Murine Haematopoiesis: Studies using X Chromosome-Inactivation Mosaics.

by

Lesley M. Forrester

A thesis submitted for the degree of
Doctor of Philosophy
University of Edinburgh

1986

To

Mum and Dad

ABSTRACT

Blood erythrocytes and leukocytes were serially sampled over many months from female mice that were heterozygous at the X-chromosomal locus encoding the glycolytic enzyme phosphoglycerate kinase (PGK-1). PGK-1A and PGK-1B alloenzymes were identified and quantified electrophoretically. There was little variation in PGK-1 phenotype between serial samples from individual mice. This small amount of variation was discussed in terms of the number of clones participating in haematopoiesis and the contribution of technical factors.

Similar studies were performed using radiation chimaeras, repopulated with either a high dose (10^7 cells) or a low dose (10^5 cells) of PGK-1AB bone marrow. The variation in PGK-1 phenotype between serial samples taken from the animals repopulated with a high dose of bone marrow was comparable to that seen in normal animals. In contrast, the variation observed in the low-dose chimaeras was relatively large. These animals were used to study the clonal organisation of the haematopoietic system.

The development of B lymphocytes carrying the X-linked immunodeficiency mutation (xid) was studied in mice that were heterozygous at both the xid and the Pgk-1 loci.

An abnormallly large population of B lymphocytes, possessing an characteristic membrane phenotype, was observed in the peripheral blood of a group of experimental mice. This behaved as a transplantable neoplasia. Subsequently, similar populations were found in several aged (>2 years) CBA/Ca mice. A preliminary characterisation of these cells was carried out and their possible relevance to human chronic lymphocytic leukaemia (CLL) was discussed.

DECLARATION.

The experiments reported in this thesis were done by myself unless otherwise stated. The thesis was composed by myself and has not been submitted in any previous application for a degree.

February, 1986.

ACKNOWLEDGEMENTS

I would like to thank my two supervisors Drs. Spedding Micklem and John Ansell. Their help and their constant encouragement and support, throughout the course of this study, has been invaluable.

My thanks are also due to: Robert Gray and Andrew Sanderson for skilled FACS work; Norman Anderson and Helen Taylor for technical assistance; Helen Lauder for washing glassware; the animal house staff for looking after my mice; Anthea Springbett for compiling the Genstat computer programme and all my friends in the Zoology Department for their friendship and advice. I am very grateful to the staff at the ERCC Advisory for their help with my last-minute printing problems.

Finally, a very special thankyou to Chris, John and my parents for their support, particularly during the last few months.

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ALD	aldolase
AMP	adenosine monophosphate
ANOVA	analysis of variance
AO/EB	acridine-orange/ethidium-bromide solution
ATP	adenosine triphosphate
BCL-1	B cell tumour cell line
BFU-E	erythroid burst-forming cells
BLA	B cell antigen
BPA	burst-promoting activity
BPB	bromophenol blue
BSA	bovine serum albumin
CFC-mix	mixed haematopoietic colonies in culture
CFU-S	colony-forming units - spleen
CGD	chronic granulomatous disease
CLL	chronic lymphocytic leukaemia
CSF-1	colony-stimulating factor
DF	degrees of freedom
DMBA	dimethyl-benzanthracene
DTE	dithioerythritol
EDTA	ethylene-diamine tetra-acetic acid
EGF	epidermal growth factor
FACS	fluorescence-activated cell sorter
FITC	fluorescein-isothiocyanate
FITC-GARIg	FITC goat-anti-rat immunoglobulin
FITC-RAMIg	FITC rabbit-anti-mouse immunoglobulin
5 FU	5-fluoro-uracil
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDH	glycerol deydrogenase
G6PD	glucose-6-phosphate dehydrogenase
GPI	glucose phosphate isomerase
Gy	Gray (100rads)
Hba	haemoglobin alpha-chain locus
Hbb	haemoglobin beta-chain locus

HBSS	hanks balanced salt solution
HCGF	haematopoietic cell growth factor
HEV	high endothelial venules
HGFs	haematopooietic growth factors
HGPRT	hypoxantine-guanine-phosphoribosyl transferase
HK	hexokinase
HU	hydroxyurea
IL-3	interleukin-3
IMS	isopropyl-methan-sulphonate
L-C	leucocyte-common antigen
LPS	lipopolysaccharide
mIg	membrane immunoglobulin
MS	mean square
MTT	3-4,5-dimethylthiazoyl-2-yl-2,5-diphenyl tetrazolium bromide: thiozolyl blue
ND	not done
NAD	nicotineamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide, reduced form
Na_3-F-1,6-DP	fructose-1,6-diphosphate, trisodium salt
PBS	phosphate buffered saline
PBS-EB	PBS supplemented with EDTA and BSA
PDGF	platelet-derived growth factor
PEI-TLC	polyethylene-imine, thin-layer-chromatography sheets
PGK-1	phosphoglycerate kinase
PLL	prolymphocytic leukaemia
PSC	pluripotent stem cell
RBC	red blood cells
SS	sums of squares
TEM	triethylenemelamine
T-eth-HCL	triethanolamine hydrochloride
TI	thymus independent
TR	texas red
TRF	T cell replacing factor
Xce	X-chromosome controlling element
XID	X-linked immunodeficiency
XLA	X-linked agammaglobulinaemia

XLR	X-linked lymphocyte regulated
X^M	maternally-derived X-chromosome
X^P	paternally-derived X-chromosome

CONTENTS

CHAPTER 1. GENERAL INTRODUCTION

The Haematopoietic System

Cell-renewal systems such as haematopoiesis, spermatogenesis and epithelial growth have been used experimentally in the study of mechanisms involved in development and cell differentiation (Potten et al, 1979; Till & McCulloch, 1980). The haematopoietic system is particularly useful because the mature blood cells are easily distinguishable and accessible for analysis. The system exists as a hierarchy of pluripotent, committed and maturing cell populations continually differentiating into at least eight different cell types; erythrocytes, granulocytes, B and T lymphocytes, macrophages, platelets, mast cells and osteoclasts. These differentiated end cells have a finite lifespan and, with the exception of lymphocytes, have only limited capabilities for cell division. They must, therefore, be continually replaced by stem cells throughout the lifespan of the animal. Haematopoietic stem cells are capable of maintaining their numbers by self-renewal and, in response to appropriate stimuli, differentiating into functionally mature end cells (Lajtha et al; 1962; Lajtha, 1979). Little is known about the organisation of the stem cell pool in steady-state conditions.

The first quantitative assay for haematopoietic stem cells was the spleen colony assay (Till & McCulloch, 1961; McCulloch et al, 1964). Bone marrow cells injected into heavily irradiated or genetically anaemic recipients form macroscopic colonies in the spleen within 7-14 days. Individual spleen colonies contain in the order of 10^6 recognisable haematopoietic cells and have been shown to arise from a single cell (colony forming unit - spleen (CFU-S)) (Becker et al, 1963; Wu et al, 1967). CFU-S include multipotent cells giving rise to cells of the myeloid lineage (Till & McCulloch, 1961; Fowler et al, 1967; Kitamura et al, 1981) and the lymphoid lineage (Trentin et al, 1967; Nowell et al, 1970; Abramson et al, 1977). The self-renewal capacity of some CFU-S was demonstrated by injecting excised colonies into a second irradiated host and observing spleen colony formation in these animals (Siminovitch et al, 1963).

The spleen colony assay can be used to estimate the number of potential stem cells (CFC-S) in a given cell suspension if a seeding factor "f" is taken into account (Siminovitch et al, 1963). The "f" number has been calculated by

several workers (Siminovitch et al, 1963; Playfair & Cole, 1965; Fred & Smith, 1968; Metcalf & Moore, 1971) and an average figure of 10% has been estimated. Thus approximately 10% of the CFU-S present in the injected inoculum will form spleen colonies. Using this approach it has been calculated that "stem" cells exist at a frequency of 10^{-3} in bone marrow.

Spleen colonies were shown to differ in size (Till & McCulloch, 1961) which was the first indication that the stem cell pool might be heterogeneous. There is now substantial experimental evidence in favour of an "age structure" within the stem cell pool; those with a longer mitotic history having an increased chance of proliferation and differentiation but a reduced chance of self-renewal (Schofield, 1970; Rosendaal et al, 1976; Hellman et al, 1978). Hodgson & Bradley (1979) showed that more primitive stem cells formed "late" colonies (10-12 days after injection) whereas "early" colonies (8 days) were formed from a more mature population of stem cells. This, and other evidence (see Chapter 4 for a fuller discussion), has raised doubts about the spleen colony technique as an assay for stem cells capable of long-term maintainance of haematopoiesis.

In vitro studies

Various culture methods have been developed for the growth of haematopoietic stem cells and committed progenitors. The regulatory processes involved in stem cell differentiation can, therefore, be studied more directly (Lord, 1983). The first such culture method was a semi-solid agar system which supported the growth of granulocyte and macrophage progenitors (Pluznik & Sachs, 1965; Bradley & Metcalf, 1966). Subsequently, culture methods have been developed for erythropoiesis (Stephenson et al, 1971), megakaryopoiesis (Metcalf et al, 1975a; Nakeff & Daniels-McQueen, 1976) as well as lymphopoiesis (Whitlock & Witte, 1982). In addition, it is possible to grow mixed colonies (CFC-mix) that contain more than one haematopoietic lineage (Johnson & Metcalf, 1978; Metcalf & Johnson, 1978; Fauser & Messner, 1979). Perhaps the most important development in vitro came with the ability to grow bone marrow in long-term culture (Dexter & Lajtha, 1974; Dexter et al, 1977). This system is capable of supporting growth of bone marrow cells, including stem cells, over a period of several months. The method involves the establishment of an

adherent layer which is made up of many different cell types (fibroblast-like cells, fat cells, endothelial cells and macrophages) (Reinke et al, 1981; Allen & Dexter, 1983) reflecting the structure of bone marrow stroma in vivo (Allen, 1978). The stromal layer can be seeded with stem cells by the addition of bone marrow; haematopoietic activity can then be maintained over long periods. Such cultures have been used in many haematopoietic investigations including the nature of some genetic defects and the effects of various factors on differentiation.

Stimulators and inhibitors which act at the stem cell level have been isolated from bone marrow (Lord et al, 1976; Lord et al, 1977) and from long term bone marrow cultures (Tokzoz et al, 1980; Cronkite et al, 1983) and their effects on stem cell proliferation have been assessed (Burgess & Nicola, 1983; Cronkite et al, 1983; Dexter et al, 1983; Wright et al, 1985). The proliferation and differentiation of haematopoietic precursors in vitro have been shown to be regulated by multi-lineage and lineage-specific haematopoietic growth factors (HGFs) (Stanley & Jubinsky, 1984). Lineage-specific HGFs include erythropoietin (Miyake et al, 1977) and colony stimulating-factor (CSF-1) (Guilbert & Stanley, 1980) and are restricted to the erythroid and macrophage lineages respectively. Multi-lineage HGFs include haematopoietic-cell growth factor (HCGF) (Bazill et al, 1983), interleukin-3 (IL-3) (Ihle et al, 1982) and a factor with erythroid burst-promoting activity (BPA) (Iscove et al, 1982). It is thought that lineage-specific HGFs regulate developmentally late cells while multi-lineage HGFs regulate the developmentally early precursors (Iscove et al, 1982; Stanley & Jubinsky, 1984). Several studies indicate that the pleiotropic response to HGFs is similar to the effects caused by other growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) on their respective target cells (Stanley & Jubinsky, 1984). Relationships between growth factors, growth factor receptors and proto-oncogene products have been described (Martinet et al, 1986; Bargmann et al, 1986). For example, the structural gene for the CSF-1 receptor has been shown to be related to the c-fms oncogene (Sherr et al, 1985). These and other observations support the theory that similar mechanisms are involved in the normal differentiation and proliferation of cells and in the uncontrolled proliferation that leads to malignancy (Hunter, 1984).

While the in vitro colony-forming techniques have been useful, it is actually the progeny that are being observed rather than the stem cells themselves. Despite their unique biological properties, attempts to obtain pure stem cell populations have proved difficult since they share many physical properties with other haematopoietic cells. However, fractions of bone marrow enriched in their CFU-S content have been separated by velocity sedimentation (Worton et al, 1969) and density gradient centrifugation (Turner et al, 1967; van Bekkum et al, 1971; Nijhof & Wierenga, 1983).

Cell surface markers on stem cells have also helped in their isolation. Nicola et al (1981) used fluorescein-conjugated pokeweed mitogen (PWM), rhodamine-conjugated anti-neutrophil sera and three-parameter cell sorting to obtain cell populations enriched for CFU-S. Two subsets of CFU-S were identified which differed in their time-course of spleen colony formation, the size and progenitor content of the spleen colonies they generated and in their capacity for generating CFU-S and CFC-mix in suspension culture (Nicola & Johnson, 1982). Harris et al (1984) found a difference in Qa-m2 antigen expression between subpopulations of CFU-S forming early and late spleen colonies. They used this property to purify multipotential haematopoietic stem cells from lymphocyte-depleted bone marrow. More recently, Spooncer et al (1985) sorted populations of CFU-S approaching 100% purity from wheat germ agglutinin labelled bone marrow cells using a modification of the electronic sorting technique described by Visser et al (1984). However, the ability of purified stem cell populations to establish long-term repopulation in direct competition with normal marrow has not been demonstrated.

Cell Markers

The validity of in vitro studies must ultimately be assessed in vivo. This generally requires the use of cell markers to define particular cell populations. Many different cell markers exist and the choice of marker depends on the questions being asked, the cells under study and the degree of precision required. However they should ideally meet the criteria discussed by McLaren (1975), Oster-Granite & Gearhart (1981), West (1984) and Ansell & Micklem (1986). The marker should be: (1) cell localised, not secreted extracellularly; (2) cell autonomous, not transferred between cells or affecting other cells; (3)

stable for the lifetime of the cell and its daughters; (4) ubiquitous - in all cells under study and at all stages of development; (5) easy to detect and, where applicable, to measure with some precision; and (6) developmentally neutral - not affecting a cell's behaviour or conferring any physiological advantage or disadvantage.

Various markers have been used in the study of haematopoiesis. Over 25 years ago, Barnes et al (1959) identified unique chromosome abnormalities in haematopoietic tissue of mice surviving large doses of ionizing radiation. These chromosome markers, generated at random, provide unequivocal markers for every metaphase cell descended from the cell in which the marker was induced. Studies using such radiation-induced chromosome markers provided evidence for the clonal nature of the spleen colony (Becker et al, 1963; Wu et al, 1967) and the pluripotency of the CFU-S (Barnes et al, 1959; Wu et al, 1967, 1968; Nowell et al, 1970; Abramson et al, 1977). In addition Abramson et al (1977) found evidence for a myeloid-lineage restricted and a T lymphocyte-lineage restricted stem cell using these markers.

The T6 chromosome marker (T14:15/6Ca) was originally derived from a reciprocal translocation between chromosomes 14 and 15 in an irradiated male mouse (Carter et al, 1956). This marker has been used to investigate haematopoiesis in whole-body or part-body-irradiated mice repopulated with bone marrow and in parabiosed animals (Ford et al, 1956; Harris et al, 1964; Micklem et al, 1966; Ford et al, 1966; Micklem et al, 1975a,b; Wallis et al, 1975; Micklem & Ross, 1978). Brecher et al (1981, 1982) used the presence or absence of the Y chromosome after injection of male bone marrow into female recipients and vice versa. Although chromosome markers have been useful, their restriction to dividing cells limits their application. The fraction of cells available for study may not be representative of the whole tissue.

There are a number of markers that are restricted to particular cell types and, although this property can be a limiting factor, it is often useful when analysing specific cell types within a heterogeneous population. The beige mouse mutation (bg^J/bg^J) has the effect of clumping and/or enlarging cytoplasmic granules in mast cells, granulocytes and osteoclasts (Oliver & Essner, 1975; Ash et al, 1980). The beige marker has been used to study the differentiation and

developmental origin of these cell types (Murphy et al, 1973; Kitamura et al, 1977,1981; Ash et al, 1980). Monoclonal antibodies or antisera to cell membrane alloantigens have also been used to follow particular cell populations. For example, the alloantigens coded for by alleles of the Thy-1 locus (Thy-1.1 and Thy-1.2) can be used to study T lymphocytes (Ezine et al, 1984) and strain-specific variants of immunoglobulin allotypes can be used as markers for B lymphocytes (Bosma et al, 1980; Green, 1981). Haemoglobin variants arise from polymorphisms at the alpha-chain (Hba) and beta-chain (Hbb) structural loci (Russell & McFarland, 1974) and can be separated electrophoretically (Whitney, 1978). The many variants have been used as markers for erythrocytes in several experimental systems (Murphy et al, 1973; Russell & Bernstein, 1968).

Marked animals are generally constructed by experimental manipulation. In haematological research the most common method is to transplant bone marrow or isolated haematopoietic cell populations into normal, X-irradiated or otherwise preconditioned animals (Micklem & Loutit, 1966; van Bekkum & de Vries, 1967; Brecher et al, 1982). Tetraparental mice (or aggregation chimaeras) can be constructed by the aggregation of two or more genetically distinct, preimplantation embryos (Mintz, 1964; Bowman & McLaren, 1970). Up to sixteen embryos, as well as isolated blastomeres or parts of embryos, have been combined in this way (Mintz, 1975). Haematopoietic chimaeras can also be made by injecting foetal liver haematopoietic cells into the placental circulation of W-mutant mice at 11 days of gestation (Fleischman & Mintz, 1979; Fleischman et al, 1982). Since both recipient foetus and the donor cells are immunologically immature, allogeneic combinations can be used without complications resulting from graft-versus-host or host-versus-graft reaction. Donors cell were shown to give rise to several cell lineages (erythrocytes, granulocytes, B and T lymphocytes) using lineage-specific cell markers (Mintz et al, 1984).

Glucose phosphate isomerase (GPI) and phosphoglycerate kinase (PGK) are enzymes of the glycolytic pathway and are present in all cells. The structural locus for GPI-1 is on chromosome 7 and four alleles have been identified. Two of these (Gpi-1s^a and Gpi-1s^b) exist in common inbred strains (Green, 1981) and have been used in this study to distinguish between donor and host cells

in bone marrow transplantation studies.

A variant of phosphoglycerate kinase (PGK-1A) (Nielsen & Chapman, 1977) is particularly useful because the structural locus for PGK-1 is on the X chromosome (Kozak et al, 1974; Chapman & Shows, 1976). Early in development one of the two X chromosomes in female cells is inactivated (Lyon, 1961; 1974). Consequently, in females heterozygous at the PGK-1 locus individual cells will express either the variant (PGK-1A) or the normal (PGK-1B) form of the enzyme, not both. This results in heterozygous females being natural mosaics; they will consist of two distinguishable cell types without the need for experimental manipulation. X chromosome-inactivation mosaics are useful in cell-lineage analysis and in the estimation of founder cell numbers in normal animals (see Chapter 4). Variants of the X-linked enzyme glucose-6 phosphate dehydrogenase (G6PDH) have been found in man and used to analyse clonality of tumours and to establish founder cell numbers for different tissues (Fialkow, 1973; Fialkow, 1983).

Genetic Mutants

Animals with mutations affecting the development and/or regulation of specific blood cell lineages have been valuable tools in haematopoietic research. They have provided information on the normal development and function of these cells as well as helping in the understanding of analogous human diseases. Perhaps the best defined mouse haematological defects are W/W^v and Sl/Sl^d caused by mutations at the W (chromosome 5) and the Steel (chromosome 10) loci respectively (Russell, 1979). Despite superficial similarities, these mutations cause separate but complementary haematological defects. The chronic, moderate anaemia of W/W^v mice is cured by a transplant of normal marrow (Russell et al, 1956), which suggests that there is a defect in the stem cells. When transplanted, W/W^v stem cells fail to form spleen colonies (McCulloch et al, 1964) although they can populate and save lethally irradiated recipients (Harrison, 1972). These recipients display the haematological defects typical of the W/W^v donor. The stem cells in Sl/Sl^d mice appear to function normally (McCulloch et al, 1965), but their tissues give defective support to stem cell proliferation and differentiation (Altus et al, 1971). An intact spleen graft from a normal or W/W^v donor significantly alleviates the Sl/Sl^d anaemia although

injection of normal cells is completely ineffective (Altus et al, 1971). The properties of W/W^v and Sl/Sl^d defects in vitro mimic the in vivo results. W/W^v marrow can provide an adherent layer for Sl/Sl^d stem cells in long term bone marrow cultures, but the reverse combination fails (Dexter & Moore, 1977).

Not all genetic variations are immediately obvious in the intact animal because compensation processes can operate to assure a balanced production of blood cells. Experimental manipulations in vivo and in vitro have revealed variants at several genetic loci involved in haematopoietic regulation. An example of this is the Fv-2 locus in the mouse which affects the percentage of primitive erythroid precursors (erythroid burst-forming cells, BFU-E) synthesizing DNA (Suzuki & Axelrad, 1980). The Fv-2-mediated differences were discovered by treating animals with ^{3}H-Thymidine then assaying for surviving BFU-E in vitro. In mouse strains carrying the Fv $2^{r/r}$ allele, less than 10% of BFU-E were engaged in DNA synthesis compared to 60% in Fv-$2^{s/s}$ strains. In spite of this significant effect, no conspicuous haematological differences have been reported for strains bearing the different Fv-2 alleles (Russell & Bernstein, 1966; Suzuki & Axelrad, 1980). This indicates the extent to which compensatory processes can operate.

Some mutations affecting B lymphocyte differentiation are only detected using in vivo or in vitro experimental manipulation. Most of these have been identified by abnormal response to antigen stimulation (Amsbaugh, et al, 1972; Shinohara et al, 1978; Marsh et al, 1977). Perhaps the best characterised is the X-linked immunodeficiency mutation (xid) of CBA/N mice (Scher, 1982), which is more fully discussed in Chapter 6.

The following chapters describe experiments using alloenzyme markers to study some aspects of haematopoietic differentiation.

CHAPTER 2. MATERIALS & METHODS

This section will describe methods that are common to several chapters. Specific materials and methods will be described in the relevant chapters.

1. Mice.

The X chromosome-encoded enzyme PGK-1 exists in two electrophoretically distinct allelic forms, PGK-1B (present in all standard laboratory strains) and the variant PGK-1A (Neilson & Chapman, 1977). Mice carrying the variant on a C3H/HeHa background were originally obtained from Dr. J.D. West (Sir William Dunn School of Pathology, Oxford). Congenic CBA/Ca-*Pgk-1*^{a} mice were produced by backcrossing the *Pgk-1*^{a} allele onto the CBA/Ca background for 20 generations. This was carried out by D. Burton, J. Lennon and the author. All mice used were bred and maintained in the Zoology Department mouse house.

CBA/Ca-*Pgk-1*^{ab} females were produced by mating CBA/Ca males with CBA/Ca-*Pgk-1*^{a} females or *vice versa*. Breeding mice were maintained with one male and one female per cage (size M1, NKP mouse cages) on a diet of BP Rat and Mouse No. 3 Stock breeding diet (BP, Special Diet Services) and chlorinated tap water *ad libitum*. Offspring were weaned at 21 days *post partum* and were transferred to stock cages (size MB1, NKP mouse cages). Each cage contained up to 20 mice. Stock and experimental animals were allowed access to BP Rat and Mouse No.1 Standard Maintainance Diet (BP, Special Diet Serivices) and chlorinated tap water *ad libitum*.

The CBA/Ca-*Gpi-1s*^{a} mice used in these experiments were descendants of a breeding nucleus of mice provided by Dr. M.J. Marshall (Charles Salt Research Centre, Oswestry, U.K.). Our other stocks of CBA/Ca mice are *Gpi-1s*^{b}.

2. PGK-1 alloenzyme analysis.

(a) *Electrophoresis*. The electrophoretic technique described below was based on the work of Bucher *et al* (1980) using modifications described fully by Ansell & Micklem (1986). The two PGK alloenzymes (PGK-1A and PGK-1B) were

separated electrophoretically using Titan III cellulose acetate membranes (Helena Labs. M.I. Scientific) in the following electrophoresis buffer: 20mM sodium barbital, 10mM sodium citrate, 5mM magnesium sulphate and 2mM EDTA (pH 8.8). Cellulose acetate membranes (hereafter referred to as gels) were soaked in electrophoresis buffer for 20 minutes and excess buffer was blotted before use. Cell lysates (7.5ul) were placed in the applicator wells and a "super Z" applicator (Helena Labs.) was used to load samples at the cathode end of the gel. The gels were placed on a Helena electrophoresis tank (bridge width - 7cm.) which had 50ml. of electrophoresis buffer containing adenosine monophosphate (AMP)(0.25mg/ml) in both cathodal and anodal chambers. A 10gm weight, placed on top of the gel, ensured an even contact with the filter paper wicks (Helena Labs.). Electrophoresis was carried out at 15mA per gel for 50 mins at 4°C to prevent PGK denaturation.

(b) PGK enzyme assay. The enzyme assay for PGK is shown in Figure 2:1. The unstable substrate of PGK, 1,3 diphosphoglycerate, was prepared in situ by linking the reactions catalysed by aldolase (ALD: E.C. 4.1.2.13), glycerol-1 phosphate dehydrogenase (GDH: E.C. 1.1.1.8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH: E.C. 1.2.1.12). In the presence of fructose-1,6 diphosphate and nicotinamide adenine dinucleotide (NAD) this system catalyses the formation of 1,3-diphosphoglycerate. PGK catalyses the conversion of 1,3 diphosphoglycerate to 3-phosphoglycerate and the phosphate produced is transferred to adenosine diphosphate (ADP) producing adenosine triphosphate (ATP). ATP couples the test system to one of two indicator systems via the reaction catalysed by hexokinase (HK: E.C.2.7.1).

Tetrazolium indicator system. NADPH produced from the reaction catalysed by glucose-6-phosphate dehydrogenase (G6PDH: E.C. 1.1.1.49) is linked through phenazine methosulphate (PMS) to reduce dimethyl thiazolyl-diphenyl tetrazolium bromide (MTT). A blue colour indicates the presence of PGK in the system.

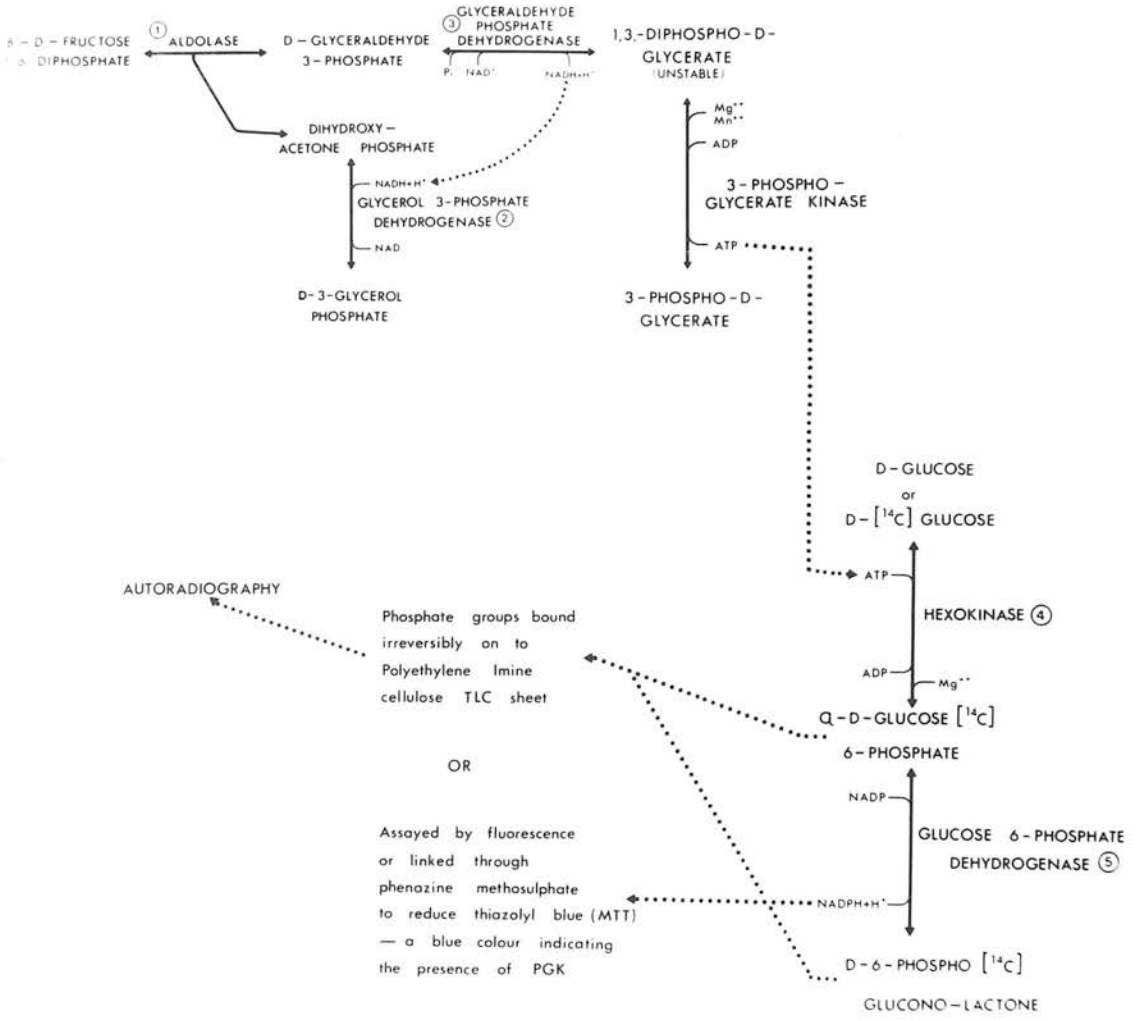
^{14}C indicator system. The addition of ^{14}C-glucose as a substrate for the reaction catalysed by hexokinase results in the incorporation of ^{14}C into the indicator system. The ^{14}C labelled products of the hexokinase and G6PDH

Figure 2:1

Diagram of biochemical pathways involved in the phospho-glycerate kinase assay.

(PGK)

PHOSPHO – GLYCERATE KINASE ASSAY



reactions (glucose-6-phosphate and 6-phosphogluconate) bind irreversibly to polyethylene imine cellulose TLC sheets via their phosphate groups. ^{14}C can then be visualised by autoradiography.

(c) PGK staining mixtures

PGK assay stock and indicator stock solutions were aliquoted (500ul and 100ul respectively) and stored at -20°C. The assay stock contained in electrophoresis buffer: 1.2mM NAD, 40mM K_2HPO_4 and 40mM trisodium fructose,1-6 diphosphate. The indicator stock contained in 0.1M triethanolamine-HCl (pH 7.6); 150mM glucose (or 15mM glucose for the ^{14}C system), 25mM ADP, 40mM NADP, and 130mM MgSO4. The enzyme mixture was prepared immediately before use and contained (for each gel) 10ul G6PDH, 10µl ALD (Sigma), 7.5µl GDH (Boehringer), 5µl HK and 5µl GAPDH (Sigma).

Tetrazolium method. The following mixture was freshly made for each analysis: 800µl electrophoresis buffer, 75µl sample buffer, 37.5ul enzyme mixture, 500µl assay stock and 100µl indicator stock (containg 150mM glucose). Immediately before staining 500ul MTT, 500µl PMS and 2.4ml of 1.2% Agar Noble (previously boiled then cooled to 56°) was added. The mixture was poured over the gel and (once the agar had set) incubated at 37° until the bands appeared. Gels were fixed in a solution of 15% glycerol and 3% acetic acid for approximately 24 hours before the alloenzyme bands were quantified.

^{14}C method. PEI TLC sheets (Linton Products, Hysol) were cut to the same size as the gel and soaked in distilled water for 20 mins at 4°C. The following staining mixture was prepared immediately before the end of the electrophoresis run: 500µl electrophoresis buffer, 30µl sample buffer, 37.5µl enzyme mix, 500µl assay stock, 100µl indicator stock (containing 15mM glucose) and 15µl ^{14}C-D-Glucose (1mCi/ml)(New England Nuclear). This mixture was spread evenly over a pre-soaked and blotted PEI sheet then the electrophoresed gel was carefully placed on top. Gentle pressure was applied to this "sandwich" to ensure maximum contact and to avoid air bubbles. After incubation for 15 mins at 37°C the gel was discarded and the PEI sheet rinsed and washed for 4 hours in 0.008M Trizma Base (Sigma) then dried at room temperature. PEI sheets were exposed for 3 days to Kodak X-Omat S film.

Films were developed for 2.5 minutes in Kodak LX 24 X Ray developer (1:5 dilution), washed in 2% acetic acid, fixed for 2.5 minutes in Kodak FX 40 X Ray liquid fixer (1:5 dilution) then washed for 20 mins in tap water. The ^{14}C method is approximately 10X more sensitive than the MTT/PMS system.

(d) Quantification of PGK-1 alloenzymes.

Tetrazolium-stained gels or autoradiographs were scanned using a Joyce Loebl densitometer (Chromoscan 3) using reflected or transmitted light respectively. The chromoscan was linked to a Sirius microcomputer which was programmed to analyse the profiles produced. The programme allowed quantification of the PGK-1 alloenzymes, giving percentages of each present, and the data were stored on a floppy disc (Minidisks, 1S/4D; Verbatim, Ireland). An example of a stained gel and the scan profiles of two tracks are shown in Figure 2:2.

Linearity of the PGK-1 system. Extensive linearity experiments have been performed in the laboratory by Dr. J.D. Ansell and Mrs. H. Taylor. Artificial mixtures of PGK-1A- and PGK-1B- expressing thymocytes were prepared and analysed using both the MTT/PMS and ^{14}C staining systems. Their results are shown in Figure 2:3 by kind permission. The figure shows that an approximately linear relationship exists between the volumetric and electrophoretic measurement of %PGK-1A over the range 20-80%. Minor components tended to be overestimated outside this range.

3. Electrophoresis of glucose phosphate isomerase (GPI).

GPI-1 alloenzymes were separated using the gels and electrophoresis tanks desribed for PGK. Samples were loaded at the cathodal end of the gels and electrophoresis was performed for 80 mins. at 350 volts (constant voltage) in Supreheme buffer (Helena Labs.), at pH 8.2-8.6. Gels were then stained with 1000µl 1.0M Tris-HCl (pH 8.0) containing 0.3mg NADP and 6.5mg fructose-6 phosphate (Sigma); 15µl G6PDH (300u/ml); 500µl MTT (10mg/ml); 500µl PMS (2.5 mg/ml); 400µl 500mM Tris-HCl, pH 8.0 and 2.4ml of 1.2% agarose (Ansell & Micklem, 1986).

Figure 2:2

An example autoradiograph of a PGK gel stained using the ^{14}C system. Scans of tracks 3 and 5 are shown.

track 3 = 46% PGK-1A

track 5 = 82% PGK-1A

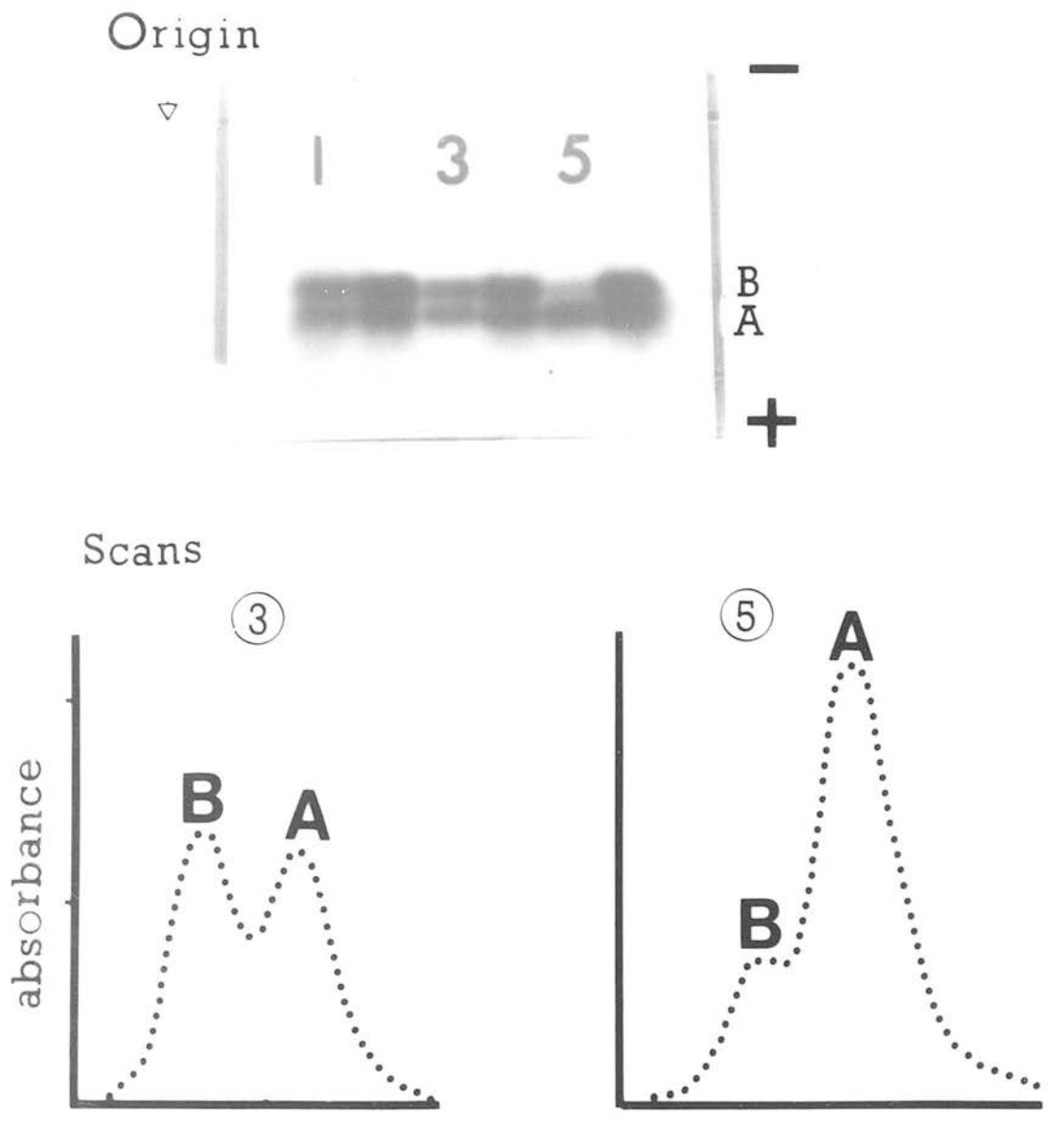
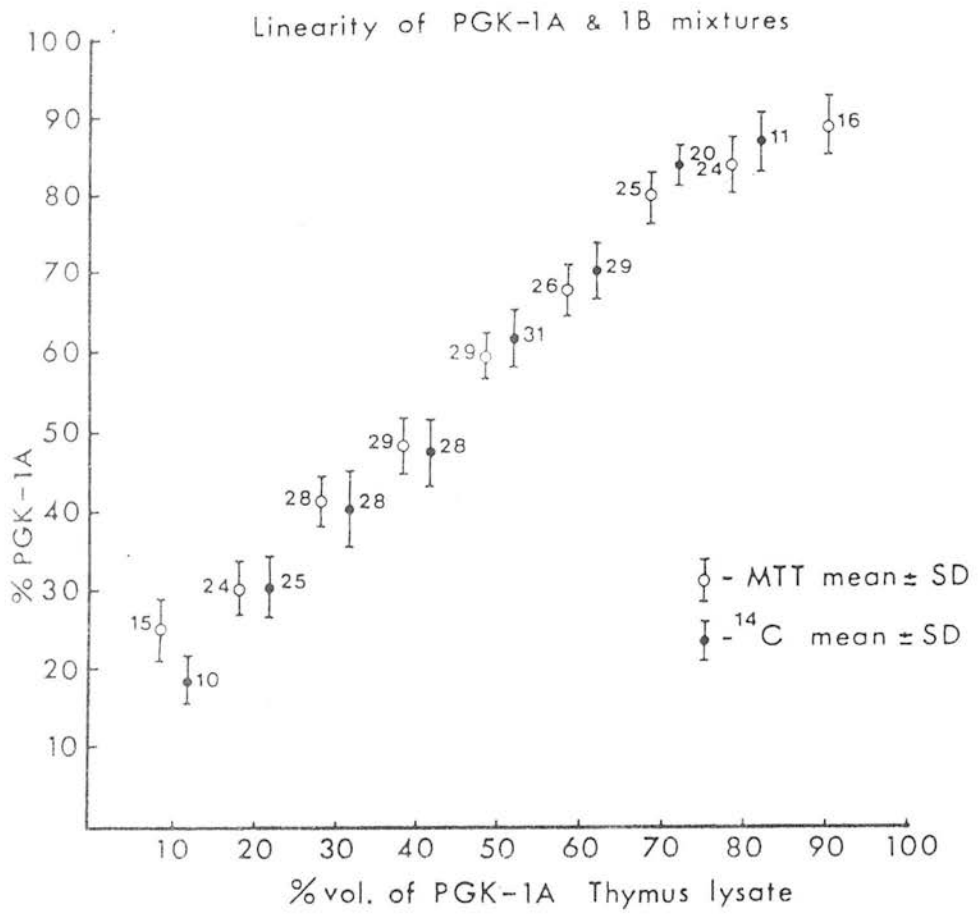

Origin
1
3
5
B
A
−
+
Scans
③
⑤
absorbance
B
A
B
A

Figure 2:3



Figures adjacent to error bars are the number of samples analysed per point.

4. Fluorescence-activated cell sorter.

This section describes the principles of the fluorescence-activated cell sorter (FACS) which has been used in this study to analyse and separate heterogeneous cell suspensions (Herzenberg & Herzenberg, 1978).

Suspended cells are injected into the centre of a fluid stream then ejected from the nozzle at a speed of approximately 10m/sec. A short distance below the nozzle, cells are illuminated by a laser beam. Light pulses emitted are directed through focusing lenses and appropriate filters to photodetectors. Optical signals are translated into electrical pulses which are amplified, processed and stored for display and analysis. A measure of cell size, an indication of cell structure and the degree of fluorescent labelling can be determined.

(a) Cell size and structural complexity. Laser light is highly collimated (waves are essentially parallel) which permits measurement of light scattered at small angles from the incident beam. This "forward scattered light" ($1-13^{o}$) is a measure of cell size. There is a strong correlation between cell size as measured in this way by the FACS and by other methods (Herzenberg & Herzenberg, 1978). Light scattered at right angles to the laser beam can be used to derive information about cell structure.

(b) Fluorescence. Cells can be stained, directly or indirectly, with fluorochromes which are excited by light of specified wavelength and emit light of a different wavelength. For example fluorescein-isothiocyanate (FITC) is excited by a variety of wavelength with a peak of 488nm and emits a spectrum with a peak of 530nm.

(c) Cell sorting. Cell populations required for isolation are identified by FACS analyis and criteria for sorting are set. Two populations can be sorted at any one time. The nozzle is vibrated (35-45 kHz.) causing the stream to break into droplets a short distance below the detection point. Each droplet is approximately 1000X the volume of a single cell and about 1/6 of the droplets will contain a cell. Just before the droplet forms, the optical signals generated by a cell are compared to the preset criteria for sorting. If a desired cell is present in a droplet, a charge generator is switched on, marking the droplet

with a trapped +ve or -ve charge. The droplets then pass through a constant electrical field created by a pair of charged deflecting plates and charged droplets are deflected to the left or right. Uncharged droplets are not deflected and continue directly downwards to a discard vessel. Before each cell-sorting session, fluorescein-labelled beads were sorted then reanalysed to check the purity of the sorted sample. After each session, at least two sorted cell samples were reanalysed. Samples that had >10% of cells outwith the defined gates were not used in further analyses.

5. May-Grunwald/Giemsa Staining.

Cells were smeared onto a clean glass slide, air-dried, fixed in 95% methanol for 2 mins. then washed in running tap water for 30 mins. The following buffer was prepared: 42.5ml 0.1M citric acid was added to 57.5ml 0.2M Na_2HPO_4 adjusted to pH 5.75 and then made up to 500ml with distilled water. Slides were rinsed in buffer, stained with May-Grunwald stain (diluted 1:2 with buffer) for 3.5 mins. then rinsed again in buffer. Slides were then stained with Giemsa (diluted 1:5 with buffer) for 10 mins, rinsed and air dried (method from Hudson & Hay, 1976).

CHAPTER 3. X CHROMOSOME EXPRESSION

Introduction

X chromosome inactivation results in individual cells of female mammals having only one X chromosome active (Lyon, 1961; 1974). Thus females, heterozygous for X-linked gene products provide a situation where two distinguishable cell populations exist within an individual. These natural mosaics are particularly useful in developmental biology for determining origins and lineages and for estimating the numbers of cells founding particular tissues during differentiation and organogenesis. Such studies using artificially constructed mosaics (or chimaeras) are limited because of the problems caused by experimental manipulation and cell selection. The information about developing systems that can be obtained from the study of X chromosome inactivation mosaics will be discussed fully in Chapter 4. The present chapter will discuss the factors that influence the X chromosome inactivation process *per se* and examine some of these factors within our own experimental system.

In the mouse, X chromosome inactivation occurs at the late blastocyst stage of embryogenesis. Rastan (1982) used a cytological method to determine the time more precisely and concluded that X inactivation was complete by 5.5 days of gestation. Takagi *et al* (1982), studying late replication, found this time to be slightly later (6 days) agreeing with Monk and Harpers' (1979) data on the expression of the X-linked enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). The classical theory about the mechanism of X chromosome inactivation is that there are two processes involved (Lyon, 1983): initiation at a particular inactivation centre then a process of spreading along the chromosome to bring about inactivation and/or activation. The range of time estimates for X-inactivation is small (0.5 days) which suggests that any intermediate state of inactivation between the two processes is short-lived and that X-inactivation takes place relatively quickly. The inactive X chromosome is condensed and is known to begin its replication later in the S phase of the cell cycle than does the active X and the autosomes. Its genetic inactivity results in dosage compensation so that there are similar levels of X-linked gene products in male and female cells. Once X chromosome inactivation has ocurred it is generally thought to be complete and the active or inactive X

chromosome is stably inherited throughout somatic growth and differentiation (Lyon, 1983). This fact makes X-linked markers particularly useful for cell lineage analyses.

The inactivation of the maternally derived X chromosome (X^M) or the paternally derived X chromosome (X^P) is not random as was originally suggested (Lyon 1961). The process is influenced to varying degrees by genetic and parental factors.

Genetic Factors. There is a locus present on the X chromosome designated the X chromosome controlling element (Xce) which is thought to be the initiation centre for X inactivation (Cattanach, 1975). Three alleles of Xce have been described and they affect the probability that a particular X chromosome will become inactivated. Analyses of females heterozygous at the Xce locus (and also at other X linked marker loci) have shown that the alleles confer an increasing probability of X chromosome expression in the order $Xce^a < Xce^b < Xce^c$ (Johnston & Cattanach, 1981). In mice homozygous for Xce, the two X chromosomes have an equal probability of inactivation.

The effect of the different Xce alleles on the X linked marker system used in this work was analysed by looking at the phenotypes of heterozygous females. We analysed the PGK-1 phenotypes of a large series of heterozygous mice to find the probability of inactivation of a particular X chromosome when two different allelic combinations of Xce were used (Xce^a/Xce^c and Xce^b/Xce^c).

Parental Factors. Parental factors influencing the X inactivation process are most obvious in studies of the extraembryonic membranes (trophectoderm and primitive endoderm) where X^P is preferentially inactivated (Takagi & Sasaki, 1975; West et al, 1977). Embryo transfer and oocyte transplantation experiments have shown that the maternal reproductive tract does not exert a selection pressure in favour of cells expressing X^M (Frels & Chapman, 1980; Papaionnou & West, 1981). It is thought that this differential expression is due to an intrinsic difference between X^M and X^P, imprinted before the X inactivation process. Parental effects on X chromosome inactivation in somatic tisues have been studied by comparing the phenotypes of females, heterozygous for X linked gene products derived from reciprocal crosses.

Some studies indicated a paternal effect ($X^P > X^M$) (Falconer et al, 1982) while others showed no significant reciprocal cross differences (Johnston & Cattanach, 1981). Falconer et al (1982) and Cattanach and Papworth (1981) found a maternal effect ($X^M > X^P$) in selection experiments with brindled (Mo^{br}) and viable brindled (Mo^{vbr}) respectively. However, they attributed this to abnormal copper transport in the heterozygous mothers rather than a chromosomal effect.

As there were confusing reports in the literature about parental effects, we felt it necessary to define our own system in this respect. All the mice used in the cell lineage studies were $\underline{Xce}^c/\underline{Xce}^a$ heterozygotes and the probability of inactivation of maternally derived and paternally derived X chromosomes were compared for this genotype. Parental effects in $\underline{Xce}^b/\underline{Xce}^c$ mice were also examined. No parental effect was observed in the $\underline{Xce}^a/\underline{Xce}^c$ heterozygotes, but a reciprocal cross difference was found in $\underline{Xce}^b/\underline{Xce}^c$ heterozygotes. The probability of inactivation of a particular X chromosome was lower if it was maternally derived.

Materials and Methods

Mice. The mouse strains C3H/HeHa-*Pgk-1*^{a} (backcross generation 9) and CBA/Ca-*Pgk-1*^{a} (backcross generation 16) were used as the source of the X chromosome carrying the *Pgk-1*^{a} and *Xce*^{c} alleles, the C57BL/6J bg^J strain for the X chromosome carrying the *Pgk-1*^{b} and *Xce*^{b} alleles and the CBA/Ca strain for the *Pgk-1*^{b} and *Xce*^{a} alleles. The *Xce* locus is very closely linked to our marker locus (*Pgk-1*) and no recombination has been observed between them in an extensive backcrossing programme in our laboratory.

Blood samples. One drop of blood was taken from the retro-orbital sinus of 3-4 week-old female progeny and mixed with 100µl sample buffer (Appendix I).

Tissue samples. Mice were killed by cervical dislocation and the thymus, brain and femurs were removed to ice. Brains and thymi were dissociated in a ground glass homogeniser in approximately 0.5ml Hanks Balanced Salt Solution (HBSS)(Gibco) then poured through a fine stainless steel sieve. Bone marrow cavities were flushed with HBSS and the marrow plug was aspirated through a 25g needle to obtain a single cell suspension. Cells were centrifuged at 1600rpm for 10mins, the supernatant discarded and the appropriate volume of sample buffer was added (50µl for bone marrow, 50µl for thymus and 100µl for brain).

PGK-1 Analyses. Samples were frozen and thawed then diluted to the appropriated concentration. Details of the electrophoresis and quantification of the two alloenzymes are described in Chapter 2. The MTT/PMS staining system was used in this experiment and the majority of the samples were run once. Some of the blood samples from each group were also analysed using the ^{14}C staining system to ensure that the haemaglobin was not interfering with the results (data not shown).

Results

Xce^a/Xce^c heterozygotes.

A large series of reciprocal crosses between sublines of CBA/Ca mice were performed and the results are shown in Table 3:1. The sublines, CBA/Ca-$Pgk\text{-}1^bXce^a$ and CBA/Ca-$Pgk\text{-}1^aXce^c$ differed only at the region of the Pgk-1 and Xce loci. In this allelic combination, when the $Xce^cPgk\text{-}1^a$-bearing X chromosome was maternally derived the mean proportion of PGK-1A in the heterozygous progeny was 69%. This was not significantly different ($p>0.05$) from the progeny produced when the $Xce^cPgk\text{-}1^a$ X chromosome was paternally derived (72%). Thus, there was no reciprocal cross difference observed when this "strong" allelic combination of Xce was used.

Xce^b/Xce^c heterozygotes.

Table 3:2 summarizes the results of phenotypic analyses of heterozygous females derived from five different crosses. Crosses 1 and 2 were the reciprocal crosses between C3H/HeHa-$Pgk\text{-}1^a$ and C57BL/6J-bg^J strains. In cross 1 where the $Xce^cPgk\text{-}1^a$ bearing X chromosome was maternally derived, the mean proportion of PGK-1A alloenzyme present in the blood of heterozygous progeny was 61%. When that chromosome was paternally derived (cross 2) the mean proprtion of PGK-1A was 54%. The difference between these two crosses was statistically significant ($p<0.05$). The F1 males ($Xce^cPgk\text{-}1^a/Y$ produced from cross 1 were backcrossed onto the C57BL/6J-bg^J strain (cross 3). In this case the $Xce^cPgk\ 1^a$ X chromosome was paternally derived and the proportion of PGK-1A present in the heterozygous progeny was 51%. The F1 females ($Xce^cPgk\ 1^a/Xce^bPgk\text{-}1^b$) produced from cross 2 were also backcrossed onto the C57BL/6J-bg^J strain (cross 4). The proportion of PGK-1A in the heterozygotes produced from these matings, where the $Xce^cPgk\text{-}1^a$ X chromosome was maternally derived, was 58%. This was significantly different from cross 3 ($p<0.05$). The heterozygotes produced from cross 3 were further backcrossed and the proportion of PGK-1A in heterozygous progeny was 60%.

In summary, there was no significant difference between the PGK-1 phenotypes

TABLE 3:1.

Mean % PGK-1A present in erythrocyte samples from progeny derived from reciprocal crosses. The crosses were between sublines of CBA/Ca mice differing only at the region of the *Pgk-1* and *Xce* loci. There was no significant difference in the proportion of PGK-1A between the offspring of these crosses. In these crosses $Pgk\text{-}1^{a}$ segregates with Xce^{c} and $Pgk\text{-}1^{b}$ with Xce^{a}.

Xce allele of parental X chromosome		Mean % PGK-1A in progeny ± S.E.	No. progeny
X^{M}	X^{P}		
c	a	69 ± 1.5	27
a	c	72 ± 1.4	44

TABLE 3:2

Mean %PGK-1A present in erythrocyte samples from progeny derived from five different crosses. Analyses of variance showed that the progeny of crosses 1, 4 and 5 were significantly different from crosses 2 and 3 ($p<0.01$). In these crosses *Pgk-1*^{a} segregates with *Xce*^{c} and *Pgk-1*^{b} with *Xce*^{b}.

CROSS	*Xce* allele of parental X chromosome X^{M}	X^{P}	Mean % PGK-1 in progeny ± S.E.	No. progeny analysed
1	c	b	61 ± 1.5	36
2	b	c	53 ± 1.1	36
3	b	c	51 ± 1.3	56
4	c	b	58 ± 1.8	32
5	c	b	60 ± 1.7	32

of the heterozygous progeny of crosses 1,4 and 5. In all these cases the $\underline{Xce^{c}Pgk\text{-}1^{a}}$-bearing X chromosome was maternally derived and the proportion of PGK-1A present was 58-61%. Also, there was no significant difference in the heterozygous phenotypes of the progeny produced from crosses 2 and 3. The $\underline{Xce^{c}Pgk\text{-}1^{a}}$ X chromosome was paternally derived and the proportion of PGK-1A present was 50-53%. There was always a significant difference when the two crosses being compared had the $\underline{Xce^{c}}$ allele derived from different parental X chromosomes.

Results of tissue analyses

Tissues from a few progeny from crosses 1,2 and 3 were analysed for their PGK 1 phenotypes. The results are shown in table 3:3. There was a significant difference ($p<0.05$) between the bone marrow samples derived from reciprocal crosses. When the $\underline{Xce^{c}}$ X chromosome was paternally derived the mean %PGK-1A was 54% and when it was maternally derived, 63%. There was not a significant difference ($p=0.06$) in the thymi of the progeny derived from reciprocal crosses, but there was a trend for preferential expression of X^{M}. The probability of expression of the $\underline{Xce^{c}}$ X chromosome was reduced from 62% to 54% when maternally or paternally derived respectively.. A similar result was obtained for the brain samples - the result was not significant ($p=0.15$) but the "maternal trend" was seen. It is very likely that significant differences would be seen if more samples were used in the analyses.

TABLE 3:3.

Mean % PGK-1A present in bone marrow, thymus and brain samples from progeny derived from reciprocal crosses.

