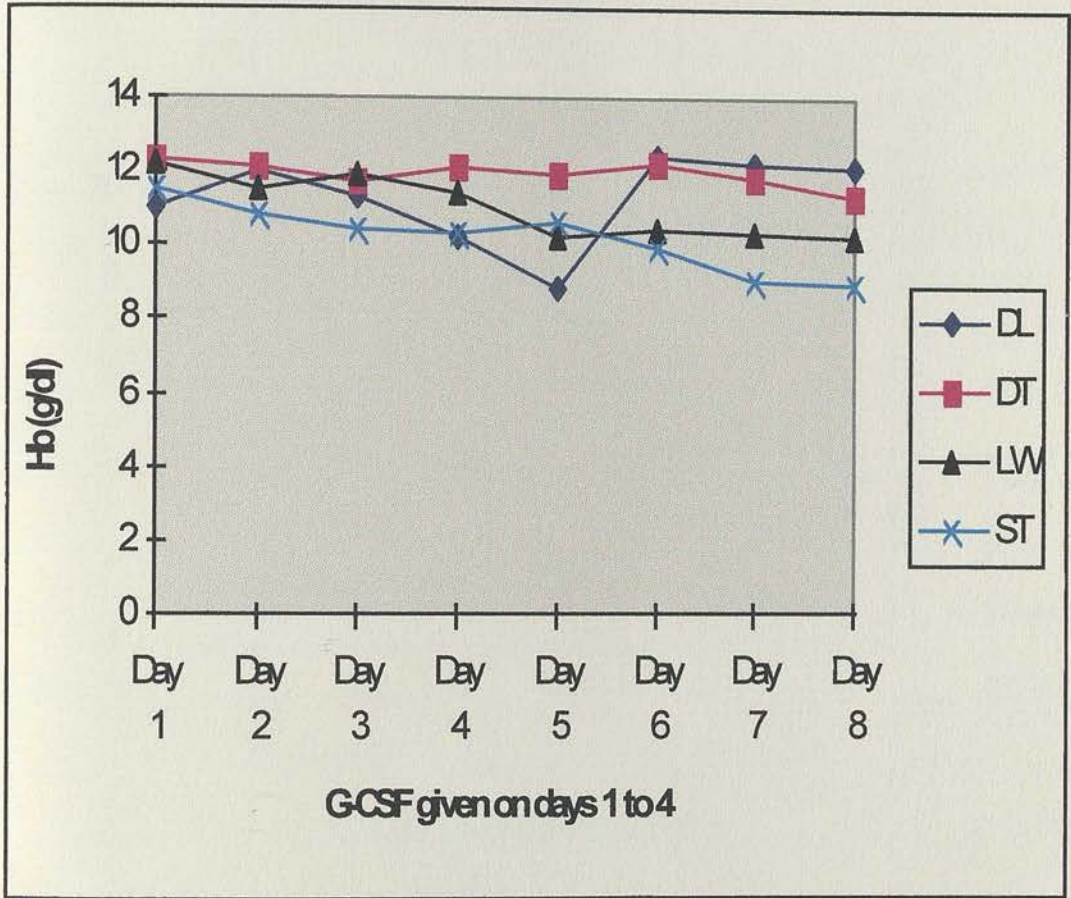
Figure 7.4

Graph showing the changes in reticulocyte percentage in the 4 PNH patients during G-CSF treatment and for 4 days following the end of treatment.

Figure: % Retics. vs Day 1 to Day 8. G-CSF given on days 1 to 4. Legend: DL, DT, LW, ST.
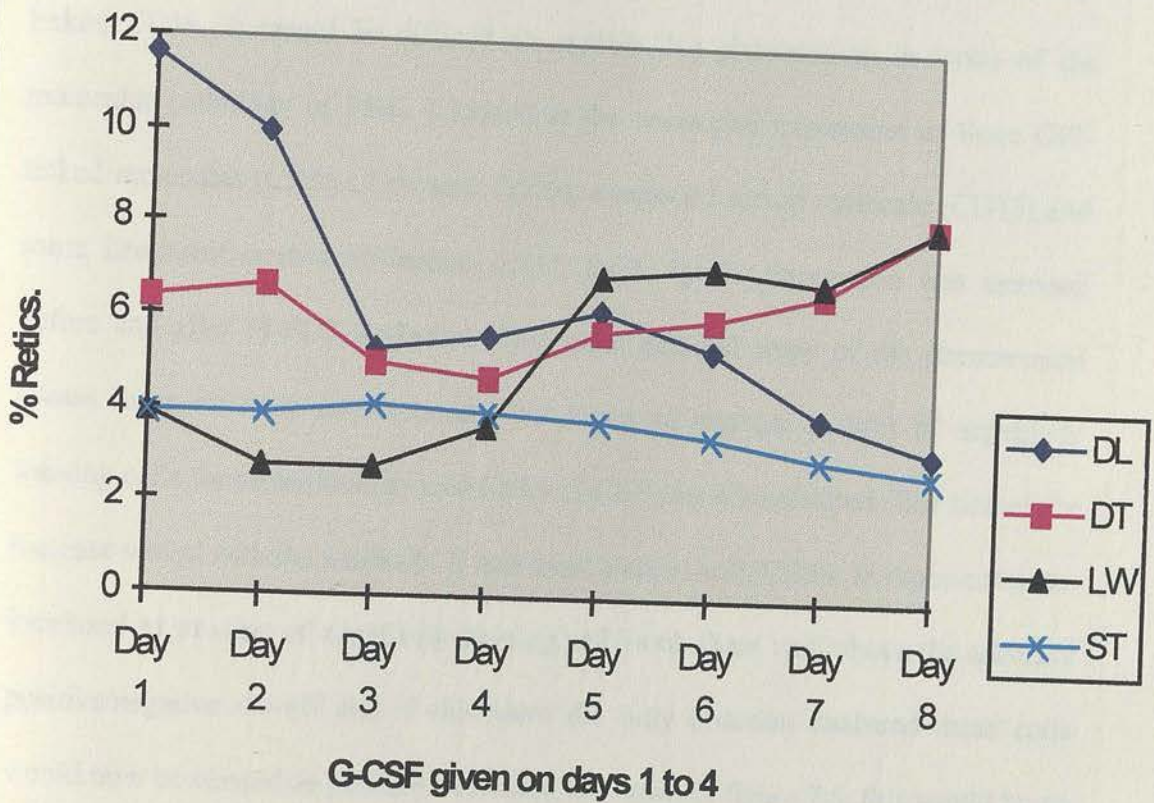
7.4   Phenotypic changes in peripheral blood neutrophils on G-CSF

It had been previously reported that PNH patients given G-CSF showed an increase in the expression of CD16 (a GPI-linked molecule) by FACS on neutrophils (Ninomiya *et al* 1993). Furthermore, this increased expression was said to be sensitive to treatment with PI-PLC implying that it was due to the presence of GPI linked CD16. It would be difficult to explain this phenomenon in terms of the molecular pathology of PNH. I looked at the neutrophil expression of three GPI-linked molecules (CD55,CD59 and CD16), a non-GPI linked molecule (CD15) and some irrelevant control antibodies (CD3, IgG1, IgG2). Expression was assessed before and after PI-PLC treatment. This more detailed study of the phenomenon allows for a different interpretation. We found an increase in MFI of negatively staining cells for all antibodies tested after G-CSF was administered. The size of the increase varied with the antibody. It was most marked with CD59. In many cases the increased expression of negatively staining cells took them well above the arbitrary positive/negative cut-off and if this were the only criterion analysed these cells would now be classed as positive. However, as shown in figure 7.5, this would be an inaccurate interpretation of the results. This figure shows that the CD16 negative peak increases in MFI on day 4 of G-CSF but that there is still a clearly discernible 'true' positive peak of similar size to baseline. Six days later, the MFI of both peaks has returned nearly to normal. Figure 7.6 shows the same cells before and after G-CSF but in addition they are shown treated and untreated with PI-PLC. As seen, the true positive peak remains sensitive to PI-PLC and is effectively abolished (from 5-10%+ve down to 0.5-1%+ve) after incubation with the enzyme. There is however no

effect of PI-PLC on the negative peak even after it has increased in MFI post G-CSF. Figure 7.7 shows essentially the same data for CD59. Here the increase in MFI of the negative peak is more dramatic and reaches the same levels as the positive peak which is swamped. PI-PLC does not reduce the MFI of the negative peak post G-CSF but cleaves the positive peak pre-G-CSF. Figure 7.8 shows the effects of G-CSF treatment on the same cells stained with irrelevant antibodies. Just as the CD16 and CD59 negative peaks increase in MFI, so do the negative staining antibodies CD3, IgG1 and IgG2.

