ANALYSIS OF MURINE HAEMATOPOIETIC STEM CELL ACTIVITY USING THE X-LINKED ENZYME PHOSPHOGLYCERATE KINASE - 1

by

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ABSTRACT

Until recently it has been virtually impossible to study stem cell activity in normal animals. Previous studies have generally employed chromosome markers, entailing the need to lethally irradiate hosts and then to inject appropriately marked cells to repopulate the host (radiation chimaeras). X-chromosome-linked electrophoresis markers can be used to study both repopulation following irradiation and normal cell turnover as females heterozygous for a particular marker are natural mosaics. This study makes use of the X-linked enzyme Phosphoglycerate kinase (PGK-1).

Stem cell numbers active at any given time in normal animals were compared with stem cell numbers repopulating the depleted haematopoietic system following total body irradiation and bone-marrow rescue or following treatment with cytotoxic drugs.

The number of haematopoietic clones estimated to be active each day in the blood of normal mice was considerably smaller than the previously estimated daily number of CFU-S entering cell cycle. Following administration of the erythrolytic drug phenylhydrazine hydrochloride (PHZ) clone numbers of a similar range to normal were found.

Following irradiation and repopulation or treatment with the cytotoxic drug hydroxyurea repopulation was examined in a much wider range of haematopoietic tissues. In irradiated and hydroxyurea-treated animals clone numbers were comparable to those reported previously using chromosome markers. Differences between tissues and within tissues in both irradiated and hydroxyurea-treated animals also paralleled earlier findings using chromosome markers.

The X-linked enzyme PGK-1 in the mouse has allowed a new approach to the study of stem cell activity in the mouse. Insights gained by

studying haematopoiesis in normal animals provide an essential background for the study of events associated with malignancy in PGK-heterozygotes.

DECLARATION

This thesis has been composed by myself and it has not been submitted in any previous application for a degree. The work reported within was executed by myself, unless otherwise stated.

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LIST OF ABBREVIATIONS

ACFU	cells capable of forming colonies in an agar medium
ADP	adenosine diphosphate
ALD	aldolase (E.C.4.1.2.13)
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BFU-e	burst forming unit - erythroid
BSA	bovine serum albumin
CFC-S	colony forming cell - spleen
CFU	colony forming unit
CFU-C	colony forming unit - culture
CFU-S	colony forming unit - spleen
DNA	deoxyribonucleic acid
DTE	dithioerythritol
EDTA	ethylenediaminetetra-acetic acid
GAPDH	glyceraldehyde-3-phosphate dehydrogenase (E.C.1.2.1.12)
GDH	glycerol dehydrogenase (E.C.1.1.1.6)
G-6-PD; G-6-PDH	glucose-6-phosphate dehydrogenase
HBSS	Hanks buffered saline solution
HGPRT; HPRT	hypoxanthine-guanine-phosphoribosyl transferase
HK	hexokinase (E.C.2.7.1.1)
^{3}H-TdR	tritiated thymidine
HU	hydroxyurea
I.P.	intra-peritoneally
K_2HPO_4	dipotassium phosphate
LPS	lipopolysaccharide
MTT	3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide: thiozolyl blue
NA	not applicable
NaCl	sodium chloride
NAD	β-nicotinamide adenine dinucleotide
NADH	β-nicotinamide adenine dinucleotide, reduced form
Na_3-F-1, 6-PD	fructose-1, 6-diphosphate, trisodium salt (E.C.3.1.3.11)

ND	not done
NH_4Cl	ammonium chloride
NT	not tested
PBS	phosphate buffered saline
PCV	packed cell volume
PGK; PGK-1	X-linked phosphoglycerate kinase (E.C.2.7.2.3)
PHZ	phenylhydrazine hydrochloride
PMN	polymorphonuclear leucocyte
PMS	phenazine methosulphate
PSC	pluripotent stem cell
T-eth-HCl	tri-ethanolamine hydrochloride
Xce	X-chromosome controlling element

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CHAPTER 1 INTRODUCTION

The Haematopoietic Stem Cell

The blood forming and transporting tissues form the largest organ of the mammalian body. Blood cells are responsible for carrying oxygen and other essential nutrients to the body tissues and also form our first defence against infection and injury.

The differential cell types found in circulating blood are incapable of reproduction and have a finite lifespan. Therefore the turnover of blood cells is quite vast, for instance, the average man produces approximately 2×10^{11} new red cells per day, while in the mouse erythrocyte production is estimated to be about 5×10^{8} cells per day.

It is now well known that haematopoiesis (the production of blood cells) is a continuous process from very early in embryonic life, where the yolk sac is thought to be the only original site of haematopoiesis (Moore and Metcalf, 1970; Weissman et al, 1978). During foetal development the onus of blood cell production falls upon the liver, spleen and gradually is switched to the major post-natal haematopoietic site, the bone marrow.

The term "haematopoietic stem cell" has been used to describe those cells whose descendents populate the circulating blood. Although mature blood cells have been cytologically and functionally well defined, haematopoietic stem cells continue to evade description by conventional histological methods. Attempts have been made to isolate progenitor cells according to their physical properties, for example by separating cells by sedimentation velocities (Miller and Phillips, 1969; Worton, McCulloch and Till, 1969) and, more recently, by density gradient centrifugation (Nijhof and Wierenga, 1983) coupled with functional characteristics. Recent advances in cell surface

marker studies and in cell sorting techniques may provide a reliable method of categorising stem cells and their early descendents which have so far evaded identification and separation. For example, thymic precursor cells have been studied using fluorescent markers (Butcher and Weissman, 1980) and B cell ontogeny has been studied by measuring changes in surface immunoglobulin density (Scher et al, 1976). Recently a mouse monoclonal antibody raised in response to quail immunoglobulin u chain has been found to react with a defined surface marker on haematopoietic stem cells and their progeny (with the exception of mature erythrocytes). This marker is also found on endothelial cells in the quail (Peault et al, 1983).

In addition to being unable to identify haematopoietic stem cells, haematologists also faced the dilemma of not knowing whether the same cell was responsible for the production of all known blood cell types (a pluripotent stem cell) or if a variety of stem cells was necessary (unipotent stem cells). There is now much evidence supporting the view that a pluripotent stem cell is the common precursor of erythrocytes, granulocytes, monocytes, platelets, T and B lymphocytes, mast cells and osteoclasts (Ford and Micklem, 1963; Lewis and Trobaugh, 1964; Abramson et al, 1977; Phillips, 1978; Ash et al, 1980; Matsuda et al, 1981; Kitamura et al, 1981). In other words it would appear that the haematopoietic stem cell is pluripotent (Metcalf and Moore, 1971), although there is evidence (Abramson et al, 1977; Phillips, 1978) that some stem cells are only capable of limited differentiation.

Pluripotent stem cells (PSC) are, by definition, able to maintain their numbers by self-renewal and, in response to appropriate stimuli, to differentiate into one of the known haematopoietic lines (Lajtha et al, 1962; Becker et al, 1963).

A major breakthrough in the study of haematopoiesis occurred in the late 1940's when Jacobson and his colleagues observed that by protecting the spleens of experimental mice during potentially lethal whole body

irradiation then their chances of survival were enhanced whether or not the spleen was later removed from the mice (Jacobson et al, 1949; 1951). Other workers soon determined that similar restoration could be effected by injection of bone marrow or splenic cells which were able to re-establish haematopoiesis in the irradiated animals (Lorenz et al, 1951; Ford et al, 1956). It was another few years, however, before a quantitative method for studying stem cell function became available. In 1961, Till and McCulloch noted that when bone marrow cells were injected into lethally irradiated host mice, some of these cells migrated to the host spleens and there divided to form nodules or colonies of cells on the surface of the spleen. These colonies contained erythroid and/or granulocytic precursor cells, allowing functional and quantitative studies of haematopoietic stem cells to be attempted for the first time. Till and McCulloch called these repopulating cells colony forming units - spleen (CFU-S). More recently cells which have the potential to initiate spleen colonies have been called colony forming cells - spleen (CFC-S), while the term CFU-S is reserved for those CFC-S which actually lodge in the spleens of recipients and form colonies there. For a number of years these cells have been regarded by many as the most likely candidate for the much sought after PSC.

Spleen colonies were found to arise from individual cells (i.e. they were of clonal origin) by studying the chromosomal make-up of cells within colonies originating from CFU-S containing specific radiation-induced chromosome aberrations (Becker et al, 1963; Wu et al, 1967; Chen and Schooley, 1968). These individual colonies could produce descendents of only one or of mixed haematopoietic classes (Lewis and Trobaugh, 1964; Fowler et al, 1967). More recently studies have shown that CFU-S from different tissues vary in their ability to self-renew, to form colonies and the manner in which cells in such colonies differentiate (Siminovitch et al, 1965; Silini et al, 1968; Lahiri and van Putten, 1969; Thomas, 1971; Gidali et al, 1974; Micklem et al, 1975). CFU-S were also shown to be able to repopulate the bone marrow, thymus and lymph nodes, as well as the spleens, of

irradiated animals (Trentin et al, 1967; Wu et al, 1968; Edwards et al, 1970).

Siminovitch, McCulloch and Till (1963) were able to demonstrate that, in addition to their ability to undergo variant pathways of differentiation, CFU-S could maintain their numbers by self-renewal. They showed this by injecting cells from excised colonies into a second irradiated host and demonstrating spleen colony formation in these animals also, a procedure which was to become standard practice in many assay systems. One can estimate the probability, p, that a CFU-S will self-renew to allow qualitative comparisons of CFU-S in bone marrow sources. This has been done by injecting bone marrow cells into irradiated recipients in a dose which will produce approximately eight colonies per spleen. Recipients were killed at a standard time and their spleen colonies excised individually. Suspensions of equal numbers of these cells were then injected into irradiated recipients and their spleen colonies were also counted after a standard period. Using this method Schofield (1979) found p to be 0.69 for normal bone marrow CFU-S. This is slightly higher than that of 0.63 found by Vogel and his colleagues using a rather different technique (Vogel et al, 1968). A value of p of 0.69 means that even under maximum demand for differentiated cells (repopulation of irradiated recipients) the proportion of CFU-S which continue to self-renew is 69%. The higher the value of p, the greater is the degree of self-renewal. If p is less than 0.5 then the CFU-S population will gradually decrease.

Repeated transplantation or repeated sub-lethal irradiation (Siminovitch et al, 1964; Hendry and Lajtha, 1972; Wu and Lajtha, 1975) lead to a rapid decline in the repopulating ability of CFU-S, demonstrating that CFU-S are unable to self-renew indefinitely. Unfortunately, damage to DNA by sub-lethal irradiation renders this method unsuitable for studying the self-renewal ability of normal CFU-S. Do haematopoietic stem cells have a finite life span? Other

studies suggest that CFU-S are able to self-renew and to support recovery from lethal irradiation for much longer periods of time than the average lifespan of the mouse (Silini and Andreozzi, 1974; Silini et al, 1974; Hellman et al, 1978; Harrison, 1979). These studies also suggest that there is no advantage of CFU-S from young donors over those from old donors in their ability to repopulate (also Lajtha and Schofield, 1971). Such apparent contradictions with regard to the repopulating ability of CFU-S suggest that CFU-S are not true haematopoietic stem cells. They may have lost some of their potential to self-renew while retaining their ability to mature into different cell types. The CFU-S may be a heterogeneous population, or the cells which are able to maintain their repopulating ability beyond the normal lifespan of the mouse may indeed not be CFU-S but pre-CFU-S which still retain a greater ability to self-renew.

The inability of CFU-S to self-renew indefinitely as shown by repeated transfer of CFU-S over a number of irradiated host generations resulting in an eventual inability of the transferred cells to repopulate the host could, however, be an artifact of serial transplantation rather than an effect of ageing (Ogden and Micklem, 1976; Harrison et al, 1978; Ross et al, 1982). For example, it is probable that after a number of transfers there are simply too few stem cells in the inoculum to be able to expand adequately to fill the haematopoietic system, rather than that the self-renewal potential of these cells had run out. A similar effect could occur in vitro (Holliday et al, 1977).

As previously mentioned Siminovitch, McCulloch and Till (1963) were able to show the colony-forming ability of transplanted CFU-S. The number of spleen colonies which will be produced by a given number of injected cells can easily be established and hence the number of colony-forming cells which come to rest in the spleen following irradiation is known. If these clones are reinjected into a second irradiated mouse as a spleen suspension the actual number of colony-forming cells is known and the proportion of these which produce

further colonies can be determined. This seeding factor or "f-number" was determined as 0.17 (17% of the injected colony-forming cells formed spleen colonies) by Siminovitch and his colleagues. A number of authors have since estimated the seeding factor (Playfair and Cole, 1965; Fred and Smith, 1968; Hendry, 1971; Metcalf and Moore, 1971) and an average figure of about 10% has been estimated. Although a direct method of measuring the ability of CFU-S to home to other tissues in the body does not exist, it has been estimated that about 1% of injected CFU-S lodge in a single femur (or about 20% in the entire bone marrow, Valeriote and Bruce, 1967).

The thymidine suicide technique (Smith et al, 1962; Becker et al, 1965) has been used to estimate the rate of turnover of CFU-S from many sources. Cell suspensions to be tested are incubated for an appropriate period of time with a high concentration of high specific activity tritiated thymidine (^{3}H - TdR). Those cells which are engaged in DNA synthesis during the incubation period incorporate ^{3}H - TdR into their DNA strands which receive a lethal dose of beta-radiation. As a result, these cells are unable to form spleen colonies when injected into irradiated recipients. By comparing the number of spleen colonies produced following injection of normal cells with the colony numbers found following injection of ^{3}H-thymidine treated cells, an estimate can be made of the number of CFU-S undergoing DNA synthesis, and hence in cell cycle at the time of incubation. Becker and his colleagues (1965) found that only a very small proportion of CFU-S were in S-phase in normal adult bone marrow and spleen, but that a large proportion of CFU-S were cycling in foetal liver and proliferating marrow transplants. More recently (Schofield, 1979) the figure for CFU-S undergoing DNA synthesis has been found to be about 10% under normal conditions. Most CFU-S are thought to be in an extended resting (G_1) phase of the cell cycle or not to be cycling at all (G_0 phase).

CFU-S are most probably a heterogeneous population which differ in their ability to self-renew, a most important point when one considers

their relationship to stem cells. Although some CFU-S may be stem cells, it is evident that not all are. In the same way, not all stem cells may reveal themselves as CFU-S. It is already clear that some highly self-renewing CFU-S may be missed in a standard eight-day spleen colony assay, appearing only by day 10 - 12 (Hodgson and Bradley, 1979; Magli et al, 1982). It is very possible that others do not form spleen colonies at all, either because they are reluctant to divide or for purely technical reasons: difficulty of obtaining in cell suspensions (see below) or failure to locate in the spleen, for example. Lord (1971) has shown that cells which initially locate in the spleen following irradiation may quite quickly be expelled from it.

Although mice are the only species in which spleen colonies have been found to grow, studies of haematopoiesis have been carried out in the rat (Blackett et al, 1964; Blackett, 1967; Tarbutt, 1967). The ontogeny of erythropoiesis in the mouse has also been studied (Rich and Kubanek, 1979; 1980) using the spleen colony forming assay.

There are however several obvious disadvantages of using the spleen colony-forming assay. These include the inability to look at a whole cell population, the necessity of perturbation to the system (irradiation) to allow investigation, and the likelihood that CFU-S are not the ultimate progenitor cells of the haematopoietic system. Since its conception, however, the spleen colony-forming assay has allowed us to expand our knowledge of haematopoiesis greatly.

More recently workers have developed methods of growing haematopoietic progenitor cells in culture (Dexter et al, 1977; Dexter et al, 1978; Gregory and Eaves, 1978), and the importance of the haematopoietic microenvironment (Wolf, 1979) has been shown both in vivo and in vitro. La Pushin and Trentin (1977) identified stromal elements within spleen colonies and more recently Lanotte and his colleagues (1981; 1982) have shown the importance of the stroma for haematopoiesis to occur in vitro. The importance of

cellular and humoral factors for the maintenance of erythropoiesis has also been shown (Reissmann et al, 1972; Lord and Schofield, 1973; Staber and Metcalf, 1980; Wagemaker and Visser, 1980).

The Phenomenon of X-Chromosome Inactivation and its Relevance to Stem Cell Studies

In mammals the female cells contain two X- chromosomes (XX) while male cells contain one X and one Y chromosome (XY). In germ cells both X-chromosomes are active, but in somatic cells one of the X-chromosomes becomes inactive and condensed. The inactive X-chromosome may be recognised by a number of properties, for example, the presence of facultative heterochromatin within interphase cells (the Barr body in human cells, for example). The inactive X-chromosome also replicates its DNA strands later than the active X and so can be observed using appropriate staining methods. Absence of a gene product is a third way of recognising the inactive X-chromosome and it is this method which will be studied in greater detail below. The phenomenon of X inactivation in female somatic cells serves as a dosage compensation mechanism, reducing the effective X-chromosome dosage to that of the XY male.

Lyon (1961) proposed the theory that one X-chromosome is randomly inactivated within somatic cells and that this inactivation is permanent, the females being mosaics with respect to genes carried on the X-chromosome. There are three principal ways in which genetic mosaicism arises. Aggregation of genetically distinct embryos (Tarkowski, 1961; Mintz, 1962) or injection of genetically distinct cells into blastocysts (Gardner, 1968) produces aggregation and injection chimaeras. X- inactivation in female mammals heterozygous for X-linked genes causes a naturally occurring functional mosaicism.

Studies of genetic mosaics have established firmly many of the events which take place during development (Lyon, 1972; 1974; Cattanach, 1975).

Using single cell injection chimaeras, Gardner and Lyon (1971) were able to show that X-inactivation had not yet occurred in embryos 3.5 days after fertilization.

Monk and Harper (1979) electrophoresed inner cell masses from female blastocysts then simultaneously stained gels for the X-chromosome encoded enzyme hypoxanthine phosphoribosyl transferase (HPRT) and the autosomally coded adenine phosphoribosyl transferase (APRT) to assess whether X-inactivation had occurred. In XX tissues prior to X-inactivation the ratio of HPRT to APRT will be twice as great as that seen in XX tissues following X-inactivation and control XY tissues. The data were consistent with the presence of two active X-chromosomes. Tissue studies of 6 day conceptuses suggested that X-inactivation had already occurred in the extra-embryonic ectoderm and primary endoderm by this stage, but that two populations with respect to X-chromosome activity were present in epiblast regions. These findings provide strong evidence for the hypothesis that X-chromosome inactivation is linked to cellular differentiation during early development (Monk, 1978; 1981).

McMahon (PhD thesis, London) used electrophoretic studies of the X-linked enzyme phosphoglycerate kinase (PGK) to estimate that the earliest time when X-inactivation can take place in the epiblast cells is between 4.5 days and 5.5 days post coitum, when the cell population has been calculated to be about 47 cells.

Electrophoretic variants of the X-linked enzyme glucose-6-phosphate dehydrogenase (G-6-PD) have been used to study cell lineages in humans. Gandini and Gartler (1969) used this technique to infer a common lineage of all blood cell precursors. Fialkow (1973) suggested a common lineage between blood, muscle and skin cells by correlation of the proportion of cells expressing G-6-PD isozymes in heterozygous human females. He estimated that these

cells arose from a shared precursor pool of about 10 - 25 cells, which he suggested might represent the number of cells from which the mesoderm or ectoderm (from which the mesoderm differentiates) arose, or even perhaps the total number of embryoblasts present at the time of X-chromosome inactivation.

Using a different approach Nesbitt (1971) also studied cell lineage relationships. Late replication of the elongated translocated X-chromosomes in female mice heterozygous for Cattanach's translocation (Cattanach, 1961; Is (In 7,X)ICt) allowed cytological studies to be made of such heterozygotes. Her results suggested that all the tissues studied (of ectodermal and mesodermal origin) were derived from the same pool of cells after X-inactivation. From the covariance in mosaic composition among tissues, the size of this pool was estimated to be approximately 21 cells. Nesbitt concluded that this was the number of cells at the time of X-inactivation. The sizes of precursor pools for the different tissues calculated from the independent variance among tissues was shown to be not less than 20 - 30 cells.

As previously mentioned it was initially thought that X-inactivation was a random event, but this would appear to be a gross oversimplification. In metatherian mammals (the marsupials) the paternal X-chromosome is always inactivated in certain tissues such as the liver, kidney, lymphocytes, and nucleated erythrocyte precursors (Cooper et al, 1971; Vandeberg et al, 1973; Cooper et al, 1975; Vandeberg et al, 1977a; Donald and Cooper, 1977). However evidence of paternal X-chromosome activity has been found in the muscle cells of the pretty-face wallaby (Macropus parryi; Vandeberg et al, 1973). Later studies produced similar results in cultured fibroblasts of M. parryi and M. giganteus (the eastern grey kangaroo; Cooper et al, 1977) and also in various tissue samples from M. giganteus and M. parryi (Vandeberg et al, 1977b).

In eutherian mammals an even more complex picture has been built up. Most studies in eutherian mammals have been carried out on mice (Mus musculus), but rats and interspecific hybrids have also been used. Studies show that the paternal X-chromosome is preferentially inactivated in the yolk sac of mice (West et al, 1977a), rats and Mus musculus x Mus caroli hybrids (West et al, 1978). Preferential inactivation of the paternal X-chromosome has also been found in the extraembryonic membranes of the mouse (Tagaki and Sasaki, 1975), while only maternal X-chromosome activity has been found in the mural trophoblast cells of the mouse (Frels and Chapman, 1980). Non-selective X-inactivation has been found within the tissues derived from the epiblast (Papaioannou and West, 1981). Similarly, non-selective X-inactivation also occurs in the germ cells of female embryos, but here the inactivated chromosomes are reactivated prior to the onset of meiosis (McMahon et al, 1981; Monk and McLaren, 1981).

A controlling element for X-chromosome inactivation has been described in the X-chromosome by a number of authors (Grahn et al, 1970; Cattanach et al, 1970; Cattanach and Williams, 1972; Ohno et al, 1973). Although the various authors have given different names to this element they were probably all describing the X-chromosome controlling element (Xce) designated by Cattanach and his colleagues. The gene locus for the controlling element is situated on the long arm of the X-chromosome close to the gene loci for the markers Tabby (Ta) and Mottled (Mo) and also close to the IsCt breakpoint of Cattanach's translocation and the locus for phosphoglycerate kinase (Cattanach and Papworth, 1981).

As a result of X-chromosome inactivation all somatic cells after the time of inactivation in female mammals contain only one active chromosome. Because of the permanent nature of X-inactivation all offspring of a particular cell will carry the same active X as that cell. In this way females are mosaics with regard to genes carried

on the X-chromosomes. Such natural X-inactivation mosaics should be valuable experimental tools for studies of development and stem cell renewal as they may carry inbuilt markers.

Theories of Stem Cell Renewal

Krohn (1962) raised the question of whether or not normal diploid cells have a finite lifespan in vivo. In 1965 Hayflick showed that normal human diploid cells (foetal lung fibroblasts) are unable to survive in culture through more than about fifty serial passages (subcultivation ratio 2:1). Daniel and his colleagues (1968) described a similar finite lifespan for normal diploid cells in the mouse. They serially transplanted normal and pre-neoplastic mammary gland tissue into the mammary fat pads (gland free tissue) of recipient animals and found that the growth rate of the normal cells decreased rapidly and no cells grew for more than 7 serial passage generations. By comparison, the pre-neoplastic cells showed no decline in growth rate and were still growing after 8 years and 30 serial passages. Viral transformed (non-malignant) human cell lines, for example lymphoblastoid cells from infectious mononucleosis patients, also show apparent immortality in culture (Moore and McLimans, 1968).

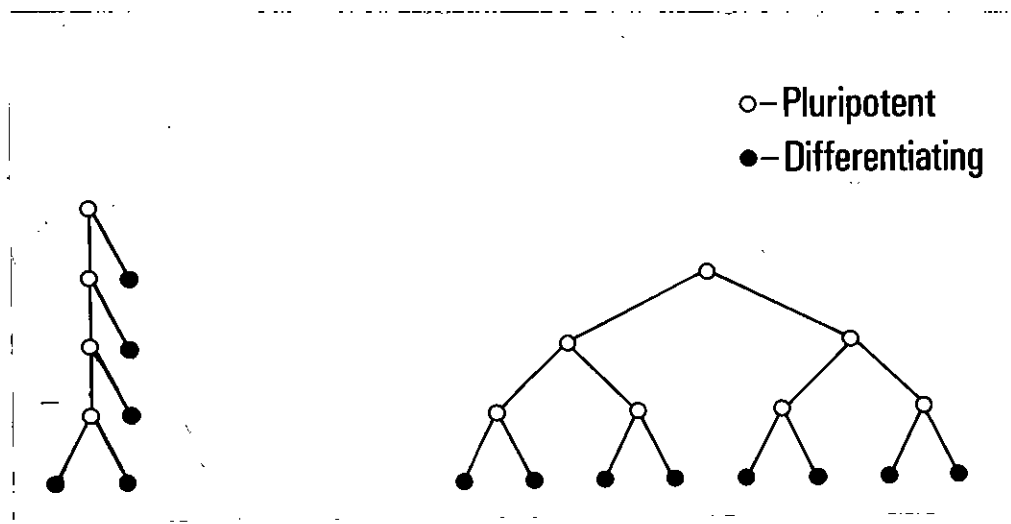
Such findings and the lack of obvious effects of senescence in tissues in which there is a rapid cell turnover throughout life (bone marrow, intestinal epithelium, skin and testis) have prompted hypotheses on the methods by which non-dividing end cells in many body systems are renewed.

There are basically two extreme ways in which cell multiplication can be represented. Kay (1965) referred to these as tangential and logarithmic types of cell growth and suggested that the actual mode of cell multiplication within many systems was to be found somewhere between these two extremes, a view which is also held by

other authors (Lajtha et al, 1962; Till et al, 1964).

The tangential type of cell growth (Figure 1.1a) is a maintenance type in which the homogeneous stem (progenitor) cells divide to produce supplies of cells both for differentiation and to maintain the stem cell pool.

Figure 1.1 Models of cell multiplication



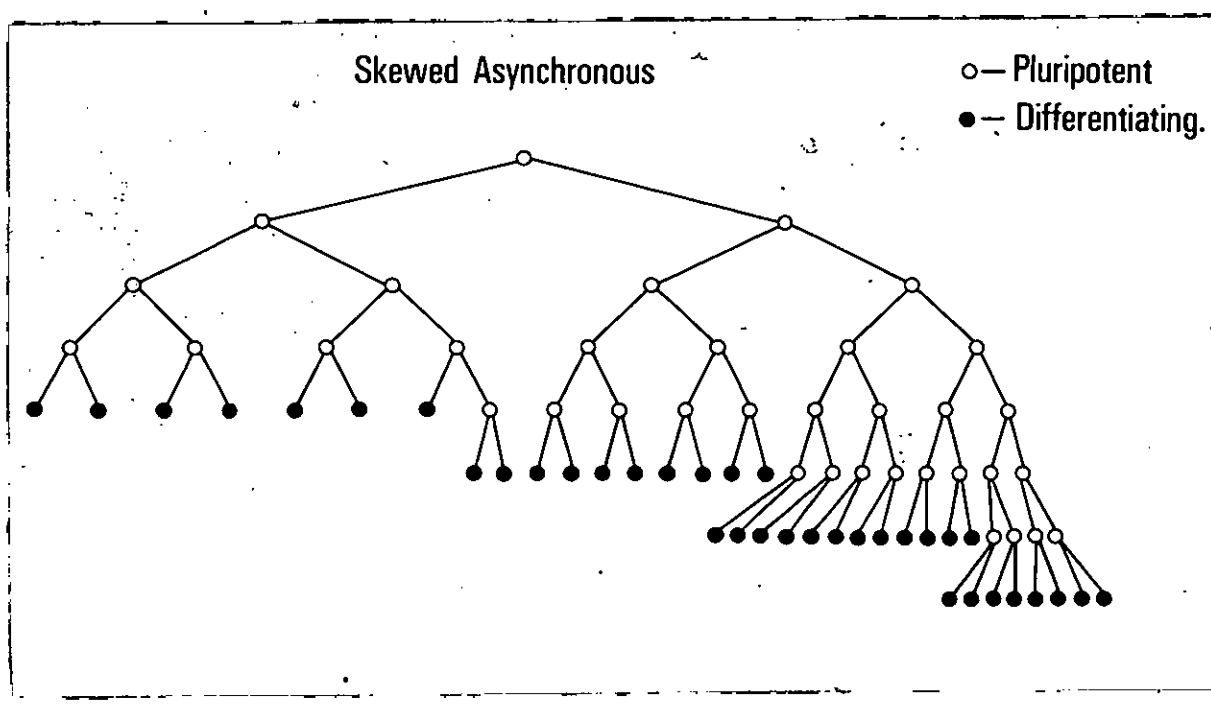
a) Tangential b) Logarithmic

On the other hand, in a perfect logarithmic (growth, Figure 1.1b) type all differentiated cells would be developed synchronously with no survival of stem cells. A maintained output of cells could be achieved within a logarithmic system if the stem cells did not divide and differentiate in a synchronous fashion. Kay suggested that this is indeed how cells proliferate within many tissues and he used the term "clonal succession" to describe the variation of rate of cell division and differentiation in the primitive stem cell pool necessary to achieve a maintained cell output using the logarithmic method. Kay estimated that to produce, from a single

stem cell, the entire blood cell population necessary for a human life (60 years) would require only 54 cell generations. Using the tangential system 12,000 cell generations would be necessary to support the blood system for sixty years (assuming a stem cell pool of 4×10^{10} cells).

At about this time variations in radiosensitivity of haematopoietic precursor cells capable of initiating colonies of cells in the spleens of lethally-irradiated host mice (CFU-S:colony forming units - spleen, Siminovitch et al, 1965) had suggested that CFU-S may comprise a heterogeneous population. Kay described a "skewed asynchronous" growth pattern for cells (figure 1.2) in which a generation of dormant ancestral cells would be successively promoted to form clones of maturing cells.

Figure 1.2 Skewed asynchronous cell growth



If at each stage of development cells were more responsive to the need to divide than to differentiate then the stem cell pool need never be exhausted. In such a system it is easy to imagine how humoral factors could promote cells into a particular differentiation pathway (Lajtha et al, 1962).

A major question is how the mixed population of stem cells necessary for the skewed asynchronous pattern of growth could evolve. Kay suggested that at some point during early embryogenesis a group of cells, by chance or perhaps due to their location, are able to divide faster than the others and may differentiate first. Differentiation at this stage could be due to genetic restriction (Monk, 1981) or to environmental factors. The very rapid division and differentiation of cells within the embryo at the time of organogenesis (Snow, 1981) could produce large changes in levels of humoral factors which could check the proliferation of stem cells within a particular tissue. From then on stimuli to differentiate could ensure a regulated outlet of cells from the stem cell pool.

The theory of clonal succession allows for cell populations to be renewed by a relatively small number of stem cells without the need for a large number of differentiating cell generations. The smaller the number of generations necessary for maturation the less likely it is that errors of transcription will arise (transcriptional errors perhaps giving rise to mutant cell populations such as those seen in neoplastic tissues).

If one defines a clone of cells as those cells which are derived from a cell at the point where it ceases to have "stem" characteristics, then the size of a clone will depend on the number of divisions that occur during differentiation to the non-dividing end cell. Testa (1979) estimated this number to be about 12 - 14 in the erythron. Such a clone would yield about 10^4 cells. If one assumes 2×10^{10} erythrocytes in a mouse, each with a lifespan

of 45 days (Russell and Bernstein, 1966), the daily production of red cells has to be 4 - 5 x 10^8. At this level there must be clonal succession within the stem cell pool as each clone has a finite life-span.

Rosendaal, Hodgson and Bradley (1976; 1979) suggested a theory, based on observations of the functional capacity of normal versus hydroxyurea or fluorouracil (Hodgson et al, 1982) treated stem cells, that haematopoietic stem cells are organised for use on the basis of their generation age. These authors suggested that young stem cells which have undergone few generations since their origin have a greater capacity for self-renewal and the production of committed precursors than do those stem cells which have undergone more generations. They substantiated this claim by showing that cells which could survive repeated doses of cycle-active drugs (i.e. those cells which would not normally be cycling, by inference those which had undergone the fewest generations) were functionally more efficient than those which could not (also Ross et al, 1982). These findings, and the inherent implication that "older" (by generation) stem cells will undergo differentiation divisions in preference to "young" stem cells add weight to Kay's theory of clonal succession.

An hypothesis to explain how stem cells are prevented from dividing was proposed by Schofield (1978). He suggested that the stem cell is associated with other cells which determine its behaviour. The importance of cellular interactions on stem cell growth and differentiation have already been shown to be of importance in vitro (Dexter et al, 1977). This cellular association, in effect, causes the stem cell to become a fixed tissue cell, preventing its maturation and, as a result, assuring its proliferation as a stem cell. When these cells divide, their progeny may do one of two things. They may occupy a similar spatial association (a stem cell niche) as the cell from which they were generated, and hence become a stem cell, or, if there are no niches available they may proliferate

and mature, acquiring an increasing probability of differentiation. Schofield suggests that colony-forming cells are stem cells which have been set on the pathway to differentiation. This theory provides an answer to the problem of why CFU-S, as shown by many workers, do not always fulfil the necessary criteria to be regarded as stem cells. The stem cell niche is not defined by Schofield, but the results of other workers (Patt and Maloney, 1972; Chamberlain et al, 1975; Lord et al, 1975; Maloney et al, 1982) suggest that the cellular environment within the bone marrow is important in determining whether or not stem cells multiply and differentiate.

One important feature of Schofield's hypothesis is that it implies an "age structure" within the haematopoietic stem cell pool. This is in accordance with the findings of Rosendaal and his colleagues. This hypothesis also allows an explanation for the often mystifying variations found in myeloproliferative disorders. In such disorders the impairment of haematopoiesis is not necessarily due to a defect of the stem cells themselves (Thomas et al, 1971; Dexter and Moore, 1977) but could be caused by a defect in one of the other cells involved in the niche.

Are stem cell niches de rigeur in all haematopoietic (and other) tissues and, if so, are they consistent with respect to the cells involved, or is there a heterogeneity of niches? Similarities in the manner in which cells are replaced have been found in epithelia, bone marrow and testis (Potten et al, 1979) which could suggest a uniformity of regulatory principles throughout the whole spectrum of tissues with factors involved in regulation varying from tissue to tissue.

The necessity for special proliferative sites of haematopoiesis has recently been called into question by Brecher and his co-workers (1982). They have shown, using chromosome and enzyme markers, that normal mice transfused with large numbers of bone marrow cells

($20 - 200 \times 10^6$) will support haematopoiesis by donor cells. 2 - 13 weeks after transfusion donor cells accounted for up to 25% of total marrow cells, depending on the number of cells transfused. One can imagine that transfused stem cells will divide and differentiate due to the normal stimuli in the recipient mice. If one takes into account Schofield's stem cell niche theory, transfused stem cells will have to compete for spaces within niches along with newly formed host daughter cells. One would assume that as greater numbers of stem cells are transfused they will be able to compete for a greater number of niches. Having acquired a site within a niche the transfused stem cells could, however, initiate repopulation in the same way as host stem cells.

The importance of the physical distribution of cells within the bone marrow has been shown by Lord and his colleagues (1975) and by Gong (1978). Local and systemic factors have been suggested (Chan and Metcalf, 1973) for the control of haematopoiesis following irradiation. Even when the system is greatly stressed (e.g. following irradiation) haematopoiesis has been shown by several authors to be controlled locally (Maloney and Patt, 1969; Gidali and Lajtha, 1972; Maloney et al, 1978).