	Xce allele of parental X chromosome		Mean % PGK-1A in progeny ± S.E.	No. progeny analysed
	X^M	X^P		
Bone marrow	c	b	63 ± 4.1	5
	b	c	54 ± 5.2	14
Thymus	c	b	62 ± 6.0	5
	b	c	54 ± 7.9	15
Brain	c	b	60 ± 5.6	5
	b	c	54 ± 7.5	15

Discussion

The three alleles of Xce have been defined by other workers with respect to their effect on the probability of X chromosome expression ($Xce^c > Xce^b > Xce^a$) (Johnston & Cattanach,1981). As the precise probability of expression in heterozygotes varies with different genetic backgrounds, this value was calculated for different Xce allelic combinations within our own experimental system. In Xce^a/Xce^c heterozygotes the mean probability of expression of the Xce^c bearing X chromosome was approximately 70% and this probability did not vary with the parental origin of the Xce^c X chromosome. In the "weaker" allelic combination, Xce^b/Xce^c, the mean probability of expression of the Xce^c X chromosome was 56% and a parental effect was observed. When the Xce^c X chromosome was maternally derived the probability of expression was 58-61%. This probability was reduced to 51-53% when it was paternally derived.

This clear maternal effect was observed in erythrocytes which have a limited lifespan and are constantly being replenished. However they can still be regarded as having a stable phenotype as mice bled for up to two years show no change in PGK-1 ratios (Micklem et al 1983; this thesis, Chapter 4). The results obtained for bone marrow in this report support the idea that the erythrocytes are representative of haematopoietic tissues. Since all tissues are derived from the same pool of X inactivation cells (McMahon et al, 1983), analyses of any tissue should show similar reciprocal cross differences. The preliminary results for brain samples show that the same "maternal trend" was observed but this difference was not statistically significant due to the small sample number.

The parental effect observed here is different to that found by Cattanach & Perez (1970) and Falconer et al (1982) who reported a slight preference in X^P expression. This effect, however, was small and large numbers of animals were needed to demonstrate it convincingly. Johnston & Cattanach (1981) analysed the heterozygous phenotypes of embryonic and adult tissues derived from reciprocal crosses. They found no reciprocal cross difference in adult tissues but the 7.5 day embryos showed a trend towards preferential expression of X^M. The sample number however was too low for statistical analyses. Interestingly, this trend was seen in both Xce^b/Xce^c and Xce^a/Xce^c heterozygotes whereas

we only found a maternal effect in $\underline{Xce}^{b}/\underline{Xce}^{c}$ animals.

The mechanism by which parental effects could operate has been suggested (Chandra & Brown, 1975). The male and female germ line are differentially modified during gametogenesis and this imprinted difference could remain until after the differentiation of the trophectoderm and primary endoderm where X^{P} is preferentially inactivated. Maternal effects observed in adult tissues may reflect residual effects of this imprinting process.

Attempts have been made to understand the molecular mechanisms involved in the X inactivation and imprinting process. Studies have indicated that the DNA of the active and inactive X chromosome is different in its ability to transform cells (Liskay & Evans, 1980), thus implying that X inactivation involves modification of DNA. This modification could involve DNA methylation (Riggs, 1975) and data in support of this idea came from experiments in which genes on the inactive X chromosome were reactivated by treatment of cells with 5 azacytidine (Mohandas, et al, 1981). This cytidine analogue leads to hypomethylation when incorporated into DNA. However, the treatment results in the reactivation of individual genes, but not the whole X chromosome, and there is no direct evidence that the reactivated DNA sequences themselves are hypomethylated. Sanford et al (1984) found a difference in the amount of total genomic DNA methylation between sperm and oocyte, which supports the hypothesis that this is the basis for the differential marking of X^{M} and X^{P} in the imprinting process.

It is probable that different factors that influence the X inactivation process interact and that if one factor is particularly strong it could override the others. In this report, the $\underline{Xce}^{a}/\underline{Xce}^{c}$ allelic combination may be too strong for the more subtle maternal effects to influence the X inactivation process to a detectable extent. Using cytological markers, Rastan & Cattanach (1983) were able to show that the strong Xce genotype could moderate the effect of imprinting on X chromosome expression in the yolk sac. However, Bucher et al (1986) could find no evidence using biochemical markers to support this claim.

It is likely that other, as yet unidentified moderator genes influence the X inactivation process and that the expression of these genes differs between

mouse strains. This could explain the discrepancies in the results, as different workers were using mice of varied genetic backgrounds.

The mechanism by which parental and genetic factors interact is unknown but could be envisaged as West (1982) proposed. The physiological modification of X^M and/or X^P would presumably include the Xce locus and the modification of one allele could affect its interaction with the other in a heterozygous female. This would have either an additive or a complementary result. If, for example, the modification during oogenesis results in the Xce^b allele on X^M behaving in a similar way to an Xce^c allele, the heterozygous female produced (if X^P carries Xce^c) would behave as if it were homozygous at the Xce locus. In this hypothetical example X^M and X^P would have an equal probability of expression.

This chapter has discussed the factors influencing the process of X chromosome inactivation and their effects on our experimental system. In the cell lineage studies described in Chapter 4, Xce^a/Xce^c heterozygotes were used. The studies described here show that, in these mice, the probability of expression of the Xce^c X chromosome was approximately 70% and that this did not alter with its parental origin.

CHAPTER 4.

STUDIES ON THE CLONAL ORGANISATION OF HAEMATOPOIESIS IN X-INACTIVATION MOSAICS.

Introduction

Hayflick (1965) showed that serially-subcultured diploid human fibroblasts had a restricted capacity for proliferation. They were found to go through only 50 doublings before the cultures died. Reincke and her colleagues (1982) showed that similar limitations existed for murine haematopoietic cells in vitro. Such experiments have been interpreted as showing that proliferating cell populations undergo an "ageing" process. It has been suggested that either there is an inherent, genetically programmed limit to the number of divisions through which a cell and its descendents can pass or that there is an accumulation of errors in or damage to macromolecules (Orgel,1973; Holliday, 1975). Such proliferative limitations may also exist in cell-renewal processes such as haematopoiesis, spermatogenesis and epithelial growth in vivo.

The mature cells of the haematopoietic system are constantly replenished from the stem cell compartment in the adult bone marrow (Lorenz, 1951; Ford et al, 1956; Till & McCulloch, 1961; Wu et al, 1968; Abramson et al, 1977; Kitamura et al,1981). An understanding of the dynamics and limitations of the haematopoietic stem cell pool is important in the theoretical considerations of experimental haematology and in the clinical use of drugs, irradiation and bone marrow transplantation.

The spleen colony assay (Till & McCulloch, 1961) provided a means of quantifying a proportion of the stem cell pool - the colony-forming units (CFU-S) (see Chapter 1). Lord (1983) calculated that 20% of CFU-S entered cycle each day. Thus, if all CFU-S were identical each one would divide, on average, once every 5 days. In the lifetime of a mouse (approximately 1000 days) 200 divisions would be required of each CFU-S (Micklem et al, 1983). These theoretical considerations already give greater limits than those suggested by Hayflick (1965).

Experimental evidence suggests that there is no loss of efficiency of stem cells in very old mice compared to those of young mice (Lajtha & Schofield, 1971; Ogden & Micklem, 1976; Harrison & Astle, 1982; Harrison, 1983). In addition the system is capable of handling massive demands over and above that of the steady state. Ross et al (1982) exposed mice to 25 pairs of hydroxyurea (HU) injections. This drug is an inhibitor of ribonucleotide-reductase and selectively kills cells in S phase of the cell cycle (Hodgson et al, 1975). The dose used reduced bone marrow cellularity and CFU-S numbers by 70%. There was no discernible effect on the number of stem cells present 3 weeks after the final injection or on their capacity to self-renew. Comparable results were obtained with the alkylating agent triethylenemelamine (TEM) (Valeriote & Tolen, 1983) and with isopropyl-methan-sulphonate (IMS) (Schofield & Lajtha, 1973).

A possible explanation for the apparent discrepancy between in vitro and in vivo results could be a system of clonal succession, first described by Kay (1965). He suggested that, at any one time, the marrow was supplied by a relatively small number of stem cells each producing a "clone" of haematopoitic cells. When exhausted they are replaced by succeeding clones derived from stem cells of shorter lineage. The model assumes a hierarchial heterogeneity of the stem cell pool, a small subset of the most primitive stem cells (possibly pre-CFU-S) being the true self-maintaining population (Hodgson & Bradley, 1979).

There is substantial experimental evidence in favour of such an "age structure" within the stem cell pool - those with a longer mitotic history having an increased chance of proliferation and differentiation but a reduced chance of self-renewal (Schofield, 1970; Rosendaal et al, 1976; Hellman et al, 1978). The first cicumstantial evidence was the fact that individual spleen colonies were of different sizes (Till & McCulloch, 1961). Rosendaal et al (1979) showed that cells with a high capacity for self-renewal were spared during treatment with the cycle-active drugs, HU or 5-fluoro-uracil (5FU). Those stem cells were presumably non-cycling. Stem cells also differ in their capacity to "home" to either the bone marrow or spleen (Hodgson & Bradley, 1979). These authors suggest that a class of "primitive" stem cells (pre-CFU-S) accumulate in the bone marrow before giving rise to cells capable of producing spleen colonies. Such pre-CFU-S would be responsible for the production of "late" colonies that

form after several days delay (day 10-12) rather than the standard 8-day ("early") colonies (Hodgson & Bradley, 1979; Magli et al, 1982). An extension of this finding would suggest the existence of stem cells which do not form colonies at all, either because they are reluctant to divide, tend not to be transferred in the bone marrow suspension or fail to locate in the spleen (Micklem & Ross, 1978; Schofield, 1978; Harrison, 1979).

The heterogeneity of the stem cell pool is also evident in vitro. Mauch et al (1980) found that the CFU-S within the adherent layer of long-term bone marrow cultures had a higher self-renewal capacity than did non-adherent CFU S. During the initial establishment of the cultures the self-renewal capacity of the adherent CFU-S decreased as the total number of CFU-S per flask increased. After 3 weeks in culture the self-renewal capacity of the adherent CFU-S stabilised and was maintained.

Serial transplantation in vivo has suggested that the potential lifespan of bone marrow, although considerably greater than that of an individual animal is nevertheless finite (Barnes et al, 1959; Harrison, 1975; Ogden & Micklem, 1976). This observation supports the idea of a "Hayflick" limit but appears to contradict the results of in situ haematopoiesis. It has however been suggested that the limitations revealed by serial transplantation are artefactual (Harrison et al, 1978). It is possible that the most primitive and highly self-renewing stem cells are either not transplanted or fail to establish in the correct microenvironment of the host (Wolf et al, 1983; Micklem, 1983).

In summary the experiments referred to above suggest an "age-structure" within the stem cell compartment, yet the average "age" of the CFU-S population does not increase. To resolve this paradox Schofield (1978) proposed that there was a more primitive stem cell than the CFU-S, which was immortal and existed in association with a cellular environment (the "stem cell niche"). While the stem cell is "fixed" in the niche its further maturation is prevented. At division only one daughter remains in the niche while the other becomes a "free" CFU-S which matures and differentiates in an "age-structured" fashion.

The need for a specific proliferative niche for stem cells has been challenged

by Brecher et al (1982). They found that 2-13 weeks after injection of 20-200 x 10^6 bone marrow cells, donor cells accounted for up to 25% of the total marrow cells. Such donor cell populations, identified by alloenzymes, have been stable for up to 2 years after transplantation (LMF, unpublished data). Comparable results to those reported by Brecher et al have been found by Saxe et al (1984) using chromosome markers. These findings are not necessarily inconsistent with the "niche" hypothesis. The transfusion of large cell numbers may increase the probability of donor cells gaining entry into a stem cell niche. It is also possible that in steady state conditions niches are not fully occupied or that in certain circumstances more than one cell can colonise a niche.

Until recently it has not been possible to study the in vivo clonal organisation of the haematopoietic system of normal mice. Chromosomally marked aggregation and radiation chimaeras have been used to analyse lineage relationships (Ford et al, 1975; Gornish et al, 1972). In addition, radiation chimaeras have been used to estimate the numbers of clones contributing to haematopoiesis (Wallis et al, 1975; Micklem & Ross, 1978). However, as the serial transplantation data suggest (Ogden & Micklem, 1976) experimentally manipulated animals may not entirely compare with normal animals.

The discovery of the electrophoretic variant of the X-encoded enzyme, phosphoglycerate-kinase (PGK-1) (Nielsen & Chapman, 1977) has made possible the study of normal mice. X chromosome inactivation (Lyon, 1961) results in animals heterozygous at the Pgk-1 locus being natural mosaics; individual cells express either the normal form of the enzyme (PGK-1B) or the variant form (PGK-1A), not both. This system has the potential to analyse all cell types whereas the chromosomal markers are restricted to dividing cells. In addition the mature haematopoietic cells of the periphery can be studied longitudinally. Such an analysis would be expected to yield information about the clonal activity of the bone marrow progenitors. If a cell population was derived from one progenitor cell, only one PGK-1 alloenzyme would be expressed in the mature compartment. At the other extreme if many progenitor cells contribute to the final population the two alloenzymes would be present in proportions predicted by the X-inactivation process itself (see Chapter3). Intermediate situations may be interpreted using binomial statistics (Wallis et al, 1975).

An initial attempt to do this appeared to show large and frequently sudden changes in the relative amounts of the two alloenzymes in serial samples of peripheral erythrocytes (Burton et al, 1982). The results were comparable to a computer simulation that assumed there were 3 clones producing erythrocytes over a 14 day period. Such changes were consistent with the clonal succession hypothesis (Kay, 1965). An obvious progression from the studies of Burton et al was to serially sample other mature cells (eg. granulocytes and lymphocytes) from the peripheral blood of heterozygous females. A parallel PGK-1 analysis of the different cell types would be expected to yield information on the clonal relationship between these lineages.

The mature cell types have widely differing lifespans in the peripheral blood. The average lifespan of murine erythrocytes is between 40 and 50 days (Russell & Bernstein, 1968). This has been calculated using ^{14}C-glycine as a haemoglobin precursor (von Ehrenstein, 1958) and by transfusing syngeneic normal ^{59}Fe-labelled or ^{51}Cr-labelled erythrocytes into mice (Davies et al, 1955; Alpen & Cranmore, 1959; Goodman & Smith, 1961). In contrast, granulocytes are thought to have a much shorter lifespan. In humans this has been calculated to be 6-8 hours (Cronkite et al, 1959; Cartwright et al, 1964) which is slightly longer than that calculated for mice (Bryant & Kelly, 1958; Cronkite et al, 1959). Cross circulation studies using chimaeric calf twins measured the survival time for granulocytes in the blood to be 9-11 hours (Vincent et al, 1974).

The length of time lymphocytes spend in the peripheral blood is less clear cut. The majority of T lymphocytes are thought to be relatively long-lived (4 6 months) (Sprent & Basten, 1973; Ropke et al, 1975). There is, however, a wide range of estimates for B lymphocytes' lifespan ranging from only a few days (de Freitas & Coutinho, 1981; Strober, 1972) to several weeks (Sprent & Basten, 1973; Kumararatne & MacLennan, 1981). It is likely that the B lymphocyte population is heterogeneous with respect to lifespan (Ropke, et al, 1975) and it has been suggested that virgin B cells are short-lived and memory B cells are long-lived (Strober, 1972). However, it is probably not as simple as this because short-lived memory cells have also been reported (Feldbush, 1973). A further point to note is that 6-8% of small lymphocytes from the peripheral blood remained unlabelled after continuous infusion of isotope for nearly 10

months (Robinson et al, 1965). This suggests the existence of cells with a very long lifespan.

This study set out to analyse the PGK-1 phenotypes of (and, by inference, lineage relationships between) serial samples of erythrocytes, granulocytes, and lymphocytes from the peripheral blood of normal CBA/Ca-Pgk-1^{ab} mice.

Materials & Methods.

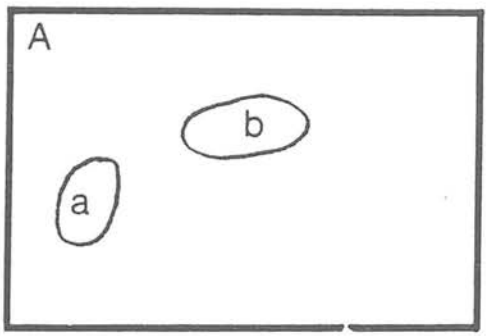
In all experiments one drop of peripheral blood, mixed with 100μl sample buffer was used as the erythrocyte sample. In the peripheral blood of a CBA mouse there are 8-10 x 10^6 rbc/mm^3 and 5-7 x 10^3 wbc/mm^3. Thus in a whole blood sample rbc outnumber wbc by a factor of more than one thousand.

1. Isolation of granulocytes.

Granulocytes were isolated from the peripheral blood using the FACS. 10-12 drops of blood were taken from the retro-orbital sinus and mixed with 8ml of phosphate-buffered saline (PBS) containing 0.2mg/ml EDTA, 0.5mg/ml BSA, 0.1% sodium azide and 25 units/ml heparin (PBS-EB). Cells were centrifuged for 10 mins at 450g and the supernatant was carefully removed with a Pasteur pipette. Red blood cells (rbc) were removed by hypotonic shock - 4.5ml distilled water was added for 12 seconds then 0.5ml 10X MEM (Eagle) medium (Gibco). Cells were centrifuged at 250g for 10mins and white blood cells were resuspended in 0.3ml PBS-EB for FACS analysis and sorting. Any remaining rbc and dead cells were excluded from further analysis using a forward light scatter gate. Figure 4:1A shows the FACS dot plot of peripheral white blood cells analysed on forward light scatter vs. right-angle light scatter. The two populations indicated (a and b) were collected in a plastic, flat-bottomed receptacle, previously coated with serum (approximately 50,000/well). After centrifugation the supernatant was removed and a hair-drier was used to evaporate the remaining liquid. The well was shaken during this evaporation process to ensure an even "lawn" of cells prior to staining with May-Grunwald and Giemsa (Chapter 2, Section 5). The bottom of the well was then mounted,

Figure 4:1

A. FACS dot plot of unstained peripheral white blood cells analysed using forward and right angle scatter. Populations "a" and "b" were sorted and stained with May-Grunwald/Giemsa. Differential cell counts (Table 4:1a) showed that Population "b" was enriched for granulocytes.

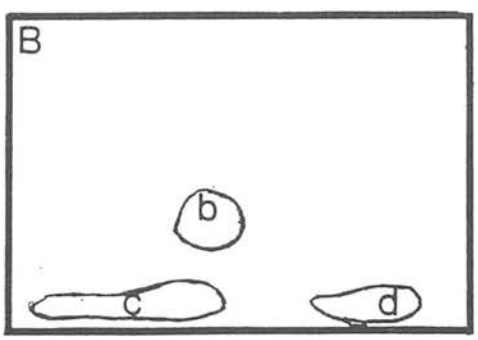


B. FACS dot-plot of FITC-RAMIg-stained peripheral white blood cells.

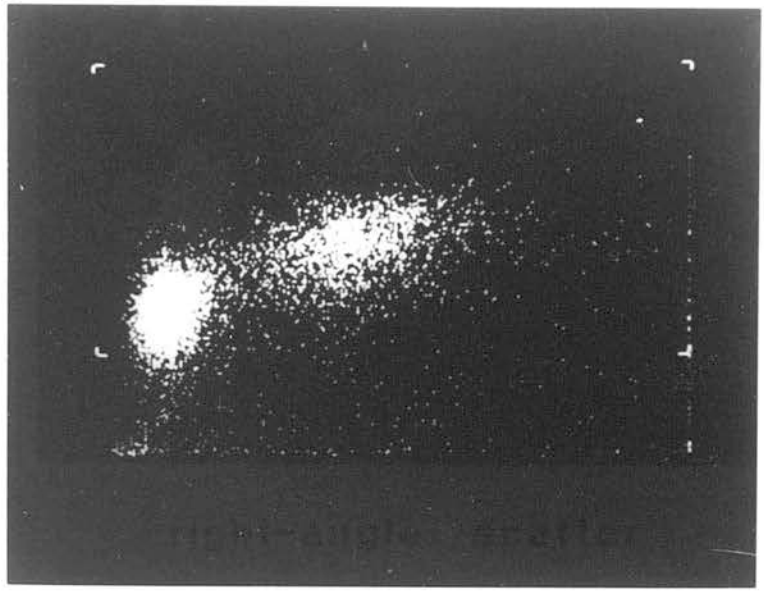
Population "b" : granulocytes

"c" : T lymphocytes

"d" : B lymphocytes



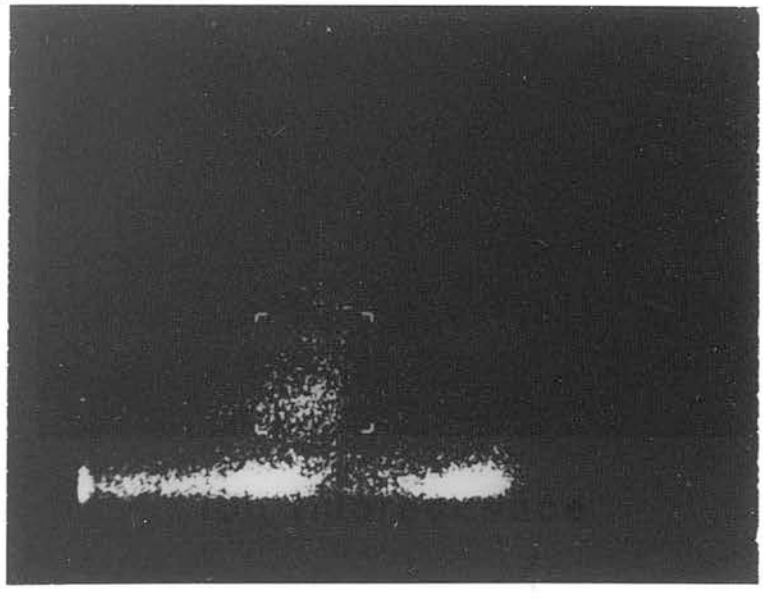
A



forward scatter

right-angle scatter

B



right-angle scatter

log fluorescence

Table 4:1(a)

Differential cell counts of sorted populations defined in Figure 4:1A. The mean percentage (±SD) of lymphocytes and granulocytes in 13 independent sorts is shown. The remaining cells were monocytes.

Population	Lymphocytes	Granulocytes
"a"	73± 9	1± 1
"b"	4± 5	89± 8

"cells up", onto a glass slide using sellotape. Differential cell counts were performed on the collected samples. Table 4:1a shows that population b was enriched for granulocytes. These cells have a higher forward light scatter (indicating that they are slightly larger) and a higher right-angled scatter (indicating that they are structurally more complex) than lymphocytes. Granulocytes usually accounted for 10-15% of the total white blood cell population.

2. Isolation of B lymphocytes.

10-12 drops of peripheral blood were collected and rbcs lysed as described above. There were approximately 10^6 viable white blood cells remaining after the osmotic lysis and they were then stained with 3µl fluorescein labelled rabbit antisera to mouse immunoglobulin heavy and light chains (FITC-RAMIg) in 1ml PBS-EB for 30 mins at 4°C. Cells were washed twice with PBS-EB and resuspended in 0.3ml for FACS analysis and sorting. A typical FACS dot plot of FITC-RAMIg-stained peripheral white blood cells is shown in Figure 4:1B. B cells were identifed by their positive staining (mIg^+) with FITC-RAMIg and granulocytes by their high right-angled light scatter. Unstained lymphocytes were thought to be predominantly T cells. On one occasion sorted mIg^- lymphocytes were reanalysed after staining with 30-H12 - a rat monoclonal antibody to mouse Thy-1.2 surface antigen followed by a FITC-labelled goat anti-rat IgG (whole molecule) (Miles-Yeda Ltd, Israel). 30-H12 reacts with the Thy-1.2 antigen on all mature T cells and thymocytes (Ledbetter & Herzenberg, 1979). The mIg^- population was found to consist of 80% Thy-1.2 positive cells.

1-2 $x10^5$ lymphocytes and 3-5 x 10^4 granulocytes were sorted, centrifuged and resuspended in sample buffer ($5x10^4$ cells/l and 10^4/l respectively). The PGK-1 analysis was performed as described in Chapter 2. The different experiments in this chapter varied with respect to the type of staining system used. Details are given in the text of the results section.

3. Enumeration of clones.

The mice used in this series of experiments were mosaics; they consisted of two distiguishable cell populations expressing either PGK-1A (A) or PGK-1B (B).

This was the case for all tissues, including the haematopoietic system. It is theoretically possible to analyse the clonal activity of the bone marrow stem cells of these mice. I will consider the erythrocyte lineage as an example. It is proposed that the stem cells involved in erythropoiesis at any one time are eventually exhausted and erythropoiesis is taken over by another set of stem cells (Kay, 1965). If the number of stem cells involved is small (eg. 1 or 2) there will be a high probability that the identity (ie A or B) of the stem cells active at different points in time will differ. Thus there will be a relatively large variance in the PGK-1 phenotype between serial samples of mature cells of the peripheral blood. On the other hand, if the number of cells involved is large (eg. 1000) the variance in the PGK-1 phenotype of serially sampled peripheral blood cells will be small. The binomial formula (Wallis et al, 1975) can be used to estimate clone numbers from the variance in PGK-1 phenotype:

ETC.

$$n = \frac{p(1-p)}{s^2}$$

where n = number of clones

p = mean proportion of PGK-1A

(1-p) = mean prportion of PGK-1B

s^2 = the variance of p

This is the simplest form of the equation and assumes that all clones are equal in size. This is probably not the case and Stone (1984) has considered the analysis of unequal clone sizes. He discusses the fact that if clones are unequal in size and if this is not considered in the analysis the number of clones will be underestimated. A correction factor could, theoretically, be used but this cannot be calculated from our data.

The variance between samples was calculated in two ways. In experiments 1 and 2 the variance (s^2) was calculated from the standard deviation (s). In experiment 3, a nested analysis of variance was performed on the data. This apportioned the variation between samples into 3 categories: mean square between mice, mean square between serial samples and mean square between replicate samples. The analysis was done using a computer programme the details of which are given in Appendix VII. An example of an ANOVA table is shown in the results section of this chapter. The variance between serial samples can be calculated from the following equation:

$$s^2 = \frac{\text{MS between serial samples} - \text{MS between replicate}}{m}$$

where : s^2 = variance between serial samples

MS = Mean square

m = number of replicates (Sokal & Rohlf, 1969)

Results

1. Experiment 1.

Erythrocytes were sampled from 6 (CBA/Ca x C3H-Pgk-1^{a}) F1 females every 2 weeks for 6 months then monthly thereafter for a total period of 26 months. The proportion of PGK-1A in each sample was analysed once using the MTT/PMS staining technique. Some (53/104) were analysed twice and in these cases the mean PGK-1A value was used in the variance calculations (see Appendix III for full results).

Erythrocytes have a lifespan of 40-50 days (Russell & Bernstein, 1966) thus only the samples that are separated by more than this period of time can be considered as "independent" samples. I have used the term "independent" to define the relationship between two erythrocyte samples that are separated by a period of time such that no erythrocyte present at sampling time n will be present at sampling time n+1. Table 4:1 shows the PGK-1 phenotypes of one series of "independent" samples (at least 56 days apart) for each of the six mice.

The variance between the samples of this "independent"series (which started at the first sample) was calculated. In addition, another 3 "independent" sets, which started at the 2nd, 3rd and 4rth samples, were calculated and used in clone number estimations (Table 4:2). The variance between samples within a series ranged from 3.7 to 24.0 and estimated clone numbers ranged from 106 to 551.

2. Experiment 2.

Erythrocytes and granulocytes were sampled from the peripheral blood of 6 CBA/Ca-Pgk-1^{ab} females at two-week intervals for 6 months, then at varying intervals over the following 18 months. Most granulocyte samples were run only once using the ^{14}C system, but when they were replicated the mean PGK-1A value was used in the variance/clone number calculation. The majority of erythrocyte samples were run once using the MTT/PMS stain and once using the ^{14}C stain, the mean value being used in further analyses. The full data are

Table 4:1

%PGK-1A present in one set of "independent" erythrocyte samples (56 days apart) from 6 (CBA/Ca x C3H-*Pgk-1*^{a}) F1 females.

Mouse / Day	B3	B5	C1	C5	D1	D5
0	84	-	70	68	73	68
65	81	78	73	81	78	67
121	82	80	65	76	79	54
180	80	75	70	72	78	63
255	78	73	64	69	77	55
324	83	73	64	72	76	58
387	79	72	63	68	77	58
446	79	70	68	68	78	58
502	82	70	63	73	83	57
636	89	76	69	71	-	62
703	83	75	64	68	80	61
789	82	-	64	66	-	60

Table 4:2

Variance/clone number calculations from 4 sets of independent erythrocyte samples from 6 (CBA/Ca x C3H-*Pgk-1*^{a}) F1 females.

Mouse	Mean (p)	Variance (s^2)	Clones (n)
B3	81.8	7.3	204
	80.4	5.5	286
	81.4	10.4	146
	80.7	11.0	142
B5	74.2	10.6	181
	73.9	10.5	184
	74.5	24.0	106
	74.0	8.4	229
C1	66.3	10.4	215
	65.9	9.6	234
	66.6	16.2	137
	66.4	6.4	349
C5	71.0	17.5	118
	70.2	12.2	171
	70.4	12.5	167
	70.8	11.0	188
D1	77.9	6.4	269
	77.0	3.7	479
	77.1	5.4	327
D5	60.2	19.9	120
	58.6	15.7	154
	58.7	4.4	551
	58.2	14.2	171

in Appendix III. Three sets of "independent" samples per mouse were used in an estimate of clone numbers involved in erythropoiesis (Table 4:3). Variances ranged from 4.7 to 44.3 and clone numbers from 50 to 438.

Granulocytes have a relatively short lifespan (less than 9 hours) and so it can be assumed that granulocyte samples taken 14 days apart are "independent". Table 4:4 shows the variance/clone number calculations for the 6 mice. Variances ranged from 10.0 to 20.2; thus clone numbers estimated to be involved in granulopoiesis ranged from 79 to 170.

3. Experiment 3.

Experiments 1 and 2 cast doubt on the results reported by Burton et al (1982). Variances calculated here are very much less and even some of this must be attributed to technical error. Some of the samples were analysed twice but this was not done routinely.

The following experiment was set up and analysed in an attempt to exclude technical variance from the clone number calculation. In addition, lymphocyte samples were analysed. Six CBA/Ca-Pgk-1^{ab} (C1-C6) were bled every two weeks for 22 weeks and the PGK-1 phenotype of erythrocytes, granulocytes and B and T lymphocytes was determined. All samples were analysed twice using the ^{14}C staining system. The mean %PGK-1A present (ie. mean of two replicates) for all samples are shown graphically in Figures 4:2(a) and 4:2(b). The complete data are given in Appendix III.

The results for each cell type were analysed using nested analyses of variance which apportioned the variance into 3 categories: variance between mice; variance between samples within mice and variance between replicates (i.e. technical variance). Table 4:5 is a typical ANOVA table. In this example 6 mice were analysed, 4 erythrocyte samples/mouse and 2 replicates/sample. The significance of the sample variations within mice was assessed by calculating the variance ratio, F. Thus, in the example given, F= 30/5 = 6. From F tables this value is significant ($F_{[18,24]}(p<0.001) = 3.7$). Abbreviated results of the ANOVA analyses are shown in Table 4:6. There are significant differences ($p<0.05$) in the PGK-1 phenotype between "independent" serial samples of all

Table 4:3

Variance/clone number calculations for 3 sets of "independent" erythrocyte samples for erythrocytes from 6 CBA/Ca-*Pgk-1*^{ab} females.

Mouse	Mean (p)	Variance (s^2)	Clones (n)
A2	69.8	9.2	229
	67.5	9.6	228
	68.8	4.9	438
A4	81.2	4.7	325
	80.8	13.2	118
	79.6	13.3	122
B7	75.1	35.8	52
	74.8	24.6	77
	74.0	21.5	89
C11	79.8	11.6	139
	76.5	13.7	131
	78.5	8.3	203
C13	81.0	15.0	103
	78.2	34.0	50
	77.8	8.9	194
C14	65.6	44.3	51
	63.5	23.0	101
	60.0	41.0	58

Table 4:4

Variance/clone number calculations for serial samples of granulocytes from 6 CBA/Ca-Pgk-1^{ab} females.

Mouse	Mean (p)	Variance (s^2)	Clones (n)
A2	70.5	12.2	170
A4	80.0	20.2	79
B7	78.5	10.0	169
C11	76.1	19.8	92
C13	78.7	10.5	160
C14	67.0	17.0	130

Table 4:5

An example of an ANOVA table

Source of variance	DF	SS	MS	F
Mouse stratum	5	2154	431	
Mouse.sample.stratum	18	556	30	6
Mouse.sample.gel stratum	24	112	5	

Grand Mean = 75.6
Total no. observations = 48

DF = degrees of freedom
SS = sums of squares
MS = mean square

Figure 4:2 (a) and (b)

% PGK-1A present in serial samples of 4 cell types isolated from the peripheral blood of 6 PGK-1AB females.

Figure 4:2(a): Mice C1, C2 and C3

4:2(b): Mice C4, C5 and C6

Colour code:

——— erythrocytes

B lymphocytes

——— T lymphocytes

——— Granulocytes

Figure 4.2(a)

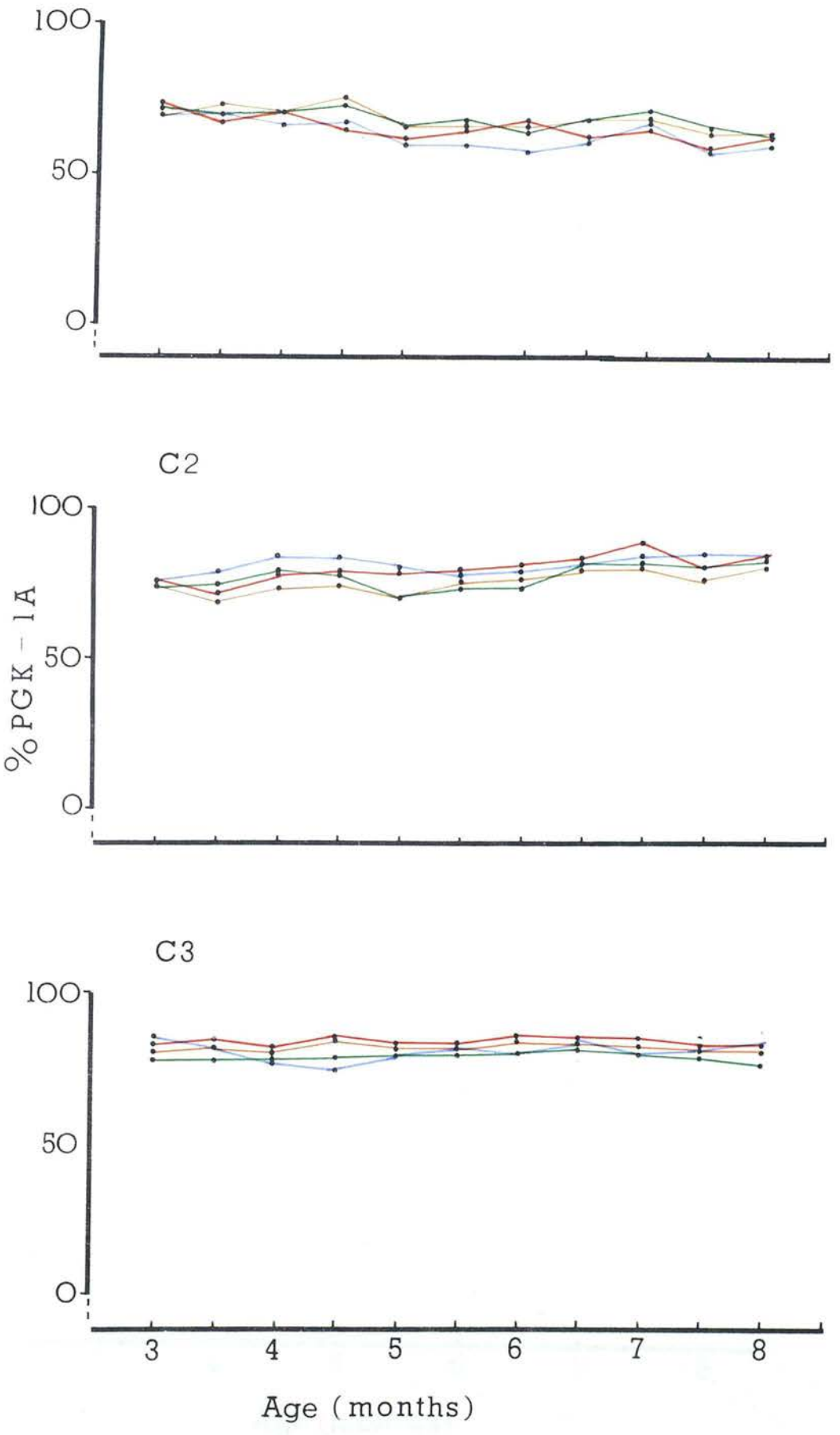


Figure 4.2(b)

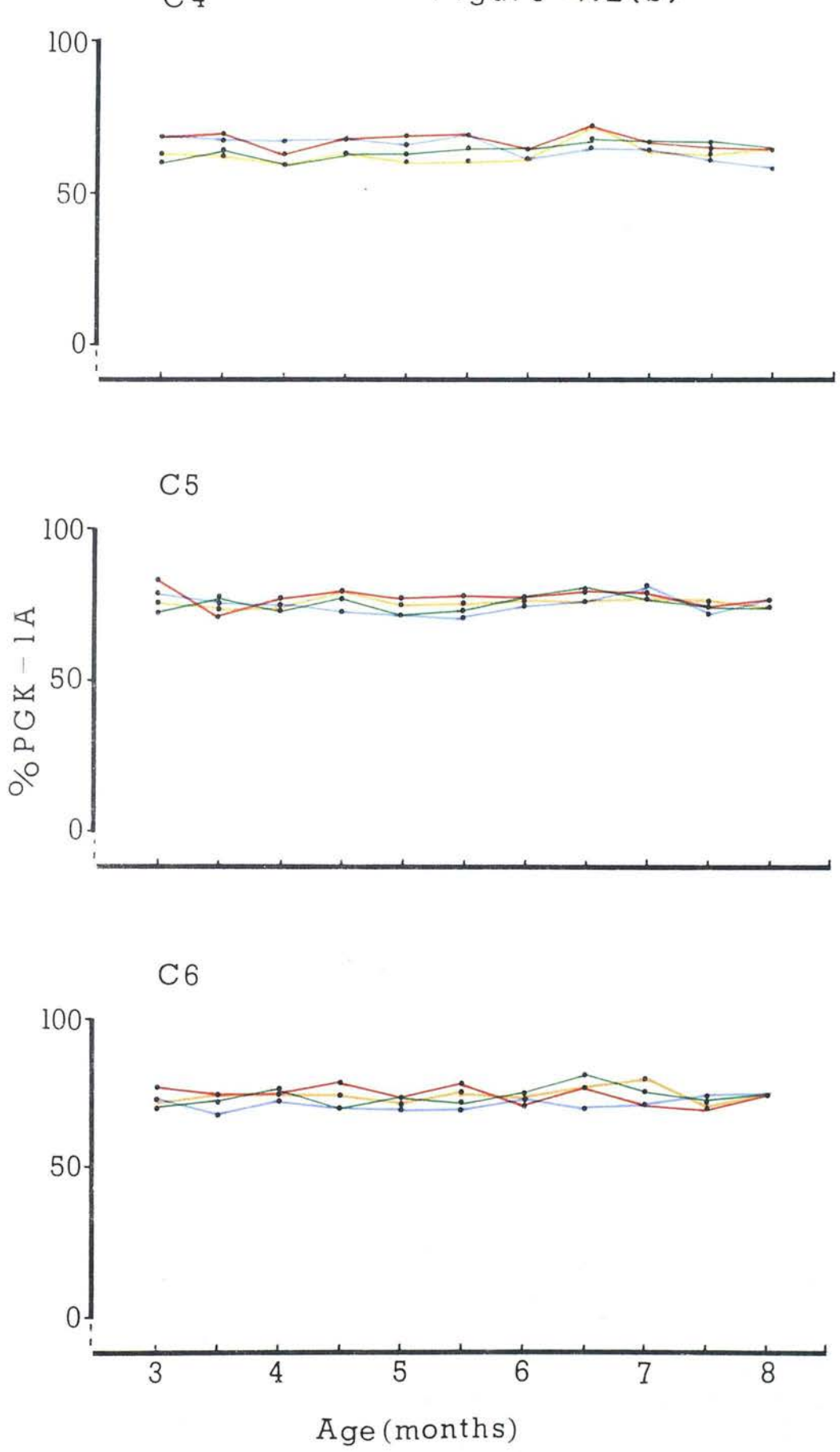


Table 4:6

Summary of ANOVA analyses of serial samples of different cell types from the peripheral blood of 6 CBA/Ca-Pgk-1^{ab} mice.
* ( ) shows the independent samples that were used in the calculation.

$F_{[18,24]}$ ($p<0.05$) = 2.0 ; ($p<0.01$) = 2.7
$F_{[48,54]}$ ($p<0.05$) = 1.7 ; ($p<0.01$) = 2.1

Cell type	MS b/w samples	MS b/w replicates	F
Eryth.(2,4,7,11)*	29.2	4.9	6.0
(1,4,7,10)	30.9	4.7	6.6
(2,5,8,11)	21.0	5.9	3.6
B lymphocytes	18.7	6.7	2.8
T lymphocytes	16.0	8.1	2.0
Granulocytes	21.5	8.0	2.7

cell types studied: erythrocytes, granulocytes, B lymphocytes and T lymphocytes.

Although some technical variance has been taken into account in this analysis, it is likely that there are additional sources that have not been included. A preliminary analysis suggested that there is a significantly ($p<0.05$) greater amount of technical error involved when replicates are run on different days than when they are run on the same day (J.D. Ansell, pers. comm.). In the experiment reported here, all replicates were run on the same day; thus some of the variance between samples could be attributed to day-day variation. In an effort to assess this source of variance, the following analysis was performed. The variance ratios (F values) were calculated for several pairs of erythrocyte samples that were 14 days apart ("adjacent"pairs). These two samples would be derived from cell populations that would presumably have one quarter to one-third of their cells in common. Biological variance between such samples would, therefore, be less than between "independent" samples (56 days apart), but technical variance should be the same. F values from "independent" and "adjacent" pairs were compared. The results are shown in Table 4:7. A Mann-Whitney U test showed that "independent" F values were significantly higher ($0.01<p<0.02$) than "adjacent" F values. Technical variance ($MS_{repl.}$) for "independent" samples was not significantly different ($p=0.5$) to that for "adjacent" samples, as expected. These results suggested that there was true biological variance between serial samples of erythrocytes.

There was no significant difference between "adjacent" and "independent" pairs for the other cell types. This could be explained by the fact that all pairs of granulocytes could be considered "independent" pairs because of their relatively short lifespan (<1 day). The long lifespan of T cells (4-6 months) would result in none of these pairs being "independent". The lifespan of B lymphocytes in the peripheral blood is not clear. As discussed in the introduction estimations have ranged from several days to several weeks.

The number of clones thought to be active in each of the cell lineages was calculated as described in the materials and methods. Clones numbers for the erythrocyte lineage ranged from 141 to 549 (Table 4:8). It should be noted that most of the "independent" erythrocyte samples in this experiment were 42 days

Table 4:7

Variance-ratios (F and F') for "adjacent" and "independent" pairs of erythrocyte samples. F (and F') values that are significant ($p<0.01$) are underlined. A Mann-Whitney U test showed that F'_{ind} values were significantly ($0.01<p<0.02$) higher than F_{adj} values.

Adjacent samples (14 days apart)					Independent samples (>70 days apart)				
Samples A	Samples B	MS samples	MS repl.	F	Samples A	Samples B	MS samples	MS repl.	F'
2	3	21.5	5.4	4.0	2	7	26.8	4.9	<u>5.5</u>
3	4	10.9	4.1	2.6	3	8	21.2	5.2	4.1
4	5	8.1	6.6	1.2	4	9	17.3	3.2	<u>5.4</u>
5	6	7.0	6.9	1.0	5	11	10.8	6.4	1.7
6	7	14.8	4.9	3.0	2	11	35.7	5.1	<u>7.0</u>
7	8	21.6	4.8	4.5	3	11	14.0	4.9	2.8
8	9	9.4	4.8	2.0	4	11	14.6	3.8	3.8
9	10	18.9	3.8	<u>5.0</u>	4	10	21.4	3.5	<u>6.1</u>
10	11	9.0	3.9	2.3	2	10	32.7	4.4	<u>7.4</u>
					3	10	19.9	4.2	4.7
					2	9	50.1	4.9	<u>10.2</u>
					3	9	15.8	4.8	3.3
					2	8	34.4	4.1	<u>8.4</u>

Table 4:8

Variance/clone number calculations for different cell lineages of 6 CBA/Ca-Pgk-1^{ab} females.

Cell Type	Mean (p)	Variance (s^2)	Clones (n)
Eryth. (2,4,7,11)	75.8	12.2	150
(1,4 7 10)	75.6	13.1	141
(2,5 8 11)	74.8	7.6	248
*(2,6,11)	74.6	8.1	234
*(3,7 11)	74.7	3.4	549
B lymphocytes	73.2	6.0	327
T lymphocytes	73.6	4.0	486
Granulocytes	73.2	6.8	288

* these sets of independent samples were 56 days apart whereas the other sets were 42 days apart.

apart - possibly a little short of the cells' lifespan. However the number of samples that were separated by the full 56 days were limited as the experiment only ran for 22 weeks. The number of estimates is too small to make a valid comparison between these two sets of figures. Clone numbers of 288, 327 and 486 were calculated for granulocytes, B lymphocytes and T lymphocytes respectively (Table 4:8).

Figures 4:2(a) and 4:2(b) show that all cell types within an individual mouse have similar %PGK-1A values. The covariances between the different cell types for 4 of the samples were calculated and mean correlation coefficients are shown in Table 4:9. High correlation coefficents were calculated between all pairs indicating that they are all derived from a common precursor pool. It is, however, likely that this is primarily representing the pool of cells present at the time of X chromosome inactivation, so no conclusions can be drawn concerning the pluripotency, or otherwise, of the stem cells in the adult.

Table 4:9

Mean correlation coefficients between the different cell types of the peripheral blood of CBA/Ca-Pgk-1[ab] mice.

	E	B	T
B	0.912		
T	0.856	0.929	
G	0.883	0.822	0.879

E - erythrocytes
B - B lymphocytes
T - T lymphocytes
G - Granulocytes

Females that are heterozygous for X-linked gene products consist of two genetically distinct cell populations. By estimating the proportions of the two cell types present in different tissues, precursor cell numbers and cell lineage relationships can be established. This form of variance/covariance analysis has been discussed in detail by Nesbitt (1971), Kane (1978) and Stone (1983). In relatively simplistic terms, the number of precursor cells is inversely related to the variance between tissues from different individuals. The covariance between tissues within an individual gives an indication of lineage relationships - a high covariance indicating a common precursor cell pool.

In a sense, all tissues within a mosaic individual are ultimately derived from a common precursor pool - the pool of cells present at the time of X inactivation. McMahon, Fosten and Monk (1983) calculated that there was a small number (approximately 47) of cells present in the mouse embryo at this time. The degree of expansion of this pool and the size of subsequent sampling events in tissue formation will affect the observed variance between and within adult tissues. The smallest sampling event will have the greatest effect and it is possible that this could be the X-inactivation process itself.

Various sampling events take place between the X-inactivation process and the formation of the adult haematopoietic system. Soon after mesoderm allocation, cells are set aside to form the yolk sac; the first site of haematopoiesis (Metcalf & Moore, 1971). It is possible that this involves a relatively small number of cells because the yolk sac appears to arise from a small area of mesodermal tissue at a time when little cell mixing could have occurred. Subsequent sampling events occur when haematopoiesis moves to the foetal liver, then to the bone marrow (Metcalf & Moore, 1971). It is likely that these events involve larger numbers of cells.

The variance in PGK-1 phenotype of haematopoietic tissue from different mice has been used to estimate a precursor cell number of 18-20 for the erythrocyte lineage (Micklem et al, 1983; Lennon, 1985), 12-15 for thymic lobes and 11 19 for individual femurs (Lennon, 1985). These numbers are in the same order of magnitude as those calculated for human haematopoietic lineages

using females heterozygous for the X-linked marker glucose-6-phosphate dehydrogenase (G6PD) (Fialkow, 1973). A precursor pool of 13 was calculated for erythrocytes, 16 for granulocytes and 19 for peripheral blood lymphocytes. It is worth noting that these numbers are less than that calculated for the number of cells present at X-inactivation.

The large variance between serial samples of erythrocytes reported by Burton et al (1982), suggested that a very small sampling event takes place during adult erythropoiesis. This could account for a proportion of the variance between individuals. Thus founder cell numbers, quoted above, would be underestimated. However, in a preliminary report (Micklem et al, 1983) we have cast doubt on this source of variance. Reanalysis of samples used in the original experiment using improved methods showed that the apparently sudden changes were attributable to technical problems. Extensive repeatability experiments (Ansell & Micklem, 1986; Lennon, 1985) stress that the technical variance within each experiment must be assessed and that rigorous quality control must be exercised.

The present study shows that there is a relatively small amount of variance between serial samples of erythrocytes, B and T lymphocytes and granulocytes. Attempts were made to exclude technical variance by routinely replicating samples. In addition the day-day variance in the technique was taken into account to some extent. However this source of variance is not necessarily constant. It is possible that there would be less technical variance between identical samples analysed two weeks apart than samples analysed 2-3 months apart. This is almost impossible to assess as the storage of such samples would provide yet another source of variance. Experimental samples were analysed within 2-3 days of collection, but obviously several months separated the analysis of the first sample and that of the last.

It remains unclear whether the small amount of variation between serial samples can be attributed to true biological variance. Clearly many clones are active in haematopoiesis in any unit time and it is, therefore, difficult to assess whether any changes in clonal activity occur. I have tried to calculate the number of clones that are active in the different cell lineages. The calculations assume that a form of clonal succession exists and this may or may not be

justified. A further assumption (probably unjustified) is that the clones are equal in size and longevity. It is possible that any variation between serial samples is the result, not of changes in clonal identity, but of changes in clone size. A combination of these two processes may exist but it must be noted that differences in clone size will lead to an underestimation of clone numbers (Stone, 1984).

Experiments 1 and 3 had different merits in the calculation of erythrocyte clone numbers. All samples were replicated in experiment 3 and thus some technical variance could be excluded. However this experiment ran for a limited period of time which meant that a maximum of four "independent" samples were available for clone number analysis. In experiment 1 at least 11 "independent" samples were used but the assesment of technical variance was less rigorous. "Independent" samples were separated by 56 days in all calculations from experiment 1 whereas in experiment 3 most of the "independent" samples were only 42 days apart. 42 days will not be completely "independent". Immediately after a sample (n) there will be a lot of erythropoiesis to replace the lost erythrocytes. Erythrocytes made immediately after sample n will, therefore, be there at sample n+1 and these will probably be products of some of the same clones analysed in sample n. This will result in an underestimation of variance and an overestimation of clone number. The mean clone number for the erythrocyte lineage (using the results of these two experiments) was calculated to be 230 (121). Experiment 2 was excluded from the calculation of the mean because only 6 "independent" samples were available per mouse and it was difficult to assess the extent to which the technical variance had been excluded.

In experiment 3 granulocyte clone numbers were calculated to be 288. This may be a more accurate number than the 133 calculated in experiment 1 as technical variance was excluded. Similar calculations have been done using human granulocytes from females heterozygous for the X-linked chronic granulomatous disease (CGD) (Buescher, Alling & Gallin, 1985). Two types of granulocytes are distinguished by their ability or lack of ability to reduce nitroblue tetrazolium dye. These authors found little variation between serial samples and, excluding technical variance, calculated the number of active clones to be 432. This value appears higher than ours. However, it is

meaningless to compare the actual clone numbers calculated from two separate experimental systems when dealing with such small variances. When variances are low additional changes (which could be due to undetectable technical problems) result in relatively large changes in clone numbers.

The clone number calculated for B and T lymphocytes was 327 and 486 respectively. The T lymphocyte value is unlikely to reflect a true clone number because of the longevity of these cells (Sprent & Basten, 1973). It is possible that cells derived from the same clones are sampled at each sample and so, in a sense, this cell lineage could be regarded as an internal control for longitudinal variance in the technique. It is unclear what the clone number for the B lymphocyte lineage represents as they are heterogeneous with respect to lifespan (Ropke et al, 1975).

Summary.

Different cell types were serially sampled from the peripheral blood of normal mice which were heterozygous at the Pgk-1 locus. The variance in PGK-1 phenotype between serial samples was small, indicating large clones numbers (200-500). It is evident from this study that too many clones are active in haematopoiesis in any unit time to clearly identify changes in clonal activity using this two-marker system. One solution to this problem would be to increase the number of markers in the system. Another approach available to study clonal activity of the haematopoietic system is to reduce the stem cell numbers artificially. This will be discussed in the following chapter.

CHAPTER 5.

STUDIES ON THE CLONAL ORGANISATION OF THE HAEMATOPOIETIC SYSTEM IN RADIATION CHIMAERAS.

Introduction

Irradiation of mice results in aplasia of the bone marrow. However, as early as 1949, Jacobson showed that shielding the spleens of mice during the irradiation process enhanced their survival rate. At that time it was unknown whether the protection was of a cellular or a humoral origin. Lorenz and his colleagues (1951;1952) later obtained survival of lethally irradiated mice by injection of bone marrow either intravenously or intraperitoneally. The protection conferred by xenogeneic (guinea pig) bone marrow suggested that a humoral factor was responsible. On the other hand, the superior protection observed with syngeneic bone marrow indicated a cellular origin. Finally, using cytological markers, it was shown that the repopulation of host bone marrow was in fact due to the proliferation of donor cells (Ford et al, 1956; Nowell et al, 1956).

The mature cells present in the bone marrow inoculum presumably act as a temporary infusion before the stem cells proliferate and differentiate to repopulate the system. The stem cells are a heterogeneous population of cells (see Chapter 4) ranging from those with a high capacity for self-renewal to those with low self-renewal capacity but a high tendency to differentiate. Also present are precursor cells restricted to particular lineages. Using radiation-induced chromosome translocations, Abramson et al (1977) obtained direct evidence for the existence of pluripotent, myeloid lineage-restricted and T lymphocyte lineage-restricted stem cells. No evidence was found for a lymphoid stem cell which had the capacity to give rise to both B and T lymphocytes although the authors point out that such negative results cannot be taken as conclusive. In children with severe combined deficiency disease the myeloid system is normal but there is an absence of both B and T lymphocytes (Cooper et al, 1973). These children can be cured by a graft of normal bone marrow cells and so it has been proposed that they have a defect in their lymphoid stem cells. It is possible that lymphoid stem cells (or B

restricted stem cells) exist in much lower frequencies than the other stem cells so they are both more rarely marked and more easily diluted out. Alternatively, they may have a greater radiation sensitivity than other stem cell classes and thus be selectively depleted by the doses of irradiation used to induce chromosome translocations.

It has become clear that haematopoiesis in the mouse can be supported by a very small number of stem cells. The first evidence for this came from studies on long term survivors of a near lethal dose of ionizing radiation (Barnes et al, 1959; Micklem & Loutit, 1966). Some of these animals carried cells with unique karyotypes in their bone marrow and in one individual virtually all the dividing haematopoietic cells belonged to a single clone. Attempts were subsequently made to estimate clone numbers in irradiated mice transfused with bone marrow cells (Wallis et al, 1975; Micklem & Ross, 1978). Using the T6 chromosome-marker system, it was possible to set up radiation chimaeras with two donor cell populations (eg. 1-T6 and 2-T6 markers) distinguishable from each other and from the host (eg. 0-T6). The binomial formula (Wallis et al, 1975) was then used to estimate the number (n) of proliferating clones present in a given tissue. Both laboratories found that n varied with the number of cells injected. For a single femur, n was calculated to be 8-15 after a cell dose of 2×10^6 and 43 after 5×10^7. The T6 marker has the disadvantage of being limited to dividing cells and so the mature, nondividing end cells are not represented in the analysis. However, comparable results in the calculation of clone numbers have been obtained by Lennon (1985) using the PGK-1 system. She found that 2 3 clones were responsible for the repopulation of a single femur after a cell dose of 10^5, 9-16 after 10^6 and 24-33 after 10^7.