7.5   Progenitor mobilisation

It was evident from the pre and post mobilisation progenitor cell numbers that all of the four patients under investigation had an element of marrow hypoplasia as is to be expected in most, if not all, PNH patients. Figure 7.9 shows the absolute CD34 numbers in the cohort during administration of G-CSF. In normal subjects (n=6), we found steady state values for peripheral blood CD34 cells ranging from $0.9 \times 10^6/l$. to $11.3 \times 10^6/l$. with a median of $2.5 \times 10^6/l$. In a larger series of normals who were given G-CSF, the mean baseline CD34 count was $8 \times 10^6/l$. and this rose to $55 \times 10^6/l$. after G-CSF (Stroncek et al 1996). In our PNH patients, the baseline figure was $0.5 \times 10^6/l$. (range 0.1-0.8) and after G-CSF it rose to only $4.2 \times 10^6/l$. (range 1.3-9). In both normal and PNH this represents a similar proportionate increase (7-9 fold) despite the baseline CD34 count in the PNH patients being 5-16 fold less than normal ranges. The kinetics of CD34 mobilisation appear to be similar in normal and PNH patients with the CD34 rise beginning around day 3-5 of G-CSF and falling rapidly

back to baseline a few days post G-CSF. This contrasts with the rise in WBC which occurs on day 2 and this may reflect the more complicated distribution of neutrophils in the body.

CFU-GM and BFU-E numbers were also measured. These assays are at best only semi-quantitative and the figures vary dramatically between laboratories. For normal steady state peripheral blood (n=12), the method used here (see section 2.16) gave values of 8.2 CFU-GM/10^6 MNC plated (range 1.7-33.3) and 27.7 BFU-E/10^6 MNC plated (range 15.3-114.3). One of the PNH patients failed to grow any colonies, the other three did but the numbers were small. Pre G-CSF the CFU-GM numbers ranged from 1 to 4 with a median of 3/10^6 MNC plated and the BFU-E from 5 to 10 with a median of 6. After G-CSF there was no significant change detectable at these low levels (3 to 5 CFU-GM/10^6 MNC and 5 to 11 BFU-E). Again, this assay underlines the hypoplastic nature of the disease

Figure 7.5

This figure shows peripheral blood neutrophils from one of the PNH patients (ST) before, during and after administration of G-CSF. The cells have been stained for the GPI-linked molecule CD16 to differentiate normal from PNH. The increased MFI for CD16 on the negative cells is clearly demonstrated during G-CSF (middle plot) and returns to normal after the drug is discontinued (bottom plot). The small proportion of normal cells on the right of the plots remains largely unchanged (see text, section 7.4)

PRE G-CSF

PNH NEUTROPHILS

NORMAL NEUTROPHILS

CD16-PE

/26/ROD1605005\FL2-H\CD16

DAY 4 OF G-CSF
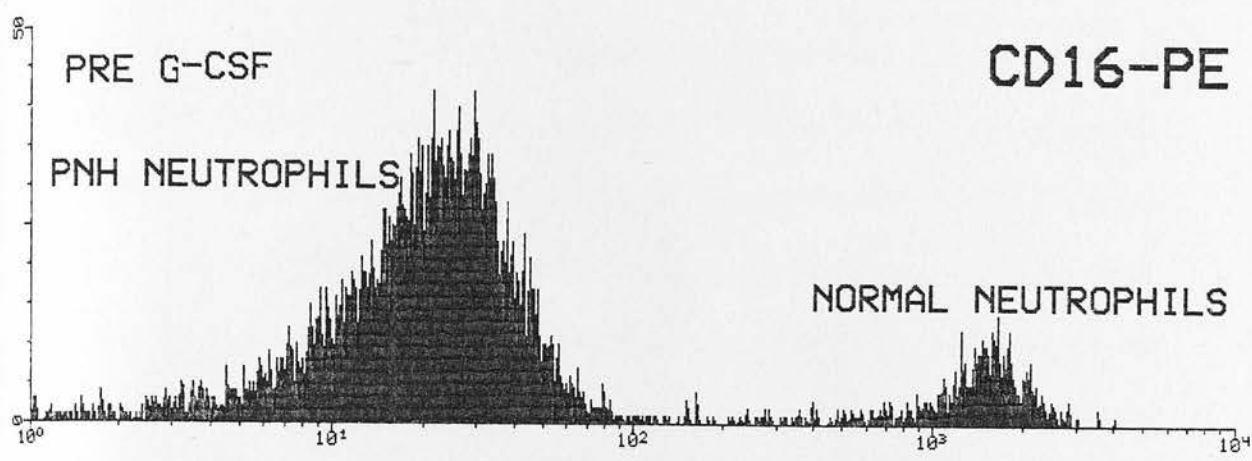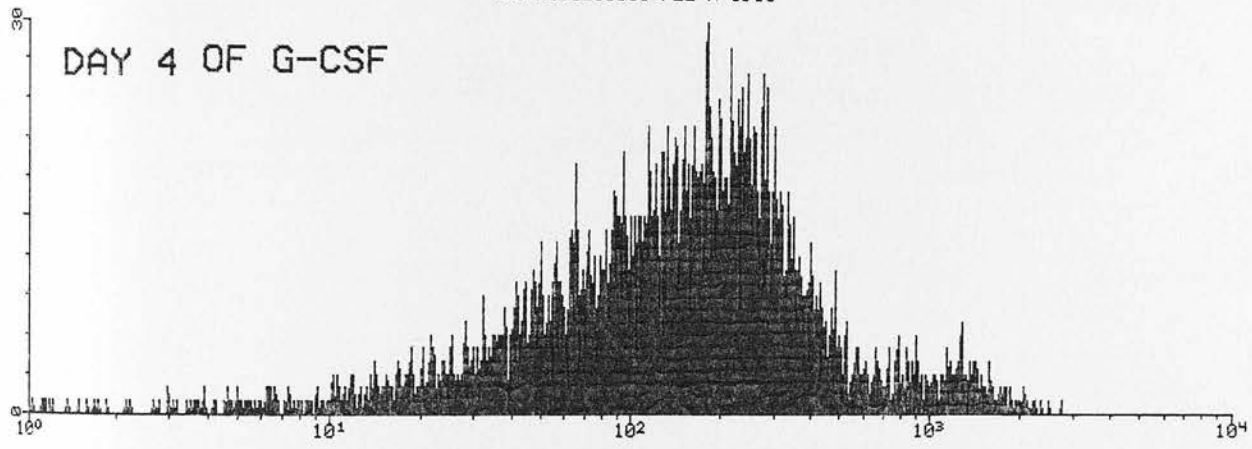
/26/ROD2405001\FL2-H\CD16

6 DAYS POST G-CSF
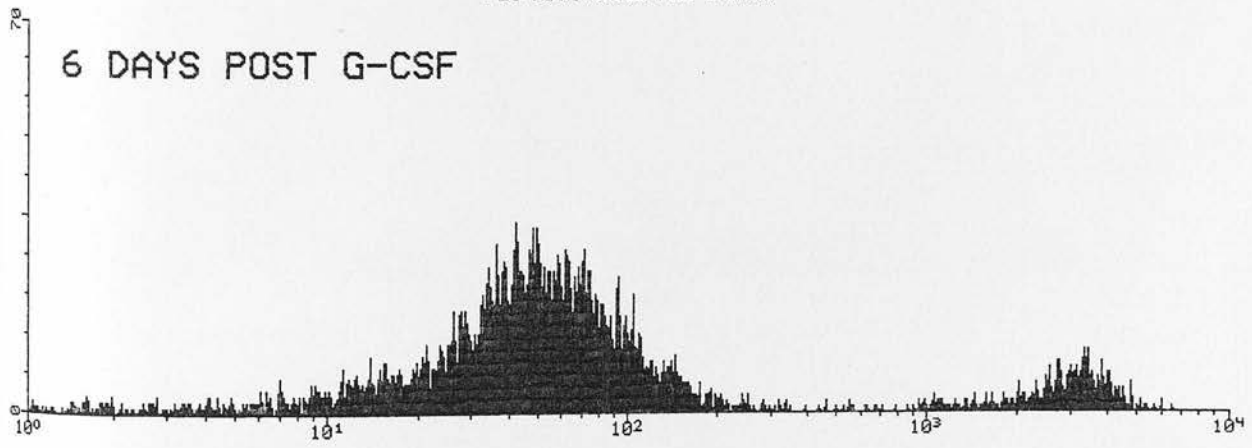
Figure 7.6

This is a FACS plot showing the effect of the enzyme PI-PLC on CD16 staining on the neutrophils from PNH patient ST before and after G-CSF. This enzyme cleaves GPI anchors and if the increase in CD16 staining following G-CSF were due to a real increase in CD16 expression on the cell surface it should be abolished by PI-PLC treatment. The top 2 plots show that normal neutrophils (right hand peak, CD16+ve) lose CD16 staining after PI-PLC but the PNH cells (left hand peak, CD16-ve) are unaffected as they do not express this marker. In the bottom 2 plots, the same experiment is shown but G-CSF has increased the MFI for CD16 of the negative (PNH) cells in the left hand peak. As shown, this apparent increased expression is not PI-PLC sensitive indicating that it is not due to true CD16 expression.