Arguing against the case for specialised stem cell niches, one could assume that there are very few niches available for newly formed stem cells in the normal animal. This would appear to be borne out by the observation that circulating CFU-S only rarely re-enter the bone marrow (Micklem et al, 1975). This could, however, be a result of the lack of stemness of the blood CFU-S (i.e. their commitment to differentiation is greater than their commitment to self-renew) rather than a lack of available niches. Such a lack of niches could also push into differentiation donor cells which would otherwise remain pluripotent stem cells.

A much longer term study of the type carried out by Brecher and his

co-workers (1982) may help to throw light on this matter by assessing the long term repopulating ability of the injected cells. Treatment of the injected hosts with cytotoxic drugs or sub-lethal irradiation might also serve to clarify whether the injected cells are CFU-S or stem cells.

I believe the evidence is now too great to discount the importance of a specialised haematopoietic microenvironment controlling the ordered release of differentiating cells from a quiescent stem cell pool. Evidence that only a very small proportion of CFU-S are in cell cycle under normal circumstances, with the vast majority of the stem cells appearing to be in a state of "no cell cycle" (Lajtha et al, 1962), suggests that rather small numbers of stem cells may be contributing to haematopoiesis at any time. Methods used to estimate the numbers of stem cells involved in haematopoiesis will now be discussed.

Stem Cell Marker Studies

The basic approach in many of the methods to be discussed has been to estimate the proportions of two distinct cell types in tissue samples. Statistical analysis of such data may provide answers to certain questions of development and repopulation. Such methods allow some extra insights which cannot be attained using the CFU-S assay alone. The origin of the repopulating cells within a tissue can be ascertained, as can the methods by which tissues are repopulated. By applying relevant statistical tests (Wallis et al, 1975; Ogden and Micklem, 1976) to the data, an estimate of the number of stem cells involved in repopulation can be made without having to look directly for the stem cells themselves. Similar statistical methods can also be used to estimate the number of stem cells involved in the process of organogenesis during embryonic development and also the size of the embryo when organogenesis begins.

There are two types of markers which can produce distinct cell types within tissues, those which occur naturally and artificial markers (i.e. markers which are introduced experimentally into the animal).

Chromosome markers may occur naturally, the trisomy of chromosome 21 found in Down's Syndrome in humans being a good example. Many chromosome markers are also found occurring naturally in other disease processes, particularly in malignant diseases. For experimental purposes, however, induced chromosome abnormalities are normally used. Such aberrations are usually radiation induced and may be of a random nature (Becker et al, 1963). Randomly-produced chromosome aberrations will however be passed on to descendents at mitosis, thus allowing the study of two or more distinct cell populations in individuals carrying such markers.

The T6 marker is a reciprocal translocation derived from an irradiated male mouse. By selective breeding animals carrying 0, 1 or 2 T6 chromosomes can be produced and cells from such animals can be injected into irradiated recipients to study haematopoiesis. This triple marker system has been used to study haematopoietic stem cells by a number of authors (Micklem et al, 1975 I, II; Wallis et al, 1975; Micklem and Ross, 1978; Ross et al, 1982).

Other authors have used different chromosome markers to study haematopoiesis. Brecher and his co-workers have injected male bone marrow cells into female recipients and vice versa. By examining the resultant cell populations for the presence or absence of the Y chromosome, repopulation has been studied in both unirradiated and X-irradiated recipients (Brecher et al, 1981; 1982).

Such methods do give rise to a number of difficulties, however. It is difficult, and very tedious, to look at cell populations of any great size as only dividing cells can be studied using chromosome markers. Therefore the difficulty inherent in using CFU-S of only examining a

small proportion of the whole population is still experienced and could lead to inaccurate estimates of stem cell numbers. In all radiation-induced marker studies only irradiated animals can be studied, while other chromosome marker studies rely on the injection of genotypically variant cells into host animals again causing possible perturbation of the normal events of haematopoiesis.

Chromosome markers can, on the other hand, be used to study individual cells and the T6 marker in particular has proved a useful tool in transplantation studies by allowing the incorporation of three distinct tissue populations in experimental animals to be studied.

Chediak-Higashi Syndrome in man is thought to be a lysosomal disease (White, 1966). The murine equivalent, which is found in Beige mice, results in the appearance of abnormal granules in the polymorpho-nuclear leucocytes (polymorphs) of these animals. When stained with Sudan Black B (Sheehan, 1939; Sheehan and Storey, 1946) Beige polymorphs are clearly distinguishable from their normal equivalents due to the inclusion within the cells of very many fewer granules which are grossly enlarged. Proportions of Beige versus normal cells can easily be counted in stained smears of peripheral blood. Indeed this system was to be incorporated into the present study. However, blastocyst chimaeras (kindly made by Dr John West) were difficult to obtain and two-thirds of those which survived were found to have non-chimaeric blood cells although they were chimaeras by coat-colour marker analysis. The advantages of being able to study normal animals longitudinally by using markers such as Beige polymorph granules are reduced by the technical difficulties involved in blastocyst fusion and embryonic injection techniques.

Studies of parabiosed animals (animals joined naturally or experimentally) can also produce chimaerism within the haematopoietic tissues of the individuals involved. Naturally occurring parabiosis is often found in dizygotic twins who may share a placenta, thus allowing mixing of

the embryonic blood systems. Chimaeric cattle twins from such circumstances have been studied by Stone and his co-workers (Stone et al, 1964; Stone and Cragle, 1964). Animals can be experimentally parabiosed either as embryos (Moore and Owen, 1965) or post-natally (Harris et al, 1964; Kitamura et al, 1979b) using animals with specific cell markers.

In general cell surface markers have so far not proved useful for studying stem cell numbers as none has yet been found which is specific to haematopoietic stem cells. They have, however, been invaluable tools in studying the ontogeny of lymphocytes. Levels of immunoglobulin molecules on the surface of B lymphocytes have been shown by various authors to vary during the process of cell maturation (Rowe et al, 1973; Gelfand et al, 1974; Osmond and Nossal, 1974; Sidman and Unanue, 1975; Scher et al, 1976; Spira et al, 1981). Cell surface receptors for insulin have been suggested as universal markers of T and B lymphocytes (Helderman and Strom, 1978), the density of such receptors varying with the degree of maturation (Spira et al, 1981). Cell surface complement receptors also vary with the maturity of the cells (Gelfand et al, 1974).

X-Chromosome Linked Enzyme Markers

Variant expression of X-chromosome-encoded enzymes provide easily measurable distinct cell populations within heterozygous individuals and have proved useful for studying X-inactivation in early embryos and also in studies of the clonality of populations later in development. To this end scientists have gone to great lengths to find allelic forms of such enzymes. According to Ohno's hypothesis of X-chromosome homology (Ohno, 1967; 1973), loci known to be on the X-chromosome in one mammal are likely to reside on the X-chromosome in others also. This has indeed been found to be the case.

Phosphoglycerate kinase (PGK-1) and glucose-6-phosphate dehydrogenase

(G-6-PD) were shown to be X-linked in the mouse by Kozak and her colleagues (Kozak et al, 1974) by studying the activity of these enzymes in the offspring of XX and XO mothers. A similar approach was also used to prove the X-linkage of these two enzymes plus hypoxanthine-guanine-phosphoribosyl transferase (HGPRT; HPRT) in the Chinese Hamster (Westerwald et al, 1972). Chapman and his colleagues independently proved that the genes which code for the enzymes G-6-PD, HGPRT and PGK are located on the X-chromosome in the mouse by studying the activity of these enzymes in interspecific mouse hybrids (M. musculus x M. caroli and M. musculus x M. dunni), making use of variations in electrophoretic mobility of these enzymes between the species used (Chapman and Shows, 1976; West et al, 1977b; West et al, 1978). The same approach was used to prove the X-linkage of PGK-1 in man-mouse hybrids (Boone and Ruddle, 1969; Khan et al, 1971) and in man-chinese hamster hybrids (Khan et al, 1971).

Kozak and her colleagues (1975) showed that ∝-galactosidase is an X-linked enzyme in the mouse by studying the activity of this enzyme in hybrid somatic cells with an X-autosome translocation. In the same manner (using man-mouse hybrid cells with a similar translocation) PGK-1, G-6-PD and HGPRT have been assigned to the long arm of the X-chromosome (Ricciutti and Ruddle, 1973). Gene mapping studies by Shows and his colleagues (1976) reaffirmed the X-linkage of these three enzymes in man, mouse and muntjac deer.

A number of investigators have studied the X-chromosome linkage of PGK-1 in man by detailed studies of the distribution within the population of individuals suffering from haemolytic anaemia associated with a deficiency of this enzyme in humans (Valentine et al, 1969; Chen et al, 1970; 1971). A similar association of anaemia to G-6-PD deficiency provided further evidence of X-linkage of this enzyme in man (Gandini et al, 1968). Indeed, one individual heterozygous for both PGK-1 and G-6-PD further proved this linkage as non-independent linkage of the two enzymes was found in this person (Gartler et al, 1972).

Although the X-linkage of all these enzymes is well documented, until recently they have not been widely used as investigational tools due to a lack of suitable polymorphisms within experimental animals. For many years the search has gone on to find such variants.

Lusis and West described a polymorphism of α-galactosidase in a feral population of Mus molossinus (Lusis and West, 1976; Adler et al, 1977). Polymorphisms of PGK-1 have been found in marsupials (Cooper et al, 1971; Vandeberg et al, 1973) and of PGK-1 and G-6-PD in humans (Chen et al, 1970; 1971; Chen and Giblett, 1972; Fialkow, 1973). However, until recently, a similar polymorphism had not been found in Mus musculus.

Phosphoglycerate Kinase as a Marker

In 1977, Nielsen and Chapman described an electrophoretic variant of the X-linked enzyme PGK in a small group of feral mice (Mus musculus) trapped in Denmark. The variant (PGK-1A) migrates faster than the allozyme (PGK-1B) found in all other types of Mus musculus so far tested. Mice carrying the marker enzyme were backcrossed onto their existing laboratory stock and since then mice heterozygous for this marker have become quite widely used as experimental tools.

PGK catalyses the glycolytic pathway reaction in which 1, 3-diphosphoglycerate (1, 3-DPGA) is reversibly converted to 3-phosphoglycerate (3-PGA). In the forward reaction energy is generated as adenosine triphosphate (ATP). PGK can therefore be used as a marker system in all metabolically active cells. It should be an excellent marker for studying the red blood cell which, because it has extruded its nucleus during maturation, cannot be studied using conventional chromosome marker techniques. In red blood cells the ATP generated in the conversion of 1, 3 - DPGA to 3 - PGA is used to maintain red cell viability. Thus, PGK deficiency (in humans, at least) is associated with haemolytic anaemia (Valentine et al, 1969; Strauss et al, 1974; Piomelli

and Corash, 1976). It is not yet known if a similar PGK-deficiency-associated anaemia exists in mice.

PGK was first isolated from brewers yeast by Bücher (1955) and has been isolated from mammalian cells by affinity chromatography (Kuntz et al, 1978, Lee, 1982). Bücher and his colleagues have carried out extensive biochemical tests on the murine variant PGK-1A and its normal counterpart PGK-1B and have found them to be virtually identical (Mulbacher et al, 1983).

The studies to be described have made use of the murine polymorphism of PGK-1 as a tool to facilitate the study of cell renewal within the blood forming tissues and the repopulation of these tissues following various stresses to the haematopoietic system. By using an X-linked marker which is present in all metabolically active cells, it is hoped that a clearer view of haematopoiesis in normal animals may be obtained. As animals carrying both PGK-1 allozymes are natural mosaics, there is little difficulty in producing large numbers of experimental animals (the main problem facing workers using man-made chimaeras). Using female mice heterozygous for this marker haematopoiesis can be studied in all cells, not just those in metaphase at the time of sampling, although it is impossible to look at individual cells.

A marker present in red cells, as PGK-1 is, also allows a longitudinal study of haematopoiesis within individual animals to be undertaken. By repeatedly bleeding individuals and comparing the proportions of PGK-1A and PGK-1B in each sample changes in the stem cell population responsible for replenishing the blood may be observed. Such longitudinal studies may also allow questions about events during development, for example the size of the initial pool of cells forming the entire blood-forming tissues, to be answered.

In addition PGK-1 may be used as a transplantation marker in the same

way as the T6 chromosome marker has been used in the past. In this respect PGK-1 may not provide such a useful marker system as only two markers are available as compared to the three T6 markers (two, one or no T6 chromosomes).

The performance of PGK-1 as a marker for haematopoietic studies will be assessed and compared with previous marker studies.

CHAPTER 2 GENERAL MATERIALS AND METHODS

Livestock Maintenance and Phenotyping

All mice used in the experiments to be described were descendents of a stock of mice provided by Dr John West (Sir William Dunn School of Pathology, Oxford). These mice showed the variant (A) type of the X-linked enzyme PGK-1 on a C3H background (West and Chapman, 1978). They were subsequently backcrossed onto our CBA stock background bearing the common PGK-1B allele.

First generation backcross matings were set up as follows:

(a) PGK-1A (C3H) male x CBA (1B) female
(b) CBA (1B) male x PGK-1A (C3H) female

Offspring of (a)-type matings are B males and AB females, and of (b)-type are A males and AB females.

For future generations A(C3H/CBA) males were mated with CBA females, yielding B male and AB female offspring; and CBA males were mated with AB (C3H/CBA) females yielding A male, B male, B female and AB female offspring.

Breeding mice were maintained with one male and one female per cage (size M1, NKP mouse cages) on a diet of Rat and Mouse No 3 Stock Breeding Diet (BP nutrition diet - Special Diet Services) and chlorinated water ad libitum.

All offspring were weaned at 21 days post partum and were typed for PGK-1 phenotype by electrophoresis of blood samples on 12% starch gels (see Appendix 1). Five drops of blood (approximately 150 µl) from each mouse were mixed with two drops of 1% EDTA (Ethylenediaminetetra - acetic

acid - disodium salt, BDH Chemicals Ltd, Poole) in distilled H_2O and were stored at -60°C overnight before a small quantity was applied to starch gels on methyl cellulose applicator strips of appropriate size.

Prior to electrophoresis the gel was removed from its mould, placed on a glass plate and blotted with absorbent tissue. Using a metal comb, a line of slots 4.5 mm long were cut in the gel 2.5 cm from the cathodal end. In this way nineteen slots were cut in each gel. Blood samples were applied to cellulose acetate strips (4.5 x 1.5 mm, Oxoid Ltd), which were then blotted on absorbent tissue before being inserted into the slots. Triple-folded Whatman No 1 filter paper wicks were applied to the gels.

The gels were run at 160 V (constant voltage) for 2½ - 3 hours in tris-citrate buffer (see Appendix 1) in electrophoresis tanks (LKB 2117, Multiphor tanks) cooled with water and ice. Prior to electrophoresis 0.1 mg/ml dithioerythritol (DTE; Sigma D-8255) was added to the buffer. Under these conditions, PGK-1 migrates about 3 cm from the cathodal end towards the anode with PGK-1A travelling further than PGK-1B.

Following electrophoresis, gels were stained using one of two enzyme mixtures (Beutler, 1969; Bucher et al, 1980) in 2% agar at 56°C. For staining mixture details see Appendix 1. After incubation at 37°C for 20 - 30 minutes gels stained using the method of Beutler were viewed under long wavelength ultra-violet illumination. This staining reaction makes use of the reverse reaction pathway shown in figure 2.1 (page 32) Dark bands of NAD (corresponding to the bands of PGK-1 activity) were observed against a fluorescent background of NADH. Alternatively, a second, histochemical staining mixture was added in 2% agar to form a coloured product. Using this stain (see Appendix 1) PGK-1 bands were observed as dark blue bands on a green/blue background. Staining by the method of Bücher et al was carried out as described for staining Cellogels (see below). Double thickness (3 mm) gels, as used in early

experiments, were sliced in half and the cut surfaces were stained. Later gels (1.5 mm thick) were left whole to be stained.

After being assessed for PGK-1 phenotype, mice were transferred to stock cages (size MB1, NKP mouse cages) containing up to twenty animals per cage. Mice from appropriate generations of backcross offspring were used as experimental animals and were caged in conditions similar to those of stock mice. Stock and experimental mice were maintained on a diet of Rat and Mouse no. 1 Standard Maintenance Diet (BP nutrition diet, Special Diet Services) and chlorinated water ad libitum.

Preparation of Experimental Samples

Prepared experimental tissue samples and haemolysates were assayed for PGK-1 phenotype using a modification of the technique of Bücher et al, 1980).

Blood samples (5 drops added to 2 drops 1% EDTA) were diluted 1 in 15 with sample and freezing buffer (see Appendix 1) - 10 µl blood/EDTA + 40 µl sample buffer + 100 µl freezing buffer - and stored at -20°C prior to electrophoresis. Duplicate, undiluted, blood/EDTA mixtures were stored at -60°C.

Tissue samples were treated in one of two ways to lyse contaminating red cells. After obtaining a cell suspension by maceration and sieving of experimental tissues, the following techniques were used.

(a) After washing cells in RPMI-1640 medium (RPMI-Gibco) and spinning at 2000 rpm (650 g) for 10 minutes, cells were resuspended in 4.5% glycerol in RPMI, left at room temperature for 25 minutes, then spun down and resuspended in excess RPMI.

(b) After washing, the cell pellet was resuspended in a very small volume of RPMI. 4.5 ml distilled water was added and the centrifuge

tube shaken vigorously. After ten seconds 0.5 ml 10 times concentrated RPMI was added and mixed rapidly before adding excess RPMI. If necessary the distilled water shock was repeated (Chandler et al, 1979).

Following either procedure the cells were washed once more in RPMI before being resuspended in appropriate volumes of sample buffer (see results) and stored at -60°C.

Electrophoresis of Experimental Samples

Appropriately diluted samples were electrophoresed for 3½ - 4 hours at 200 V (constant voltage) on 16 x 17 cm Cellogel sheets (Whatman Laboratory Sales, Maidstone) at an ambient temperature of 4°C using a bridge distance of 9 cm (Del Campo, 1968; Khan, 1971). Whatman no. 3 chromatography strips (Whatman Laboratory Sales, Maidstone) were used as wicks. Electrophoresis buffer (see Appendix 1) had DTE (Sigma no. D-8255) added at 0.01% and AMP (free acid, Sigma no. A-2127) added to the cathodal buffer at 0.036% before use.

Cellogel electrophoresis strips (Cellogels) were stored at 0 - 4°C in 20% methanol solution. Before use Cellogels were blotted with absorbent tissue to remove excess moisture, soaked in electrophoresis buffer for 10 minutes and then re-blotted. Electrophoresis buffer was pre-run into the Cellogels for 10 minutes at about 200 V (constant current) in home-made electrophoresis tanks (based on the Shandon Universal Electrophoresis Tank, Shandon Scientific Co Ltd) with Whatman no. 3 chromatography paper strips (3 x 17 cm) as wicks.

Samples were applied to the gel on Whatman no. 3 chromatography paper strips (1 x 4 mm). Twelve samples per gel were loaded 1 cm from the cathodal end of the gel, and were run at 4°C for 3½ - 4 hours at 200 V (constant voltage). After 5 - 10 minutes of the run, the sample strips were removed to prevent overloading. PGK-1 migrates towards the anode

under such conditions with PGK-1A travelling about 3 cm and PGK-1B travelling about 0.5 cm less to produce two distinct bands of activity.

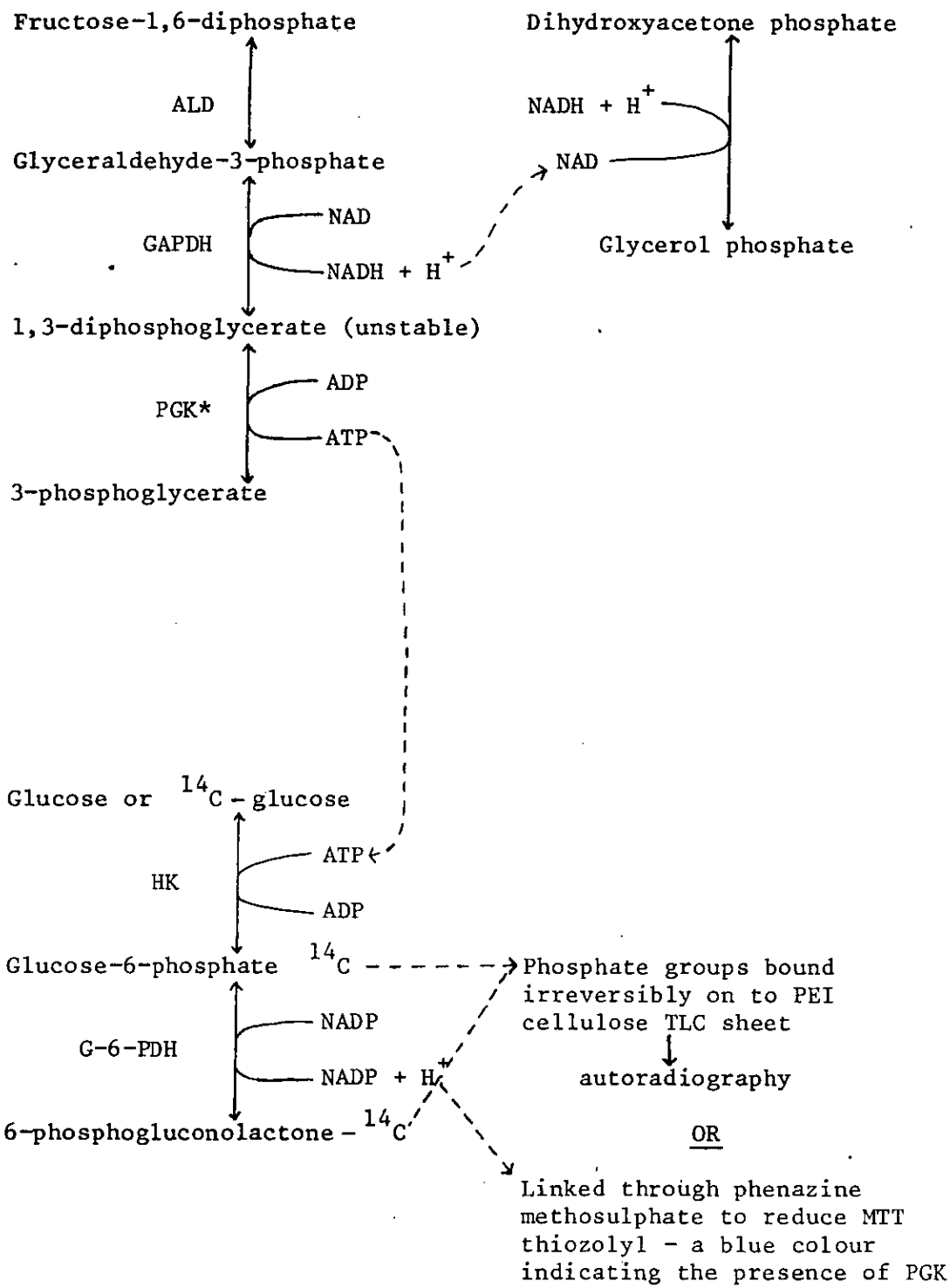
After electrophoresis, gels were cut to 6 x 17 cm, and were exposed to a staining mixture essentially as described by Bucher and his colleagues (Bücher et al, 1980) but with ^{14}C-labelled glucose (Amersham International, specific activity 230 mCi/mmol 8.5 GBq/mmol) added at 15 µl per gel. (See Appendix 1). Polyethyleneimine (PEI) cellulose thin layer chromatography (TLC) sheets (Bakerflex, Linton Products, Hysol, Harlow) were cut to the same size as the gel strips to be stained and were soaked in distilled water for 30 minutes then blotted to remove excess moisture before being used.

The radioactive staining mixture was spread evenly over the absorptive surface of the PEI sheet (using a glass rod spreader) and gels were inverted and carefully placed on the prepared PEI sheets. The staining sandwich was transferred to a 37°C incubator for 15 minutes, after which the PEI sheets were washed in 0.008 M Tris buffer (Trizma 7 - 9, Sigma no. T-1378) for at least 4 hours. The washed PEI sheets were then left to dry at room temperature.

The principle of the staining method is to provide the unstable substrate 1,3-diphosphoglycerate for PGK-1 (see reaction pathway, figure 2.1), and to feed the ATP formed during its conversion to 3-phosphoglycerate into the hexokinase/glucose-6-phosphate dehydrogenase pathway, catalysing the production of ^{14}C-labelled glucose-6-phosphate and 6-phosphogluconolactone which are covalently bound onto the PEI sheets via their phosphate groups.

By adding excess substrate for the reaction to occur the quantities of ^{14}C-labelled glucose-6-phosphate and 6-phosphogluconolactone produced are dependent on the levels of PGK-1 activity within the substrate. As the reaction can only go ahead where there is PGK-1 present and the quantity of end-product is dependent upon the available PGK-1, then

Figure 2.1 Phosphoglycerate Kinase Assay



end products will only be produced over the bands of PGK-1A and -1B, and the quantity of radioactively-labelled end-product bound to the PEI sheets will be directly proportional to the PGK-1 activity. The ^{14}C-activity over each band of PGK-1 was found to be proportional to the enzyme activity present by staining duplicate gels with MTT/PMS or ^{14}C-glucose.

The ^{14}C-labelled staining method is particularly useful as the reaction can be stopped quickly by removal of the gel substrate and washing of the PEI sheet, thus preventing dispersion of the end-products throughout the gel. This dispersion proved to be a major obstacle to using MTT/PMS stained cellogels for quantitative work as the slow fixation method (see Appendix 1) allowed dispersal of stain and loss of sharpness of bands. The fixing and drying methods also bleached out a large proportion of the stain. These difficulties introduced large inaccuracies into any quantitative analysis carried out on these gels.

Autoradiographs were prepared on X-ray (Kodak XS) film and were developed after 3 days exposure to the radioactive PEI sheet at room temperature. X-ray films were developed for 5 minutes in Kodak DX 80 developer (Kodak Ltd) and fixed for 5 minutes in Kodak FX 40 X-ray liquid fixer (Kodak Ltd). After washing in running water for one hour, the film was rinsed in distilled water and allowed to dry at room temperature.

Autoradiographs were scanned by transmitted light using a 3 mm slit on a Joyce-Loebl semi-automated gel scanner (Joyce-Loebl, Vickers Instruments, Gateshead, England) using wedges B915 or D1510. Each track was carefully marked to ensure that when scanned the slit would pass over the PGK-1 bands perpendicular to them. The area under each peak was calculated by dropping a perpendicular from the lowest point between the two peaks to the baseline (figure 2.2) then calculating the area under each peak using a computerised integrator (Olivetti P040 minicomputer). A typical gel and scan are shown in figure 2.2.

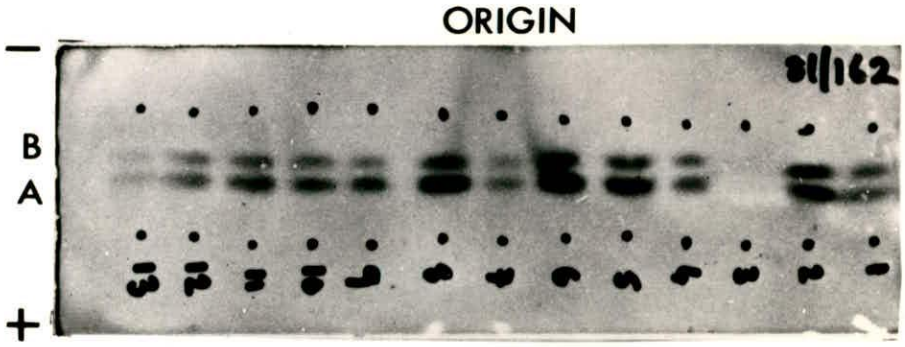
facing page 34

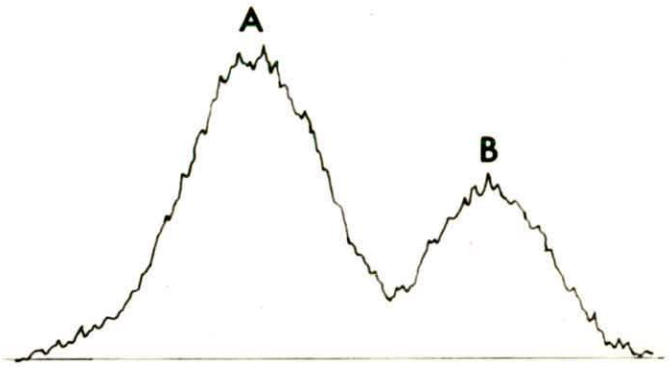
Figure 2.2 A Typical Cellogel Autoradiograph

Top:- a specimen autoradiograph

Bottom:- a typical scan made on the Joyce-Loebl scanner

Specimen Autoradiograph





Scan of track 5

The figures thus obtained were corrected to allow for the log-linear response of the photographic emulsion. A series of gels was run using known mixtures of A and B enzyme. Measurements of each variant after radiography were found to differ from the known values of each variant but were found to be related semi-logarithmically to the real values (figure 2.3). From this data a table of observed-real values was drawn up and was used to correct all experimental results obtained from autoradiographs (table 2.1).

Towards the end of the period of study a series of samples was run using the Helena Zip-Zone electrophoresis system (Helena Laboratories, Beaumont, Texas). Prior to electrophoresis Helena gels (Titan 111 Zip-Zone cellulose acetate plates, 94 x 76 mm; Helena Laboratories) were soaked in electrophoresis buffer (see Appendix 1) for twenty minutes. AMP (25 mg/100 ml) was added to electrophoresis buffer before use. To enhance cooling of the gel during electrophoresis two coolant sponges (Helena Laboratories) were placed in the centre of the electrophoresis tank.

8 µl of appropriately diluted sample (prepared as for Cellogel electrophoresis) was added to each well of a 12-well Super Z well plate (Helena Laboratories). The applicator (Super Z applicator, 12 sample; Helena Laboratories) was wetted in detergent (Zip Zone Prep, 10% W/V Sodium Lauryl Sulphate; Helena Laboratories) then rinsed well in distilled water and blotted on absorbent tissue. The gel was firmly blotted with absorbent tissue then placed on a Super Z CPK aligning base (Helena Laboratories) in the correct position for cathodal application. The applicator was wetted ten times with sample then blotted and rewetted ten times with sample. The applicator was transferred to the gel where the samples were automatically applied to the gel by pressing firmly on the applicator for ten seconds.

Disposable wicks for use in Zip Zone chambers were placed on the gel supports in the electrophoresis chamber (Zip Zone electrophoresis

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Figure 2.3 Cellogel Correction Curve

Data obtained from cellogels (Y-axis) are plotted against the known phenotypes of standard dilutions (X-axis).

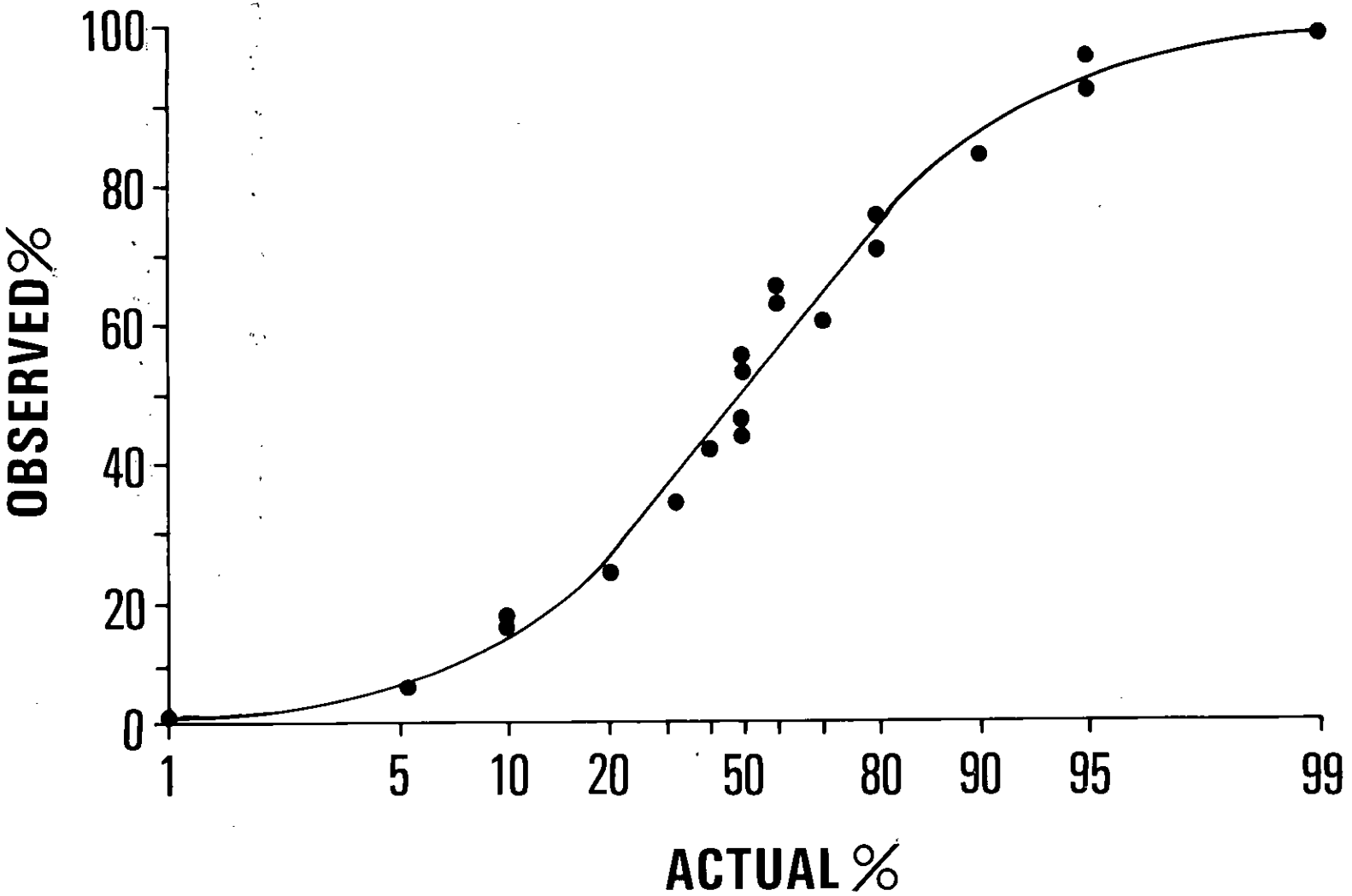
100
80
60
40
20
0
OBSERVED%
1
5
10
20
50
80
90
95
99
ACTUAL %

TABLE 2.1

Correction Table for PGK-1 Estimations

OBSERVED	REAL	OBSERVED	REAL	OBSERVED	REAL	OBSERVED	REAL
1	1	26	19	51	51	76	82
2	2	27	20	52	53	77	83
3	2	28	21	53	55	78	84
4	3	29	22	54	57	79	85
5	3	30	23	55	58	80	86
6	4	31	24	56	60	81	87
7	4	32	25	57	61	82	88
8	5	33	26	58	63	83	89
9	6	34	27	59	64	84	89
10	6	35	28	60	65	85	90
11	7	36	29	61	67	86	91
12	8	37	30	62	68	87	92
13	9	38	31	63	69	88	93
14	9	39	33	64	71	89	93
15	10	40	34	65	72	90	94
16	11	41	36	66	73	91	94
17	11	42	37	67	74	92	95
18	12	43	39	68	75	93	96
19	13	44	40	69	76	94	96
20	14	45	42	70	77	95	97
21	14	46	43	71	78	96	97
22	15	47	45	72	79	97	98
23	16	48	47	73	80	98	98
24	17	49	49	74	81	99	99
25	18	50	50	75	81	100	100

chamber, Helena Laboratories). With the line of samples at the cathodal end the gel was inverted before being placed in the chamber and pressed firmly at the edges to ensure contact with the wicks. A two pence coin was placed in the centre of each gel to weight the gel and ensure continued contact with the wicks during electrophoresis. Gels were run at 250 V (constant voltage) for 45 minutes in a refrigerator. To prevent overheating during electrophoresis the current was not allowed to rise above 20 mA per gel - if necessary the voltage being reduced slightly during the run.