In these experiments it was not clear what was being measured. Wallis et al (1975) assumed that the estimated number of proliferating clones reflected the number of stem cells contributing to bone marrow regeneration. This would be the number which initially seeded the marrow minus the number which suffered early terminal differentiation. However, as stated by Micklem & Ross (1978) it may instead, or in addition, reflect the number of clones which happen to be active at the time when the animal is killed for cytological examination. This problem could be resolved by longitudinal analysis of such individuals. A preliminary report has been published on the results of serial sampling of

erythrocytes from radiation chimaeras established with low (10^5) or high (10^7) doses of CBA/Ca-Pgk-1^{ab} bone marrow cells (Micklem et al, 1983). In the animals repopulated with a low dose of bone marrow the variance between serial samples was obviously greater than that due to technical error and, therefore, greater than the variance observed between serial samples of blood cells taken from normal mice (Chapter 4). This suggested that there were changes in clonal activity throughout the test period. However, Micklem et al (1983) were unable to rule out the possibility that the changes observed between serial erythrocyte samples reflected regeneration of host bone marrow.

In the experiments reported in this chapter the two alloenzymes of the autosomally encoded enzyme glucose phosphate isomerase (Gpi-1^a and Gpi-1^b) were used to distinguish host and donor cells in radiation chimaeras given high or low doses of bone marrow. Different cell types from the peripheral blood were serially sampled and the variance in PGK-1 phenotype between individuals and between serial samples within individuals was analysed. It was possible, therefore, to distinguish between the number of repopulating clones and the number of clones active at any point in time. In addition, the low-dose chimaeras appeared to provide a system where a small number of stem cells was involved in haematopoiesis in vivo. This is potentially useful for analysing clonal activity and for identifying lineage relationships between the different haematopoietic cell lineages. Attempts to do this using normal animals was unsuccessful (Chapter 4) due to the apparently large numbers of stem cells involved.

Materials & Methods.

1. Radiation Chimaeras.

Recipient mice were exposed to gamma radiation from a 137-Caesium source at a dose rate of 0.343 Gy./minute (±3.4%). The whole body dose was 10.5 Gy. Mice were irradiated in a ventilated round plastic container fitted with a perspex restrainer 2.5cm from the floor.

Donor bone marrow was flushed from femurs with cold phosphate-buffered saline (PBS) using a 25g needle. Cells were washed and a viability cell count was performed using AO/EB fluid (see Appendix I). Cell suspensions were then diluted to the appropriate concentration for intravenous injection into irradiated recipients.

In the low-dose experiment, 20 CBA/Ca-Gpi-1^{a} females were injected with 10^{5} viable CBA/Ca-Pgk-1^{ab} bone marrow cells in 0.4ml PBS. The femurs from four donors were pooled and an aliquot of the cell suspension was prepared for PGK-1 analysis. 100ul of the diluted cell suspension was centrifuged then 10ul sample buffer was added to the pellet. The bone marrow inoculum was found to be 62% PGK-1A.

In the high-dose experiment, 15 CBA/Ca-Gpi-1^{a} females were injected with a mixture of CBA/Ca-Pgk-1^{a} and CBA/Ca-Pgk-1^{b}. The total cell dose was 10^{7} cells in 0.4ml and the proportions of the two donors present were 2:3 respectively.

2. Preparation of Samples.

Erythrocytes, B and T lymphocytes and granulocytes were isolated as described in Chapter 4. The PGK-1 phenotype of all samples was analysed twice using the ^{14}C staining system. GPI-1 analysis was performed on some of the samples and replicates were always run.

3. Enumeration of Clones.

A nested analysis of variance was performed on the data from this chapter. This has been described in full in Chapter 4. Briefly, using data from the ANOVA table, the variance between serial samples (s^{2}_{A}) can be calculated from the following formula:

$$s^2_A = \frac{\text{MS between samples} - \text{MS between replicates}}{m}$$

where MS = mean square (from ANOVA table)

m = number of replicates

The variance (s^2_R) between different mice can be calculated from the following formula:

$$s^2_R = \frac{\text{MS between mice} - \text{MS between samples}}{mb}$$

where MS = mean square (from ANOVA table)

m = number of replicates

b = number of samples per mouse

Variances calculated in this way can then be used in the binomial equation:

$$n = \frac{p(1-p)}{s^2}$$

where n = clone number

p = mean proportion PGK-1A

1-p = mean proportion PGK-1B

The number of active clones can be calculated by using s^2_A in the above equation and the number of repopulating clones by using s^2_R.

Results

A. Low-dose Chimaeras

Erythrocytes, B and T lymphocytes and granulocytes were serially sampled from 6 radiation chimaeras repopulated with a low dose (10^5 cells) of bone marrow. The mice were named LR1, LR2, LR3, LR4, LR5 and LR7. The first sample was taken 13 weeks after irradiation and bone marrow repopulation. Samples were subsequently taken at 2 week intervals for 24 weeks then again 1 month later. Figures 5:1(a) and 5:1(b) show the %PGK-1A present (mean of the two replicates) in serial samples of the different cell lineages. The full set of data is given in Appendix IV. GPI-1 analysis was performed on all erythrocyte samples and in sorted samples that are marked by an asterisk - no host GPI-1 (i.e., GPI-1A) was detected in any of the samples tested.

The results have been divided into 3 sections: (1) PGK-1 phenotypes of the different cell lineages were compared within individuals; (2) the variance of serial samples within individuals was used to estimate the number of clones active at any one time; (3) the variance between different mice was used to estimate the number of repopulating clones.

1. Cell-lineage Comparisons. From Figures 5:1(a) and 5:1(b) it is apparent that there are consistent differences in PGK-1 phenotypes between the cell lineages of individual mice. Paired t tests (Table 5:1) showed that there was a significant difference between B lymphocytes and granulocytes in all 6 mice. Also, significant differences were seen between erythrocytes and B lymphocytes, between erythrocytes and T lymphocytes and between granulocytes and T lymphocytes in all mice, except LR1. However, when the analysis of this mouse (LR1) was restricted to samples taken between 6 and 10 months after repopulation, the differences between these lineages were significant. The reason for this is evident from the graph. The erythrocytes and granulocytes at sample 1 had a significantly lower proportion of PGK-1A than the lymphocytes, but this gradually increased over the next few samples until 6 months after repopulation when the phenotypes were reversed. Differences between cell types are therefore dampened when all samples are pooled.

Figure 5:1 (a) and (b)

% PGK-1A present in serial samples of 4 cell types isolated from the peripheral blood of 6 low-dose radiation chimaeras.

Figure 5:1 (a): Mice LR1, LR2 and LR3
(b): Mice LR4, LR6 and LR7

Colour code:
——— Erythrocytes
B lymphocytes
——— T lymphocytes
Granulocytes

Figure 5.1(a)

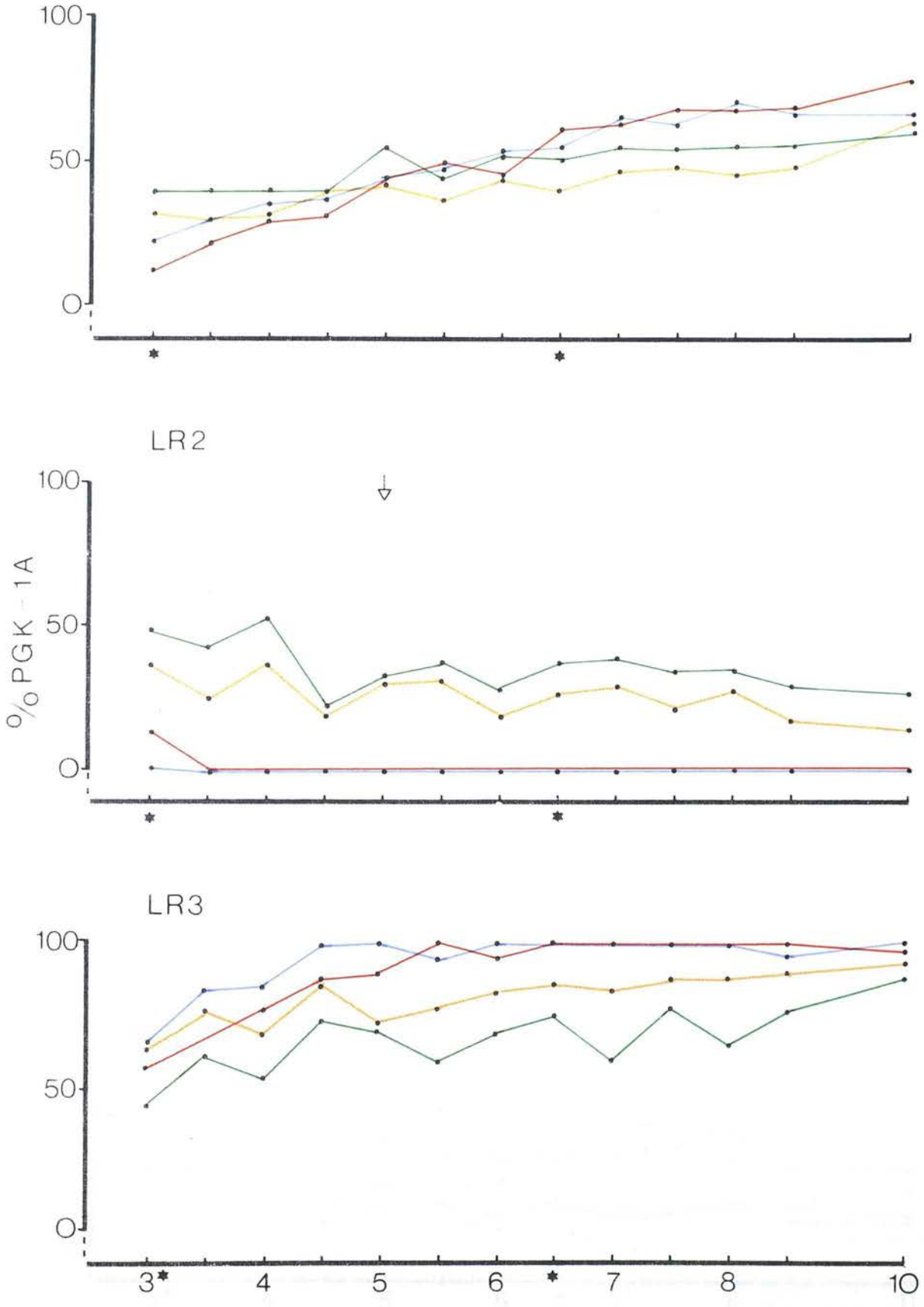

LR1
100
50
0
LR2
100
50
0
% PGK - 1A
LR3
100
50
0
3
4
5
6
7
8
10
Time after repopulation (months)

Figure 5.1(b)

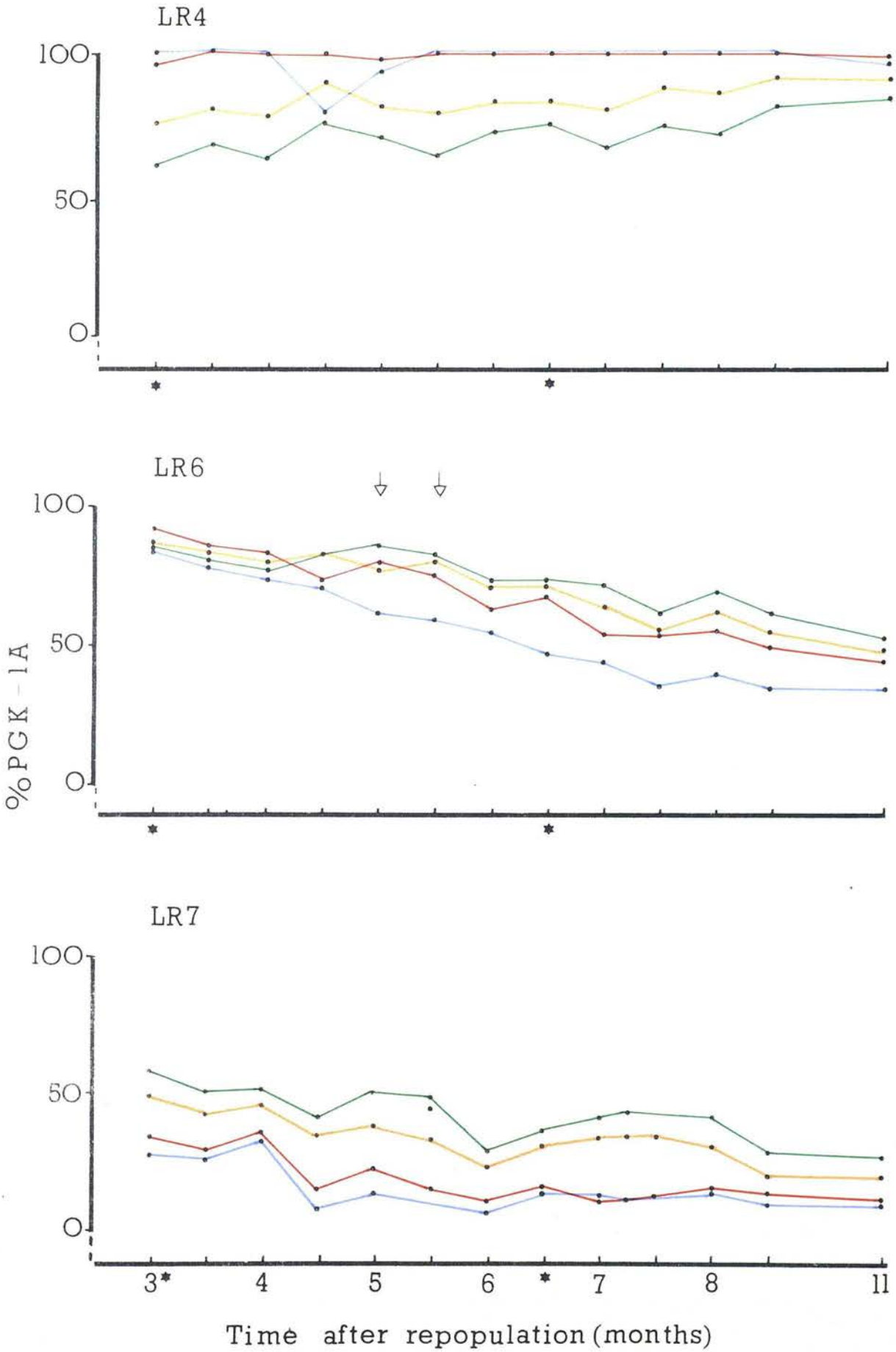

LR4
100
50
0
LR6
100
50
0
%PGK-1A
LR7
100
50
0
3*
4
5
6
*
7
8
11
Time after repopulation (months)

Table 5:1

Results of paired t tests between cell lineages of low-dose radiation chimaeras.

Mouse	E-B	B-T	T-G	E-G	E-T	G-B
LR1	NS	S**	NS	NS	NS	S*
LR2	S**	S**	S**	NS	S**	S**
LR3	S**	S**	S**	NS	S**	S**
LR4	S**	S**	S**	NS	S**	S**
LR6	S*	NS	S**	S**	S*	S**
LR7	S**	S**	S**	NS	S**	S**

E = erythrocytes
B = B lymphocytes
T = T lymphocytes
G = granulocytes
S = significant: S = $p < 0.05$
S* = $p < 0.01$
S** = $p < 0.001$
NS = not significant ($p > 0.05$)

In all mice, except LR6, there was no significant difference between erythrocytes and granulocytes. At sample 5 of LR6 the proportion of total leukocytes that were in the granulocyte population increased from approximately 20% to 35% and remained high (32%) at sample 6. Such a high granulocytosis (indicated by a black arrow (↑)) is not uncommon in radiation chimaeras. A change in the PGK-1 phenotype of the granulocytes, but not the erythrocytes or lymphocytes coincided with this granulocytosis. This suggested that there were changes in clonal activity in the granulocyte lineage during this period which probably coincided with exceptional demands for granulopoiesis. A high proportion of granulocytes (52%) was also seen in mouse LR2 at the 5th sample. However, no change in PGK-1 phenotype was observed since both the erythrocytes and granulocytes of this mouse expressed only one PGK-1 alloenzyme (PGK-1B). A single PGK-1 phenotype was also seen in the erythrocytes and granulocytes of LR3 and LR4 whereas both alloenzymes were expressed in the lymphocyte lineages of these mice.

In general, erythrocytes and granulocytes had a similar PGK-1 phenotype. This was different to the lymphocytes, although some degree of correlation was observed. Changes in PGK-1 in erythrocytes were paralleled by changes in granulocytes. Similarly, for B and T lymphocytes, a change in PGK-1 in one of these lineages was usually paralleled by a change in the other, although in most of the mice these two cell types were significantly different.

2. Serial Samples Comparison. Figures 5:1(a) and 5:1(b) show that there were differences between serial samples within individual mice. This was apparent for all cell types tested. In general, serial samples of B and T lymphocytes varied less than serial samples of erythrocytes and granulocytes. This can be assessed crudely by calculating the difference between the highest and lowest values. For lymphocytes, this difference was never greater than 37% (LR1;B lymphocytes). This difference was often higher for erythrocytes: LR1-62%; LR3-42% and LR6-47%. Similar large variations were seen between serial samples of granulocytes.

These changes in PGK-1 phenotypes between serial samples could support the clonal succession hypothesis. The variance between samples can be used to estimate the number of clones active in any unit time. The variance (s^2_A)

between serial samples of B and T lymphocytes can be calculated using the nested ANOVA analysis described in the materials and methods. This type of analysis cannot, however, be used for the calculation of clones active in the erythrocyte and granulocyte lineages. Three of the mice expressed only one PGK-1 alloenzyme in these lineages for most of the period of study; thus binomial calculations were not feasible. To estimate the number of active clones in the erythrocyte and granulocyte lineages, the %PGK-1A (mean of two replicates) for each sample from the other 3 mice (expressing both PGK-1 alloenzymes) was used to calculate the variance between serial samples. "Independent" samples for erythrocytes were at least 42 days apart and three sets were analysed for each of the three mice. Variances and estimated clone numbers are shown in Table 5:2. Clone numbers for B and T lymphocytes were estimated in two ways. First, by using the variance (s^2) calculated from the nested ANOVA and also by the same method as the granulocyte calculation.

The number of clones estimated to be active in the erythrocyte lineage of the different mice ranged from 3 to 21 with a mean of 8.8. Granulocyte clone numbers were estimated to be within the same range (mean 9.9). Results for B and T lymphocytes were comparable, they ranged from 13 to 46 with a mean of 28. There was no obvious difference between the outcomes of the the two types of calculation.

3. Inter-mouse Comparisons. Figures 5:1(a) and (b) indicated that there was a large amount of variation in PGK-1 phenotype between individual mice. Nested analyses of variance were performed on the different cell lineages from these six mice. This allowed a calculation of the variance s^2_R between mice for each lineage, excluding intramouse and technical variance. The number of repopulating clones was estimated from s^2_R (Table 5:3). A small number of clones appeared to be responsible for the repopulation of the myeloid lineages (approximately 2) and slightly higher numbers, 4 and 10, for the B and T lymphocytes respectively.

On closer examination of Figures 5:1(a) and (b) it was apparent that the variance between mice became greater as the experiment progressed. This was particularly true of the lymphocyte lineages. For example, at the first sample, lymphocyte values ranged from 40-80% whereas by the final sample

Table 5:2

Estimated numbers of active clones in different cell lineages of low-dose radiation chimaeraras. The mean clone number, calculated for each lineage, is given (±S.D.).

Cell Type			Mean (p)	Variance (s^2)	Clones (n)	Mean Clone number
Erythro-cytes	LR1	(i)	49.8	800	3.1	
		(ii)	47.7	704	3.5	
		(iii)	48.6	435	5.7	
	LR6	(i)	68.6	451	4.8	
		(ii)	66.1	310	7.2	8.8 ± 5.8
		(iii)	71.2	241	8.5	
	LR7	(i)	20.2	124	13.0	
		(ii)	16.5	105	13.0	
		(iii)	19.6	76	21.0	
B Lympho-cytes.	ANOVA		56.9	92	26.8	
	LR1		43.2	90	27.4	
	LR2		26.7	57	34.2	
	LR3		82.2	69	21.2	28.0 ± 11.5
	LR4		83.8	29	46.3	
	LR6		71.9	159	12.7	
	LR7		33.3	85	26.0	
T lympho-cytes.	ANOVA		56.7	103	23.8	
	LR1		49.7	62	40.3	
	LR2		35.9	84	27.4	
	LR3		67.8	136	16.0	28.2 ± 10.4
	LR4		71.7	50	40.6	
	LR6		74.3	100	19.1	
	LR7		41.4	94	25.8	
Granulo-cytes.	LR1		50.5	244	10.2	
	LR6		56.6	303	8.1	9.9 ± 1.7
	LR7		17.6	127	11.4	

Table 5:3

Variances and estimates of repopulating clone numbers for low-dose radiation chimaeras (using ANOVA data).

Cell Type	MS b/w mice	MS b/w samples	mb	Variance (s^2_R)	Mean (p)	Clones (n)
Erythrocytes.	14269	471	10	1380	53.3	1.8
B lymphocytes.	11053	193	18	603	56.9	4.1
T lymphocytes.	4658	217	18	247	56.7	9.9
Granulocytes.	26098	258	18	1436	53.6	1.7

MS = mean square
m = number of replicates (2)
b_2 = number of samples per mouse
s^2_R = variance between mice

see materials and methods (p.61.) for details of calculations.

this range was 8-94% PGK-1A.

An additional 5 mice (LR8-12), set up at the same time as LR1-7, were analysed at four selected times during the experiment and all 12 mice were used in the estimation of clone numbers. The estimations from all 4 samples which were taken at 3, 5, 7.5 and 10 months after repopulation are shown in Table 5:4 and Figure 5:2. There was virtually no difference between the number of clones estimated at different times in the erythrocyte and in the granulocyte lineages. However, the variance in the lymphocyte samples was greater at later samples, resulting in a lower estimate of clone number. Possible reasons for this difference are suggested in the discussion.

B. High-dose Chimaeras.

Erythrocytes, B and T lymphocytes and granulocytes were sampled from 6 radiation chimaeras repopulated with a high dose (10^7 cells) of bone marrow. (These mice were named HR1-HR6.) This was repeated every 2 weeks for a total period of 24 weeks. The PGK-1 phenotype of each sample was analysed twice using the ^{14}C staining system. The results are shown in Figure 5:3 (a) and (b); the full data in Appendix V. A description of the results is divided into three sections as in the low-dose chimaeras.

1. Cell-lineage Comparisons. Differences between the cell lineages within individual mice were not as dramatic as in the low-dose radiation chimaeras. However, in mice HR2 and HR3, there was a small but consistent difference between the PGK-1 phenotypes of the myeloid and lymphoid lineages. This was also true for samples taken from mouse HR1 between 5-7.5 months after repopulation and samples taken from HR4 between 5.5 and 8.5 months. Paired t tests were performed on the data and the results are shown in Table 5:5. Generally, there was no significant difference between the PGK-1 phenotypes of erythrocytes and granulocytes or between B and T lymphocytes. Significant differences were seen between erythrocytes and B lymphocytes, between erythrocytes and T lymphocytes and between T lymphocytes and granulocytes in the majority of mice. In mouse HR5 there was no significant difference between any pairs of cell types.

Table 5:4

Estimated numbers of repopulating clones in 12 low-dose radiation chimaeras.

Cell Type	Time after repopulation	Mean (p)	Variance (s^2)	Clones * (n)
Erythrocytes	3	58.7	901	2.7
	5	56.5	1292	1.9
	7.5	56.4	1231	2.0
	10	55.5	1268	1.9
B lymphocytes	3	63.3	304	7.6
	5	60.2	344	7.0
	7.5	61.3	543	6.6
	10	60.1	742	3.2
T lymphocytes	3	60.8	194	12.3
	5	63.6	235	9.8
	7.5	62.2	241	9.8
	10	61.8	447	5.3
Granulocytes	3	56.3	1070	2.3
	5	55.1	1383	1.8
	7.5	55.9	1319	1.9
	10	53.7	1423	1.7

* Means for each cell type are plotted in Fig. 5.2.

Figure 5:2

Estimated number of clones involved in the repopulation of 4 cell types isolated from 12 low-dose radiation chimaeras at 3, 5, 7.5 and 10 months after irradiation and bone marrow transplantation.

Colour code:

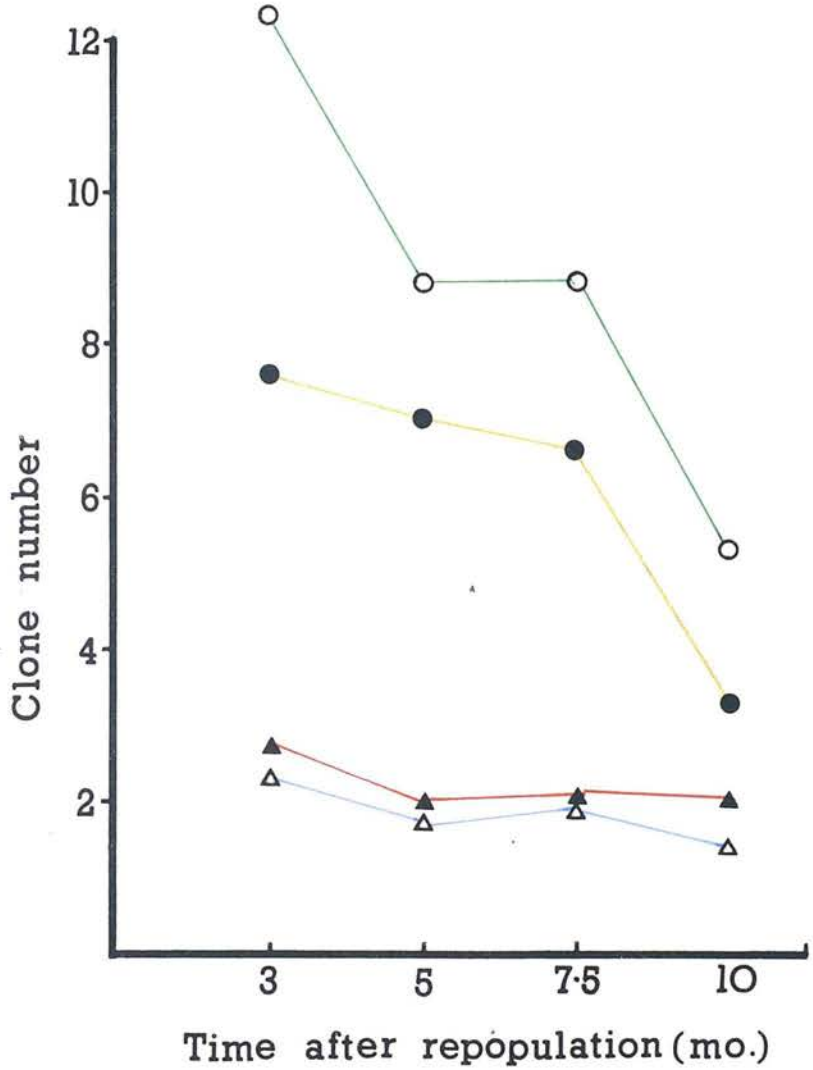
——— Erythrocytes

——— B lymphocytes

——— T lymphocytes

——— Granulocytes

Figure 5:2



2. Serial Samples Comparisons. The variance (s^2_A) between serial samples was calculated for the different cell lineages from the ANOVA data (Table 5:6). The technical variance in this particular experiment was larger than in others. This can be assessed by comparing the $MS_{repl.}$ in table 5:6 with that in table 4:6 of the previous chapter. The majority of the samples in this high-dose experiment were between 30 and 40% PGK-1A and samples of this phenotype are particularly difficult to integrate accurately. Linearity/repeatability experiments performed by J.D. Ansell and H. Taylor (unpublished) in this laboratory also indicate that the system is less "repeatable" within this range compared to higher PGK- 1 values. This, however, was not appreciated when the experiment was set up.

The variance between samples, excluding this high technical variance was calculated and used in the estimation of the number of active clones (Table 5:6).

3. Inter-mouse Comparisons. The number of repopulating clones was estimated from the variance (s^2_R) between mice, excluding intramouse and technical variance (Table 5:7). Somewhat more clones appeared to be involved in the repopulation (400-700) than were active at any point in time (200-500).

Figure 5:3 (a) and (b)

% PGK-1A present in serial samples of 4 cell types isolated from the peripheral blood of 6 high-dose radiation chimaeras.

Figures 5:1 (a): Mice HR1, HR2 and HR3
(b): Mice HR4, HR5 and HR6

Colour code:
- Erythrocytes
- B lymphocytes
- T lymphocytes
- Granulocytes

Figure 5.3(a)

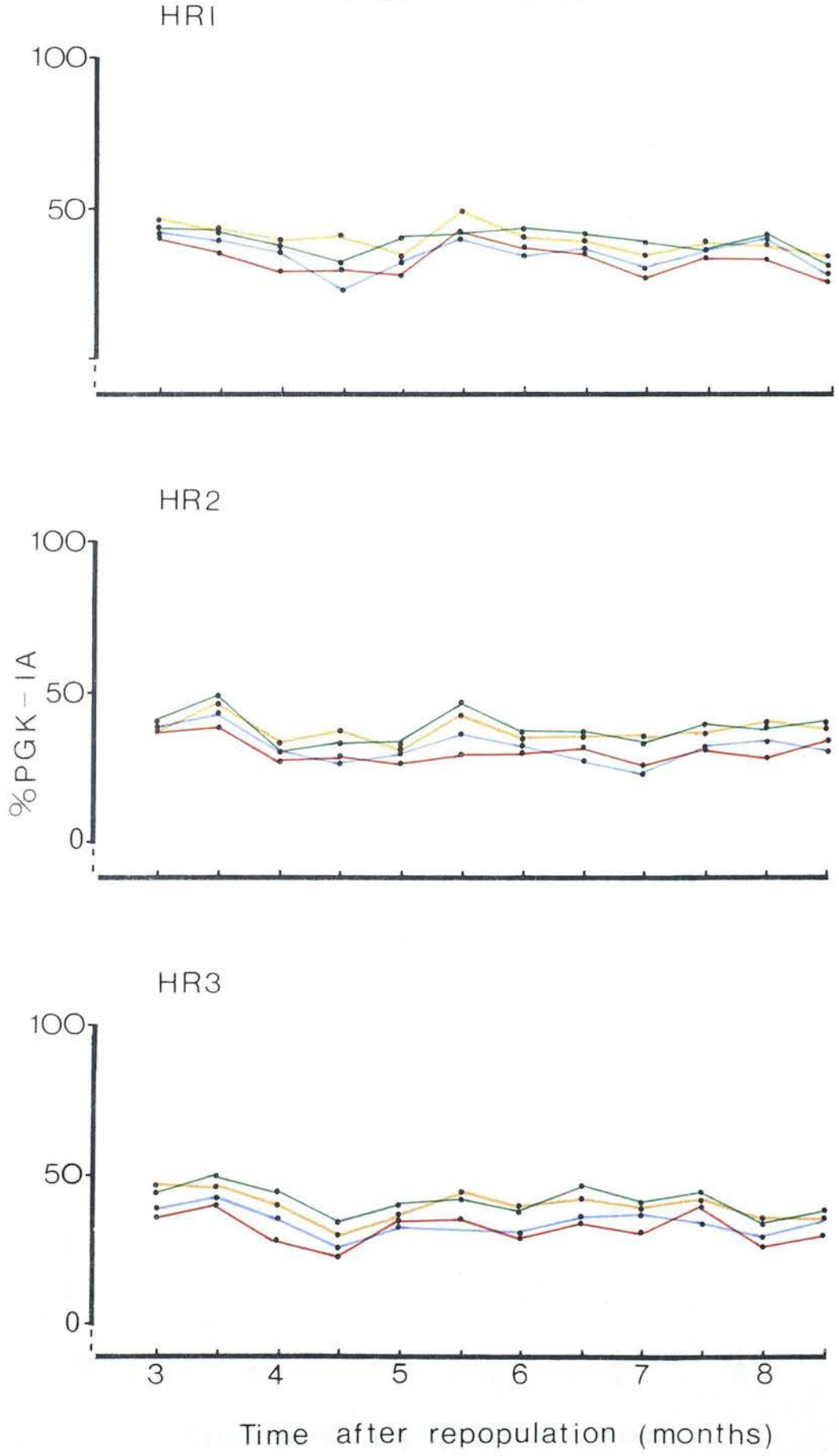

HR1
100
50
HR2
100
50
0
%PGK-IA
HR3
100
50
0
3
4
5
6
7
8
Time after repopulation (months)

Figure 5.3(b)

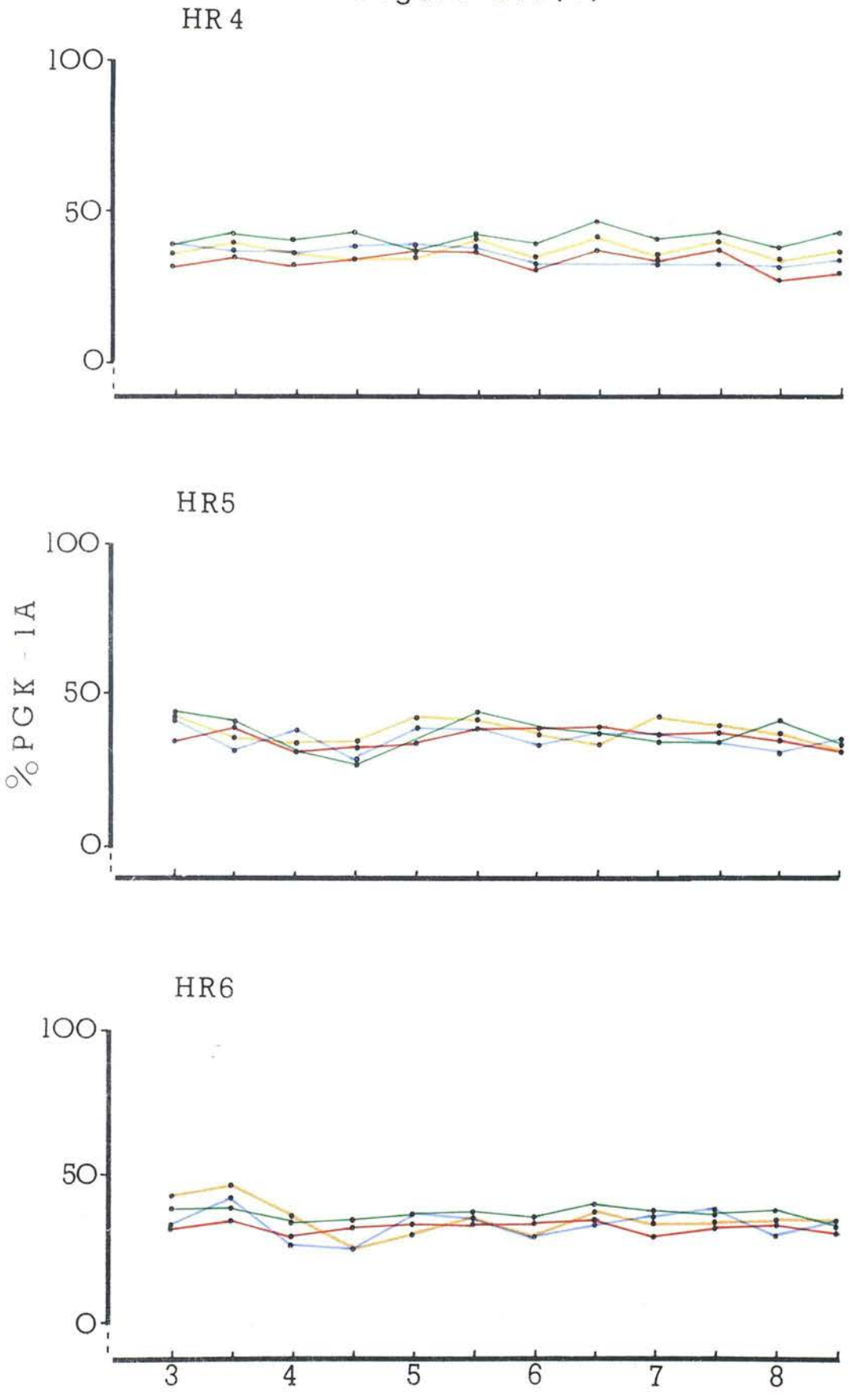

HR4
100
50
0
HR5
100
50
0
% PGK - 1A
HR6
100
50
0
3
4
5
6
7
8
Time after repopulation (months)

Table 5:5

Results of paired t tests between cell lineages of high-dose radiation chimaeras.

Mouse	E-B	B-T	T-G	E-G	E-T	G-B
HR1	S**	NS	S*	S	S**	S
HR2	S**	NS	S*	NS	S*	S*
HR3	S**	NS	S**	NS	S**	S**
HR4	S*	S**	S*	NS	S**	NS
HR5	NS	NS	NS	NS	NS	NS
HR6	S	NS	NS	NS	S**	NS

E = erythrocytes
B = B lymphocytes
T = T lymphocytes
G = granulocyte
S = significant S = $p < 0.05$
S* = $p < 0.01$
S** = $p < 0.001$
NS = not significant

Table 5:6

Estimated number of active clones in high-dose radiation chimaeras.

Cell Type	MS b/w samples	MS b/w repl.	Variance (s^2_A)	Mean (p)	Clones (n)
Erythrocytes	29.7	9.8	9.9	32.6	221
B Lymphocytes	38.2	16.6	10.8	38.1	218
T Lymphocytes	26.2	11.0	7.6	39.3	314
Granulocytes	31.3	21.7	4.8	35.2	476

repl. = replicates
s^2_A = variance between serial samples

Table 5:7

Estimated numbers of repopulating clones in high-dose radiation chimaeras.

Cell Type	MS b/w mice	MS b/w samples	mb	Variance (s^2_R)	Mean (p)	Clones (n)
Erythrocytes	62.3	29.7	10	3.26	32.6	674
B lymphocytes	93.8	38.2	18	3.09	38.1	763
T lymphocytes	130.4	26.2	18	5.79	39.3	412
Granulocytes	86.2	31.3	18	3.05	35.2	748

m = number of replicates
b_2 = number of samples per mouse
s^2_R = variance between mice

See materials and methods p._ for details of caculations.

Several workers have attempted to estimate the number of clones responsible for the repopulation of various haematopoietic sites in radiation chimaeras (Wallis et al, 1975; Micklem & Ross, 1978; Lennon, 1985). The number of clones was apparently related to the size of the bone marrow inoculum. Using the T6 marker system, Wallis et al (1975) and Micklem & Ross, (1978) calculated that a single femur was repopulated by approximately 7-15 clones after a cell dose of 2.5×10^6 and 35-54 after $4-5 \times 10^7$. Comparable results were obtained in a more extensive study using the PGK-1 system (Lennon,1985); 2-3 clones were estimated to repopulate a femur after 10^5 cells; 9-16 after 10^6 cells and 24-33 after 10^7 cells.

All these studies showed that the number of repopulating clones did not increase proportionally with cell dose. A high dose of bone marrow appeared to result in proportionally fewer clones. An explanation for this has been provided by Lennon (1985). She found that in animals repopulated with a low dose (10^5) of bone marrow, there was a high correlation between the PGK-1 phenotypes of femurs, humeri, thymus lobes and spleen. This suggested that all haematopoietic sites were repopulated by clonally-expanded progenitors derived from a common source. In contrast, there was virtually no correlation between different haematopoietic sites from animals repopulated with a high dose (10^7) of bone marrow, suggesting that each site was repopulated independently.

Thus, in the low-dose animals, the number of clones calculated to repopulate a single femur represented the number repopulating the whole haematopoietic system. However, this was not the case for animals receiving a high dose of bone marrow. Assuming that a single femur represents 5% of total bone marrow, and that there was no migration between bones, the number of clones responsible for repopulating the whole system was estimated to be 480-560. When the differences in clonal dispersal between mice that received different cell doses were taken in account, the estimated number of repopulating clones was related to cell dose in a reasonably linear fashion. The clone number extrapolated back to approximately one repopulating clone for every 4×10^4 cells injected. This is very close to the minimal cell dose reported to be

capable of saving lethally irradiated mice (van Bekkum & Vos, 1957; Micklem & Loutit, 1966) and curing W/W^v mice of their macrocytic anaemia (Boggs et al, 1982).

Some of the variance calculated in the above experiments would perhaps be due to temporal changes in clonal activity. A bone might be repopulated by a relatively large number of clones, but if only one or two were active at any one time then this would contribute greatly to the observed variance. In the study reported here the PGK-1 phenotypes of serial samples of peripheral blood cells from high- and low-dose radiation chimaeras were analysed. An appropriate analysis of variance test on the data made it possible to assess how much of the variance seen between mice was actually due to changes in clonal activity within individuals.

The serial analysis of peripheral blood was not, however, able to indicate how much variance between individual haematopoietic sites was due to changes in clonal activity within these sites. Estimations of repopulating and active clone numbers derived from serial peripheral blood samples represent changes in the total marrow pool. Any small changes in clonal activity within, say one femur, could be dampened by an opposing change in another bone. Serial sampling of individual bone marrow sites would be necessary to solve this problem, and this is probably not practicable. Even if serial sampling of an individual bone were achieved, there remains the possibility of clonal inhomogeneity of marrow even within the bone.

The number of repopulating clones in the mice receiving a high dose (10^7) of bone marrow was estimated to be between 400 and 700 for the different cell lineages. These results therefore correspond well with Lennon's (1985) estimate of 480-560 obtained by a different method. The number of "active" clones was estimated, from the between-sample variances, to be between 200 and 500. This suggested that there were less clones active at any one time than the number that originally repopulated the system. However, as discussed in the previous chapter, when variances are low, small changes result in relatively large changes in clone number; thus the inaccuracy of the estimates probably increases with the clone number. For this reason it cannot be confidently asserted that the number of repopulating clones exceeds that of

active clones. For the same reason it was not possible to compare accurately the clone numbers in the different cell lineages. In this respect these animals are similar to the normal animals described in Chapter 4. In that chapter, serial analyses of normal mice, heterozygous at the Pgk-1 allele, were described. Small changes in the PGK-1 phenotype between serial samples were apparent, but it was unclear whether this reflected changes in clonal activity; these changes could, as previously discussed, have been due to technical variance. In some of the high-dose chimaeras the PGK-1 phenotypes of B and T lymphocytes were similar to each other, but significantly different from erythrocytes and granulocytes. This was not seen, however, in the analysis of normal animals in Chapter 4. It is interesting, in this respect to compare Figures 4:1(a) and (b) to Figures 5:1(a) and (b).

In the low-dose chimaeras there was a relatively large amount of variance in PGK-1 phenotype between animals, between serial samples within an individual and between cell lineages in all animal studied.

The stem cells responsible for the repopulation of the haematopoietic system are heterogeneous; those with a longer mitotic history having an increased chance of proliferation and differentiation but a reduced chance of self renewal (Reinke et al, 1975; Micklem et al, 1976; Rosendaal et al, 1976; Hellman et al, 1978). Presumably, those with the highest self-renewal capacity will ultimately have the greatest potential for repopulation. However, these cells will be present in relatively small numbers and there is evidence to suggest that the most primitive cells are more reluctant to divide (Schofield, 1978). It is possible that a relatively large pool of "older" stem cells are responsible for the initial proliferation. The resultant clones will become exhausted in due course, perhaps more quickly than under steady-state conditions. New stem cells with a shorter mitotic history and present in smaller numbers would then take over. Eventually, the most "primitive" stem cells would become dominant and proliferate to produce progeny capable of long-term repopulation and dispersal. Such a scheme of repopulation corresponds, essentially, to the clonal succession hypothesis advanced by Kay (1965) to describe steady-state haematopoiesis and could explain various aspects of our data.

The variance in PGK-1 phenotypes between mice was used to estimate the

number of clones repopulating the different cell lineages in the low-dose chimaeras. Cells of the myeloid lineages (erythrocytes and granulocytes) were the products of very small numbers of clones (2-3). In some of the animals these cell types were exclusively of one phenotype, although this does not mean that they were necessarily monoclonal. Thus, these lineages were presumably repopulated by the progeny of the few most primitive (and therefore most highly self-renewing) stem cells discussed above; the clones derived from "older" stem cells having, meanwhile, become exhausted. We estimated the number of such primitive cells to be 2-3, which is similar to that calculated by Lennon (1985) for the repopulation of the entire bone marrow. Such a small number of repopulating clones is compatible with the observation of monotypic (PGK-1A or PGK-1B) granulocytes and erythrocytes in mice LR2, LR3 and LR4. The bone marrow inoculum was found to be 62% PGK-1A. Thus, the probability of 2 clones being the same PGK-1 phenotype is $0.62^2 + 0.38^2 = 0.53$; for 3 clones, $0.62^3 + 0.38^3 = 0.29$, etc. Monotypy in three out of six mice, therefore, is not compatible with large clone numbers.

The number of clones involved in the repopulation of the B and T lymphocyte populations of the low-dose radiation chimaeras was estimated to be 4 and 10 respectively; slightly higher than that estimated for the repopulation of the myeloid cells. However, accurate binomial estimates are only obtained when hundreds of samples are used (Stone, 1984); clearly, our scale of operations leaves much room for error. On the other hand, it is reassuring that the relationships of clone numbers to cell dose, of clone numbers in blood (this thesis) to clone numbers in marrow (Lennon, 1985) and of clonogenic cell frequency to the minimum repopulating dose for lethally irradiated mice (van Bekkum & Vos, 1957; Micklem & Loutit, 1966) or W/W^V mice (Boggs et al, 1982) seem consistent. If this small difference in repopulating clone numbers between myeloid and lymphoid lineages is a true biological difference, it could be explained by the fact that the lymphocytes have a relatively long lifespan. T lymphocytes have been shown to have a lifespan of 4-6 months (Sprent & Basten, 1973; Ropke et al, 1975); this means that progeny of the initial repopulating clones will be present for a considerable length of time. As discussed above, the initial repopulation is likely to be by a relatively large pool of "older" stem cells. It is interesting that the number of clones estimated from the earliest samples was higher than that calculated from later samples

(Table 5:4; Figure 5:2). This could suggest that the clones producing lymphocytes during the initial repopulation become exhausted and a smaller pool of more primitive stem cell take over. However, many more samples would be required to establish the reality of these small changes in clone number with confidence.

In the majority of low-dose chimaeras there was no significant difference between the PGK-1 phenotypes of erythrocytes and granulocytes which suggested that these lineages were the progeny of a common stem cell or precursor. There was one exception to this. In mouse LR6, a high granulocytosis was followed by a change in the PGK-1 ratio of the granulocyte but not the erythrocyte population. It is possible that this exceptional demand for granulocytes was met by the proliferation of a more primitive pool of stem cells.

There was a persistent and consistent difference between B and T lymphocytes in the majority of the low-dose chimaeras studied. This is unlikely to be due to technical differences such as variations in the way the B- and T-cell lysates run on the electrophoretic system. Such as explanation would be expected to result in one of the cell types having a consistently higher (or lower) %PGK-1A and this is clearly not so: in some mice the B lymphocytes showed a higher %PGK-1A while in others the reverse was true. It is conceivable that the presence of a few percent of host cells (too few to be detected by the GPI analysis) was responsible for the observed differences. Alternatively, it is possible that the B and T cell pools are the progeny of different stem cells or progenitors.

The variance between serial samples of erythrocytes or granulocytes from low-dose chimaeras presumably reflects temporal changes in clonal activity, providing support for the clonal succession hypothesis (Kay, 1965). The mean number of clones active at any one time was estimated to be between 3 and 21 with a mean of 9. This variance was higher than for T or B cells, as would be expected on the basis of the relatively long lifespan of lymphocytes.

There are two types of fluctuations in the PGK-1 phenotypes of the lymphocyte populations - long-term trends over the whole sampling period and short-term

fluctuations between adjacent samples. As discussed above, the long-term changes can possibly be explained by the exhaustion of "older" stem cell pools and the subsequent take-over by more primitive stem cells. The PGK-1 phenotypes of both B- and T- lymphocyte populations approach that of the myeloid lineages over the period of study in several mice (LR2, LR3 and LR4) and do so at a similar rate. If this is indeed due to replacement from a common stem-cell pool, the results suggest that B and T lymphocytes in the peripheral blood have similar life-spans. The short-term, inter-sample variation in the lymphocyte populations, apparent mainly in the low-dose chimaeras, is difficult to explain. Some of the fluctuations are small enough to be explained by technical variance of the electrophoretic technique, but some are slightly larger than this. It is possible that larger variations are due to differences in the cell-sorting purity from day to day. On the other hand, short-term fluctuations in PGK-1 phenotype may reflect the inputs of a small number of relatively short-lived cells, from currently active clones, into the main pool of long-lived peripheral blood lymphocytes. B lymphocytes have been shown to be heterogeneous with respect to lifespan (Ropke et al, 1975), ranging from only a few days (de Freitas & Coutinho, 1981; Strober, 1972) to several weeks (Sprent & Basten, 1973; Kumararatne & MacLennan, 1981). They are produced in the bone marrow in very large numbers (Opstelten & Osmond, 1983) but it has been suggested that only a small proportion of these enters the mature B lymphocyte pool (Kumararatne et al, 1985). This is similar to that reported for T lymphocytes; newly-formed lymphocytes that migrate from the thymus have been reported to account for only 1% of the total thymocyte pool per day compared with the daily thymocyte turnover of about 30% per day (Scollay et al, 1983).

Summary. In the high-dose radiation chimaeras the number of repopulating clones was estimated to be between 400 and 700. The number of active clones was estimated to be between 200 and 500 which was comparable to that estimated for normal mice. There was a significant difference between the PGK-1 phenotypes of lymphoid and myeloid lineages in some of the high-dose radiation chimaeras. This had not been observed in normal animals. In the low-dose radiation chimaeras, all cell lineages were repopulated by a small number of stem cells; 2 for the myeloid cells 4 for B lymphocytes and 10 for T lyphocytes. In the majority of mice there was no significant difference between

erythrocytes and granulocytes, indicating that these cell types were derived from a common stem cell pool. In contrast, the PGK-1 phenotypes of B and T lymphocytes were significantly different from one another and from the myeloid cells which could suggest that they were the progeny of different stem cells or progenitors. Temporal changes in the PGK-1 phenotypes of all lineages were evident in the low-dose radiation chimaeras. These results support a system of clonal succession in the haematopoietic system. (Kay, 1965).

CHAPTER 6.

THE DEVELOPMENT OF B LYMPHOCYTES CARRYING THE XID MUTATION.

Introduction

Murine B lymphocytes, like other haematopoietic cells, arise ultimately from multipotential stem cells (Wu et al, 1968; Abramson et al 1977; Till & McCulloch, 1980). Stem cells are first detected in the yolk sac of the 7 day old mouse foetus (Moore & Metcalf, 1970). They are later found in the foetal liver and by 12 days of gestation they give rise to progeny capable of synthesizing immunoglobulin heavy chains (cμ) (Raff et al, 1976). Surface IgM-bearing B lymphocytes appear in the liver and spleen of 16-17 day old embryos (Spear et al, 1973; Owen et al, 1974) but cells expressing surface IgA and IgG only appear later, during the first few days of life (Abney et al, 1978). Between 10 and 15 days after birth, IgD appears and increases in amount until 3 months of age when it is the predominant immunoglobulin on the surface of most B cells (Vitetta et al, 1975; Vitetta & Uhr, 1977). Ia glycoproteins, encoded by genes within the I region of the H-2 complex are expressed on all B cells within the first few days of life (Kearney et al, 1977) and are present on 95% of splenic B lymphocytes by the ninth day (Huber, 1982). Qa-2 antigens, also encoded in the H-2 region, are present on both B cells and their immediate precursors in the adult but not the embryo (Kincade et al, 1980). It is well established that neither Ia nor Qa-2 expression is restricted to the B lineage (Hammerling, 1976; Kincade et al, 1980).

Adult B lymphocytes are heterogeneous in many respects including their expression of distinct differentiation antigens (Ahmed et al, 1977a; Huber, 1982); their qualitative and quantitative membrane immunoglobulin isotypes (Abney et al, 1978; Scher et al, 1976; Vitetta & Uhr, 1977); their reactivity to different mitogens (Gronowicz & Coutinho, 1975) and antigens (Lafleur et al, 1973); their aquisition of immunocompetence during ontogeny (Mosier et al, 1977) and their selective inactivation following exposure to tolerogenic signals (Cambier et al, 1977; Metcalf et al, 1980).

The developmental basis of this functional heterogeneity remains to be

established. Functionally distinct B lymphocytes could represent the sequential maturational stages of a single B cell lineage (Cambier et al, 1977; Gronowicz & Coutinho, 1975) or the distinct sublines of independently maturing B cells that have diverged from a common stem cell early in B lymphocyte development (Paige et al, 1979; Slack et al, 1980). The extent to which intrinsic and/or extrinsic factors play in the generation of functional heterogeneity is also unclear (McKearn et al, 1982). Control of B lymphocyte-antigen expression has been claimed to be dependent on qualitative properties inherent in the signals activating a particular B cell (Augustin & Coutinho, 1980; Cambier et al, 1977) or differential involvement of certain accessory cells (Kishimoto & Ishizaka, 1973; Richman et al, 1981). Such models, based exclusively on extrinsic control mechanisms, generally view B cell development within a single lineage. Differential B cell function would be attributed to regulatory influences exerted upon B cells comprising consecutive sequential stages within one B lymphocyte differentiation pathway. There is, however, evidence for the existence of at least two distinct B lymphocyte subpopulations (Wortis et al, 1982; Sprent & Bruce, 1984a; Hardy et al, 1984).

There are several promising approaches to understanding B lymphocyte heterogeneity and development. Among these is the use of monoclonal antibodies directed to cell surface antigens on B cell precursors (Coffman & Weissman, 1981a,b; Dessner & Loken, 1981; Kincade et al, 1981a). One such antigen, B220, the B cell-specific form of the T200 glycoprotein family, is present on all B lymphocytes including their precursors. There are apparently two subpopulations of $B220^+$ pre-B cells with respect to the expression of another antigen ThB (Eckhardt & Herzenberg, 1980); the small $B220^+$, ThB^+, sIg^- cells are thought to be a more mature than the larger $B220^+$, ThB^-, sIg^- cells (Coffman, 1983 (review)). Monoclonal antibodies against these antigens can be used to isolate B lymphocyte precursors allowing their further maturation to be studied in vitro (Kincade et al, 1981a, b) and in irradiated or immunodeficient mice (Paige et al, 1979; Kincade et al, 1978; Landreth, et al, 1983).

The discovery of mouse strains that are genetically defective in B lymphocyte function has helped in the analysis of B lymphocyte subpopulations and their functional and lineage relationships. Perhaps the most informative model has been the CBA/N mouse strain. Amsbaugh et al (1972) first recognised that

CBA/N mice were unresponsive to several pneumococcal polysaccharide antigens and that their unresponsiveness was transmitted in an X-linked fashion. Subsequent work has revealed that the immune deficit is characterised by unresponsiveness to certain thymus-independent (TI-2) antigens (Scher et al, 1975), hyporesponsiveness to some B cell mitogens and unresponsiveness to others (Scher et al, 1975; Goodman et al, 1978), and inability to produce B lymphocyte colonies in soft agar culture after mitogen stimulation (Kincade, 1977). The unresponsiveness of CBA/N mice to TI-2 antigens can be corrected by the transfer of B cells from normal histocompatible mice (Scher et al 1975), which suggests that the xid mutation is a defect within B cells and/or their precursers.

Studies have shown that the CBA/N mouse is in fact deficient in at least one functional subset of mature B lymphocytes (Scher et al, 1975). This subpopulation is first detected in the spleen of normal mice at 2-3 weeks after birth and has been characterised as (a) bearing Lyb-5 antigens; (b) functionally expressing minor lymphocyte-activating determinants (Ahmed & Scher, 1976) and (c) expressing a high level of surface IgD and a low level of surface IgM (Hardy et al, 1982). It has been suggested that the xid mutation causes a maturational block in B lymphocyte development prior to the differentiation of this mature subpopulation. If this is the case, and if B lymphocyte differentiation occurs along a single lineage, then adult CBA/N B cells might resemble immature B cells seen in normal, neonatal mice (Scher, 1982). This is true for some properties, IgM/IgD (Finkelman et al, 1975), Lyb-5 expression (Scher et al, 1975) and their response to antigen-non-specific T cell replacing factor (TRF) (Parker et al, 1979), but not others. Hardy et al (1983) showed that CBA/N B cells did not express the antigen BLA-1 whereas all B cells from normal 2 week old mice were BLA-1^{+}.

The other suggestion has been that there is more than one lineage in B lymphocyte development and that the xid mutation results in a block in one or more of these lineages (Kincade, 1977; Kincade et al, 1978). If this were the case, Lyb-5^{-} cells in adult CBA/N mice should be indistiguishable from Lyb-5^{-} cells in normal adults. However, B cells that emerge in CBA/N mice appear to be a unique population not represented in normal mice and may also be functionally defective (Ono et al, 1983; Sprent & Bruce, 1984b).