L2-H\CD16

CD16-PE

PRE G-CSF
NO PI-PLC

M1

10%
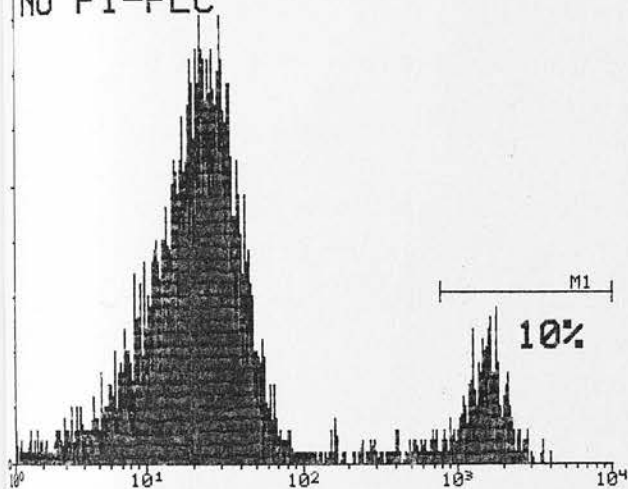
/26/ROD1305015\FL2-H\CD16

PRE G-CSF
AFTER PI-PLC

M1

1%
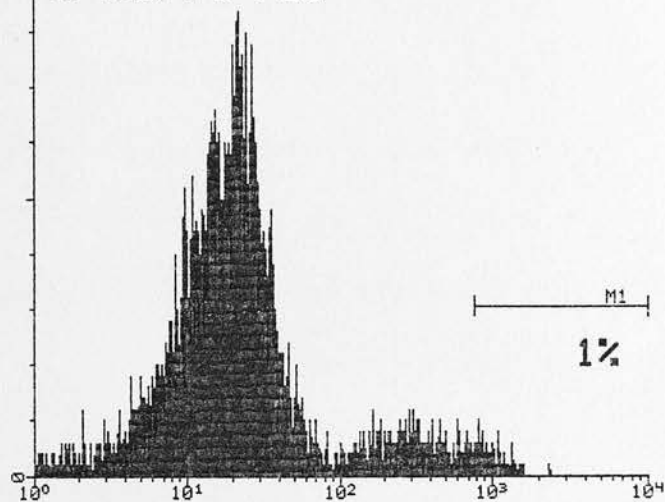
/26/ROD1405001\FL2-H\CD16

POST G-CSF
NO PI-PLC

M1

5%
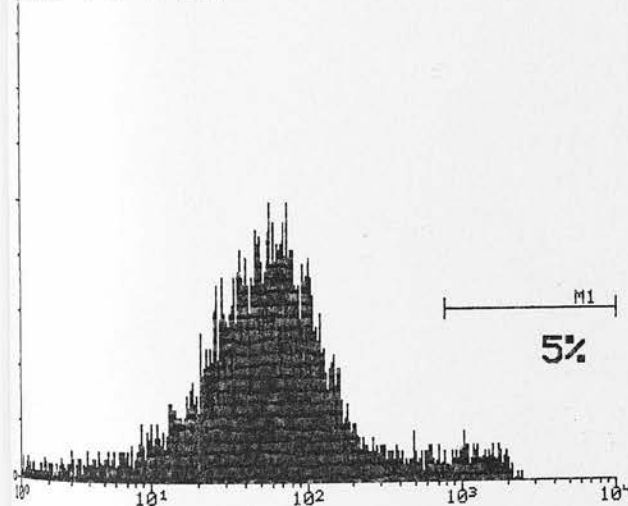
/26/ROD1405003\FL2-H\CD16

POST G-CSF
AFTER PI-PLC

M1

0.5%
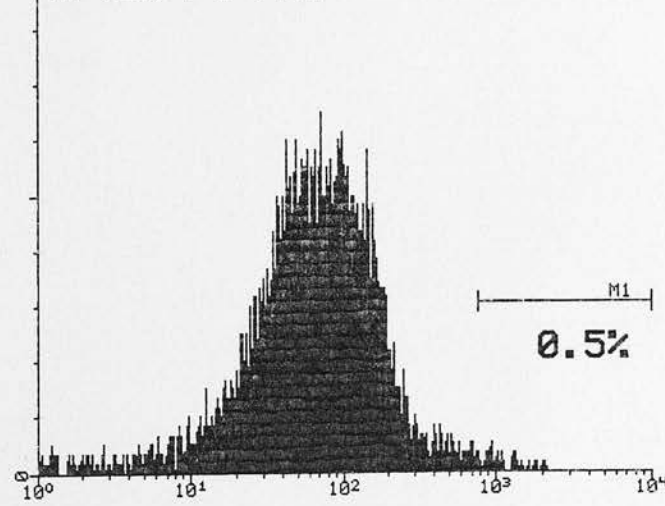
Figure 7.7

This FACS plot is essentially the same as for figure 7.6 but shows the results with another GPI-linked molecule, CD59. In the upper plot, comparison of the red (pre-PI-PLC) and grey (post PI-PLC) traces shows the cleavage of the CD59+ve peak by the enzyme and the lack of effect on the negative (PNH) peak. The lower plot shows the greatly increased MFI of the negative cells which now swamp the positive peak. These cells are not sensitive to PI-PLC cleavage however (grey trace) indicating that the MFI is not due to true increases in CD16 expression.