Following electrophoresis gels were stained as for cellogels using MTT/PMS staining mixture (see Appendix 1). Gels were scanned wet either while the staining reaction proceeded or after being fixed (see Appendix 1). All Helena gels were scanned using an automated gel scanner (Joyce-Loebl Chromoscan 3, Vickers Ltd, Gateshead). Gels were scanned by reflected light using a 3 mm slit and a green filter (530 nm, 1-FII-2134). The gels were realigned before each track was scanned to ensure that the slit would pass over the PGK-1 bands perpendicular to them. No corrections were made for values of PGK-1 obtained using this method. A typical Helena gel and scan are shown in figure 2.4.

Results for repeated samples of known PGK-1 phenotype run on Helena gels are shown in table 2.2.

In the experiments to be described using Helena gels, no linearity correction, comparable to that made when Cellogels were used, was made.

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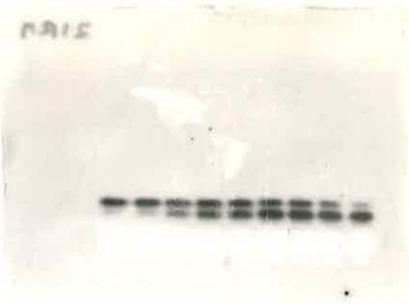
Figure 2.4 A Typical Helena Gel

Top:- a typical MTT/PMS stained Helena gel

Bottom:- a typical scan produced on the Joyce-Loebl Chromoscan 3 automated scanner.



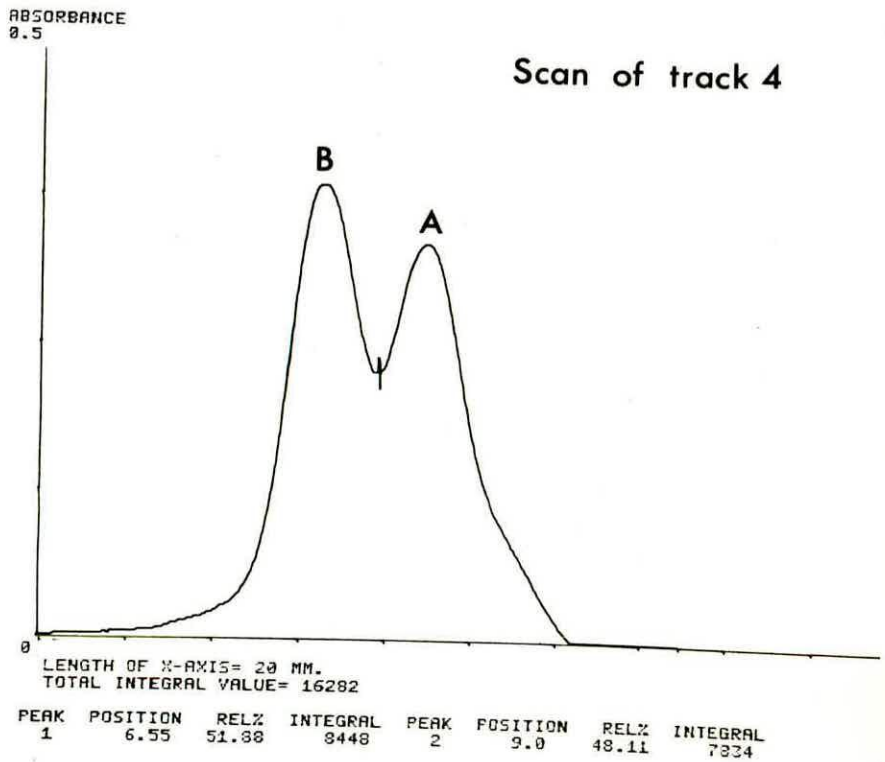

ABSORBANCE
0.5
Scan of track 4
B
A
0
LENGTH OF X-AXIS= 20 MM.
TOTAL INTEGRAL VALUE= 16282
PEAK POSITION RELX INTEGRAL PEAK POSITION RELX INTEGRAL
1 6.55 51.88 8448 2 9.0 48.11 7834

Table 2.2 Linearity of Samples using Helena Gels

Actual % PGK-1A	Calculated mean % PGK-1A
0	0
1	*
5	*
10	13
20	22
30	32
40	38
50	46
60	58
70	73
80	83
90	88
95	*
99	*
100	100

* In these samples the minor PGK-1 band could not be successfully separated from the major band.

Enumeration of Clone Numbers

In most of the experiments to be reported estimates were made of the number of stem cells (or clones of cells) seeding the haematopoietic system at any given time. Estimates were made by applying the binomial theorem to either a series of samples from an individual, or a series of comparable samples within a population. Calculations were made using the equation

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

where n = number of active stem cells
p = mean proportion of PGK-1A
$1-p$ = mean proportion of PGK-1B
s^2 = the variance of PGK-1A within the series of samples being tested.

Repeatability tests were carried out in which replicate samples of both known and unknown mixtures of PGK-1A and-1B were electrophoresed on up to six different occasions (17 samples over a total of 61 electrophoresis runs on cellogels). As a result a mean standard deviation of 0.026 (2.6%) and a mean variance of 0.000676 was found within replicate samples. Therefore, when clone numbers were being calculated, a second calculation was made using a corrected variance (observed variance - mean variance due to experimental error, M. Stone, 1983). When small clone numbers were being considered (i.e. when large variances were involved) the corrected value of n varied very little, if at all, from the original value. The corrected value of n became increasingly different from the original value as the method approached the limits of its usefulness - as the variance found between samples approached the value of the variance found between duplicate samples.

Duplicate electrophoresis of 45 samples on Helena gels revealed a mean

standard deviation of 0.035 (3.5%) and a mean variance of 0.001225 in duplicate samples.

All results obtained by integration of areas under curves were rounded to the nearest whole number (percent), as were all numbers in the correction table for evaluation of samples run on Cellogels. The accuracy of the correction table itself is also limited by the accuracy of the prepared samples of standard proportions of PGK-1A and-1B. The standard samples used throughout were measured using Gilson Pipetman adjustable pipettes, p20, p200 and p1000.

It should be noted that although the utmost care was taken to produce accurate results, the values presented for clone numbers should be regarded as estimates only of the minimum number of active stem cells and not in absolute terms.

Irradiation and Repopulation Protocol

For three series of experiments recipient mice were lethally irradiated and subsequently rescued by injection of appropriate donor bone marrow cells. In each experiment the following general procedures were followed.

Recipient mice were irradiated with 1050 rads (10.5 Gy., dose rate = 34.3 rads per minute +/- 3.4% from a ^{137} caesium source). Twenty mice were irradiated at a time in a special jig comprising a ventilated round plastic container fitted with a perspex restrainer 2.5 cm from the floor to immobilise the animals.

Long bones were dissected out of donor mice and the bone marrow was flushed out using a 23G or 25G needle and 1 ml Hanks Buffered Saline Solution (HBSS, Gibco). The bone marrow cells were washed twice in HBSS and spun down at 2000 rpm (650 g) for 10 minutes. Contaminating red cells were lysed as described previously, the cells were then

washed for a final time and resuspended in HBSS. Cells were counted using an Improved Neubauer haemocytometer and cell suspensions were then diluted to the desired concentration. Donor bone marrow cells were injected intravenously into the previously irradiated recipients within seven hours from irradiation.

Statistical Analysis of Hydroxyurea and Repopulation Data

All data were initially presented as PGK-1A activity as a percentage of total PGK-1 activity against day after irradiation or injection. The following parameters were analysed statistically. In repopulation experiments the left and right femora and left and right thymic lobes were treated as single variates. In hydroxyurea-treated animals left and right femora, left and right humeri and left and right thymic lobes were analysed as single variates. In each case differences between right and left organs were also treated as single variates.

Using the original data, the arithmetic mean was calculated for each variable at each separate time.

The data were analysed to see if there were any significant trends in PGK-1A with time. In irradiated and repopulated animals data from PGK-1AB repopulated animals were compared to data from the control group repopulated with PGK-1A cells only to ensure that any trends found in the experimental (PGK-1AB-repopulated) animals did not merely reflect the rate of repopulation in a particular tissue. Differences between left and right organs were also analysed with respect to time to see if any significant changes occurred with time. The time trends for each variable were investigated by fitting of cubic polynomials, and the contributions to the variation by the linear, quadrate and cubic elements investigated by analysis of variance. The data were transformed to angles by $\sin^{-1}\sqrt{pc/100}$ in order to homogenise between group variances.

All data were analysed by Genstat on the ICL 2980 at the Edinburgh Regional Computing Centre. Statistical analyses were kindly carried out by Peter Teague of the Medical Research Council's Clinical and Population Cytogenetics Unit, Western General Hospital, Crewe Road, Edinburgh.

Particular techniques relevant to individual experiments are described in the relevant results chapters.

CHAPTER 3 ANALYSIS OF VARIATION OF PGK-1A AND -1B IN NORMAL HETEROZYGOUS FEMALES

Until recently it has been virtually impossible to study haematopoiesis in normal individuals. The naturally occurring polymorphism of the X-linked enzyme G-6-PD has proved a useful tool in studies of stem cell numbers in humans (Fialkow, 1973). Such studies in humans are, however, hampered by the rarity of subjects suitable for study, with most subjects presenting with medical disorders.

Stem cell studies in experimental animals have generally been undertaken by introducing cell markers into the subjects being studied. Chromosomes with random aberrations have been produced by sub-lethal irradiation (Becker et al., 1963). More recent investigators have, however, introduced cells containing known chromosome abberations into animals whose own haematopoietic systems have been ablated by lethal irradiation (Micklem et al., 1975, I and II; Wallis et al., 1975; Micklem and Ross, 1978; Ross et al., 1982). Other authors have studied haematopoiesis by injecting cells with known chromosome markers into irradiated recipients (Brecher et al., 1981; 1982). Experimental parabionts have also been used to study haematopoiesis (Harris et al., 1964; Moore and Owen, 1965; Kitamura et al., 1979).

All the above methods of studying haematopoiesis in experimental animals involve perturbation of the haematopoietic system of the animal being studied, essentially making it impossible to study normal haematopoiesis.

The discovery of a polymorphism of the X-linked enzyme PGK-1 in mice (Nielsen and Chapman, 1977) has allowed investigation of haematopoiesis in normal mice to be undertaken for the first time. In order to interpret the events of haematopoiesis in experimentally compromised animals and during neoplastic change an understanding of the events of normal haematopoiesis is necessary. Haematopoiesis in normal animals was

studied by serially-bleeding groups of mice heterozygous for PGK-1.

Serial Blood Samples from Normal Heterozygous Mice

A long-term study of PGK-1 variations in heterozygous mice was set up with the intention of ascertaining the background of natural variation within animals, upon which to base future experimental results. Fifteen PGK-1AB female mice (C3H x CBA backcross generations 3 and 4) were used for the study. The animals were divided into 5 groups of 3, according to age (3 to 7 months at the time of the first bleed) and were bled at 14 day intervals for 112 days and thereafter at 28 day intervals for up to 672 days from the start of the experiment.

At each interval 5 drops of blood were removed and added to 2 drops of 1% EDTA (this bleeding regime was used for all experimental animals to maintain a standard quantity). This degree of blood loss did not cause any prolonged anaemia as measured by packed red cell volumes (see figure 5.3 on page 108). Undiluted samples were stored at -60°C while diluted samples (1:4:10 blood:sample buffer: freezing buffer) were stored at -20°C prior to electrophoresis.

Whole blood samples were electrophoresed and gels treated using the standard methods described in the previous chapter.

Repopulation studies using PGK-1 as a marker show the lifespan of erythrocytes to be between 42 and 56 days (see figure 4.1, page 84) which is in accord with the previously published figure of 45 days (Russell and Bernstein, 1966). As a result of this finding, only bleeds 56 days apart were used for statistical analysis to ensure that the cell samples were independent. Of the 15 mice in the experiment, 3 were drowned in a watering system accident after only 9 bleeds (112 days). As there were insufficient data from the drowned animals to allow reliable statistical testing, these

animals are not included in the results shown below. In addition two further mice were found to be exclusively of phenotype B over the first nine bleeds and so were excluded from later sampling.

If a large number of stem cells are active at any point in time, little or no variation around the expected mean proportions of PGK-1A and -1B should be seen. If only small numbers of stem cells are involved in replenishing the haematopoietic tissues, then fluctuations in the levels of PGK-1A and -1B would be expected. The size of such fluctuations should allow estimates to be made of the average numbers of stem cells seeding the haematopoietic system at any one time. The frequency with which such fluctuations occur should also allow estimations of the frequency, if any, of stem cell turnover.

Initially it was decided that differences found between duplicate runs of individual samples were small enough to allow experimental samples to be run only once. At that stage preliminary data from these mice were presented elsewhere (Burton et al., 1982). As the study progressed, however, it became apparent that the large changes in PGK-1 phenotype found at early sample times were no longer occurring. As a result the entire series of samples was repeated on cellogels and verified using Helena gels. A small number of samples were repeated using only one of the gel systems and these are appropriately marked in Appendix 2, Table 1. Most of the repeated samples had results within the range for duplicate runs, and therefore within the variance deducted from the total variance as a correction factor (see Materials and Methods, page 41). As a result only one set of data is presented here. For repeated samples with differences greater than that expected for duplicates, the range of values obtained is shown in the appropriate tables in Appendix 2. Verification of cellogel data using Helena gels allows comparisons to be made between these experiments and those reported later on mice treated with the drug phenylhydrazine (carried out

using Helena gels only).

Although considerable changes in the proportions of PGK-1A and -1B are still seen in the repeated series, these are much smaller than those initially found. Estimates of active stem cell numbers are therefore correspondingly greater than those previously reported.

Results from the ten mice are shown in figure 3.1 (full details for individual mice can be found in Appendix 2, table 1). From figure 3.1 it can be seen that the proportions of PGK-1A and -1B in each individual are constantly changing around a mean value, and that changes of as much as 20% in PGK-1 phenotype can occur in as little as fourteen days. From these points we can deduce that the PGK-1 phenotype of erythroid progenitors fluctuates. There are two possible explanations for such fluctuations. First, the fluctuations may be caused by the erythron being maintained by a small number of erythroid progenitors (BFU-e). In the mouse, however, approximately 5×10^8 erythrocytes are produced each day and it has been estimated that there are only 12 - 14 divisions between BFU-e and red blood cells (Testa, 1979). If this is true then of the order of 10^4 BFU-e/day are needed to maintain erythropoiesis. The observed changes in phenotype cannot, therefore, be caused by small BFU-e numbers. The second, and most probable, explanation is that the phenotype and therefore the identity of the clones from which the BFU-e are drawn changes with time. The second explanation suggests that clonal succession is acting in erythropoiesis.

Clonal succession may be acting in one of two ways. The haematopoietic system appears to be replenished either by a relatively small pool of stem cells which are switched into and out of production at random, or by a large pool of stem cells from which a small number of cells are selected to be switched into production for a relatively short period of time, after which they are either switched off and enter a "rest" period or lost to the pluripotent stem cell pool by

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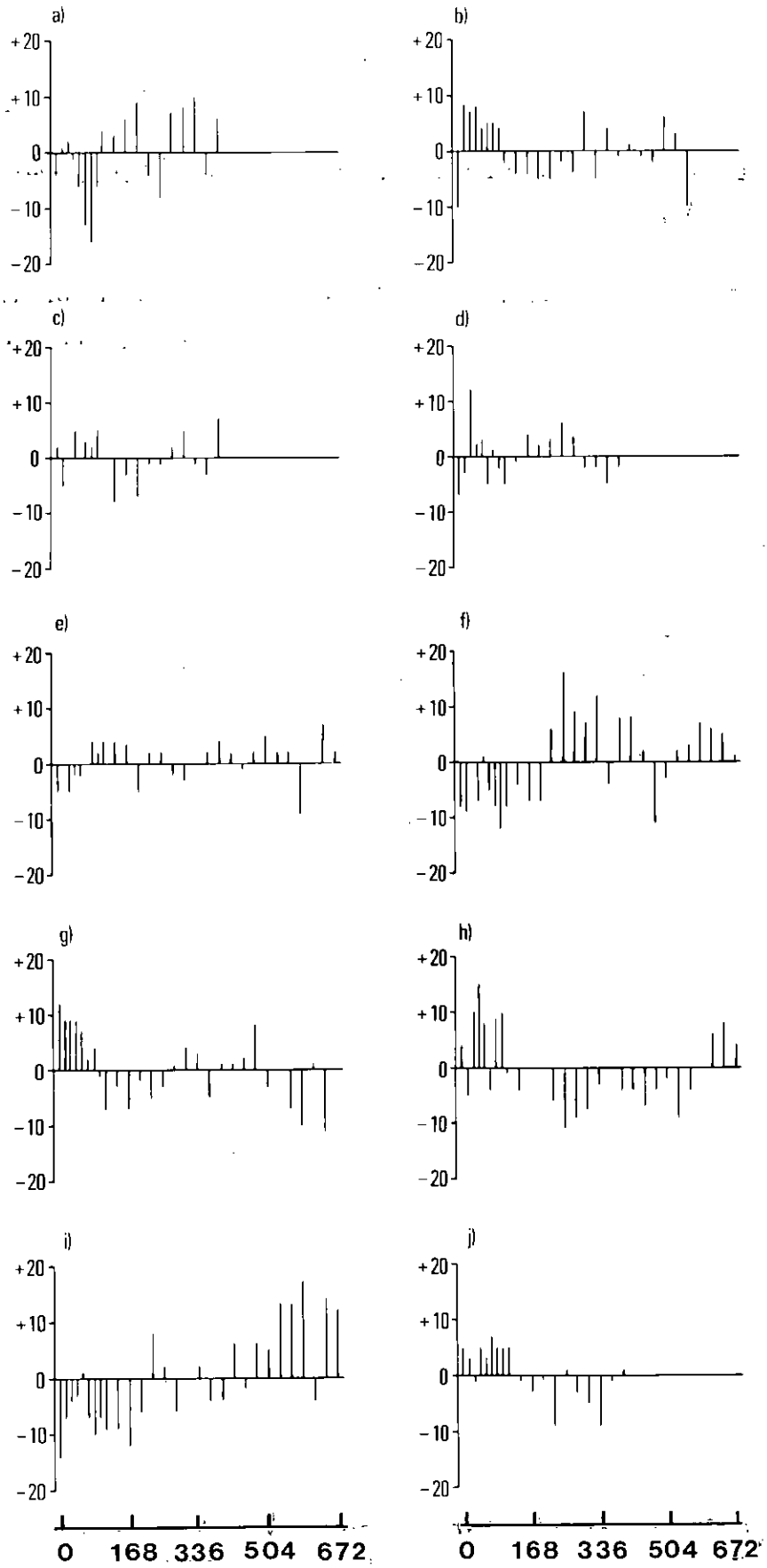
Figure 3.1 Variations in Blood PGK-1 Phenotype in Heterozygous Individuals

The y-axis shows variation (in % PGK-1A) from the mean of all bleeds. (Mean = 0). Individual bleeds are represented along the x-axis.

(a) Mouse 1, mean = 71
(b) Mouse 2, mean = 83
(c) Mouse 3, mean = 87
(d) Mouse 4, mean = 70
(e) Mouse 5, mean = 86
(f) Mouse 6, mean = 72
(g) Mouse 7, mean = 74
(h) Mouse 8, mean = 78
(i) Mouse 9, mean = 67
(j) Mouse 10, mean = 48


a)
b)
c)
d)
e)
f)
g)
h)
i)
j)
+20
+10
0
-10
-20
0
168
336
504
672
Days
Days

differentiation or exhaustion.

The average number of stem cells active at any time was calculated using the binomial formula

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

as described in Chapter 2. The calculated figures are shown in table 3.1. "Corrected n" figures are derived by subtracting the mean variance in duplicate samples (0.000676) from the variance calculated for individual series.

These numbers are 3 - 4 orders of magnitude smaller than those quoted for CFU-S numbers in the haematopoietic system (Smith et al., 1968; Rencricca et al., 1970). Many CFU-S are known to be in G_o or in very slow cycle, however, and are presumably not contributing to haematopoiesis. If one assumes the fraction of CFU-S in rapid cell cycle to be 10% of the total, the difference is only 2 - 3 orders of magnitude, or about 6 - 10 doublings.

If the number of clones present in a blood sample is representative of the number of stem cells responsible for replenishing the blood system at that time, then these figures suggest that at least some CFU-S are not stem cells, but are in fact members of clones derived from the stem cells. These clones are probably used sequentially to maintain haematopoiesis. In figure 3.1 an almost sinusoidal pattern is apparent in some of the animals. One would expect to find some evidence of gradual shifts from A to B clones and back again, resulting in waves around the mean, if new clones are sequentially activated. As each new clone is likely to be switched into production randomly there is no guarantee that an A clone will be followed by a B, and vice versa, so it is unlikely that a sine

Table 3.1 Serial Bleeds of Normal Heterozygous Mice

Calculation of n, the average number of clones present in a blood sample (assuming all clones are of equal size).

Mouse	n (to nearest integer)	corrected n
1	54	67
2	64	87
3	111	373
4	120	194
5	133	676
6	44	52
7	54	66
8	60	79
9	30	33
10	72	89

wave pattern will persist throughout the test period. In addition, it is likely that individual cells will have unequal repopulating potential, giving rise to variously sized clones. The resulting asynchrony of clone renewal will further break down expected patterns in individuals. As can be seen in figure 3.1, this is indeed what happens, with the wave form only being apparent at times and then only in some individuals.

Studies involving sequential samples from individual animals have been difficult to attempt in the past due to a lack of suitable markers. Variations within individual mice, and within groups of mice, heterozygous for PGK-1 have, however, previously been found by West and Chapman (1978). These authors analysed PGK-1 expression within groups of up to 10 mice over a period of 210 days. They tested blood samples using starch gel electrophoresis and scored them for PGK-1 phenotype according to a five point scale (1 = no PGK-1A; 2 = PGK-1A $\ll$ B; 3 = PGK-1A = B; 4 = PGK-1A $\gg$ B; 5 = only PGK-1A; West et al., 1977). Even using this scale these authors were able to see marked fluctuations in the proportions of the two erythrocyte populations in individual mice. The most extreme change in phenotype was found in a mouse bled prior to dissection and 6 days previously. During these 6 days the PGK score changed from 2 to 4, which the authors suggested represented a minimum increase from 40% to 60% PGK-1A.

Although the proportions of the two isozyme bands were only measured by eye in this experiment, the changes observed within individuals appear to be of a similar size and frequency to those observed in the present study. West and Chapman (1978) stated that these changes might reflect the mitotic activity of only a proportion of the haematopoietic stem cells during the intervals between bleeds. Repeated sampling of several animals over a long period of time, as in the present study, suggests that only a fraction of the haematopoietic stem cells are dividing and giving rise to differentiated progeny

at any time.

The mean proportions of PGK-1A:1B are not 1:1 as might be expected on the hypothesis of random X-inactivation (Lyon, 1961), but are closer to 7:3 both in individual animals (see figure 3.1 and Appendix 2, table 1) and within the population in general (table 3.2). This is, in fact, the expected ratio of PGK-1A:1B which is caused by non-random X-chromosome activity as described by Cattanach and his colleagues (Johnston and Cattanach, 1981; Cattanach and Papworth, 1981).

The Pgk-1 locus in the mouse lies on the X-chromosome close to the loci for the coat colour markers Ta and Mo, and also close to the region of crossover suppression caused by Cattanach's translocation (Cattanach and Papworth, 1981). By carrying out linkage tests with Ta^{vbr} and Ta these authors were able to show that the Xce locus lies very close to Ta and Pgk-1. The non-randomness of X-chromosome activity in the somatic cells of females caused by Xce derives from a primary non-randomness of the X-inactivation process itself (Johnston and Cattanach, 1981). Three alleles of the Xce locus are now known, Xce^a, Xce^b and Xce^c. Appropriate X-chromosomal marker genes (Cattanach, Pollard and Perez, 1969; Johnston and Cattanach, 1981) can be observed in heterozygotes (e.g. Xce^a/Xce^b, Xce^b/Xce^c). Cells with an active Xce^bX tend to predominate over those with an active Xce^aX, and those with an active Xce^cX tend to predominate over those with an active Xce^bX. The Xce^c allele was found in the group of feral mice carrying the $Pgk\text{-}1^a$ allele (Nielsen and Chapman, 1977). Mice of PGK-1A genotype therefore also have the Xce^c allele. Because of the proximity of the two loci it is very unlikely that any crossovers will occur between them during meiosis. As a result, PGK-1A will tend to predominate over PGK-1B in PGK-1AB heterozygotes, as the X-chromosome carrying the $Pgk\text{-}1^a$ and Xce^c alleles will tend to remain active in the majority of cells.

Mouse 10 (figure 3.1 j), with an overall mean percentage of PGK-1A of 48% (47% when calculated from 56 day interval bleeds), is an exception to the expected Xce allele activity. This mouse may be a rather "low" normal individual going through a phase of cell replenishment from predominantly B clones during the experiment. However it is unlikely that B clones would predominate over such a long period.

Two other explanations can be given for the consistently low relative levels of PGK-1A in mouse 10. They could be due to a crossover event taking place during meiosis at a point on the X-chromosome between the Pgk-1 and Xce loci. The frequency of crossovers in this position has not been calculated but, due to the closeness of the two loci, is probably an extremely rare event.

Another explanation for the low levels of PGK-1A found in the blood of mouse 10, is that the overall phenotype of the blood-forming tissues may be directly dependent on the number and phenotype of the cells forming these tissues during embryogenesis. If small numbers of cells are seeding individual tissues it is likely that tissue phenotypes will show some variation. On the other hand, if large numbers of precursor cells were responsible for tissue formation one would expect to see very little variation in PGK-1 ratios from tissue to tissue. Such an explanation, however, depends on the relationship between X-inactivation and tissue formation in the embryo. Considerable clonal expansion may occur after X-inactivation and cell mixing might also take place before tissue formation commences. In addition, measurement of primordial stem cell pool sizes for different tissues may well determine the number of coherent clones present at the time of tissue differentiation rather than the actual cell number. Ignorance about the relationship between X-inactivation and tissue formation compounds theories on the importance of patch size (i.e. the number and spatial distribution of cells which give rise to an organ or tissue) at the time

of tissue formation. Previous estimates of primordial cell pool sizes (Nesbitt, 1971; McMahon, Ph.D Thesis, London) suggest that tissue formation commences soon after X-inactivation, and that small numbers of cells are responsible for laying down the tissues.

Unfortunately phenotypic analysis of various tissues in mouse 10 gave equivocal results (see table 3.3, page 61). Only in the right lobe of thymus was the PGK-1A activity substantially greater than 50%, and in two tissues it was substantially less than 50%. The mean phenotype of the animal, from all the tissues studied, was 47% PGK-1A. This is consistent with a single crossover event during meiosis in a parent or grandparent, which would lead to a ratio of 1:1 (PGK-1A:1B). The variety of phenotypes throughout the tissues are, however, consistent with what would be expected if all tissues arise from small precursor pools.

Several workers have used naturally occurring chimaeras or tetra-parental mice to estimate primordial pool sizes in mice and humans. Nesbitt (1971) used 5-7 day old female mice heterozygous for Cattanach's translocation. At this age Cattanach's heterozygotes can be identified because of the spotty pigmentation of the skin which results from X-inactivation (Ohno and Cattanach, 1962). By scoring S-phase cells as containing an inactive X^n or X^t chromosome (Nesbitt and Gartler, 1970) Nesbitt estimated the number of cells making up the embryo at the time of X-inactivation and also the precursor cell pool size for a variety of tissues (lung, abdominal fascia, spleen, thymus and melanocytes). Assuming that the observed covariances among the various tissues arose from random X-inactivation occurring in a few cells ancestral to all tissues Nesbitt estimated that at least 13, and probably 21, embryoblast cells are present at the time of inactivation.

McMahon (Ph.D Thesis, London) used 12½ day post-coitum female foetuses heterozygous for PGK-1 to make similar estimates. Using

samples of yolk sac endoderm, yolk sac mesoderm, neural ectoderm, heart (mesoderm) and liver (endoderm), he used the covariance among tissues and the independent variance of the tissues within individuals to estimate the number of cells represented in the common pool from which the embryo is formed after X-inactivation and the number of cells sampled at tissue formation. These he estimated to be 47 and 193 respectively.

Wegmann and Gilman (1970) used tetraparental mice with genetic markers for $7SG_{2a}$-globulin, the beta chain of haemoglobin and coat colour to estimate the primordial cell pool for the haematopoietic and coat-colour determining systems in mice. They estimated that not less than 5 cells were responsible for the formation of these systems, and that they probably shared a common precursor pool. If this is the case though, the cells seeding the different systems must have diverged at some point during development as these authors found large shifts in the proportions of marker haemoglobin in some animals with no concurrent change in globulin markers. Similar findings have been reported in chimaeric cattle twins (Stone et al., 1964). Nesbitt (1971) estimated the following precursor cell pools for various tissues in mice: lung 41 and 43, abdominal fascia 58, spleen 21, thymus 23 and melanocytes 22. Fialkow also studied precursor pool sizes in humans heterozygous for the X-linked enzyme G-6-PD (Gd^B/Gd^A, Fialkow, 1973). He estimated that the number of cells responsible for the formation of the erythrocyte pool is 13, for granulocytes 16, for blood lymphocytes 19, for lymph nodes 10, for skin 15 and for skeletal muscle 10. His estimates for erythrocytes are comparable to those obtained by other workers using female humans heterozygous for G-6-PD. Nance (1964) estimated a precursor pool of 10 - 18 cells and Gandini and his colleagues (1968) suggested a precursor pool of less than 8 cells for erythrocytes.

In the present study the precursor pool size for blood cells was

estimated by calculating the variance between blood samples taken from all the animals tested on each day. Results are shown in table 3.2.

As can be seen from table 3.2, precursor pool size estimates were only made from bleeds in which all ten mice were still alive. The smaller numbers of mice bled at later dates would lead to less accurate estimations of cell numbers. The results shown agree well with the estimates of precursor cell pool size made by Wegmann and Gilman (1970) in the mouse and by Nance (1964), Gandini et al., (1968) and Fialkow (1973) in humans. Unfortunately, although Nesbitt (1971) presented extensive data, she did not analyse blood cell samples. Her estimation of spleen precursor pool size (the spleen samples will contain large numbers of blood cells) is, however, also in agreement with the data presented here. The limitations of a small breeding colony precluded the collection of tissue samples from a large number of mice heterozygous for PGK-1 to compare precursor pool sizes for a variety of tissues with those presented by Nesbitt.

From the data presented here is appears that following X-inactivation in the foetus a small number of cells (about 10 - 20) are stimulated in some way to differentiate and expand to form the haematopoietic system. This expansion results in an excess of stem cells (assayed as CFU-S), many of which remain quiescent most of the time, as judged by the large proportion of non-cycling or slowly cycling stem cells found in normal haematopoietic tissues (Lajtha et al., 1962; Schofield, 1979). It is impossible at this stage to say whether normal haematopoiesis is being maintained by CFU-S. Although some CFU-S are able to self-renew and to differentiate, it is probable that not all are stem cells. It is possible that an even more primitive cell than the CFU-S is the true stem cell.

Table 3.2 Precursor Pool Sizes for Haematopoietic Tissues

Bleed Number	Mean % PGK-1A ± s.d.	Number of precursor cells
1	71.1 ± 13.1	12
2	72.8 ± 12.7	12
3	76.6 ± 13.3	10
4	76.8 ± 13.8	9
5	75.5 ± 11.8	13
6	71.9 ± 12.9	12
7	73.2 ± 15.1	9
8	73.4 ± 14.3	10
9	71.7 ± 12.3	13
10	70.9 ± 11.9	15
11	71.3 ± 13.5	11
12	71.4 ± 10.9	17
13	72.5 ± 13.6	11
14	73.8 ± 12.6	12
15	73.4 ± 12.7	12
16	75.0 ± 14.0	10
17	74.3 ± 13.9	9
18	71.6 ± 12.8	12
19	75.0 ± 13.1	11

Bone Marrow Repopulation Studies using Marrow from Mice of Known PGK-1 Phenotype

After normal heterozygous mice had been bled 19 times it was thought that a fair estimation of the overall blood phenotype of the initial series of heterozygotes could be made. Four of these animals were killed and the spleen, left and right thymic lobes, and pooled brachial and axillary lymph nodes removed and tested for PGK-1 phenotype. Results are shown in table 3.3.

In general estimates of tissue phenotypes within individuals were similar (with the exception of 3 thymus samples). This broad similarity among tissues was also found by Nesbitt (1971) using Cattanach's translocation as a marker and by West and Chapman (1978) using the PGK-1A marker.

Mice 1, 3, 4, and 10 had all long bones removed and the marrow was flushed out using a 23G or 25G needle and 1 ml Hanks Buffered Saline Solution (HBSS, Gibco). These cells were spun down at 2000 rpm (650 g) for 10 minutes and contaminating erythrocytes were lysed as described previously. Cells were then rewashed and counted (using an Improved Neubauer haemocytometer) and diluted to a final concentration of 2×10^7 cells per ml.

1×10^7 bone marrow cells were injected into lethally irradiated PGK-1B recipients. 10^7 cells was the largest practicable dose which allowed a reasonable number of recipients per donor. Biased repopulation due to a limiting dilution of stem cells in the inoculum cannot, however, be ruled out as a possibility. The cell dose used is, however, greater than one hundred times the minimum repopulating dose as defined by the early mouse marrow transplanters (van Bekkum and Vos, 1957). Recipients were grouped as follows:

Group A : 4 mice, donor mouse 1 (71% PGK-1A)
Group B : 4 mice, donor mouse 3 (87% PGK-1A)
Group C : 5 mice, donor mouse 4 (70% PGK-1A)
Group D : 5 mice, donor mouse 10(48% PGK-1A)

Following repopulation mice were bled at 28 day intervals from day 56 post transfusion. By day 56 post transfusion, all blood cells are derived from donor stem cells (see PGK-1A repopulation results, page 84) and so all ratios observed from then on should reflect the activity of donor stem cells only. Blood samples were continued up to day 308 post transfusion and were processed as described previously. All results were verified using both cellogel and Helena electrophoresis.