Comparative studies of B lymphocytes from congenitally athymic (nu/nu) and CBA/N mice have provided some evidence for a multi-lineage model of B lymphocyte development. Nude mice have a defect in B cell development (Griffith et al, 1976; Parks et al, 1977), complementary to the xid defect (Wortis, 1974). Double mutants (CBA/N nu/nu) are profoundly deficient in B lymphocytes compared to CBA/N mice (Wortis et al, 1982; Mond et al, 1982; Sprent & Bruce, 1984a). This suggests that mature B cells belong to two subpopulations: thymic influence is critical for the normal development of mature Lyb-5^- B cells whereas Lyb-5^+ cells are relatively independent of such influences (Mond, et al, 1982).

The level of expression of the antigens BLA-1 and BLA-2 is thought to be inversely related to the degree of B cell maturity - cells expressing BLA-1 and BLA-2 include the pre-B cells that contain cμ (Hardy et al, 1984). The increase in Lyb-5^+ during ontogeny is paralleled by a decrease in cells expressing the BLA antigens, suggesting that BLA-1^-2^- cells are a more mature population. BLA-1^+2^- cells were diminished ten-fold in CBA/N mice and BLA-1^-2^+ were 2-3 times reduced in nudes. Three- and four-colour FACS analysis of the relative expression of BLA-1, BLA-2, IgD and IgM in normal, CBA/N and nude mice suggested that the order in which the BLA antigens were lost during B lymphocyte differentiation distinguished two distinct B cell lineages.

We have studied the development of B lymphocytes carrying the xid mutation in mice that were heterozygous both for xid and for the X-linked enzyme phosphoglycerate kinase (PGK-1). The two allelic forms of PGK-1, PGK-1A and PGK-1B, provide an appropriate marker system in CBA/N x CBA/Ca-Pgk-1^a female mice because X chromosome inactivation results in individual cells having only one X chromosome active. Thus, cells will carry either Pgk 1^b and xid OR Pgk-1^a and the normal (+) allele at the xid locus. Females heterozygous at an X-linked locus are balanced mosaics for the products of neutral polymorphic genes. If a mutation at an X-linked locus directly affects a cell population by being lethal at a particular developmental stage or by affecting the cells' ability to proliferate, the mosaicism will become unbalanced. If on the other hand, the mutation acts indirectly via microenvironmental factors, one would not expect the mosaicism in the final target cells to be affected. The degree of imbalance in the PGK 1 phenotype of different cell types will reflect

the extent of the effect of the xid mutation.

The main objectives of this study were as follows.

(1) To confirm that the effect of the xid mutation is restricted to the B lymphocyte lineage. In addition to B cells, a series of different cell types, including erythrocytes, thymocytes, granulocytes and non-haematopoietic cells were isolated from xid/+ and +/+ mice and their PGK-1 phenotypes compared.

(2) To find out whether all B lymphocyte subpopulations were affected by the mutation. It is known that one particular subpopulation (Lyb- 5^{+};high IgD, low IgM) is missing in CBA/N mice, but is unclear whether other B lymphocytes that are present are also defective in some way. As stated previously, there is evidence that xid Lyb-5^{-} B cells are not functionally equivalent to normal Lyb-5^{-} B cells (Ono et al 1983; Sprent & Bruce, 1984b).

(3) To find out at what age and at what stage of B lymphocyte development the xid mutation acts. Bone marrow pre-B and B cells and splenic B lymphocyte subpopulations were isolated from xid/+ mice of different ages.

(4) to assess whether the xid defect is life-long. There is evidence to suggest that the defect is partially restored in old mice (Fidler et al. 1980) but other workers (Kincade et al, 1982 (review)) have found no clonable B cells in mice up to 2 years old. PGK-1 analysis was performed on B lymphocytes from old xid/+ mice.

Materials & Methods

1. Mice.

CBA/Ca-Pgk-1^{a} males were mated to either CBA/N females to produce xid/+;Pgk-$1^{b/a}$ F1 hybrids or to CBA/Ca-Pgk-1^{b} females to produce control +/+;Pgk-$1^{b/a}$ mice. The reciprocal crosses were used to examine the parental effects on X chromosome inactivation (see Chapter 3). Peripheral blood and whole tissue samples were prepared as described in Chapter 3.

2. Preparation of Cell Suspensions.

Cell suspensions of subcutaneous lymph node, thymus and spleen were made by dissociating tissues in a ground glass homogeniser in approximately 1ml RPMI BSA medium (see Appendix I) and poured through a fine stainless steel sieve to remove stroma. Erythrocytes were removed from the spleen cell suspension by osmotic lysis (see Chapter 4). Bone marrow cavities were flushed with RPMI-BSA medium and the marrow plug was aspirated through a 25g needle to obtain a single cell suspension. Cell viability counts were performed using a solution of acridine orange and ethidium bromide (see Appendix I). Suspensions were adjusted to a concentration of 10^7 viable cells per ml.

3. Isolation of B Lymphocytes.

(a) Mature B Lymphocytes. FITC-RAMIg antiserum (Chapter 4) was used to isolate mature B lymphocytes from lymphoid cell suspensions. A total of $2.5x10^6$ lymph node or spleen cells were stained with 5µl FITC-RAMIg for 40 mins. at 4°C at a concentration of 5 x 10^6 cells per ml of RPMI-BSA medium. $7.5x10^6$ bone marrow cells were stained with 15µl FITC-RAMIg. Cells were washed three times with cold medium and resuspended in an appropriate volume (approx. 10^7 cells/ml) for FACS analysis and sorting. Figure 6:1 (A) shows a FACS dot plot of bone marrow cells stained with FITC-RAMIg. Ig^+ and Ig^- cells were sorted into 1.5ml Sarstedt tubes containing 0.25ml RPMI-BSA medium. Collected samples of $1-2x10^5$ cells were centrifuged for 5 mins. at 250g. The supernatant was carefully removed and the inside of the Sarstedt tube dried with tissue. Sample buffer was added to the pellet at a concentration of 1µl/ $5x10^4$ lymphocytes. Granulocyte-lineage cells were identified by their higher 90° scatter profile (Chapter 4) and $3-5x10^5$ were isolated from some of the bone marrow samples.

(b) B-Cell Precursors. Pre-B cells were isolated using a rat monoclonal antibody (14.8) against a 220 kd molecular weight glycoprotein, present on all B lineage cells including their precursors. $6x10^6$ bone marrow cells were stained with 30µl of 14.8 (10X concentrated, purified culture supernatant, kindly given to us by Dr. P.W. Kincade). Staining was performed for 40 mins at 4°C at a

Figure 6:1

A. FACS dot plot of bone CBA/Ca bone marrow cells stained with FITC-RAMIg. 8-12% (on average) of nucleated marrow cells were positively stained.

B. FACS dot plot of CBA/Ca bone marrow cells stained with monoclonal rat anti-mouse 14.8 + FITC-GARIg. 14.8 dull and 14.8 bright populations are shown.

A

right-angle scatter

B

log fluorescence

concentration of $5x10^6$ cells per ml. 80µl fluorescein labelled goat antiserum to rat immunoglobulin (whole molecule) (FITC-GARIg) was added for 40 mins at 4°C. Cells were washed four times after each staining step and finally resuspended in the appropriate volume of RPMI-BSA medium for FACS analysis and sorting. Figure 6:1 (B) shows a FACS dot plot of bone marrow cells stained with 14.8. This monoclonal antibody is useful because it differentially stains B and pre-B lymphocytes. B cells stain brightly and these cells have been shown by 2-colour FACS analysis to express surface-membrane Ig. Cells that stain dully with 14.8 are considered to be B lymphocyte precursors: they do not express surface-Ig but a proportion contain cytoplasmic u (Kincade et al, 1981). Populations of 14.8-dull and 14.8 bright cells were defined, sorted and prepared for PGK analysis as described above.

(c) B Lymphocyte Subpopulations. $4x10^6$ spleen cells were stained with 200µl Texas Red-labelled goat antiserum to mouse IgM (TR-GAMIgM) (E.Y.labs.) and 25µl fluorescein-labelled goat antiserum to mouse IgD (FITC-GAMIgD) (Nordic) for 40 mins at 4°C in 1ml RPMI-BSA medium. Cells were washed three times in 5ml RPMI-BSA medium and then resuspended in 1ml for FACS analysis and sorting. Non-lymphocytes were gated out using forward scatter; lymphocytes were analysed and sorted using green and red fluorescence simultaneously.

4. PGK-1 Analysis.

Whole tissue samples were diluted to the appropriate concentration immediately before PGK-1 analyses (sorted cell samples were undiluted). Blood samples for tests of mosaicism were diluted 1:1 and run on the MTT/PMS system. All other samples were run at least twice on the ^{14}C system (Chapter 2) and the mean value used in final results.

Results.

1. X Chromosome-Inactivation Effects.

The probability of expression of the $Xce^{c}Pgk\text{-}1^{a}$-bearing X chromosome in mice used in this chapter was compared to that in the mice described in Chapter 3. These included CBA/Ca-$Pgk\text{-}1^{a}$ (backcross generation 16-18) and CBA/Ca x C3H/HeHa-$Pgk\text{-}1^{a}$ F1 progeny.

Table 6:1 shows the PGK-1 phenotypes of erythrocyte samples from these mice. The probability of expression of the $Xce^{c}Pgk\text{-}1^{a}$ X chromosome in xid/+ mice was 71% and this was not significantly different from that found for the other Xce^{a}/Xce^{c} heterozygotes. Also, there was no significant difference between the xid/+ progeny of reciprocal crosses (Table 6:2). This shows that no parental effects on the X-inactivation process are evident in the xid/+ mice.

The aim of this chapter was to analyse the effect of the xid mutation on different cell types by looking at imbalances in X chromosome expression. The $Pgk\text{-}1^{b}$ allele was a convenient marker for xid bearing cells; any reduction in the proportion of PGK-1B present in a cell population would indicate that it was affected by xid. There is a large range of "baseline" PGK-1A values (35-90%) between mice. If a mouse had a baseline level of mosaicism at the top of the range it would be difficult to detect any reduction in PGK-1B alloenzyme. To resolve this problem, all mice were pre-screened and only those with a phenotype of between 35 and 75% PGK-1A were used for further analysis.

2.Analyses of Different Cell Lineages.

The mean PGK-1 phenotypes of a number of different cell types isolated from several xid/+ mice and several control mice (+/+) are shown in Figure 6:2. % PGK-1B is plotted and this reflects the % xid-bearing cells present in the sample. Control mice were heterozygous for PGK-1 but did not carry xid. There was no significant difference between the two sets of mice for most of the cell populations, i.e. erythrocytes, thymocytes, non-B cells of the lymph node and spleen, granulocytes and kidney cells. Development of these cell

Table 6:1

Mean % PGK-1A present in erythrocytes of xid/+ and +/+ mice.

Mouse strain xid genotype	CBA/N x CBA.PGK-1A xid/+	CBA/Ca +/+	CBA/Ca x C3H +/+
Mean	71	72	73
SD	10	6	9
no. samples	92	100	100

Table 6:2

Mean % PGK-1A present in erythrocytes of xid/+ mice derived from reciprocal crosses.

Cross	CBA.PGK-1A x CBA/N	CBA/N x CBA.PGK-1A
Mean	74	76
SD	11	9
no. samples	27	27

Figure 6:2

Mean % xid-bearing cells (inferred form %PGK-1B) present in different cell populations from xid/+ (○) and +/+ (●) mice. The mean % PGK-1B value (± SD) is plotted and the number of animals used in the estimation is given. When only 1 or 2 values were obtained, individual points are plotted.

E = erythrocytes

T = thymus

LN^- = sIg^- (non-B cells) from lymph node

LN^+ = sIg^+ (B cells) from lymph node

SPL^- = sIg^- (non-B cells) from spleen

SPL = unsorted spleen

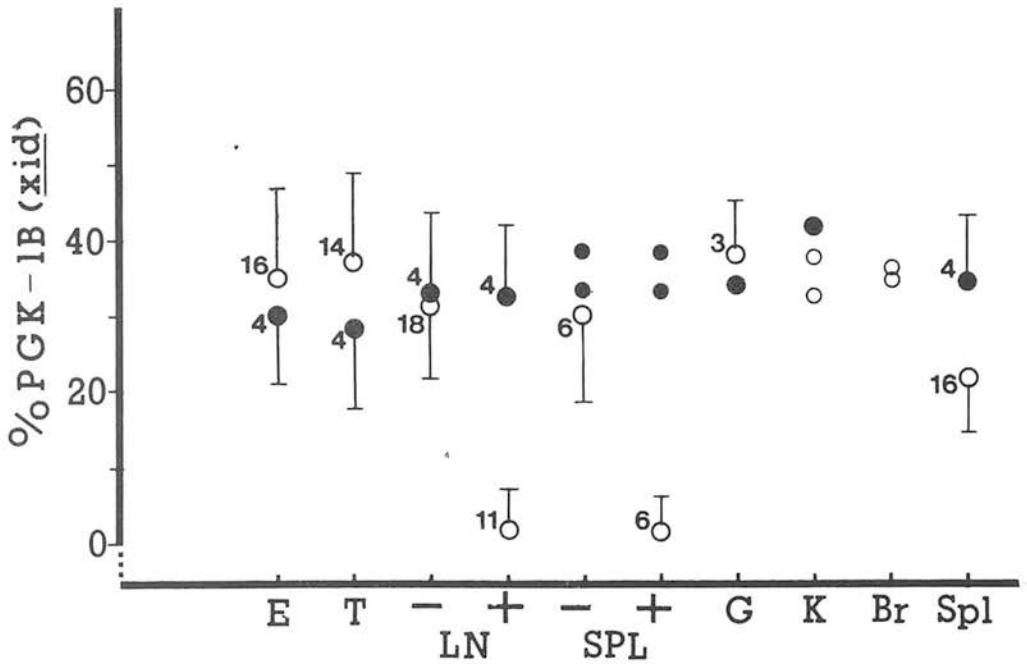
SPL^+ = sIg^+ (B cells) from spleen

G = granulocytes

K = kidney

Br = brain

Figure 6:2



types is thus unaffected by the xid mutation. There were virtually no xid bearing cells present in B lymphocyte populations isolated from the lymph node and spleen of xid/+ mice . Taken together these data suggest that the developmental effects of the xid gene(s) are restricted to the B lymphocyte lineage. B lymphocytes and their subpopulations were analysed in more detail.

3. Peripheral Lymphoid Organs.

Figure 6:3 shows the %PGK-1B(xid) present in B cells and non-B cells isolated from the peripheral lymph nodes and spleen of several xid/+ heterozygotes and control (+/+) mice (2-18 months old). There was no significant difference in the %PGK-1B present in non-B cells and B cells isolated from individual control mice. This shows that the PGK-1 marker is neutral; cells are not selected against if they carry either one of the PGK-1 alloenzymes.

In xid/+ mice the non-B cell compartment had comparable levels of PGK-1B to the control mice, whereas the B cell compartment had virtually no PGK-1B activity. In nearly all cases (13/14) there was less than 4% PGK-1B; the one exception to this (mouse 41) had 16% PGK-1B, but this was still less than the corresponding non-B cell population (44%). As PGK-1B marks xid-bearing cells, these data indicate that cells carrying the xid lesion are not present among the B cell populations normally found in the peripheral lymph nodes and spleen of adult mice.

4. Bone Marrow B cell Populations.

Figure 6:4 shows the %PGK-1B present in non-B cells, pre-B cells and mature B cells isolated from the bone marrow of xid/+ and +/+ mice (2-6 months old). In individual control mice there was no significant difference between any of the cell populations studied. Also there was no significant difference between non-B cells and pre-B cells isolated from xid/+ bone marrow. This indicates that, like non-B cells, the pre-B cells are unaffected by the xid mutation. There were significantly less xid-bearing cells present in B compared to non-B cells isolated from individual xid/+ mice ($p=0.01$). Also, B cells had a consistently smaller xid-bearing component than pre-B cells. This difference reflects the degree of imbalance in the bone marrow B cell population and thus the extent

Figure 6:3

% xid-bearing cells present in B cells and non-B cells isolated from lymph node (A and B) and spleen (C and D) of xid/+ and +/+ mice. Figures on the left of the broken line (A and C) are xid/+ mice, those on the right (B and D), +/+ mice.

● - sIg^- (non-B cells)

○ - sIg^+ (B cells)

Figure 6:3

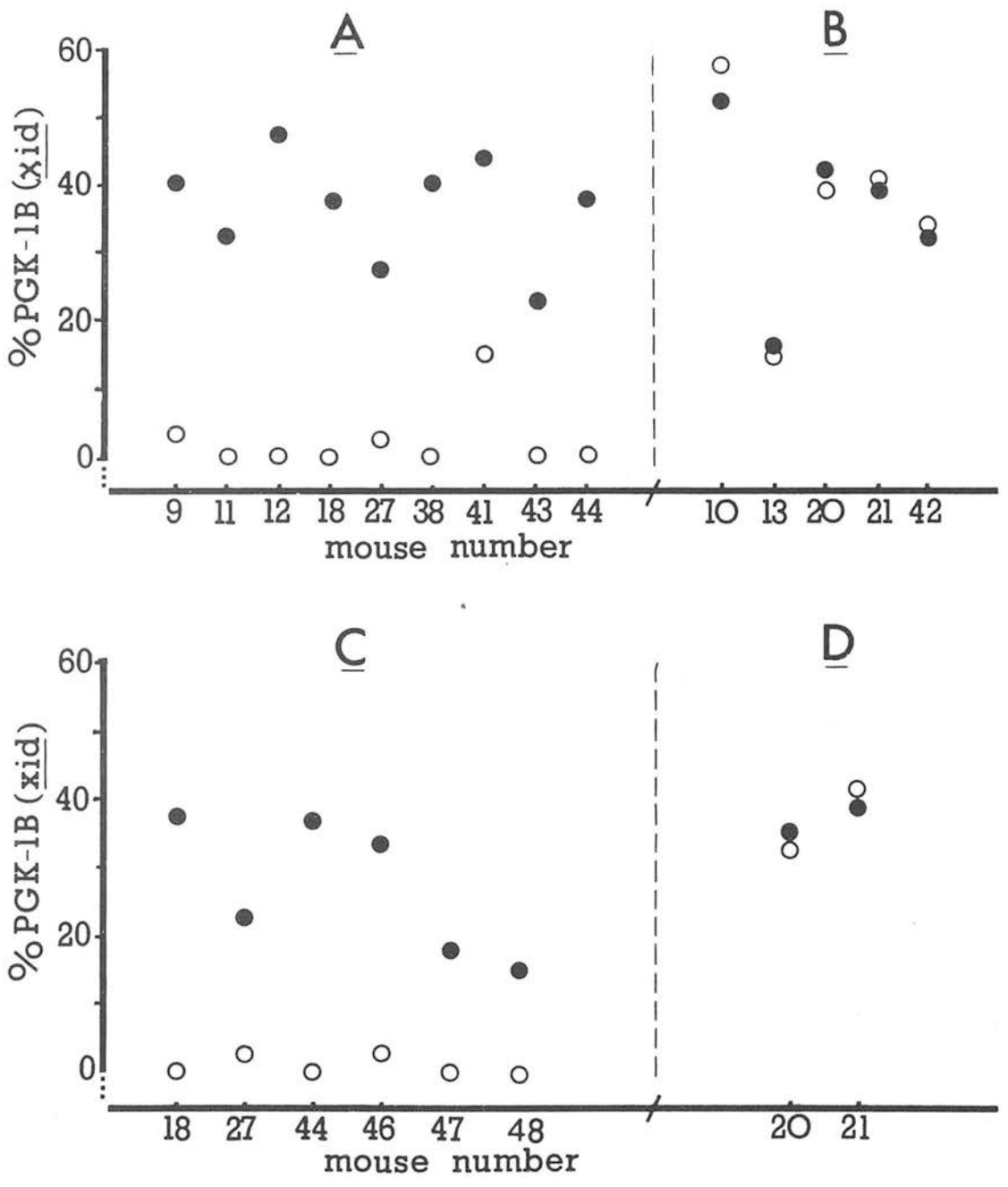

A
B
C
D
60
40
20
0
% PGK-1B (xid)
9 11 12 18 27 38 41 43 44
10 13 20 21 42
mouse number
18 27 44 46 47 48
20 21

Figure 6:4

% xid-bearing cells present in non-B cells, pre-B cells and B cells from the bone marrow of xid/+ and +/+ mice. Figures on the left of the broken line are xid/+ mice, those on the right, +/+ mice.

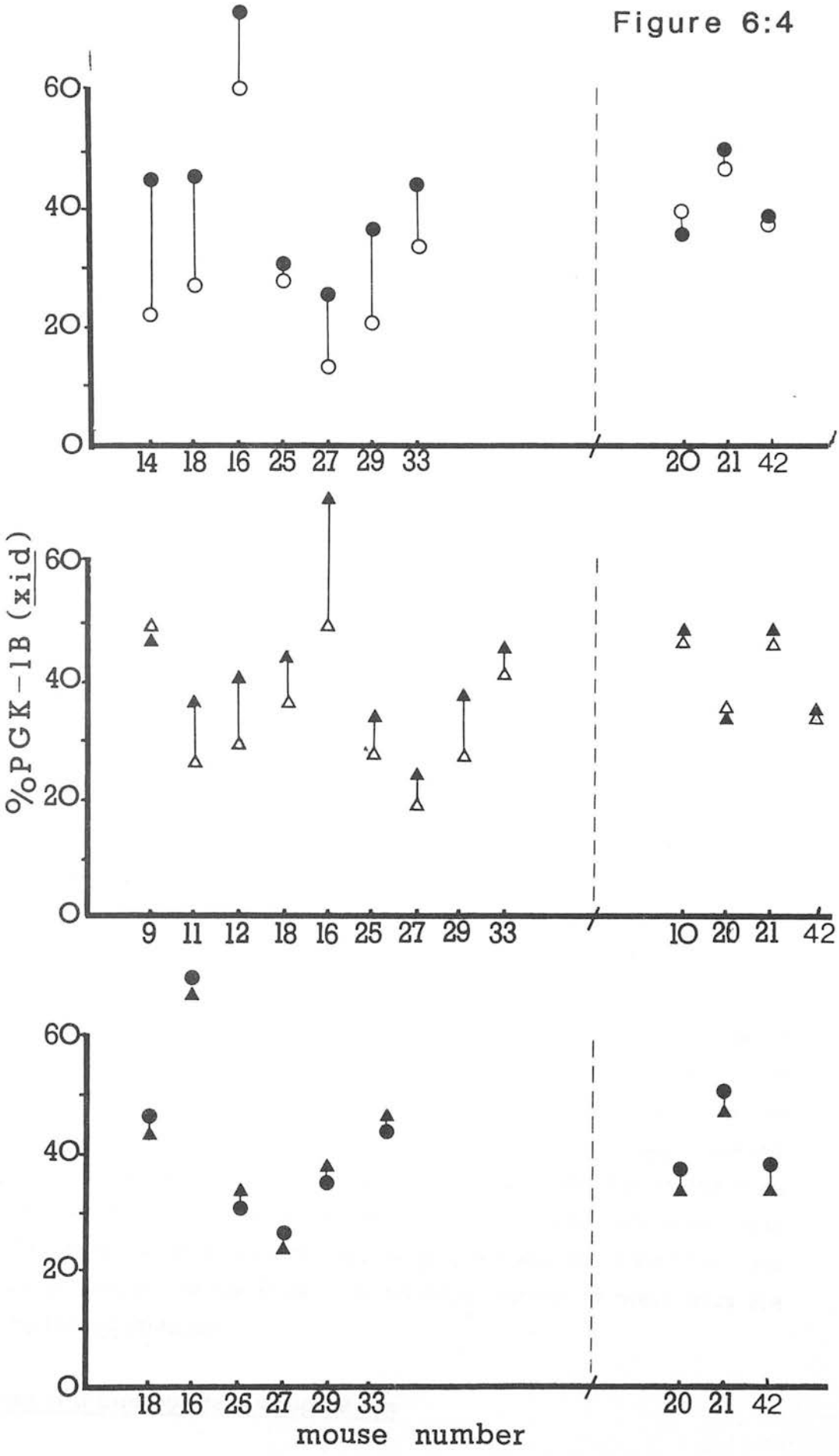
● - sIg^- (non-B cells)

○ - sIg^+ (B cells)

▲ - 14.8 dull (pre-B cells)

△ - 14.8 bright (B cells)

Figure 6:4


60
40
20
0
14 18 16 25 27 29 33
20 21 42
%PGK-1B (xid)
9 11 12 18 16 25 27 29 33
10 20 21 42
18 16 25 27 29 33
20 21 42
mouse number

of the effect of the xid mutation on that population.

5. B Cells from Bone Marrow of Different Ages.

Non-B cells, pre-B cells and mature B cells were isolated from the bone marrow of younger (2-6 weeks) and older (12-20 months) xid/+ mice (Figures 6:5 and 6:6). The % xid-bearing cells present was compared to the adult (2-6 months) mice described above. Appropriated age-matched controls (+/+) were also analysed. The variation in the baseline level of mosaicism between mice made it difficult to pool PGK-1 values for specific cell populations. To overcome this, the difference in PGK-1A values between cell populations was calculated for individual mice and the mean difference calculated for each age group. A large difference between two cell populations will indicate that one or other of them is greatly affected by the xid mutation. Small differences will show that neither or, alternatively, both of the cell populations are affected. Mean differences between cell populations from different age groups are shown in Table 6:3. As expected, none of the differences calculated from control mice was significant. In the 2 week old xid/+ heterozygotes there was no significant difference between B cells and non-B cells, indicating that at this age the B cells of the bone marrow are unaffected by xid. (It was not possible to sort cells on the basis of their 14.8 staining in these animals due to the low numbers of cells present). At 6 weeks of age the mean differences between non-B cells and B cells was 6.8 and between pre-B cells and B cells, 5.2. Both these differences were significant. These results suggest that the xid mutation has some effect on the B cells in the bone marrow at 6 weeks of age but the relatively small imbalance shows that the effect is small. The difference between cell populations of 2-6 month old mice was greater; 12.6 for non-B minus B and 11.1 for pre-B minus B cells. In the 12-20 month age group this difference was increased to 24.5 and 21.9 respectively. The xid mutation, therefore, has a greater effect on B cells of the bone marrow as the mice mature. Figures 6:5 and 6:6 show that in some of the older mice (Nos. 38,43 and 48) the proportion of B cells carring the xid allele was less than 10%. This suggests that virtually all the B cells in the bone marrow of these mice are affected by the xid mutation.

6. Analyses of B Lymphocyte Subpopulations.

Figure 6:85

% xid-bearing cells present in pre-B cells and B cells isolated from the bone marrow of xid/+ mice of different ages.

▲ - 14.8 dull (pre-B cells)

△ - 14.8 bright (B cells)

Figure 6:5

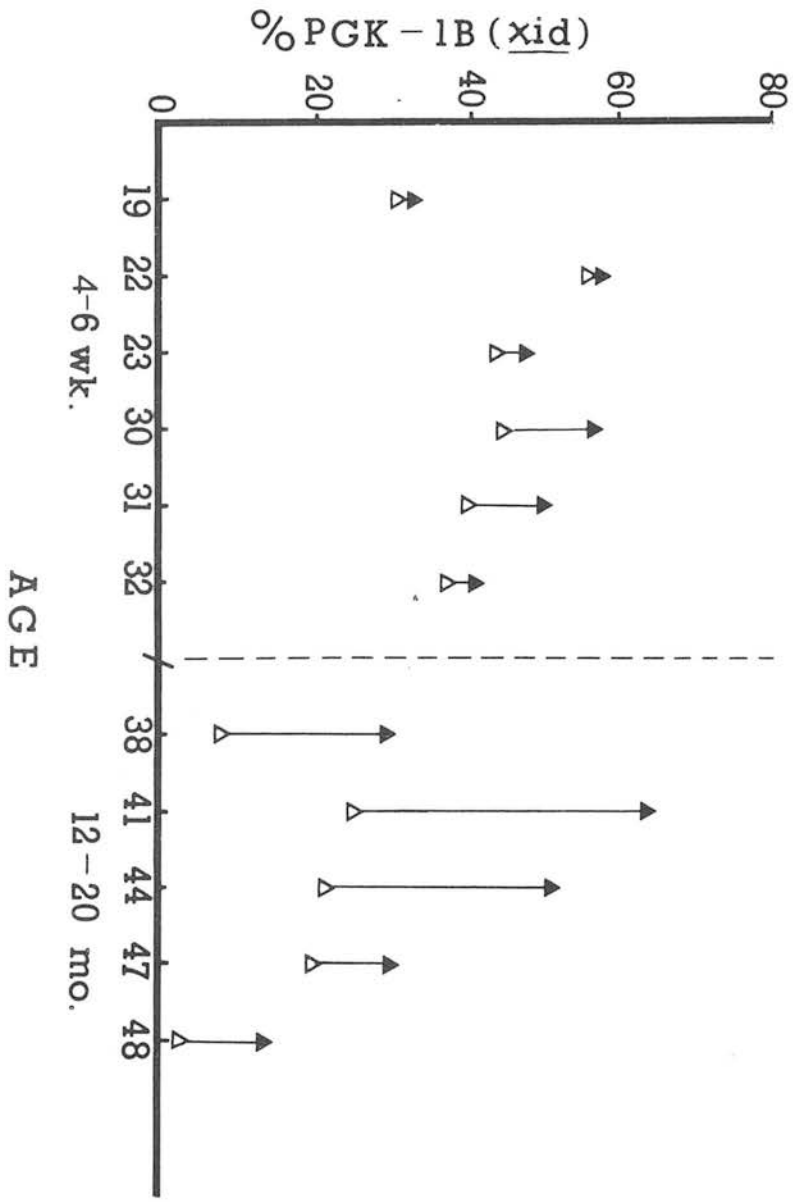

% PGK – 1B (xid)
0
20
40
60
80
19
22
23
30
31
32
38
41
44
47
48
4-6 wk.
12–20 mo.
AGE

Figure 6:6

% xid-bearing cells present in B cells and non-B cells isolated from the bone marrow of xid/+ mice of different ages.

● - sIg^- (non-B cells)

○ - sIg^+ (B cells)

Figure 6:6

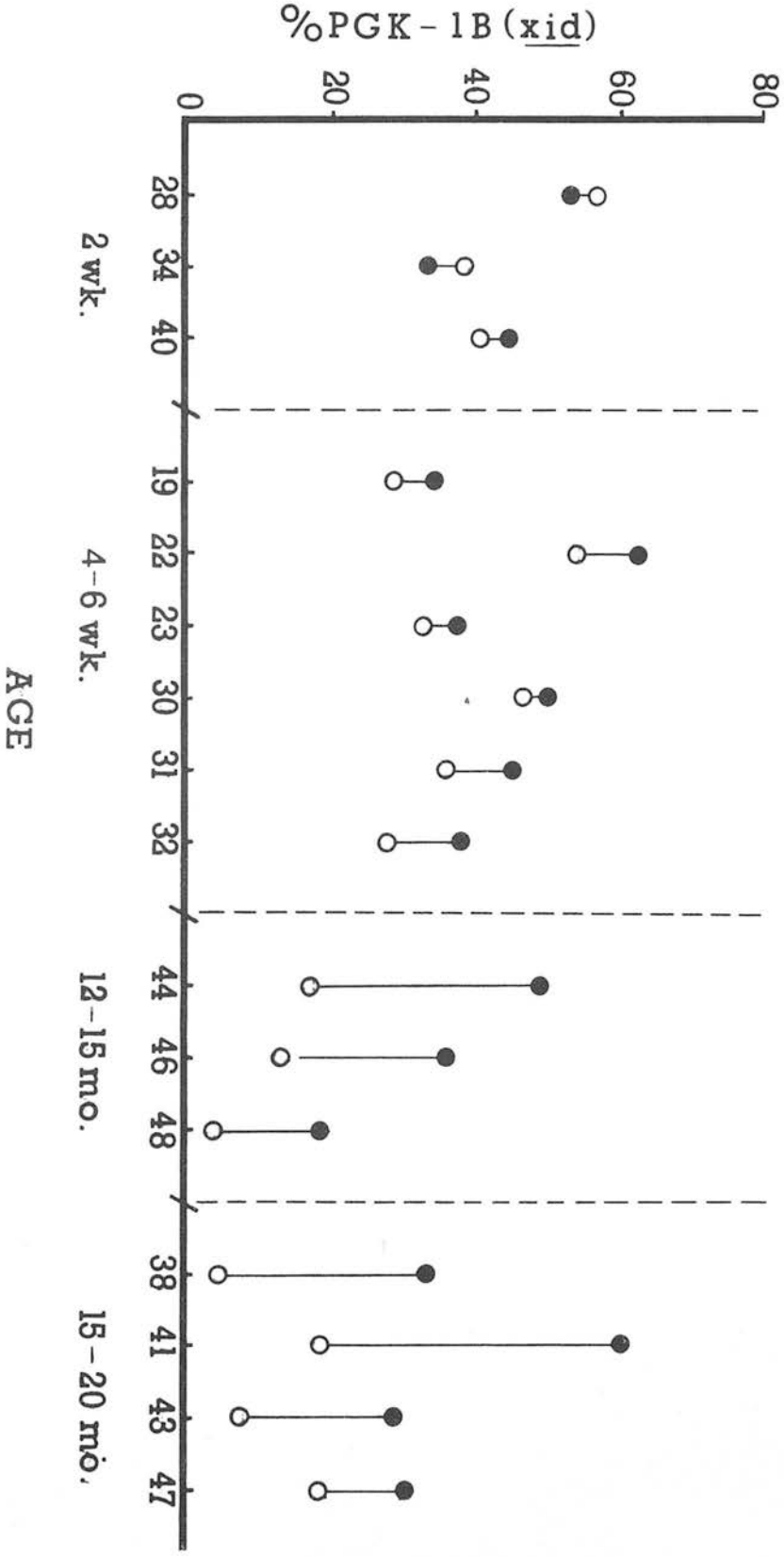

%PGK-1B(xid)
0
20
40
60
80
28
34
40
19
22
23
30
31
32
44
46
48
38
41
43
47
2 wk.
4-6 wk.
12-15 mo.
15-20 mo.
AGE

Table 6:3

Mean difference in %PGK-1A (± SD) between different cell populations isolated from the bone marrow of xid/+ and +/+ mice. The number in square brackets [] refers to the number of mice analysed.
The results of a paired t test are shown in parenthesis.

NS = not signicant
S* = $p<0.05$
S** = $p<0.01$
S*** = $p<0.001$

Age	non minus B	preB minus B	nonB minus preB
Xid/+ Mice			
2 wks [3]	2.4± 2.1 (NS)	N.D.	N.D.
4-6 wks [6]	6.8± 2.6 (S**)	5.6± 4.2 (S*)	-2.5± 4.8 (NS)
2-6 mo [8]	12.6± 6.1 (S**)	11.1± 9.0 (S*)	-0.5± 1.9 (NS)
12-20 mo [7]	24.5± 10.8 (S***)	21.9± 12.0 (S*)	-0.1± 4.9 (NS)
+/+ Mice			
2 weeks [2]	-5.5± 4.4 (NS)	N.D.	N.D.
> 2 mo. [3]	-0.9± 3.2 (NS)	0.62± 1.1 (NS)	0.5± 1.6 (NS)

B lymphocytes can be divided into 3 functionally distinct subpopulations with respect to their relative expression of surface IgD and IgM (Hardy et al, 1982); Population I - high IgD, low IgM, Population II - high IgD, high IgM and Population III - low IgD, high IgM. It has been reported that Population I is missing in CBA/N mice.

Two-colour FACS analysis was used to isolate these three subpopulations from the spleen of xid/+ heterozygotes to examine the effect of the xid mutation on the different B lymphocyte subpopulations.

FACS dot plots of IgM- and IgD-stained splenic lymphocytes from (+/+), (xid/xid) and (xid/+) mice are shown in Figure 6:7. Table 6:4 indicates the percentage of the total lymphocyte population that are present in Populations I,II and III. The results may be compared to those reported by Hardy et al (1982) which are shown in brackets. It is difficult to calculate accurate values for each population as the positioning of the gates to define the populations is decided by the experimenter. In addition, the values obtained in this study were taken from samples where the gates were set up for cell-sorting. To ensure a minimum amount of contamination from other subpopulations it was inevitable that some cells were not included in any of the populations. This may be the reason why values obtained for Population II in this study are substantially lower than that reported by the Stanford group and indeed why the total percentage of B lymphocytes is less than expected. Both studies show that there is a substantial reduction in the proportion of Population I present in CBA/N mice compared to CBA/Ca whereas the other two populations are present in comparable amounts.

The results of the FACS analyses indicate that population I is affected by the xid lesion, but Populations II and III are able to develop in CBA/N (xid/xid) mice. However, the previous results of this section indicate that the mutation effects all B cells in the spleen of heterozygotes: there are virtually no xid-bearing cells present in the B cell fraction. It is possible, but unlikely, that xid-bearing cells of Populations II and III are present in numbers too low to be detected.

To resolve this apparent discrepancy, the 3 subpopulations were isolated from xid/+ mice. Table 6:5 shows the PGK-1 analyses of splenic subpopulations

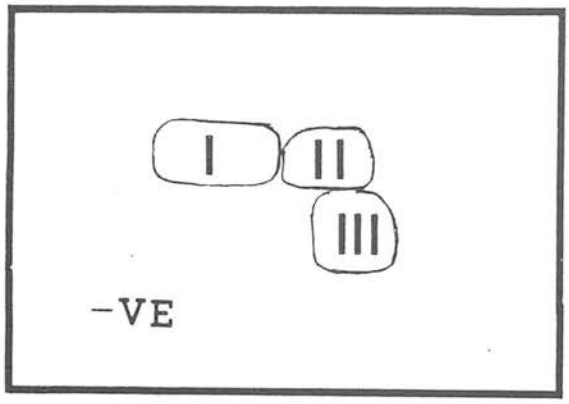
Figure 6:7

FACS dot plot of splenic lymphocytes stained with FITC-RAMIgD (green fluorescence) and TR-RAMIgM (red fluorescence) from three different mice:

Figure	Mouse Strain	*xid* genotype
A	CBA/Ca (control)	+/+
B	CBA/N	*xid*/*xid*
C	CBA/N x CBA/Ca-*Pgk-1*[a]	*xid*/+

Diagrammatic representation of these dot plots, showing:

Population I = high IgD, low IgM
Population II = high IgD, high IgM
Population III = low IgD, high IgM



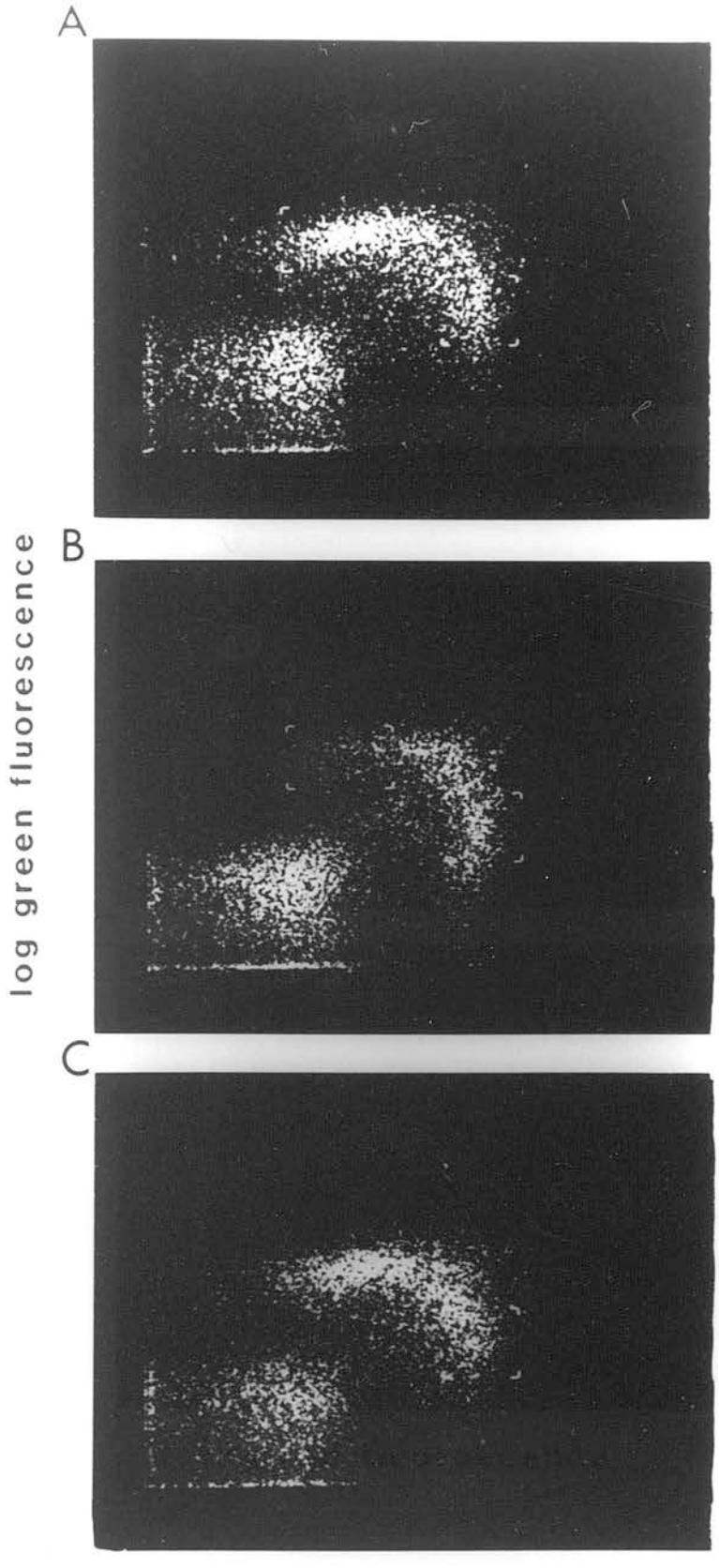

A
B
C
log green fluorescence
log red fluorescence

Table 6:4

Number of cells present in B lymphocyte subpopulations as a proportion of total splenic lymphocytes. See text for definitions of Pops. I, II and III. The value in parenthesis is the value calculated by Hardy *et al*, (1982).

Mouse strain *xid* genotype	CBA/Ca +/+	CBA/N *xid*/*xid*	CBA.PGK-1AxCBA/N *xid*/+
Population I	18 (23)	6 (<4)	19
Population II	9 (18)	9 (20)	10
Population III	10 (8)	11 (15)	10

Table 6:5

% PGK-1B (*xid*) present in erythrocyte, thymocytes, and splenic non-B and B cell subpopulations from *xid*/+ and +/+ mice.

Mouse No.	25	29	33	41	42
Age	3mo.	3.5mo.	6mo.	18mo.	16mo
xid genotype	*xid*/+	*xid*/+	*xid*/+	*xid*/+	+/+
erythrocytes	39	35	38	51	36
thymocytes	28	29	33	56	26
SPL non-B cells	28	30	32	41	34
Pop. I	0	0	0	0	35
Pop. II	4	0	0	0	36
Pop. III	6	10	0	0	38

from 4 adult mice. The results of whole blood and thymi are also given as a comparison. In all 4 xid/+ mice, no xid-bearing cells were detectable in Population I. Populations II and/or III had very low levels in the two of youngest mice (no. 25 and 29) but none in the others. In contrast non-B cells from the spleen, whole blood and thymus samples had a relatively large proportion of xid-bearing cells. It is clear that Populations II and III, as well as Population I, are affected by the xid mutation. Xid-bearing cells in all three populations are at a selective disadvantage in the presence of cells carrying the normal allele for xid.

7. Analyses of B Cell Subpopulations from Young Mice.

Data presented earlier in this chapter (section 5) suggested that bone marrow B cells were unaffected by the xid mutation in young (2 week old) mice and that they became progressively more affected as the mice matured. Splenic B cell subpopulations were analysed to see if the same phenomenon was observed. It is known (Hardy et al, 1982) that normal 2 week old mice are deficient in Population I but Populations II and III are present.

Two-colour FACS analysis was performed to compare the proportions of cells present in the spleens of young xid/+ and adult xid/+ mice (Tables 6:6). Population I is present in very low proportions in 2 week old mice (6%) and rose to a mean of21% in the adult. Population III was present in slightly higher proportions in young mice (17%) and this fell to 11% in the adult. There was no detectable change in the relative proportions of population II as the mice matured.

The proportions of xid-bearing cells present in the B lymphocyte subpopulations of young mice were analysed (Table 6:7). Pooled values for these results and the adult values (from Table 6:5) are shown graphically in Figure 6:8. Population I was present in such low numbers in the 2 week old spleen that it was difficult to get enough cells for accurate PGK-1 analysis. So far, a value for only one mouse has been obtained. There was no detectable xid-bearing cells in Population I of mouse 34, indicating that at this stage these cells were affected by xid, but more results are needed to demonstrate this convincingly. There were virtually no xid-bearing cells in Population I of the 6

Table 6:6

Number of cells present in splenic B lymphocyte subpopulation as a proportion of total splenic lymphocytes.

Mouse No.	Age	Pop.I	Pop.II	Pop.III
34	2wks	7	8	12
39	2wks.	6	9	20
40	2wks.	5	10	19
31	6wks.	12	12	12
32	6wks.	11	10	16
-	4mos.	27	ND	11
-	5mos.	19	10	10
33	6mos.	18	8	12

Table 6:7

% PGK-1B (xid) present in erythrocyte, thymocytes and splenic subpopulation of young mice.

Mouse No.	34	39	40	30	31	32
Age	2wks	2wks	2wks	6wks	6wks	6wk
Erythrocytes	33	38	-	46	37	36
Thymocytes	26	-	52	40	39	40
Spl non-B	36	36	44	42	36	42
Pop. I	0	-	-	2	2	0
Pop. II	9	17	19	21	18	18
Pop. III	16	41	42	29	12	14

Figure 6:8

% xid-bearing cells present in non-B cells and three splenic subpopulations from mice of different ages.

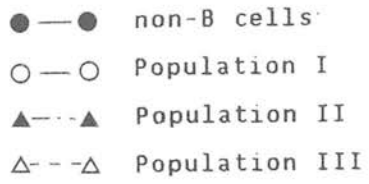
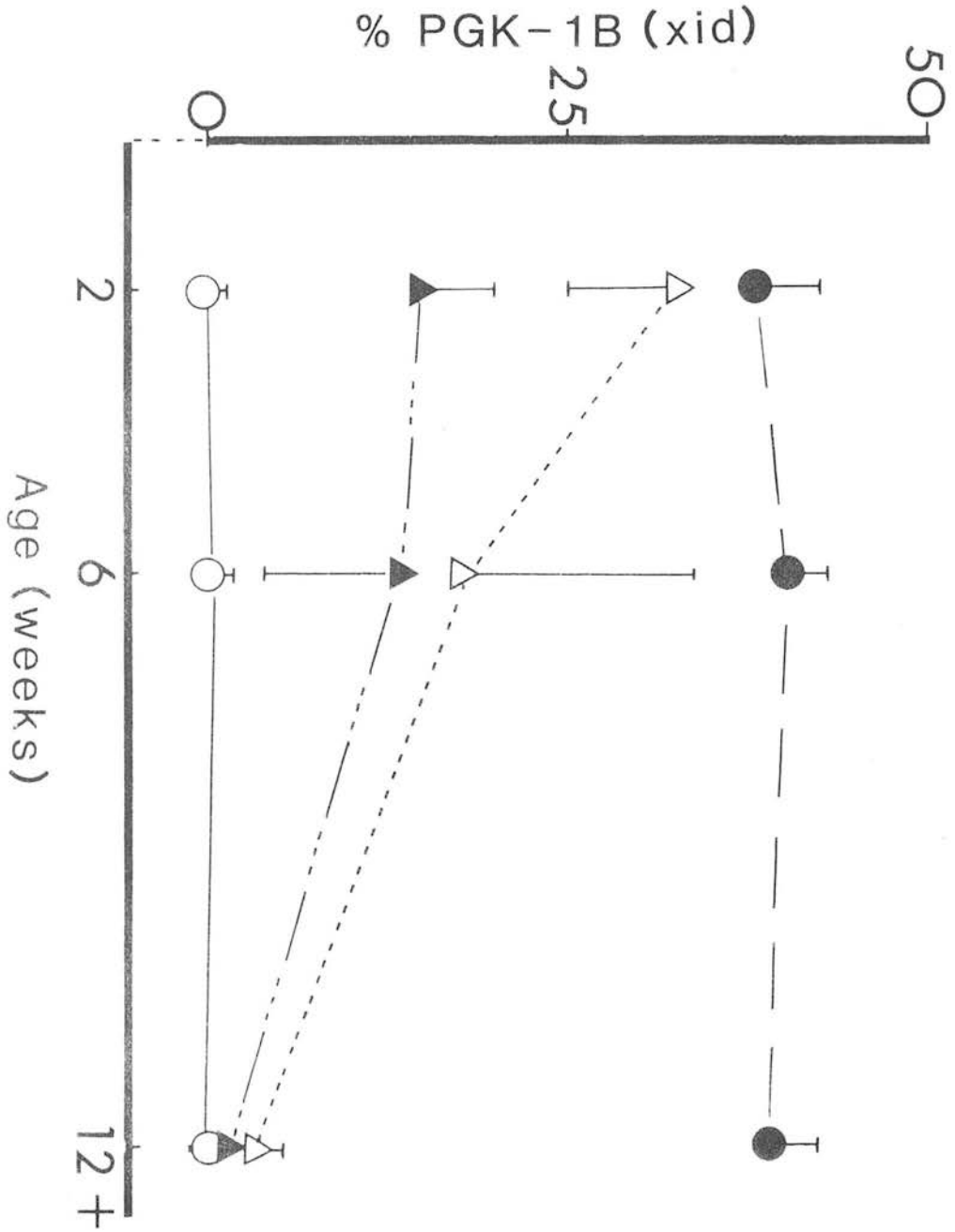


Figure 6:8



week-old mice. At 2 weeks of age Population II had about 15% xid bearing cells and this was not significantly different from the amount at 6 weeks of age (mean, 19%). In the adult this fell to virtually zero. Population III was interesting in that it was present in relatively larger proportions compared to the other subpopulations in 2 week old mice. The PGK-$\sqrt{}$1 value for this population at 2 weeks did not differ significantly from the non-B cells in mice 39 and 40, indicating that it was unaffected by the mutation at this stage. By 6 weeks of age the proportion of xid-bearing cells in Population III fell, which suggests that they were being selected against in favour of cells carrying the normal allele. Clearly, more animals will need to be analysed before any definite conclusions can be drawn about the timing of xid-related effects on these subpopulations.

Discussion.

The immunological defect of CBA/N mice has been shown to involve an intrinsic abnormality of B lymphocytes and/or their progenitors rather than a microenvironmental defect (Scher et al, 1975; Volf et al,1978; Nahm et al, 1983). In this report, B lymphocytes from different tissues and B lymphocyte subpopulations from xid/+ mice have been analysed and compared to non-B cell populations. As the marker system used here was quantitative (Ansell & Micklem, 1986), it was possible to detect different degrees of imbalances in B cell populations. The effect of the xid mutation on different stages of B lymphocyte development in the adult bone marrow and during ontogeny could therefore be assessed.

The erythrocyte lineage is not directly affected by the xid mutation: the mean PGK-1 phenotype of red blood cells from a large number of xid/+ mice was not significantly different from +/+ mice. There was a large and similar variation between individual mice in the two groups, but the mean probability of expression of the Pgk-1^a- X chromosome was 70%. This value reflects the nonrandom nature of the X-inactivation process, which is influenced by various genetic and parental factors. There was no detectable difference in these factors between the xid/+ and +/+ mice.

As the erythrocytes were unaffected by the xid mutation, it was reasonable to use the PGK-1 phenotype of this population as the baseline level of mosaicism for an individual. Any imbalances in other cell populations were assessed by comparing them to this baseline level. T lymphocytes were not directly affected by the xid mutation; this agrees with reports that T cell function is normal in CBA/N mice (Scher et al, 1975; Benca-Kaplan & Quintans, 1979; Janeway & Barthold, 1975). Also, in lethally-irradiated mice reconstituted with a mixture of normal and CBA/N bone marrow, peripheral B lymphocytes were, in the long term, repopulated by the normal marrow only, whereas both donors contributed to the T cell lineage (Sprent & Bruce, 1984b; Witkowski et al, 1985). Granulocytes as well as non-haematopoietic cells were apparently unaffected by the xid mutation.

B cells isolated from the peripheral lymph node and spleen of xid/+ mice were

virtually devoid of xid-bearing cells confirming the view that the xid defect has a direct effect on the B-lymphocyte lineage.

Adult CBA/N mice are deficient in a subpopulation of B lymphocytes that has been designated 'Population I' (Lyb-5^+, high IgD and low IgM) (Hardy et al, 1982). Other splenic B lymphocyte subpopulations, Populations II (Lyb 5^-, high IgD and high IgM) and III (Lyb-5^-, low IgD and high IgM) are present in CBA/N mice in comparable numbers to normal mice so they can obviously develop in the presence of the xid mutation (Hardy et al, 1982). However, our results show that xid-bearing cells in Populations II and III, like Population I, are selected against in the presence of normal cells suggesting that they are also affected. This explains why the imbalance in sIg^+ B cells from the spleen in my experiments and those of Nahm et al (1983) is greater than expected if only Population I cells were defective. Ono et al (1983) have shown that normal adult Lyb-5^- and CBA/N Lyb-5^- B cells differ in their response to phenol-extracted lipopolysaccharide. This agrees with the suggestion that CBA/N B cells are a unique population, not represented in normal mice (Sprent & Bruce, 1984b; Sprent et al, 1985).

The effects of the xid mutation on splenic B lymphocyte subpopulations of young mice (2-6 week old) differed from those of adult mice; the mutation had no apparent effect on population III in 2 week old spleens. This could be interpreted as meaning that xid acts during the maturation of population III. In 2 week-old mice this population could consists of cells at a stage prior to the "block" in the differentiation pathway, while in the adult post "block" cells predominate. This scheme is in agreement with Ono et al (1983) who suggested the existence of at least two Lyb-5^- subpopulations; the xid mutation blocking the differentiation of Lyb-5^-_1 B cells into Lyb-5^-_2 B cells. Population I (Lyb-5^+) is thought to represent a different lineage to Populations II and III, but it is unclear how Populations II and III are related to one another. We have found a slight difference in the effect of the xid mutation between these two cell populations in 2 week-old mice but this does not necessarily reflect a lineage difference.

Kincade et al (1982) have shown that B cell precursors (14.8^+sIg^- and $c\mu^+sIg^-$) appear in normal numbers in the marrow of adult CBA/N mice suggesting that

the xid mutation blocks B lymphocyte development at a later stage. This is confirmed by unpublished results from our laboratory suggesting that CBA/N bone marrow contains a normal number of 14.8-dull cells. However, the fact that they are present does not prove that they are totally unaffected by the xid mutation (as PGK-1 analyses of splenic subpopulations have shown). Data presented in this study show that pre-B cells (14.8 dull, sIg^-) carrying the xid mutation are not selected against in xid/+ bone marrow, indicating that they are unaffected. This is in agreement with Reid & Osmond (1985) who report that pre-B cell proliferation and small lymphocyte production proceed at a similar rate in the bone marrow of CBA/N and normal mice.

Paige and his colleagues (1979, 1981) found that sIg^+-depleted adult bone marrow could regenerate the B cell compartment of unirradiated CBA/N mice and suggested that CBA/N mice were deficient in at least a subpopulation of pre-B cells. The rationale behind this conclusion comes from the, perhaps over simplified, view that it is only possible to engraft unirradiated mice with cell types that are deficient in the recipient (Paige et al, 1979). Foetal liver cells were more efficient than bone marrow in grafting unirradiated CBA/N mice which may suggest that the pre-B cells affected by xid are prevalent in foetal liver and only represent a subset of the pre-B cells of the adult marrow. This would indicate that B lymphocyte subpopulations diverge at an early stage. In this report the imbalance in the PGK-1 phenotype of the total pre-B cell population might be too small to be detected if only a small subpopulation were affected. It would be interesting to analyse the PGK-1 phenotype of pre-B cells of xid/+ foetal liver to see if any imbalance is detected there.