CD59 NEG NO PIPLC
CD59 NEG + PIPLC

CD59 POS NO PIPLC
CD59 POS + PIPLC

PRE PIPLC
POST PIPLC
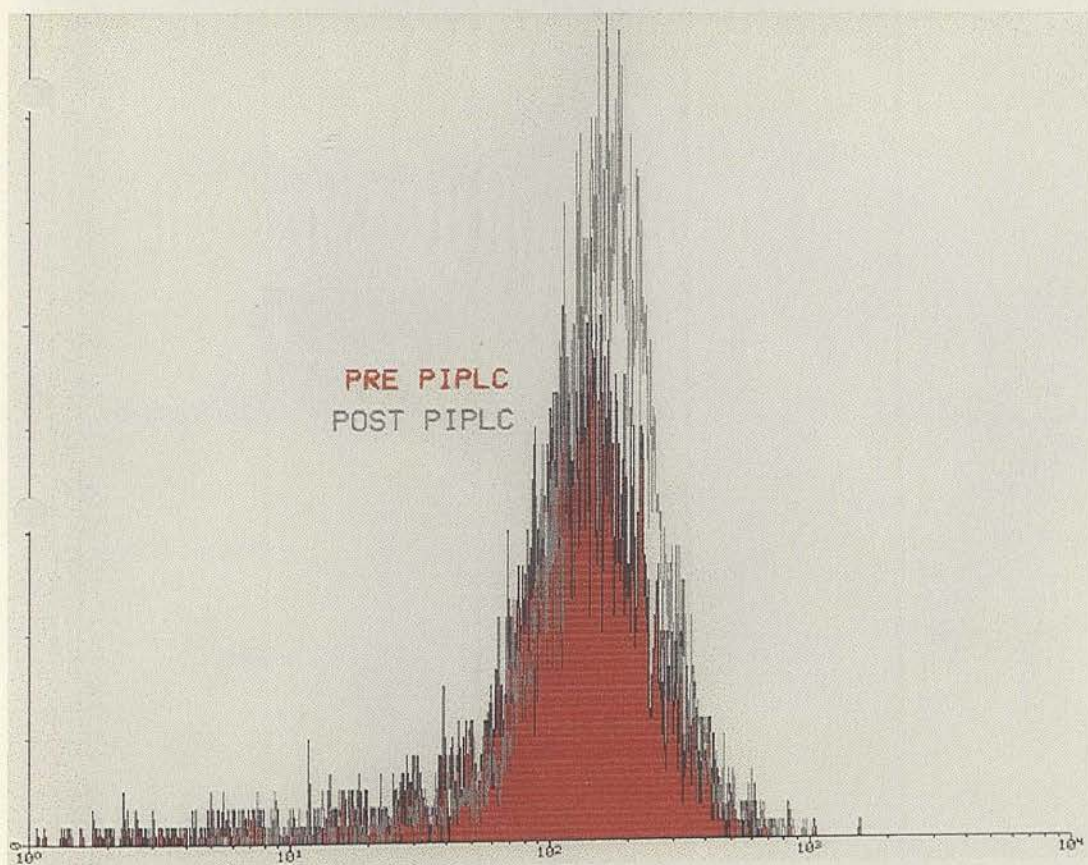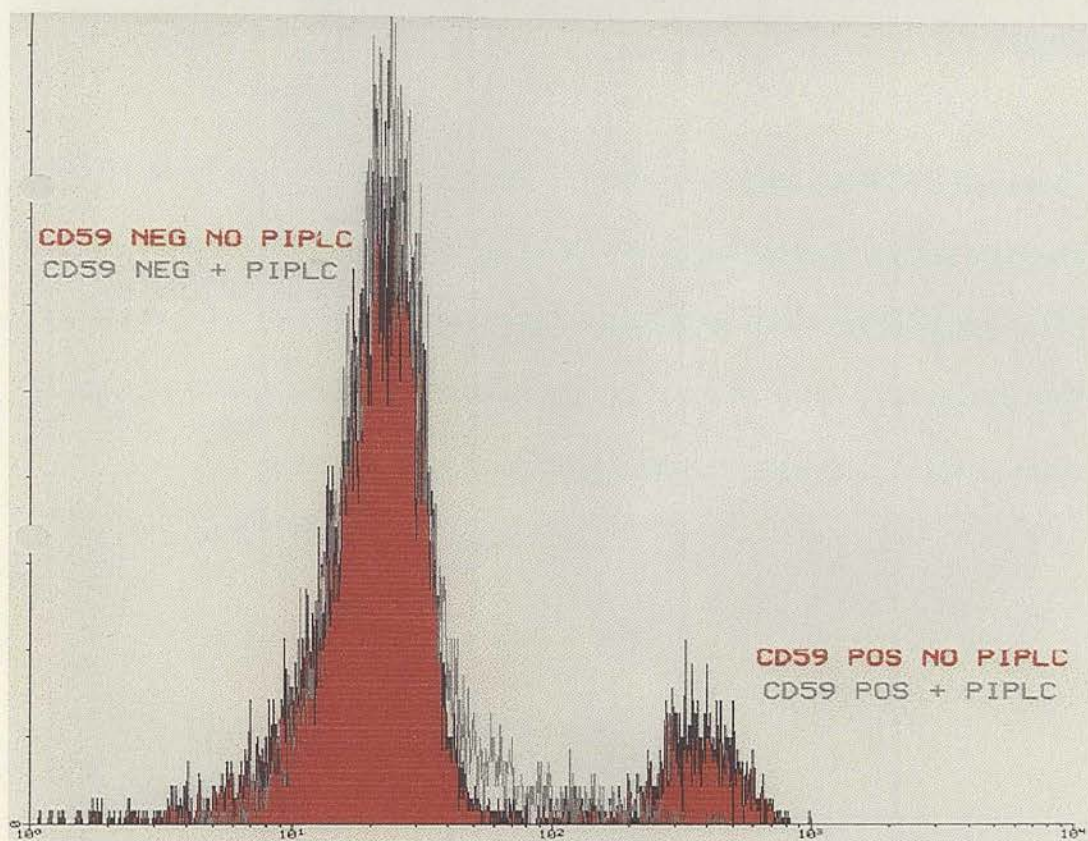
Figure 7.8

These FACS plots are controls for the experiments shown in figures 7.5 to 7.7 using irrelevant MABs. Four antibodies are shown: a FITC and a PE conjugated CD3 and IgG1, none of which should be expressed on neutrophils. The cells are from the same PNH patient (ST). They all show the same pattern of increase in MFI after G-CSF which serves to underline the artefactual nature of the observation.

PRE G-CSF

IGG1-PE   IGG1-FITC

/24/ROD1705004\FL1-H\IGG1

POST G-CSF

IGG1-FITC   IGG1-PE

PRE G-CSF

CD3-PE   CD3-FITC

/24/ROD1705003\FL1-H\CD3

POST G-CSF

CD3-FITC   CD3-PE
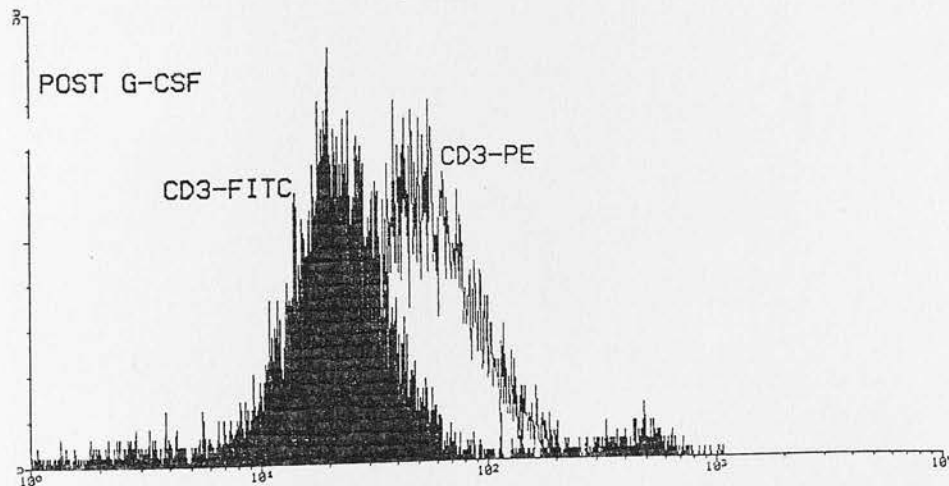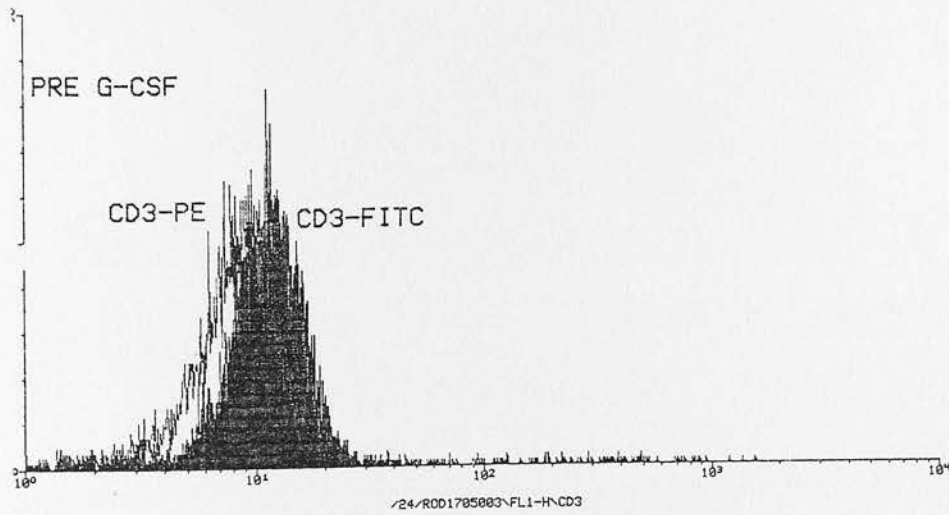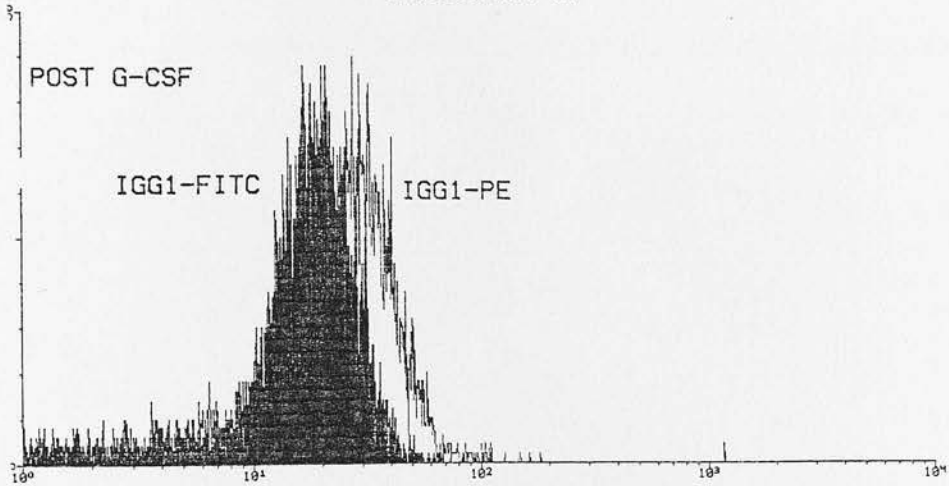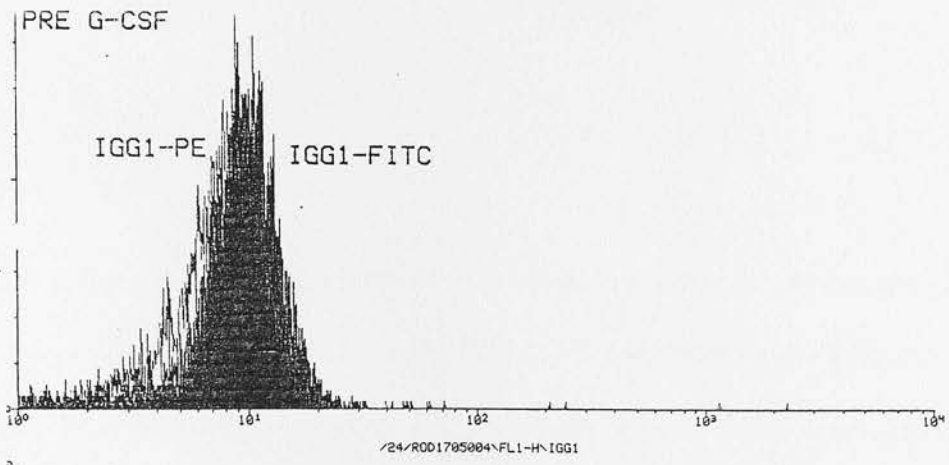
Figure 7.9