Results are shown in figures 3.2 - 3.5 (full results are shown in Appendix 2, table 3). Table 3.3 shows that the mean % PGK-1A in the blood for each group of recipient mice is similar to the mean % PGK-1A for the blood of the respective donors. This finding probably reflects the fact that the major site of haematopoiesis in the adult mouse is the bone marrow and that the phenotype of the circulating blood reflects the phenotype of the bone marrow of that individual. In particular the mice of group D, recipients of bone marrow from mouse 10, all show a mean % PGK-1A of less than 50%. This experiment shows that the phenotype of the donor marrow reflects the phenotype of the repopulating stem cells. This in turn suggests that the primordial marrow pool was predominantly B. This "B"-ness might reflect a cross-over event close to the Pgk-1 locus, or sampling from a small number of primordial cells, or both. The data suggest that all blood cells may be descendents of a relatively small pool of stem cells which establish the haematopoietic system early in embryogenesis.

In this experiment clone numbers were calculated from as few as 2 or 3 observations, and thus a large degree of inaccuracy can be

Table 3.3 Tissue Phenotypes of Donors for Repopulation Studies

Tissue * (% PGK-1A)	Mouse 1	Mouse 3	Mouse 4	Mouse 10
Blood	77	94	67	48
Spleen	74	83	68	51
Right Thymus	78	38	ND	64
Left Thymus	67	82	ND	24
Lymph Nodes	67	86	75	50
Mean % A	73	77	70	47
Mean % A of recipients	71	89	70	36

* No bone marrow samples were tested. Marrow from all long bones was washed out and used to repopulate irradiated recipients.

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Figure 3.2 Variations in Blood PGK-1 Phenotype in Animals Repopulated with Marrow Cells of Known Phenotype

Group A donor mouse = 1

Individual bleeds are shown as variations from the mean percent PGK-1A for each mouse.

(a) mouse A1, mean = 66

(b) mouse A2, mean = 72

(c) mouse A3, mean = 76

Figure 3.3 as figure 3.2

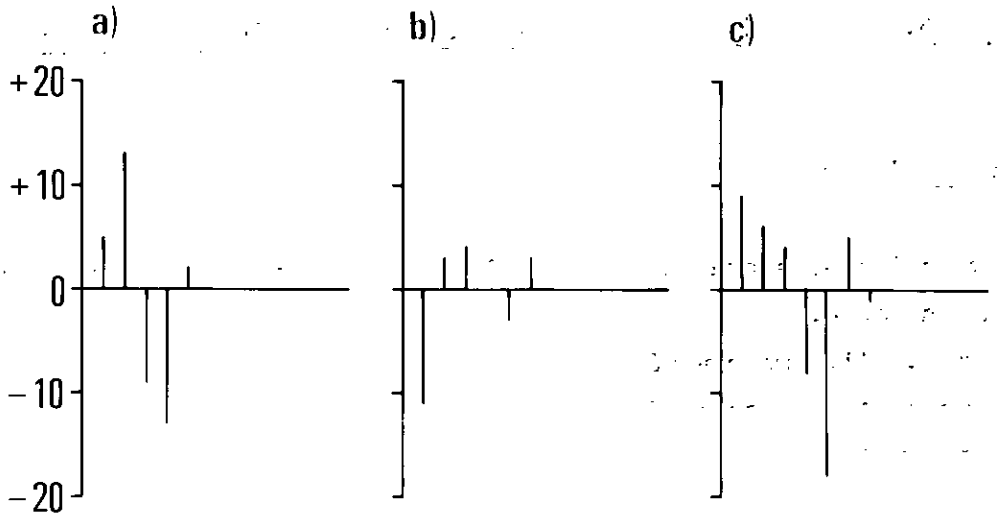
Group B donor mouse = 3

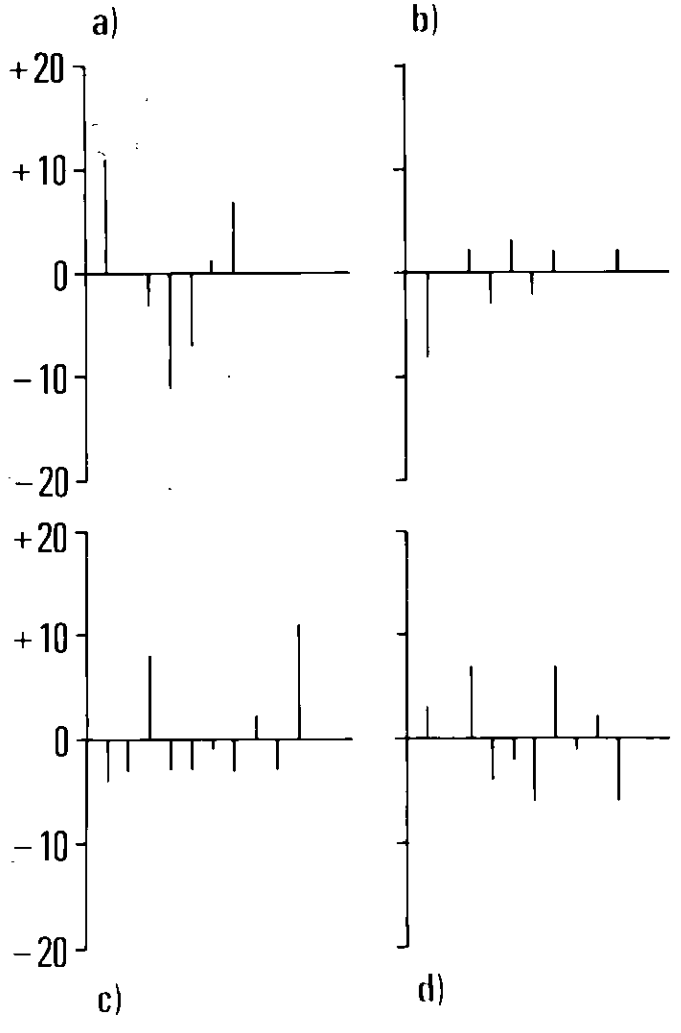
(a) mouse B1, m = 89

(b) mouse B2, m = 92

(c) mouse B3, m = 89

(d) mouse B4, m = 87


a)
b)
c)
+20
+10
0
−10
−20

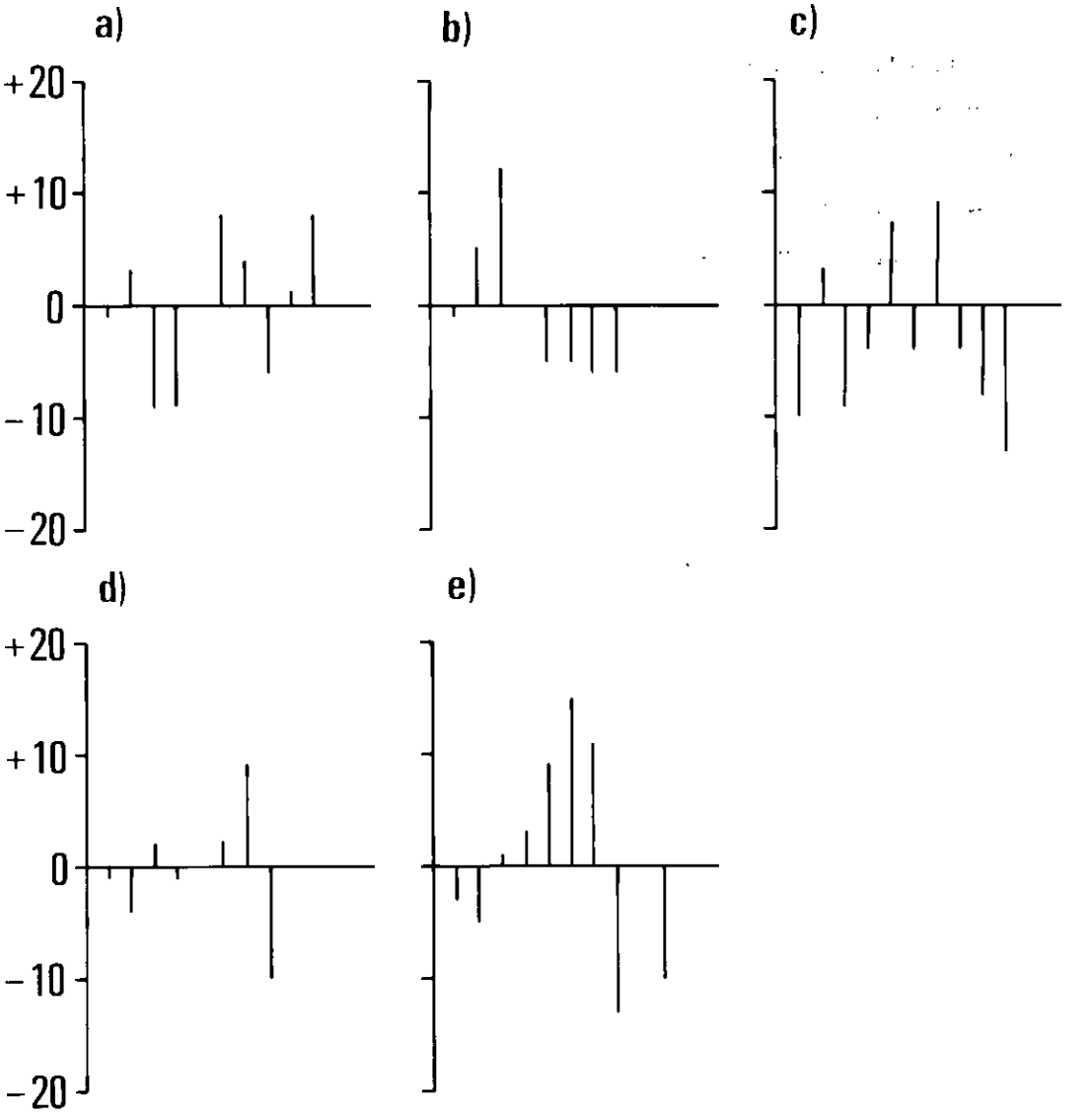

a)
b)
+20
+10
0
−10
−20
+20
+10
0
−10
−20
c)
d)

facing page 63

Figure 3.4 as figure 3.2

Group C donor mouse = 4

(a) mouse C1, mean = 64
(b) mouse C2, mean = 66
(c) mouse C3, mean = 73
(d) mouse C4, mean = 73
(e) mouse C5, mean = 77

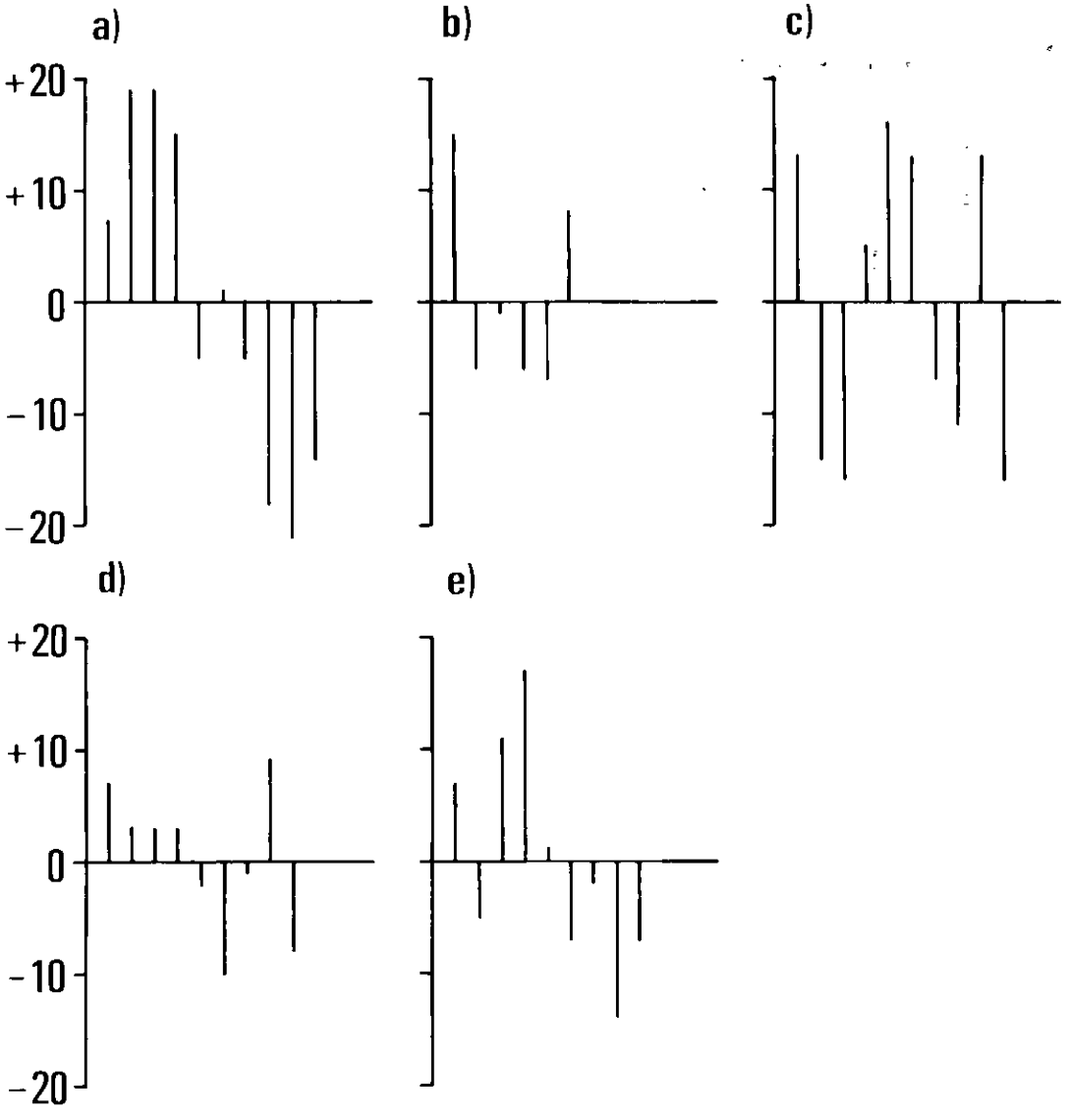

a)
b)
c)
+20
+10
0
−10
−20
d)
e)
+20
+10
0
−10
−20

facing page 64

Figure 3.5 as figure 3.2

Group D donor mouse = 10

(a) mouse D1, mean = 36
(b) mouse D2, mean = 28
(c) mouse D3, mean = 37
(d) mouse D4, mean = 44
(e) mouse D5, mean = 36


a)
b)
c)
d)
e)
+20
+10
0
-10
-20

expected. Estimates in some individuals vary considerably depending on the chosen starting observation. To avoid biasing results, all clone number calculations have been made using the first observation as the starting point. The estimated numbers of clones actively replenishing the blood of these animals (table 3.4) are generally smaller and more variable than those recorded for normal heterozygotes (table 3.1) and may represent a limiting dilution of stem cells in the inoculum. However, the estimations of clone numbers in several mice are of the same order of magnitude as those for normal heterozygotes.

In the present experiment irradiated mice were injected with 10^7 bone marrow cells, only about 5% of the total marrow. Although estimates of "active clone" numbers are smaller in the repopulated mice than in normal animals, they are much greater than 5% of "active clone" numbers in normal mice. Assuming that these estimates are reliable two conclusions can be reached. First, that normal mice use only a small proportion of the available stem cells to maintain haematopoiesis, as repopulated mice with only 5% of the available stem cells have almost as many active clones as normal mice. Also, repopulating marrow is able to switch extra clones into production in response to the need for greater numbers of cells, as recipients have more clones for a given number of available stem cells than do normal mice.

Serial Blood Samples from Heterozygotes of Known Pedigree

As the skewed ratio of PGK-1A:1B was noted before the publication of data showing the existence of different Xce alleles (Johnston and Cattanach, 1981), a second series of normal heterozygotes was studied to investigate the effects, if any, of parental PGK-1 phenotype on that of the offspring (West and Chapman, 1978). Any such effect would obviously affect results on population variance.

Table 3.4 Serial Bleeds of Repopulated Mice

Calculation of n, as for serial bleeds of normal heterozygotes.

Mouse	n (to nearest integer)	corrected n
A1	41	47
A2	40	46
A3	14	15
B1	12	13
B2	40	64
B3	43	59
B4	62	123
C1	71	89
C2	33	37
C3	20	22
C4	93	143
C5	14	15
D1	10	10
D2	16	17
D3	12	12
D4	78	100
D5	47	54

All mice used were C3H x CBA backcross generation 8 females (PGK-1AB phenotype) whose pedigrees had been recorded over eight generations. By studying offspring of well-recorded breeding stock, any preferential inactivation of a parental X chromosome during embryonic and foetal life should be apparent in the long-term ratios of PGK-1A:1B during these animals' adult lives.

The mice were set up in groups consisting of single litter siblings as follows:

Group	No. of individuals	Parental Blood Phenotypes	
A	3	A	B
B	2	B	AB
C	2	B	AB
D	3	B	AB

The mice were bled at 28 day intervals from the age of 7 weeks.

A fifth group of 4 siblings from an A x B mating was also set up. These mice were accidently drowned after only three bleeds and are therefore not included in the results shown in figure 3.6 (see also Appendix 2, table 2).

The parental PGK-1 phenotype had no influence on the relative amounts of PGK-1A and PGK-1B. PGK-1A was the predominant type in all the animals investigated.

The frequency and size of the changes in PGK-1 phenotype are comparable to those found in the original group of normal heterozygotes. The estimations of active stem cell numbers (table 3.5) are also of the same order of magnitude as those previously observed. The smaller numbers observed in this group are probably due to the smaller number of observations used to calculate n.

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Figure 3.6 Variations in Blood PGK-1 Phenotype in Heterozygotes of Known Pedigree

Individual bleeds are shown as variations (% PGK-1A) from the mean (0) for each animal.

(a) mouse A1, mean = 74
(b) mouse A2, mean = 65
(c) mouse A3, mean = 71
(d) mouse B1, mean = 68
(e) mouse B2, mean = 69
(f) mouse C1, mean = 70
(g) mouse C2, mean = 78
(h) mouse D1, mean = 81
(i) mouse D2, mean = 93
(j) mouse D3, mean = 86

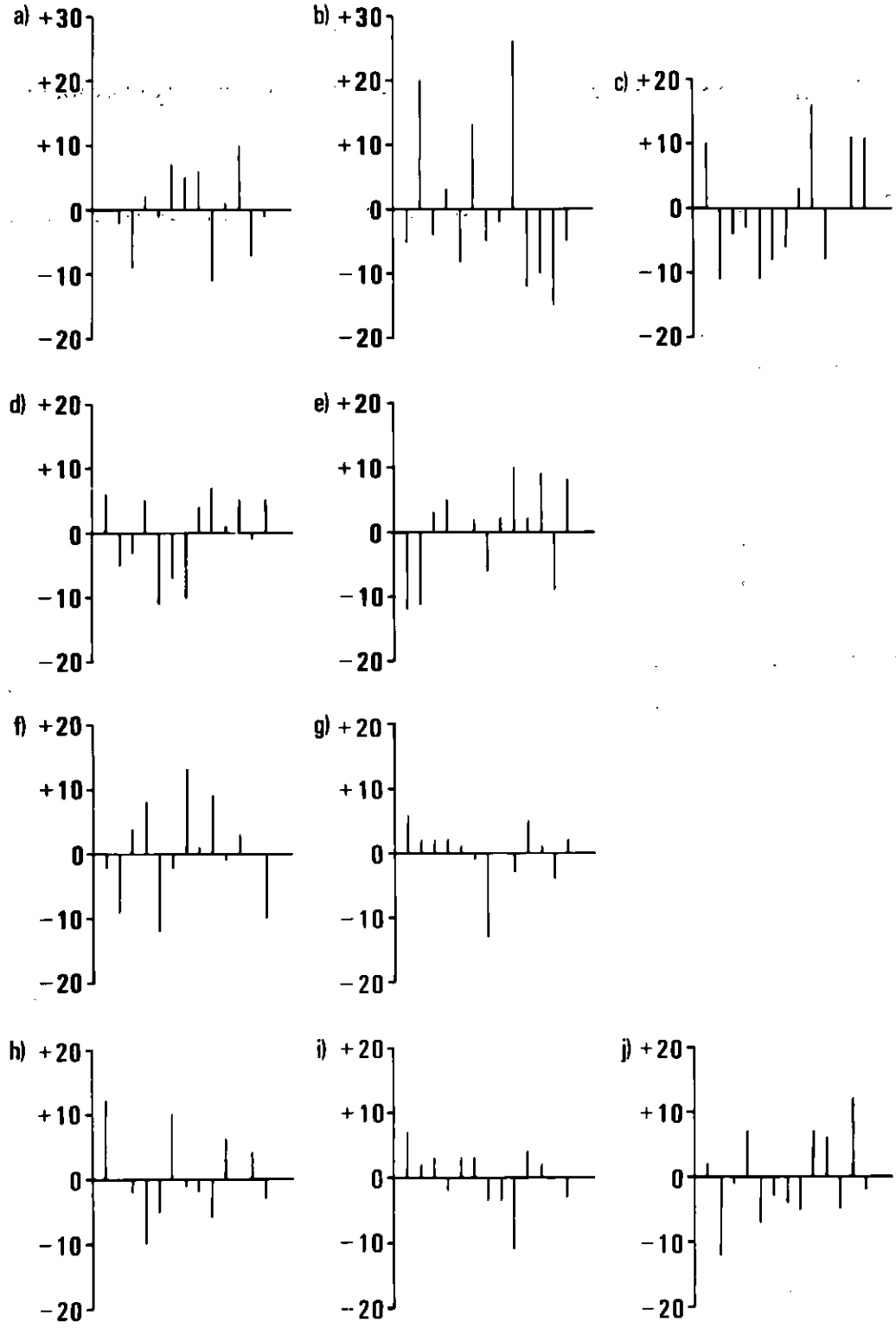
a) +30
+20
+10
0
−10
−20
b) +30
+20
+10
0
−10
−20
c) +20
+10
0
−10
−20
d) +20
+10
0
−10
−20
e) +20
+10
0
−10
−20
f) +20
+10
0
−10
−20
g) +20
+10
0
−10
−20
h) +20
+10
0
−10
−20
i) +20
+10
0
−10
−20
j) +20
+10
0
−10
−20

Table 3.5 Serial Bleeds of Normal Heterozygotes of Known Pedigree

Calculation of n, as for serial bleeds of normal heterozygotes

Mouse	n (to nearest integer)	corrected n
A1	19	20
A2	11	12
A3	19	20
B1	17	18
B2	30	33
C1	24	26
C2	37	43
D1	44	54
D2	20	26
D3	61	90

Table 3.6 Precursor pool number for haematopoietic tissues

Bleed Number	Mean % PGK-1A ± s.d.	Number of precursor cells
1	77.9 ± 14.0	9
2	72.9 ± 12.4	13
3	74.4 ± 10.8	16
4	77.2 ± 8.7	23
5	67.9 ± 11.9	15
6	76.9 ± 11.4	14
7	72.5 ± 11.4	15
8	75.8 ± 7.2	35
9	79.9 ± 8.9	20
10	75.9 ± 13.7	10
11	77.0 ± 10.3	17
12	75.1 ± 15.7	8
13	75.7 ± 9.7	20

Precursor pool sizes for the blood system were estimated as for the first group of normal heterozygotes. The results for precursor cell pool size (table 3.6) are also comparable to those calculated for the first group (shown in table 3.2).

Clonal Succession Studies in Granulocyte Enriched Populations

In all the experiments described so far, results were obtained by electrophoresis of whole blood samples. Attempts were also made to study haematopoiesis by electrophoresis of enriched and purified blood cell populations. Of the enrichment techniques used the most successful was the induction of an intraperitoneal granulocytes with calcium caseinate (Casilan (90% calcium caseinate); Farley Health Foods, Plymouth). It should be advantageous to study a highly purified cell population, rather than a mixture as found in whole blood samples. Granulocytes which have a short lifespan (3 - 4 days, van Bekkum and de Vries, 1967) and blood lymphocytes (B lymphocyte half-life 7 days, Elson et al., 1976) are extremely useful if enriched populations can be obtained in numbers great enough to allow analysis of samples. The short lifespan of these cells allows more independent samples to be studied in a shorter interval of time than in erythrocyte-rich whole blood.

20 three-month-old male CBA mice were injected I.P. with 2 ml 0.2% calcium caseinate (Watt et al., 1979). The mice were killed by cervical dislocation 3 hours later and 5 ml 0.168 M NaCl was injected I.P. into each mouse. After gentle peritoneal massage, peritoneal exudate was drawn out using a 19 G needle. Cells were spun down at 2000 rpm (650 g) for 5 minutes then washed three times in 0.168 M PBS. After the third wash the cells were resuspended in 0.168 M NH_4Cl and left to stand at room temperature for 10 minutes to lyse erythrocytes (Shortman et al., 1972). After washing, the cells were resuspended in a final solution of 0.168 M PBS containing 1% BSA. The cells were spread onto pre-cleaned microscope slides and stained

for 20 minutes with Leishman's stain. At least 100 cells per smear were counted to establish the percentage of polymorphonuclear leucocytes (PMNs) present in each exudate. Results for each mouse are shown in table 3.7 (PMN numbers are presented as a percentage of total white blood cells).

Repeated Induction of Intraperitoneal Granulocytosis

To ensure that intraperitoneal granulocytosis could be induced repeatedly with no ill-effects, 4 male CBA mice, aged 3 months at the time of the first injection, were treated with calcium caseinate as before. Peritoneal exudates were drawn off under ether anaesthesia and the mice were allowed to recover afterwards. Five collections of peritoneal exudate were made in this way at various intervals, and cells were treated as described previously. In order to eliminate biased reporting of PMN numbers due to preferential counting of specific areas of smears, cell concentrations were calculated and after the final resuspension 2.5 - 3.0 x 10^6 cells were spun down in a Cytospin (Shandon Elliot) to produce easy-to-read and well separated cell preparations.

To prepare cytospin samples, slides were covered with two thicknesses of cytospin filter paper, with holes punched out, and placed in the cytospin with the sample wells. 0.1 ml of serum was added to the base of each well before the cell sample, suspended in 0.2 ml 0.168 M PBS was added. Samples were spun at 1000 rpm for 2 minutes. Slides and sample wells were removed together and the slides were air-dried (Blackett, personal communication). The slides were then stained with Leishman's as described previously.

PMN numbers, as a percentage of total white cells, are shown in table 3.8. Results are shown graphically in figure 3.7.

These results showed that even with repeated peritoneal washing,

Table 3.7 Induction of Intraperitoneal Granulocytes

Mouse	% PMN in peritoneal exudate
1	78
2	77
3	60
4	50
5	50
6	63
7	52
8	50
9	59
10	42
11	69
12	57
13	60
14	18
15	70
16	54
17	74
18	56
19	57
20	79

Table 3.8 Repeated Induction of Intraperitoneal Granulocytosis

Polymorphonuclear leucocyte numbers are presented as a percentage of the total number of nucleated cells.

Day	% PMN	mean % PMN	S.E
0	82 80 53 80	73.8	6.9
9	88 84 88 86	86.7	1:2
30	73 87 88 81	82.2	3.4
60	80 88 80 91	84.8	2.8
80	89 83 88 87	86.8	1.3

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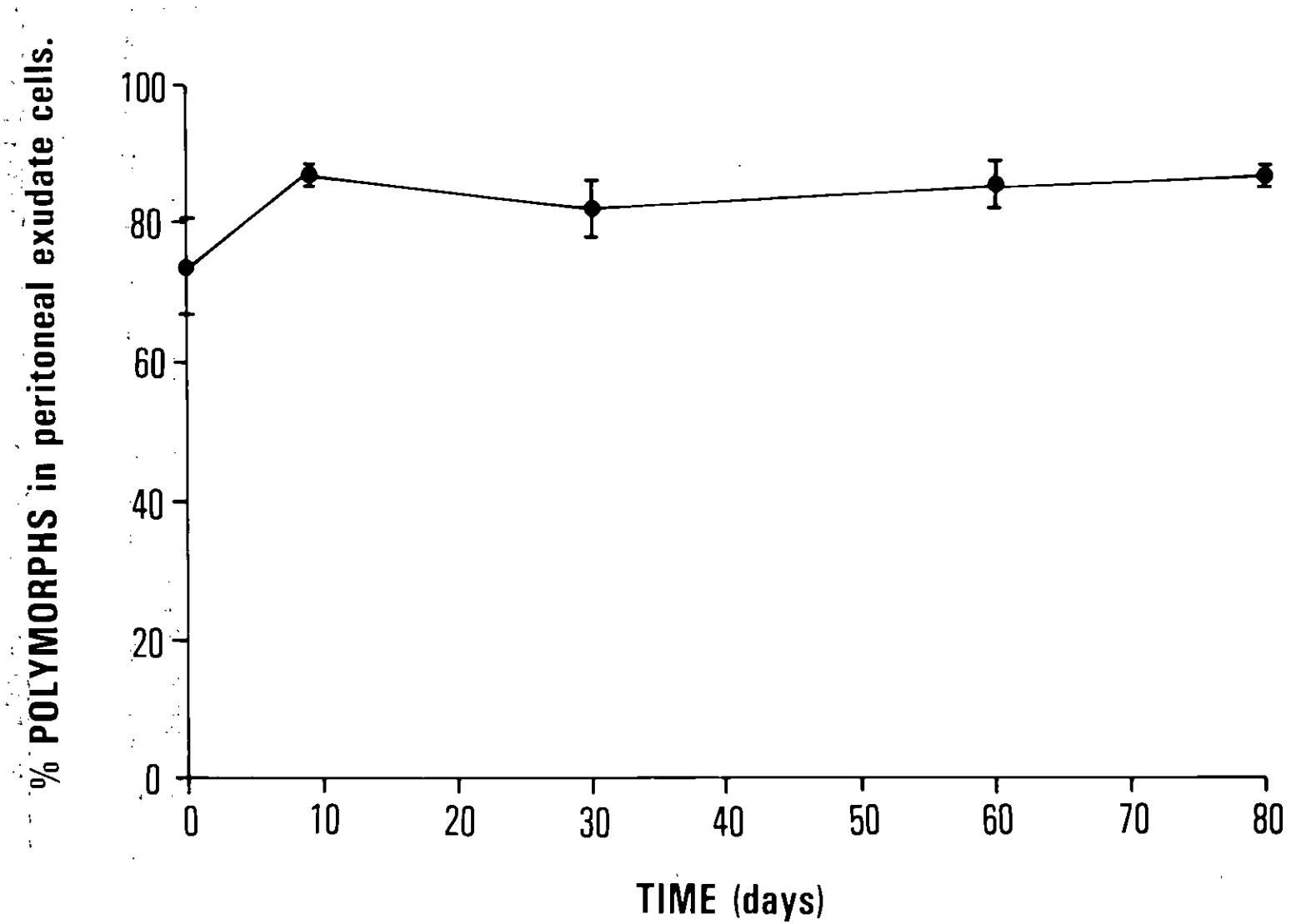
Figure 3.7 Induction of Intra-Peritoneal Granulocytosis Using Calcium Caseinate

Polymorphonuclear leucocytes (y-axis) are shown as a percentage of total nucleated cells. Each point represents the mean and standard error for 4 mice.


% POLYMORPHS in peritoneal exudate cells.
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
TIME (days)

The fat must have a saturation limit – did you try adding KS Nall

12 hr point seems OK?

– that's it

exudates containing a high concentration of PMNs were induced.

Induction of Intraperitoneal Granulocytosis in PGK-1 Heterozygotes

Ten heterozygous females C3H/CBA mice (backcross generation 6) were treated with calcium caseinate as described above. Peritoneal exudates were treated as before and finally resuspended in sample buffer at a final concentration of 3×10^6 cells/100 ul. These samples were stored at $-60^{\circ}C$ before being electrophoresed on Helena gels. Peritoneal drainage was carried out at 14 day intervals.

Recovery of peritoneal exudates from female mice proved very unsatisfactory as compared to results using males. Fatty tissues protecting the female reproductive organs absorbed most of the NaCl injected. As a result only 3 exudates were collected, too few to make statistical analysis of the data profitable. Results (PGK-1A activity is shown as a percentage of total PGK-1 activity) are shown in table 3.9.

Proportions of PGK-1A in granulocyte enriched populations are similar to those recorded for whole blood samples from heterozygotes (see Appendix 2, tables 1 and 2). Variations within individuals are also comparable to those of whole blood samples. This suggests that active stem cell numbers for granulocytes would probably be comparable to those estimated for maintenance of whole blood.

Induction of leucocytosis was also attempted using lipopolysaccharide (LPS) stimulation. Leucocyte numbers in circulating blood were increased but the peaks gained were, on the whole, neither large nor sharp enough to allow a regular regime to be established for large scale experiments (see figure 3.8). LPS leucocyte induction never resulted in enrichment for PMN of more than 30% of the total white cell count. Further purification after enrichment would be necessary to make this technique worthwhile.

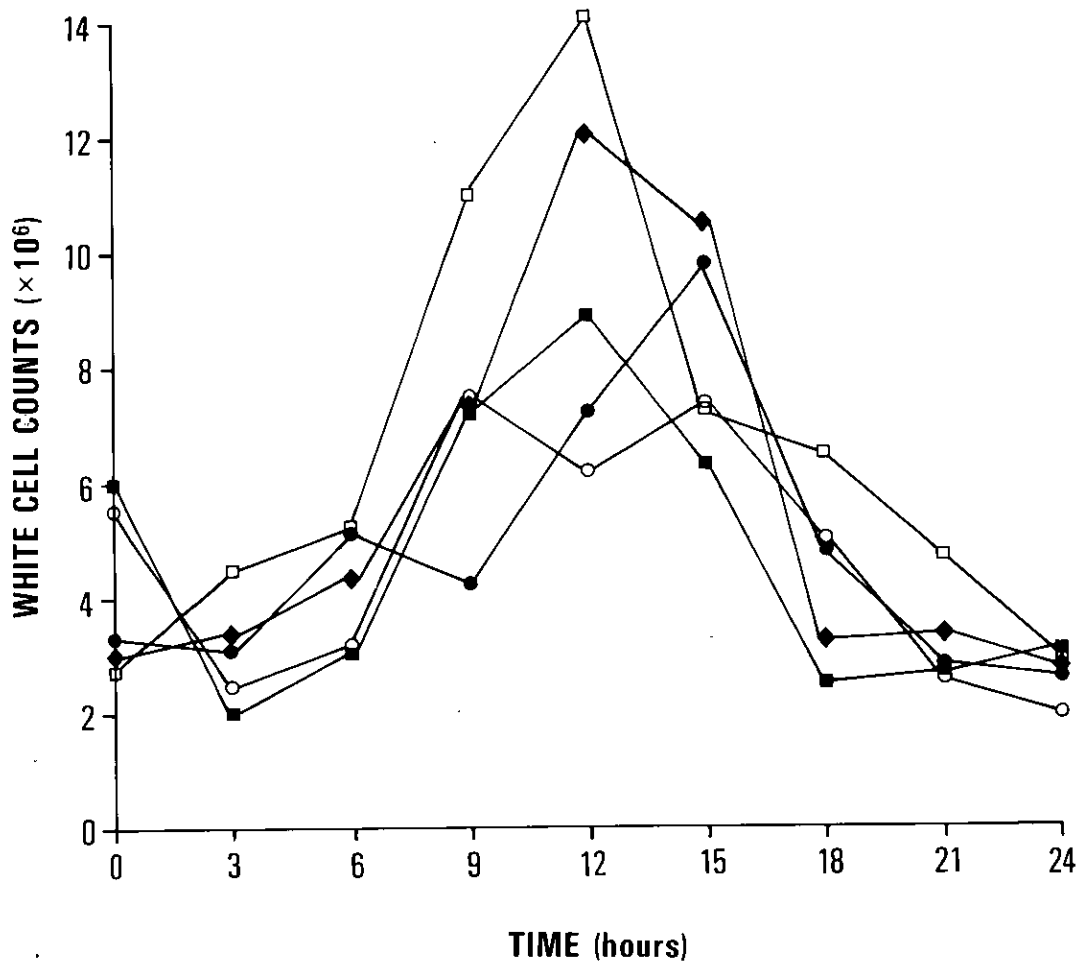
Table 3.9 PGK-1 Phenotype in Peritoneal Granulocytes of Heterozygous Mice

Mouse	1st exudate	2nd exudate	3rd exudate	mean % A ± s.d.
1	82	78	76	78.7± 3.1
2	89	76	82	82.3± 6.5
3	66	86	86	79.3±11.5
4	86	82	82	83.3± 2.3
5	NT	86	84	85.0± 1.4
6	82	79	75	78.7± 3.5
7	64	78	76	72.7± 7.6
8	81	79	85	81.7± 3.1
9	74	74	NT	74
10	NT	88	NT	88

facing page 78

Figure 3.8 Leucocytosis Induced by Injection of Lipopolysaccharide

The total white cell count (y-axis) was measured at 3 hourly intervals (x-axis) after injection of lipopolysaccharide. Each set of points represents data from one individual.