An alternative explanation for the higher efficiency of engraftment with foetal liver cells could be the fact that these cells have an increased ability to proliferate and self-renew (Micklem et al, 1972) at least in CBA mice. Quans and his colleagues (1981) demonstrated that the engraftment of the B lineage of CBA/N mice with foetal liver cells was greatly affected by the number of cells transferred and was increased by a low dose of irradiation. This suggested that it was not simply a question of rapidly filling a defective cell compartment. The authors suggest that the donor cells have to wait for the appropriate microenvironment which the host cells still occupy even though they are arrested at some stage in their maturation. Transplanted populations

must compete to establish themselves in these spaces before they can differentiate.

There was a consistent imbalance in the sIg^+(14.8-bright) cell population in the bone marrow of adult xid/+ mice indicating that some of these cells were affected by xid. The affected cells could represent (a) the most mature bone marrow B cells of single or multiple lineages or (b) the descendants of a pre-B cell subpopulation (Paige et al, 1979) that eventually gives rise to the peripheral lymphoid B cells. The high rate of B lymphocyte production in the rodent bone marrow (sufficient to replenish the peripheral pool in only 4 days (Opstelten & Osmond, 1983; Bazin et al, 1985)) and the apparent longevity of B cells within secondary lymphoid organs (Kumararatne & MacLennan, 1981; Sprent & Basten, 1973) implies that only a small proportion of B cells produced in the adult bone marrow become part of the mature lymphocyte pool (Kumararatne et al, 1985). A proportion of B cells produced in the bone marrow (and possibly unaffected by xid) may then be rapidly eliminated.

The xid mutation may, therefore, act on a subpopulation of B cells as they mature within the marrow. Alternatively, it is possible that the mutation acts after the cells have moved to the periphery and the imbalance seen in the bone marrow sIg^+ population may represent recirculating B cells. The imbalance becomes greater as the mice mature, possibly reflecting the increased number of recirculating cells in the bone marrow.

A number of reports that have indicated that certain immune defects of CBA/N mice improve with advanced age (>12 months) (Rosenberg, 1979; Fidler et al, 1980; Scher, 1982 (review)). Fidler et al (1980) found that 12-14 month old CBA/N splenocytes gave a positive response to TNP-Ficoll. We found no evidence in favour of this view, but it is possible that the relative number of cells involved in the unstimulated animal could be small, beyond the limits of detection. There is, however, a substantial amount of confusion in the literature on this issue (Nariuchi & Kakiuchi, 1981; Scher, 1982 (review)).

Eldridge et al (1984) identified a subpopulation of mature lymphocyte in Peyers patches (PP) of CBA/N mice that expressed the surface markers Lyb-5 and low IgM (therefore comparable to Population I) and could respond to TNP-ficoll.

The transfer of CBA/N PP B cells into CBA/N recipients resulted in the ability of these adoptive hosts to produce splenic PFC against this antigen. This suggested that a B cell population with the surface phenotype and functional properties of Population I can develop in CBA/N mice, but they appear to be restricted to Peyers patches. It would be interesting to examine the PGK-1 phenotype of these cells in our xid/+ animals. It is possible that mobilization of these Peyers patch cells increases with age, providing an explanation for their appearance in the spleen of some old mice (Fidler et al, 1980).

The effect of the xid mutation on B lymphocyte development is therefore not as clear-cut as was originally suggested. Different subpopulations, possibly representing separate lineages, are obviously affected by the mutation to different extents. The mutation may act at different stages of maturation in the different lineages.

In the mouse a gene family, XLR (X-linked lymphocyte-regulated) has recently been identified (Cohen et al, 1985a) and there is evidence to suggest that the xid defect occurs within a member of this gene family (Cohen et al, 1985b). Studies using a cDNA clone showed that plasmacytomas from normal mice expressed XLR-RNA, but plasmacytomas carrying the xid mutation did not. The results of studies on tumours of different stages of B lymphocyte development from normal mice suggest a timing of xid gene product expression that is consistent with the results reported. XLR RNA was detected in presecretory-B cell tumours carrying sIgD but not in pre-B cell tumours (cu^+,sIg^-) nor tumours of immature B cells such as those found in neonatal mice (Cohen et al, 1985b). These could possibly be analogous to Population III of 2 week old mice.

Functional genes mapping to the X chromosome in one mammal are often found on the X chromosome in all other mammals (Ohno, 1969). This would suggest that the relatively large number of human immunodeficiencies reflects mutations within the XLR gene family (Darling & Goodfellow, 1985). Bruton's X-linked agammaglobulinaemia (XLA) disease shows similarities to the xid defect in the mouse. The disorder appears to result in a failure of pre-B cells to differentiate into B cells; pre-B cells are present in normal numbers in the bone marrow of XLA patients (Pearl, 1978) but mature B cells are not found in the peripheral circulation or lymph nodes (Vogler et al, 1976). Further studies

using murine X-linked immunodeficiencies may help in the understanding of analogous human disorders.

Summary.

I have used mice which were heterozygous at the xid and Pgk-1 loci to examine the effects of the xid mutation on pre-B cells and B lymphocyte subpopulations. The mutation has no apparent affect on pre-B cells (14.8 dull) in the bone marrow. Ig^+ B cells of the bone marrow are unaffected in young mice but become progressively more defective as the mice mature. All splenic B lymphocyte subpopulations are affected by the xid mutation in adult mice. However, in young mice (2 weeks old), the mutation has differential effects on the B lymphocyte subpopulation; at 2 weeks of age Population III (low IgD, high IgM) is apparently unaffected.

CHAPTER 7.

AN ANIMAL MODEL FOR HUMAN CHRONIC LYMPHOCYTIC LEUKAEMIA ?

Introduction

Human leukaemias can be classified according to their apparent cellular origins by virtue of their cell surface markers and intracellular enzymes (see Foon et al, 1982 for review). In addition to the traditional cell surface markers such as surface membrane immunoglobulin (mIg) and receptors for sheep erythrocytes that define B and T lymphocytes, highly specific monoclonal antibodies that distinguish surface membrane antigens have been used to establish their specific phenotype. Such classification may be important for both diagnosis and the direction of treatment.

Human chronic lymphocytic leukaemia (CLL) is generally a disease of mid-old age; the average age for diagnosis is 55 years with a range of 45-75 years (Catovsky et al, 1979). It is a clinically heterogeneous disease ranging from a relatively benign, non-progresssive form to an aggressive type with severe clinical consequences.

Until recently, the diagnosis of the major categories of CLL was relatively straightforward. The majority of CLLs represent monoclonal proliferation of mIg positive B lymphocytes and are thus considered to be a neoplastic expansion of the B cell system (Jaffe, 1980). The mIg is most often mu or delta heavy chain (Fu et al,1975; Aisenberg et al, 1980) and the density of Ig molecules on the membrane is very low with a uniform distribution (Slease et al, 1979). B-CLL cells usually have C'3 and Fc receptors and the B1, BA-1 and Ia antigens (Foon et al, 1982).

Prolymphocytic leukaemia (PLL) is probably a variant of CLL. Patients with PLL generally have extremely high blast counts and splenomegaly, but lack significant lymphadenopathy. PLL cells appear morphologically immature with a fine lacy nuclear chromatin and 1-2 nucleoli; they may contain cytoplasmic granules. These cells generally have a higher density of mIg than CLL cells and express Ia and B1 antigens (Koziner et al, 1980).

Approximately 5% of cases of CLL and PLL result from malignant proliferation of T cells rather than B cells. These cells react with T antisera and anti-T monoclonal antibodies and form rosettes with sheep erythrocytes; they lack mIg (Brouet et al, 1975; Catovsky et al, 1973).

The classification of CLLs has, however, become blurred by reports on the ability of B-CLL and B-PLL cells to react with T cell antisera (Foon et al, 1980) and anti-T monoclonal antibodies (Foon et al, 1982 (review)). It was considered that the ambiguities surrounding the precise cellular origins of the disease might also be reflected in its clinical heterogeneity. Smith et al, (1985) examined the surface glycoproteins of lymphocytes isolated from patients suffering from B-CLL. Abnormalities found in the expression of polypeptides of the leukocyte-common (L-C) antigen were examined. They found that this group of polypeptides could, within a panel of B-CLL patients, range from a typical B cell pattern to a pattern resembling that normally found in T cells. A T lymphocyte pattern was associated with a poor prognosis. It was unclear whether the heterogeneity of the L-C profiles represented a progression of the disease within an individual or was a consequence of a defective differentiation of the B cells - variations between patients resulting from differences in the precise point of maturational arrest. The observation that ,in one patient a change in the glycoprotein profile towards that of the T cell coincided with a clinical deterioration, tended to favour the former explanation.

An animal model for CLL would be useful in the study of the disease and in its treatment. Most spontaneous lymphomas and leukaemias in mice predominantly express T lymphocyte markers (Schevach, Stobo & Green, 1972): an obvious example is the AKR mouse strain (McGrath et al, 1980). B lymphocyte leukaemias have been produced experimentally using the Abelson virus (Abelstein & Rabstein, 1970), Rauscher virus (Schevach, Stobo & Green, 1972) and using the carcinogen, dimethyl-benzanthracene (DMBA) (Haran-Ghera & Peled, 1973; Bergman & Haimovich, 1977). Cell lines grown from such experimentally induced tumours have been important in the biochemical analysis of immunoglobulins and other complex molecules. These tumours, however, are not necessarily good models for human leukaemias of unknown etiology. Leukaemias with a B lymphocytes phenotype have been reported to occur spontaneously in guinea pigs (Congdon & Lorenz, 1954) and mice (Slavin

& Strober, 1978) producing the L_2C and BCL-1 cell lines respectively. These lines have been maintained in vitro and by transplantation and have been thought of as analogues to human CLL. However, care must be taken when interpreting results of transplanted tumours and tumour cells in vitro as they often have different properties to the primary tumour. B and T lymphomas may become progressively more acute as they are passaged (Denton & Symes, 1968) and the ability to grow tumour cell lines in vitro increases as the cells are passaged in vivo (Dexter & Allen, 1983). This suggests that passaging uncouples the primary tumour from some growth restraint. The regular occurence of a spontaneous B cell leukaemia in a laboratory animal might well provide a better model for the human disease.

This chapter describes the appearance of an abnormal cell population in old CBA/Ca experimental mice and in normal old (>2 years) mice of the same strain. A preliminary characterisation of this cell population has been carried out and its possible relationship with human B-CLL discussed.

Materials and Methods.

1. Mice. CBA/Ca-Pgk-1^a mice received five daily intravenous injections of CBA/Ca (PGK-1B) bone marrow cells; $8x10^7$ nucleated cells were injected on the first day and $5x10^7$ on each of the following 4 days. No irradiation or other prior conditioning was given to the recipients. Both host and donor mice were 3-5 months of age. These mice were bled monthly and the PGK-1 phenotype of the erythrocytes were analysed. All mice showed the continuing presence of approximately 40-50% donor cells.

2. FACS analyses and sorting. Nucleated peripheral blood cells were stained with a fluorescein-labelled antiserum to mouse immunoglobulin heavy and light chains (FITC-RAMIg) and sorted as described in Chapter 4.

In the analyses of cell surface antigen expression, the number of viable, nucleated cells were counted using AO/EB fluid (Appendix 1) and made to a concentration of 10^7 per ml. $2x10^5$ cells (20ul) were stained with the appropriate volume of reagents which were directly labelled with fluorochromes

(0.5µl FITC-RAMIg, 1.25µl fluorescein-labelled goat anti-mouse IgD (FITC-GAMIgD) (Noric), 10µl Texas Red-labelled goat anti-mouse IgM (TR GAMIgM) (E-Y Labs.). Staining was performed in 50µl RPMI-BSA in a microtitre plate (96 well). Cells were washed in 4 drops of RPMI-BSA (three times) then resuspended in 100µl for analysis. 50µl of monoclonal rat anti-mouse-Ly1 (53 7.2 supernatant) was incubated with $2x10^5$ cells for 40 min., washed, then incubated with 2µl FITC labelled goat anti-rat Ig (FITC-GARIg) for another 40 mins. The cells were washed again and resuspended in 100µl for analysis. In the two-colour FACS analyses all cells, except lymphocytes, were gated out on the basis of forward and right-angle light scatter.

PGK-1 analysis was performed as described in Chapter 2. All samples were run twice on the ^{14}C system.

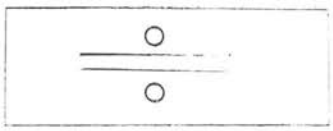
3. Serum protein analysis.

Serum collection. 10 drops of blood were collected in a 1.5ml Sarstedt tube and left for 2 hours at 4°C before centrifugation at 250g for 10 mins. The supernatant serum was removed, aliquoted (50µl) and stored at -20°C until analysed.

Serum protein gels. Serum proteins were separated electrophoretically using Titan III cellulose acetate membranes (Helena Labs.) at pH 8.2 in 0.05M sodium barbital (veronal buffer). The membrane was soaked for 20 mins. in this buffer, blotted then serum samples containing 0.05% bromophenol blue (BPB) were loaded at the cathodal end of the membranes. Electrophoresis was performed at a constant voltage of 200 volts (initially 5mA/membrane) for 40 mins at 4°C. Membranes were stained for 5 mins. in Napthalene black, blotted then destained for 20 mins. in a solution of 10% acetic acid and 24% alcohol. After clearing in 29% acetic acid, 67% methanol and 4% Clearaid (Helena Labs) for 5 mins the membranes were dried at 37°C.

4. Immunoelectrophoresis .

Agar gels were made by coating glass slides with 1.2% Noble agar in Veronal buffer (0.05M sodium barbital). A trough and wells were cut out as indicated below after agar had set.



Test serum was placed in one well and control serum in the other. Electrophoresis was performed at 250 volts for 80 mins. at room temperature in veronal buffer. The trough was then filled with the appropriate antiserum and incubated overnight in a cold (4°C), humid chamber. The gels were washed in veronal saline (0.25M NaCl, 0.02M sodium barbital) for 24 hours, changing the veronal saline 3 or 4 times during this time. Gels were soaked in distilled water for 5 mins, covered with damp filter paper and dried at 37°C overnight. Gels were stained in Napthalene black for 5 mins and destained for 20 mins. Immunoglobulin precipitin arcs were viewed using a light box.

Results.

1. Original observation.

FACS analysis of FITC-RAMIg stained peripheral blood cells from six experimental animals revealed an unusual staining profile. These CBA/Ca-Pgk-1^a mice had been given a total of 2.8 x 10^8 CBA/Ca (PGK-1B) bone marrow cells without prior irradiation 14 months previously and the donor contribution to the erythrocyte lineage had been monitored monthly by PGK-1 analysis.

Figure 7:1(A) shows a typical FACS dot-plot of peripheral leukocytes from a normal, young CBA/Ca mouse stained with FITC-RAMIg; an Ig^+bright population is present, together with a small number (<5%) of duller-staining Ig^+ cells. Two of the experimental mice had a profile similar to that of normal mice. However, in the other 4 mice there was a distinct cell population which had a Ig^+dull phenotype and a slightly higher right angle scatter than normal lymphocytes (Figures 7:1(B) and 7:1 (C)). This cell population formed a variable proportion of the total lymphocytes: two mice were as Figure 7:1(B) and two were as Figure 7:1(C). These mice were bled again at various time intervals and the amount of Ig^+dull cells present, as a proportion of the total peripheral white blood cell (wbc) population, was recorded (Table 7:1). From each blood sample, the four different cell populations indicated in Figure 7:1(D) (Ig^-, granulocytes, Ig^+dull and Ig^+bright) were sorted and the PGK-1 phenotypes were analysed (Figure 7:2).

The results showed that there was a consistently larger donor component in the Ig^+dull population than in any other cell type of the peripheral blood. There had been 8 mice (Br1 - Br8) in this group originally. Br6 showed no increase in Ig^+dull cells before it died, Br1 died before testing and the other six are recorded below (see also Figure 7:2).

Br 2. No Ig^+dull cells were detected in the first sample from this mouse but, four months later 19% of the total white blood cells (wbc) were Ig^+dull. PGK-1 analysis showed that there was slightly more donor present in this population than in others. There was no apparent change in the cell number or donor contribution in the next four months.

Figure 7:1

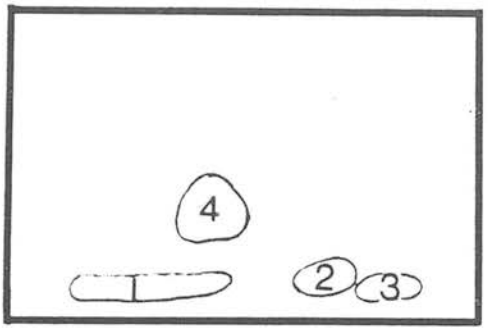
FACS dot plots of FITC-RAMIg-stained peripheral white blood cells from 3 different mice. All three samples were stained and analysed on the same day (19.11.84; see Table 7:1)

Figure	Mouse	% Ig^+dull
A	Br2	<5%
B	Br4	Approx. 30%
C	Br7	Approx. 50%

Figure 7:1(d)

Diagrammatic representation of these FACS dot plots.

Population 1 = Ig^- (T cells)
2 = Ig^+dull
3 = Ig^+bright (normal B cells)
4 = granulocytes



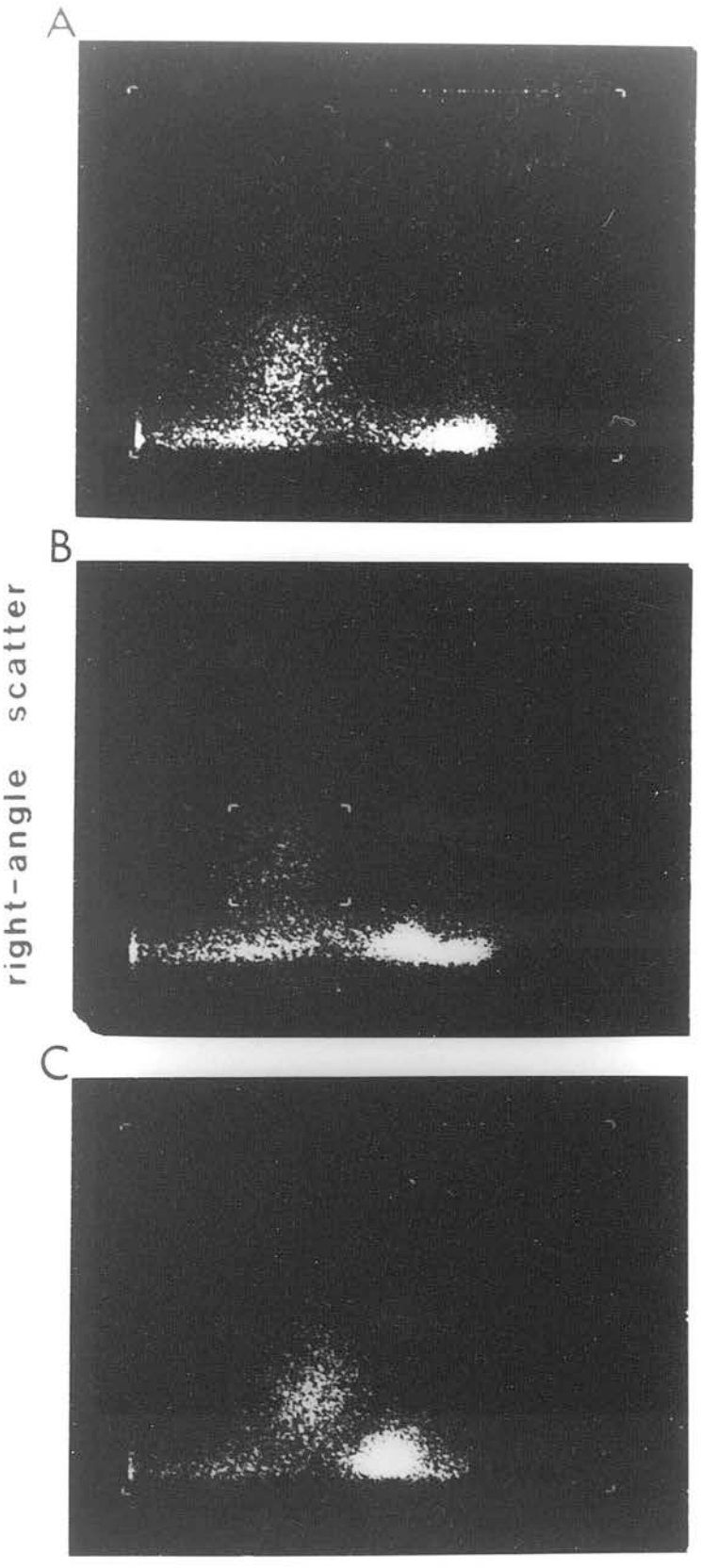

A
B
C
right-angle scatter
log fluorescence

Table 7:1

Number of Ig^+dull cells present as a percentage of the total peripheral white blood cells of 6 mice. The PGK-1 phenotypes of different cell populations of these mice are shown in Figure 7:2.

Sample	Date	Br2	Br3	Br4	Br5	Br7	Br8
a	19.11.84	<5%	20%	ap.30%	37%	ap.50%	N.D.
b	19.12.84	N.D.	N.D.	ap.30%	N.D.	ap.50%	17%
c	18.2.85	N.D.	N.D.	N.D.	49%	75%	N.D.
d	11.3.85	19%	33%	52%	N.D.	N.D.	N.D.
e	8.7.85	17%	N.D.	N.D.	N.D.	N.D.	24%

N.D. = not done
ap. = approximately

Figure 7:2

% PGK-1B (donor) present in different cell populations isolated from 6 different mice. (a - e refers to the sampling date.)

a = 19.11.84
b = 19.12.84
c = 18.2.85
d = 11.3.85
e = 8.7.85

See Table 7:1 for proportions of Ig^+dull cells present at different sampling times.

Colour code:

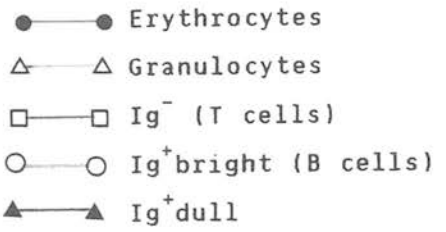
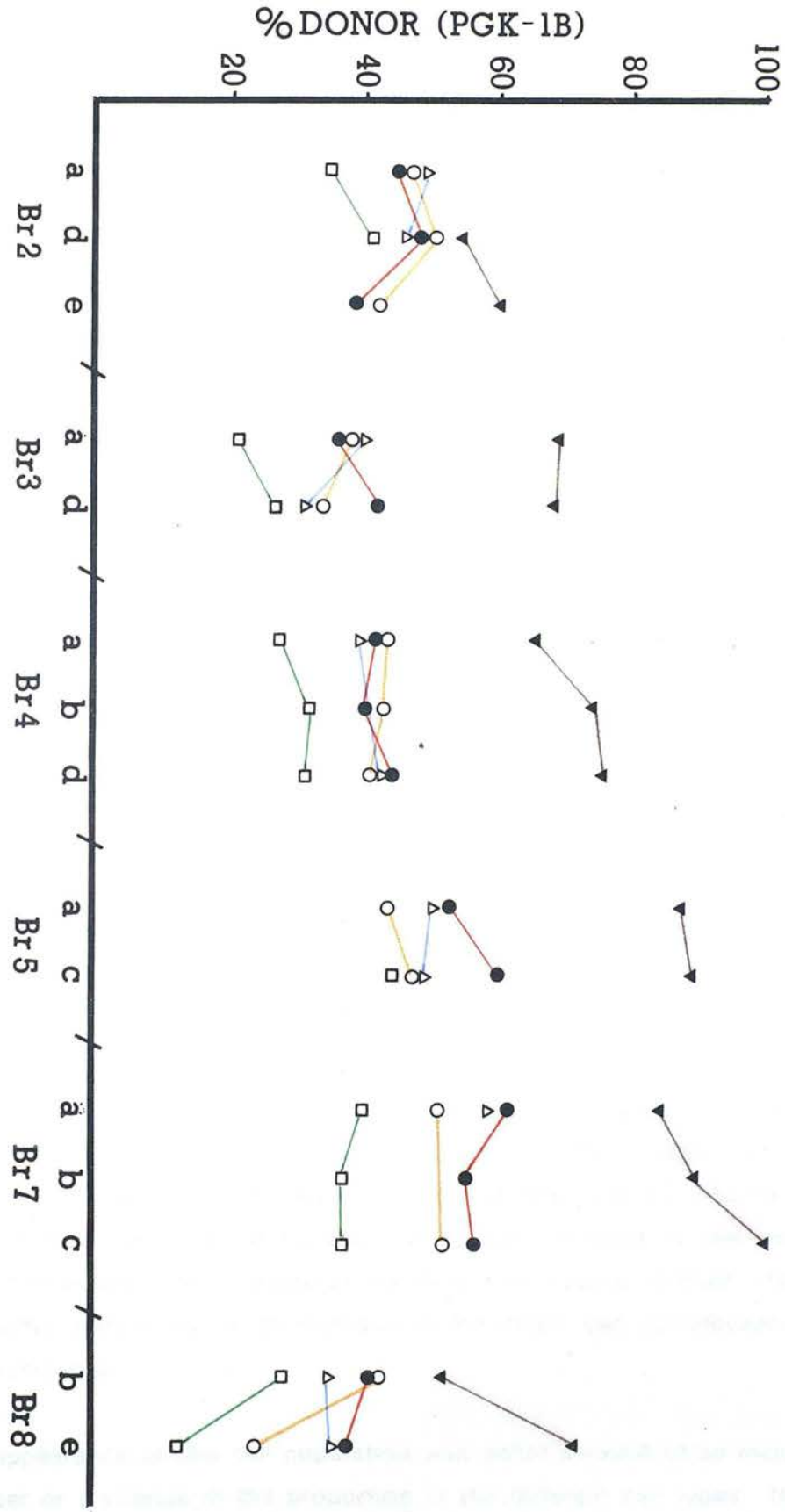


Figure 7:2


%DONOR (PGK-1B)
20
40
60
80
100
a d e
Br2
a d
Br3
a b d
Br4
a c
Br5
a b c
Br7
b e
Br8

Br 3. The proportion of Ig^+dull cells present in the peripheral blood of this mouse increased from 20% to 30% of the total wbc in the four months between the first and second samples. The donor contribution to this population was consistently higher (70%) than to any of the other cell types (30-40%).

Br 4. In the first sample the proportion of cells in the Ig^+dull population was estimated from the FACS dot plots to be approximately 30%. This rose to 52% in the next four months. The donor contribution to this cell population rose from 65-75% during the test period whereas other cell types remained constant at about 40%.

Br 5. 37% of the total wbc of this mouse were Ig^+dull at the first sample and this rose to 49% in the following three months. The donor contribution was 85-90% compared to 50-60% for the other cell types.

Br 7. There was a large proportion (approx. 50%) of Ig^+dull cells present in the first two tests of this mouse and this rose to 75% over the next four months. There was no detectable host present in the tumour cell population. At this point Br7 was dissected and its spleen and bone marrow transplanted (see later, Sections 2 and 3).

Br 8. There was a relatively small number of Ig^+dull cells in the first test (15-20%). This rose to 24% after seven months. The donor contribution rose from 50 to 70% in this population.

The main conclusion drawn from the analyses of these mice was that an abnormally high proportion of a cell population that carried a low level of surface immunoglobulin appeared in the peripheral blood. These cells were derived from donor bone marrow. A gradual increase in the proportion of Ig^+dull cells was seen in some of the mice over several months. This increase was often parallelled by an increase in the donor cell contribution within the cell population.

The appearance of this cell population was either a result of an increase in cell number or a change in the proportion of the different cell types. Normal mice

have a small proportion (<5%) of these cells in the peripheral blood. A slight-moderate elevation of circulating wbc numbers was noted in mice that had raised proportions of Ig^+dull cells (Table 7:2).

2. Tissue distribution.

Spleen, lymph node and bone marrow of Br 7 were stained with FITC-RAMIg and analysed on the FACS (Table 7:3). The results show that the Ig^+dull cells were present in the bone marrow (22% of the nucleated cells in the bone marrow of Br 7 were Ig^+dull compared to 5% in controls) and were the predominant population (93%) in an obviously enlarged spleen. Only 8% of the lymph node cells of Br 7 were Ig^+ and the nodes were not enlarged; this suggests that that the Ig^+dull cells did not populate the lymph nodes in this mouse.

Figure 7:3 shows the results of PGK-1 analyses of unsorted bone marrow and spleen and Ig^-, Ig^+dull, Ig^+bright and granulocytes isolated from these organs. The results show clearly that Ig^+dull cells were largely, if not entirely, donor-derived. The unsorted spleen cell preparation was used in the following transplantation experiments.

3. Transplantation.

10^7 spleen cells (predominantly PGK-1B) from Br 7 were injected intra venously into 4 irradiated (2.0 Gy) CBA/Ca-Pgk-1^a recipients (SP1,2,3 and 4). The peripheral blood of one of the recipients (SP1) was tested at various intervals for the presence of Ig^+dull cells (Table 7:4). The alloenzyme phenotype of the different cell populations was also determined. One month after transplantation 11% of the wbc of the recipient were Ig^+dull and whereas there was no donor PGK-1 (ie. PGK-1B) in the erythrocytes or normal B cells there was 68% PGK-1B in the Ig^+dull population. Three months later, 37% of the recipient's wbc were Ig^+dull and this population was 60% PGK-1B. By 8 months after transplantation 71% of the peripheral wbc were Ig^+dull cells. This showed clearly that it was possible to transplant Ig^+dull cells and that they could proliferate in the recipient. PGK-1 phenotypes and FACS profiles of the other recipients were monitored and showed similar results.

Table 7:2

Comparitive cell counts from several "leukaemic" mice and a young CBA/Ca mouse.

	Mouse	Cells/ml* ($\times 10^{-7}$)	Factor of increase (£)
Exp.1	CBA/Ca	0.4	-
	Br2	0.9	2.2
	Br3	0.8	2.0
	Br4	1.7	4.2
	Br5	0.8	2.0
	SP1	1.3	3.0
	SP2	0.8	2.0
Exp.2	CBA/Ca	2.4	-
	Br2	4.4	1.8
	SP1	8.2	3.5

* Ten drops of blood were taken from each mouse and, after osmotic lysis, wbc were resuspended in 0.2ml. Cell counts of the "leukaemic" mice were compared to that of the control mouse for that particular experiment.

(£) Factor of increase = $\frac{\text{Cell count for "leukaemic" mouse}}{\text{Cell count for control}}$

Figure 7:3

% PGK-1B (donor) present in different cell populations isolated from bone marrow and spleen of Mouse Br7.
See Table 7:3 for FACS analysis.

U = unsorted tissue
sIg^- = non-B cells
sIg^+dull = "neoplastic" cells
$sIg^+bright$ = B cells
G = granulocytes

Figure 7:3

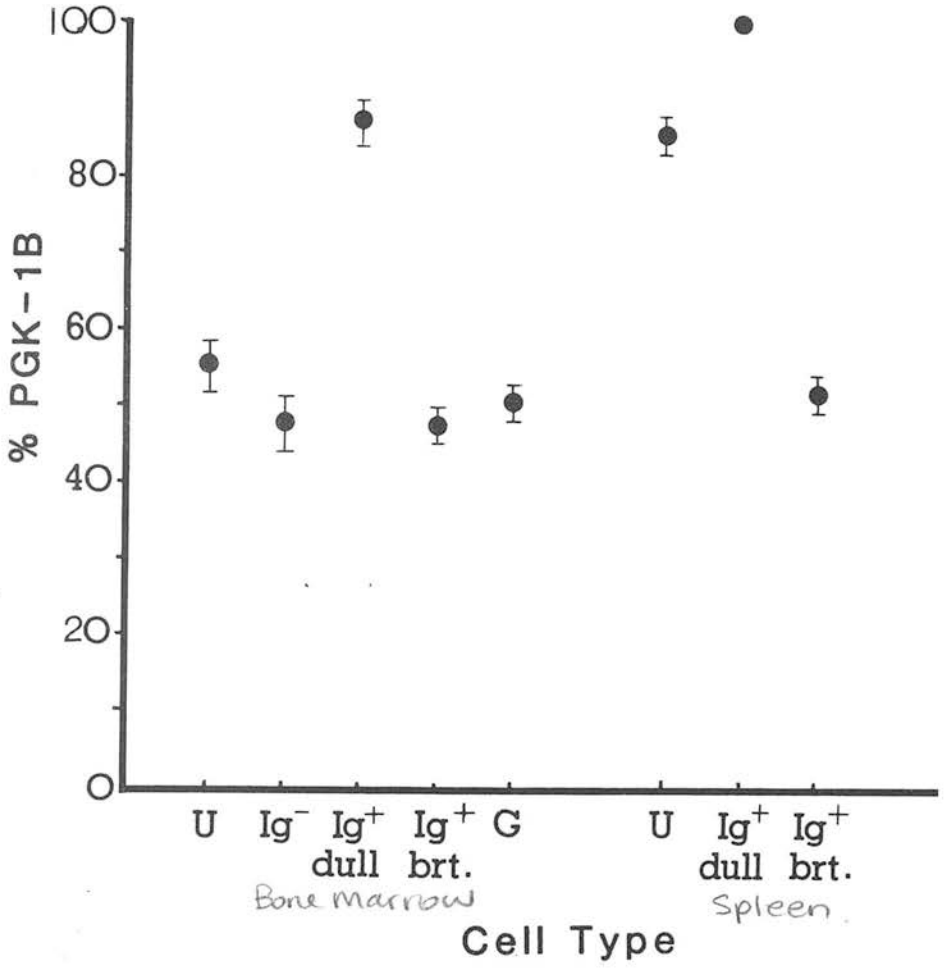

100
80
60
40
20
0
% PGK-1B
U
Ig⁻
Ig⁺ dull
Ig⁺ brt.
G
U
Ig⁺ dull
Ig⁺ brt.
Bone Marrow
Spleen
Cell Type

Table 7:3

FACS analyses of tissues from mouse Br 7 and a normal young CBA/Ca mouse. Cell suspensions were stained with FITC-RAMIg.

Cell Type		Br 7	Control (CBA/Ca)
Bone marrow	Ig^+dull	22%	5%
	Ig^+bright	3%	12%
Spleen	Ig^+dull	93%	5%
	Ig^+bright	2%	47%
Lymph node	Ig^+dull	5%	5%
	Ig^+bright	2%	13%

Table 7:4

Percentage of Ig^+ cells present in the peripheral blood of mouse SP1 - a recipient of 10^7 spleen cells from Br 7 (donor contribution as measured by PGK-1 analysis is shown in brackets).

Time after transplant (months)	% Ig^+dull	% Ig^+bright
1	11 (68%)	12 (0%)
4	37 (60%)	5 (0%)
5	65 (N.D.)	3 (N.D.)
8	71 (N.D.)	3 (N.D.)

N.D. = not done

4. Morphology.

Spleen cells from mouse Br7 were smeared, fixed and stained with May-Grunwald and Giemsa (Chapter 2, Section 5). The cells had one, or occasionally two, distinct nucleoli and basophilic cytoplasm. There was a considerable variation in size (Figure 7:4).

5. Occurrence in old CBA/Ca mice.

It seemed important to ascertain whether the phenomenon reported in the group of mice reported in Section 1 was a feature of normal, old CBA/Ca mice or was confined to bone marrow transplant recipients. The abnormal cells were detected when the recipient mice and the donor marrow were about 18 months old. However, it was possible that the cells in bone marrow transplant itself or the cell loss incurred at the monthly sampling of blood caused the neoplasia.

The peripheral blood of twelve 2.5 year old CBA/Ca mice was tested for the presence of Ig^+dull cells. The full results of the FACS analyses of FITC RAMIg stained peripheral blood from these mice are shown in Table 7:5 and the FACS histograms of 4 old mice are compared to a young CBA/Ca and a transplanted mouse (SP3) in Figure 7:5. The profiles of three old mice (Nos. 6,7 & 8) were similar to those of young CBA/Ca mice; one peak of bright Ig^+ cells was present. In five of the old mice (Nos. 3,4,5,10 & 12) Ig^+dull cells were present and represented 15-52% of the total wbc. Nos.2 and 11 had Ig^+dull populations which accounted for 54-78% of the wbc (cf. Br4); no Ig^+bright cells were detected in these two individuals. One mouse (No. 1) had a very high percentage of granulocytes and monocytes and too few lymphocytes for analysis and mouse No.9 had a smear of Ig^+ cells but no obvious peaks could be discerned. These results indicate that an Ig^+dull cell population is present in some old CBA/Ca mice.

6. Clonality study.

Ig^+dull cells were observed in an old CBA/Ca-Pgk-1^{ab} heterozygous female (C13). PGK-1 is X-linked and X chromosome inactivation results in individual

Figure 7:4

Spleen cells from Mouse Br7 stained with May-Grunwald/giemsa (Chapter2, Section 5).

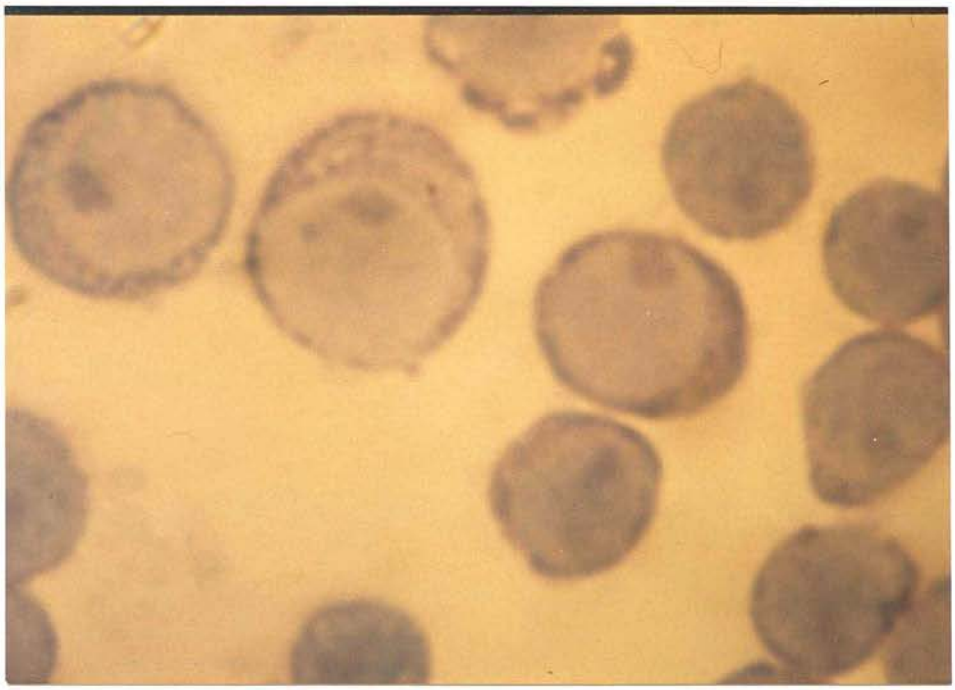


Table 7:5

FACS analyses of peripheral wbc from 1 young CBA/Ca, 3 known "leukaemic" mice and 12 old CBA/Ca (1-12) stained with FITC-RAMIg. The FACS histograms for some of these mice are shown in Figure 7:5.

Mouse	% Ig^+dull	peak	% Ig^+bright	peak
CBA/Ca	3	-	41	187
Br4	73	163	10	189
SP3	23	166	20	188
SP4	20	166	22	190
1	*		*	
2	54	166	<5	-
3	27	162	23	174
4	52	171	19	182
5	21	173	24	186
6	<5	-	55	178
7	9	147	50	179
8	6	150	51	187
9	*		*	
10	49	167	11	191
11	78	159	<5	-
12	15	161	35	184

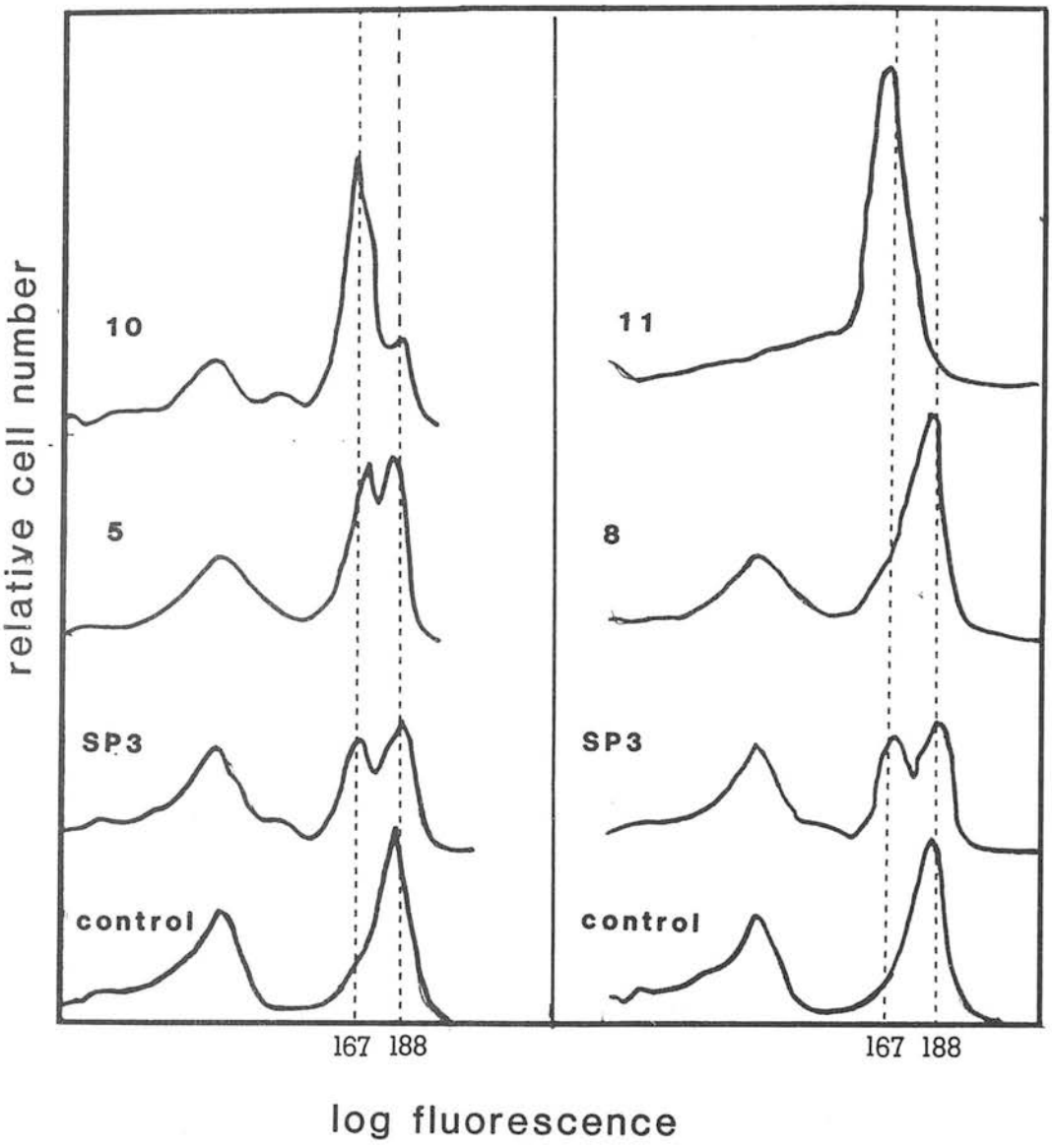
* = reasons for lack of data from these mice is given in the text.

Figure 7:5

FACS histograms of FITC-RAMIg-stained peripheral white blood cells of 4 old mice (No. 5, 8, 10 and 11), one young CBA/Ca control mouse and a known "leukaemic" animal (SP3). The position of the Ig^+dull and Ig^+bright peaks are shown; they were, on average, 20 channels apart.

The results of the FACS analyses are given in Table 7:5.

Figure 7:5


relative cell number
10
5
SP3
control
11
8
SP3
control
167 188
167 188
log fluorescence

cells of heterozygous females expressing either PGK-1A or PGK-1B. Thus if a neoplastic cell population is derived from a single cell it will express only one of the PGK-1 alloenzymes.

This mouse had been bled, at two-weekly intervals, for 5 months and the PGK-1 phenotype of all peripheral blood cells tested had been approximately 80% PGK 1A (see Chapter 4, Experiment 2). The first evidence of the neoplasia was observed when the PGK-1 phenotype of the total lymphocyte population changed from 80% to 60% PGK-1A while the red blood cell and granulocyte phenotype remained unchanged at 80%. Peripheral blood cells were then stained with FITC RAMIg and it was found that 71% of the wbc were Ig^+ (compared to 40% in control mice). The PGK-1 phenotype of this population was 27% PGK- 1A compared to 80% for the other peripheral blood cell types. Two months later 72% of the wbc were Ig^+ and the PGK-1 phenotype of these cells was 15% PGK 1A. This suggests that the neoplasia was derived from a single cell (or at the most, very few cells) expressing the $\underline{Pgk\text{-}1^b}$ X chromosome. The small amount of PGK-1A present was possibly derived from Ig^+bright cells as these cells were not analysed and sorted independently.

These result support the hypothesis that abnormal, possibly monoclonal, expansion of Ig^+dull cells is a feature of old age in CBA/Ca mice. However the PGK-1 heterozygous mouse was similar to Br1-8 in that it had been bled several times prior to the appearance of the neoplasia. It is conceivable that the cell loss at each sampling and/or the ether and trauma could have been causative factors. The screening of old CBA/Ca-$\underline{Pgk\text{-}1^{ab}}$ mice is underway to find out the incidence of neoplasias and to investigate their clonality further.

7. Serum proteins.

The electrophoretic profiles of the serum proteins from three mice (Br3, Br5 and C13) which had a high proportion of Ig^+dull cells in the peripheral blood was indistinguishable from that of normal, young CBA/Ca controls (Figure 7:6). This similarity suggested that the Ig^+dull cells were not secreting any substantial amounts of immunoglobulins. Immunoelectrophoresis confirmed the absence of monoclonal gammopathy (data not shown).

Figure 7:6

Serum protein profiles of several mice which had a high proportion of Ig^+dull cell present in their peripheral blood and several control mice. The proportion of Ig^+dull cells present is given in parenthesis.

A = normal young CBA/Ca control (<5%)

B = Br3 (30%)

C = C13 (70%)

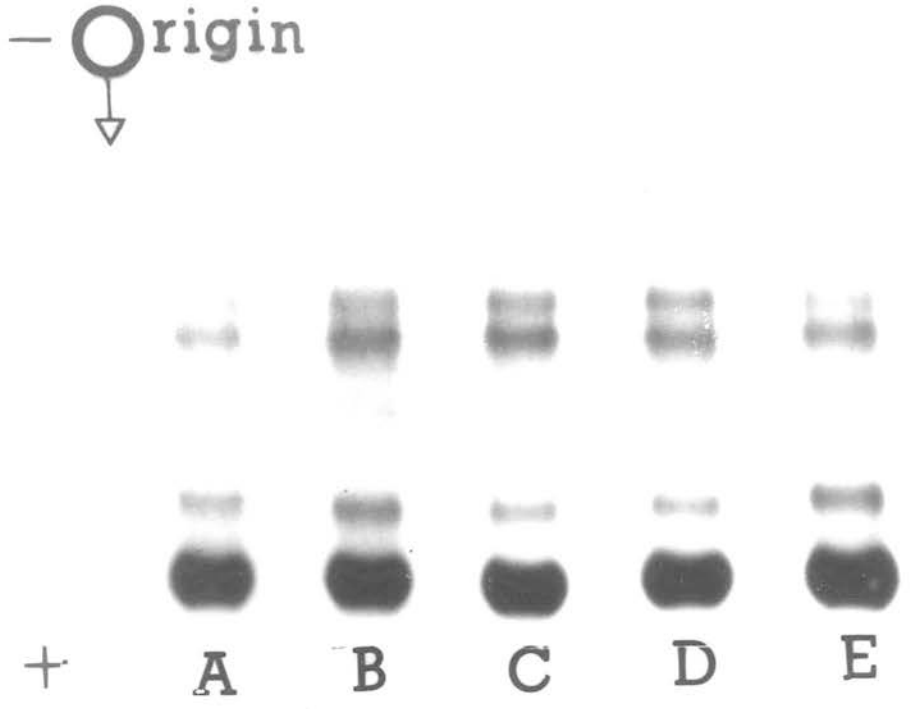
D = C13 (70%)

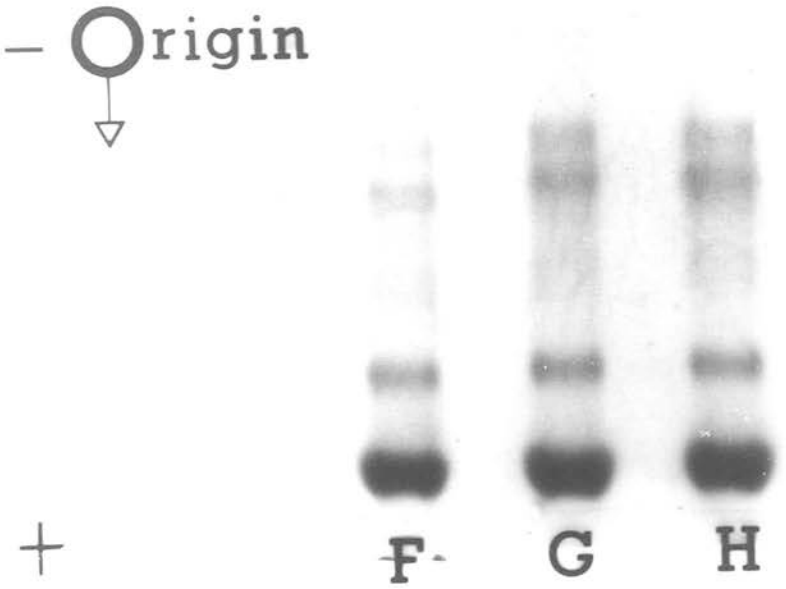
E = CBA/Ca

F = CBA/Ca

G = Br5 (50%)

H = Br3 (30%)


– Origin
A
B
C
D
E
+


– Origin
F
G
H
+

8. Cell surface characterisation.

The relative expression of the surface markers IgD and Ly-1 was studied in a series of 4 mice which possessed various proportions of Ig^+dull cells in the peripheral blood (Table 7:6). The increase in Ig^+dull cells within the series was paralleled by an increase in cells expressing low levels of IgD.

B lymphocytes of normal mice can be divided into subpopulations with respect to their relative expression of IgD and IgM (see Chapter 6). B lymphocytes expressing a low level of IgD and a high levels of IgM belong to "Population III". Preliminary two-colour FACS analysis of the peripheral blood of the above series of mice indicated that the neoplastic cells also belonged to this population (Figure 7:7). In addition these Ig^+dull cells also expressed low levels of Ly-1. Thus they appeared similar to some other B lymphomas previously desribed (Lanier et al, 1981) and to normal Ly-1 B cells (Hayakawa et al, 1983).

Table 7:7 shows that the neoplastic cells of old CBA/Ca mice (No.10 and C13) and the transplanted mouse, SP1, also expressed a low level of IgD. In this respect they are similar to the cells in the original experimental group.

Table 7:6

FACS analyses of peripheral lymphocytes from 3 known "leukaemic" mice and one young normal CBA/Ca mouse. Cells were stained with (a) FITC-RAMIg; (b) FITC-RAMIgD and (c) RAM Ly1 + FITC-GARIg.

	Cell Population	CBA/Ca (control)	Br2	Br3	Br5
(a)	Ig^+ dull	<5%	6%	32%	50%
	Ig^+ bright	41%	56%	30%	17%
(b)	IgD dull	6%	13%	19%	37%
	IgD bright	30%	34%	28%	18%
(c)	Ly-1 dull	0%	N.D.	12%	30%
	Ly-1 bright	48%	N.D.	24%	18%

Table 7:7

FACS analyses of four "leukaemic" mice and one normal young CBA/Ca. Cells were stained with reagents (a) and (b) described in Table 7:6.

	Cell Population	CBA/Ca (control)	Br4	old 10	C13	SP1
(a)	Ig^+ dull	<5%	80%	53%	84%	73%
	Ig^+ bright	20%	6%	13%	<5%	<5%
(b)	IgD dull	<5%	69%	55%	77%	60%
	IgD bright	18%	11%	11%	6%	<5%

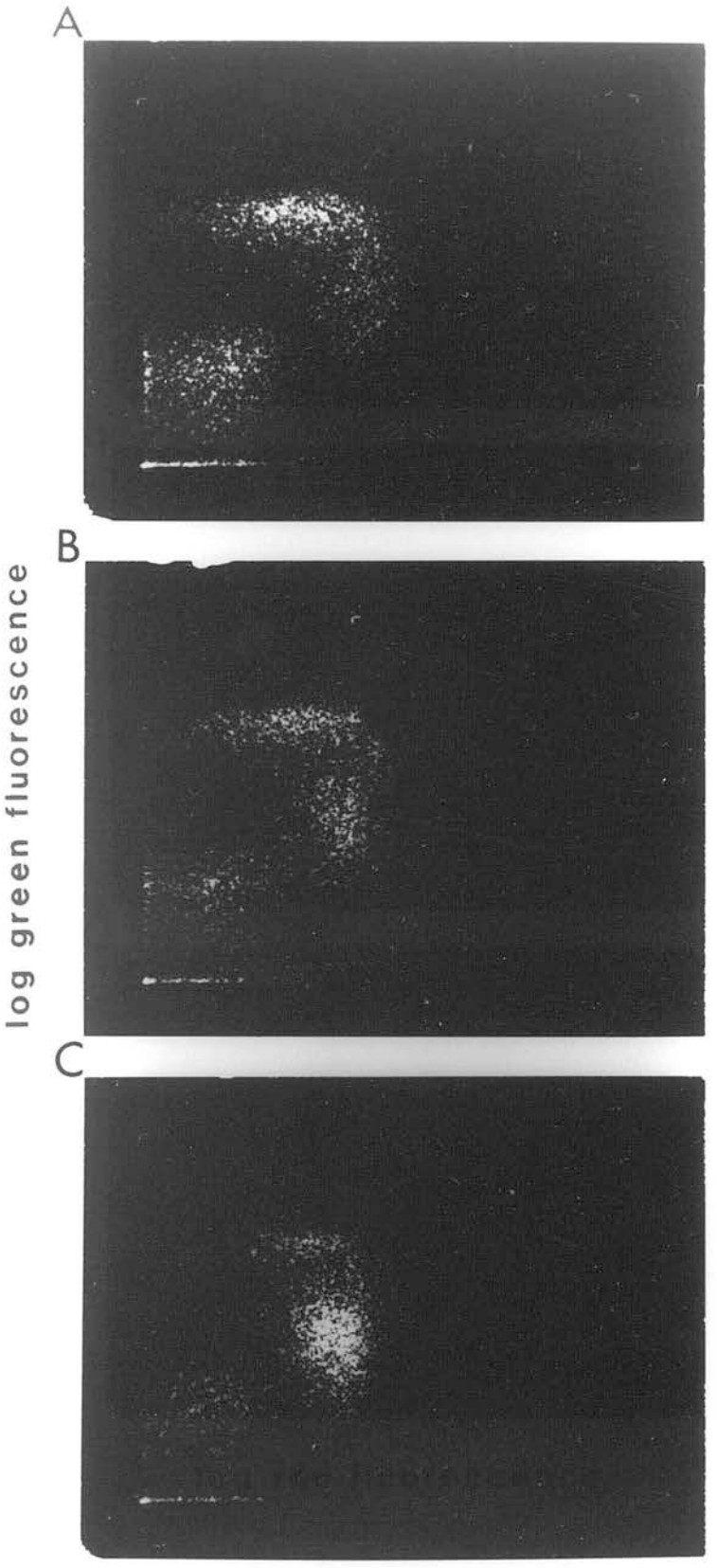
Figure 7:7

FACS dot plots of peripheral white blood cells stained with FITC-RAMIgD (green) and TR-RAMIgM (red). The three dot plots represent three different mice. The proportions of Ig^{+}dull cells were analysed on the same day (3.12.84) and are given in parenthesis.

A = Mouse Br2 (6%)

B = Mouse Br3 (32%)

C = Mouse Br5 (50%)


A
B
C
log green fluorescence
log red fluorescence

Discussion.

This chapter reports the appearance of an unusual cell population in the peripheral blood of a group of experimental mice and subsequently in several normal, aged (>2 years) CBA/Ca mice.

The cell surface characteristics of these cells are similar to human B-CLL cells in that they have a low density of surface IgM and IgD (Foon et al, 1982). The cells also express low levels of the antigen, Ly-1. This was originally thought of as a T cell marker, but was subsequently found to be present also on a minor subpopulation of B cells (Ledbetter et al, 1980; Hayakawa et al, 1984). Several B cell tumour cell lines also express Ly-1 (Lanier et al, 1981; Hayakawa et al, 1985) and human B-CLL cells express low levels of the human analogue Leu-1 (Caligaris-Cappio et al, 1982).