Graph showing the increase in peripheral blood CD34 positive cells in the 4 PNH patients during the administration of G-CSF (given on days 1 to 4 inclusive). The kinetics of mobilisation is very similar to that in normal individuals with the rise beginning after 2 to 3 days of treatment. The absolute numbers are much lower than normal individuals due to the hypoplastic nature of the disease. See text for discussion.

Figure with Y-axis labeled "Abs. CD34x106/l." ranging 0 to 9, X-axis labeled "G-CSF given on days 1 to 4" with points Day 1, Day 2, Day 3, Day 4, Day 5. Legend: DL, DT, LW, ST.
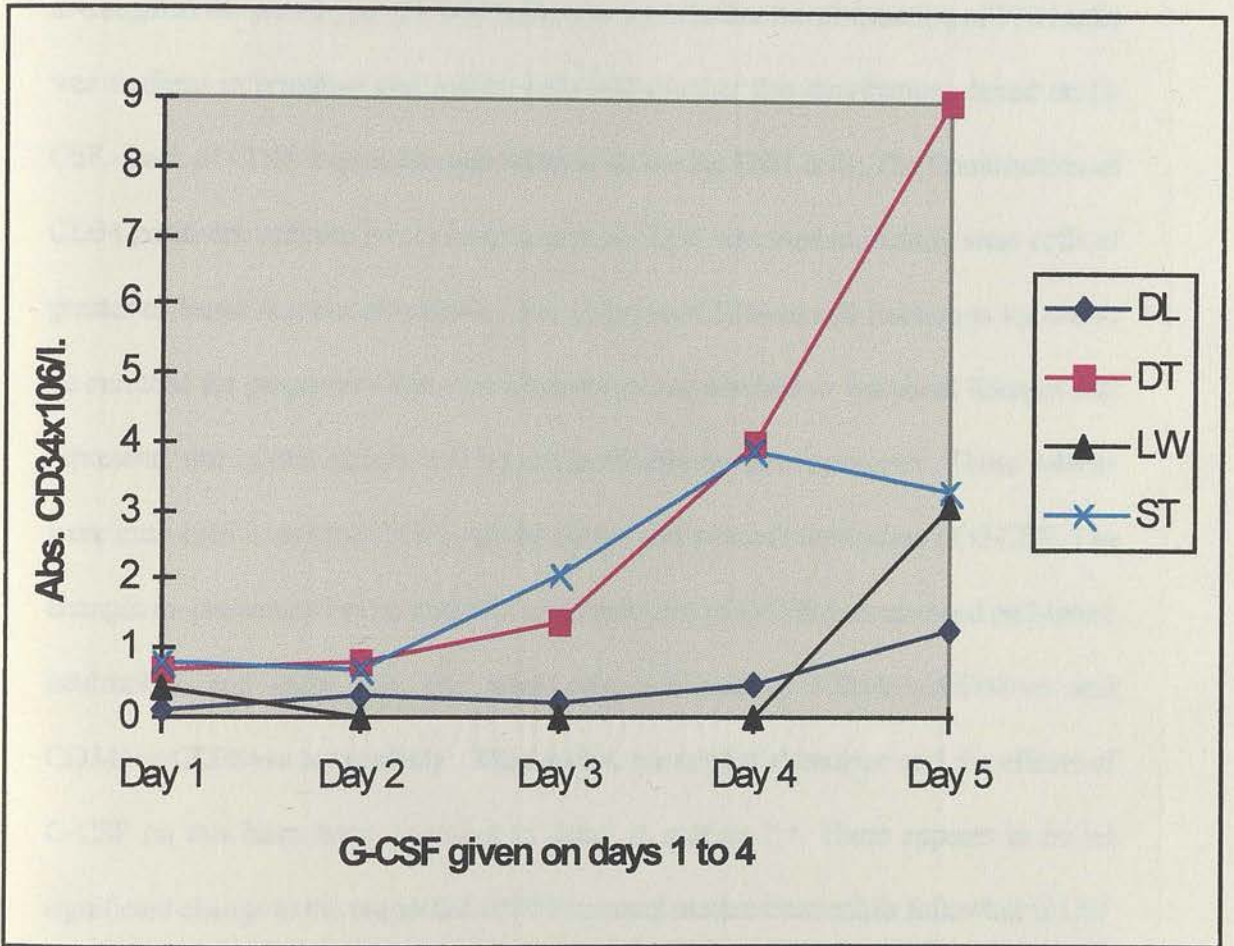
7.6   Stem cell subset analysis before and after G-CSF