WHITE CELL COUNTS (×10⁶)
14
12
10
8
6
4
2
0
0
3
6
9
12
15
18
21
24
TIME (hours)

Are you suggesting monoparental stem cells? If so that's ok

Percoll gradients (Pharmacia Fine Chemicals Ltd) were used to separate individual cell types from blood samples (Pertoft et al., 1968) with reasonable success. Cell numbers recovered following gradient centrifugation and cell washing were so small that PGK-1 activity was not measurable in samples tested.

The addition of a fluorescence activated cell sorter (FACS IV, Becton Dickinson) to this laboratory's equipment has allowed more simple and successful separation of cell types from whole blood samples, making repeated sampling of individual cell lineages possible.

It is instructive to study whole blood samples to estimate the numbers of stem cells responsible for blood maintenance at any time. This, however, does not give any indication of the relationships between different cell lineages.

We know that the pluripotent haematopoietic stem cell is the common precursor of the whole variety of blood cells (Ford and Micklem, 1963; Lewis and Trobaugh, 1964; Abramson et al., 1977; Phillips, 1978; Ash et al., 1980). We also know that stem cells injected into irradiated mice will form clonal spleen colonies of mixed cell types (Lewis and Trobaugh, 1964; Fowler et al., 1967). We are still unable, however, to assess the amount of diversification stem cells are capable of under conditions of normal repopulation. When measuring PGK-1 proportions in whole blood, one is, in effect, looking at a predominantly erythrocytic population. The fluctuations one sees in serial samples may, indeed, not be due to new stem cells taking over total blood cell production, but may be a reflection of stem cell involvement in the production of individual cell lines. Primordial cell pools for each individual cell type, rather than for general haematopoiesis, may be set up during embryogenesis, and these may maintain cell numbers of each lineage independently under normal conditions.

Analysis of a number of different cell types from individual blood samples over a long period of time using PGK-1 allozymes would produce parallel results for each cell lineage. Hence independent variations in PGK-1 phenotype could be seen. In addition, analysis of a large group of animals would allow estimations of primordial pool size for each blood cell type and also truer estimates of the number of stem cells actively repopulating the haematopoietic system at any time to be made.

CHAPTER 4 RECOVERY OF HAEMATOPOIETIC TISSUES IN IRRADIATED AND REPOPULATED MICE

Although previous investigators have been unable to study haematopoiesis in normal animals, they have commonly used irradiated and repopulated mice as tools in their studies. Since the discovery of Jacobson and his colleagues (Jacobson et al., 1949; 1951) that by protecting the spleens of mice during lethal whole-body irradiation their chances of survival were enhanced, many investigators have used irradiated mice to study the properties of CFU-S, cells which form colonies in the spleens of irradiated recipients (Till and McCulloch, 1961).

By injecting cells from excised spleen colonies into a second irradiated host and demonstrating spleen colony formation in these animals, Siminovitch, Till and McCulloch (1963) were able to demonstrate that CFU-S could maintain their numbers by self-renewal. This, and the ability of CFU-S to undergo variant pathways of differentiation, has made the CFU-S an excellent candidate for the role of the haematopoietic stem cell. More recent studies have, however, shown that CFU-S vary in their ability to self-renew and to differentiate (Siminovitch et al., 1965; Silini et al., 1968; Lahiri and van Putten, 1969; Gidali et al., 1974; Micklem et al., 1975).

Several authors have repopulated irradiated mice with cells carrying T6 markers to study haematopoiesis (Micklem et al., 1975, I, II; Wallis et al., 1975; Micklem and Ross, 1978; Ross et al., 1982). These authors have been able to estimate the numbers of stem cells involved in repopulation by repopulating animals with mixed cell populations (i.e. cells carrying 0, 1 or 2 T6 chromosomes) and by applying relevant statistical tests to the data.

During the present study recovery of haematopoiesis in irradiated

animals was examined by repopulating the animals with cells whose PGK-1 phenotype was distinct from that of the recipient. Lethal irradiation should destroy all dividing haematopoietic cells in, and perturb the haematopoietic microenvironment of the treated animals. Differences in stem cell repopulating patterns were noted in a series of tissues concerned with haematopoiesis. Estimates of stem cell numbers repopulating various tissues were also made.

Repopulation with PGK-1A Bone Marrow

Fifty sixteen-week-old female CBA (PGK-1B) mice were irradiated with 1050 rads as described in Chapter 2.

Bone marrow was flushed from the long bones of generation 8 back-cross CBA x C3H, PGK-1A donors as described previously. These cells were washed and resuspended in HBSS and each recipient was injected I.V. with 2.5×10^6 nucleated bone marrow cells in 0.33 ml. Injections were carried out within seven hours of irradiation. Groups of 5 mice were killed on each of days 10, 14, 18, 21, 24, 28, 35, 42, 56, and 70 post irradiation. From these mice the following organs were removed and treated as described below.

Blood samples were collected and diluted as for normal heterozygotes. Spleens, left and right thymic lobes (separately), pooled brachial and axillary lymph nodes were homogenised in a small volume of RPMI (variable according to sample size) using a ground glass pestle, and sieved through a stainless steel mesh (size 3) in RPMI 1640 at pH 7. Cells were spun down in siliconised centrifuge tubes at 2000 rpm (650 g) for ten minutes and, after contaminating erythrocytes had been lysed as described previously, were suspended in sample buffer as follows (all volumes are in µl):

/over

Where, then, do they come from??

0.1% of 10^6 CFC-s = 10^3 !!!

(9)

Day post-transplantation	Spleen*	Left * Femur	Right* Femur	Left Thymus	Right+ Thymus	Lymph nodes
10	200	100	100	100		100
14	200	100	100	100		100
18 - 70	200	100	100	100	100	100

* These tissues were treated to remove contaminating erythrocytes.
\+ On days 10 and 14 left and right thymic lobes were pooled.

All samples were stored at -60°C and electrophoresed on cellogel sheets. PGK-1AB ratios were calculated from autoradiographs as described previously.

The rate of donor cell repopulation in each tissue is shown in figure 4.1 (for full tables of results see Appendix 2, table 4). Figure 4.1 shows that in femora, donor cells were responsible for more than 95% of haematopoiesis from day 10 onwards. In the thymus over 95% of PGK-1 activity was of donor origin from day 18 onwards and in the spleens over 90% of PGK-1 activity was of donor origin by day 42. In these tissues the very small, but in some cases detectable, contribution of PGK-1B to the total enzyme activity is unlikely to be caused by surviving host haematopoietic cells.

Haematopoietic stem cells are able to survive and proliferate following low doses of whole-body irradiation (Brecher et al., 1948) or part-body lethal irradiation (Swift et al., 1954; Gidali and Lajtha, 1972). Studies of bone marrow after in vitro and in vivo irradiation have shown, however, that the number of stem cells capable of surviving decreases as the radiation dose increases (Till and McCulloch, 1961; McCulloch and Till, 1962). Extrapolation of survival curves following radiation doses of up to 700 rads shows that fewer than 0.1% of host bone marrow cells would survive an X-ray

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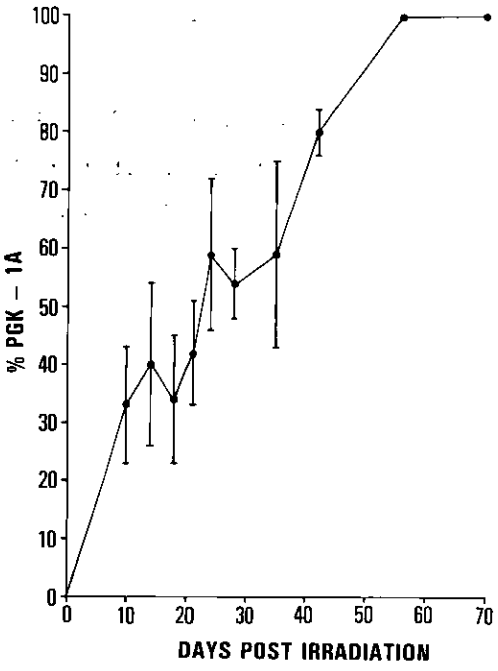
Figure 4.1 Rate of Repopulation of Haematopoietic Tissues Following Lethal Irradiation and Bone Marrow Transfer

Lethally irradiated PGK-1B mice were repopulated with 5×10^6 PGK-1A bone marrow cells. Donor PGK-1A is shown as a percentage of total PGK-1 activity. The mean and standard deviation for 5 mice are shown on each sample day.

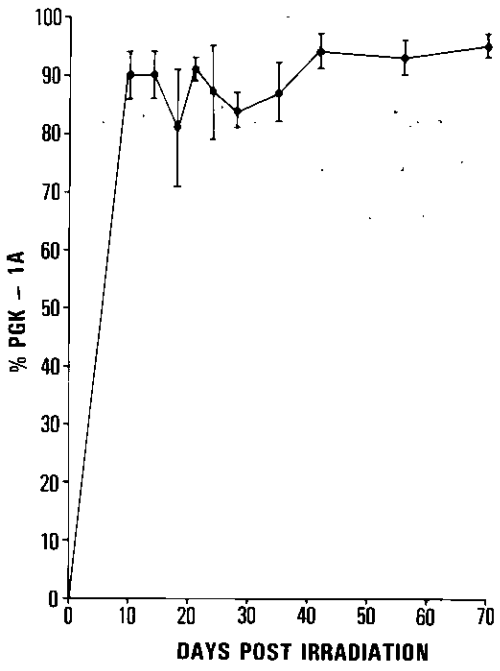
(a) Blood
(b) Spleen
(c) Femora (pooled left and right)

Continued overleaf

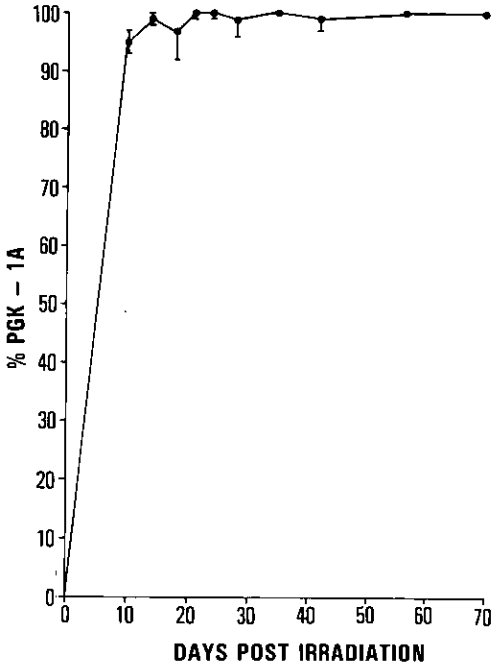
a) BLOOD



b) SPLEEN



c) FEMORA (left + right pooled)



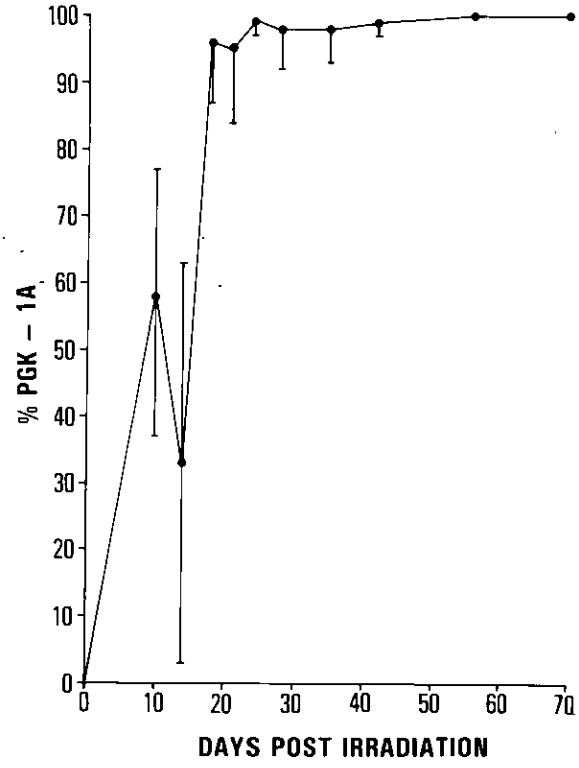
facing page 85

Figure 4.1 continued

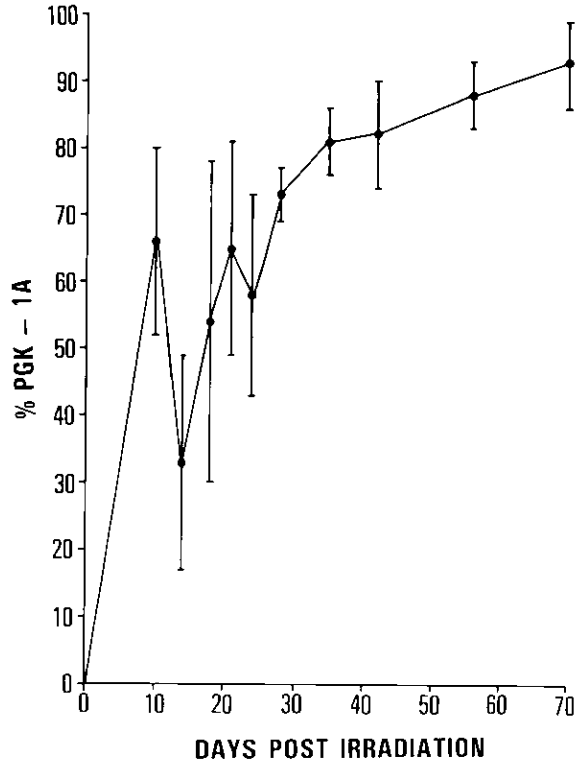
(d) Thymus Lobes (pooled left and right)

(e) Lymph Nodes (pooled axillary and brachial)

d) THYMUS (pooled lobes)



e) LYMPH NODES



— But not in blood up to 60 days, surely.

— Gregory's results of PFC would agree with this.

dose of 1050 rads. Even if pluripotent stem cells were able to survive a lethal dose (X-ray dose lethal to mice: 950 rads, Mitchison, 1956; 1000 rads, Micklem et al., 1975 I) it is unlikely that they would remain undamaged (Ogden and Micklem, 1976). As a result they would probably be unable to compete successfully with donor stem cells and so their contribution to haematopoiesis would be extremely small and would decline with time.

The most likely explanation for the continued small proportion of host tissue is the presence of reticular and stromal cells in the samples. No attempts to obtain pure cell populations from the various tissues tested were made other than to remove erythrocytes by selective lysis.

In general, only extremely low levels of PGK-1 activity were found in thymus samples on days 10, 14 and 18 after irradiation. Although bands of activity were found on autoradiographs from these days, in many cases they were too faint to scan. This was particularly so in the PGK-1AB repopulation results presented in the following section. Reanalysis of samples using Helena gels showed no PGK-1 activity. As a result, the data presented for early thymic repopulation probably incorporate larger measurement errors than the data for other tissues. These data do, however, suggest that the PGK-1 method is more sensitive than the T6 marker system for measuring early thymic repopulation. Micklem and his colleagues found few or no dividing donor cells in thymus samples from animals killed 10 - 14 days after repopulation (Micklem et al., 1966; 1972) and suggested that this was because repopulation of the thymus was only beginning at that time. Results of recent experiments using fluorescence-labelled donor bone marrow cells suggest that a significant number (about 10^4 cells within twenty-four hours after injection of 10^7 bone marrow cells) migrate to the thymus within hours of injection (Lepault and Weissman, 1981). These authors also showed that donor cells lodging in the thymus begin to express T-cell-specific markers

Gilbert et al (~1956) found shortening of ~~life~~ RBC lifespan after mid-lethal irradiation in Rhesus monkeys

shortly thereafter. The apparent increased sensitivity of the PGK-1 assay in this case may be accounted for by the continued presence in the thymus of differentiating T lymphocytes during the early sample times in the present study. Lepault and Weissman (1981) did not, however, present data to show how long these differentiating cells remain in the thymus. Such cells, though undetectable with the T6 markers, will be found with the PGK-1 system.

In the blood, a progressive increase in percent donor activity is apparent from day 10, with all cells being donor-derived by day 56. Erythrocyte lifespan in CBA mice has previously been estimated to be about 45 days (Russell and Bernstein, 1966). Recipient red blood cells already in the circulation at the time of irradiation can be expected to contribute, with declining importance, to the overall PGK-1 activity for at least 45 days after transfusion. This is indeed the situation found here, with maximum red cell lifespan falling between 42 and 56 days.

In spleen samples, although the contribution of donor cells to repopulation remains less than 100% throughout the test period, there is a persistent high percentage of PGK-1A activity in this organ. The most reasonable explanation for the almost constant contribution of host PGK-1, is that spleens were ground and sieved to provide cell suspensions. It is very likely that cells of the reticular framework of the spleen have been included in the samples throughout the test period.

The participation of host and donor cells in lymph node repopulation is more complex than in the other tissues studied. The contribution of donor progeny to lymph node repopulation (figure 4.1e) increases much more slowly than in any of the other tissues. Donor repopulation remains incomplete even 70 days after transfusion in animals whose other tissues are maintained totally by donor cells at this time (full data are shown in Appendix 2, table 4). As with the thymus, lymph

node samples from the first three test days (days 10, 14 and 18) had so little PGK-1 activity that many electrophoresis runs were necessary to obtain scannable gels. In general the results from these days are from single analyses.

The large amounts of PGK-1B observed in lymph node samples could occur for a number of reasons. First, the lymphocytes may not be completely ablated by irradiation. Lymphocytes have, however, been shown to be particularly radio-sensitive even at comparatively low doses (Henshaw, 1944). It is therefore unlikely that a radioresistant population of lymphocytes would be large enough to maintain the large B proportion of the total PGK-1 activity observed. Brecher and his colleagues (Brecher et al., 1948) found that early lymphoid damage was very pronounced in mice following a single dose of 400 rads, and lymphocyte numbers were suppressed for at least three weeks following irradiation, although numerous macrophages and plasma cells were found in the regenerating areas. Following a dose of 1050 rads., a very much smaller proportion of lymphocytes would survive than did following a 400 rad dose. In addition, it is likely that the cells regenerating lymph nodes following 400 rads were not locally derived, but were immigrants, probably from the marrow. In this case, as the marrow of animals in the present study showed almost complete donor repopulation by day 10, one would expect cells immigrating to repopulate lymph nodes to be of donor origin.

Osmond has estimated that approximately 10^8 B lymphocytes per day are produced in the bone marrow, the primary site of B lymphocyte production, in the adult mouse (Osmond, 1975); that is, that lymphocyte production is of the same order of magnitude as erythrocyte production. However, whereas the red cell lifespan is about 45 days, virgin B lymphocytes have been estimated to have a half-life of only 7 days (Elson et al., 1976). After lethal irradiation and marrow injection the rate of entry of lymphocytes into lymph nodes may be limited by the rate of B cell production in the regenerating marrow.

7. B-lymphocyte stem cells
in lymph nodes.

If lymphocyte repopulation is slow then the stromal elements of the lymph nodes would continue to make a large contribution to the total PGK-1 activity for a long period. As no attempts were made to obtain a pure lymphocyte population for analysis, this is the most likely reason for the large amounts of PGK-1B activity found in lymph nodes.

As donor-derived lymph node repopulation is incomplete by the end of the experimental period, the results obtained following PGK-1AB bone marrow repopulation could not be interpreted in the same manner as the results for the other tissues considered, as will be seen below.

Repopulation with PGK-1AB Bone Marrow

After acquiring a series of control data on the rate and degree of donor repopulation of various haematopoietic tissues by transfusing PGK-1A donor cells into lethally-irradiated PGK-1B hosts, an attempt was made to estimate the number of clones involved in repopulation by rescuing lethally-irradiated PGK-1B hosts with marrow cells from heterozygous donors.

One hundred 16-week-old female CBA mice were irradiated as before and repopulated using 2.5×10^6 nucleated bone marrow cells from PGK-1AB donors. Ten mice were killed on each of days 10, 14, 18, 21, 24, 28, 35, 42, 56 and 70. Dissections and sample treatments were as for the previous experiment.

For electrophoresis cells were resuspended in sample buffer as follows (all volumes are in µl):

/over

days post transplantation	Spleen*	Left* Femur	Right* Femur	Left Thymus	Right+ Thymus	Lymph nodes
10	200	100	100	50		50
14	200	100	100	50		50
18	200	100	100	50	50	50
21	200	100	100	50	50	50
24	200	100	100	100	100	100
28 - 70	200	100	100	100	100	100

* These tissues were treated to remove contaminating erythrocytes.
\+ On days 10 and 14, left and right thymic lobes were pooled.

Graphs showing the differences between PGK-1A and PGK-1AB donor repopulation are shown in figure 4.2 (for full data on PGK-1A repopulation, see Appendix 2, table 5). In all the tissues examined in recipients of heterozygous donor bone marrow cells, the percentage of PGK-1A activity present reaches a plateau at values of about 70 - 80% of the total PGK-1 activity. This is approximately the level of PGK-1A activity found in the normal heterozygotes studied and is thus the level of PGK-1A activity one would expect to achieve in mice repopulated with cells from such animals.

The variation in the rate of repopulation of different tissues when repopulated using PGK-1A or -AB cells is in agreement with repopulation studies carried out using different marker systems (Brecher et al., 1948; Micklem et al., 1975, I; Wallis et al., 1975). This variation between tissues makes it difficult to calculate the number of stem cells responsible for the repopulation of the whole animals, due to the variable contributions made by host tissues. In addition, clones of cells responsible for the repopulation of certain tissues may be subclones of the stem cells initially repopulating the animal. For example, injected stem cells may home to the bone marrow of the host

Figure 4.2 Comparison of Haematopoietic Repopulation in PGK-1B Mice Irradiated and Repopulated with PGK-1A or PGK-1AB Bone Marrow Cells

Lethally irradiated PGK-1B mice were repopulated with 5×10^6 PGK-1A or PGK-1AB bone marrow cells. Donor PGK-1A is shown as a percentage of total PGK-1 activity.

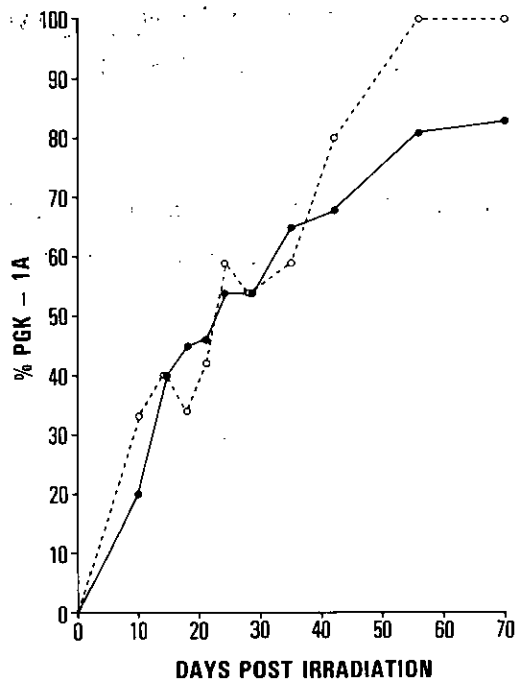
O - PGK-1A repopulation, mean of 5 mice

● - PGK-1AB repopulation, mean of 10 mice (9 on day 42)

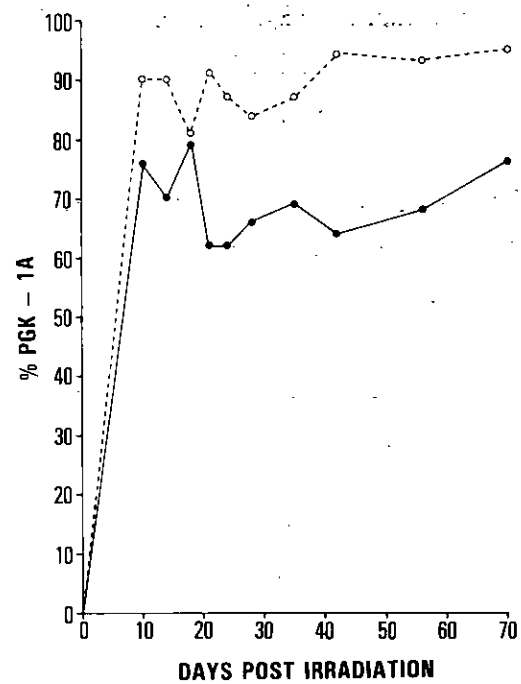
(a) Blood
(b) Spleen
(c) Left Femur
(d) Right Femur

Continued overleaf

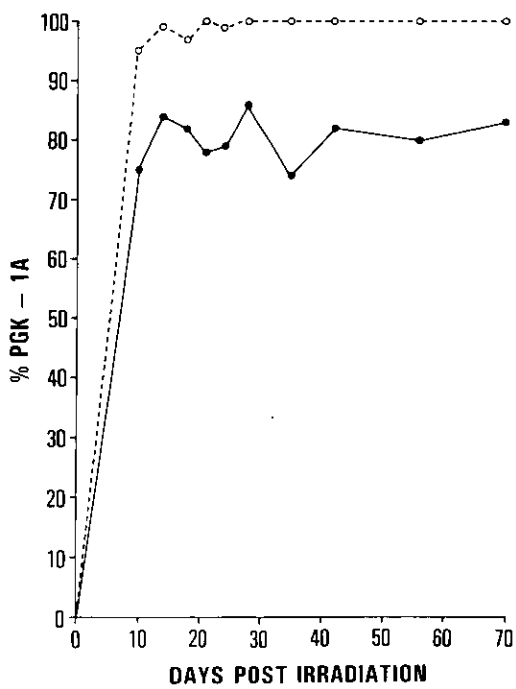
a) BLOOD



b) SPLEEN



c) LEFT FEMORA



d) RIGHT FEMORA

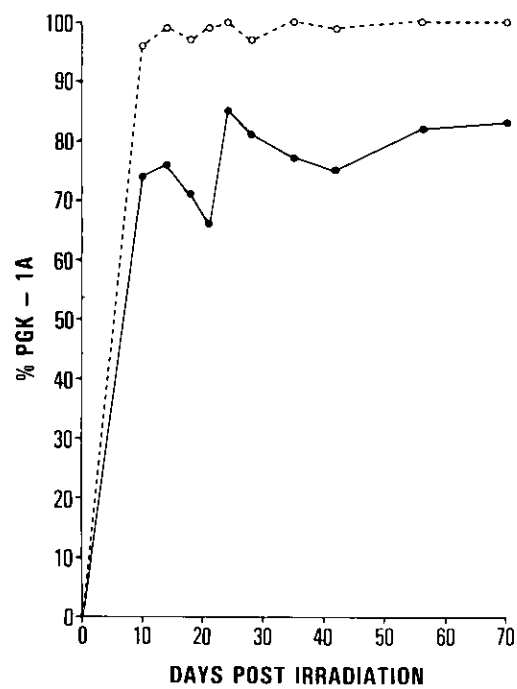


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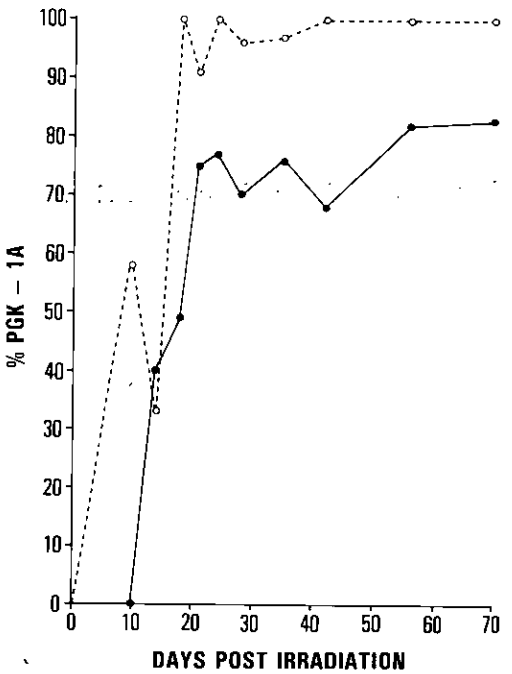
Figure 4.2 continued

(e) Left Thymus

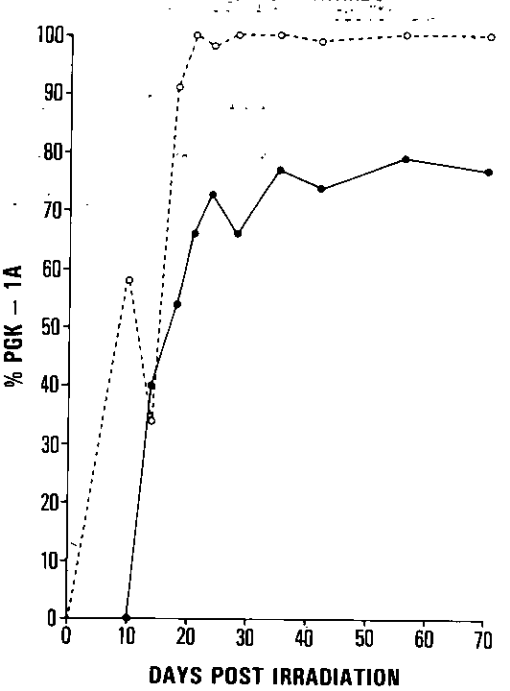
(f) Right Thymus

(g) Lymph Nodes (pooled axillary and brachial).

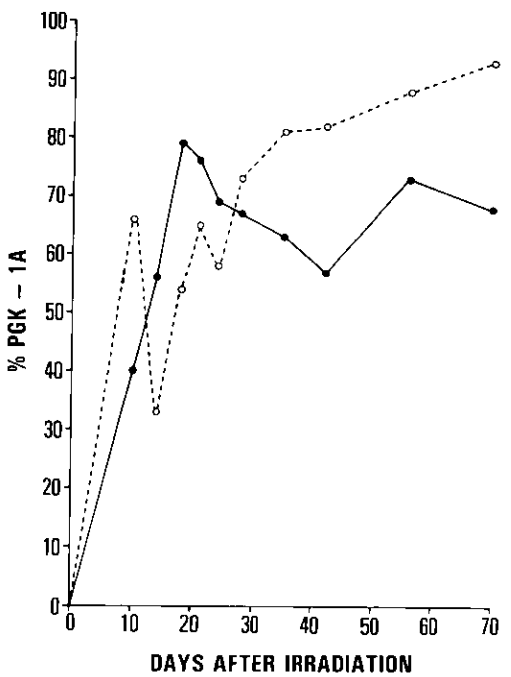
e) LEFT THYMUS



f) RIGHT THYMUS



g) LYMPH NODES



T6 subject to
same conditions

where they undergo clonal expansion. Thereafter selected stem cells, perhaps of only limited differentiating ability, may migrate to other organs such as the thymus or lymph nodes to initiate repopulation there. If this is the case, estimations of stem cell numbers responsible for overall repopulation would be invalid. It is extremely unlikely that the ratio of A:B subclones would always be the same as in the population from which they arose, therefore giving rise to variations between tissues which would artificially lower estimates of stem cell numbers responsible for repopulation.

A more accurate view of repopulation will be achieved by estimating the numbers of cells repopulating individual tissues. Because the host contribution to each tissue varies considerably between animals on any day (see figure 4.1 and Appendix 2, table 4), estimates of stem cell numbers were only made in tissues where the host contribution was negligible. Estimates of clone numbers repopulating individual tissues are shown in table 4.1. Such estimates will still give an inaccurate view of whole-body repopulation because of the lack of knowledge of inter-organ cell flows in animals recovering from X-irradiation and, therefore, duplication of clones which may be seeding more than one organ.

The estimates of clone numbers presented here for individual tissues do, however, agree with comparable studies using the T6 marker system. Wallis and her colleagues (Wallis et al., 1975) reported that an average of 7 stem cells (or stem cell clones) were responsible for the repopulation of a single femur following injection of 5×10^6 bone marrow cells (present data: mean number of stem cells = 12 following injection of 2.5×10^6 bone marrow cells). Micklem and his colleagues (Micklem et al., 1975 I; Micklem and Ross, 1978) have also estimated clone numbers repopulating individual femora. Their data showed 8 - 15 stem cells repopulating each femur following injection of 2×10^6 bone marrow cells. The results of both these groups were also comparable when much larger cell numbers were

Table 4.1 AB Repopulation of B Mice

Estimated Number of Clones Repopulating Each Tissue Each Day.

Days post-irradiation	Tissue						
	Blood	Spleen *	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph Nodes
10	(25)	18	8	11	N.A.	N.A.	(4)
14	(30)	21	27	18	(6)		(10)
18	(17)	17	10	6	5	8	(26)
21	(39)	12	12	6	8	3	(18)
24	(25)	24	6	9	7	20	(18)
28	(20)	62	15	8	15	18	(27)
35	(19)	15	24	15	5	15	(19)
42	(18)	47	18	10	13	16	(30)
56	62	34	8	9	12	10	(40)
70	22	15	7	6	22	9	(8)

Bracketed figures are those in which the host contribution is not negligible.

* In spleen samples, although the host contribution is not negligible

/continued

Table 4.1 continued

it is consistent. Host contamination should therefore make a constant contribution to the measured PGK phenotype, thus affecting all calculations equally.

inoculated (Wallis et al., 43 clones after 5 x 10^7 cells; Micklem et al., 35 - 54 clones after 4 x 10^7 cells).

Wallis and her colleagues also estimated clone numbers repopulating individual thymus lobes following injection of 5 x 10^6 bone marrow cells. These were 4, 6 and 8 cells on days 16, 18 and 20 respectively after injection. These agree very well with the present numbers of 6 on day 14 (estimated from incompletely repopulated tissues), 5 and 8 on day 18 and 3 and 8 on day 21. Wallis and her colleagues further reported that the number of stem cells responsible for repopulation increased at later times after irradiation, but that the number involved was still relatively small, probably fewer than 20. A similar tendency to larger numbers of stem cells at later sample times was also found in the present study (see table 4.1). The data presented here also suggest that fewer stem cells are responsible for thymus and femur repopulation following irradiation than in the other tissues investigated.

The large range of values for the proportion of repopulation instigated by cells of PGK-1A phenotype in individual tissues is shown graphically in figure 4.3 (for full details see Appendix 2, table 5). The overall impression gained by studying the whole population is that repopulation within individual bones and separate thymic lobes is very similar (see figures 4.2 c-f and 4.3 c-f). However, differences within individuals are often found which are much greater than can be accounted for by inaccuracies in the electrophoretic technique used.