The unusual cells found in the peripheral blood of these mice also appeared in the spleen (causing splenomegaly) and bone marrow, but not in the lymph nodes. Tissue distribution was analysed in only one mouse in this particular study, but subsequent work in our laboratory has shown similar results for several other animals (J.D. Ansell, pers. comm.). These included normal old mice, where the cells appeared spontaneously, and animals that had been transplanted with spleen cells from the original experimental mice. Thus, with respect to tissue distribution, the cells appear similar to the murine BCL-1 cell line Slavin & Strober, 1978) and human PLL cells. It is possible that the cells are unable to traverse the high endothelial venules (HEV) of the lymph node, perhaps because they lack a necessary receptor (Krolick,et al, 1979). It is worth noting that Ly-1^+ B cells are not found in the lymph node in normal young mice (Hayakawa et al, 1985).

There was apparently an increase in peripheral white blood cell number during the progress of these hyperplasias. This suggested that proliferation was taking place outwith the normal growth control. However, the increase in cell number was very gradual, suggesting that some control still existed. Dexter et al (1981) described the progression of erythroleukaemia induced by the Friend virus (Friend, 1957). In the first stage of the disease erythroid precursors showed a reduced requirement for erythropoietin. Later, primitive

cells emerged which had a reduced maturational capacity. These cells would induce leukaemia if injected in vivo and produced tumour-type colonies in soft agar but they required stromal adherent cells for sustained growth in vitro. They were, therefore, dependent on regulatory signals from the stromal cells. In the final stage of the disease truly autonomous cells emerged which were able to grow independently in vitro. A similar sequence of events was found in leukaemias induced by the Abelson leukaemia virus which produces, predominantly B-cell leukaemias and methylnitrosourea, MNU, which produces "null" cell leukaemia (Dexter & Allen, 1983). It is possible that such a progression exists in all types of leukaemias and particularly in chronic cases, the disease may never progress beyond the first or second stage.

This neoplasia was observed in experimental animals that had been transplanted with bone marrow cells and/or bled several times. It is conceivable that the transplantation and/or the repeated cell loss were causative factors, but the appearance of the neoplasm in normal old mice is not consistent with this idea.

Neoplastic cells in the majority of patients with B-CLL have a surface Ig restricted to one light and one heavy chain class and to one idiotype (Fialkow, 1980 (review)). This suggested that, at the time of diagnosis, CLL cells were monoclonal. Fialkow et al (1978) confirmed this using the human X-linked enzyme marker glucose-6-phosphate dehydrogenase (G6PD). Similar results have been found in the murine neoplasm reported here using the PGK-1 marker system. In the one mouse studied (C11) the abnormal cells appeared to be derived from one (or, at the most, very few) cell expressing PGK-1B. Neoplasms from other PGK-1 heterozygotes have been analysed and they also appear to be monoclonal (J.D. Ansell, pers. comm.). Evidence for the monoclonality of the murine BCL-1 tumour was obtained using an anti-idiotype antibody (Vitteta et al, 1979). When these cells were stimulated in vitro with lipo- polysaccharide (LPS) they secreted IgM bearing the same idiotype as the cells' surface Ig. However, BCL-1 idiotype determinants were not detected in the sera of tumour-bearing mice, indicating that very few neoplastic B cells, if any, differentiated into IgM-secreting cells in vivo. In our small series of observations, there was no detectable difference in the serum protein profiles of normal mice and mice bearing a high proportion of Ig^{+}dull cells in the

peripheral blood. However, novel serum paraproteins have been found in a more recent series of old mice analysed (J.D.Ansell, pers.comm.) but their relationship to the B- cell neoplasia is not yet clear.

Using the G6PD marker, Fialkow et al (1978) showed that erythrocytes, granulocytes, platelets and at least some T lymphoytes did not arise, in large numbers, from the leukaemic progenitor cells. They therefore concluded that the CLL was expressed in progenitors with differentiative expression restricted to the B lymphocyte pathway. The data of Arnold et al (1983) suggest that B lymphomas (including some that carry Ly-1) are inducible by antigenic hyperstimulation. This would suggest that the basic lesion is, indeed, within B lymphocytes.

This chapter has reported the appearance of a neoplastic cell population and its preliminary characterisation. This murine neoplasm could prove to be a useful model for several reasons. If similar neoplasms occur with a high frequency in normal, old CBA/Ca mice there could be a constant supply of primary tumours. This would overcome the problems encountered when attempting to relate the behaviour of transplanted tumours and tumour cells in vitro to that of primary tumours. The initial appearance of the abnormal cells is easy to detect in the peripheral blood by FACS analysis and the "chronic" nature of the both the primary and the transplanted neoplasm would allow long term studies to be performed on the pattern and progression of the disease. Work is in progress to analyse the leukocyte-common (L-C) polypeptides (Smith & Maddy, 1984) of these murine neoplastic cells and to compare them the the human profiles. There is some evidence in the human disease that the profiles change as the disease progresses (Smith et al, 1985).

The model could be used to study the effects of different types of therapy that are currently being used to treat CLL patients and possibly to develop new therapeutic methods. It could also prove useful the the study of regulatory mechanisms that occur in normal cell growth, particularly in the lymphocyte lineage.

CHAPTER 8

CONCLUDING COMMENTS.

X chromosome-inactivation mosaics have been used to study various aspects of murine haematopoiesis. The alloenzymes of PGK-1 (PGK-1A and PGK-1B) were used as markers for the two X chromosomes. At the outset, factors affecting the X inactivation process per se were examined. It was known that the process was non-random in females that were heterozygous at the Xce locus and could also be influenced by the parental origins of the X chromosomes. We demonstrated that in Xce^{b}/Xce^{c} heterozygotes parental effects could influence the X-inactivation process, but in mice used in subsequent experiments (Xce^{a}/Xce^{c}) no such effects could be detected. In these mice, regardless of the parental origins of the X chromosome, it was established that the mean probability of expression of the X chromosome carrying the Xce^{c} and $Pgk\text{-}1^{a}$ alleles was 70% (range: 35-95%).

PGK-1 heterozygotes were used to analyse the clonal organisation of the haematopoietic system under steady-state conditions. The number of clones actively involved in haematopoiesis between sequential sampling points was estimated to be large. It was not possible, therefore, to draw any conclusions concerning the structure of the haematopoietic stem cell pool or the lineage relationships between the different cell types in normal animals. There were two possible solutions to this problem: (1) to reduce the stem cell number by experimental manipulation or, (2) to increase the number of cell markers within an individual.

An attempt to reduce stem cell number was made by producing radiation chimaeras which were repopulated with a low dose (10^{5}) of PGK-1AB bone marrow cells. The results of this experiment showed that, in these mice, the erythrocytes and granulocytes were the progeny of a common stem cell pool. The data for B and T lymphocytes appeared to suggest that these lineages were derived from separate precursors. The number of repopulating clones was estimated to be 2-3 for myeloid cells, 4 for B lymphocytes and 10 for T lymphocytes. Temporal variations in the PGK-1 phenotype of all lineages were apparent, which provided support for the clonal succession hypothesis

advanced by Kay (1965). The number of active clones, used during a given sampling period, was estimated to be 2-3 in the myeloid lineages. It was, however, difficult to estimate the number of clones active in the lymphocyte lineages due to the longevity of these cells. Results of this study suggested that the initial repopulating events involved "older" stem cells with a relatively low capacity for self-renewal; when exhausted, they were succeeded by clones derived from a more primitive stem cell population. How do these observations relate to normal haematopoiesis ? It may be possible to approach these questions using two experimental systems:-

(1). Mintz & Fleischman (1979) have devised a technique which involves the introduction of haematopoietic stem cells into early foetuses by microinjecting a small inoculum of foetal liver cells into a placental blood vessel. Using mixtures of two normal strains of foetal liver cells as donors and mildly-afflicted W mutants (W^f/W^f) as recipients, they found that some individuals were seeded by a single donor cell (Mintz et al, 1984). This method could, therefore, provide a means of reducing stem cell numbers. The technique allows stem cell pedigrees to be examined during the normal developmental progression. In some mice, there was a complementary rise and fall in proportions of cells of different genotypes (Mintz et al, 1984) which supported the clonal succession hypothesis. The major disadvantage to the system, however, is the lack of efficiency of engraftment. Only 5-6% of foetuses injected showed a stable engraftment (Fleischman et al, 1982; Mintz et al, 1984). The efficiency of production of low-dose chimaeras, described in this thesis, can approach 100% when done under carefully controlled conditions. The foetal microinjection technique does not solve the problem of using a "disrupted" population as a donor, but it could provide a method for increasing cell marker numbers. The immunological immaturity of both donor and host cells allows allogeneic grafts to be accepted with impunity. Foetuses could, therefore, be engrafted with combinations of marked cells which do not necessarily have to be of the same genetic background. However, it is possible that cell-selection processes could then operate.

(2). The most recent advance in cell markers has involved the use of retroviral vectors to transfer exogenous DNA sequences into pluripotent stem cells in vitro (Williams et al, 1984; Keller et al, 1985). Viral integration sites

(identified by restriction-enzyme digests of the DNA) were used as clonal markers for identifying haematopoietic precursors and for following cell lineages in irradiated mice repopulated with the modified stem cells. The foetal-injection technique, described above, will undoubtedly be used in the near future as a system for studying the progeny of these genetically modified cells. In addition, the combination of these two systems will provide a unique opportunity to analyse the expression and function *in vivo* of a variety of different genes transferred *in vitro*.

The study of normal animals described in Chapter 4 showed that the proportions of the two X chromosomes active were similar in all cell types studied. This agrees with the results of McMahon *et al* (1983) who showed that all cell lineages were derived from a common pool of X-inactivated cells. These results provided the necessary background for the analysis of the X-linked immunodeficiency (*xid*) mutation (Chapter 6). Imbalances in the expression of the *xid*/*Pgk-1*^{b}-bearing X chromosome in *xid*/+ mice were, therefore, due to the genetic defect and not an inherent feature of normal B lymphocyte populations. Using the PGK-1 marker system, we established that B cell precursors in the bone marrow were unaffected by the *xid* mutation. All splenic B-lymphocyte subpopulations in the periphery were defective in the adult, but the analyses of immature mice suggested that the stage in development at which the *xid* mutation acts is not the same for the different subpopulations. It is conceivable that these subpopulations belong to different lineages. Future experiments using three- and four-colour FACS analysis and sorting in combination with the PGK-1 system should help to clarify the nature of the defect itself and some of the processes of normal B lymphocyte differentiation. The marker system, in conjunction with FACS analysis and sorting, can also be used to identify the B-cell subpopulations involved in other, generally milder, X linked immunodeficiencies that have been described. For example, genetic differences in the IgG response to denatured DNA between mouse strains is apparently X-chromosome linked (Mozes & Fuchs, 1974). Similarly, the immune response to LDH-C4 is controlled by an X linked locus (Marsh *et al*, 1974). There is some evidence to suggests that these two loci are identical (Green, 1981). Analysis of such deficiencies could further help in the understanding of normal B lymphocyte function.

Finally, during the course of the studies described in this thesis a B-cell neoplasm was found in a group of experimental mice. A series of superficially similar neoplasms were then found in old CBA/Ca mice. The PGK-1 marker was used to analyse clonality and the data were compatible with the view that some of the neoplasms of the old mice were monoclonal. A preliminary characterisation suggested that this could possibly be an animal model for human chronic lymphocytic leukaemias. FACS analysis and the PGK-1 marker may prove useful in establishing the validity of the model and in analysing the early development of such neoplasms.

BIBLIOGRAPHY.

ABELSON, H.T. & RABSTEIN, L.S. (1970) Lymphosarcoma: virus-induced thymus independent disease in mice. Cancer Res., 30, 2213-2222.

ABRAMSON, S., MILLER, R.G. & PHILLIPS, R.A. (1977) The identification in adult bone marrow of pluripotent and restricted stem cells of myeloid and lymphoid series. J. Exp. Med., 145, 1567-1579.

ABNEY, E.R., COOPER, M.D., KEARNEY, J.F., LAWTON, A.R. & PARKHOUSE, R.M.E. (1978) Sequential expression of immunoglobulin on developing mouse B lymphocytes: A systemic survey that suggests a model for the generation of immunoglobulin isotype diversity. J. Immunol., 120, 2041-2049.

AHMED, A. & SCHER, I. (1976) Studies on non-H-2-linked lymphocyte-activating determinants. II. Nonexpression of MIs determinants in a mouse stain of an X linked B lymphocyte immune defect. J. Immunol., 117, 1922-1926.

AHMED, A., SCHER, I. & SELL, K.W. (1977b) Studies on non-H-2-linked lymphocyte-activating determinants. IV. Ontogeny of the MIs product on murine B cells. Cell. Immunol., 30, 122-134.

AHMED, A., SCHER, I. SHARROW, S.O., SMITH, A.H., PAUL,W.E., SACHS,D.H. & SELL, K.W. (1977a) B lymphocyte heterogeneity: development and characterisation of an alloantiserum which distinguishes B lymphocyte differentiation alloantigens. J. Exp. Med., 145, 101-110.

AISENBERG, A.C., WILKES, B.M., LONG, J.C. & HARRIS, N.L. (1980) Cell surface phenotype in lymphoproliferative disease. Am. J. Med., 68, 206-213.

ALLEN, T.D. (1978) Ultrastructural aspects of in vitro haemopoiesis. In Stem Cells and Tissue Homeostasis (eds. Lord, B.I., Potten, C.S., and Cole, R.J.). Cambridge University Press, Cambridge.

ALLEN,T.D. and DEXTER, T.M. (1983) Long-term bone marrow cultures: an

ultrastructural review. Scanning Electron Microscopy, IV, 1851-1866.

ALPEN, E.L. & CRANMORE, D. (1959) Cellular kinetics and iron utilization in bone marrow as observed by Fe^{59} radioautography. Annals. N.Y. Acad. Sci., 77, 753-765.

ALTUS, M.S., BERNSTEIN, S.E., RUSSELL, E.S., CASTEN, A.L., and UPTON, A.C. (1971) Defect extrinsic to stem cells in spleens of steel mutant mice. Proc. Soc. Exp. Biol. & Med. 138, 985-988.

AMSBAUGH, D.F., HANSEN, C.T., PRESCOTT, B.. STASHAK, P.W., BARTHOLD, D.R. & BAKER, P.J. (1972) Genetic control of the antibody response to Type III pneumococcal polysaccharide in mice. Evidence that an X-linked gene plays a decisive role in determining responsiveness. J. Exp. Med., 136. 931-949.

ANSELL, J.D. and MICKLEM, H.S. (1986) Genetic markers for following cell populations. In Handbook of Experimental Immunology (4rth Edition) (eds. Weir, D.M., Blackwell, C.M. & Herzenberg, L.A.) Blackwell Scientific Publications, Oxford (in the press).

ARNOLD, L.W., LoCASCIO, N.J., LUTZ, P.M., PENNELL, C.A., KLAPPRE, D. & HAUGHTON, G. (1983) Antigen-induced lymphomagenesis: identification of a murine B cell lymphoma with known antigen specificity. J. Immunol., 131, 2064-2068.

ASH, P., LOUTIT, J.F. and TOWNSEND, K.M.S. (1980) Osteoclasts derived from haematopoietic stem cells. Nature, 283, 669-670

AUGUSTIN, A.A. & COUTINHO, A. (1980) Specific T-helper cells that activate B cells polyclonally. In vitro enrichment of cooperative function. J. Exp. Med., 151, 587-601.

BARGMANN, C.I., HUNG, M-C. & WEINBERG, R.A. (1986) The neu oncogene encodes an epidermal growth factor receptor-related protein. Nature, 319, 226-300.

BARNES, D.W., FORD, C.E., GRAY, S.M. & LOUTIT, J.F. (1959) Spontaneous and induced changes in cell populations in heavily irradiated mice. In Progress in Nuclear Energy series VI, 2, 1-10 (eds. Bugher, J.G., Coursaget, J. & Loutit, J.F.) Pergamon Press, London.

BAZILL, G.W., HAYNES, M., GARLAND, J. & DEXTER, T.M. (1983) Characterisation and partial purification of a hemopoietic growth factor in WEHI-3 cell conditioned medium. Biochem. Journal 210, 747-759.

BAZIN, H., PLATTEAU, B. MACLENNAN, I.C.M., STUART, N.S.A., KHAN, M. & JOHNSON, G.D. (1985) B Cells production in adult rats. In Microenvironments in the Lymphoid System. (ed. Klaus, G.G.B.) Adv. Exp. Med. Biol., 186, 65-71.

BECKER, A.J., McCULLOCH, E.A. & TILL, J.E. (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse cells. Nature, 197, 452-454.

van BEKKUM, D.W. & VOS, O. (1957) Immunological aspects of homo- and hetero-logous bone marrow transplantation in irradiated animals. J. Cell. Comp. Physiol., 50 (Suppl. 1), 139-156.

van BEKKUM, D.W. & de VRIES, M.S. (1967) Radiation Chimaeras. Academic Press.

van BEKKUM, D.W., van NOORD, M.J., HAAT, B. & DICKE, K.A. (1971) Attempts at identification of haematopoitic stem cells in the mouse. Blood, 38, 547-558.

BENCA-KAPLAN, R. & QUINTANS, J. (1979) Phosphorylcholine-specific helper T cells in mice with an X-linked defect of antibody production to the same hapten. J. Exp. Med., 149, 267-272.

BERGMAN, Y. & HAIMOVICH, J. (1977) Characterization of a carcinogen-induced murine B lymphocyte cell line of C3H/eB origin. Eur. J. Immunol., 7, 413-417.

BOGGS, D.R., BOGGS, S.S., SAXE, D.F., GRESS, L.A. & CANFIELD, D.R. (1982)

Hematopoietic stem cells with high proliferative potential: Assay of their concentration in marrow by the frequency and duration of cure of W/W^v. J. Clin. Invest., 70, 242-253.

BOSMA, G.C., OWEN, J., EATON, G., MARSHALL, G., deWITT, G. & BOSMA, J. (1980) Concentration of IgG1 and IgG2a allotypes in serum of nude and normal allotye congenic mice. J. Immunol., 124, 879-884.

BOWMAN, P. & McLAREN, A. (1970) Viability and growth of mouse embryos after in vitro culture and fusion. J. Embryol. Exp. Morph., 23, 693-704.

BRADLEY, T.R. & METCALF, D. (1966) The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci., 44, 287-300.

BRECHER, G., LANCE,H. & TJIO, J-H. (1981) Bone marrow transfusion in previously irradiated, hematologically normal syngeneic mice. Proc. Soc. Exp. Biol. Med., 166, 389-393.

BRECHER, G., ANSELL, J.D., MICKLEM, H.S., TJIO, J-H. & CRONKITE, E.P. (1982) Special proliferative sites are not needed for seeding and proliferation of transfused bone marrow cells in normal syngeneic mice. Proc. Natl. Acad. Sci. USA., 79, 5085-5087.

BROUET, J.C., FLANDRIN, G., SASPORTES, M., PREUD-HOMME, J.L. & SELIGMAN, M. (1975) Chronic lymphocytic leukaemia of T cell origin. Immunological and clinical evaluation of eleven patients. Lancet, 2, 890-893.

BRYANT, B.J. & KELLY, L.S. (1958) Autoradiographic studies of leukocyte formation. Proc. Soc. Exp. Biol. Med., 99, 681-684.

BUCHER, T., BENDER, W., FUNDELE, R., HOFNER, H. & LINKE, I. (1980) Quantitative evaluation of electrophoretic allo- and isoenzyme patterns for developmental genetics. FEBS Letters, 115, 319-324.

BUCHER, T., LINKE, I.M., DUNNWALD, M. WEST, J.D. & CATTANACH, B.M. (1986) Xce genotype has no impact on the effect of imprinting of X-chromosome

expression in the mouse yolk sac endoderm. Genet. Res. (In Press).

BUESCHER, E.S., ALLING, D.W. & GALLIN, J.I. (1985) Use of an X-linked human neutrophil marker to estimate timing of Lyonisation and size of dividing stem cell pool. J. Clin. Invest., 76, 1581-1584.

BURGESS, A. & NICOLA, N. (1983) Growth Factors and Stem Cells. Academic Press.

BURTON, D.I., ANSELL, J.D., GRAY, R.A. & MICKLEM, H.S. (1982) A stem cell for stem cells in murine haematopoiesis. Nature, 298, 562-563.

CALIGARIS-CAPPIO, F., GOBBI,M., BOFILL, M. & JANOSSY, G. (1982) Infrequent normal B lymphocytes express features of B-chronic lymphocytic leukaemia. J. Exp. Med., 155, 623- 628.

CAMBIER, J.C., VITETTA, E.S., UHR, J.W. & KETTMAN, J.R. (1977) B cell tolerance. II. Trinitrophenyl human gammaglobulin-induced tolerance in adult and neonatal murine B cells responsive to thymus-dependent and independent forms of the same hapten. J. Exp. Med., 145, 778-783.

CARTER, T.C., LYON, M.F. & PHILLIPS, R.J.S. (1956) Further genetic studies of eleven translocations in the mouse. J. Genet., 54, 462-473.

CARTWRIGHT, G.E., ATHENS, J.W. & WINTROBE, M.M. (1964) The kinetics of granulopoiesis in man. Blood, 24, 780-803.

CATOVSKY, D., OKOS, A., WILTSHAR, F., GALETTO, J. GALTEN, D.A.B. & STRATHOPOULOS, G. (1973) Prolymphocytic leukaemia of B and T cell type. Lancet, 2, 232-234.

CATOVSKY, D., PITTMAN, S., O'BRIAN, N. et al (1979) Multiparameter studies in lymphoid leukaemia. Am. J. Clin. Pathol., 72, 736-745.

CATTANACH, B.M. (1975) Control of X-chromosome inactivation. Ann. Rev. Genet., 9, 1-18.

CATTANACH, B.M. & PAPWORTH, D. (1981) Controlling elements in the mouse. V. Linkage tests with X-linked genes. Genet. Res., Camb., 38, 57-70.

CATTANACH, B.M. & PEREZ, J.N. (1970) Parental influence on X-autosome translocation-induced variegation in the mouse. Genet. Res., Camb., 15, 43-53.

CHANDRA, H.S., & BROWN, S.W. (1975) Chromosome imprinting and the mammalian X chromosome. Nature, 253, 165-168.

CHAPMAN, V.M. & SHOWS, T.B. (1976) Somatic cell genetic evidence for X chromosome linkage of three enzymes in the mouse. Nature, 259, 665-667.

COFFMAN, R.L. (1983) Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. Immunol. Rev., 69, 5-23.

COFFMAN, R.L. & WEISSMAN, I.L. (1981a) A monoclonal antibody that recognises B cells and B cell precursors in mice. J. Exp. Med., 153, 269-279.

COFFMAN, R.L. & WEISSMAN, I.L. (1981b) B220: a B cell-specific member of the T200 glycoprotein family. Nature, 289, 681-683.

COHEN, D.I., HEDRICK, S.M., NIELSEN, E.A., D'EUSACHIO, P., RUDDLE, F., STEINBERG, A.D., PAUL, W.E., DAVIES, M.M. (1985a) Isolation of a cDNA clone corresponding to tge X-linked gene family (XLR) closely linked to the murine immunodeficiency disorder xid. Nature, 314, 369-372.

COHEN, D.I., STEINBERG, A.D., PAUL, W.E. & DAVIES, M.M. (1985b) Expression of an X-linked gene family (XLR) in late-stage B cells and its alteration by the xid mutation. Nature, 314, 372-374.

CONGDON, C. & LORENZ, E. (1954) Leukemias in guineapigs. Am. J. Path., 30, 337-359.

COOPER, M.D., KEIGHTLEY, R.G., WU, L-Y.F. & LAWTON, A.R. (1973) Developmental defects of T and B cell lines in humans. Transplant. Rev., 16, 51-84.

CRONKITE, E.P., FLIEDNER, T.M., BOND, V.P., RUBINI, J.R., BRECHER, G. & QUASTLER, H. (1959) Dynamics of hemopoietic proliferation in man and mice studied by ^{3}H-thymidine incorporation into DNA. Annals. N.Y. Acad. Sci., 77, 803-820.

CRONKITE, E.P., MILLAR, M.E., GARNETT, H. & HARIGAYA, K. (1983) Regulation of hemopoiesis: inhibitors and stimulators produced by a murine bone marrow stromal cell line (H-1). In Haemopoietic Stem Cells: Characterisation, Proliferation, Regulation . Alfred Benzon Symp. 18, 266-282. (eds., Killmann, Sv-Aa., Cronkite, E.P. & Muller-Berat, C.N.) Munksgaard, Copenhagen.

DARLING, S. & GOODFELLOW, P. (1985) Lymphocyte development genes and immunodeficiency disease. Nature, 314, 318

DAVIES, W.M., ALPEN, E.L. & DAVIS, A.K. (1955) Studies of radio-iron utilization and erythrocyte lifespan in rats following thermal injury. J. Clin. Invest., 34, 67-74.

DENTON, P.M. & SYMES, M.O. (1968) Observations on the changing behaviour of AKR mouse lymphomas serially transplanted in the strain of origin. Immunol., 15, 371-380.

DESSNER, D.S. & LOKEN, M.R. (1981) DNL 1.9: a monoclonal antibody which specifically detects all murine B lineage cells. Eur. J. Immunol., 11, 282-285.

DEXTER, T.M. & ALLEN, T.D. (1983) The regulation of growth and development of normal and leukaemic cells. J. Pathol., 141, 415-433.

DEXTER, T.M., ALLEN, T. & LAJTHA, L.G. (1977) Conditions controlling the proliferation of haematopoietic stem cells in vitro. J. Cell. Physiol. 91, 335-344.

DEXTER, T.M., ALLEN, T.D., TESTA, N.G. & SCOLNICK, E. (1981) Freind disease in vitro. J. Exp. Med., 154, 594-608.

DEXTER, T.M., GARLAND, J., SCOTT, D., SCOLNICK, E. & METCALF, D. (1980)

Growth of factor-dependent hemopoietic precursor cell lines. J. Exp. Med., 152, 1036-1047.

DEXTER, T.M. & LAJTHA, L.G. (1974) Proliferation of haematopoietic stem cells in vitro. Br. J. Haemotol., 28, 525-530.

DEXTER, T.M. & MOORE, M.A.S. (1977) In vitro duplication and "cure" of haemopoietic defects in genetically anaemic mice. Nature, 269, 412-414.

DEXTER, T.M., SPOONCER, E., VARGA, J., ALLEN, T.D. & LANOTTE, M. (1983) Stromal cells and diffusable factors in the regulation of haemopoietic cell development. In Haemopoietic Stem Cells: Characterisation, Proliferation, Regulation. 303-318. Alfred Benzon Symposium. (eds. Killmann, Sv-Aa., Cronkite, E.P. & Muller-Berat, C.N.). Munksgaard, Copenhagen.

ECKHARDT, L.A. & HERZENBERG, L.A. (1980) Monoclonal antibodies to ThB detect close linkage of Ly-6 and a gene regulating ThB expression. Immunogenet., 11, 275-291.

von EHRENSTEIN, G. (1958) The lifespan of the erythrocytes of normal and of tumour-bearing mice as determined by glycine-2-^{14}C. Acta. Physiol. Scand., 44, 80-91.

ELDRIDGE, J.H., YAFFE, L.J., RYAN, J.J., KIYONO, H., SCHER, I. & McGHEE, J.R. (1984) Expression of Lyb-5 and Mls determinants by Peyers patch B lymphocytes of X-linked immunodeficient mice. J. Immunol., 133, 2308-2311.

EVERETT, N.B., CAFFREY, R.W. & RIEKE, W.O. (1964) Recirculation of lymphocytes. Annals. N.Y. Acad. Sci., 113, 887-897.

EZINE, S., WEISSMAN, L.R. & ROUSE, R.V. (1984) Bone marrow cells give rise to distinct clones within the thymus. Nature, 309, 629-631.

FALCONER, D.S., ISAACSON, J.H. & GAULD, I.K. (1982) Non-random X chromosome inactivation in the mouse: Difference of reaction to imprinting. Genet. Res., Camb., 39, 237-259.

FAUSER, A.A. & MESSNER, H.A. (1979) Identification of megakaryocytes, macrophages and eosinophils in colonies of human bone marrow containing neutrophilic granulocytes and erythroblasts. Blood, 53, 1023-1027.

FELDBUSH, T.L. (1973) Antigen modulation of the immune response. Cell. Immunol., 8, 435-444.

FIALKOW, P.J. (1973) Primordial pool size and lineage relationships of five human cell types. Ann. Hum. Genet., London, 37, 39-48.

FIALKOW, P.J. (1980) Clonal and stem cell origin of blood cell neoplasms. In Contemporary Haematology and Oncology. 1-46. Plenum Press, New York.

FIALKOW, P.J. (1983) Hierarchal haematological stem cell relationships studied with glucose-6-phosphate dehydrogenase alloenzymes. In Haemopoietic Stem Cells. Charaterisation, Proliferation, Regulation. Alfred Benzon Symp. 18 (eds. Killman, Sv-Aa., Cronkite, E.P. & Muller-Berat, C.N.). Munksgaard, Copenhagen.

FIALKOW, P.J., NAJFIELD, V., REDDY, A.L., SINGER, J. & STEINMANN, L. (1978) Chronic lymphocytic leukaemia: clonal origin in a committed B lymphocyte progenitor. Lancet, 2, 444-446.

FIDLER, J.M., MORGAN, E.L. & WEIGLE, W.O. (1980) B lymphocyte differentiation in the CBA/N mouse: a delay in maturation rather than a total arrest. J. Immunol., 124, 13-19.

FINKELMAN, F.D., SMITH, A.H., SCHER, I. & PAUL, W.E. (1975) Abnormal ratios of membrane immunoglobulin class in mice with X-linked B lymphocyte defect. J. Exp. Med., 142, 1316-1321.

FLEISCHMAN, R.A., CUSTER, R.P. & MINTZ, B. (1982) Totipotent haemopoietic stem cells: normal self-renewal and differentiation after transplantation between mouse fetuses. Cell, 30, 351-359.

FLEISCHMAN, R. & MINTZ, B. (1979) Prevention of genetic anemias in mice by

micro-injection of normal hemopoietic stem cells into the fetal placenta. Proc. Nat. Acad. Sci. USA., 76, 5736-5740.

FOON, K.A., BILLING, R.J. & TERASAKI, P.I. (1980) Dual B and T markers in acute and chronic lymphocytic leukaemia. Blood, 55, 16-20.

FOON, K.A., SCHROFF, R.W. & GALE, R.P. (1982) Surface markers on leukaemia and lymphoma cells: Recent advances. Blood, 60, 1-19.

FORD, C.E, EVANS, E.P. & GARDNER, R.L. (1975) Marker chromosome analysis of two mouse chimaeras. J. Embryol. Exp. Morphol., 33, 447-457.

FORD, C.E., HAMERTON, J.L., BARNES, D.W. & LOUTIT, J.F. (1956) Cytological identification of radiation chimaeras. Nature, 177, 452-454.

FORD, C.E., MICKLEM, H.S., EVANS, E.P., GRAY, J.G. & OGDEN, D.A. (1966) The flow of bone marrow cells to the thymus: studies with part-body irradiated mice injected with chromosome-marked bone marrow and subjected to antigenic stimulation. Annals. N.Y. Acad. Sci., 129, 283-296.

FOWLER, J.H., WU, A.M., TILL, J.E. & McCULLOCH, E.A. (1967) The cellular composition of hematopoietic spleen colonies. J. Cell. Physiol., 69, 65-72.

FRED, S.S., & SMITH, W.W. (1968) Induced changes in transplantability of haematopoietic colony-forming cells. Proc. Soc. Exp. Biol. Med., 128, 364-366.

de FREITAS, A.A. & COUTINHO, A. (1981) Very rapid decay of mature B lymphocytes in the spleen. J. Exp. Med., 154, 994-999.

FRELS, W.I. & CHAPMAN, V.M. (1980) Expression of the maternally-derived X chromosome in mural trophoblast of the mouse. J. Embryol. Exp. Morph., 56, 179-190.

FRIEND, C. (1957) Cell-free transmission in adult swiss mice of a disease having the character of a leukaemia. J. Exp. Med., 105, 307-312.

FU, S.M., WINCHESTER, R.J. & KUNKEL, H.G. (1975) Similar idiotypic specificity of the membrane IgD and IgM of human B lymphocytes. J. Immunol., 114, 250-254.

GOODMAN, M.G., FIDLER, J.M. & WEIGLE, W.O. (1978) Nonspecific activation of murine lymphocytes IV. Proliferation of a distinct, late maturing lymphocyte subpopulation induced by 2-mercaptoethanol. J. Immunol., 121, 1905-1913.

GOODMAN, J.W. & SMITH, L.H. (1961) Erythrocyte lifespan in normal mice and in radiation bone marrow chimaeras. Amer. J. Physiol., 200, 764-778.

GORNISH, M., WEBSTER, M.P. & WEGMANN, T.G. (1972) Chimaerism in the immune system of tetrparental mice. Nature, 237, 249-251.

GREEN, M.C., (1981) Genetic variants and strains of the laboratory mouse. Gustav Fischer Verlag., Stuttgart, New York.

GRIFFETH, A.L., CATSIMPOOLA, M.K., DEWANJEE, M.K. & WORTIS, H.H. (1976) Electrophoretic separation of radioisotopically-labelled mouse lymphocytes. J.Immunol., 117, 1949-1952.

GRONOWICZ, E. & COUTINHO, A. (1975) Functional analysis of B cell heterogeneity. Transplant. Rev., 24, 3-40.

GUILBERT, L.J. & STANLEY, E.R. (1980) Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J. Cell. Biol., 85, 153-159.

HAMMERLING, G.J. (1976) Tissue distribution of Ia antigens and their expression on lymphocyte subpopulations. Transplant. Rev., 30, 64-82.

HARAN-GHERA, N. & PELED, A. (1973) Thymus and bone marrow derived lymphatic leukaemia in mice. Nature, 241, 396-398.

HARDY, R.R., HAYAKAWA, K., PARKS, D.R., HERZENBERG, L.A. & HERZENBERG, L.A. (1984) Murine B cell differentiation lineages. J. Exp. Med., 159, 1169-1188.

HARDY, R.R., HAYAKAWA, K., HAAIJMAN, J. & HERZENBERG, L.A. (1982) B cell subpopulations identified by two color fluorescence analysis. Nature, 297, 589-591.

HARDY, R.R., HAYAKAWA, K., PARKS, D.R., HERZENBERG, L.A. (1983) Demonstration of B cell maturation in X-linked immunodeficient (xid) mice by simultaneous three-color immunofluorescence. Nature, 306, 270-272.

HARRIS, J.E. & FORD, C.E. (1964) Cellular traffic of the thymus: experiments with chromosome markers. Evidence that the thymus plays an instructive part. Nature, 201, 884-885.

HARRIS, J.E., BARNES, D.W.H., FORD, C.E. & EVANS, E.P. (1964) Cellular traffic of the thymus: experiments with chromosome markers. Evidence from parabiosis for an afferent stream of cells. Nature, 201, 886-887.

HARRIS, R.A., HOGARTH, P.M., WADESON, L.J., COLLINS, P. McKENZIE, I.F.C. & PENINGTON, D.G. (1984) An antigenic difference between cells forming early and late haematopoietic spleen colonies (CFU-S). Nature, 307, 638-640.

HARRISON, D.E. (1972) Lifesparing ability (in lethally irradiated mice) of W/W^{V} mouse marrow with no macroscopic colonies. Rad. Res., 52, 553-563.

HARRISON, D.E. (1975) Normal function of transplanted marrow cell lines from aged mice. J. Gerontol., 30, 279-285.

HARRISON, D.E. (1979) Proliferative capacity of erythropoietic stem cell lines and ageing: an overview. Mech. Age. Dev., 9, 409-426.

HARRISON, D.E. (1983) Long-term erythropoietic repopulating ability of old, young and fetal stem cells. J. Exp. Med., 157, 1496-1504.

HARRISON, D.E. & ASTLE, C.M. (1982) Loss of stem cell repopulating ability upon transplantation: effects of donor age, cell number and transplantation procedure. J. Exp. Med., 156, 1767-1779.

HARRISON, D.E., ASTLE, C.M. & DeLAITTRE, J.A. (1978) Loss of proliferative capacity in immunohemopoietic stem cells caused by serial transplantation rather than ageing. J. Exp. Med., 147, 1526-1531.

HAYAKAWA, K., HARDY, R.R., HONDA M., HERZENBERG, L.A., STEINBERG, A.D. & HERZENBERG, L.A. (1984) LY-1 B cells: Functionally distinct lymphocytes that secrete IgM autoantibodies. Proc. Nat. Acad. Sci. USA., 81, 2494-2498.

HAYAKAWA, K., HARDY, R.R., HERZENBERG, L.A. & HERZENBERG, L.A. (1985) Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J. Exp. Med., 161, 1554-1568.

HAYFLICK, L. (1965) The limited in vitro lifetime of human diploid cell strains. Exp. Cel. Res., 37, 614-635.

HELLMAN, S. BOTNICK, L.E., HANNON, E.C. & VIGNEULLE, R.M. (1978) Proliferative capacity of murine hematopoietic stem cells. Proc. Nat. Acad. Sci. USA., 75, 490-494.

HERZENBERG, L.A. & HERZENBERG, L.A. (1978) Analysis and separation using the fluorescence-activated cell sorter (FACS). In Handbood of Experimental Immunology (3rd Edition) (ed. Weir, D.M.) Blackwell Scientific Publications.

HODGSON, G.S. & BRADLEY, T.R. (1979) Properties of haematopoietic stem cells surviving 5-fluorouracil treatment: evidence for pre-CFU-S cell. Nature, 281, 381-382.

HODGSON, G.S., BRADLEY, T.R., MARTIN, R.F., SUMNER, M. & FRY, P. (1975) Recovery of proliferating haemopoietic progenitor cells after killing by hydroxyurea. Cell. Tiss. Kinet., 8, 51-60.

HOLLIDAY, R. (1975) Growth and death of diploid and transformed human fibroblasts. Fed. Proc., 34, 51-55.

HUBER, B.T. (1982) B cell differentiation antigens as probes for functional B cell subsets. Immunol. Rev., 64, 57-79.

HUDSON, L. & HAY, F.C. (1976) Practical Immunology. Blackwell Scientific Publications.

HUNTER, T. (1984) Oncogenes and proto-oncogenes: how do they differ. J. Nat. Can. Inst., 73, 773-786.

IHLE, J.N., KELLER, J., HENDERSON, L. KLEIN, F. & PALASZYNSKI, E. (1982) Procedures for the purification of interleukin-3 to homogeneity. J. Immunol., 129, 2431-2435.

ISCOVE, N.N., ROITSCH, C.A., WILLIAMS, N. & GUILBERT, L.J. (1982) Molecules stimulating early red cell, granulocyte, macrophage, megakaryocyte precursors in culture: similarity in size, hydrophobicity and charge. J. Cell. Physiol. (Suppl. 1), 65-78.

JACOBSON, L.O., MARKS, E.K., ROBSON, M.J., GASTON, E. & ZIRKLE, R.E. (1949) The effect of spleen protection on mortality following X-irradiation. J. Lab. Clin. Med., 34, 1538-1543.

JAFFE, E.S. (1980) Non-hodgkin's lymphomas as neoplasms of the immune system. In A Multidisciplinary Approach to Non-hodgkin's Lymphoma. (Moderator, Berard, C.W.) Ann. Intern. Med., 94, 218-235.

JANEWAY, C.A. & BARTHOLD, D.R. (1975) An analysis of the defective response of CBA/N mice to T-dependent antigens. J. Immunol., 115, 898-900.

JOHNSON G.R. & METCALF, D. (1978) Nature of cell-forming erythroid colonies in agar after stimulation by spleen conditioned medium. J.Cell. Physiol., 94, 243-252.

JOHNSTON, P.G. & CATTANACH, B.M. (1981) Controlling elements in the mouse. IV. Evidence of non-random X-inactivation. Genet. Res., Camb., 37, 151-160.

KANE, V.E. (1978) Data analysis of four X chromosome-inactivation experiments. In Genetic Mosaics and Chimaeras in Mammals. (ed. Russell, L.B.). Basic Life Sci., 12. Plenum Press.

KAY, H.E.M. (1965) How many cell generations? Lancet, 2, 418-419.

KEARNEY, J.F., COOPER, M.D., KLEIN, J., ABNEY, E.R., PARKHOUSE, R.M.E. & LAWTON, A.R. (1977) Ontogeny of Ia and IgD on IgM-bearing B lymphocytes in mice. J. Exp. Med., 146, 297-301.

KELLER, G., PAIGE, C., GILBOA, E. & WAGNER, E.F. (1985) Expression of a foreign gene in myeloid and lymphoid cells derived from multipotent haematopoietic precursors. Nature, 318, 149-154.

KINCADE, P.W. (1977) Defective colony-formation by B lymphocytes from CBA/N and C3H/HeJ mice. J. Exp. Med., 145, 249-263.

KINCADE, P.W., FLAHERTY, L. LEE, G., WATANABE, T. & MICHAELSON, J. (1980) Qa antigen expression on functional lymphoid, myeloid and stem cells in adult mice. J. Immunol., 124, 2879-2885.

KINCADE, P.W., JYONOUCHI, H., LANDRETH, K.S. & LEE, G. (1982) B Lymphocyte precursors in immunodeficient, autoimmune and anemic mice. Immunol. Rev., 64, 81-98.

KINCADE, P.W., LEE, G., PAIGE, C.J. & SCHEID, M.P. (1981b) Cellular interactions affecting the maturation of murine B lymphocyte precursors in vitro. J. Immunol., 127, 255-260.

KINCADE, P.W., LEE, G., WATANABE, T., SUN, & SCHEID, M.P. (1981a) Antigens displayed on murine B lymphocyte presursors. J. Immunol., 127, 2262-2268.

KINCADE, P.W., MOORE, M.A.S., LEE, G. & PAIGE, C.J. (1978) Colony-forming cells in F1 hybrid and transplanted CBA/N mice. Cell. Immunol., 40, 294-302.

KISHIMOTO, T. & ISHIZAKA, K. (1973) Regulation of antibody responses in vitro IV. Carrier-specific helper T cells for IgG and IgE antibody responses. J. Immunol., 111, 720-732.

KITAMURA, Y., SHIMADA, M., HATANAKA, K. & MIYANO, Y. (1977) Development

of mast cells from grafted bone marrow cells in irradiated mice. Nature, 268, 442-443.

KITAMURA, Y., YOKOYAMA, M. MATSUDA, H., OHNO, T. & MORI, K.J. (1981) Spleen colony-forming cells as a common precursor for tissue mast cells and granulocytes. Nature, 291, 159-160.

KOZAK, L.P., McLEAN, G.K. & EICHER, E.M. (1974) X-linkage of phosphoglycerate kinase in the mouse. Biochem. Genet., 11, 41-47.

KOZINER, B., KEMPIN, S., PASSE, S., GEE, T., GOOD, R.A. & CLARKSON, B.D. (1980) Characterisation of B cell leukaemias: A tentative immunological scheme. Blood, 56, 815-823.

KROLICK, K.A., ISAKSON, P.C., UHR, J.W & VITETTA, E.S. (1979) BCL-1. A murine model for chronic lymphocytic leukaemia. Use of sIg idiotype for detection and treatment of tumours. Immunol. Rev., 48, 81-106.

KUMARARATNE, D.S., GRAY, D., MACLENNAN, I.C.M., LORTAN, J., PLATTEAU, B. & BAZIN, H. (1985) The paradox of high rates of B cell production in bone marrow and the longevity of most mature B cells. In Microenvironments in the Lymphoid System (ed. Klaus, G.G.B.) Adv. Exp. Med. Biol., 186, 73-80.

KUMARARATNE, D.S. & MACLENNAN, I.C.M. (1981) Cells of the marginal zone of the spleen are lymphocytes derived from recirculating precursors. Eur. J. Immunol., 11, 865-869.

LAFLEUR, L., MILLER, R.G. & PHILLIPS, R.A. (1973) Restriction of specificity in the precursors of bone marrow-associated lymphocytes. J. Exp. Med., 137, 954-966.

LAJTHA, L.G. (1979) Stem cell concepts. Differentiation, 14, 23-34.

LAJTHA, L.G., OLIVER, R. & GURNEY, C.W. (1962) Kinetic model of a bone marrow stem cell population. Brit. J. Haematol., 8, 442-460.

LAJTHA, L.G. & SCHOFIELD, R. (1971) Regulation of stem cell renewal and differentiation: possible significance in ageing. Adv. Gerontol. Res., 3, 131-146.

LANDRETH, K.S., KINCADE, P.W., LEE, G. & MEDLOCK, E.S. (1983) Phenotypic and functional characterisation of murine B lymphocyte precursors isolated from fetal and adult sites. J. Immunol., 131, 572-580.

LANIER, L.L., WARNER, N.L., LEDBETTER, J.A. & HERZENBERG, L.A. (1981) Expression of Lyt-2 antigen on certain murine B cell lymphomas. J. Exp. Med., 153, 998-1003.

LEDBETTER, J.A. & HERZENBERG, L.A. (1979) Xenogenic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol. Rev., 47, 63-89.

LEDBETTER, J.A., ROUSE, R.V., MICKLEM, H.S. & HERZENBERG, L.A. (1980) T cell subsets defined by exprssion of Lyt-1,2,3 and Thy-1 antigens. Two-parameter immunofluorescence and cytotoxicity analysis with monoclonal antibodies modifies current views. J. Exp. Med., 152, 280-295.

LENNON, J.E. (1985) Studies of haematopoiesis in the mouse. PhD. Thesis, Edinburgh University.

LISKAY, R.M. & EVANS, R.G. (1980) Inactive X chromosome DNA does not function in DNA-mediated cell transformation for the hypoxanthine phospho-ribosyltransferase gene. Proc. Natl. Acad. Sci., 77, 4895-4898.

LORD, B.I. (1983) Haemopoietic stem cells. In Stem Cells. Their Identification and Characterisation. (ed. Potten, C.S.) Churchill Livingston.

LORD, B.I., MORI, K.J. & WRIGHT, E.G. (1977) A stimulator of stem cell proliferation in regenerating bone marrow. Biomedicine, 27, 223- 226.

LORD, B.I., MORI, K.J., WRIGHT, E.G. & LAJTHA, L.G. (1976) An inhibitor of stem cell proliferation in normal bone marrow. Brit. J. Haematol., 34, 441- 445.

LORENZ, E., UPHOFF, D., REID, T.R. & SHELTON, E. (1951) Modification of irradiation injury in mice and guinea pigs by bone marrow injections. J. Nat. Cancer. Inst., 12, 197-201.

LORENZ, E., CONGDON, C. & UPHOFF, D. (1952) Modification of acute irradiation injury in mice and guinea pigs by bone marrow injections. Radiology, 58, 863-877.

LYON, M.F. (1961) Gene action in the X-chromosome of the mouse (Mus musculus L.). Nature, 190, 372-373.

LYON, M.F. (1974) Review Lecture: Mechanisms and evolutionary origins of variable X-chromosome activity in mammals. Proc. Roy. Soc. London, B, 187, 243-268.

LYON, M.F. (1983) The X-chromosome and their levels of activation. In Cytogenetics of Mammalian Chromosome, 187-204.

MAGLI, M.C., ISCOVE, N.N., ODARTCHENKO, N. (1982) Transient nature of early haematopoietic spleen colonies. Nature, 295, 527-529.

MALONEY, M.A. & PATT, H.M. (1968) Granulocyte transit from the bone marrow to blood. Blood, 31, 195-201.

MARSH, J.A., WHEAT, T.E. & GOLDBERG, E. (1977) Temporal regulation of the immune respnse to LDH-C_4 by an X-linked gene in C3H/HeJ and SJL/J mice. J. Immunol., 118, 2293-2298.

MARTINET, Y., BITTERMAN, P.B., MORNEX, J-F., GROTENDORST, G.R. MARTIN, & CRYSTAL, R.G. (1986) Activated human monocytes express the c-sis proto-oncogene and release a mediator showing PDGF-like activity. Nature, 319, 158-160.

MAUCH, P., GREENBERGER, J.S., BOTNIK, L., HANNON, E. & HELLMAN, S. (1980) Evidence for structured variation in self-renewal capacity within long-term bone marrow cultures. Proc. Nat. Acad. Sci., 77, 2927-2930.

McCULLOCH, E.A., SIMINOVITCH, L.E. & TILL, J.E. (1964) Spleen colony-formation in anemic mice of genotype W/W^v. Science, 144, 844-846.

McCULLOCH, E.A., SIMINOVITCH, L.E., TILL, J.E., RUSSELL, E.S. & BERNSTEIN, S.E. (1965) The cellular basis of genetically determined hemopoietic defect in anemic mice of genotype Sl/Sl^d. Blood, 26, 399-410.

McGRATH, M.S., PILLEMER, E., KOOISTRA, D. & WEISSMAN, I.L. (1980) The role of MuLV receptors on T-lymphoma cells in lymphomas cell proliferation. In Contemporary Topics in Immunobiology. Vol. 11, 157-184. (ed. Warner, N.L.) Plenum Publishing Corporation, New York.

McKEARN, J.P. PASLAY, J.W., SLACK, J., BAUM, C. & DAVIE, J.M. (1982) B cell subsets and diffefential responses to mitogens. Immunol. Rev., 64, 5-23.

McLAREN, A. (1975) Mammalian Chimaeras. Cambridge Univ. Press, Cambridge & London.

McMAHON, A., FOSTEN, M. & MONK, M. (1983) X-chromosome inactivation mosaicism in the three germ layers and the germ-line of the mouse embryo. J. Embryol. Exp. Morph., 74, 207-220.

METCALF, D. & JOHNSON, G.R. (1978) Production by spleen and lymph node cells of the conditioned medium with erythroid and other hemopoietic colony-stimulating activity. J. Cell. Physiol., 96, 31-42.

METCALF,D. MacDONALD, H.R., ODARTCHENKO, N. & SORDAT, B. (1975a) Growth of mouse megakaryocyte colonies in vitro. Proc. Nat. Acad. Sci., 72, 1744-1748.

METCALF, D. & MOORE, M.A.S. (1971) Haemopoietic Stem Cells North Holland Publishing Company, Amsterdam.

METCALF, E.S., SCHER, I. & KLINMAN, N.R. (1980) Susceptibility to in vitro tolerance induction of adult B cells from mice with an X-linked B cell defect. J. Exp. Med., 151, 486-491.

MICKLEM, H.S. (1983) Factors affecting serially transplanted bone marrow. A commentary. Blood Cells, 9, 527-531.

MICKLEM, H.S., ANSELL, J.D., WAYMAN, J.E. & FORRESTER, L.M. (1983) The clonal organisation of hematopoiesis in the mouse. Prog. Immunol. V., 633-644. (eds. Yamamura, Y. & Tada, T.) Academic Press, Japan.

MICKLEM, H.S., ANDERSON, N. & ROSS, E.A.M. (1975c) Limited potential of circulating haematopoietic stem cells. Nature, 256, 41-43.

MICKLEM, H.S., FORD, C.E., EVANS, E.P. & GRAY, J. (1966) Interrelationship of myeloid and lymphoid cells: studies with chromosome-marked cells transfused into lethally irradiated recipients. Proc. Roy. Soc. B, 165, 78-102.

MICKLEM, H.S., FORD, C.E., EVANS, E.P. & OGDEN, D.A. (1975a) Compartments and cell flows within the mouse haematopoietic system: I. Restricted interchange between haematopoietic sites. Cell Tiss. Kinet., 8, 219-232.

MICKLEM, H.S., FORD, C.E., EVANS, E.P., OGDEN, D.A. & PAPWORTH, D.S. (1972) Competitive in vivo proliferation of foetal and adult cells in lethally irradiated mice. J. Cell. Physiol., 79, 293-298.

MICKLEM, H.S. & LOUTIT, J.F. (1966) Tissue Grafting and Radiation, Academic Press.

MICKLEM, H.S., OGDEN, D.A., EVANS, E.P., FORD, C.E. & GRAY, J.G. (1975b) Compartments and cell flows within the mouse haematopoietic system. II. Estimated rates of interchange. Cell Tiss. Kinet., 8, 233-248.

MICKLEM, H.S. & ROSS, E. (1978) Enumeration of haematopoietic clones in mice. In Human lymphocyte differentiation: its applications to cancer. INSERM Symp. 8, 15-22. North Holland Publishing Company, Amsterdam.

MINTZ, B. (1964) Formation of genetically mosaic mouse embryos and early development of "lethal" (t^{12}/t^{12}) - normal mosaics. J. Exp. Zool., 157, 273-285.

MINTZ, B. (1975) Experimental genetic mosaicism in the mouse. In Preimplantation Stages of Pregnancy, (eds. Wolstenholm, G.E.W. & O'Connor, M.) Churchill, London.

MINTZ, B., ANTHONY, K. & LITWIN, S. (1984) Monoclonal derivation of mouse myeloid and lymphoid lineages from totipotent hematopoietic stem cells experimentally engrafted into fetal hosts. Proc. Nat. Acad. Sci. USA., 81, 7835-7839.

MIYAKE, T., KUNG, C.K.H. & GOLDWASSER, E. (1977) Purification of human erythropoietin. J. Biol. Chem. 252, 5558-5564.

MOHANDAS, T., SPARKS, R.S. & SHAPIRO, L.J. (1981) Reactivation of inactive human X chromosome: evidence for X inactivation by DNA methylation. Science, 211, 393-396.

MOND, J.J., SCHER, I., COSSMAN, J., KESSLER, S., MONGINI, P.G.A., HANSEN, C., FINKELMAN, F.D. & PAUL, W.E. (1982) Role of the thymus in directing the development of a subset of B lymphocytes. J. Exp. Med., 155, 924-936.

MONK, M. & HARPER, M. (1979) Sequential X-chromosome inactivation coupled with cellular differentiation in early mouse embryos. Nature, 281, 311-313.

MOORE, M.A.S. & METCALF, D. (1970) Ontogeny of the haematopoietic system: yolk sac origin of in vivo and in vitro colony-forming cells in the developing mouse embryo. Br. J. Haematol., 18, 279-296.

MOSIER, D.E., MOND, J.J. & GOLDINGS, E.A. (1977) The ontogeny of thymic-dependent antibody responses in vitro in normal mice and with an X-linked B cell defect. J. Immunol., 119, 1874-1878.

MOZES, E. & FUCHS, S. (1974) Linkage between immune response potential to DNA and X chromosome. Nature, 249, 167-168.

MURPHY,E.D., HARRISON, D.E. & ROTHS, J.B. (1973) Giant granules of biege mice: a quantitative marker for granulocytes in bone marrow transplantation.

Transplantation, 15, 526-530.

NAKEFF, A. & DANIELS-McQUEEN, S. (1976) In vitro colony assay for a new class of megakaryocyte precursors: colony-forming unit megakaryocyte (CFU-M). Proc. Soc. Exp. Biol. Med., 151, 587-590.

NARIUCH, H. & KAKIUCHI, T. (1981) Response of spleen cells from mice with X linked B-cell activators, purified protein derivative of tuberculin, and dextran sulphate. Cell. Immunol., 61, 375-385.

NAUHM, M.N., PASLAY, J.W. & DAVIE, J.M. (1983) Unbalanced X chromosome mosaicism in B cells of mice with X-linked immunodeficiency. J. Exp. Med., 158, 920-931.

NESBITT, M.N. (1971) X Chromosome-inactivation mosaicism in the mouse. Devel. Biol., 26, 252-263.

NICOLA, N.A., METCALF, D., von MELCHNER, H. & BURGESS, A.W. (1981) Isolation of murine fetal hemopoietic progenitor cells and selective fractionation of various erythroid precursors. Blood, 58, 376-386.

NIELSEN, J.T. & CHAPMAN, V.M. (1977) Electrophoretic variation for X chromosome-linked phosphoglycerate kinase (PGK-1) in the mouse. Genetics, 87, 319-325.

NIJHOF,W. & WIERENGA, P.K. (1983) Isolation and characterisation of the erythroid progenitor cell CFU-E. J. Cell. Biol., 96, 386-392.

NOWELL, P.C., COLE, L.T., HABERMEYER, J.G. & ROAN, P.L. (1956) Growth and continued function of rat marrow cells in X-irradiated mice. Cancer Res., 16, 258-261.