PNH arises in conditions of marrow hypoplasia and there is evidence for a growth advantage of the PNH clone over normal haemopoiesis in such states. The mechanism of this selection and the level of cell maturity at which it operates is unknown. I investigated the phenotype of CD34 subsets to see whether the distribution of PNH cells was uniform in primitive and mature cells and whether this distribution altered on G-CSF. Lack of CD59 expression was taken to define the PNH cells. The Combination of CD34 positivity with the level of expression of CD38 was used to identify stem cells of greater or lesser degrees of maturity. The CD34+ve/CD38-ve cell fraction is known to be enriched for progenitors that can differentiate into myeloid or lymphoid lineages and represents one of the earliest cell types identifiable by flow cytometry. These subsets were examined in our four PNH patients before and after administration of G-CSF. The changes in phenotype before and after administration of G-CSF was assessed on Mature neutrophils and early and late stem cells (defined as CD34+ve/CD38-ve and CD34+ve/CD38+ve respectively. The baseline neutrophil phenotype and the effects of G-CSF on this have been discussed in detail in section 7.4. There appears to be no significant change in the proportion of PNH:normal mature neutrophils following G-CSF (p>0.1). The situation for the more primitive subsets is however very different. One of the patients (ST) showed around 75% PNH cells in all subsets examined and this percentage did not change significantly after G-CSF administration. The other three patients showed considerable differences in the proportion of PNH cells in different cell compartments and a dramatic change following G-CSF. There was no obvious clinical distinction between patient ST and the others to explain this observation. In steady state

peripheral blood, these three patients were found to have almost no detectable PNH cells in the most primitive (CD34+ve/CD38-ve) subset (median 0%, range 0-7%). The more mature CD34+ve/CD38+ve fraction had a proportion of PNH cells intermediate between the primitive CD34+ve/CD38-ve cells and the mature neutrophils (median 47%, range 29-50). Following the administration of G-CSF, the pattern changed dramatically. Both the early and more mature stem cells became phenotypically 'more PNH' (median 87%, range 68%-90% for the CD34+ve/CD38+ve cells and median 88%, range 51%-91% for the CD34+ve/CD38-ve cells). The changes in the CD38-ve fraction were significant at $p<0.05$ but those for the CD38+ve fraction were not ($p<0.1$). These changes reverted to steady state values within a few days of discontinuing G-CSF. Only one marrow sample was available in these patients (DT) and this was taken post G-CSF. The proportions of PNH to normal cells in the patient's marrow was very similar to their peripheral blood with the majority of cells being PNH in all subsets. Unfortunately there was no available marrow pre G-CSF but other studies have suggested that the numbers of CD34+ve/CD38-ve cells in the marrow of PNH patients in steady state are similar to that of their mature neutrophils. The individual values are shown in table 7.2.

Table 7.2 Percentage of PNH Cells at Different Stages of Maturation

Patient		Neutrophils	CD34+/CD38+ cells	CD34+/CD38- cells
DL	Pre G-CSF	96	50	0
	Post G-CSF	94	87	91
DT	Pre G-CSF	97	29	7
	Post G-CSF	96	90	88
	Marrow post G-CSF	-	79	97
LW	Pre G-CSF	50	47	0
	Post G-CSF	55	68	51
ST	Pre G-CSF	79	78	71
	Post G-CSF	76	80	73

(The figures represent the percentage of peripheral blood cells in each subgroup that are of PNH phenotype as defined by lack of CD59 expression. CD34+/CD38+ cells are more mature stem cells while CD34+/CD38- cells are more primitive.)

7.7   Discussion: prospects for autografting and gene therapy

The changes in haematological parameters and cell surface phenotype in 4 PNH patients before and after G-CSF are reported. The WBC rose in all patients to a peak mean of 15.3×10^9/l. This is less than that seen in normal volunteers on G-CSF whose absolute WBC reaches mean peak levels of around 28×10^9/l. on similar regimens (Stroncek *et al* 1996). These findings reflect the underlying hypoplasia in PNH. There is wide variation in the numbers of peripheral blood CD34 cells in normal individuals but all 4 PNH patients had baseline values that were 10 to 20 fold less than those generally reported. The mean baseline CD34 count in the peripheral blood was 0.5×10^6/l. for the PNH patients compared to 8×10^6/l. in a series of normals. After G-CSF, the mean figures were 4.2×10^6/l. for the PNH patients and 55×10^6/l. for the normals. In both PNH and normal blood this represents a similar proportionate increase (7-9 fold).

The rise in NAP score in normal neutrophils on exposure to G-CSF is well documented and represents one of a range of stimulatory or priming effects of this cytokine (Avalos, 1996). It was interesting to observe this phenomenon in the PNH patients as this enzyme is known to be GPI-linked and consequently the NAP score in PNH is classically low. Since two of these patients had >95% PNH neutrophils circulating before and after G-CSF, I can be certain that the increase in NAP score occurred in both PNH and normal neutrophils as the NAP scoring by light microscopy confirmed that the majority of cells showed increased granulation. Increase in the NAP score in PNH patients post G-CSF has not been previously reported.

The relative proportions of normal and PNH cells occurring at different stages of cell maturity in steady state and G-CSF mobilised peripheral blood in these patients was studied. All four had substantial PNH clones as assessed by neutrophil phenotype which implies that the marrow precursors are also largely PNH. Previous studies of PNH marrow have supported this view and shown a similar proportion of affected CD34+ve cells to neutrophils (Terstappen et al 1993). Despite this it has been previously noted that the great majority of the most primitive peripheral blood stem cells are in fact of normal phenotype in sharp contrast to the marrow and the more mature cell types (Prince et al 1995; Musto et al 1997). My observations confirm this phenomenon in three out of the four patients. In addition I have shown a gradient of expression with the most primitive cells being normal, the more mature stem cells (CD34+ve/CD38+ve) having an intermediate proportion of PNH cells and the mature neutrophils being largely PNH. It is not clear why normal pluripotent cells should circulate selectively in the peripheral blood of these patients when they are only a small minority of the marrow precursors. Normal stem cells may be selectively released, perhaps due to a GPI-linkage deficiency involving adherence or homing, or they may have a survival advantage over PNH cells when in the peripheral blood which is not present in the marrow miroenvironment. This phenomenon is marked in the majority of patients and it is intriguing to consider that it may have some relevance to the pathogenesis of PNH.

It has been suggested that these normal stem cells could be collected from the blood and used as a source of normal progenitors for autologous transplantation in PNH (Musto et al 1997; Prince et al 1995) but the absolute numbers of peripheral CD34+ve cells in PNH patients is generally very small. A logical approach would be to mobilise stem cells from such patients with growth factors as is done in the management of other

haematological malignancies. However, my work shows that the number of stem cells mobilised by G-CSF in this condition is disappointing and the vast majority that are released are of PNH phenotype unlike those that naturally circulate. I found that after G-CSF the absolute number of CD34+ve cells in the peripheral blood of these patients was a mean of 4.2×10^6/l. (range 1.3-9.0) and in the important CD38-ve fraction only a minority (median 12%) remained of normal phenotype. Contrasting this with recommendations for harvesting in haematological malignancy where minimum post G-CSF levels of around $10-20 \times 10^6$ CD34+ve cells/l. of peripheral blood are preferred it suggests that it would be difficult to mobilise sufficient numbers of normal progenitors into the peripheral blood of PNH patients to support transplantation.

In summary, conventional mobilisation techniques do not achieve adequate stem cell collections for autografting in this disease and far from the hoped for 'in vivo purge' of PNH cells the reverse is true with a substantial increase in abnormal cells after G-CSF. Prospects for gene therapy are also poor due to the difficulties in obtaining adequate stem cells for transduction. For these approaches to be pursued, other technological advances might be required such as selection of normal stem cells in vitro, perhaps using high speed multi-parameter cell sorting and subsequent expansion of the poor stem cell numbers in order to achieve sufficient numbers for practical, clinical scale cell manipulation.

Chapter 8.

General Discussion

8.1   Haemopoietic stem cells
8.2   Stem cell mobilisation
8.3   Stem cell selection
8.4   Stem cell modification by gene therapy
8.5   PNH: a disease model for the manipulation of stem cells

8.1   Haemopoietic stem cells

For many years, it has been accepted that there is a marrow pool of primitive

cells defined by their ability both to self renew without differentiation and to

differentiate to produce cells of any of the haemopoietic lineages (Gordon &

Blackett, 1994; Leitman & Read, 1996).   Despite an extensive literature on the

subject and the introduction of therapies based on the use of these cells into routine

clinical practice, our understanding of them is still limited.   There is no one test that

can precisely define them and, perhaps for this reason, their biology and behaviour

remains controversial (Gordon & Blackett, 1994; Morrison *et al* 1995).

The manipulation of haemopoietic stem cells for clinical use is of major interest in

haematology but their elusive nature as targets for research has been a difficult

hurdle which continues to delay the development of therapeutic strategies that have

long held out promise but have yet to have a clearly defined place in patient

management. These include: the collection (mobilisation and harvesting), selection, expansion and modification of stem cells. These techniques are by no means exclusive. A complete understanding of stem cell biology might allow the controlled collection and selection of the precise cell population required for each clinical application. They could then be expanded and/or genetically modified and reinfused to achieve the desired therapeutic goal.