Disregarding thymic samples from mice sacrificed on days 10 and 14 following irradiation, the following observations (see figure 4.4 and table 4.2) regarding differences between left and right femora and left and right thymic lobes of individual animals were made. The data were examined statistically to ascertain whether the large variations in PGK-1 phenotype between paired tissues were affected by the length of time which had elapsed since irradiation. For

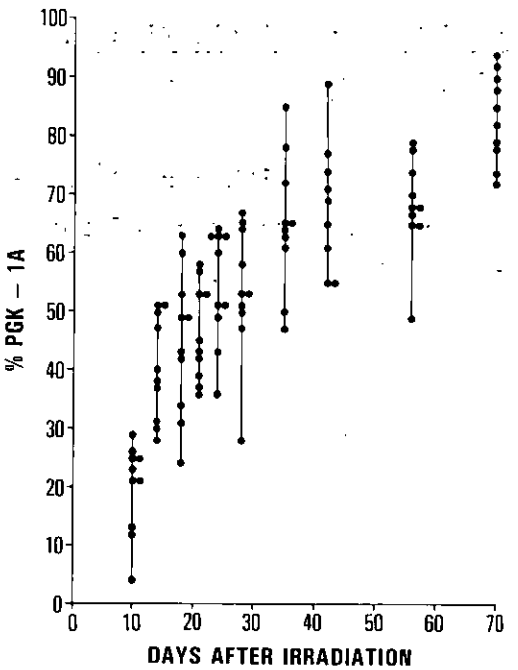
Figure 4.3 Variation in Repopulation of Haematopoietic Tissues Following Lethal Irradiation and Repopulation

Lethally irradiated PGK-1B mice were repopulated with 5 x 10^6 PGK-1AB bone marrow cells. Donor PGK-1A is shown as a percentage of total PGK-1 activity. Each point represents one individual.

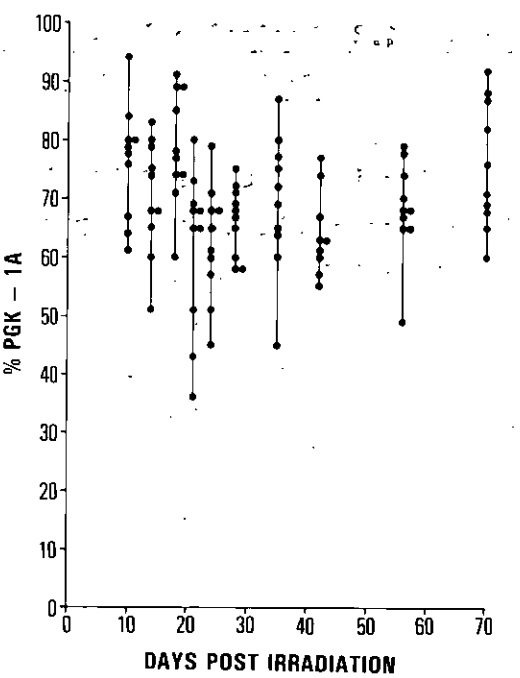
(a) Blood
(b) Spleen
(c) Left Femur
(d) Right Femur

Continued overleaf

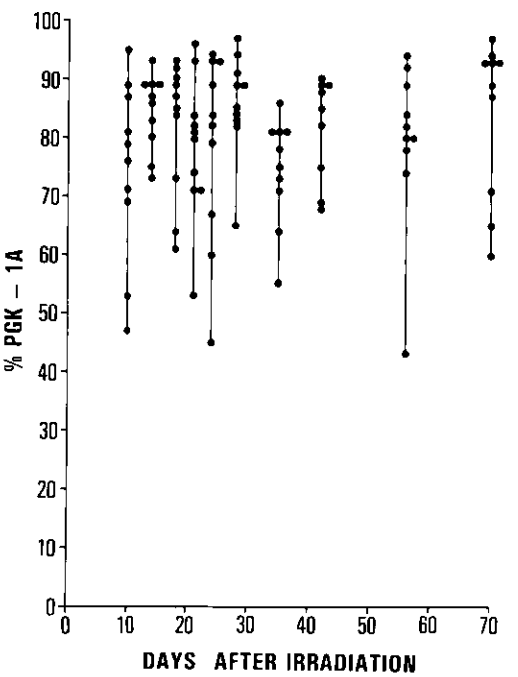
a) BLOOD



b) SPLEENS



c) LEFT FEMORA



d) RIGHT FEMORA

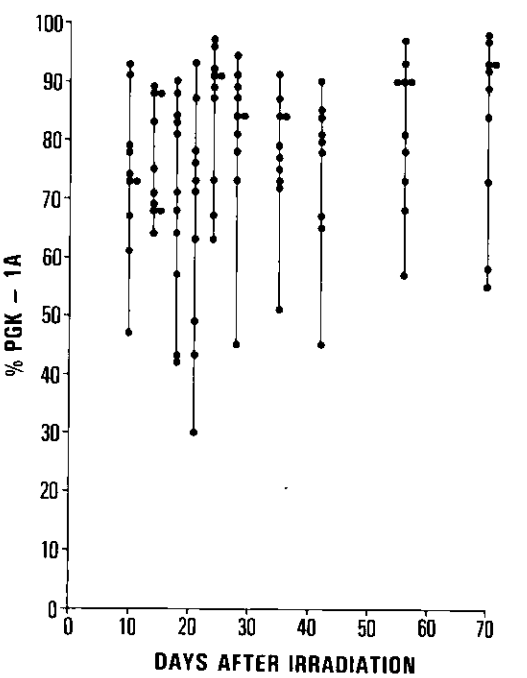


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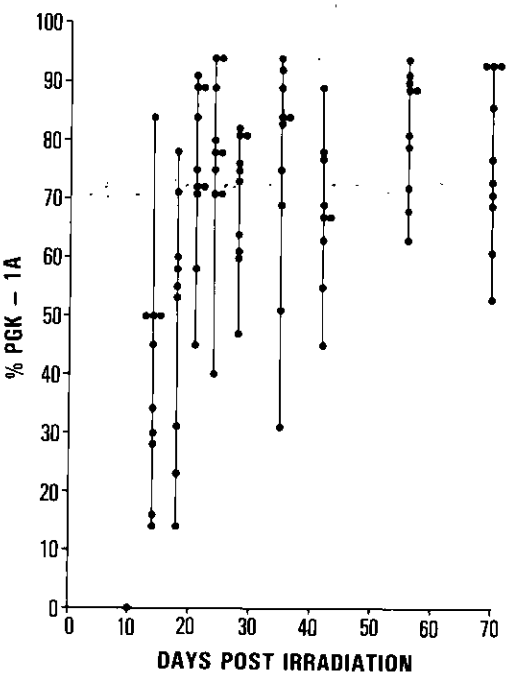
Figure 4.3 continued

(e) Left Thymus

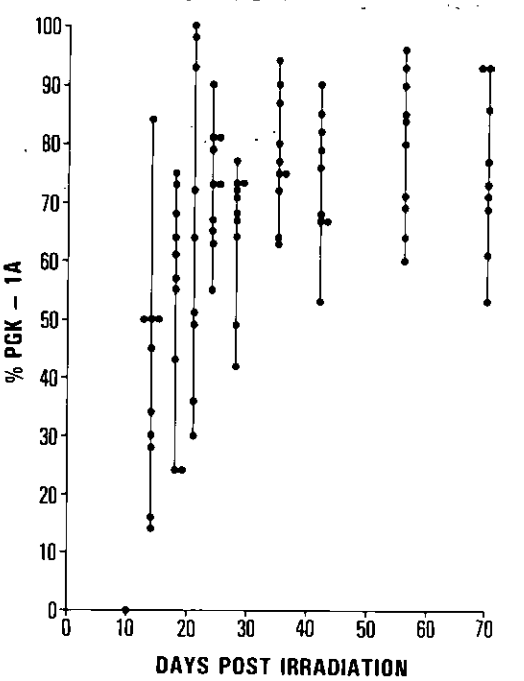
(f) Right Thymus

(g) Lymph Nodes (Pooled axillary and brachial).

e) LEFT THYMII



f) RIGHT THYMII



g) LYMPH NODES

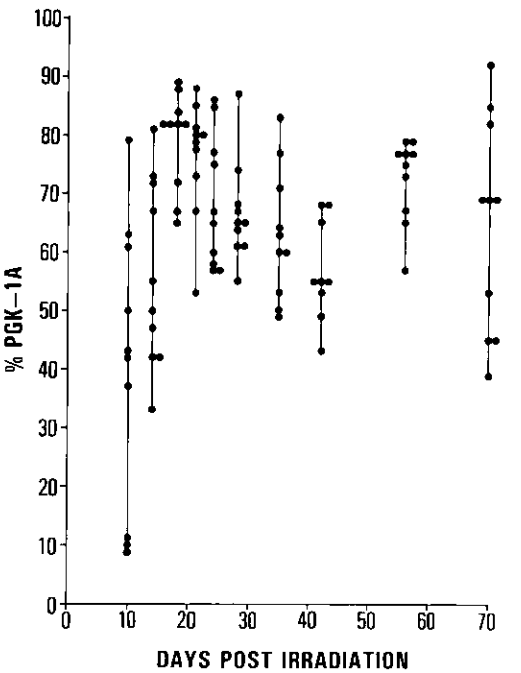


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Figure 4.4 Differences between Comparable Tissues in Irradiated and Repopulated Mice

PGK-1B mice were lethally irradiated and repopulated with 5×10^6 PGK-1AB bone marrow cells. Differences in % PGK-1A between (a) left and right femora and (b) left and right thymus lobes are shown. In all cases the % PGK-1A in the right tissue is subtracted from the left, hence the negative results in some cases. Each point represents a single animal.

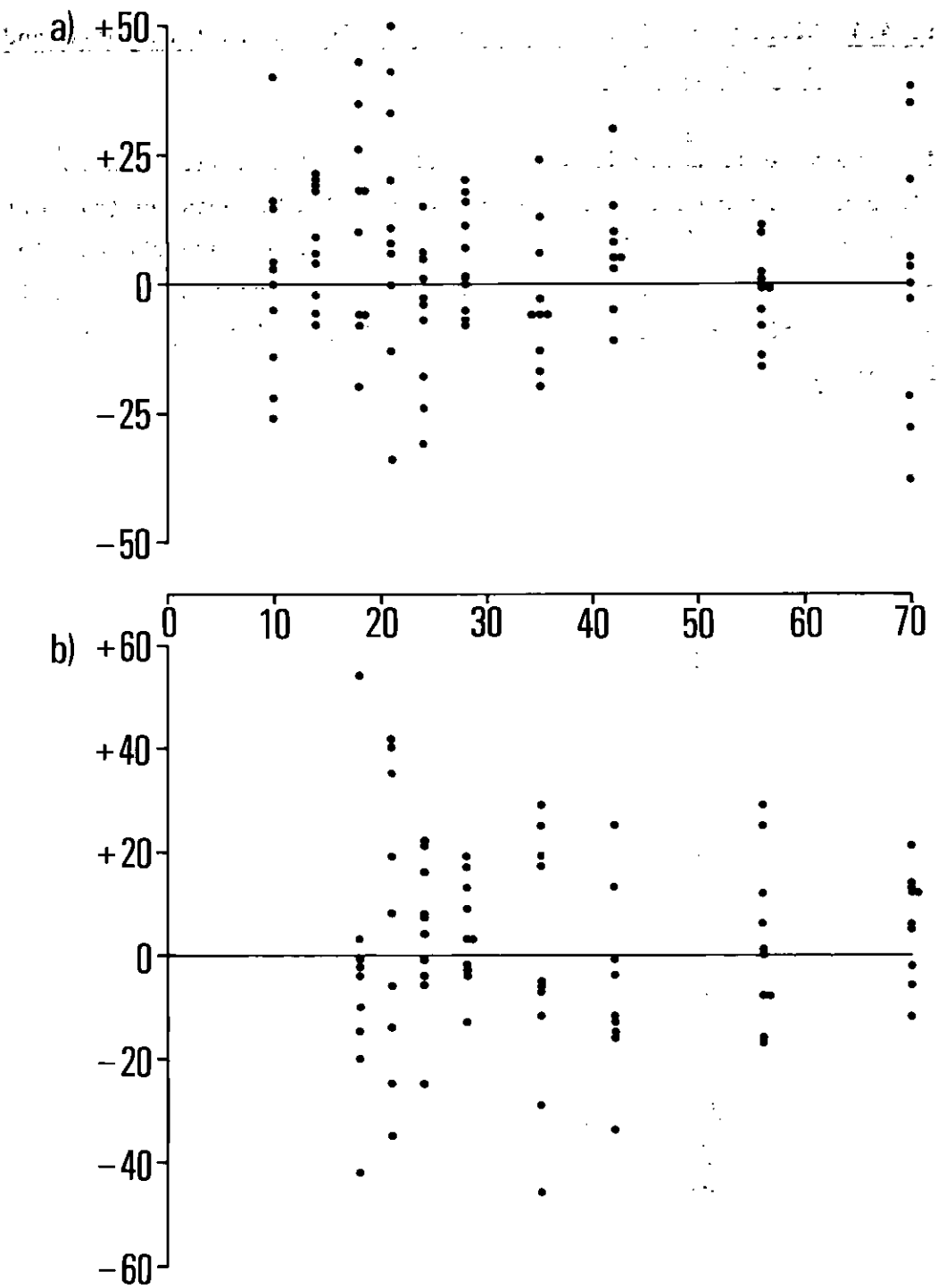
a)
+50
+25
0
−25
−50
0
10
20
30
40
50
60
70
b)
+60
+40
+20
0
−20
−40
−60

Table 4.2 Differences Between Individual Tissues Studied

Size of Difference (% PGK-1A)	% animals showing differences between	
	Left and right femora	Left and right thymus
0 - 9	48	42
10 - 19	26	33
20 - 29	14	15
30 - 39	8	4
40 - 49	3	5
≥ 50	1	1

example, if the differences between paired tissues decreased with time, this might suggest an increase in migrating stem cell numbers as time passes.

For statistical analysis, each variate was examined separately and the derived variates calculated:

$$\text{dfemur} = \text{left femur} - \text{right femur}$$
$$\text{dthymus} = \text{left thymus} - \text{right thymus}$$

Since the data were in the form of percentages, the angular transformation $\sin^{-1}\sqrt{pc/100}$ was also applied in order to homogenise the error variances within each group.

In the left femora there was no evidence of time-related variations in the proportion of repopulation instigated by PGK-1A stem cells. In the right femora, however, there was some evidence ($p < 0.05$) of an upward trend in the proportion of PGK-1A with time, with a rise of about 15% in PGK-1A activity over the test period. Examination of the paired differences table points to a significant difference between left and right femora (left > right, $p < 0.02$) at days 18 and 21, disappearing in subsequent days.

Although the proportion of PGK-1A in both left and right thymic lobes showed an upward trend ($p < 0.001$) which is most obvious at early times (up to day 21), there were no significant differences between left and right and no time-based variations in the differences.

The observed decrease in the differences between left and right femora with time is probably a reflection of the rise in percent PGK-1A contributing to haematopoiesis, rather than an indication of increased inter-tissue cell traffic.

The large differences between equivalent tissues within many of the

How do you visualise
repopulation by 10^7 vs 10^5 NSC
cells?

individuals tested are in agreement with the findings of Micklem and his colleagues (Micklem et al, 1972; 1975 I) and Wallis and her colleagues (1975). Both these groups used the T6 marker system and were able to inject two distinguishable donor cell types, T6/+ and T6/T6 (i.e. cells with one or two T6 chromosomes), into recipients with a third distinguishable chromosome complement (CBA, no T6 chromosome). Using such a marker, the proportions of the 2 donor cell types can be compared at all times. In this respect the T6 marker has a great advantage over PGK-1, which cannot be used in a similar fashion until repopulation is completely donor-based.

As a result of this limitation of the PGK-1 marker system, the only days on which a comparison between all tissues tested (with the exception of lymph nodes) could be made were days 56 and 70. The range of values found for percent PGK-1A donor present in each animal on these days is shown in table 4.3 (for full details see Appendix 2, table 5). The large range of PGK-1A values within individuals suggests that a small number of stem cells are responsible for repopulation in these animals. Further experiments on irradiated and repopulated animals in this laboratory have shown that in animals rescued with large (10^7) or small (10^5) numbers of bone marrow cells and subsequently bled at regular intervals, the larger bone marrow cell dose was associated with smaller variations between bleeds (Micklem et al., 1984). These findings support the view that the number of stem cells responsible for repopulation is at least partly determined by the number of bone marrow cells injected. In animals rescued by larger doses of donor bone marrow, observed differences between tissues are therefore likely to be smaller than in the present experiment. In addition, the large differences found between opposite femora and thymic lobes suggest that even following lethal irradiation there is relatively little interflow of regenerating cells between organs at this cell dose. Such findings may also be dependent upon the number of stem cells injected.

Table 4.3 Differences within Individual Animals

Day 56		Day 70	
Mouse	Range of PGK-1A (%)	Mouse	Range of PGK-1A (%)
1	43 - 93	1	55 - 93
2	60 - 90	2	71 - 94
3	63 - 81	3	60 - 93
4	69 - 90	4	82 - 93
5	68 - 97	5	53 - 93
6	49 - 89	6	58 - 94
7	68 - 91	7	65 - 93
8	78 - 94	8	72 - 98
9	67 - 84	9	73 - 92
10	68 - 81	10	71 - 93

CHAPTER 5 MAINTENANCE OF HAEMATOPOIESIS IN PHENYLHYDRAZINE-TREATED MICE

The drug phenylhydrazine hydrochloride (PHZ) causes haemolysis by denaturing haemoglobin and other proteins in red cells (Hughes-Jones, 1979) and has been shown to produce a transient increase (greater than ten-fold) in blood and spleen CFU numbers in the mouse (Hodgson et al., 1972; 1975; Rencricca et al., 1970) following three daily doses of 1 mg PHZ.

PHZ was injected into mice to attempt to stimulate a synchronised erythrocyte population by lysing the majority of red cells and so inducing a sudden increase in circulating reticulocytes. A synchronised erythrocyte population may present a clearer view of the involvement of individual clones of cells in red cell turnover.

Optimum dose of PHZ

This experiment was designed to calculate the optimum dose of PHZ per mouse for future experiments. Mice were injected with various doses of PHZ (Sigma no. P-7126) dissolved in distilled water (5 mg/ml) and adjusted to pH 7.0 with 2N NaOH. This mixture was made up fresh for each injection.

Four mice per group were injected intra-peritoneally (I.P) with the following doses on days 0, 1 and 2: group A, 0.8 mg; group B, 1.0 mg; group C, 1.2 mg; group D, 1.4 mg PHZ. Two drops of tail blood were taken from each mouse on days 0, 2, 5, 7, 8 and 10, and mixed with 10 µl citrate saline (1 vol. 30 g/L tri-sodium citrate (BDH) to 4 vol. 9 g/L sodium chloride (BDH)) containing 4% (w/v) bovine serum albumin (BSA, Sigma). 15 µl of New Methylene Blue (1g NMB (George T. Gurr Ltd.) in 100 ml citrate saline) was added and the mixture was incubated at 37°C for 10 minutes. Smears of the stained cells were made and reticulocyte numbers were estimated by counting

Not much difference between B, C, D at 7 days is there?

at least 100 cells on each smear. With NMB staining, erythrocytes are shown as pale blue cells while reticulocytes contain dark blue staining precipitates of RNA (Dacie and Lewis, 1968; Hughes-Jones, 1979).

Maximum reticulocytosis (figure 5.1, 64 - 82% of all red cells according to PHZ dose) was observed on day 7. A similar peak has previously been found on day 6-7 in rats (Stohlman, 1961) and on day 7 in mice (Hodgson et al., 1972). Day 7 was chosen for the first post-injection bleeds in future experiments. Mice given 1.2 mg PHZ/mouse/day consistently produced the highest reticulocytosis with 100% survival (figure 5.1, group C) and this was adopted as the standard dose for all future PHZ experiments.

Packed Cell Volumes Following PHZ Treatment

Measurements of packed cell volume (PCV) were made to establish the degree of anaemia suffered by individuals following PHZ treatment. Ten mice were injected with 1.2 mg PHZ as described previously and were bled, in rotation, on days 0 - 5, 7 and 9. Blood was taken up in plain capillary tubes (Gelman-Hawksley Ltd.), sealed and spun down for 5 minutes in a Hawksley microhaematocrit centrifuge. Results are shown in figure 5.2. Maximum anaemia of about 23% PCV was found on day 3 (20% in rats, Stohlman, 1961; 22% in mice, Rencricca et al., 1970). PCVs returned to normal by day 7 following the first PHZ injection.

Long term measurements of PCV were made on 8 PGK-1 heterozygotes (2 groups of 4, given identical treatments) given up to 6 sets of PHZ injections at 28 day intervals. PCV results are shown in figure 5.3. Although PCVs were greatly reduced after each set of injections no persistent anaemia was found in any of these animals even after repeated doses of phenylhydrazine. The haematopoietic tissues are well able to cope with the increased red cell output demanded of

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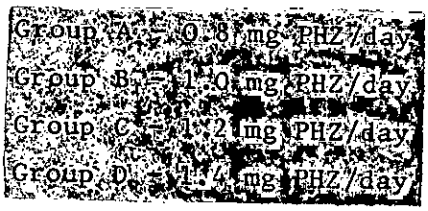
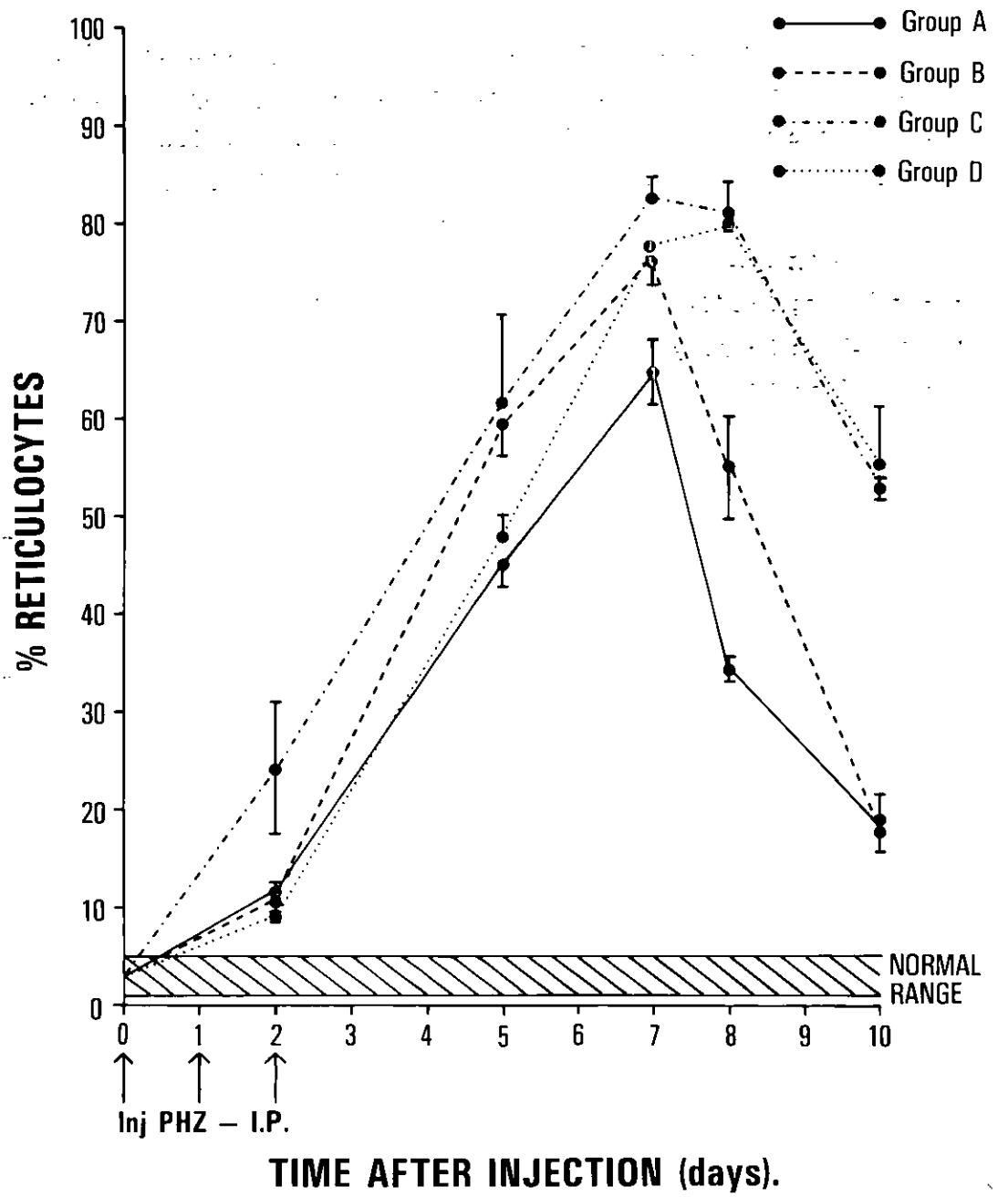
Figure 5.1 Induction of Reticulocytosis using Varying Quantities of Phenylhydrazine

The y-axis shows the number of reticulocytes as a percentage of all red cells. Means and standard deviations for each group are shown. The normal range was calculated from the percentage reticulocytes shown in all animals prior to treatment with phenylhydrazine.

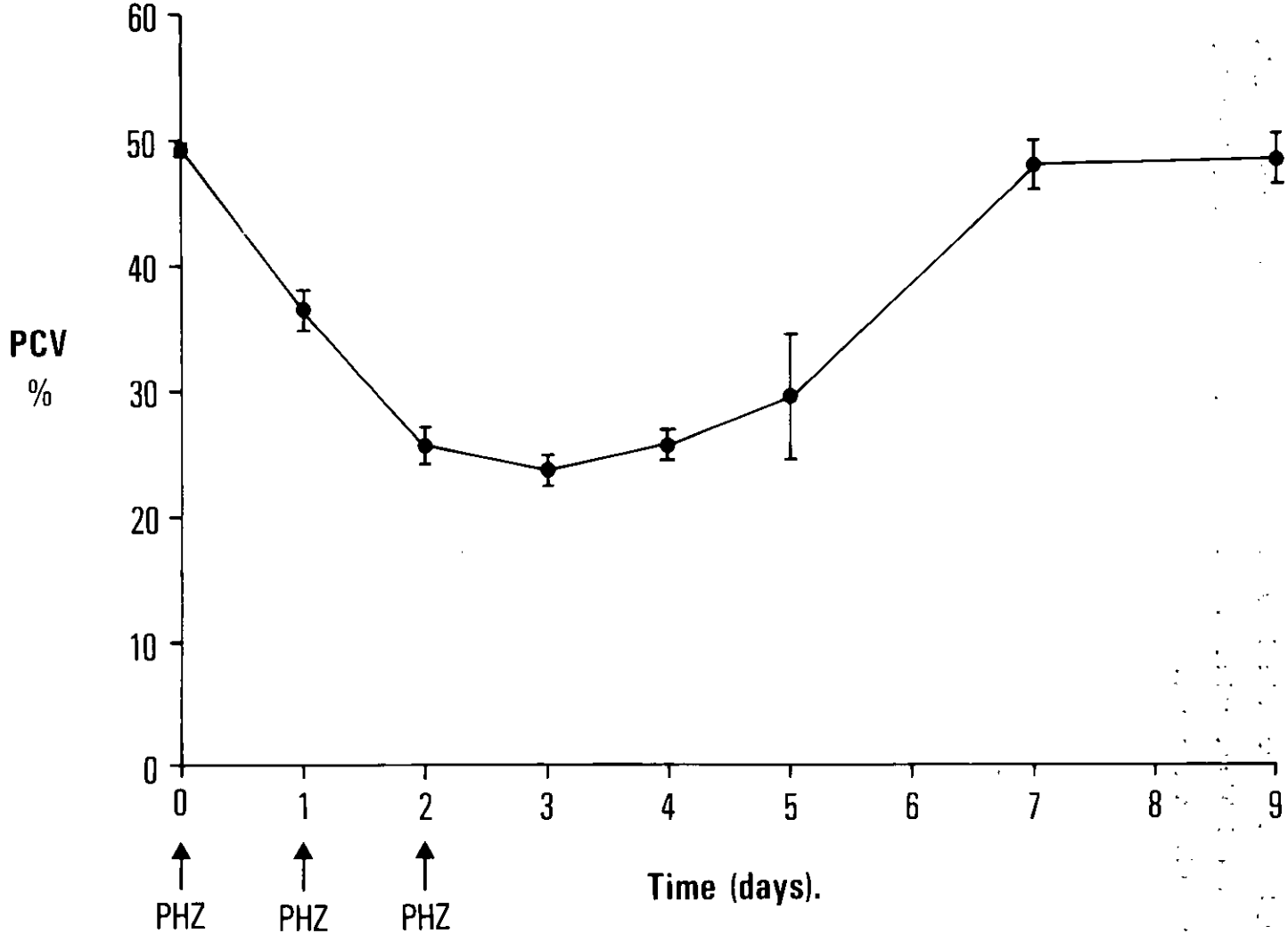



Group A
Group B
Group C
Group D
% RETICULOCYTES
100
90
80
70
60
50
40
30
20
10
0
NORMAL
RANGE
0
1
2
3
4
5
6
7
8
9
10
Inj PHZ — I.P.
TIME AFTER INJECTION (days).

facing page 107

Figure 5.2 Degree of Anaemia in Mice Treated with Phenylhydrazine

The degree of anaemia is shown by the packed cell volume (y-axis, % of total blood volume). On each day the mean and standard deviation for 5 mice are shown.


60
50
40
30
20
10
0
PCV
%
0
1
2
3
4
5
6
7
8
9
PHZ
PHZ
PHZ
Time (days).

facing page 108

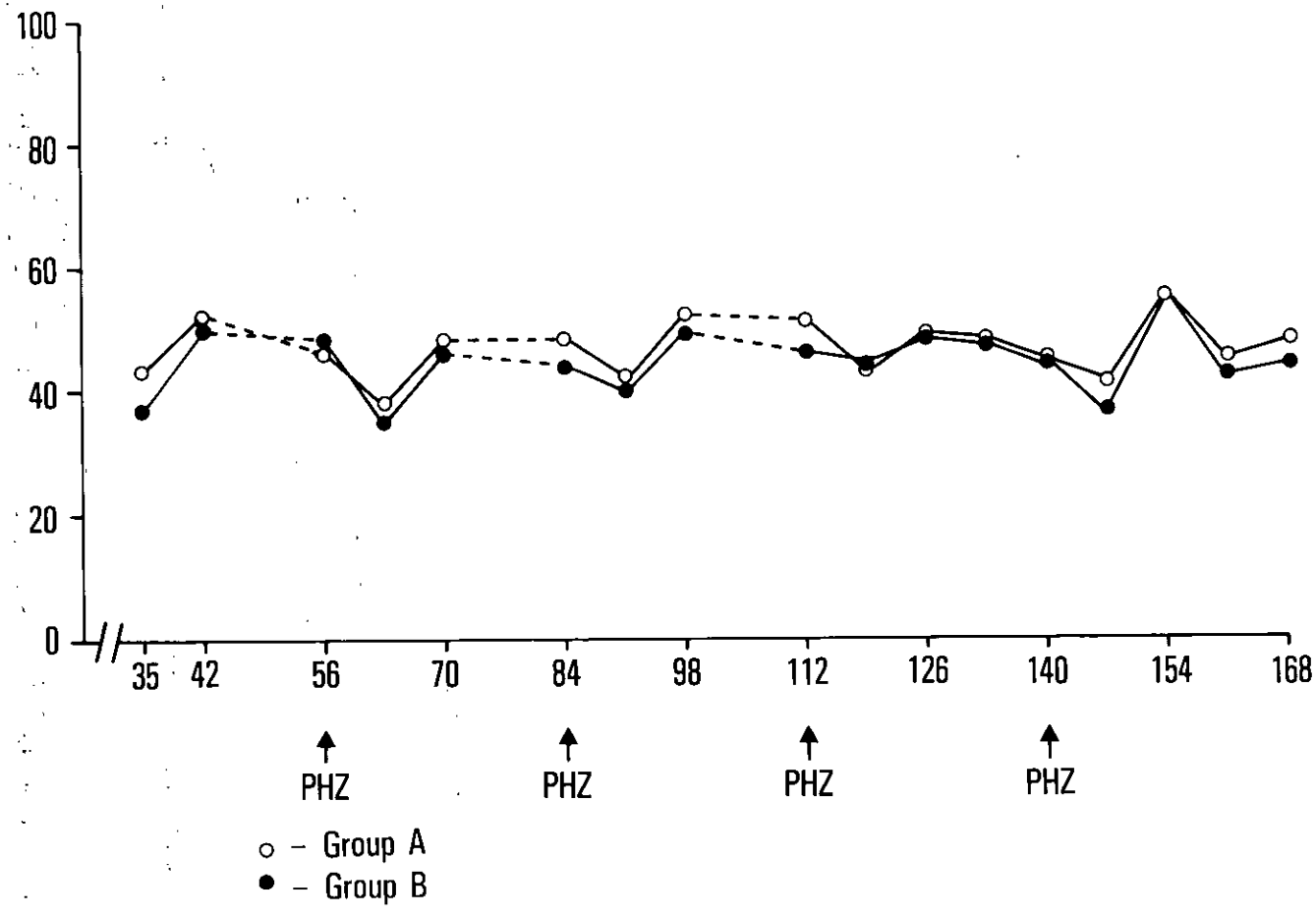
Figure 5.3 Variation in Packed Cell Volumes after Repeated Phenylhydrazine Treatments

The packed cell volume (y-axis) is shown as a percentage of the total blood volume

O - Group A - mean PCV of 4 mice

● - Group B - mean PCV of 4 mice.

Groups A and B were treated in an identical manner.



Days after beginning of expt.

PHZ injections are given AFTER the bleeds for that day.

stem cell pool — did you establish that production comes from SC pool?

them by repeated PHZ treatments.

Effects of Repeated PHZ Treatments on Red Cell Maintenance

Eight PGK-1AB mice (backcross generation 8, those in whom long term PCV measurements above were made) were used to study the effects of repeated PHZ treatments on erythrocyte turnover. This experiment was undertaken to investigate the effects on the haematopoietic system of having to maintain an increased production of red cells from the stem cell pool over a long period of time.

Having shown the marked effects of a single 3-injection regime of PHZ (i.e. up to 82% reticulocytosis) on the circulating blood, we decided to carry out a similar regime of injections at 28 day intervals. Animals were treated with PHZ for up to 168 days. They were injected with 1.2 mg PHZ/day on days 0, 1 and 2 of each 28 day cycle and blood samples were taken on days 0 (before injection), 7, 14 and later day 21 of each PHZ treatment cycle.

Results are shown in figure 5.4 (full tables of % PGK-1A/mouse/day can be found in Appendix 2, table 6) as deviations in percent PGK-1A from the mean for each sample in all the animals tested. In these animals (and in all other PHZ experiments) results were obtained from Helena gel electrophoresis. Estimates of the mean numbers of active stem cells in mice given repeated PHZ injections are shown in table 5.1. Calculations of corrected n were made by subtracting the variance found within duplicate samples (0.001225) from the experimental variance.