NOWELL, P.C., HIRSCH, B.E., FOX, D.H. & WILSON, D.B. (1970) Evidence for the existence of multipotential lympho-hematopoietic stem cells in the adult rat. J. Cell. Physiol., 75, 151-158.

OGDEN, D.A. & MICKLEM, H.S. (1976) The fate of serially transplanted bone marrow cell populations from young and old donors. Transplant., 22, 287-293.

OHNO, S. (1969) Evolution of sex chromosomes in mammals. Ann. Rev. Genet., 3, 495-524.

OLIVER, C. & ESSNER, E. (1975) Formation of anomalous lysosomes in monocytes, neutrophils and eosinophils from bone marrow of mice with Chediak-Higashi syndrome. Lab. Invest., 32, 17-27.

ONO, S., YAFFE, L.J., RYAN, J.L & SINGER, A. (1983) Functional heterogeneity of the Lyb-5^{-} B cell subpopulation: mutant xid B cells and normal, Lyb-5^{-} - B cells differ in their responsiveness to phenol-extracted lipopolysaccharide. J. Immunol., 130, 2014-2021.

OPSTELTEN, D. & OSMOND, D.G. (1983) Pre-B cells in mouse bone marrow: immunofluorescence stathmokinetic studies of the proliferation. J. Immunol., 131, 2635-2640.

ORGEL, L.E. (1973) Ageing of clones of mammalian cells. Nature, 243, 441-445.

OSTER-GRANITE, M.L. & GEARHART, J. (1981) Cell lineage analysis of cerebellar cells in mouse chimaeras. Devel. Biol., 85, 199-208.

OWEN, J.J.T., COOPER, M.D. & RAFF, M.C. (1974) In vitro generation of B lymphocytes in mouse foetal liver, a mammalian "bursa equivalent". Nature, 249, 361-363.

PAIGE, C.J., KINCADE, P.W., SHINEFELD, L.A. & SATO, V.L. (1981) Precursors of murine B lymphocytes. Physical and functional charaterisation and distinctions from myeloid stem cells. J. Exp. Med., 153, 154-165.

PAIGE, C.J., KINCADE, P.W., MOORE, M.A.S., LEE, G. (1979) The fate of fetal and adult B cell progenitors grafted into immunodeficient CBA/N mice. J. Exp. Med., 150, 548-563.

PAPAIOANNOU, V.E. & WEST, J.D. (1981) Relationship between the parental origin of the X-chromosomes, embryonic cell lineage and X-chromosome expression in mice. Genet. Res., Camb., 37, 183-197.

PARKER, D.C., FOTHERGILL, J.J. & WADSWORTH, D.C. (1979) B lymphocyte activation by insoluble immunoglobulin. Induction of immunoglobulin secretion by a T cell-dependent soluble factor. J. Immunol., 123, 931-941.

PARKS, D.E., DOYLE, M.V. & WEIGLE, W.O. (1977) Effect of lipopolysaccharide on immungenicity and tolerogenicity of HGG in C57BL/6J nude mice; evidence for a possible B cell deficiency. J. Immunol., 119, 1923-1932.

PEARL, E.R., VOGLER, L.B., OKOS, A.J., CRIST, W.M., LAWTON, A.K. & COOPER, M.D. (1978) B lymphocyte precursors in human bone marrow: an analysis of normal individuals and patients with antibody-deficiency states. J. Immunol., 120, 1169-1175.

PLAYFAIR, J.H.L. & COLE, L.J. (1965) Quantitative studies on colony-forming units in isogenic chimaeras. J. Cell. Comp. Physiol., 65, 7-17.

PLUZNIK, D.H. & SACHS, L. (1965) The cloning of normal "mast" cells in tissue culture. J. Cell. Comp. Physiol., 66, 319-324.

POTTEN, C.S., SCHOFIELD, R. & LAJTHA, L.G. (1979) A comparison of cell replacement ine bone marrow, testis and 3 regions of surface epithelium. Biochim. Biophys. Acta., 560, 281-299.

QUAN, Z.W., DICK, R.F., REGUEIRO, B. & QUINTANS, J. (1981) B cell heterogeneity. II. Transplantation resistance in xid mice which affects the ontogeny of B cell subpopulations. Eur. J. Immunol., 11, 643-649.

RAAB, S.O., ATHENS, J.W., HAAB, O.P., BOGGS, D.R., ASHENBRUCKER, H., CARTWRIGHT, G.E. & WINTROBE, M.M. (1964) Granulocytokinetics in normal dogs. Am. J. Physiol., 206, 83-88.

RAFF, M.C., MEGSON, M., OWEN, J.J. & COOPER, M.D. (1976) Early production of

intracellular IgM by B lymphocyte precursors in the mouse. Nature, 259, 224-226.

RASTEN, S. (1982) Primary non-random X-inactivation caused by controlling elements in the mouse demonstrated at the cellular level. Genet. Res., Camb., 40, 139-147.

RASTEN, S. & CATTANACH, B.M. (1983) Interaction between the Xce locus and imprinting of the paternal X chromosome in the mouse yolk sac endoderm. Nature, 303, 635-636.

REID, G.K. & OSMOND, D.S. (1985) B lymphocyte production in the bone marrow of mice with X-linked immunodeficiency (xid). J. Immunol., 135, 2299-2302.

REINCKE, U., BURLINGTON, H., CRONKITE, E.P. & LAISSUE, J. (1975) Hayflick's hypothesis; an approach to in vivo testing. Fed. Proc., 34, 71-75.

REINCKE, U., HANNON, E.C., ROSENBLATT, M. & HELLMAN, S. (1982) Proliferative capacity of murine haematopoietic stem cells in vitro. Science, 215, 1619-1622.

REINKE, U., HSIEH, P. MAUCH, P. HELLMAN, S. & CHEN, L.B. (1981) Cell types associated with fibronectin in long-term mouse bone marrow cultures. J. Histochem. Cytochem., 30, 235-244.

RICHMAN, L., GRAEFF, A.E., YARCHOAN, R. & STROBER, W. (1981) Simultaneous induction of antigen-specific IgA helper T cells and IgG suppressor T cells in the murine Peyers patches after protein feeding. J. Immunol., 126, 2079-2083.

RIGGS, A.D. (1975) X-inactivation, differentiation and DNA methylation. Cytogenet. Cell. Genet., 14, 9-25.

ROBINSON, S.H., BRECHER, G., LOWRIE, I.S. & HALEY, J.E. (1965) Leucocyte labelling in rats during and after continuous infusion of ^{3}H-thymidine: implications for lymphocyte longevity and DNA reutilization. Blood, 26, 281-288.

ROPKE, C., HOUGEN, H.P. & EVERETT, N.B. (1975) Long-lived T abd B lymphocytes in the bone marrow and thoracic duct lymph of the mouse. Cell. Immunol., 15, 82-93.

ROSENBERG, Y.J. (1979) Influence of the sex-linked defect in CBA/N mice on autoimmune responses to isologoûs erythrocytes. Ability to overcome the defect with age. J. Exp. Med., 150, 1561-1566.

ROSENDAAL, M., HODGSON, G.S. & BRADLEY, T.R. (1976) Haemopoitic stem cells are organised for use on the basis of their generation-age. Nature, 264, 68-69.

ROSENDAAL, M., HODGSON, G.S. & BRADLEY, T.R. (1979) Organisation of haemopoietic stem cells: the generation-age hypothesis. Cell. Tiss. Kinet., 12, 17-29.

ROSS, E.A.M., ANDERSON, N & MICKLEM, H.S. (1982) Serial depletion and regeneration of the murine haematopoietic system. Implications for haematopoietic organisation and the study of cellular ageing. J. Exp. Med., 155, 432-444.

RUSSELL, E.S. (1979) Hereditary anaemias of the mouse: a review for geneticists. Adv. Genet., 20, 357-459.

RUSSELL, E.S. & BERNSTEIN, S.E. (1966) Blood and blood formation. In Biology of the Laboratory Mouse, 351-372 (ed. Green, E.L.) Dover, New York.

RUSSELL, E.S. & BERNSTEIN, S.E. (1968) Proof of whole-cell implant in therapy of W-series anemias. Arch. Biochem. & Biophys., 125, 594-597.

RUSSELL, E.S. & McFARLAND, E.C. (1974) Genetics of mouse hemoglobin. Annals. N.Y. Acad. Sci., 241, 25-38.

RUSSELL, E.S., SMITH, L.J. & LAWSON, F.A. (1956) Implantation of normal blood-forming tissue into radiated genetically anemic hosts. Science, 124, 1076-1077.

SANFORD, J., FORRESTER, L., CHAPMAN, V., CHANDLEY, A. & HASTIE, N. (1984)

Methylation patterns of repetitive DNA sequences in germ cells of Mus musculus. Nucl. Acid. Res., 12, 2823-2836.

SAXE, D.F., BOGGS, S.S. & BOGGS, D.R. (1984) Trabsplantation of chromosomally marked syngeneic marrow cells into mice not subjected to hematopoietic stem cell depletion. Exp. Hematol., 12, 227-283.

SCHER, I. (1982) CBA/N immune defective mice; evidence for the failure of a B cell subpopulation to be expressed. Immunol. Rev., 64, 117-136.

SCHER, I., SHARROW, S.O., WISTAR, R., ASOFSKY, R.& PAUL, W.E. (1976) B lymphocyte heterogeneity: ontogenetic development and organ distribution of B lymphocytes defined by their density of surface immunoglobulin. J.Exp. Med., 144, 494-506.

SCHER, I., STEINBERG, A.D., BERNING, A.K. & PAUL, W.E. (1975) X-linked B lymphocyte immune defect in CBA/N mice II. Studies on the mechanisms underlying the immune defect. J. Exp. Med., 142, 637-650.

SCHOFIELD, R. (1970) A comparitive study of the repopulating potential of grafts from various haematopoietic sources. CFU repopulation. Cell. Tiss. Kinet., 3, 119-130.

SCHOFIELD, R. (1978) The relationship between the spleen colony-forming cell and the haematopoietic stem cell. Blood Cells, 4, 7-25.

SCHOFIELD, R. & LAJTHA, L.G. (1973) Effect of isopropyl methane sulphonate (IMS) on haemopoietic colony-forming cells. Br. J. Haem., 25, 195-202.

SCOLLAY, R.A., WILSON, A. & SHORTMAN, K. (1983) Thymocyte subpopulations: an experimental review, including flow cytometric cross-correlations between the major thymocyte markers. Thymus, 5, 245-295.

SHERR, C.J., RETTENMIER, C.W., SACCA, R., ROUSSEL, M.F., LOOK, A.T. & STANLEY, E.R. (1985) The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell, 41,

665-676.

SHEVACH, E.M., STOBO, J.D. & GREEN, I. (1972) Immunoglobulin- and theta-bearing murine leukaemias and lymphomas. J. Immunol., 108, 1146-1151.

SHINOHARA, N., HANSEN, T.H., SCHURKO, E. McKENZIE, I.F.C. & SACHS, D.H. (1978) Genetic control of the immune response to the H-2D^{K} private specificity, H-2.32. II Genetic linkage analysis of a backcross generation. J. Immunol., 121, 2472-2476.

SIMINOVITCH, L., McCULLOCH, E.A. & TILL, J.E. (1963) The distribution of colony-forming cells among spleen colonies. J. Cell. Comp. Physiol., 62, 327-336.

SLACK, J., der BARLIAN, G., NAHM, M. & DAVIE, J.M. (1980) Subclass-restriction of murine antibodies II. The IgG plaque-forming cell response to TI-1 and TI-2 antigens in normal and CBA/N mice. J. Exp. Med., 151, 853-862.

SLAVIN, S & STROBER, S. (1978) Spontaeous murine B cell leukaemia. Nature, 272, 624-626.

SLEASE, R.B., WISTAR, R. & SCHER, I. (1979) Surface immunoglobulin density on human peripheral blood mononuclear cells. Blood, 54, 72-86.

SMITH, S.K., BROWN, V.A., DEWAR, A.E., STOCKDILL, G. COHEN, B. & MADDY, A.H. (1985) Abnormalities in the expression of the leukocyte-common antigen in chronic lymphocytic leukaemia. Clin. Exp. Immunol., 59, 55-63.

SMITH, S.K. & MADDY, A.H. (1984) Surface glycoproteins in chronic lymphocytic leukaemia in 'glyco-conjugates and tumour cell surfaces. Biochem. Soc. Trans., 12, 535.

SOKAL, R.R. & ROHLF, F.J. (1969) Biometry: the principles and practice of statistics in biological research. Freeman and Co. San Fransisco.

SPEAR, P.G., WANG, A., RITISHAUSER, U. & EDELMAN, G. (1973) Characterisation

of splenic lymphoid cells in fetal and newborn mice. J. Exp. Med., 138, 557-573.

SPOONCER, E., LORD, B.I. & DEXTER, T.M. (1985) Defective ability to self- renew in vitro of highly purified primitive haematopoietic cells. Nature, 316, 62-64.

SPRENT, J. & BASTEN, A. (1973) Circulating T and B lymphocytes of the mouse. III. Lifespans. Cell. Immunol., 7, 40-59.

SPRENT, J. & BRUCE, J. (1984a) Physiology of B cells in mice with X-linked immunodeficiency. II. Influence of the thymus and mature T cells on B cell differentiation. J. Exp. Med., 160, 335-340.

SPRENT, J. & BRUCE, J. (1984b) Physiology of B cells im mice with X-linked immunodeficiency. III. Disappearance of xid B cells in double bone marrow chimaeras. J. Exp. Med., 160, 711-723.

SPRENT, J., BRUCE, J., RON, Y. & WEBB, S.R. (1985) Physiology of B cells in mice with X-linked immunodeficiency. I. Size, migratory properties and turnover of the B cell pool. J. Immunol., 1442-1448.

STANLEY, E.R. & JUBINSKY, P.T. (1984) Factors affecting the growth and differentiation of haematopoietic cell in culture. Clinics in Haematol., 13, 329-348.

STEPHENSON, J.R., AXELRAD, A.A., McLEOD, D.L. & SHREEVE, M.M. (1971) Induction of colonies of hemoglobin-synthesising cells by erythropoietin in vitro. Proc. Natl. Acad. Sci. USA., 68, 1542-1546.

STROBER, S. (1972) Initiation of antibody responses by different classes of lymphocytes. V. Fundamental changes in the physiological characteristics of virgin thymus-independent (B) lymphocytes and B memory cells. J. Exp. Med., 136, 851-871.

STONE, M. (1983) A general statistical model for clone-tissue studies, using X chromosome-inactivation data. Biometrics, 39, 395-409.

STONE, M. (1984) Variance/covariance modelling with chromosome markers. J. Theor. Biol., 107, 275-286.

SUZUKI, S. & AXELRAD, A.A. (1980) Fv-2 locus controls the proportion of erythropoietic progenitor cells (BFU-E) synthesizing DNA in normal mice. Cell, 19, 225-236.

TAKAGI,N. & SASAKI, M. (1975) Preferential inactivation of the paternally-derived X chromosome in the extraembryonic membranes of the mouse. Nature, 256, 640-642.

TAKAGI, N. SUGAWARA, O. & SASAKI, M. (1982) Regional and temporal changes in the pattern of X chromosome replication during the early postimplantation development of the female mouse. Chromosoma, 85, 275-286.

TILL, J.E. & McCULLOCH, E.A. (1961) A direct measure of radiation sensitivity of normal mouse bone marrow. Rad. Res., 14, 213-222.

TILL, J.E. & McCULLOCH, E.A. (1980) Haematopoietic stem cell differentaion. Biochim. Biophys. Acta., 605, 431-459.

TOKZOZ, D., DEXTER, T.M., LORD, B.I., WRIGHT, E.G. & LAJTHA, L.G. (1980) The regulation of hemopoiesis in long-term bone marrow cultures. II. Stimulation and inhibition of stem cell proliferation. Blood, 55, 931-936.

TRENTIN, J.J., WOLF, N., CHENG, V., FAHLBERG, W., WEISS, D. & BONHAG, R. (1967) Antibody production by mice repopulated with limited numbers of clones of lymphoid precursors. J. Immunol., 98, 1326-1337.

TURNER, R.W.A., SIMINOVITCH, L., McCULLOCH, E.A. & TILL, J.E. (1967) Density gradient centrifugation of hemopoietic colony-forming cells. J. Cell. Physiol., 68, 73-82.

VALERIOTE, F. & TOLEN, S. (1983) Extensive proliferative capacity of haematopoietic stem cells. Cell. Tiss. Kinet., 16, 1-5.

VINCENT, P.C. (1977) Granulocyte kinetics in health and disease. Clinics in Haematol., 6, 695-717.

VINCENT, P.C., CHANANA, A.D., CRONKITE, E.P. & JOEL, D.D. (1974) The vascular survival of neutrophils. Blood, 43, 371-377.

VISSER, J.W.M., BAUMAN, J.G.J., MULDER, A.H., ELIASON, J.R. & de LEEUW, A.M.J. (1984) Isolation of murine pluripotent hemopoietic stem cells. J. Exp. Med., 159, 1576-1590.

VITETTA, E.S. & MELCHERS, U., McWILLIAMS, M., LAMM, M.E., PHILLIPS-QUAGLIATA, J. & UHR, J.W. (1975) Cell surface immunoglobulin XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. J. Exp. Med., 141, 206-215.

VITETTA, E.S. & UHR, J.W. (1977) IgD and B cell differentiation. Immunol. Rev., 37, 50-88.

VITETTA, E.S., YUAN, D., KROLICK, K., ISAKSON, P., KNAPP, M., SLAVIN, S. & STROBER, S. (1979) Characterisation of the spontaneous murine B cell leukaemia (BCL-1). III. Evidence for monoclonality by using anti-idiotype antibody. J. Immunol., 122, 1649-1654.

VOGLER, L.B., PEARL, E.R., GATHINGS, W.E., LAWTON, A.R. & COOPER, M.D. (1976) B lymphocyte precursors in bone marrow in immunoglobulin-deficient disease. Lancet, 2, 376.

VOLF, D., SENSENBRENNER, L.L., SHARKIS, S.J., ELFENBEIN, G. & SCHER, I. (1978) Induction of partial chimaerism in nonirradiated B lymphocyte-deficient CBA/N mice. J. Exp. Med., 147, 940-945.

WALLIS, V.J., LEUCHARS, E., CHWALINSKI, S. & DAVIES, A.J. (1975) On the sparse seeding of bone marrow and thymus in radiation chimaeras. Transplant., 19, 2-10.

WEST, J.D. (1982) X chromosome expression during mouse embryogenesis. In

Genetic Control of Gamete Production and Function. (eds. Crosignani, P.G., Rubin, B.L. & Fraccaro, M.). Academic Press.

WEST, J.D. (1984) Cell markers. In Chimaeras in Developmental Biology. (eds. Le Douarin, N. & McLaren, A.). Academic Press.

WEST, J.D., FRELS, W.I. & CHAPMAN, V.M. (1977) Preferential expression of the maternally-derived X chromosome in mouse yolk sac. Cell, 12, 873-882.

WHITNEY, J.B. (1978) Simplified typing of mouse hemoglobin (Hbb) phenotypes using cystamine. Biochem. Genet., 16, 667-672.

WILLIAMS, D.A., LEMISCHKA, I.R., NATHAN, D.G. & MULLIGAN, R.C. (1984) Introduction of new genetic material into pluripotent haematopoietic stem cells of the mouse. Nature, 310, 476-480.

WITKOWSKI, J. FORRESTER, L.M., ANSELL, J.D. & MICKLEM, H.S. (1985) Influence of the xid mutation on B lymphocyte development in adult mice. In Microenvironments in the Lymphoid System. (ed. Klaus, GGB). Adv. Exp. Med. Biol., 186, 47-56.

WHITLOCK, C.A. & WITTE, O.N. (1982) Long-term cultures of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci., 79, 3608-3612.

WOLF, N.S., MACMILLAN, J.R. & PRIESTLEY, G.V. (1983) Decline of colony-forming units with serial bone marrow cell passages: intrinsic or extrinsic causation. Blood Cells, 9, 515-525.

WORTIS, H.H. (1974) Immunological studies in nude mice. In Contemporary Topics in Immunobiology, 111, (ed. Warner, N.). Plenum Publishing Corporation. New York.

WORTIS, H.H., BURKLY, L., HUGHES, D., ROSCHELLE, S. & WANECK, G. (1982) Lack of mature B cells in nude mice with X-linked immune deficiency. J. Exp. Med., 155, 903-913.

WORTON, R.G., McCULLOCH, E.A. & TILL, J.E. (1969) Physical separation of hematopoitic stem cells differing in their capacity for self-renewal. J. Exp. Med., 130, 91-103.

WRIGHT, E.G., LORIMORE, S.A. & LORD, B.I. (1985) Regulators of stem cell proliferation in the haemopoietic tissues of W/W^{V} and Sl/Sl^{d} mice. Leuk. Res., 9, 491-496.

WU, A.M., TILL, J.E., SIMINOVITCH, L. & McCULLOCH, E.A. (1967) A cytological study of the capacity for differentiation of normal hematopoietic colony-forming cells. J. Cell. Physiol., 69, 177-184.

WU, A.M., TILL, J.E., SIMINOVITCH, L. & McCULLOCH, E.A. (1968) Cytological evidence for a relationship between normal haematopoietic colony-forming cells and cells of the lymphoid system. J. Exp. Med., 127, 455-463.

APPENDIX I. Appendix to Materials and Methods

(a) Electrophoresis Buffer

for 1 litre, in distilled water at pH 8.8

	molarity	quantity
EDTA	2mM	0.744g
sodium citrate	10mM	2.94g
magnesium sulphate	5mM	1.23g
sodium barbital	20mM	4.12g

(b) Sample Buffer

to 100ml of 50mM tri-ethanolamine-HCL (T-eth-HCL) (pH 7.6) add:

	concentration	quantity
dithioerythritol (DTE)	0.3mg/ml	30mg
bovine serum albumin (BSA)	0.5mg/ml	50mg

(c) Indicator Stock

to 10ml distilled water add:

	molarity	quantity
T-eth HCL	100mM	185.6mg
magnesium suphate	130mM	320mg
glucose	150mM	270mg
ADP (Sigma, A 8146)	25mM	112.5mg
NADP (Sigma, N 0505)	40mM	306mg

The solution is adjusted to pH 7.6 with 2N NaOH and 100µl aliquots are stored at -20°C.

(d) Assay Stock

to 40ml electrophoresis buffer add:

	molarity	quantity
NAD (Sigma, N 7004)	1.2mM	32mg
trisodium fructose 1,6-diphosphate (Sigma 752-1)	40mM	650mg
K_2HPO_4	40mM	279mg

400µl aliquots are stored at -20°C.

(e) Stock solutions of MTT and PMS

to 50ml distilled water add:

	Concentration	quantity
MTT (thiazolyl blue, Sigma M2128)	10mg/ml	500mg
PMS (phenazine methosulphate, Sigma P 9625)	2.5mg/ml	125mg

(f) Enzymes

	Volume used	concentration *
G6PDH (Sigma, G 8878)	10µl	225 units/ml
HK (Sigma, H 5625)	5µl	8000 units/ml
ALD (Sigma, A 6253)	10µl	6.5 units/ml
GDH (Sigma, 6751)	5µl	222 units/ml
GAPDH (Boehringer Mannheim)	7.5µl	10 mg/ml

* This refers to the manufacturers concentration and the volumes will have to be adjusted if the manufacturers change the concentration of the solution.

(g) GPI Supre-heme buffer

1 sachet Supre-heme buffer (Helena Labs., No. 5802) diluted with 1 litre distilled water.

(h) GPI Assay Stock

to 20ml 1.0M Tris-HCl, pH 8.0, add:

	quantity
NADP (Sigma, N050)	60mg
Fructose-6-phosphate (Sigma, F 3627)	130mg

aliquots (1ml) stored at -20°C.

(i) Phosphate-buffered saline (PBS)

for 10 litres, in distilled water

	quantity
KH_2PO_4	8.6g
Na_2HPO_4-$12H_2O$	56.9g
NaCl	56.7g

pH to 7.2

(j) RPMI-1640 medium

10X RPMI-1640 (Gibco, M405))	50ml
Hepes buffer (1M)(Gibco, 043-5639)	10ml
7.5% Sodium bicarbonate soln. (Gibco, 043-5630)	12.5ml
10% Sodium azide	5ml
distilled water	422.5ml

pH adjusted to 7.2. Before use 3.3ml 10% bovine serum albumin is added to 100ml of the above medium.

(k) Acridine-orange/Ethidium-bromide stain

Acridine-orange (BDH, No. 34001)	50mg
Ethidium-bromide (Sigma, E8751)	15mg

Dissolve in 1ml 95% ethanol, add 49ml distilled water and mix well. Store in 1ml aliquots at -20°C.
The working solution is madeby diluting one aliquot in 100ml PBS. This is stored in a dark bottle at 4°C and is usable for up to one month.

APPENDIX II-VI

Experimental Data

The following pages consists of all experimental data from Chapters 3,4,5 and 6. The results are expressed as %PGK-1A present in all experiments. Replicate samples are indicated thus 77/75 - ie the first analysis gave a result of 77 and the second, 75 %PGK-1A. Both results are shown if the sample was analysed twice.

The following abbreviations have been used in this Appendix.

E - erythrocytes

B - B lymphocytes

T - T lymphocytes

G - granulocytes

LN - lymph node

SPL - spleen

BM - bone marrow

sIg^- - surface-immunoglobulin negative

sIg^+ - surface-immunoglobulin positive

APPENDIX II

%PGK-1A present in progeny of 5 different crosses. The Xce and Pgk-1 genotype of the parental X chromosomes are given: male x female.

Cross 1. $Xce^{b}/Pgk\text{-}1^{b}$ x $Xce^{c}/Pgk\text{-}1^{a}$

69 65 66 75 64 60 73 81 57 62 64 76 60 47 71 56 56 53
59 55 50 70 64 81 55 69 58 57 56 60 55 53 52 43 52 59

Cross 2. $Xce^{c}/Pgk\text{-}1^{a}$ x $Xce^{b}/Pgk\text{-}1^{b}$

63 67 68 53 60 52 48 46 45 55 51 40 66 57 52 44 45 53
55 48 56 47 56 58 45 49 48 62 49 48 60 56 62 53 52 56

Cross 3. $Xce^{c}/Pgk\text{-}1^{a}$ x $Xce^{b}/Pgk\text{-}1^{b}$

48 49 44 48 36 48 46 42 48 45 45 33 50 42 60 40 59 53
57 47 55 51 48 53 60 51 38 50 46 56 67 33 54 50 52 57
55 62 48 63 47 66 57 50 54 43 63 52 67 46 63 40 67 46
44 53

Cross 4. $Xce^{b}/Pgk\text{-}1^{b}$ x $Xce^{c}/Pgk\text{-}1^{a}$

56 74 66 66 66 59 76 66 43 47 68 41 57 57 61 60 50 45
53 63 62 67 45 49 30 66 64 62 62 57 55

Cross 5. $Xce^{b}/Pgk\text{-}1^{b}$ x $Xce^{c}/Pgk\text{-}1^{a}$

66 65 67 56 65 78 61 58 55 64 58 42 42 57 59 75 51 66
62 60 62 46 61 52 45 68 70 83 65 54 50 56

APPENDIX III

Experiment 1

MOUSE DATE of SAMPLE	B3	B5	C1	C5	D1	D5
14.3.83	84/84	84	68/72	68	73	68
28.3.83	75/81	71/84	63/70	72	-	54/64
3	86/81	81/84	68/73	72	75	61
4	72/81	73/83	64/70	78/76	81	58
17.5.83	81/81	79/81	73/72	80/82	78	71/63
31.5.83	82/80	75/81	66/72	68	72/82	58
14.6.83	71/82	73/82	75/70	72	-	59
28.6.83	73/82	78/78	70/67	69	76	57
13.7.83	82/83	68/80	66/64	76	74/84	54
26.7.83	80/84	79/80	68/65	71	-	-
9.8.83	84	-	66	73	75	57
9.9.83	80	75	70	72	78	64/61
17.10.83	79	76	68	74	75	-
17.11.83	78	73	64	65/74	77	55
18.12.83	78	66	70	-	76	53
-.1.84	86/86	75/78	-	68	84	62
-.2.84	83	73	64	72	76	58
16.3.84	81	73	62/64	73	77	58
4.4.84	79	72	63/63	68	77	58
-.5.84	79	74	67	66	79	59
2.6.84	79	70	68	68	78	58
26.6.84	77/88	72	63	72	-	56
23.7.84	80/83	70/69	64/62	73	83/82	57
2.10.84	87/86	-	-	-	75	-
9.12.84	90/87	76	72/66	71	-	62
-.1.85	80/81	73/74	67/64	73/74	85	67
14.2.85	83	75	64	68	80	61
13.3.85	79	DEAD	60	64	80	59
11.5.85	82		64	66	DEAD	60

APPENDIX III

Experiment 2

MOUSE	A2		A4		B7	
DATE	E	G	E	G	E	G
2.12.82	69/79	-	81	84	85	83
15.12.82	66/66	71	81/86	82	82/79	80
7.1.83	66	67	75	-	72	-
21.1.83	66/71	69	82	82	75/76	-
4.2.83	63/65	70	76/76	70	74/72	81/79
18.2.83	66/71	70	77	82	73	77/74
4.3.83	71/68	66	78/84	86	71/79	77
18.3.83	67/75	-	78/87	-	77	-
31.3.83	69/72	73	84/79	-	82	80
24.4.83	71	78	-	80	77	-
13.5.83	65/73	73	-	-	77/82	-
27.5.83	71	68	80	79	73	79
	66	-	-	-	-	-
	-	-	-	-	-	-
14.5.84	-	-	84	79	70	-
1.10.84	-	-	73/82	76	66/69	68/75

APPENDIX III

Experiment 2, continued

MOUSE	C11		C12		C13		C14	
DATE	E	G	E	G	E	G	E	G
10.1.83	75	73	79	74	80/77	73	74	73
24.1.83	72/76	70	74/73	73	69/75	-	68/60	71
7.2.83	76	-	78	77	77	-	69	70
21.2.83	80/81	78	82/77	71	83/76	79	74/65	68
7.3.83	80/77	74	79/82	-	73/82	80	70	-
21.3.83	75/78	-	74	-	77	-	59	-
4.4.83		-	-	82	77	79	66	62
2.5.83	76/70	77	75/80	-	77	78	58/64	65
16.5.83	83/79	-	74	80	75	-	57/60	69
	83	77	-	72	-	-	-	-
29.10.84	81/81	86/81	-	-	86/85	83/84	60/58	62
26.11.84	83	-	-	-	82	78	54	-
28.1.85		-	-	-	86/82	-	59	63

APPENDIX III

Experiment 3

Mouse C1

Age (months)	E	B	T	G
3.0	82/73	78/71	75/68	72/70
3.5	66/70	72/75	69/72	70/69
4.0	68/71	70/69	70/69	65/70
4.5	65/65	75/75	76/72	66/69
5.0	59/63	62/68	62/68	58/59
5.5	64/65	66/66	65/72	60/60
6.0	69/67	67/65	64/64	60/56
6.5	64/66	68/68	70/68	63/61
7.0	66/66	69/70	73/70	74/62
7.5	59/62	65/62	64/64	59/60
8.0	66/60	66/62	65/62	62/59

Mouse C2

Age (months)	E	B	T	G
3.0	77/74	76/74	74/75	78/74
3.5	73/72	69/71	75/75	80/80
4.0	80/78	72/77	79/79	85/84
4.5	76/80	75/75	79/-	84/-
5.0	77/80	66/72	68/71	80/81
5.5	79/81	73/82	71/77	70/86
6.0	83/79	77/75	74/74	77/82
6.5	84/83	81/77	82/78	85/78
7.0	89/86	85/79	86/80	85/84
7.5	81/82	74/78	80/82	86/83
8.0	86/83	80/81	79/84	84/84

APPENDIX III

Experiment 3, continued

Mouse C3

Age (months)	E	B	T	G
3.0	84/83	80/83	78/79	82/87
3.5	85/86	82/85	79/78	85/78
4.0	84/83	79/80	80/80	76/79
4.5	87/84	83/85	83/76	75/75
5.0	81/86	81/82	81/80	81/80
5.5	84/84	82/82	81/78	80/78
6.0	88/85	86/84	82/78	80/80
6.5	87/85	84/83	80/81	85/-
7.0	86/84	80/86	81/80	80/79
7.5	85/85	83/86	79/85	85/80
8.0	82/83	83/80	77/-	83/84

Mouse C4

Age (months)	E	B	T	G
3.0	68/70	62/64	61/62	69/70
3.5	71/68	64/61	61/65	67/70
4.0	62/64	57/62	57/63	61/71
4.5	66/69	65/64	65/63	68/67
5.0	66/72	64/56	62/64	66/66
5.5	65/71	60/63	64/65	68/68
6.0	65/62	61/63	61/67	59/65
6.5	68/75	68/74	67/69	64/68
7.0	63/68	64/66	68/64	62/66
7.5	64/64	62/63	63/71	63/-
8.0	65/66	62/66	63/65	59/-

APPENDIX III

Experiment 3, continued

Mouse C5

Age (months)	E	B	T	G
3.0	83/-	76/77	78/72	80/-
3.5	73/71	73/74	78/77	78/74
4.0	81/75	78/73	73/76	79/75
4.5	80/80	79/81	77/80	74/74
5.0	79/76	76/77	74/71	74/71
5.5	80/77	74/77	72/75	73/73
6.0	77/80	77/77	69/78	68/75
6.5	80/79	75/78	80/84	76/84
7.0	78/80	78/77	77/78	78/85
7.5	75/77	77/75	76/74	75/72
8.0	77/75	77/73	79/70	80/73

Mouse C6

Age (months)	E	B	T	G
3.0	77/-	73/-	71/-	72/-
3.5	77/71	76/73	73/72	67/72
4.0	77/74	72/78	71/83	70/76
4.5	79/80	74/74	68/75	71/72
5.0	74/72	73/70	71/75	70/72
5.5	76/80	75/78	73/74	74/66
6.0	70/72	74/73	74/75	69/75
6.5	77/79	75/80	81/-	84/71
7.0	75/78	80/78	79/75	78/68
7.5	73/68	74/68	73/72	74/74
8.0	77/75	72/76	74/74	72/77

APPENDIX IV

Mouse LR1

Months after repopulation	E	B	T	G
3.0	17/16	32/33	41/38	24/23
3.5	24/19	30/29	39/41	30/31
4.0	27/30	31/33	40/39	36/32
4.5	37/28	39/40	38/42	34/41
5.0	46/43	46/40	57/53	43/-
5.5	49/51	35/38	47/46	50/50
6.0	43/50	43/48	53/51	51/53
6.5	61/61	42/39	51/50	54/57
7.0	65/62	47/47	56/59	59/69
7.5	66/71	52/45	57/52	65/61
8.0	66/71	44/47	52/60	71/68
8.5	69/67	52/45	59/51	69/65
10.0	78/79	63/63	62/62	66/68
11.0	72/77	69/70	70/69	77/69

Mouse LR2

Months after repopulation	E	B	T	G
3.0	18/11	41/35	48/50	6/0
3.5	0/0	24/28	44/42	0/0
4.0	0/0	39/37	54/54	0/0
4.5	0/0	17/23	19/23	0/0
5.0	0/0	31/32	33/32	0/0
5.5	0/0	35/29	38/38	0/0
6.0	0/0	18/21	25/29	0/0
6.5	0/0	27/32	35/42	0/0
7.0	0/0	32/30	38/39	0/0
7.5	0/0	19/23	33/37	0/0
8.0	0/0	30/27	35/34	0/0
8.5	0/0	18/17	27/31	0/0
10.0	0/0	16/13	28/27	0/0
11.0	0/0	7/6	20/18	0/0

APPENDIX IV, continued

Mouse LR3

Months after repopulation	E	B	T	G
3.0	56/56	60/65	42/42	70/64
3.5	-/-	78/78	64/58	84/85
4.0	79/76	67/73	54/56	82/88
4.5	90/88	87/85	79/72	100/100
5.0	92/89	72/75	72/70	100/100
5.5	100/100	80/76	61/59	95/94
6.0	100/92	84/85	70/71	100/100
6.5	100/100	88/86	76/74	100/100
7.0	100/100	85/84	58/64	100/100
7.5	100/100	87/90	76/80	100/100
8.0	100/100	88/88	67/63	100/100
8.5	100/100	90/92	73/81	97/96
10.0	100/96	92/95	88/88	100/100
11.0	100/90	92/91	81/81	100/100

Mouse LR4

Months after repopulation	E	B	T	G
3.0	100/90	68/74	56/60	100/100
3.5	100/100	81/80	64/72	100/100
4.0	100/100	78/78	64/61	100/100
4.5	100/100	89/92	78/76	80/79
5.0	100/97	82/81	69/74	89/100
5.5	100/100	82/76	62/65	100/100
6.0	100/100	80/87	71/76	100/100
6.5	100/100	83/83	73/78	100/100
7.0	100/100	79/82	68/70	100/100
7.5	100/100	88/88	75/76	100/100
8.0	100/100	85/88	66/76	-/-
8.5	100/100	88/95	78/84	100/100
10.0	96/100	90/94	81/87	100/95
11.0	100/100	100/100	90/88	-/-

APPENDIX IV, continued

Mouse LR6

Months after repopulation	E	B	T	G
3.0	83/100	82/100	84/86	87/85
3.5	85/-	86/84	82/81	79/80
4.0	82/85	82/79	75/82	78/75
4.5	78/70	85/82	86/80	73/70
5.0	78/83	76/81	84/88	56/69
5.5	79/76	82/81	83/84	62/59
6.0	63/63	70/72	71/74	56/58
6.5	69/68	75/72	76/72	48/48
7.0	58/55	69/64	78/68	48/43
7.5	59/54	54/59	63/62	34/39
8.0	57/57	64/62	72/69	43/40
8.5	50/51	56/57	63/62	39/33
10.0	48/43	52/47	54/52	40/33

Mouse LR7

Months after repopulation	E	B	T	G
3.0	33/36	48/50	58/58	27/29
3.5	30/29	44/41	50/52	28/23
4.0	39/33	47/44	52/51	37/27
4.5	15/14	35/33	48/43	16/0
5.0	23/-	34/42	50/51	16/13
5.5	15/-	34/53	45/56	44/57
6.0	12/9	28/18	27/29	7/10
6.5	16/14	27/35	36/37	9/19
7.0	12/12	36/30	42/36	11/12
7.5	12/13	36/34	44/40	10/13
8.0	14/13	31/30	42/40	11/17
8.5	12/8	18/20	28/29	7/-
10.0	13/8	21/17	26/29	13/7
11.0	0/8	24/20	21/26	0/0

APPENDIX V

Mouse HR1

Months after repopulation	E	B	T	G
3.0	42/-	49/46	45/44	40/48
3.5	36/37	44/43	46/43	46/36
4.0	28/30	35/40	38/36	42/33
4.5	34/29	44/38	-/-	-/-
5.0	27/28	32/33	35/45	32/45
5.5	42/42	49/50	42/42	40/39
6.0	36/38	40/39	43/41	36/36
6.5	34/31	40/38	44/40	42/35
7.0	24/30	35/33	40/38	30/-
7.5	32/36	35/43	34/42	38/36
8.0	35/32	40/36	40/43	33/46
8.5	33/25	38/30	33/31	29/25

Mouse HR2

Months after repopulation	E	B	T	G
3.0	41/-	36/45	37/41	36/44
3.5	39/-	47/39	47/39	45/33
4.0	26/27	31/32	30/31	30/30
4.5	26/-	37/-	31/39	28/28
5.0	27/-	31/46	33/31	27/32
5.5	31/28	46/42	40/-	36/37
6.0	30/32	33/34	34/36	30/36
6.5	32/32	36/37	-/-	-/-
7.0	26/25	36/31	34/35	27/-
7.5	29/31	36/36	37/40	30/33
8.0	29/-	38/-	36/39	34/32
8.5	33/34	37/39	41/37	35/24

APPENDIX V, continued

Mouse HR3

Months after repopulation	E	B	T	G
3.0	36/38	46/48	46/45	37/41
3.5	42/-	46/-	49/35	44/33
4.0	28/30	37/44	45/44	35/40
4.5	28/25	31/31	32/35	24/28
5.0	33/37	36/38	36/45	29/37
5.5	35/35	43/46	42/43	-/-
6.0	31/29	38/40	38/40	30/29
6.5	37/35	48/38	49/43	39/36
7.0	35/27	40/39	40/41	41/36
7.5	38/35	36/30	43/45	35/38
8.0	27/27	38/34	38/32	32/23
8.5	29/31	37/34	37/38	35/37

Mouse HR4

Months after repopulation	E	B	T	G
3.0	36/-	36/40	42/39	39/42
3.5	43/34	44/40	45/42	41/41
4.0	33/34	38/39	42/41	37/36
4.5	-/-	28/44	39/50	35/45
5.0	35/41	36/36	37/37	40/-
5.5	38/38	44/44	41/45	39/-
6.0	32/34	34/39	38/42	29/35
6.5	40/38	39/43	49/46	43/-
7.0	34/34	38/36	42/38	35/36
7.5	38/37	36/41	39/42	29/34
8.0	29/27	37/31	44/33	41/25
8.5	30/34	38/36	43/43	37/34

APPENDIX V, continued

Mouse HR5

Months after repopulation	E	B	T	G
3.0	37/37	36/44	42/47	38/47
3.5	43/37	37/38	38/43	31/36
4.0	32/34	34/34	33/39	44/35
4.5	30/38	29/39	28/-	28/41
5.0	33/37	43/42	38/-	40/-
5.5	39/38	43/42	45/42	43/37
6.0	38/38	38/34	37/37	35/34
6.5	40/38	40/-	38/37	42/34
7.0	33/36	41/46	40/33	38/32
7.5	47/30	48/33	44/27	40/31
8.0	39/33	35/39	38/45	28/34
8.5	29/36	33/33	36/32	37/34

Mouse HR6

Months after repopulation	E	B	T	G
3.0	30/35	38/44	40/37	33/34
3.5	34/-	45/-	37/43	36/39
4.0	25/33	33/37	33/34	23/33
4.5	34/-	24/-	-/-	-/-
5.0	32/27	26/34	30/37	36/-
5.5	32/34	34/36	35/37	32/36
6.0	35/31	30/26	35/32	30/27
6.5	34/33	34/36	38/38	32/33
7.0	30/27	33/32	33/40	31/39
7.5	33/28	36/33	34/-	36/-
8.0	27/36	33/32	38/34	28/30
8.5	27/29	32/33	28/33	32/33

APPENDIX VI Chapter 6 experimental data.

Mouse		9	11	12	14	16	18
Tissue	Age	4mo.	5mo.	6mo.	-	4.5mo.	3mo.
erythrocytes		61/65	73/-	63/-	60/63	37/32	62/69
Thymus		60/55	69/-	-	66/-	41/37	59/60
Bone marrow (BM)		54/56	59/-	-	62/61	32/35	60/65
Spleen (SPL)		78/74	72/-	64/-	78/78	63/61	77/75
Lymph node (LN)		65/60	69/-	-	64/66	37/41	68/70
LN mIg^-		59/62	68/-	54/-	-	-	62/65
LN mIg^+		91/100	100/-	100/-	100/-	-	100/100
SPL mIg^-		-	-	-	-	-	62/64
SPL mIg^+		-	-	-	-	-	100/100
BM mIg^-		-	-	-	57/-	25/31	52/58
BM mIg^+		-	-	-	79/-	42/39	73/74
BM 14.8 dull		51/56	64/-	60/-	-	27/30	56/58
BM 14.8 bright		48/54	74/-	70/-	-	51/53	64/63
Granulocytes		62/61	68/-	-	-	-	-
PBL sIg^-			67/-	58/-	-	-	-
PBL sIg^+			82/-	100/-	-	-	-
Kidney							62/-
Brain							63/64

APPENDIX VI, continued

Mouse		19	22	23	24	27	28
Tissue	Age	1mo.	6 wk.	1mo.	5.5mo.	6mo.	2wks
Erythrocytes		64/61	48/53	77/-	60/-	73/77	65/63
Thymus		63/58	-	-	54/-	74/73	59/61
Bone marrow		69/67	-	84/-	-	74/74	51/-
Spleen		-	59/59	84/-	67/-	76/84	60/66
Lymph node		55/56	-	84/-	60/-	78/80	42/48
LN mIg^-		56/54	46/49	83/81	67/65	72/76	48/46
LN mIg^+		87/-	88/84	86/-	82/88	100/94	60/64
SPL mIg^-		56/55	42/48	81/79	74/68	77/78	43/53
SPL mIg^+		74/-	65/75	90/86	79/79	100/95	62/65
BM mIg^-		66/68	40/35	75/75	61/62	74/76	47/44
BM mIg^+		68/74	48/45	76/77	66/-	86/90	45/46
BM 14.8 dull		60/72	42/44	78/74	53/-	73/78	
BM 14.8 bright		66/72	45/45	80/-	57/-	79/-	
Kidney		-			66/-		
Brain		65/64			-		
Granulocytes							52/52

APPENDIX VI, continued

Mouse		25	29	30	31	32	33
Tissue	Age	11wk.	3.5mo.	6wks.	6wks.	6wks.	6mo.
Erythrocytes		61/-	65/66	51/57	62/64	61/64	61/63
Thymus		73/71	74/68	59/60	62/60	61/60	62/71
Bone marrow		65/63	70/64	55/55	59/62	64/67	52/60
Spleen		74/-	82/85	67/67	74/77	64/65	86/84
Lymph node		-	71/68	61/62	62/68	56/56	68/68
LN sIg^-		-	-	-	-	-	75/70
LN sIg^+		-	-	-	-	-	89/86
SPL sIg^-		-	-	-	-	-	-
SPL sIg^+		-	-	-	-	-	-
BM sIg^-		66/73	63/68	48/51	55/55	63/61	57/56
BM sIg^+		70/72	80/81	50/56	66/65	72/70	66/-
BM 14.8 dull		67/68	62/64	43/45	52/50	57/64	54/56
BM 14.8 bright		67/68	76/69	53/59	61/61	64/61	51/62
Granulocytes		70/73	52/52	-	-	-	-
Two Col. SPL -ve		72/-	70/71	58/58	62/65	60/57	71/66
Pop. I		100/-	100/100	97/100	96/100	100/100	100/100
Pop. II		94/100	100/100	77/81	90/94	92/93	100/100
Pop. III		92/100	91/88	68/74	87/89	84/88	100/100

APPENDIX VI, continued

Mouse		34	38	39	40	41	43
Tissue	Age	2wks.	17mo.	2wks.	2wks.	18mo.	18mo.
Erythrocytes		62/72	52/-	62/-	-	48/50	-
Thymus		72/76	54/52	-	-	45/50	-
Bone marrow		66/65	-	-	-	61/53	-
Spleen		76/76	84/84	62/66	60/-	78/79	-
Lymph node		75/78	-	-	46/50	56/60	-
LN sIg^-		75/76	62/59	66/52	59/55	54/58	77/81
LN sIg^+		89/86	100/100	53/60	64/59	87/81	100/100
BM sIg^-		67/66	73/63	55/44	61/54	36/43	73/69
BM sIg^+		71/72	92/100	-	60/-	81/81	93/94
BM 14.8 dull		-	76/69	-	-	33/40	75/78
BM 14.8 bright		-	86/100	-	-	73/75	-
PBL sIg^-		-	53/55	-	-	48/51	-
PBL sIg^+		-	83/84	-	-	73/75	-
Two Col. SPL -ve		61/67		67/61	58/53	62/57	86/80
	Pop. I	100/100	-	-	-	100/100	100/-
	Pop. II	89/93	-	83/-	81/81	100/100	100/-
	Pop. III	85/82	-	66/52	58/58	100/100	100/-

APPENDIX VI, continued

Mouse		44	45	46	47	48
Tissue	Age	13mo.	3mo.	12mo.	20mo.	14mo.
Erythrocytes		70/71	71/70	74/70	87/87	84/89
Thymus		66/72	56/52	73/69	-	85/86
Bone marrow		61/60	58/59	64/69	82/83	-
Spleen		82/85	79/76	79/81	94/-	89/89
Lymph node		66/69	66/62	65/-	90/-	86/86
LN sIg^-		62/62	-	-	-	87/86
LN sIg^+		100/100	-	-	-	100/100
SPL sIg^-		63/64	-	66/65	83/82	83/86
SPL sIg^+		100/100	-	91/93	100/100	100/100
BM sIg^-		49/52	-	66/65	77/77	85/78
BM sIg^+		83/85	-	86/88	87/87	100/94
BM 14.8 dull		49/49	-	59/59	77/79	88/86
BM 14.8 bright		79/79	-	-	84/80	94/-
Granulocytes		71/66				
PBL sIg^-		70/65				
PBL sIg^+		85/80				

APPENDIX VI, continued

Control mice

Mouse	10	13	20	21	42	49	50
Tissue	6wks.	-	4mo.	4mo.	16mo.	2wk.	2wk.
Erythrocytes	53/54	87/-	75/-	67/64	60/68	64/60	84/81
Thymus	46/51	89/-	74/78	59/58	74/-	51/55	74/75
Bone marrow	52/50	89/-	71/72	52/51	70/67	63/58	78/79
Spleen	53/52	82/-	61/66	57/56	68/70	58/57	79/74
Lymph node	42/46	-	71/72	64/57	63/68	-	-
LN sIg^-	44/42	88/-	59/-	59/62	71/66	-	-
LN sIg^+	50/45	88/-	61/-	60/61	66/69	-	-
SPL sIg^-	-	-	70/66	61/61	-	58/52	74/75
SPL sIg^+	-	-	66/67	58/64	-	58/52	80/80
BM sIg^-	-	-	64/-	50/54	69/65	63/61	81/78
BM sIg^+	-	-	62/-	54/56	61/66	56/51	79/75
BM 14.8 dull	54/48	-	66/-	53/51	68/64	-	-
BM 14.8 br	54/50	-	65/-	52/54	68/66	-	-
Granulocytes	56/53	-	-	-	64/67	-	-
PBL sIg^-	-	83/-	-	-	-	-	-
PBL sIg^+	-	81/-	-	-	-	-	-
Two Col.							
SPL-ve	-	-	-	-	70/63	-	-
Pop.I	-	-	-	-	66/65	-	-
Pop.II	-	-	-	-	64/63	-	-
Pop.III	-	-	-	-	74/79	-	-

APPENDIX VII

An example of a GENSTAT input file for the analysis of the data in Chapters 4 and 5 of this thesis. This example is the analysis of 6 mice, 4 bleeds per mouse and 2 replicates per bleed. The programme can be altered according to sample number.

```
'REFERENCE' MOUSEBLOOD
'UNITS' $ 48
'VARIATE' SCORE
'FACTOR' MOUSE $ 6 : BLEED $ 4 : GEL $ 2
'INPUT' 2
'READ' MOUSE, BLEED, GEL, SCORE $ F, (1,1X) 3 , 2 , /
'INPUT' 1
'BLOCKS' MOUSE/BLEED/GEL
'ANOVA' SCORE
'RUN'
'CLOSE'
'STOP'
```

An example of a data file:

```
1 1 1 65
1 1 2 67
1 2 1 68
1 2 2 69
1 3 1 65
1 3 2 70
1 4 1 67
1 4 2 68
2 1 1 66
: : : :
: : : :
: : : :
6 3 1 69
6 3 2 62
6 4 1 65
1 4 2 67
'EOD'
```

The command on EMAS is:

```
GENSTAT INPUT FILE NAME, DATA=DATA FILENAME, LISTING=OUTPUT FILE NAME
```

Published Papers

The Clonal Organization of Hematopoiesis in the Mouse

H. S. Micklem, J. D. Ansell, J. E. Wayman, and L. Forrester

Department of Zoology
University of Edinburgh
Edinburgh, United Kingdom

I. The Hematopoietic System

The hematopoietic system produces large numbers of variously differentiated cells throughout life. These include erythrocytes, granulocytes, macrophages, platelets, osteoclasts, Langerhans cells, mast cells, and lymphocytes. Clearly, most, if not all, of these cells are related in some way to the proper functioning of the immune system. It is

ISBN 0-12-768240-6

generally believed that all of them are ultimately derived from a common ancestral cell, the so-called pluripotent stem cell.

The purpose of this paper is to discuss what is known about the conveniently termed "structure" of hematopoietic cell populations. The word "structure" is not used here in an anatomical sense. Rather, it concerns the size, turnover, and interrelationships of the various compartments. To a large extent we shall be dealing, directly or by implication, with the stem cell compartment.

II. Stem Cells

A. The Concept

A precise definition of a stem cell is difficult to formulate. A recent definition (Lajtha, 1979) is "cells with extensive self-maintaining (self-renewal) capacity, extending throughout the whole (or most) of the lifespan of the organism." The comment follows that "differentiation potential is a property of some types of stem cell but is not an essential feature of stem-ness." This definition is adequate for our present purposes. The insistence on self-renewal as the essential property of stem cells does, however, involve a possible paradox. There is nothing at present to exclude the possibility that animals are provided with a stock of pluripotent hematopoietic progenitor cells during ontogeny and simply use them up in series throughout life. Each of these cells might produce large clones (i.e., there might be amplification steps at one or several stages in the differentiation of the clone); but each clone would be doomed to extinction within some more or less precisely predetermined time. Thus, the progenitor pool would be continually reduced and non-self-maintaining, although doubtless more than adequate in size for the animal's lifetime requirements. Such progenitor cells would not be stem cells in the strict sense of the definition, and the adult animal would not in fact possess any stem cells. This is slightly more than a mere semantic quibble since it draws attention to a model of hematopoiesis which is rarely considered, yet is conceivable in the context of existing knowledge and has the attraction (Cairns, 1975) of limiting the impact of any DNA-copying errors to the lifetime of the clone. We do not wish to support this idea against any others, but merely to introduce it as part of the background for the following discussion.

B. Stem Cells and CFC-S

Since the introduction of the spleen colony technique (Till and McCulloch, 1961), it has become common to equate stem cells with cells that form spleen colonies (CFC-S). This equation, although convenient, is undoubtedly too simple. Many investigations have demonstrated that CFC-S are heterogeneous. Most important, some appear to have little or no capacity for self-renewal and can produce only small descendent clones. Conversely, the more highly self-renewing CFC-S may form colonies only after several days' delay (Hodgson and Bradley, 1979; Magli *et al.*, 1982). An extension of this latter finding would suggest the existence of stem cells which do not form spleen colonies at all—a possibility previously suggested on other grounds (Schofield, 1978; Micklem and Ross, 1975).

C. Pluripotency

1. *Pluripotency versus Sequential Bipotency*

In vitro cloning methods have confirmed older *in vivo* evidence for the existence of pluripotent stem cells (Metcalf *et al.*, 1979). It is still uncertain, however, whether any individual cell is truly pluripotent, or whether there is a programmed sequence of bipotential cells, the progression from one to the next involving cell division, a "quantal cell cycle" (Holtzer, 1978). Existing evidence is consistent with the latter hypothesis (Lord, 1983).

2. *Lymphopoiesis*

The derivation of lymphocytes from pluripotent stem cells, suggested by *in vivo* studies, has yet to be demonstrated *in vitro*. Abramson *et al.* (1977) have suggested that considerable powers of self-renewal may reside in cells already restricted to T-lymphocyte, B-lymphocyte, or nonlymphoid differentiation. Their studies were based on analysis of *in vivo* clones marked with unique radiation-induced chromosome translocations. Their data clearly show that at any one moment, different functional cell compartments may be predominantly populated by different clones. However, since the turnover rate of cells within these compartments is not the same, the clonal composition of (for example) bone marrow cells and PHA- or LPS-responsive cells in blood could get out of step. In other words, the data could be interpreted in terms of a succession of truly pluripotent clones, rather than self-maintaining unipotent clones. The question could be com-

pletely resolved only by longitudinal studies of individual animals, which would be extremely taxing to perform with the chromosome marker methods. There is reason to think, however, that the clonal makeup of hematopoiesis in these highly abnormal animals is changeable (see Section V). Thus, while there is no a priori reason why stem cell properties should necessarily be linked to pluripotency, existing evidence for *in vivo* departures from this linkage is not strong.

III. How Many Cell Generations?

A. The Hayflick Limit

This question was originally posed by Kay (1965) in the wake of Hayflick's early evidence for the limited life span of diploid fibroblasts *in vitro*. Such cells were found to be able to go through only some 50 doublings before the cultures stopped proliferating and died. Is there a "Hayflick limit" *in vivo*, and if so, how can a continuously productive system such as hematopoiesis avoid infringing it?