Cell expansion ex vivo has already been achieved using both simple liquid culture and more complex stromal-based systems (Alcorn & Holyoake, 1996). Optimal conditions are not yet fully defined but suitable combinations of growth factors (e.g. IL3, IL6, SCF and erythropoietin) with or without stromal support have been consistently shown to yield large increases in post-culture cell numbers (Bodine *et al* 1989; Ploemacher *et al* 1993; Haylock *et al* 1992a). Such products have been reinfused into patients with no observed toxicity (Silver *et al* 1993; Brugger *et al* 1995a). Despite this, it remains unclear whether the true repopulating cells are expanded by these techniques (Srour *et al* 1993; Koller *et al* 1995) and early clinical trials have reported problems with engraftment (Holyoake *et al* 1995).

Genetic modification of human blood cells can be achieved in the laboratory and in vivo (Dunbar, 1996b; Boucher *et al* 1994). Stem cells, with their capacity for renewal and differentiation have been popular targets for gene transfer protocols (Williams *et al* 1984b; Chatterjee *et al* 1995; Dunbar, 1996a) but there is still limited evidence that the currently available gene vectors can infect primitive, quiescent cells (Moore *et al* 1992; Dunbar *et al* 1995). The most studied vectors (based on retroviridae) can only integrate into host DNA at mitosis when the nuclear membrane is disrupted (Miller *et al* 1990). Other vectors are under development but

safe, stable integration over long time periods remains elusive and in vivo gene marking experiments have shown only limited expression in small proportions of cells after transplantation (Brenner, 1996).

Despite the considerable technical difficulties in cell expansion and gene therapy, progress is continuing and it may become possible in the future to combine these techniques either to expand cell populations which have been genetically modified (Fraser *et al* 1990) or to expand the most appropriate subsets of primitive cells as a starting population for genetic modification. Such holistic marrow engineering is currently beyond our technology but clinical trials have been conducted in all these individual areas with encouraging preliminary results (Brenner, 1995; Brugger *et al* 1994; Holyoake *et al* 1995). A greater understanding of the intrinsic kinetics of stem cells and their extrinsic interactions with the marrow microenvironment and soluble factors may lead to improvements in mobilisation, collection and culture expansion techniques. More precise assays for recognition of the pluripotent fraction are necessary for cell selection in systems which seek to purge grafts of contaminating tumour cells and considerable progress is needed in the field of gene therapy before effective introduction of engineered DNA into primitive cells with controlled long term expression in vivo is achieved.

8.2 Stem cell mobilisation

Despite a lack of understanding of the mechanisms involved in PBSC mobilisation, it has become a safe, effective and common clinical procedure in the last decade (Gratwohl *et al* 1996). In haematological malignancy and in solid tumours, high dose

chemo-radiotherapy is now routinely supported by reinfusion of mobilised PBSCs. The purpose of the mobilisation procedure has primarily been to harvest sufficient stem cells for safe engraftment. There are however other aspects of these cell collections that are the focus of increasing interest. Harvest composition in terms of the proportions of cells at different stages of maturity is becoming better characterised and this knowledge may impact on our ability to predict the kinetics of short and long term haemopoietic reconstitution (Rawstron *et al* 1998). Most transplants are performed for malignant disease and contamination of the harvests by clonal cells can occur and may contribute to relapse in some patients (Brenner *et al* 1994). There are a number of factors which affect the success of cell collection and the degree of tumour contamination. These include: the timing of mobilisation in relation to the treatment of the disease, the amount of pre-treatment the patient has undergone, the regimen used to mobilise cells and the timing of the harvest.

In this thesis, I have described a mobilisation regimen in CML (Johnson *et al* 1996a). This is an example of a disease-specific approach which exploits the timing of harvest to collect mainly non-clonal stem cells. This in vivo purging strategy relies on the differential mobilisation of Ph+ve/Ph-ve stem cells following myelosuppression and was evaluated in 18 patients. The Hydroxyurea/G-CSF regimen used was markedly less toxic than standard chemotherapy. 28% of the harvests were entirely Ph-ve and 56% showed a major response. This compares favourably with other more intensive regimens (Johnson & Smith, 1997). Seven of the patients have been transplanted, two showing a major cytogenetic response post autograft (Pratt *et al* 1998).

There is as yet no clear consensus regarding the optimum mode of myelosuppression or the subsequent choice of growth factor/cytokine for stem cell collection. Newer agents

are being investigated such as SCF, Flt3 ligand, IL3 and MIP-1α, all of which have been shown to have relevant activity. Combinations of these may prove more effective and less toxic but are only in early trial stages (Andrews *et al* 1994; Brasel *et al* 1995). With increasing experience, it may be possible in the future to mobilise stem cells in vivo in a more controlled manner using optimised regimens of cytotoxics and soluble factors and collect a better and more precisely characterised product for clinical use, with or without further ex vivo manipulations.

8.3   Stem cell selection

The cells harvested by traditional techniques from the marrow or the peripheral blood are of all lineages and all stages of maturation and function. The majority are of no therapeutic value in this setting. Procedures to select subsets of cells from a collection allow either the concentration of desired cell types (positive selection) or the elimination of unwanted cells from the product (negative selection). These processes are not mutually exclusive and combined positive and negative selection may provide the purest end result.

In vitro selection of stem cells most commonly relies on the presence on their surface of the CD34 antigen (Krause *et al* 1996a). In haematological malignancy and solid tumours, CD34 selection is becoming widely used to purge clonal cells from harvests (providing they themselves do not express this antigen) (Berenson *et al* 1991; Schiller *et al* 1995). Selected products may be further depleted of tumour by negative selection with antigens present on tumour cells but not on stem cells (e.g. CD19 in some B-cell malignancies) or they may be used as the starting point for further manipulations,

including cell expansion or genetic modification. Most culture expansion techniques use purified CD34+ve cells as their initial cell source with the aim of maximising the number of primitive precursors which can undergo self renewal division and lead to a true increase in repopulation capacity in the final product (Alcorn & Holyoake, 1996; Haylock *et al* 1992a). Many gene therapy protocols are aimed at the primitive stem cell in an attempt to capitalise on its abilities to self renew and repopulate a marrow. The hope is that this will allow long term survival of the new DNA after reinfusion (Apperley & Williams, 1990b; Dunbar, 1996a). The titre in which gene vectors can be produced and their infection efficiency will combine to limit the proportion of target cells that are successfully transduced in any protocol. It is thus desirable to maximise the ratio of vector particles to stem cells (increase the multiplicity of infection). CD34 selection achieves this by producing a concentrated stem cell source as a starting point for such protocols which should improve the targeting of vectors and ensure that adequate numbers of the cell type of interest are transduced (Dunbar *et al* 1995).

For these in vitro manipulations to be relevant, reinfusion of CD34 selected cells must be a clinical practicality and lead to effective short and long term haemopoietic reconstitution. I have investigated this question in patients with myeloma (chapter 4) (Johnson *et al* 1996b). PBSCHs were CD34 selected using an immunoaffinity column (Ceprate^{TM}). All but one of the patients achieved an adequate number of purified CD34s for reinfusion and the case that failed had been previously autografted and had poor marrow function. Engraftment in this cohort was similar to that seen in unselected transplants and long term follow up has shown no late haematological problems. I conclude that clinical scale CD34 selection is a safe and effective process. This allows the investigation of other additional manipulations of these cells. The question of

whether tumour purging by this means is clinically advantageous is currently being studied in many centres (Gribben *et al* 1991; Pico *et al* 1995; Williams *et al* 1996).