With the exception of mouse 5 (294 active clones) the active stem cell numbers in these PHZ treated animals are within the range found in normal animals (11 - 133, tables 3.1 and 3.5). Although the final output of red cells is greatly increased by repeated phenylhydrazine treatments these results indicate that such an increase in

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Figure 5.4 Variations in Blood PGK-1 Phenotype in Heterozygous Mice given Repeated Phenylhydrazine Treatments

Individual bleeds at 7 day intervals are shown as deviations (% PGK-1A) from the mean percent PGK-1A (0) for each mouse.

(a) Mouse 2, mean = 84
(b) Mouse 3, mean = 70
(c) Mouse 4, mean = 88
(d) Mouse 5, mean = 86
(e) Mouse 6, mean = 89
(f) Mouse 7, mean = 74

Mouse 1 showed virtually completely PGK-1A activity (see Appendix 2, table 6).

Mouse 8 showed only PGK-1A activity; therefore no data are presented for that mouse.

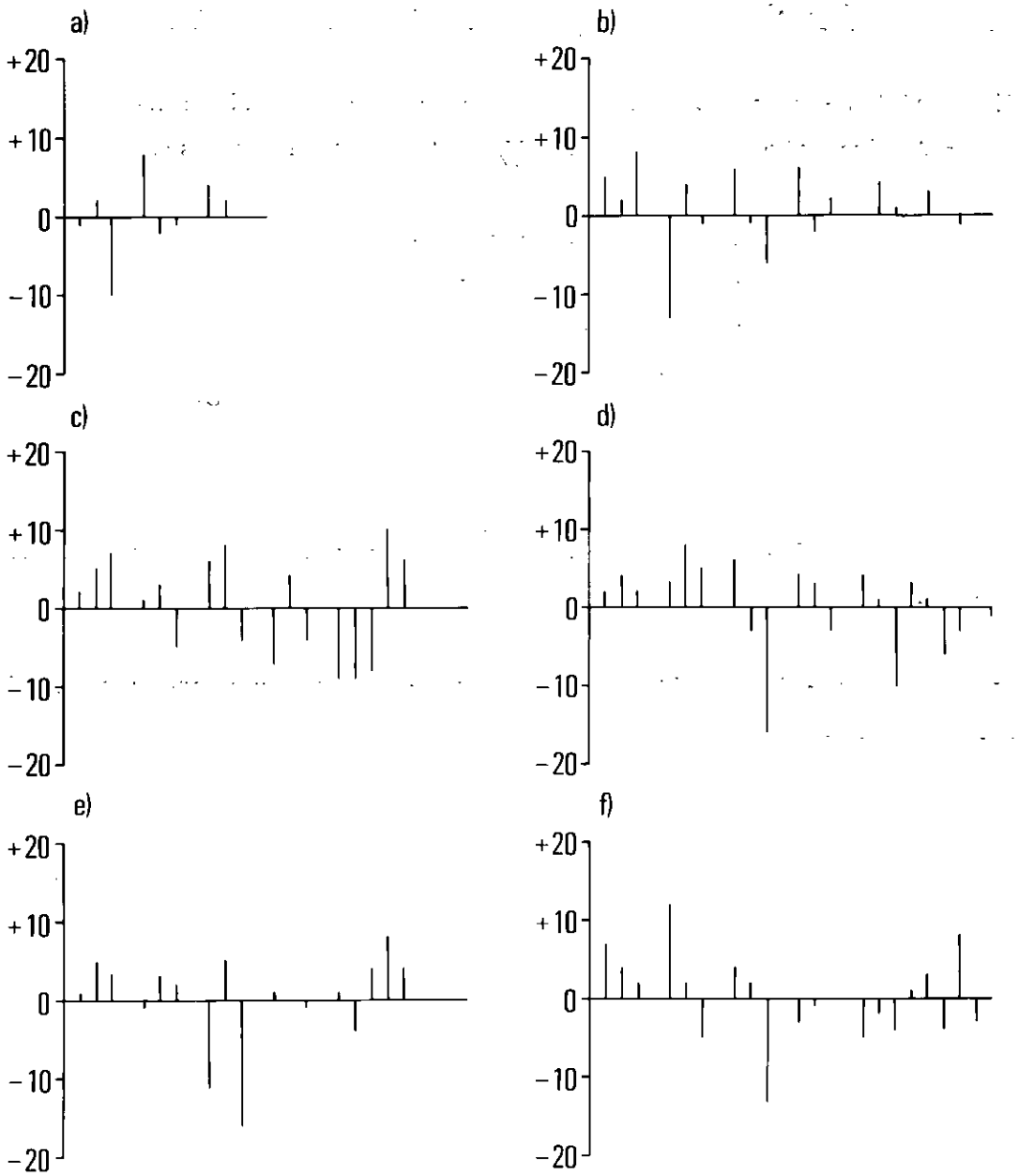
a)
b)
c)
d)
e)
f)
+20
+10
0
−10
−20

Table 5.1 Estimated Mean Numbers of Active Stem Cells Following Repeated PHZ Treatments

Mouse	n (to nearest integer)	corrected n
2	53	135
3	38	50
4	29	42
5	294	NA*
6	41	75
7	51	79

* NA - not applicable. In mouse number 5, the variance between samples is similar to that found between repeated samples. No correction can therefore be made by subtracting the variance of repeated samples from the experimental variance.

What do you make of
Wolf's data?
How does this tie up ~~with~~ ~~Ross et al~~ (the data) ?

production can be maintained by the same number of active stem cells. Increased 'end' cell production (in this case erythrocytes) may be achieved either by an expansion of the differentiating cell pool, or by a more frequent activation of primitive stem cells. Wolf (1982) claimed that repeated PHZ treatment exhausts the stem cell compartment, and this, if true, would be in agreement with a more rapid stem cell turnover in PHZ-treated mice.

Apart from erythrocytes, no other cell populations were assayed during the course of this experiment. However, because of the specific action of PHZ in denaturing haemoglobin, it is possible that no great change takes place in blood cell populations other than erythrocytes. If this is the case, the implication is that feedback control operates only on committed red cell precursors to increase their output following phenylhydrazine treatment.

Effects of a Single PHZ Treatment on Red Cell Maintenance

Eight PGK-1 heterozygous female mice (backcross generation 8) were treated with a single series (3 x 1.2 mg PHZ on consecutive days) of PHZ injections and bled at seven day intervals for up to 140 days following treatment. Graphs showing changes in phenotype within these mice are shown in figure 5.5. Estimates of active stem cell numbers are given in table 5.2 (for full results see Appendix 2, table 7). As with the previous experiment these numbers, with the exception of one individual (mouse 6) fall within the normal range. One might imagine that erythrocyte precursor activity would be synchronised at the time of treatment and that new clones become progressively less synchronised as time passes and stem cells with varying repopulating potential recover from the increased demand following treatment. This might be expected to give rise to a highly regular pattern of changing phenotype at the first few sample times following treatment with a gradual decline in regularity as less synchronised clones arise. Such a course of events cannot, however, be

Figure 5.5 Variations in Blood PGK-1 Phenotype in Heterozygotes Given One Phenylhydrazine Treatment

Individual bleeds at 7 day intervals are shown as deviations (% PGK-1A) from the mean percent PGK-1A (0) for each mouse.

(a) mouse 1, mean = 61
(b) mouse 2, mean = 71
(c) mouse 3, mean = 78
(d) mouse 4, mean = 84
(e) mouse 5, mean = 88
(f) mouse 6, mean = 88
(g) mouse 7, mean = 67
(h) mouse 8, mean = 85

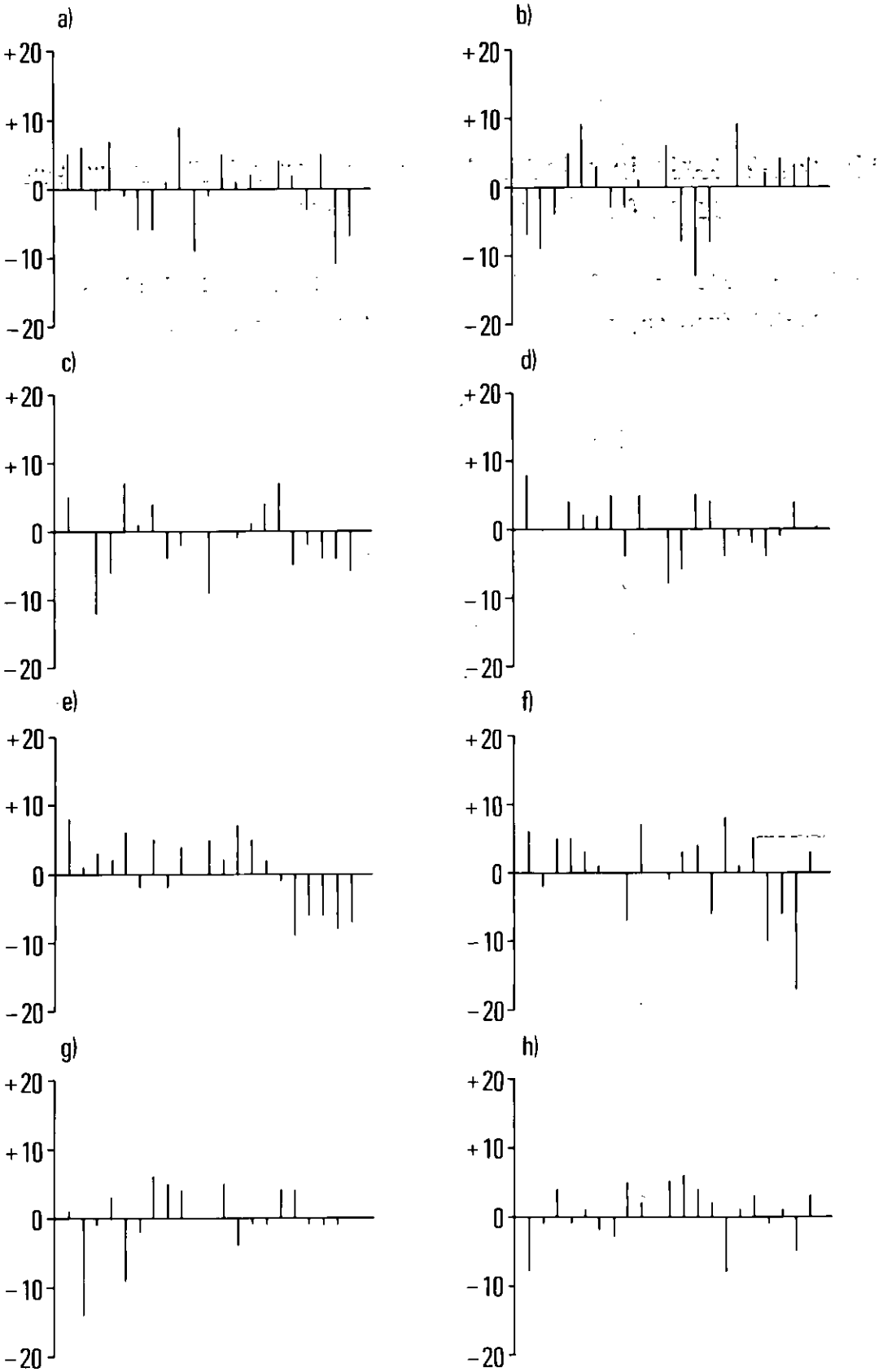
a)
b)
c)
d)
e)
f)
g)
h)
+20
+10
0
−10
−20

Table 5.2 Estimated Mean Numbers of Active Stem Cells Following Single PHZ Treatment

Mouse	n (to nearest integer)	corrected n
1	80	139
2	34	42
3	62	109
4	83	785
5	17	21
6	264	NA
7	71	117
8	42	89

NA = Not applicable

Also Wright & Co.

substantiated by the results shown in figure 5.5.

Effects of Bleeding Mice at Seven Day Intervals

Because of the frequency of bleeding required in the preceeding two experiments, it is impossible to draw direct comparisons between these and the results obtained from longitudinal studies of normal heterozygotes (bled at 14 or 28 day intervals). As a control to establish the variation induced in individuals by frequent bleeding (at least 150 ul every 7 days) a group of eight heterozygotes (back-cross generation 10) were bled at seven day intervals without PHZ treatment.

In most of these animals the results (figure 5.6 and Appendix 2, table 8) are quite similar to those found in normal heterozygotes. Active stem cell numbers were calculated from bleeds taken at 28 day intervals and these are shown in table 5.3. These figures must be viewed with some caution due both to the small numbers of samples (3) used to calculate them and to the likelihood that these samples are not independent (a problem in all the PHZ experiments) although a shortened erythrocyte lifespan has been reported in response to acute blood loss (Berlin and Lotz, 1951). A recent experiment in this laboratory (Ansell, personal communication) in which PHZ-induced reticulocytes were transfused into PGK-congenic recipients, did not, however, indicate any marked reduction in lifespan.

Previous workers have reported conflicting results in CFU numbers following PHZ treatment. Rencricca and his colleagues (Rencricca et al., 1970) reported a drop in bone marrow CFU to about 50% of their normal levels 5 days after PHZ treatment. These authors also noted a concomitant increase (to levels approximately 4 times normal) by day 3, and lasting for several days in spleen CFU numbers. They reported that the observed changes in CFU numbers were not due to CFU proliferation but to migration of CFU to the spleen following

Figure 5.6 Variations in Blood PGK-1 Phenotype in Heterozygotes Bled at Seven Day Intervals

Individual bleeds at seven day intervals are shown as deviations (% PGK-1A) from the mean % PGK-1A (0) for each mouse.

(a) mouse 1, m = 87
(b) mouse 2, m = 74
(c) mouse 3, m = 61
(d) mouse 4, m = 78
(e) mouse 5, m = 68
(f) mouse 6, m = 62
(g) mouse 7, m = 85
(h) mouse 8, m = 93

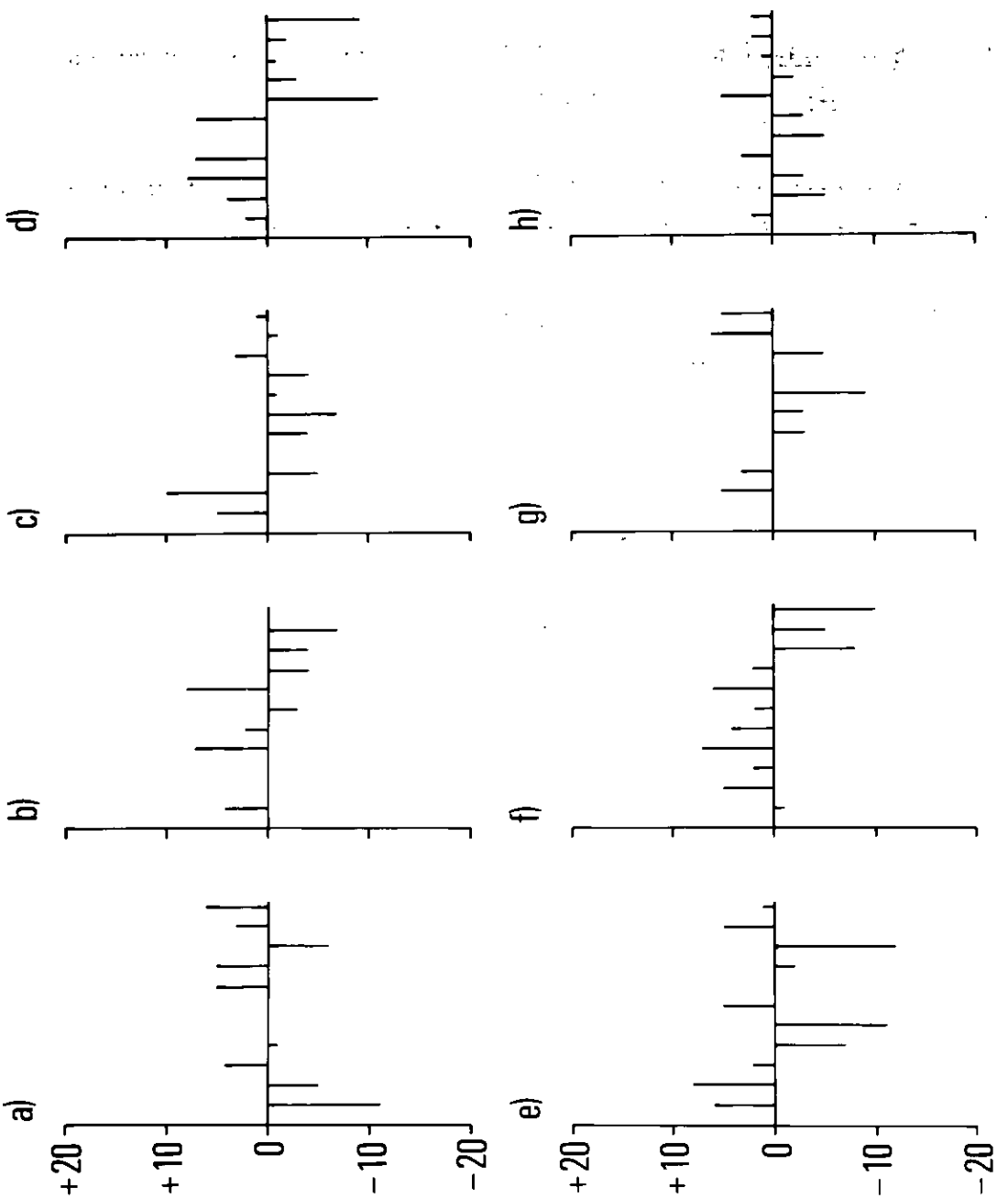
a)
b)
c)
d)
e)
f)
g)
h)
+20
+10
0
−10
−20

Table 5.3 Estimated Mean Numbers of Active Stem Cells in Mice Bled every Seven Days

Mouse	n (to nearest integer)	corrected n
1	138	NA
2	107	350
3	106	239
4	382	NA
5	23	26
6	66	101
7	233	NA
8	49	324

NA = Not applicable

severe anaemia. They also noted that CFU in the bone marrow entered cell cycle later than 5 days after PHZ treatment. As animals which receive repeated doses of erythropoietin also show an increase in splenic CFU with little or no change in bone marrow CFU, these authors suggested that the splenic and bone marrow erythroid hyperplasia observed after severe PHZ anaemia may reflect a physiological response to the severe anaemia subsequent to increased erythropoietin production.

Conversely Hodgson and his colleagues (Hodgson et al., 1972) noted a ten-fold expansion in spleen CFU, while CFU numbers in bone marrow showed no changes. ACFU (cells capable of forming colonies in an agar medium) numbers showed greater changes (splenic ACFU up to 40 times normal with a great reduction in bone marrow ACFU). These authors also noted an increase in CFU in peripheral blood following PHZ treatment (also Hodgson et al., 1968; Hodgson, 1973). This increase may indicate a migration of CFU from the bone marrow to other tissues after severe anaemia as suggested by Rencricca and his colleagues, although Hodgson (1973) reported that circulating CFU-S in PHZ-treated mice had a low self-renewal capacity and therefore doubted their role as precursors of spleen CFU-S in PHZ-treated mice which he found to have a high self-renewal capacity. Hodgson (1973) also reported an expansion of the erythropoietin-sensitive cell pool in phenylhydrazine pre-treated animals, suggesting an increase in differentiating red cell precursors.

The results of these two groups suggest that different mechanisms may be responsible for the maintenance of erythropoiesis following PHZ in different mouse strains. Rencricca's and his colleagues' studies in CF_1 mice suggest that splenic CFU-S numbers increase by migration of CFU-S from the bone marrow. In the studies of Hodgson and his colleagues, however, spleen CFU-S numbers appear to increase in situ, although there may be evidence of ACFU migration from the bone marrow. These different patterns could be due to genetic

factors, to external factors (such as infection) or to a mixture of both types influencing the splenic environment.

Rencricca and his colleagues (1970) suggested that under normal circumstances the committed compartments in haematopoietic differentiation are, in large measure, self-sustaining. However, after damage to a committed compartment (in this case erythroid following PHZ treatment) cells from the pluripotent stem cell pool (which they identified with CFU-S) must enter cell cycle to replace the cells leaving the committed compartment.

The increases in CFU numbers noted by these authors may be caused by the activation of more primitive pluripotent stem cells, with greater self-replicating and differentiating ability than those stem cells normally replenishing the haematopoietic system. If this is so, larger numbers of committed precursors and mature end cells will be seen due to increased numbers of doubling divisions during differentiation, rather than due to increased clone numbers. Feedback stimuli in response to the sudden-onset anaemia caused by PHZ may cause those stem cells which are active under normal circumstances to differentiate early in the response to PHZ-treatment, necessitating the activation of stem cells with a greater capacity to self-renew.

The generally higher numbers obtained for active stem cells in mice which have been bled at seven-day intervals may represent a situation half-way between those found in normal and PHZ-treated animals. Cell loss caused by such regular bleeding may be too great for the normal number of cells to maintain haematopoiesis adequately, without providing a strong enough stimulus to activate more primitive stem cells. In this case a greater number of stem cells of normal potency may be activated to cope with the increased demand. If this is the case, then one might expect a gradual change in the pattern of repopulation in mice treated with only one series of PHZ injections as compared to

either those treated repeatedly with PHZ or the group which were simply bled once a week. If small numbers of stem cells are responsible for the initial repopulation following PHZ one would expect to see quite large variations in PGK-1 phenotype. As normal red cell numbers are restored a change to the type of maintenance proposed in response to repeated bleeding may be perceived as a reduction in the variation of PGK-1 phenotype (caused by increased stem cell numbers) or by an increase in the rate of change of PGK-1 phenotype. There is no consistent evidence from the present data (figure 5.4) that such changes in stem cell maintenance occur.

CHAPTER 6 RECOVERY OF HAEMATOPOIESIS IN HYDROXYUREA-TREATED MICE

The drug hydroxyurea can ablate over 90% of cycling cells within the haematopoietic system after five injections. Sinclair (1965) suggested that hydroxyurea either prevents entry of cells into S-phase or inhibits, or kills, cells in S-phase. Philips and his colleagues (1967) showed that HU selectively kills cells in S-phase. It acts by inhibiting the reduction of ribonucleotides to deoxyribonucleotides (Young et al., 1967). This effect has also been demonstrated in CFU-S (Hodgson and Blackett, 1977).

Hydroxyurea is rapidly cleared from the plasma; for a dose of 1 mg/g body weight, the plasma half-life is 0.80 $\pm$ 0.09 hours (Hodgson et al., 1975). This rapid clearance ensures that the majority of cells killed are those in S-phase at the time of injection.

A number of authors have shown that hydroxyurea administration significantly increases the proliferation rate in CFU populations in vivo (Vassort et al., 1973; Nečas and Neuwirt, 1976; Nečas and Hauser, 1982). Nečas and Činátl (1982) further showed that the increased proliferation rate could be enhanced if HU was administered in two doses separated by two hours. They noted that this enhancement did not appear to be caused by more extensive bone marrow damage, although one would expect a few more CFU to be killed by the additional HU injection.

Hodgson and his colleagues (Hodgson et al., 1975) showed that two doses of HU, separated by 7 hours, would kill 90% of CFU-S in mice which had previously been irradiated (700 rads). In normal mice, however, the same regime killed only about 30% of CFU-S, but 92% of CFU-C (granulocyte/macrophage progenitors, Bradley and Metcalf, 1966) and 99% of ESC (erythropoietin sensitive cells, Filmanowicz and Gurney, 1961; Stephenson et al., 1971). Although only about 10% of CFU-S are in cycle at any given time, both CFU-C (Iscove, Till and McCulloch,

1970) and ESC (Hodgson, 1967) are actively proliferating in normal mice. Nečas and Hauser (1982) showed that hydroxyurea kills approximately 80% of CFU-S which have entered S-phase by measuring the number of cells killed by repeatedly injecting ^{3}H - TdR after HU treatment.

The mean transit time of CFU-S through S-phase has been estimated to be four hours (Vassort et al., 1973) and 4.79 $\pm$ 1.45 hr (Nečas and Hauser, 1982). This is approximately half the total cell cycle time of normal bone marrow CFU (estimated to be 7 to 8.6 hours by Schofield and Lajtha, 1969; Vassort et al., 1973; Schofield et al., 1980). Nečas and Hauser also showed that 10 - 15 hours after a single HU injection, 60 - 70% of CFU-S were synchronised in a cell cohort 1 to 2 hours wide.

A number of investigators have used five injections of HU over a thirty hour period to kill actively cycling CFU (Hodgson and Blackett, 1977; Micklem and Ross, 1978; Rosendaal et al., 1976; 1979). This regime has been shown to kill over 90% of bone marrow CFU (Rosendaal et al., 1976; Micklem and Ross, 1978). New CFU entering cell cycle after an injection of HU will be destroyed by further injections.

Whole body irradiation followed by injection of flushed bone marrow cells almost certainly causes damage to the haematopoietic microenvironment. Because HU selectively kills dividing cells, treatment with HU should allow investigation of repopulation patterns within haematopoietic tissues without destroying the haematopoietic microenvironment.

Forty female mice heterozygous for the Pgk-1 locus (3 months old, backcross generation 9) were injected I.P. with 1 mg/g HU (Sigma) in 0.2 ml saline, 5 times in 30 hours. Fourteen control heterozygous mice were similarly injected with 5 volumes of 0.2 ml saline. Mice were injected at -30, -24, -8, -5 and 0 hr to destroy the maximum

number of cycling stem cells (method modified from Rosendaal et al., 1976). Haematopoietic recovery in femora, humeri, spleen and separate lobes of thymus was investigated in groups of 5 mice on days 2, 5, 8, 14, 21, 28 and 35 following the final injection. Two control mice were also sacrificed and tested on each day. All tissue samples were treated and stored as previously described in chapter 4. On days 2 - 8 all samples were resuspended and stored in 100 µl sample buffer. From day 14 onwards, spleens were resuspended in 200 µl sample buffer and all other tissues in 100 µl. All samples were stored at -60°C prior to electrophoresis.

All spleens were weighed and bone marrow cellularity was estimated for one femur per mouse using an improved Neubauer haemocytometer. Results for spleen weights and femur cellularities are shown in figures 6.1 and 6.2. In both cases the normal range is calculated from values for each parameter found in control mice.

Femur cellularity was depressed on day 2 showing the rapid effects of HU injections on bone marrow cells. By day 5 cell numbers were increasing as recovery of haematopoiesis occurred in the bone marrow. By day 21 femur cellularity had reached a maximum of up to four times the normal level. Femur cellularity then dropped once more and stabilised at normal levels by day 28.

Splenic cellularity, as assessed by total organ weight, also decreased by day 2 after HU, but to a much smaller degree than in bone marrow reflecting the fact that the spleen is full of T and B cells, relatively few of which are cycling compared to marrow cells. After day 2 spleen weights increased and overshot the normal range reaching maximum weights by day 14 and returning to approximately normal weights by day 21. Spleen weights remained slightly above normal for the remainder of the test period.

Spleen cellularity may increase and reach maximum levels earlier than

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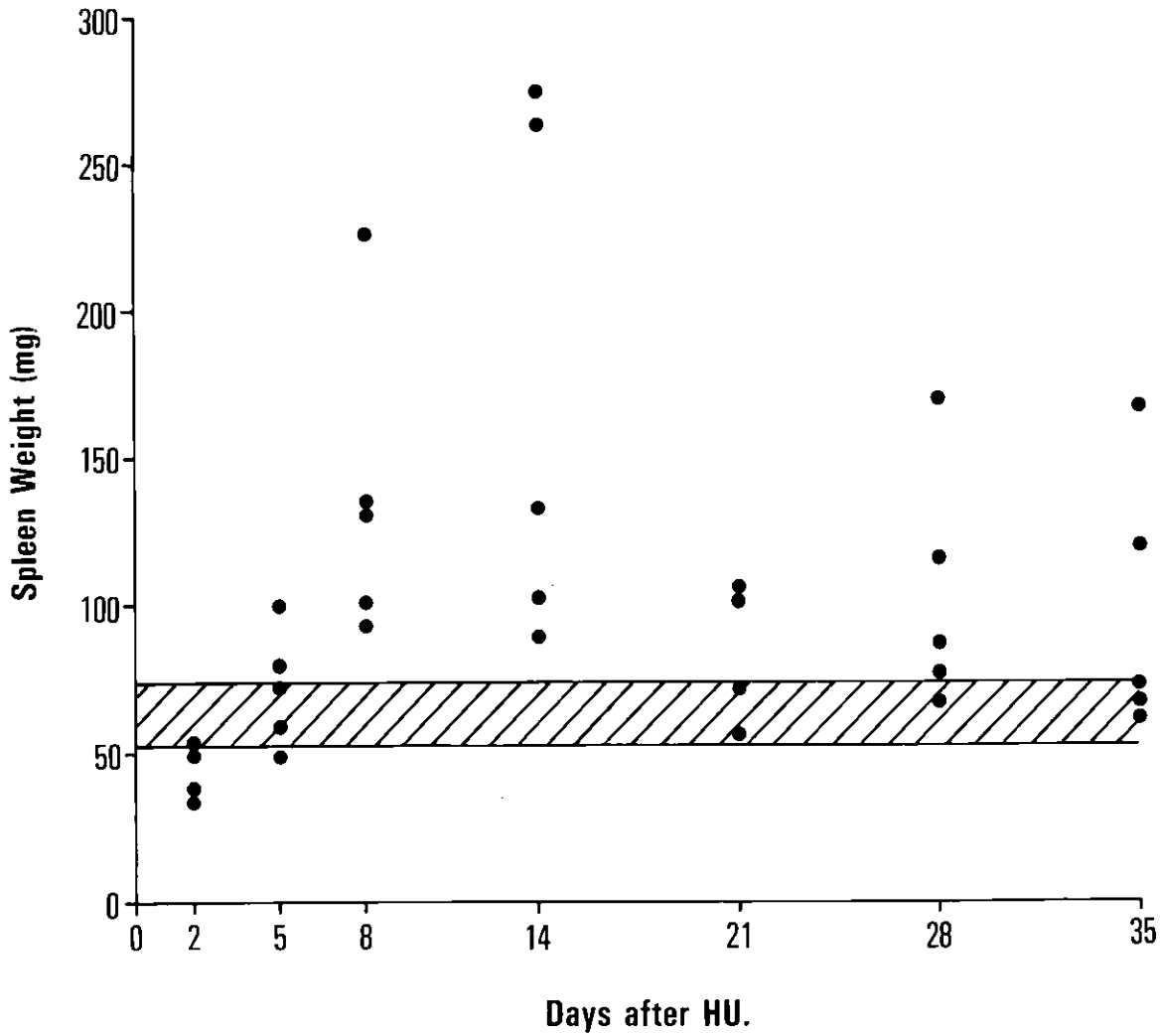
Figure 6.1 Spleen Weights in Mice Treated with Hydroxyurea

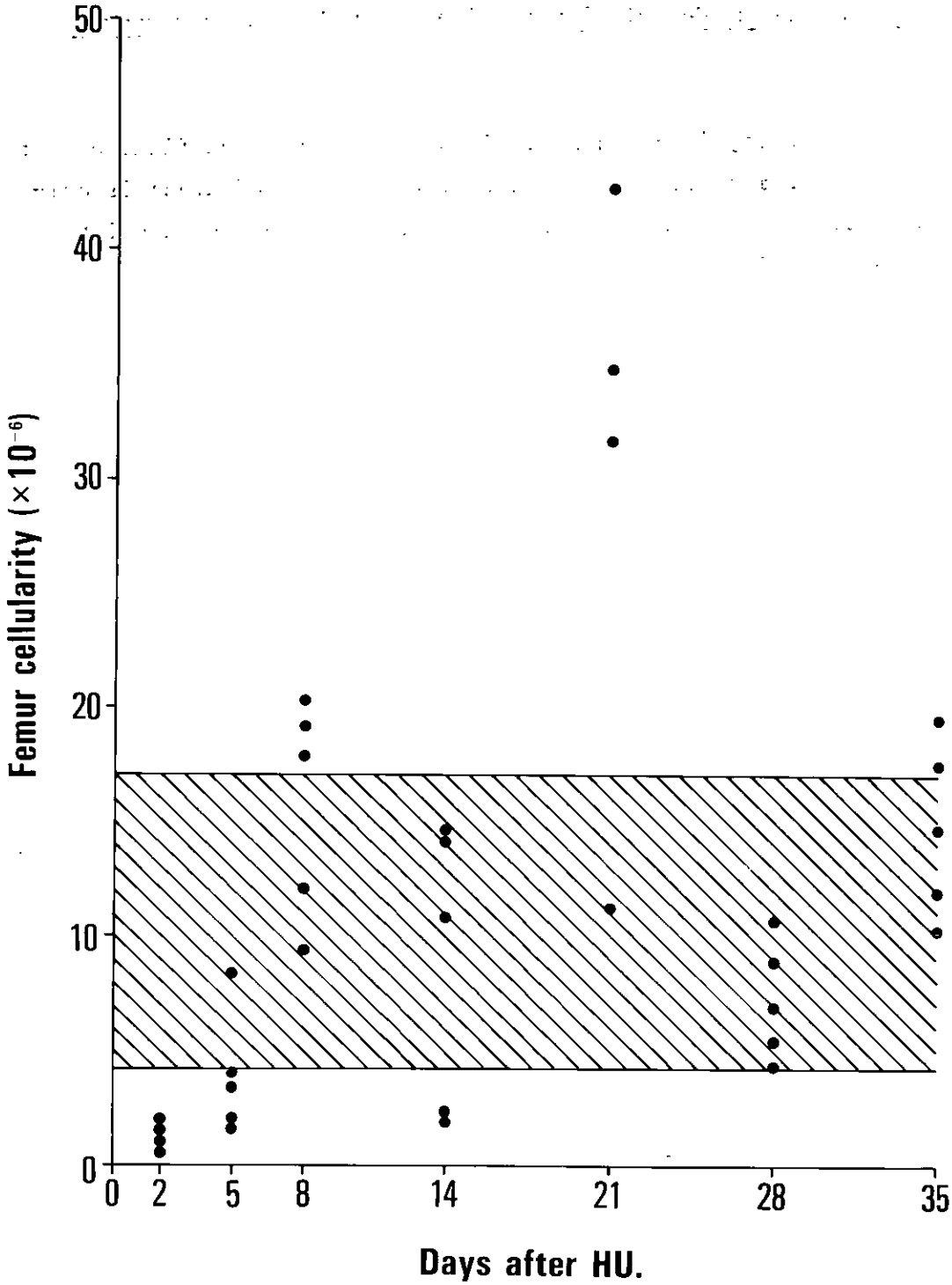
Spleen weights are shown at various times after the final one of 5 hydroxyurea injections. The normal range of spleen weight (shaded area) is calculated from control animals injected with saline. Each point refers to an individual spleen.


Spleen Weight (mg)
300
250
200
150
100
50
0
0
2
5
8
14
21
28
35
Days after HU.

facing page 125

Figure 6.2 Femur Cellularity in Mice Treated with Hydroxyurea

Femur cellularity is shown at various times after the final one of 5 hydroxyurea injections. The normal range (shaded area) is calculated from control animals injected with saline. Each point represents an individual femur.


50
40
30
20
10
0
Femur cellularity (×10⁻⁶)
0
2
5
8
14
21
28
35
Days after HU.

bone marrow for two reasons. First, although the spleen does not support haematopoiesis under normal conditions it does contain about 10^4 potential CFU-S. It is therefore possible that the spleen rapidly regenerates from its own CFU-S. Alternatively, the spleen may serve as a reservoir for more mature blood cell precursors which could expand quickly in response to severe haematopoietic stress to produce adequate numbers of end cells to support the individual while more primitive stem cells in the bone marrow proliferate to restore the bone marrow stem cell pool to its normal balance. When the spleen's resources are exhausted, the bone marrow has recovered adequately to allow migration of maturing cells to the spleen where they can complete their maturation. A third possibility is that haematopoietic recovery may begin in the bone marrow with self-renewal and differentiation of the most primitive stem cells. To allow maximum expansion of the primitive (pluripotent) stem cells, differentiating cells may migrate to the spleen to complete the maturation process.