B. Steady-State Demands on the Stem Cell Pool

The scale of the problem can be gauged by estimating either the daily output of blood cells or the turnover of CFC-S. An adult mouse needs to produce 3×10^8 erythrocytes each day under steady-state conditions. These come off a production line estimated at 12–14 divisions (Testa, 1979). Fourteen doublings from a single cell will produce 2^{14} or about 16,000 cells. Thus about 24,000 progenitors are needed each day for erythropoiesis. The CFC-S compartment in a mouse numbers about 3×10^5 cells, of which some 20% (the estimates vary considerably according to experimental conditions) enter the cycle each day (Lord, 1983). The estimates for cycling CFC-S and erythroid progenitors are thus of the same order. Indeed, these cells are probably closely related or even overlapping sets, although the CFC-S must also provide progenitors for the other lines of blood cell.

If 20% of CFC-S are renewed each day, and if the compartment is regarded as homogeneous, then each CFC-S will divide on the average of once every 5 days. A laboratory mouse (e.g., CBA) lives about 1000 days, demanding 200 divisions of each CFC-S. It has been demonstrated that the proliferative capacity of stem cells from very old mice is as good as in young mice (Lajtha and Schofield, 1971; Ogden and Micklem, 1976; Harrison, 1983).

C. Reserve Capacity of the Stem Cell Pool

Already this performance is much better than would be predicted from the *in vitro* behavior of fibroblasts, but in fact the system is also capable of handling massive demands over and above those of the steady state. Ross *et al.* (1982; E. A. M. Ross, unpublished) exposed CBA mice repeatedly to hydroxyurea at a dose which reduced bone marrow cellularity and CFC-S numbers by 70%. This was done up to 37 times at 3-week intervals and at the end the numbers of CFC-S and their proliferative capacity were as high as in saline-injected controls or in young mice. Valeriote and Tolen (1983) obtained similar results with the alkylating agent triethylenemelamine. Serial transplantation techniques also allow the hematopoietic system to be stretched far beyond its usual limits (Barnes *et al.*, 1958; Ogden and Micklem, 1976; Harrison, 1979), although here there is an eventual loss of efficiency due to the transplantation procedure itself (Ross *et al.*, 1982; Harrison and Astle, 1982).

In short, if the CFC-S compartment is homogeneous, i.e., consists of cells all equally likely to enter the cycle, then each cell must be capable of far more than 200 divisions with no apparent loss of efficiency.

D. The Clonal Succession Hypothesis

As Kay (1965) pointed out, the above number, however, would be greatly reduced if hematopoiesis were based on the successive expansion and demise of large clones. This would involve rather infrequent divisions of rare clonogenic cells. On this hypothesis, the bulk of the CFC-S would be regarded as a transit population; the true self-maintaining stem cells would be a small subset of CFC-S, or even a pre-CFC-S. The implied heterogeneity of the stem cell/CFC-S compartment is, in fact, supported by data from many laboratories (Worton *et al.*, 1969; Micklem *et al.*, 1972; Schofield and Lajtha, 1973; Rosendaal *et al.*, 1976; Hodgson and Bradley, 1979; Monette and Stockel, 1981; Ross *et al.*, 1982; Botnick *et al.*, 1982). A repeated finding has been that the cells most resistant to cycle-active drugs are those with a high capacity for self-renewal.

E. An Age Structure for Stem Cell Populations

These data have led to the now widely accepted idea that the stem cell compartment has an age structure, the clonogenic capacity of an individual cell being influenced by the number of divisions through which it has already passed (Micklem and Ogden, 1976; Hellman *et al.*,

1978; Rosendaal *et al.*, 1979). Two predictions follow from this idea: (1) The number of cell clones in the bone marrow may be relatively small, compared with the number of CFC-S in cycle; and (2) the identity of these clones may change with time.

IV. Evidence on Hematopoietic Clone Numbers

A. Bone Marrow

1. *Chromosome Marker Studies in Chimeric Mice*

The first evidence that hematopoiesis in the mouse can in fact be supported by a very small number of clones came from Barnes *et al.* (1959), who studied long-term survivors of a near-lethal dose of ionizing radiation. Some of these animals carried cells with unique karyotypes in their bone marrow and in one individual virtually all the dividing hematopoietic cells belonged to a single clone. These mice were small in number and highly abnormal in character, but they established the potential for oligoclonal hematopoiesis. Attempts were subsequently made to estimate clone numbers in irradiated mice transfused with bone marrow cells (Wallis *et al.*, 1975; Micklem and Ross, 1975). Using the T6 marker chromosome system, it was possible to set up chimeras with two donor cell populations distinguishable from each other and from the host. It was shown that the presence or absence of T6 markers did not affect the fitness of the cells, as measured by their proliferative capacity (Micklem *et al.*, 1975). The principle was to use the binomial formula $n = p(1-p)/s^2$ to estimate the number (n) of proliferating clones present in a given tissue; p and $(1-p)$ are, respectively, the proportions of donor-1 and donor-2 mitoses observed, and s^2 is the variance of p. Both laboratories found that n varied with the number of cells injected (Table I). These figures suggest the presence of about one clone per femur for each 10^6 cells (10^3 CFC-S) injected. Since various error factors beside the binomial variance will have contributed to the observed values of s^2, these experiments will have tended to underestimate n, though by how much is not clear. Another problem with the interpretation of such data is that one does not know exactly what is being measured. Both the number of stem cells that initially seed the recipient's depopulated marrow spaces, and the number of descendant clones that happen to be active at the time the animal is killed for cytological study, could be estimated by this method; the calculated value of n will mainly reflect whichever of these numbers is

TABLE I

Estimated Number of Clones Present in Femoral Bone Marrow of Irradiated/Repopulated Mice (T6 System)[a]

Number of cells injected ($\times 10^{-6}$)	Number of clones per femur	Clones per femur per 10^6 cells injected
2	8–15	4.0–7.5
5	7	1.4
40	35–54	0.9–1.4
50	43	0.9

[a]Data from Wallis *et al.* (1975) and Micklem *et al.* (1975).

the smaller. Only repeated studies of individuals can resolve this problem.

2. *Alloenzyme Marker Studies in Chimeric Mice*

The advent of an X-linked enzyme marker (Nielsen and Chapman, 1977) has made such studies practicable in the mouse. The marker is provided by an electrophoretic variant of phosphoglycerate kinase (PGK-1A). We have back-crossed this onto the CBA strain. Due to the phenomenon of X inactivation early in embryogenesis, any cell of a heterozygous female expresses either the variant or the normal (PGK-1B) form of the enzyme, not both (Lyon, 1974; Monk and Harper, 1979). Overall, approximately 70% of somatic cells in our stock express the A form and 30% the B form. This unequal expression is due to close linkage between the *PGK-1* locus and the *Xce* locus which controls the probability that a particular X chromosome will be inactivated (Johnston and Cattanach, 1981).

CBA (PGK-1B) mice were lethally irradiated (10.5 Gy) and injected with 10^5 or 10^7 nucleated bone marrow cells from CBA-PGK-1AB heterozygotes. One drop (about 50 μl) of blood was taken, normally at 2- or 4-week intervals, starting at 10 weeks after irradiation/repopulation. Lysates were prepared from the whole blood and assayed for their relative content of the two enzymes by a modification of the method of Bücher *et al.* (1980) (Ansell and Micklem, 1984). Data are shown in Table II. In confirmation of the chromosome marker data, there was clearly more variation between mice that had received the lower dose of bone marrow. Three of the mice in fact stabilized at 100% A type. Since the probability of having $\geq n$ stem cells, all of which are A type, is 0.7^n, this result strongly suggests that the entire hematopoietic, or at

TABLE II

Fluctuations in PGK Alloenzyme Ratios in CBA Mice after Lethal Irradiation (10.5 Gy) and Injection of Large (10^7) or Small (10^5) Doses of CBA-PGK-1AB Bone Marrow

Cells injected	Mouse no.	PGK-1A in blood cells at weeks postirradiation (%)					
		10	18	28	40	48	56
10^7	1	74	62	81	76	68	77
	2	71	59	71	67	76	83
	3	70	72	83	75	79	85
	4	65	69	79	85	69	74
10^5	1	92	51	26	27	53	—
	2	61	56	47	44	49	28
	3	68	100	100	100	100	100
	4	82	100	100	100	100	100

least erythropoietic, system is populated by very few clones. The tendency for the proportion of B-type enzyme to increase in some mice may be due to recovery of host hematopoiesis. Such recovery, even after supralethal irradiation, is quite common when only small numbers of restorative bone marrow cells have been injected. Its extent can be monitored in the future by the use of a second enzyme marker, phosphoglucose isomerase (PGI) (Chapman *et al.*, 1972). Recipients of the 100-times-higher cell dose showed a relatively small amount of variation around the expected figure of 70%. In principle, such data allow estimates to be made of two numbers: (1) the number of repopulating stem cells, derived from the group variance between mice; and (2) the number of hematopoietically active clones, derived from the variance around the mean for each individual mouse. From this small series of animals the numbers must be regarded as very approximate (Stone, 1983), but are 330 for the repopulating cells and 31–110 for the active clones, after repopulation with 10^7 cells. The repopulating cell figure, in particular, may be an underestimate since the variance on which it is based is within the range found in repeated analyses of a single sample (see Table III).

3. *Alloenzyme Marker Studies in Normal Mice*

The same kinds of calculation can be applied to repeatedly bled normal PGK-1-heterozygous females. Here, the inter-mouse variance represents the number of X-inactivated cells that seed the hematopoietic compartment of the early embryonic mesoderm. The intra-mouse

TABLE III

Estimates of Hematopoietic Clonality in CBA-PGK-1AB Heterozygous Female Mice

	Single bleeds from 100 mice	Serial[a] bleeds from 4 mice	Repeats of a single sample[b]
Mean PGK-1A (%)	72	68–73	71–73
Variance	98	4.0–20.0	1.2–8.3
Estimated clone number (n)	19	99–500	(239–1603)

[a]Ten bleeds at 4-week intervals.

[b]Four samples each run nine times. The range of values obtained with the four samples is shown.

variance may allow an estimate of the average number of hematopoietically active clones and perhaps indicates the rate of clonal succession. Our initial attempt to do this (Burton *et al.*, 1982) suggested the existence of a rapid succession of large hematopoietic clones. This interpretation was based on a high intramouse variance with occasional very sharp changes in the A : B enzyme ratio. Regrettably, it is now clear that the apparent sudden changes, together with a part of the overall variance, were attributable to technical problems. We now find that the intramouse variance is small and not much in excess of the variance of repeated runs of the same sample. Thus, it may prove impossible to establish any reliable estimates of hematopoietic clone numbers in normal mice. The intermouse variance is, however, substantial (Table III) and implies that roughly 20 X-inactivated cells contributed originally to the hematopoietic system, a result that agrees quite well with Fialkow's (1973) estimate for man. This figure could be a slight underestimate if X inactivation occurs before the differentiation of the hematopoietic system.

B. The Thymus

In lethally irradiated mice repopulated with bone marrow cells, Wallis *et al.* (1975) found that very few donor cells contributed to the initial repopulation of thymus, possibly as few as one per lobe. Since the number and expansion of clones in the thymus has a bearing on the generation of T-cell receptor diversity, we have tried to estimate the number of clones present in the thymus of normal mice. Seventy mice were studied, groups of five being killed at intervals from day 16 of gestation to 24 months. The two thymus lobes, as well as femora and other tissues, were assayed individually for the relative concentration of the two PGK alloenzymes. No significant differences were found

with time. For the whole series, the estimates of n were right thymus lobe 10.8, left thymus lobe 11.3, right femur 9.5, left 11.5. Much of the variance that contributes to these low estimates will be due, however, to the limited number of original X-inactivated cells seeding the hematopoietic system, so the number of active clones per thymus lobe or per bone may be much higher. Resolution of this question will demand methods for serially studying the thymus or its output of T cells.

V. Evidence for Clonal Succession

A. Chimeras

A rather dramatic example of clonal succession was observed many years ago in serially transplanted bone marrow carrying three clones individually marked with unique chromosome rearrangements (Micklem and Loutit, 1966). The data showed large changes with time in the relative proportions of the marked clones.

Although unique chromosome markers at present provide the only sure way of identifying individual clones, repeated attempts failed to produce further mice with more than one well-marked and persistent clone. Moreover, these animals are difficult or impossible to study serially as individuals, so that their marrow cells have to be transplanted.

In the PGK alloenzyme data shown in Table II, the shifting fortunes of individual clones can only be inferred from the changes in A : B alloenzyme ratio. Nevertheless, the data demonstrate that the clonal composition, at least of erythropoietic cells, must be changeable. This can only be seen clearly in recipients of small numbers of bone marrow cells where relatively few clones are present.

B. Normal Animals

The preceding reservation applies still more strongly to normal animals, in which many clones are represented in the blood. The 45-day life span of erythrocytes imposes a large "damping" factor on studies of whole blood. This can be avoided by analyzing cohorts of short-lived cells such as granulocytes or reticulocytes; but preliminary experiments with FACS-sorted granulocytes suggest that even without the damping factor the number of active clones may be inconveniently high. It will be interesting to study the pattern of hematopoietic recovery after various kinds of cytotoxic insults, and indeed such studies may provide a critical test of the age–structure hypothesis for hematopoietic cells.

VI. Conclusions and Prospects

1. About 10–20 embryonic X-inactivated cells contribute to the hematopoietic system in the mouse.
2. The number of clones contributing to hematopoiesis at any one time seems to be too large to measure satisfactorily in the unseparated blood of normal mice.
3. The existence of clonal succession in hematopoiesis, while it is implied by the idea of a stem cell hierarchy and by all the evidence that contributes to that idea, is not clearly demonstrable with present techniques in the blood of normal animals. Marked changes in clonal composition are, however, observable in irradiated/repopulated mice.

The X-linked PGK system offers scope for further development as a marker for hematological and immunological studies. For the first time it allows estimates of clone numbers to be made in normal mice. Its use in transplantation experiments will be enhanced in conjunction with the autosomally encoded PGI marker. Since assays can be performed on 1000 cells or less; the enzyme techniques lend themselves to the study of specific populations separated by fluorescence-activated sorting with monoclonal antibodies or lectins. This approach will facilitate the study of narrowly defined cohorts of cells in terms of both their clonality and their lineage interrelationships.

Acknowledgments

This work was supported by the Melville Trust, the Cancer Research Campaign, and the Medical Research Council (of which J. E. W. and L. F. are research students).

References

Abramson, S., Millar, R. G., and Phillips, R. A. (1977). *J. Exp. Med.* **154**, 1567.

Ansell, J. D., and Micklem, H. S. (1984). *In* "Handbook of Experimental Immunology" (D. M. Weir, C. M. Blackwell, and L. A. Herzenberg, eds.), 4th ed. Blackwell, Oxford (in preparation).

Barnes, D. W. H., Ford, C. E., and Loutit, J. F. (1958). *Sang* **30**, 762.

Barnes, D. W. H., Ford, C. E., Gray, S. M., and Loutit, J. F. (1959). *Prog. Nuclear Energy, Series VI* **2**, 1.

Bücher, T., Bender, W., Fundele, R., Hofner, H., and Linke, I. (1980). *FEBS Lett.* **115**, 319.

Burton, D. I., Ansell, J. D., Gray, R. A., and Micklem, H. S. (1982). *Nature (London)* **298**, 562.

Cairns, J. (1975). *Nature (London)* **255**, 197.
Chapman, V. M., Ansell, J. D., and McLaren, A. (1972). *Dev. Biol.* **29**, 48.
Fialkow, P. J. (1973). *Ann. Human Genet.* **37**, 39.
Harrison, D. F. (1979). *Mech. Aging Dev.* **9**, 409.
Harrison, D. E. (1983). *J. Exp. Med.* **157**, 1496.
Harrison, D. E., and Astle, C. M. (1982). *J. Exp. Med.* **156**, 1767.
Hellman, S. S., Botnick, L. E., Hannon, E. C., and Vigneulle, R. M. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75**, 490.
Hodgson, G. S., and Bradley, T. R. (1979). *Nature (London)* **281**, 381.
Holtzer, H. (1978). *In* "Stem Cells and Tissue Homeostasis" (B. I. Lord, C. S. Potten, and R. J. Cole, eds.), pp. 1–27. Cambridge Univ. Press, London and New York.
Johnston, P., and Cattanach, B. M. (1981). *Genet. Res.* **37**, 151.
Kay, H. E. M. (1965). *Lancet* **2**, 418.
Lajtha, L. G. (1979). *Differentiation* **14**, 23.
Lajtha, L. G., and Schofield, R. (1971). *Adv. Gerontol. Res.* **3**, 131.
Lord, B. I. (1983). *In* "Stem Cells—Their Identification and Characterisation" (C. S. Potten, ed.), pp. 118–154. Churchill, London.
Lyon, M. E. (1974). *Proc. Roy. Soc. Ser. B* **187**, 243.
Magli, M. C., Iscove, N. N., and Odartchenko, N. (1982). *Nature (London)* **295**, 527.
Metcalf, D., Johnson, G. R., and Mandel, T. (1979). *J. Cell. Physiol.* **98**, 401.
Micklem, H. S., and Loutit, J. F. (1966). "Tissue Grafting and Radiation," pp. 86–88. Academic Press, New York.
Micklem, H. S., and Ogden, D. A. (1976). *In* "Stem Cells of Renewing Cell Populations" (A. B. Cairnie, P. K. Lala, and D. G. Osmond, eds.), pp. 331–341. Academic Press, New York.
Micklem, H. S., and Ross, E. (1975). *In* "Human Lymphocyte Differentiation: Its Application to Cancer" (B. Serrou and C. Rosenfeld, eds.), pp. 15–22. Elsevier/North-Holland, Amsterdam.
Micklem, H. S., Ford, C. E., Evans, E. P., Ogden, D. A., and Papworth, D. S. (1972). *J. Cell Physiol.* **79**, 293.
Micklem, H. S., Ford, C. E., Evans, E. P., and Ogden, D. A. (1975). *Cell Tissue Kinet.* **8**, 219.
Monette, F. C., and Stockel, J. B. (1981). *Stem Cells* **1**, 38.
Monk, M., and Harper, M. I. (1979). *Nature (London)* **281**, 311.
Nielsen, J. T., and Chapman, V. M. (1977). *Genetics* **87**, 319.
Ogden, D. A., and Micklem, H. S. (1976). *Transplantation* **22**, 287.
Rosendaal, M., Hodgson, G. S., and Bradley, T. R. (1976). *Nature (London)* **264**, 68.
Rosendaal, M., Hodgson, G. S., and Bradley, T. R. (1979). *Cell Tissue Kinet.* **12**, 17.
Ross, E. A. M., Anderson, N., and Micklem, H. S. (1982). *J. Exp. Med.* **155**, 432.
Schofield, R. (1978). *Blood Cells* **4**, 7.
Schofield, R., and Lajtha, L. G. (1973). *Br. J. Haematol.* **25**, 195.
Stone, M. (1983). *Biometrics* (in press).
Testa, N. (1979). *Clin. Haematol.* **8**, 311.
Till, J. E., and McCulloch, E. A. (1961). *Rad. Res.* **14**, 213.
Valeriote, F., and Tolen, S. (1983). *Cell Tissue Kinet.* **16**, 1.
Wallis, V. J., Leuchars, E., Chwalinski, S., and Davies, A. J. S. (1975). *Transplantation* **19**, 2.
Worton, R. G., McCulloch, E. A., and Till, J. E. (1969). *J. Cell. Physiol.* **74**, 71.

* INFLUENCE OF THE XID MUTATION ON B LYMPHOCYTE DEVELOPMENT IN ADULT MICE

J. Witkowski, L.M. Forrester, J.D. Ansell and H.S. Micklem

Department of Zoology, University of Edinburgh
Edinburgh EH9 3JT, United Kingdom

INTRODUCTION

The genesis and maturation of murine B lymphocytes in the bone marrow is marked by the sequential appearance of the B-220 molecule, cytoplasmic u chains, surface IgM and IgD [1,2]. In the spleen at least three distinct populations of B cells can be identified on the basis of the relative concentrations of surface IgM and IgD [3]. One of these, carrying high IgD and low IgM concentrations, is absent in CBA/N mice, which are homozygous for the X-linked immunodeficiency (xid) trait [3]. Transplantation of normal bone marrow or foetal liver cells into CBA/N mice results in normal B cell development, implying that the xid defect is intrinsic to the cells and not microenvironmental [4]. Splenic B lymphocytes in female xid/+ heterozygotes were recently found to express mainly the non-xid carrying chromosome in a population associated with IgG3 production [4]: presumably those stem cells in which the xid-carrying X-chromosome was active showed at least a partial failure to differentiate into B lymphocytes. Thus, the defect appeared to act directly on B cells rather than on some other cell type influencing B cell development.

In xid/+ female heterozygotes, the two electrophoretically distinct forms of the X-linked enzyme phosphoglycerate kinase (PGK-1A and PGK-1B) can be used as markers of the normal or xid-carrying X-chromosome respectively [5]. Using these markers, we are now approaching the following questions: (1) how early in B cell development does the xid trait express itself? (2) Does the trait affect all B cells? (3) Is the development of other haematopoietic lineages affected? Partial answers, based on the expression of xid-carrying and normal X-chromosomes in haematopoietic tissues and in

* see Bibliography, page 175 for details of this publication.

FACS-sorted bone marrow and peripheral lymphoid cells, are advanced in this paper.

MATERIALS AND METHODS

Mice. Mice of the inbred CBA/Ca strain and certain lines derived from it were used: CBA/N, CBA/Ca-Pgk-1^a, PGK-1-heterozygous F_1 hybrids of the above, and CBA/Ca-Gpi-1^a. With the exception of the CBA/Ca-Pgk-1^a congenic line and the hybrids, all other mice have the PGK-1B phenotype. GPI-1 (glucose phosphate isomerase) is an autosomally encoded enzyme with electrophoretic variants; normal CBA/Ca and CBA/N mice, and the Pgk-1^a derivatives are Gpi-1^b [6]. All mice were bred and maintained in our own animal facilities, and used when 3-4 months old. We are indebted to Dr M. J. Marshall for the gift of a breeding nucleus of the CBA/Ca-Gpi-1^a mice.

Antibodies. The following were used. 1. Rat anti-mouse B-lineage cell antigen monoclonal antibody 14.8 (kindly provided by Dr P.W. Kincade). 2. Fluorescein isothiocyanate (FITC)-labelled goat anti-rat immunoglobulin antibody (Miles-Yeda), solid phase-absorbed with mouse immunoglobulin (used as a second step in 14.8 antibody staining). 3. FITC-labelled goat anti-mouse immunoglobulin (Miles-Yeda).

Cells. Animals were anaesthetized with chloroform vapour and blood was collected by heart puncture into a heparinized syringe. Cell suspensions were prepared from spleen, thymus, subcutaneous and mesenteric lymph nodes and bone marrow in RPMI 1640 medium supplemented with 0.3% bovine serum albumin (RPMI-BSA). Erythrocytes were eliminated from suspensions of spleen and bone marrow cells and a portion of the blood by hypotonic shock. Suspensions were made up to a concentration of 10^7 viable cells per ml in RPMI-BSA.

Fluorescent staining. 4×10^6 peripheral blood leucocytes, bone marrow, spleen or lymph node cells were stained with 10ul of FITC-anti-mouse Ig antibody to reveal cells carrying surface immunoglobulin (sIg). The same number of bone marrow cells were incubated first with 20ul of purified 14.8 monoclonal antibody and then stained with FITC-anti-rat-Ig antibody. All staining steps were performed for 40 mins on melting ice in a volume of 0.4ml RPMI-BSA containing 0.1% sodium azide and were followed by three washes in cold azide- containing medium.

Bone marrow repopulation. CBA/Ca-Gpi-1^a male mice were lethally gamma-irradiated (10.5Gy, whole body, from a ^{137}Cs source). Within 30mins 10^7 bone marrow cells were injected intravenously into the lateral tail vein . The bone marrow cell suspension was a mixture consisting either of $4x10^6$ CBA/Ca-Pgk-1^a plus $6x10^6$ CBA/N (Pgk-1^b) cells or of $4x10^6$

CBA/Ca-Pgk-1^{a} plus 6x10^{6} CBA/Ca (Pgk-1^{b}) cells. The first group is referred to as "experimental" and the second as "control". Thus, in the experimental group, the xid-carrying cells were marked with PGK-1B and were in an initial 3:2 majority. The use of the congenic strain, CBA/Ca-Gpi-1^{a}, as the recipient in these experiments allowed us to estimate the host contribution (if any) to the cell populations analysed, since all donor cell populations have a GPI-1B phenotype [6]. At two-week intervals after irradiation animals from both groups were killed and cell suspensions from bone marrow, blood, spleen and lymph nodes were prepared and stained as described above. Host activity was significant only at 2-4 weeks post-irradiation and was allowed for in calculating the ratio of the two donors.

FACS analysis and sorting. Stained cell suspensions were analysed with a fluorescence activated cell sorter (FACS- IV, Becton Dickinson, Sunnyvale, California), on the basis of forward and right angle light scattering properties [7] and fluorescence intensity. Between 50 and 100 thousand cells were then sorted for enzyme analysis.

Measurement of PGK-1 and GPI-1 alloenzymes. These techniques were performed as described in full elsewhere [8]. Briefly, cell lysates were electrophoresed on cellulose acetate membranes for 45 minutes at +4^{o}C (12.5mA/membrane, constant current) in the case of PGK-1 or for 1h 20mins (350V/membrane, constant voltage) in the case of GPI-1. The activity of both enzymes was visualized in the membranes by means of linked enzyme reactions leading to the reduction of the dye thiazolyl blue to its formazan derivative; alternatively, with PGK-1, ^{14}C-labelled glucose was included in the substrate and ^{14}C-labelled products were detected by autoradiography. Both colour and autoradiographic methods yield similar results for the measurement of PGK-1 activity; autoradiography, being more sensitive, was used for the analysis of FACS-sorted cell samples. The relative proportions of PGK-1A and B were then measured on an automated scanning and integrating densitometer (Chromoscan 3, Joyce-Loebl, Gateshead, England). Measurements of replicate samples showed an accuracy within 5% above or below the mean. All experimental samples were run twice or more.

RESULTS

The ratios of PGK-1A to B activity in spleen cells and in sorted B lymphocyte populations of xid/+ heterozygotes, were consistently higher than in sorted non-B cells and unsorted cells from several non-splenic haematopoietic tissues (representative data are shown in Table 1). This shows that disproportionately few B cells had the xid-carrying chromosome active. Some splenic and

Table 1 Percent PGK-1A present in unsorted cell suspensions and purified cell populations of +/+ heterozygotes (mice 1 and 2) and +/xid heterozygotes (mice 3 and 4).*

	+/+ Pgk-1^{ab}		+/xid Pgk-1^{ab}	
	1	2	3	4
erythrocytes	64	70	72	46
thymus	58	74	72	46
spleen (unsorted)	57	63	83	61
spleen sIg$^+$	61	70	76	ND
sIg$^-$	61	66	100	ND
lymph node (unsorted)	60	72	70	44
lymph node sIg$^+$	60	60	68	43
sIg$^-$	60	61	100	100
blood lymphocytes				
sIg$^+$	58	ND	68	41
sIg$^-$	56	ND	90	75
bone marrow	52	70	71	44
bone marrow sIg$^+$	53	64	71	34
sIg$^-$	54	62	86	59
14.8 dull	52	66	70	41
14.8 bright	53	65	86	49

* In the +/xid mice, PGK-1A was associated with the activity of the non- xid-carrying X-chromosome. Values showing a clear bias against the expression of the xid (and Pgk-1^b) X chromosome are underlined. Note that these all relate to samples containing a large proportion of B lymphocytes. Broadly similar results were obtained in a further 10 +/xid and 3 +/+ mice.
ND = not done sIg = surface immunoglobulin

lymph node B cell samples showed exclusively PGK-1A activity, indicating that cells with the xid-bearing chromosome active were virtually absent. In contrast, the sIg-positive cells in the bone marrow showed only a slight excess of PGK-1A activity compared to the sIg-negative cells from the same bones. After staining bone marrow cells with the 14.8 monoclonal antibody, two positive fluorescent populations were observed: about 30-40% of the nucleated cells stained above background and a minority (5-10% of the nucleated cells) stained brightly. We have found (unpublished data) that the 14.8- 'bright' subset includes most of the sIg- positive cells, while 14.8- 'dull' cells are nearly all sIg- negative,

presumably pre-B [9] cells. (Less than 3% of bone marrow 14.8-positive cells were T lymphocytes as judged by staining with monoclonal antibodies to Thy- 1, Ly-1 and Lyt-2.) Like marrow sIg-positive cells, the 14.8- 'bright' cells showed only a slight excess of PGK-1A activity. The 14.8-'dull' cells had the same relative PGK-1A activity as the non- stained (i.e. non B-lineage) cells from the same bones.

In contrast to the xid/+ animals, +/+ Pgk-1ab females showed little variation in PGK-1A/B ratios between the tissues studied and between sorted B and non-B cells (Table 1).

Results of PGK-1 analyses of samples from irradiated and repopulated mice are shown in Fig.1. Differences in the patterns of mosaicism between the two groups of animals reflect the repopulating abilities of the donor cells. In the control group only small differences were seen between sorted B and non-B cells and unsorted populations. In the experimental group, which received mixed CBA/N and CBA/Ca-Pgk-1^a bone marrow, the sIg-positive cells in the blood showed a progressive increase in the proportion of PGK-1A, that is, by inference, a disappearance of CBA/N-derived cells. The lymph node B cells of experimental animals showed a marked deficit of CBA/N-derived cells throughout the period of observation; this deficit was total in the animals assayed at 6 and 8 weeks after irradiation. Observations on unfractionated spleen cells suggested that the splenic B population was also deficient in CBA/N-derived cells, but sIg-positive and negative cells were not studied separately to test this. Deficits of CBA/N-derived cells were not clearly evident in sIg-negative lymphocytes of blood or lymph nodes, although the data do not rule out a marginal effect. The 14.8-'dull' (pre-B) bone marrow population in the experimental mice showed little or no difference from the 14.8-negative (non-B) cells from the same bones or from either population in controls. The numbers of the 14.8 "bright" cells in the repopulated bone marrow were too low to collect for enzyme analysis.

DISCUSSION

Due to the phenomenon of X-chromosome inactivation [10] only one X-chromosome is active in female cells. Thus, in normal female mice heterozygous for neutral polymorphisms on the X-chromosome, a balanced mosaicism exists for the products of the polymorphic genes. If a mutation on the X-chromosome directly affects a particular cell population, being, for example, lethal at a particular developmental stage or affecting the ability of the cells to proliferate, this will result in the mosaicism becoming unbalanced in the affected cell population. If, on the other hand, the mutation acts indirectly via other cells or microenvironmental factors, one would not expect mosaicism in the final target cells to be affected.

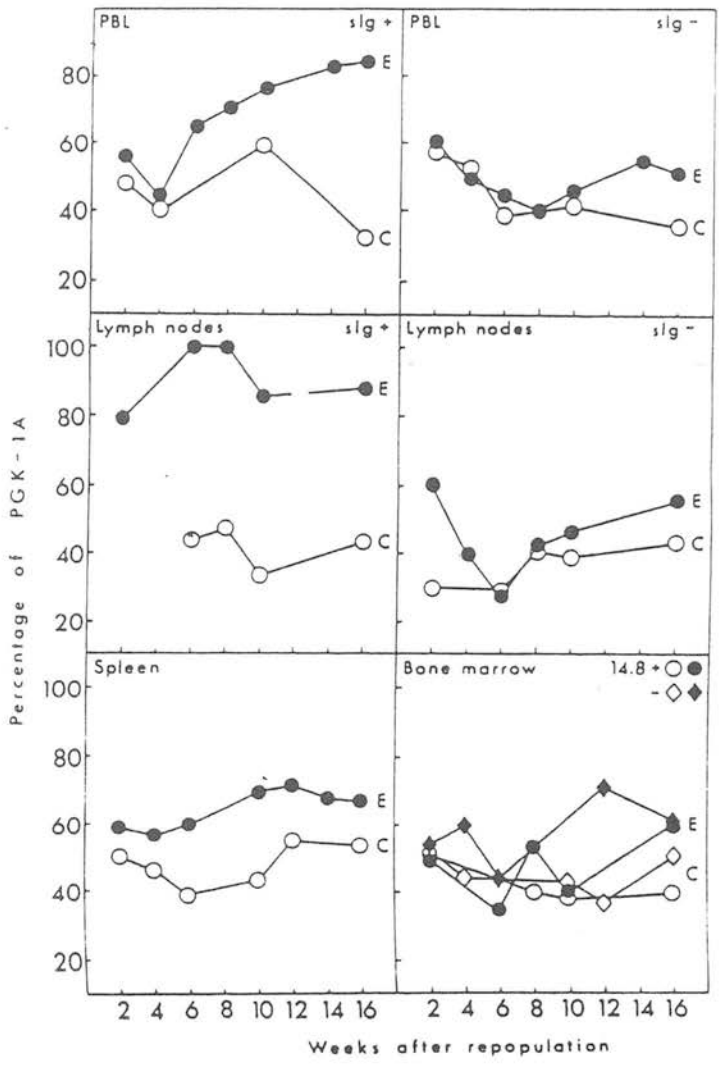


FIGURE 1.
Reconstitution of various cell populations of lethally irradiated mice injected with either a mixture of $\frac{+/+\ Pgk\text{-}1^a}{}$ and $\frac{+/+\ Pgk\text{-}1^b}{}$ (control-C) or $\frac{+/+\ Pgk\text{-}1^a}{}$ and $\frac{xid/xid\ Pgk\text{-}1^b}{}$ (experimental-E) bone marrow. In the E group PGK-1A identifies non-xid-bearing cells.

We have used variants of the X-linked enzyme PGK-1, which differ in their electrophoretic mobilities, to monitor the activity of the xid-carrying X-chromosome in lymphoid cells of xid/+ female heterozygotes. A similar strategy was adopted by Nahm et al.[5], but the present methods for visualizing and measuring the alloenzymes are more quantitative [8].The data demonstrate that in the peripheral lymphoid organs and blood, surface immunoglobulin (sIg)-bearing lymphocytes carrying an active xid gene are severely depleted compared to those with the normal gene active. In CBA/N mice, which are homozygous for xid, it has been demonstrated that the subpopulation most severely affected is one carrying high levels of IgD and low levels of IgM [3,4]. Since this is the predominant B cell type in the periphery, it is possible that the enzyme data reflect the depletion of cells carrying an active xid gene specifically from this sub-population . The presence of some xid-expressing B cells was clearly demonstrated in blood and (in some individuals) spleen and lymph nodes. However, the present data do not exclude some (presumably smaller) effects of the xid mutation on the development or function of other B cell subpopulations.

Bone marrow B lymphocytes showed relatively slight imbalances of PGK-1A/B ratio in xid/+ heterozygotes. In the 14.8-'dull'(pre-B) cells PGK-1A values were not elevated. In the 14.8-'bright' and the sIg-positive cells, some excess of cells with the normal chromosome active was apparent, but it was far from complete. These data suggest that the xid gene acts at a late stage of differentiation of B cells in the marrow, but some effect on a minor population of pre-B cells is not excluded. It is probable that some of the sIg-positive, 14.8-bright cells found in bone marrow were immigrants from the periphery, and possible that immigrants were sufficient in number to account for the imbalance of X-chromosome expression in this population.

In irradiated mice that had been repopulated with a mixture of CBA/N and normal (PGK-1A marked) marrow the patterns of mosaicism came to resemble those seen in the xid/+ heterozygotes: there was an excess in the proportion of non-xid B cells in the periphery, but not in the marrow. The observation that xid B cells decreased proportionally in blood over a period of several weeks may indicate either that relatively immature B cells were migrating out of the bone marrow during this period or that the normal numerical balance between mature B cell subpopulations was upset. The slight decrease in splenic xid cells over the same period is compatible with the idea that immature B cells migrate from marrow to spleen during the early weeks of repopulation and then gradually cease to do so. However, interpretation of the spleen data is clouded by the probable presence of splenic erythropoiesis during the post-radiation recovery period and by the absence of data on sorted splenic B cells. In the lymph nodes xid B cells were already

absent by 6 weeks, suggesting that most recirculating B cells belonged to the xid-affected subpopulation(s).

In summary, the data presented show that xid acts directly on B cells and that its expression is unaffected by the concomitant presence of non-xid cells . It apparently acts on the differentiation and development of certain B cells, probably at a stage after the acquisition of surface IgM. No effect was demonstrable on bone marrow pre-B cells or on non-B lymphocytes. Further analysis of the patterns of mosaicism of PGK-1 alloenzymes in subpopulations of B cells separated on the basis of their quantitative expression of IgM, IgD, B-220 and other markers of B cell development [11] may indicate more precisely the subpopulation(s) in which the X-linked immunodeficiency trait is active, and provide some information about the lineage relationships between these subpopulations.

ACKNOWLEDGEMENTS

This work was supported by the Cancer Research Campaign and the Medical Research Council. J.W. was the recipient of a Fellowship from the Wellcome Trust. L.M.F. holds an M.R.C. Postgraduate Studentship. We are indebted to Robert Gray and Andrew Sanderson for advice and skilled assistance with the cell sorting.

REFERENCES

1. P. W. Kincade. Formation of B lymphocytes in fetal and adult life. Adv. Immunol. 31:177 (1981).
2. R. L. Coffman and I. L. Weissman. Immunoglobulin gene rearrangement during pre-B cell differentiation. J. mol. cell. Immunol. 1:31 (1983).
3. R. R. Hardy, K. Hayakawa, J. J. Haaijman, L. A. Herzenberg and L. A. Herzenberg. B cell subpopulations identified by two-color fluorescence analysis. Nature 297:589 (1982).
4. I. Scher. The CBA/N mouse strain: an experimental model illustrating the influence of the X chromosome on immunity. Adv. Immmunol. 33:1 (1982).
5. M. H. Nahm, J. W. Paslay and J. M. Davie. Unbalanced X chromosome mosaicism in B cells of mice with X-linked immunodeficiency. J. exp. Med. 158:920 (1983).
6. M. C. Green, ed. "Genetic Variants and Strains of the Laboratory Mouse". Gustav Fischer Verlag, Stuttgart (1981).
7. A. W. S. Ritchie, R. A. Gray and H. S. Micklem. Right angle light scatter: a necessary parameter in flow cytofluorimetric analysis of human peripheral blood mononuclear cells. J. imm. Meths. 64:109 (1983).
8. J. D. Ansell and H. S. Micklem. Genetical markers for

following cell populations, in: "Handbook of Experimental Immunology", 4th ed. (D. M. Weir, L. A. Herzenberg, C. C. Blackwell and L. A. Herzenberg, eds.). Blackwells, Oxford (in press).

9. M. P. Scheid, K. S. Landreth, J. W. Tung and P. W. Kincade. Preferential but nonexclusive expression of macromolecular antigens on B-lineage cells. Immunol. Revs. 69:141 (1982).
10. M. F. Lyon. Mechanisms and evolutionary origins of variable X chromosome activity in mammals. Proc. Roy. Soc. B 187:243 (1974).
11. R. R. Hardy, K. Hayakawa, D. R. Parks and L. A. Herzenberg. Demonstration of B cell maturation in X-linked immunodeficient mice by simultaneous three-color immunofluorescence. Nature 306:270 (1983).

Genet. Res., Camb. (1985), **45**, *pp.* 95–100
Printed in Great Britain

Parental influences on *X* chromosome expression

By LESLEY M. FORRESTER and J. D. ANSELL
Dept of Zoology, University of Edinburgh, West Mains Rd, Edinburgh, EH9 3JT

(*Received 4 June 1984 and in revised form 1 October 1984*)

SUMMARY

Using mice that were mosaics for both *Xce* and phosphoglycerate kinase (*Pgk*-1) alleles, we have established that the parental source of the *Xce* gene may affect the probability with which the *X* chromosome carrying it will remain active. This effect was seen in one allelic combination of *Xce* but not in another. The relationship between these effects and other phenomena of maternal 'imprinting' is discussed.

1. INTRODUCTION

In female eutherian mammals, *X* chromosome inactivation results in individual somatic cells expressing either the maternally derived *X* chromosome (X^M) or the paternally derived *X* chromosome (X^P) (Lyon, 1961). In some circumstances genetic (Cattanach & Isaacson, 1965) and/or parental effects (Takagi & Sasaki, 1975; West, Frels & Chapman, 1977) modify the *X* inactivation process, resulting in X^M and X^P having an unequal probability of becoming inactivated.

Work in the mouse has suggested that there is an inactivation centre, present on the *X* chromosome, designated the *X* chromosome controlling element (*Xce*) (reviewed by Cattanach, 1975). Three alleles of the *Xce* locus have been described and they affect the probability that a particular *X* chromosome will become inactivated. Analyses of females heterozygous at the *Xce* locus (and also at other *X* linked marker loci) have shown that an *X* chromosome carrying the Xce^a allele is more likely to become inactivated than an *X* chromosome bearing the Xce^b allele. Similarly an Xce^b *X* chromosome has a higher probability of inactivation than an *X* chromosome bearing a third allele, Xce^c.

The existence of parental factors which influence the *X* inactivation process is most obvious in studies on the extraembryonic membranes (trophectoderm and primitive endoderm) of the mouse embryo, where the paternally derived *X* chromosome (X^P) is preferentially inactivated (Takagi & Sasaki, 1975; West *et al.* 1977). Embryo transfer and oocyte transplantation experiments have shown that the maternal reproductive tract does not exert a selection pressure in favour of cells expressing X^M (Frels & Chapman, 1980; Papaioannou & West, 1981). It is thought that the differential expression is due to an intrinsic difference between X^M and X^P, imprinted before the *X* inactivation process, although the molecular mechanism for this imprinting remains unknown.

Parental effects on *X* chromosome inactivation in somatic tissues have been

studied by comparing the phenotypes of females, heterozygous for *X*-linked gene products, derived from reciprocal crosses. Some studies indicate a 'paternal' effect i.e. X^P has a higher probability of remaining active than X^M (Falconer, Isaacson & Gauld, 1982), while others show no significant reciprocal cross differences (Johnston & Cattanach, 1981). In selection experiments with the *X*-linked gene brindled (*Mo*^{br}) (Falconer *et al.* 1982) and viable brindled (*Mo*^{vbr}) (Cattanach & Papworth, 1981), a positive correlation was observed between the expression of brindled in mothers and daughters. However, this 'maternal' effect was attributed to abnormal copper transport in the heterozygous mothers rather than a chromosomal effect. Using alleles of phosphoglycerate kinase as markers for *Xce* we have found no evidence of a parental source effect in females that are heterozygous for the *a* and *c* alleles of *Xce*. However, we show that in females that were heterozygous for the *b* and *c* alleles of *Xce* the *X* chromosome carrying the *Xce*^{c} alleles has a higher probability of remaining active if it is maternally derived than if it is paternally derived.

2. MATERIALS AND METHODS

(i) *Mice*

The strains C3H/HeHa-*Pgk-1*^{a}*Xce*^{c} (backcross generation 9) and CBA/Ca-*Pgk-1*^{a}*Xce*^{c} (backcross generation 16) were used as the source of the *X* chromosome carrying the *Pgk-1*^{a} and *Xce*^{c} alleles; the C57BL/6J-*bg*^{J} strain for the *X* chromosome carrying the *Pgk-1*^{b} and *Xce*^{b} alleles and the CBA/Ca for the *Pgk-1*^{b} and *Xce*^{a} alleles. *Pgk-1* and *Xce* are closely linked (Cattanach, Perez & Pollard, 1970; Franke & Taggart, 1980) and any recombination between these two loci would be expected to be infrequent. An extensive backcrossing programme in our laboratory supports this expectation.

(ii) *Preparation of samples*

One drop of blood taken from the retro-orbital sinus of 3–4 week-old females was mixed with 100 μl sample buffer (50 mM triethanolamine-HCl, pH 7·6, containing 0·3 mg/ml dithioerythritol, 0·5 mg/ml bovine serum albumin and 2 mg/ml digitonin). Electrophoresis and quantification of the two PGK-1 alloenzymes were carried out as described elsewhere (Woodruff *et al.* 1982; Ansell & Micklem, 1984). Repeats of a single sample within and between gels give an overall standard deviation of approximately 2·1 %. A linear relationship is observed between different artificial mixtures of PGK-1 alloenzymes over the range from 20 to 80 % PGK-1A.

3. RESULTS

Table 1 summarizes the results of phenotypic analyses of heterozygous females derived from 5 different crosses. Crosses 1 and 2 were reciprocal crosses between C3H/HeHa-*Pgk-1*^{a} *Xce*^{c} and C57BL/6J-*bg*^{J} strains. In cross 1 where the *Xce*^{c} *Pgk-1*^{a} bearing *X* chromosome was maternally derived, the mean proportion of PGK-1A alloenzyme present in the blood of heterozygous progeny was 61 %. When the *Xce*^{c}*Pgk-1*^{a} bearing *X* chromosome was paternally derived (cross 2) the mean

proportion of PGK-1A present was 54 %. The difference between these reciprocal crosses was statistically significant ($P < 0{\cdot}01$).

The F1 males (*Xce*^{c}*Pgk-1*^{a}/*Y*) produced from cross 1 were then backcrossed onto the C57BL/6J-*bg*^{J} strain (cross 3). In this case, the *Xce*^{c}*Pgk-1*^{a} *X* chromosome was paternally derived and the proportion of PGK-1A present was 51 %. The F1 females (*Xce*^{c}*Pgk-1*^{a}/*Xce*^{b}*Pgk-1*^{b}) produced from cross 2 were also backcrossed onto the C57BL/6J-*bg*^{J} strain (cross 4). The proportion of PGK-1A in the tissues of heterozygous females produced in this case was 58 %. This was significantly different from the proportion in cross 3 ($P < 0{\cdot}01$). The heterozygous females produced from cross 3 were further backcrossed onto the C57BL/6J-*bg*^{J} strain (cross 5) and the proportion of PGK-1A in the heterozygous progeny of this cross was 60 %.

Table 1. *Percentage PGK-1A in mice heterozygous for the* b *and* c *alleles of* Xce

Cross	*Xce* allele of parental *X* chromosome		Mean percentage of PGK-1A in progeny ± S.E.	No. progeny analysed
	X^M	X^P		
1	*c*	*b*	61 ± 1·5	36
2	*b*	*c*	53 ± 1·1	36
3	*b*	*c*	51 ± 1·3	56
4	*c*	*b*	58 ± 1·8	32
5	*c*	*b*	60 ± 1·7	32

Analysis of variance showed that the progeny of crosses 1, 4 and 5 were significantly different from crosses 2 and 3 ($P < 0{\cdot}01$). In these crosses *Pgk-1*^{a} segregates with *Xce*^{c} and *Pgk-1*^{b} with *Xce*^{b}.

Table 2. *Percentage PGK-1A in mice heterozygous for the* a *and* c *alleles of* Xce

Xce allele of parental *X* chromosome		Mean percentage of PGK-1A in progeny ± S.E.	No. progeny analysed
X^M	X^P		
c	*a*	69 ± 1·5	27
a	*c*	72 ± 1·4	44

These data were derived from reciprocal crosses of sublines of CBA/Ca mice differing only at the region of the *Pgk-1* and *Xce* loci. There was no significant difference in the proportion of PGK-1A between the offspring of either cross. In these crosses *Pgk-1*^{a} segregates with *Xce*^{c} and *Pgk-1*^{b} with *Xce*^{a}.

There was no significant difference between the PGK-1 phenotypes of the heterozygous progeny of crosses 1, 4 and 5. In all these cases the *Xce*^{c}*Pgk-1*^{a}-bearing *X* chromosome was maternally derived and the proportion of PGK-1A present was 58–61 %. Also, there was no significant difference in the heterozygous phenotypes of the progeny produced from crosses 2 and 3. The *Xce*^{c}*Pgk-1*^{a} *X* chromosome in these crosses was paternally derived and the proportion of PGK-1A present was 50–53 %.

A large series of reciprocal crosses between CBA/Ca (*Pgk-1*^{b} *Xce*^{a}) and CBA/Ca-

Pgk-1^a*Xce*^{c} mice were analysed (Table 2). In this case the 'strong' allelic combination of *Xce* (*Xce*^{a}/*Xce*^{c}) was involved. No reciprocal cross differences were observed in this series.

DISCUSSION

In the extraembraembryonic membranes of the mouse, X^M is preferentially expressed (Takagi & Sasaki, 1975) but the existence of parental effects are less well defined in the embryo proper and adult somatic tissues. X^M and X^P may have an equal probability of becoming inactivated (Johnston & Cattanach, 1981) or in some circumstances X^P may be preferentially expressed (Falconer *et al.* 1982). Using PGK-1 alloenzyme expression in erythrocytes, we have found parental effects on *X* chromosome expression using one allelic combination of *Xce* genes but no such effects with another. In the first case the parental source of the *Xce*^{c} allele changed the probability of it being expressed in *Xce*^{b}/*Xce*^{c} heterozygotes from approximately 0·6 to 0·5, when maternally or paternally derived respectively. However, in *Xce*^{a}/*Xce*^{c} heterozygotes the parental source of *Xce*^{c} did not affect the probability of its expression. Although erythrocytes are constantly being replenished the variance in the proportions of PGK-1 alloenzymes in the blood of individual mice, bled regularly for up to 1 year, is small (Micklem *et al.* 1984). Peripheral blood can therefore be regarded as having a stable phenotype and being representative of haematopoietic tissues. Analyses of a small number of thymus and bone marrow samples from offspring of crosses 1, 2, and 3 (data not shown) confirm this assumption. Since all tissues are derived from the same pool of *X*-inactivated cells (McMahon, Fosten & Monk, 1983), analyses of any tissue should show similar results to those described in the blood and other haematopoietic tissues. Preliminary data from kidney and brain samples tend to support this hypothesis.

It is probable that we are looking at an interaction between parental and genetic effects. Previous analyses of *Xce*^{b}/*Xce*^{c} females would predict that (in the absence of other influences) an *Xce*^{c}*Pgk-1*^{a} *X* chromosome would have a higher probability (approx. 0·6) of remaining active than an *Xce*^{b}*Pgk-1*^{b} *X* chromosome (Johnston & Cattanach, 1981). We have found that when the *Xce*^{c}*Pgk-1*^{a} *X* is maternally derived, this prediction is borne out. However, when the *Xce*^{c}*Pgk-1*^{a} *X* is paternally derived the probability of that *X* chromosome being expressed is reduced to approximately 0·5. The mechanism by which these two effects interact is unknown but could be envisaged as West (1982) proposed. The physiological modification of X^M and/or X^P would presumably include the *Xce* locus and the modification of one *Xce* allele could affect its interaction with the other in a heterozygous female. This could have either an additive or a complementary result. If for example the modification of the genome during oogenesis results in the *Xce*^{b} allele on X^M behaving in a similar way to an *Xce*^{c} allele, the heterozygous female produced (if X^P carries *Xce*^{c}) would behave as if it were homozygous at the *Xce* locus. In this hypothetical example X^M and X^P would have an equal probability of expression.

The preferential inactivation of X^P in the earliest differentiated tissues of the embryo, i.e. the trophectoderm and primary endoderm, suggests that X^M and X^P are differentially marked before the *X* inactivation process. When these lineages

differentiate this 'imprinted' difference between X^M and X^P is still present, resulting in X^P being preferentially inactivated. Cell lineages that differentiate later express both X^M and X^P, suggesting that this 'imprinting' is short-lived. The reciprocal cross difference observed in Xce^b/Xce^c heterozygotes in our experiments could indicate that residual effects of the 'imprinting' process remain. However, Rastan & Cattanach (1983) have shown that 'strong' alleles at the *Xce* locus can override the maternal effect in the extraembryonic membranes. When X^P carries the Xce^c allele, the extraembryonic membranes do not exclusively express X^M. We did not see any parental effects on X chromosome expression in the erythrocytes of mice, heterozygous for the 'strong' allelic combination (Xce^a/Xce^c). It is possible that this allelic combination is too extreme for the more subtle maternal influences to affect it. Alternatively, it is possible that other moderator genes are causing the reciprocal cross difference and the expression of these genes differs between different mouse strains. The reciprocal cross differences observed were between two different strains (C3H/HeHa-*Pgk-1*^{a} Xce^c and C57BL/6J-bg^J) whereas the crosses that did not show these differences were between mice which had been backcrossed onto the CBA/Ca strain for 16 generations and ostensibly differed only in the region of the *Pgk-1* and *Xce* loci. It is possible that the reciprocal cross differences are only seen between different strains or between strains with particular moderator genes being expressed. Backcrossing of the *Pgk-1* and *Xce* loci onto the C57BL/6J-bg^J strain would perhaps resolve this question.

This work was supported by the Cancer Research Campaign and the Medical Research Council. L. M. F. is in receipt of a Medical Research Council research studentship. The authors would like to thank Dr H. S. Micklem for his encouragement and advice during experiments and the preparation of this manuscript and Drs J. D. West, V. M. Chapman and P. Fantes for helpful discussions.

REFERENCES

Ansell, J. D. & Micklem, H. S. (1984). Genetical markers for following cell populations. In *Handbook of Experimental Immunology* 4th edn. (ed. D. M. Weir, L. A. Herzenberg, C. D. Blackwell and L. A. Herzenberg) Blackwell Scientific Publication (In the Press.)

Cattanach, B. M. (1975). Control of X chromosome inactivation. *Annual Review of Genetics* **9**, 1–18.

Cattanach, B. M. & Isaacson, J. H. (1965). Genetic control over the inactivation of autosomal genes attached to the X chromosome. *Zeitschrift fur Vererbungslehre* **96**, 313–323.

Cattanach, B. M. & Papworth, D. (1981). Controlling elements in the mouse. V. Linkage tests with X linked genes. *Genetical Research* **38**, 57–70.

Cattanach, B. M., Perez, J. N. & Pollard, C. E. (1970). Controlling elements in the mouse X chromosome. II. Location in the linkage map. *Genetical Research* **15**, 183–195.

Falconer, D. S., Isaacson, J. H. & Gauld, I. K. (1982). Non-random X-chromosome inactivation in the mouse: difference of reaction to imprinting. *Genetical Research* **39**, 237–259.

Franke, V. & Taggart, R. T. (1980). Comparative gene mapping: order of loci on the X chromosome is different in mice and humans. *Proceedings of the National Academy of Sciences, U.S.A.* **77**, 3595–3599.

Frels, W. I. & Chapman, V. M. (1980). Expression of the maternally derived X chromosome in mural trophoblast of the mouse. *Journal of Embryology and Experimental Morphology* **58**, 179–190.

Johnston, P. G. & Cattanach, B. M. (1981). Controlling elements in the mouse. IV. Evidence of non-random X-inactivation. *Genetical Research* **37**, 151–160.

LYON, M. F. (1961). Gene action in the *X* chromosome of the mouse (*Mus musculus L.*). *Nature* **190**, 372–373.

MICKLEM, H. S., ANSELL, J. D., WAYMAN, J. E. & FORRESTER, L. M. (1984). The clonal organisation of hematopoiesis in the mouse. In *Progress in Immunology*, vol. V (ed. Y. Yamamura & T. Tada), pp. 633–644. Academic Press.

MCMAHON, A., FOSTEN, M. & MONK, M. (1983). *X* chromosome inactivation in the three germ layers and the germ line of the mouse embryo. *Journal of Embryology and Experimental Morphology* **74**, 207–220.

PAPAIOANNOU, V. E. & WEST, J. D. (1981). Relationship between the parental origin of the *X* chromosomes, embryonic cell lineage and *X* chromosome expression in mice. *Genetical Research* **37**, 183–197.

RASTAN, S. & CATTANACH, B. M. (1983). Interaction between the *Xce* locus and imprinting of the paternal *X* chromosome in mouse yolk sac endoderm. *Nature* **303**, 635–636.

TAKAGI, N. & SASAKI, M. (1975). Preferential inactivation of the paternally derived *X* chromosome in the extraembryonic membranes of the mouse. *Nature* **256**, 640–642.

WEST, J. D. (1982). *X* chromosome expression during mouse embryogenesis. In *Genetic control of gamete production and function* (ed. P. G. Crosignani, B. L. Rubin and M. Fraccaro), pp. 49–91. Academic Press, Grune & Stratton.

WEST, J. D., FRELS, W. I. & CHAPMAN, V. M. (1977). Preferential expression of the maternally derived *X* chromosome in the mouse yolk sac. *Cell* **12**, 873–882.

WOODRUFF, M. F. A., ANSELL, J. D., FORBES, G. M., GORDON, J. C., BURTON, D. I. & MICKLEM, H. S. (1982). Clonal interactions in tumours. *Nature* **299**, 822–824.