8.4   Stem cell modification by gene therapy

The unique ability of stem cells to both self renew and differentiate down any or all of the haemopoietic lineages makes them an ideal target for gene therapy (Gordon & Blackett, 1994; Dunbar, 1996a). Modification of the phenotype of such a cell would theoretically ensure persistence of the new gene. CD34 selection offers a pure cell source for transduction and appropriately altered cells could be expanded ex vivo to produce an adequate product for reinfusion. This deceptively simple notion draws on several separate techniques of stem cell manipulation, only some of which have reached the clinical arena. Selection technology is well advanced and I have shown it to be clinically safe and effective in the setting of myeloma (Johnson *et al* 1996b). Gene transfer into haemopoietic progenitors is achievable with high rates of efficiency and I have been able to do this in human peripheral blood and PBSCHs using PCR on CFU-GM and BFU-E colonies to assay the result. The median transduction rate I achieved in these progenitor colonies was around 36% depending on the vector used, with a range of 17-63.5% in different experiments. The median rate for both PBSCHs and peripheral blood cells was the same at 36% (ranges: PBSCHs 25-63.5%, blood 17-50%). Despite this high transduction efficiency, there are serious problems in assessing whether the true pluripotent stem cells are effectively modified by these methods and experimental evidence suggests that retroviruses (by far the most studied vector) are unable to achieve this, despite some reports to the contrary (Miller *et al* 1990). Attempts

to 'persuade' stem cells to cycle and allow infection to take place may abolish their self renewing properties and defeat the purpose. If stem cells can be modified, the conflicting theories regarding their in vivo behaviour (Gordon & Blackett, 1994; Kay, 1965; Metcalf & Moore, 1971) cast some doubts on the predictability of the survival and expansion of the desired subset. This is to say nothing of the requirement to control expression of the new DNA in vivo. With these and other problems to overcome, it is not surprising that preliminary studies of clinical gene transfer have, in the main, shown only temporary low level expression of simple genes (Brenner, 1996; Dunbar, 1996b). For the future, better vectors and a more complete understanding of the biology of the stem cell are needed if indeed stem cells are to be a clinically useful target for therapeutic gene transfer.

8.5   PNH: a disease model for the manipulation of stem cells

PNH is an acquired stem cell disorder in which normal and abnormal cell populations co-exist in the marrow and blood. The molecular abnormality underlying the classical disease phenotype (intravascular haemolysis and a thrombotic tendency) is now well understood (Miyata *et al* 1993) although some aspects of aetiology and pathophysiology remain contentious (see chapters 1 and 7). Treatment for the more severely affected patients with PNH is unsatisfactory and mainly supportive. Some patients benefit from immunosuppression or allogeneic bone marrow transplantation (De Planque *et al* 1989; Kawahara *et al* 1992) but there is a clear need for safer and more effective approaches. The disease is interesting from the point of view of stem cell research for many reasons, including: its origin as an acquired somatic mutation in a haemopoietic stem cell, the

pressures that lead to the expansion of the resulting abnormal clone, the balance between normal and PNH stem cells and the link with aplasia and the development of AML/MDS (Young, 1992). The specific molecular lesion and the success of the few syngeneic transplants that have been performed in this disease indicate the potential for gene therapy and some authors have recently speculated that autografting may have a role because of the discovery that the most primitive PBSCs in PNH peripheral blood are normal (Musto *et al* 1997; Prince *et al* 1995). I have described clinical and laboratory findings that help to put the prospects for autografting and gene therapy in context (Johnson *et al* 1998).

A novel retroviral construct of relevance to PNH was kindly provided by Russell Rother of Alexion Pharmaceuticals, Newhaven, Connecticut. This contained an engineered insert coding for a form of the complement regulatory protein, CD59 modified to bind to cells via a transmembrane domain (CD59-TM) rather than by the natural GPI-linkage. Replacement of this gene protects PNH cells from complement, and thus has the potential to ameliorate the disease phenotype (Rother *et al* 1994). I have developed a FACS-based assay for complement sensitivity and have been able to demonstrate the relative resistance to complement of CD59+ve cell lines by this method. The construct was used to successfully transduce haemopoietic progenitors from normal individuals and patients with haematological malignancy. The conditions for gene transfer were optimised using growth factors and multiple cycles of infection to give rates of transduction up to 63% (median 36%) as assessed by single progenitor colony RT-PCR for the neomycin reporter gene. The gene was transferred to CFU-GMs and BFU-Es from peripheral blood and stem cell harvests with equal efficiency.

In an attempt to collect PNH stem cells for gene transfer and to investigate the potential of harvesting normal PBSCs for autografting in this disease, four patients were mobilised with G-CSF. The overall progenitor numbers were low, as expected in this hypoplastic condition and unsuitable for the gene transfer experiments described above. I was able to confirm in 3 out of 4 cases that the most primitive (CD34+ve, CD38-ve) PBSCs in the peripheral blood were indeed of normal phenotype but went on to show that following G-CSF, mainly PNH cells were released (Johnson *et al* 1998). I conclude that the prospects for gene therapy or autografting using conventional cell collection techniques are poor in PNH. Further manipulation of the small number of cells available is theoretically possible if they could be ex vivo expanded but the growth characteristics of PNH cells in culture are known to be poor and there would be considerable technical difficulties.

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PUBLICATIONS RELATED TO THIS WORK

Papers in peer-reviewed journals:

1. Johnson, R.J., Rawstron, A., Richards, S., Morgan, G.J., Norfolk, D.R., O'Connor, S. & Hillmen, P. (1998) Circulating primitive stem cells in PNH are predominantly normal in phenotype but G-CSF treatment mobilises mainly PNH stem cells. *Blood*, **In Press.**

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1. European Blood and Marrow Transplant group meeting, Davos, 1995.
A pilot study of oral hydroxyurea and G-CSF mobilisation of Ph-ve stem cells in patients with CGL. RJ Johnson, RG Owen, GJ Morgan, DL Barnard, JA Child and GM Smith.

2. British Society of Haematology meeting, Brighton, 1995.
Oral hydroxyurea (HU) and rhG-CSF (Neupogen) mobilisation of Ph-ve stem cells in patients with CGL. RJ Johnson, RG Owen, GJ Morgan, DL Barnard, JA Child and GM Smith.

3. British Society of Haematology meeting, Birmingham, 1996.
Outcome of PBSCT in CGL using Ph-ve stem cells collected following hydroxyurea and G-CSF (Filgrastim). RJ Johnson, RG Owen, GJ Morgan, AC Rawstron, DL Barnard, NH Russell, A Haynes, JA Child and GM Smith.

4. British Society of Haematology meeting, Harrogate, 1997.
Autotransplantation with Philadelphia negative peripheral blood stem cells mobilised with hydroxyurea and G-CSF achieves prolonged engraftment in patients with chronic myeloid leukaemia. RJ Johnson, RG Owen, GJ Morgan and GM Smith.

5. American Society of Haematology meeting, Nashville, 1994.
Detection of contaminating cells in PBPC harvests and the efficacy of CD34 selection in patients with multiple myeloma. RG Owen, JA Child, AC Rawstron, GM Smith, RJ Johnson, V Woodhead, A Elsworth and GJ Morgan.

6. European Blood and Marrow Transplant group meeting, Davos, 1995.
PCR assessment of the efficacy of CD34 selection (Cellpro) in multiple myeloma and follicular lymphoma. RG Owen, RJ Johnson, GM Smith, JA Child, MC Galvin and GJ Morgan.

7. British Society of Haematology meeting, Brighton, 1995.
PCR assessment of stem cell contamination and CD34 selection in multiple myeloma and other lymphoproliferative disorders. RG Owen, PA Evans, RJ Johnson, AC Rawstron, MC Galvin, DL Barnard, GM Smith, JA Child and GJ Morgan.

8. British Society of Haematology meeting, Brighton, 1995.
Autologous PBSCT in myeloma and Follicular lymphoma using CD34 selected cells.
RJ Johnson, RG Owen, JA Child, MC Galvin, GJ Morgan and GM Smith.

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Outcome of CD34 selected PBSCT's (Cellpro^{TM}) for patients with myeloma and follicular lymphoma. RJ Johnson, RG Owen, GM Smith, JA Child, MC Galvin and GJ Morgan.

10. British Society of Haematology meeting, Birmingham, 1996.
Mobilisation of PBSC's for ex-vivo manipulation with a view to gene therapy for PNH.
RJ Johnson, P Hillmen, RG Owen, DR Norfolk, GM Smith, SJM O'Connor, AC Rawstron and GJ Morgan.

11. British Society of Haematology meeting, Harrogate, 1997.
A large proportion of the primitive stem cells in patients with PNH can be normal even if their neutrophils are almost all of PNH phenotype. RJ Johnson, AC Rawstron, RG Owen, DR Norfolk, GJ Morgan and P Hillmen.

12. British Society of Haematology meeting, Glasgow, 1998.
Thy-1+ve progenitor cell numbers correlate with sustained engraftment following autologous stem cell transplantation. AC Rawstron, G Pratt, A English, RJ Johnson, AS Jack, GJ Morgan, GM Smith.

I declare that this MD thesis is entirely my own composition.

Where work is described which was performed by others or in collaboration with others, it is properly acknowledged in the text.

Roderick Johnson,   June 1998