Mean values of PGK-1A as a percentage of total PGK-1 activity ($\pm$ standard deviations) for each mouse on each test day following HU treatment are shown in table 6.1. Standard deviations are included to show the variation observed within individual mice (full results for all tissues in all individuals are given in Appendix 2, table 9). Although the variations within individuals suggest that different clones may be responsible for the maintenance of haematopoiesis in different organs this cannot be verified using the present data. In order to estimate stem cell numbers responsible for haematopoiesis within an individual one must obtain independent samples from that individual (for example, repeated blood sampling as in chapter 3). In this experiment only one sample from each tissue was taken. In addition individual tissue samples from each animal cannot be regarded as independent as each stem cell clone may contribute to the maintenance of haematopoiesis in a number of tissues. As a result, no estimations of the number of clones responsible for haematopoiesis in HU-treated animals are presented here.

Table 6.1 PGK-1A activity (mean ± s.d. for each animal) following HU treatment

Days after HU treatment	HU treated mice					Control mice	
	1	2	3	4	5	1	2
2	56 ± 23	82 ± 9	83 ± 8	73 ± 13	71 ± 23	47 ± 20	78 ± 11
5	76 ± 16	83 ± 6	84 ± 10	82 ± 12	78 ± 13	74 ± 14	77 ± 10
8	80 ± 6	70 ± 10	71 ± 8	74 ± 14	81 ± 10	76 ± 14	66 ± 8
14	81 ± 8	77 ± 13	82 ± 15	88 ± 3	80 ± 11	69 ± 14	87 ± 6
21	76 ± 14	NT*	83 ± 8	83 ± 7	67 ± 9	74 ± 9	86 ± 7
28	63 ± 10	89 ± 14	83 ± 14	86 ± 6	86 ± 6	79 ± 16	71 ± 17
35	90 ± 6	84 ± 8	81 ± 7	55 ± 8	82 ± 4	87 ± 7	80 ± 9

* NT - Not tested. When dissected this animal was found to have a diseased liver and no tissue samples were taken for analysis.

If individual tissues are studied within the whole group on each day then estimates of the numbers of clones responsible for maintaining haematopoiesis in each organ at any given time may be made (table 6.2). These numbers should, at least partly, reflect the number of embryonic cells responsible for founding the haematopoietic system soon after X-inactivation. In table 6.2 control mice have been treated as one group.

The clone numbers are much more variable in experimental mice than in controls although the majority of these estimations are comparable to the numbers calculated for controls. Control numbers compare reasonably well with previous estimates of primordial cell pool sizes for individual tissues. Nesbitt (1971) estimated pools in mice of 21 cells for spleen and 23 for thymus, while Fialkow (1973) a primordial cell pool of 10 cells for human lymph nodes.

Hydroxyurea treatment causes severe damage to the stem cell pool, with only those stem cells most resistant to entering cell cycle able to survive.

The more variable estimates of clone numbers in experimental animals suggest that, in some cases at least, the active clones within a population do not reflect the primordial cell pool for that population. The range of variances found in experimental animals is much greater than in the control series. This may reflect the fact that HU treatment leads to regeneration from fewer than normal clones in some animals, but from more than the normal number of clones in other animals. On the other hand, the wide spread of variances seen in experimental groups might simply reflect the relatively small group sizes.

From examination of the actual figures obtained for PGK-1 proportions within each tissue of each individual, differences between left and right femora, humeri and thymus lobes can be calculated. Results

Table 6.2 5HU treatment : clone numbers maintaining haematopoiesis in individual tissues

Day	TISSUES						
	Left Femur	Right Femur	Left Humerus	Right Humerus	Left Thymus	Right Thymus	Spleen
2	3	4	18	60	66	5	7
5	14	9	18	6	**	**	11
8	26	35	3	21	**	**	13
14	N.A.*	15	55	9	3	49	6
21	19	3	7	2	37	8	4
28	5	10	4	6	4	6	4
35	29	8	6	5	3	27	6
Controls	8	6	8	9	13	8	11

* The variance obtained within the five samples was about the same level as that found in duplicate sampling, and therefore could not be used to estimate clone numbers.

** On days 5 and 8 post-treatment, thymic samples had too little PGK-1 activity to be analysed.

are shown in figure 6.3 and table 6.3. The HU data were examined statistically (see Materials and Methods) for trends consistent with time, differences between treatment and control groups, and heterogeneity of trends between treatment and control. Each variate was treated separately and these derived variates calculated:

dfemur = l. femur - r. femur
dhumerus = l. humerus - r. humerus
dthymus = l. thymus - r. thymus

Since the data are in the form of percentages, the angular transformation $\sin^{-1}$ pc/100 was also applied in order to homogenise the error variances within each group, in keeping with assumptions for Analysis of Variance.

In the left femur there was little evidence of a clear trend in PGK-1 phenotype with time, nor was there any difference between treatment and control groups. In the right femur, however, there was slight evidence of a time trend (p 0.1) and this was borne out on examination of dfemur where there is slight evidence of a steady negative time trend (p 0.05). Results for left and right femora appear to converge. The differences between left and right femora decline steadily from day 2 (with left right, p 0.01).

In the humeri no evidence of trends in PGK-1 phenotype with time or due to treatment was found. There were no trends in the differences between left and right humeri with time.

There were no trends due to time or treatment in left thymus samples. However the right thymus showed good evidence of a time trend (p 0.02) with PGK-1A values increasing steadily, but levelling out by day 35. There were no systematic differences between treatment and control groups.

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Figure 6.3 Differences between Comparable Tissues in Heterozygotes Treated with Hydroxyurea

PGK-1AB mice were treated with 5 injections of hydroxyurea over 30 hours. Differences in percent PGK-1A between (a) left and right femur, (b) left and right humeri and (c) left and right thymic lobes are shown. Negative results occur because the result from the right tissue is always subtracted from the left. Each point represents a single animal.

● - HU-treated

O - control

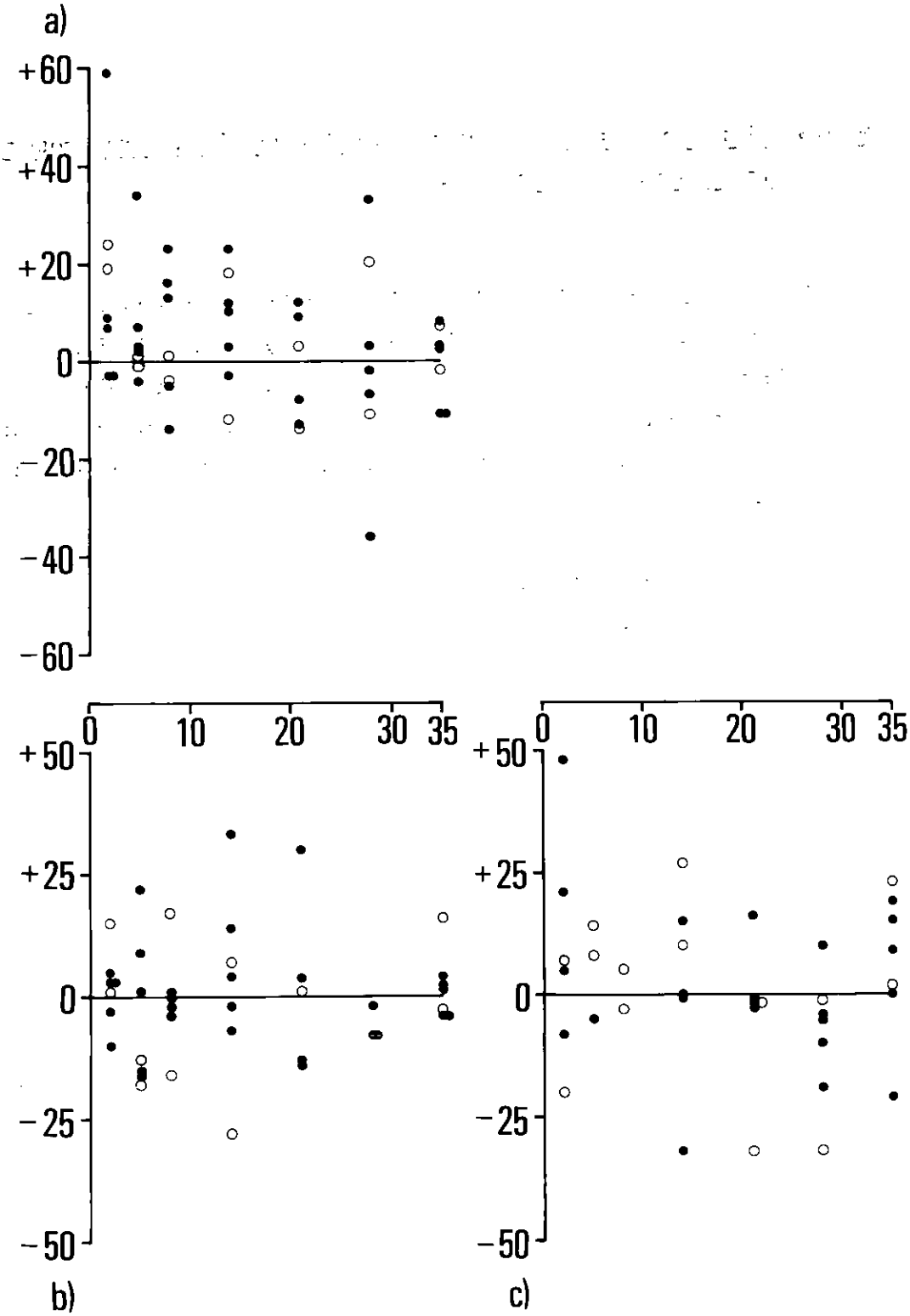
a)
+60
+40
+20
0
−20
−40
−60
0
10
20
30
35
+50
+25
0
−25
−50
b)
c)

Table 6.3 Differences (percent PGK-1A) between corresponding tissues in individuals

Percent Difference in PGK-1A	Percent of total estimations of difference between					
	Left and right femur		Left and right humerus		Left and right thymus	
	HU	Control	HU	Control	HU	Control
0 - 9	56	50	73	50	56	51
10 - 19	26	36	15	43	28	21
20 - 29	6	14	6	7	8	21
30 - 39	9	0	6	0	4	7
40 - 49	0	0	0	0	4	0
⩾ 50	3	0	0	0	0	0

Previous authors (Rosendaal et al., 1976) have suggested that "super stem cells", i.e. stem cells of much greater proliferative capacity are responsible for repopulating mice subjected to repeated hydroxyurea treatment. Both these authors and Micklem and Ross (1978) have shown that CFU from hydroxyurea-treated animals can self-renew more effectively than CFU from normal animals. If stem cells in normal animals do have a smaller proliferative capacity than those which are active in hydroxyurea-treated animals, then there must be a more rapid turnover of active stem cells in these animals. By repeatedly bleeding normal and hydroxyurea-treated mice over a long period any differences in the rate of stem cell turnover should become obvious as the rate of change of PGK-1 phenotype will vary. Alternative methods of measuring the proliferative capacity of stem cells from each group would be to measure the number of stem cells repopulating irradiated hosts, by CFU self-renewal assay or to measure the capacity of bone marrow cells of each experimental type to proliferate in vitro.

Micklem and Ross (1978) suggested that results obtained following hydroxyurea treatments represent the events of normal haematopoiesis better than experiments studying repopulation following lethal irradiation. These authors lethally-irradiated CBA mice and injected them with 10^8 T6/T6 and T6/+ cells (1:1 mix). Eight weeks later mice were injected with hydroxyurea or saline and later (3 weeks or more) killed for cytological examination of the bone marrow. Clone numbers were estimated using the binomial equation as in the present experiment. They estimated that about 5 clones were responsible for regeneration of the bone marrow in HU-treated animals while more than 100 clones were found to be active in saline-treated animals. This large difference between HU and saline-treated mice has not been found in the present experiment (table 6.2) where results for both groups are much more similar. All the animals tested in Micklem and Ross's experiments were, however, irradiated and repopulated with marked cells prior to HU treatments in order to allow the binomial equation to be used. The larger number of clones found in the

saline-treated animals as compared to lethally irradiated and repopulated animals in the present study (table 4.1, page 94) are as would be expected from the results obtained in previous repopulation studies showing that the number of stem cells active in repopulated animals is proportional to the number of cells injected (Wallis et al., 1975; Micklem et al., 1984). One would expect to find many more active clones when 10^8 cells are injected as opposed to 2.5×10^6 cells.

The PGK-1 marker system allows the effects of HU treatment to be studied in mice whose haematopoietic systems have had no previous insult. In this respect, the mice were much more "normal" than those studied by Micklem and Ross. The present data suggest that in their saline-treated animals, Micklem and Ross were merely measuring the number of stem cells responsible for repopulation of irradiated animals. In their hydroxyurea-treated mice, the previously active stem cells responsible for repopulation will have been virtually ablated leaving only those stem cells which, even under extreme repopulation stimuli, have so far remained resistant to entering cell cycle. These cells may well be ancestors of the colony forming cells destroyed by HU. Cells which are so resistant to stimuli to divide are likely to be extremely primitive stem cells, and, as such, may reflect accurately the phenotype of the haematopoietic primordial cell pool of the donor mice. To this extent HU treated mice will represent normal haematopoiesis better than irradiated animals.

CHAPTER 7 GENERAL DISCUSSION

Before discussing the PGK-1 assay system in comparison to other systems used to investigate cell maintenance within the haematopoietic system, a few general comments should be made regarding the system itself.

At the beginning of the period of study, the PGK-1 assay system was in its infancy. During the course of the experiments reported here a number of difficulties became apparent. Although at first it was thought that single electrophoresis of individual samples would be adequately accurate, it soon became clear that this was not the case, in spite of extremely good results in repeatability tests. Multiple testing of individual samples showed that repeated freezing and thawing led to a rapid decline in the overall activity of PGK-1. This made analysis of PGK-1 ratios much more difficult and increasingly inaccurate. This made apparent the importance of dividing samples into several aliquots for storage to maximise the accuracy of analyses. There was insufficient time available for a systematic comparison of storage methods to be carried out.

The use of a spectrophotometer to analyse PGK-1 activity within individual tissues would allow standardised dilutions of samples for electrophoresis to be made. In the present study dilutions were made purely according to sample size. In experiments in which the haematopoietic tissues had been depleted this led to samples being too low in PGK-1 activity to allow measurement of PGK-1 ratios to be made.

With regard to PGK-1 activity, it must be noted that throughout the present study, no allowance was made for possible differences in PGK-1 activity between different tissues sampled, although these may well exist. For the experiments in which different tissues were assayed, activity differences between tissues should have no bearing on the results as they should not affect the proportions of each

allozyme found in a particular tissue. Of much greater importance in estimating stem cell numbers is the assumption which must be made to allow statistical analysis. This is that all stem cells produce clones of equal size and longevity. It is extremely unlikely that this is the case in practice. As a result it must be emphasised that the numbers of active stem cells quoted here are only estimates.

Data presented here do, however, imply that the CFU-S may not be the ultimate progenitor cell of the haematopoietic system, but that CFU-S are themselves members of substantial clones of cells (Micklem et al., 1982). This is in agreement with the work of many previous authors whose results have proved inconsistent with the hypothesis that the CFU-S is the primary haematopoietic stem cell (Ogden and Micklem, 1976; Harrison et al., 1978; Schofield, 1978). The present data suggest that the haematopoietic stem cell may precede the CFU-S by as many as 10 doubling divisions. Such an hypothesis explains the large differences found between "stem" cell numbers estimated using CFU-S techniques (Smith et al, 1968; Rencricca et al., 1970) and those estimated using the binomial approach (Wallis et al., 1975; Micklem and Ross, 1978; Ross et al., 1982; Micklem et al., 1982). Although the use of the binomial method (using either chromosome or enzyme markers) adds weight to evidence that the CFU-S is not the primary haematopoietic stem cell, it does not allow a means of identifying this evasive cell. The binomial approach simply allows investigation of progenitor cells by studying their offspring. In this respect enzyme markers, such as PGK-1A, may prove to be a more effective tool than chromosome markers as their use allows the investigation of cell populations as a whole, rather than only a small number of dividing cells. In addition, the estimation of proportions of each PGK-1 allotype is much less tedious than the cytological investigations necessary to gain the same results using the T6 marker system.

The T6 marker system is not, however, without its advantages, not least the presence of an additional marker (3 rather than 2 as in the case of PGK-1) which greatly enhances its effectiveness in repopulation studies. Chromosome markers are applicable to individual cells, a facility as yet impossible with the PGK-1 marker, although a number of workers are presently engaged in attempting to make monoclonal antibodies to PGK-1A and 1B. Such monoclonal antibodies might allow individual cells to be examined using fluorescein-conjugated monoclonal antibodies if the enzyme could be labelled sufficiently to detect individual cells. Even if individual cells could not be labelled, monoclonal antibodies should still allow in situ investigation of cell populations within tissues, for example in serial sections of bone marrow. Such an advance would greatly enhance the ability of workers to estimate the patch size of cell populations within tissues, and also to study organogenesis during embryonic life.

It appears that although we may be creeping closer to the true haematopoietic stem cell, we are still unable to find it. Just as in the past the CFU-S compartment has been shown to comprise a heterogeneous cell population, so the "stem" cells enumerated in the present study also appear to be heterogeneous in their ability to repopulate the haematopoietic tissues as approximately similar numbers of stem cells are able to maintain haematopoiesis under normal circumstances and when an increased output is necessary. Whether we are enumerating the same cell population in each situation remains unclear. If we are in fact measuring true pluripotent stem cell numbers, then cell numbers are maintained following stress to the system, not by an increase in the number of stem cells themselves, but by an expansion of the number of descendents these cells produce. Early offspring of these cells are likely to retain at least some of the pluripotency of their parents. In recent experiments in vitro, Dexter and his colleagues showed that stem cells in culture have a tremendous proliferative reserve (Dexter,

personal communication) but that when daily feeding is given the number of stem cells in the medium is decreased while they are increased in the stroma where they undergo maturation. The total number of stem cells within the total culture remains very similar however.

In order to produce large numbers of cells in response to haematopoietic stress (either by loss of mature cells or by depletion of the stem cell pool) the cell cycle time is probably shortened. Schofield and his colleagues (Schofield et al., 1980) showed that CFU-S which were derived from marrow which was recovering or had recovered from some depopulation event had a shorter cell cycle time than CFU-S from normal marrow. These authors found that CFU-S with a shorter cell cycle time had a lower self-renewal capacity than CFU-S from normal marrow. Similarly, others have noted an association between poor self-renewal ability and a high frequency of cells in S-phase (suggesting a higher frequency of cells in cycle, Gidali et al., 1974; Micklem et al., 1975). Such results are in agreement with the suggestion of Lajtha (1979) that CFU-S undergo some sort of rejuvenation during the G_o phase of the cell cycle. If stem cells are having to undergo increased numbers of divisions to maintain cell numbers then their offspring will have no opportunity to rejuvenate themselves and hence extend their ability to self-renew. Such an hypothesis serves to explain to some extent why repeated transfer of stem cells, or repeated sub-lethal irradiation lead to a rapid decline in the repopulating ability of CFU-S (Siminovitch et al., 1964; Hendry and Lajtha, 1972; Vos and Dolmans, 1972; Wu and Lajtha, 1975).

Cairns (1975) suggested that extended self-renewal depends on being able to keep the original DNA, precluding expansion of the stem cell pool. In animals whose haematopoietic tissues have been depopulated, stem cells must divide to increase the numbers of cells in the stem cell pool. As a result, the self-renewal capacity of this pool will,

if Cairns is right, be reduced. Evidence that this happens in vitro has been reported by Mauch and his colleagues (Mauch et al., 1980). These authors found that in early cultures the self-renewal capacity of the CFU-S was reduced but after 3 weeks in culture the self-renewal capacity stabilised and was maintained. They suggested that the initial decrease in self-renewal capacity occurred while the primitive stem cell pool was established. Self-renewal capacity could however be depressed in early cultures because the haematopoietic microenvironment is stimulating cells to differentiate rather than to self-renew.

Mauch and his colleagues reported that after the initial culture period the self renewal capacity of the adherent CFU-S within the culture was maintained for at least 13 weeks. These authors suggested that CFU production and cell maintenance were achieved by successive activation of clones of cells from the established stem cell pool, that is, that clonal succession was operating in the cultures.

Some authors have attempted to estimate the mitotic lifespan of CFU-S (80 - 200, Ogden and Micklem, 1976; not less than 158, Valeriote and Tolen, 1983) on the assumption that CFU-S proliferate homogeneously and that clonal succession does not occur. These numbers are many times smaller than the number of divisions estimated by Kay (1965) to be necessary if cell numbers are to be maintained by a tangential system of cell division. Such numbers become irrelevant, however, if cell numbers are maintained by clonal succession, as the number of cell generations necessary to maintain the blood system throughout life (about 50 in the human, Kay, 1965) is far smaller.

The data presented here suggest that under normal circumstances, and also in the blood of repopulated mice, haematopoiesis is maintained by clonal succession. Unfortunately the frequency and size of swings in PGK-1 are generally not large enough to prove conclusively that clonal succession is responsible for blood cell maintenance. If

there were frequent large changes (of about 50%) in the percentage of PGK-1A present in blood samples (as early results suggested, Burton et al., 1982) then it would be impossible for the same clones to be repopulating the blood continuously. As the graphs show, however, many of the differences found within animals in longitudinal studies are only about 10% or less, and quite a few of these are not greater than the expected variations found on repeated electrophoresis runs. There are, however, a number of larger changes which are substantially greater than can be accounted for by inaccuracies in the method. Large swings in phenotype will only be seen if both the following conditions are fulfilled: (1) that clonal succession is operating and (2) that few clones are active at any time. Once the number of active clones approaches the range of 50 - 100, the expected swings become rather small (cf computer simulations, Burton et al., 1982).

In order to clarify the role of clonal succession in the maintenance of haematopoiesis, future longitudinal studies of the blood, such as those described here, should be carried out on purified cell populations. The variety of lifespans and rates of turnover of blood end cells is such that differences in the clonal composition of each cell type may be concealed by the presence of other cell types. In addition clonal expansion might occur at a variety of levels depending on the stimuli within the system at any given time. For instance, some stem cells may give rise only to erythrocytes, macrophages or lymphocytes and others might give rise to progeny of all cell types, in the same way as spleen colonies of single or mixed cell types can be found in animals repopulated with CFU-S.

In irradiated and repopulated animals, and in mice subjected to treatments with cytotoxic drugs, the results presented here appear to agree with results of previous workers that small numbers of stem cells are responsible for regeneration of the haematopoietic system (Wallis et al., 1975; Micklem et al, 1975 I; Micklem and Ross, 1978).

Even under conditions of severe stress, stem cell proliferation appears to be a local phenomenon with relatively little cell traffic between tissues. That large differences should be found in different tissues of an individual is in accord with previous reports that control of differentiation and proliferation is maintained locally by short-range factors or cell interactions (Maloney and Patt, 1969; Croizat et al., 1970; Gidali and Lajtha, 1972; Rencricca et al., 1970; Tubiana et al., 1974).

If control of haematopoiesis is local, what is the relationship between stem cells and the cells with which they are closely associated? In vitro studies have shown that in bone marrow cultures the adherent layer which supports haematopoiesis is made up of phagocytic mononuclear cells, epithelial cells and "giant fat" cells (Dexter et al., 1977; Weston and Bainton, 1979). These cells make up the stromal element of the haematopoietic system. Normal stem cells are able to grow in the absence of stromal cells if appropriate regulators are constantly present, but in normal culture circumstances the stroma is necessary for haematopoiesis to occur. The factors and intercellular relationships which control the proliferation of stem cells and their differentiation into committed precursor cells, are of fundamental importance in understanding the mechanisms controlling haematopoiesis. What is the relationship between stromal cells and haematopoietic stem cells? Do they share a common ancestral stem cell? Some authors would suggest not (Bentley et al., 1982) although mononuclear phagocytic cells are commonly found in the adherent layers of bone marrow cultures and these are derived from haematopoietic stem cells. In order to understand and treat haematopoietic dysfunction an understanding of the total haematopoietic microenvironment is necessary. Breakdown of normal haematopoiesis is not only caused by defective stem cells, but also by a defective environment, as is the case in Sl/Sl^{d} mice. Furthermore the kinetics of the environment may play a large part in the efficacy of treatments for haematopoietic disorders.

It has been generally assumed that the cells which make up the stromal element of the bone marrow are relatively radio-resistant and radiotherapy is a commonly used treatment for cancer. Recent experiments have shown, however, that this is not the case (Werts et al., 1980; Tavassoli, 1982; Ploemacher et al, 1983) with doses of as little as 500 rads causing the loss of the ability of stromal cells to support and maintain proliferation of haematopoietic stem cells in culture (Tavassoli, 1982). The effects of ionising radiation might have been missed by earlier workers because the haematopoietic stroma is made up of slowly dividing cells. Ploemacher and his colleagues (1983) suggested that stromal injury might at first be confined to the stroma which exerts a stimulus for CFU-S proliferation, but that it does not affect CFU-S maintenance, seeding and maturation until later. They found no improvement in stromal injury up to six months after total body irradiation. Maloney and her colleagues (1983), however, reported that delayed radiation damage to the marrow microenvironment was highly localised, leading to a decrease in the number of effective stem cell domains, but that growth within the remaining domains continued with normal kinetics.

In irradiated and repopulated animals, is the stroma of donor or recipient origin? Maloney and Patt (1972) showed that following partial body irradiation, cells which migrated from shielded bone marrow were responsible for recovery of haematopoiesis in the irradiated marrow. They presumed that the migrating cells were haematopoietic stem cells which found, in the irradiated site, a microenvironment conducive to proliferation.

More recently, however, Werts and his colleagues (Werts et al., 1980) carried out experiments on total body and partially irradiated mice and on mice which had one limb irradiated then, 3 hours later, were total body irradiated with the exception of the previously irradiated limb which was shielded. From their results they concluded that stromal repopulation preceded haematopoietic recovery and that

immigration of stromal cells from an unirradiated site facilitated haematopoietic recovery of a heavily irradiated site. Are stromal cells migratory? Can they be transplanted? Recent reports appear to give conflicting views.

Bentley and his colleagues (1982) concluded from their transplantation experiments using mice that the haematopoietic stroma following irradiation was of recipient origin. They transplanted 10^5 T6 +ve cells into T6 -ve mice previously given 750 rads X-irradiation. Five weeks later they set up bone marrow cultures and after a further six weeks they recharged the cultures with a further 10^7 T6 -ve cells. These authors only studied the colony-forming ability of stem cells in the cultures after they had been recharged with a large number of cells of recipient phenotype and it is quite probable that the stromal cells which were able to support haematopoiesis came from the recharging inoculum rather than the initial culture. The authors expressed difficulty in establishing satisfactory adherent layers in cultures of radiochimaeric models. That they found no cells of donor karyotype after the initial period of culture is not particularly surprising as their initial inoculum of donor cells was minimal (10^5 cells/mouse). Even if the bone marrow is composed of as many as 50% stromal cells and only 20% of injected cells home to the marrow (20% is the fraction estimated for haematopoietic stem cells seeding the marrow) then only 10^4 stromal cells would lodge in the entire marrow. It is not surprising that these cells were not isolated in the subsequent culture assays.

Keating and his colleagues (1982) also set up cultures of irradiated and repopulated bone marrow. Their cultures originated from bone marrow transplant patients and they were set up 14 - 490 days after the transplant. In this case the patients were treated with larger doses of X-irradiation and cyclophosphamide prior to being transplanted with large numbers of bone marrow cells. Their results showed that 25 days after transplantation all haematopoietic stem cells were

of donor origin. After transplantation the stromal cells became progressively donor-derived and by 45 days were almost completely of donor origin. They suggested that the delayed disappearance of host stromal cells indicated that haematopoietic stem cells are more radiosensitive than stromal cells which may retain limited proliferative capacity. The main differences between these two experiments are the pre-transplantation treatment of recipients and the cell dose administered following irradiation. It is possible that less injury was sustained by the stromal cells in Bentley's experiment.

In the present repopulation studies no attempts were made to assay purely haematopoietic stem cells. However, in bone marrow samples from PGK-1B recipients transplanted with-1A bone marrow cells, only PGK-1A activity was found as soon as 21 days after transplantation. This suggests that in the present study stromal cells as well as haematopoietic stem cells were donor derived, although if only a small proportion of bone marrow PGK-1 activity is stroma-derived this could be missed in the assay. In the present study both the irradiation dose and the donor cell inoculum were much larger than those used by Bentley and his colleagues; this may explain in part the lack of recipient stromal cells in the present study.

If haematopoietic stromal cells are indeed transplantable, the question arises whether the haematopoietic stem cells and stromal cells share a common ancestral stem cell. This early theory of Maximow (1924) may be clarified by the use of markers such as PGK-1. Although stromal cells are damaged by radiation, this damage may not be apparent straight away. The make-up of the bone marrow is therefore likely to change with time after irradiation. For instance, if damage to stromal cells only becomes apparent when these cells divide, then the effect of a marrow transplant at this stage may be quite different to that found when marrow is transplanted immediately after irradiation. When stromal cells die, because they have been unable

to divide the marrow will eventually become depopulated. If mice are re-irradiated at this time and a marrow transplant is given, then donor stromal cells will then be much more likely to be able to locate in the marrow spaces. If Bentley and his colleagues' culture experiments were carried out under these conditions it may be that a very much larger proportion of stromal culture cells would be of donor origin.

If haematopoietic stem cells and stromal cells have a shared ancestry, it is possible that stem cell domains in the bone marrow may be made up of the progeny of individual cells. If this is so one would expect to find patches of A or B cells in the bone marrow. Such population patterns might be found using monoclonal antibodies to PGK-1, though this is impossible at present.

Future experiments must elucidate the nature of the cells being examined in such circumstances, perhaps most possible by studying stem cell function in vitro and by combining studies using the PGK-1 marker to CFU-S studies to establish the true relationship between the cells being enumerated using each method. In addition, the CFU-S assay would allow the self-renewal ability of stem cells to be assessed in both normal animals and mice subjected to haematopoietic stress.

Further studies of the haematopoietic microenvironment (Curry et al., 1967; Trentin, 1970; 1971; 1976; La Pushin and Trentin, 1977; Wolf, 1979) and the importance of humoral feedback controls within the haematopoietic system (Reissman and Samorapoompichet, 1970; Reissman et al., 1972; Lord, 1979; Broxmeyer et al., 1979; Staber and Metcalf, 1980) are necessary to understand fully the effects of cellular depletion, by drugs or irradiation of the blood forming tissues.

Although the PGK-1 assay system still requires considerable refinement, this enzyme marker has already proved to be an important tool for the

investigation of haematopoiesis, allowing, for the first time, the study of normal, unmanipulated animals. In addition Dr Marilyn Monk and her colleagues have shown PGK-1 markers to be invaluable in the study of tissue formation in the embryo. These studies could be further improved by the production of monoclonal antibodies to PGK-1A and -1B. Such monoclonal antibodies, which would prove such an experimental boon, are likely to prove extremely difficult to make as biochemical tests of the two allozymes suggest that they are virtually identical (Mühlbacher et al., 1983).

PGK-1 markers may also prove to be of considerable importance in the study of neoplastic change. If a neoplasm was initiated by a rare, more-or-less random event such as "spontaneous" somatic mutation, a single cell, or clonal, origin would be expected. In contrast, a malignancy caused by a continuous cell-to-cell spread of virus would have a multicellular origin. Acquired chromosome markers (such as the Philadelphia chromosome of chronic granulocytic leukaemia) and X-linked markers (such as G-6-PD) have already proved useful for studying the origins and development of tumours in humans (Fialkow et al., 1972; Fialkow, 1974) and have shown clonal origins of the mycloproliferative disorders chronic granulocytic leukaemia (Adamson and Fialkow, 1978), polycythaemia vera (Adamson et al., 1976) and agnogenic myeloid metaplasia with myelofibrosis (Jacobson et al., 1978). It has further been shown that in chronic granulocytic leukaemia and polycythaemia vera, there is no increase in the numbers of Ph^1 negative mitotic cells during remission (Tjio et al., 1966), suggesting an alteration in the response of the abnormal clone to regulatory control, rather than replacement by normal cells. They also show clearly the need for a greater understanding of controlling factors within the haematopoietic system. These markers, however, rely on the availability of suitable patients which seriously reduces their usefulness.

The discovery of a suitable enzyme marker (PGK-1) in an experimental

animal, allows a more systematic review of the events causing neoplastic changes to be made. Reddy and Fialkow (1979) reported that methylcholanthrene-induced tumours in mice heterozygous for PGK-1 contained both PGK-1A and -1B. These results indicate that these fibrosarcomas were of multicellular origin. Tanooka and Tanaka (1982) used much smaller doses of methylcholanthrene ($^1/40$ of Reddy and Fialkow's lowest dose) to induce fibrosarcomas in PGK-1 heterozygotes. In order to verify the phenotypes of induced tumours, these authors transplanted pieces of the original tumours into PGK-1A and -1B mice. Of eight original tumours, 7 were found to be of only one PGK-1 phenotype, suggesting a single cell origin for these tumours. These authors suggested that the very much larger doses of methylcholanthrene used by Reddy and Fialkow resulted in multiple cellular transformation events leading to tumours of mixed phenotype. In collaboration with another laboratory, members of this group have also studied methylcholanthrene-induced tumours in PGK-heterozygotes (Woodruff et al., 1982; 1984). These authors have, in addition to transplanting tumours into single-phenotype hosts, cultured tumour cells over several culture generations and have transplanted cells after culture. Their results show that methylcholanthrene-induced fibrosarcomas are often pleoclonal, but that the clonal composition may vary markedly during tissue culture and on transplantation into congenic hosts. As a result, although pleoclonal, some tumours may appear to be monoclonal at a particular point in time because all clones except one have been competitively eliminated or reduced to the point of being undetectable.

PGK-1 can also be used to study the origins of the spontaneous leukaemia which arises in AKR mice during adulthood. This can be done by studying the phenotype of tumours occurring in AKR (PGK-1B) x PGK-1A F_1 progeny. Such studies using the PGK-1 marker system may provide a means of rationalising further the cellular basis of neoplasms. The events controlling normal haematopoiesis, must,

however, be more fully understood before the abnormal events of neoplastic change can be recognised.

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APPENDIX 1 APPENDIX TO MATERIALS AND METHODS

1. Starch Gels

Gels - 12% electrostarch

e.g. 12 g electrostarch (Connaught Laboratories Ltd., Willowdale, Ontario, Canada)
5 ml tris-citrate buffer pH 6.4
+ distilled water to 100 ml.

Tris-citrate buffer pH 6.4

27 g tris (Sigma 7-9, Tris (hydroxymethyl) - aminomethane)
16.2 g citric acid (May and Baker, Ltd)
+ distilled water to 1 litre.

100 ml electrostarch is sufficient to make 2 gels, 9.5 x 18.5 x 0.15 cm.

To make gels the electrostarch was heated, with constant stirring, until clear and thin. The mixture was then de-gassed using a vacuum pump. The hot starch was poured into pre-wetted perspex moulds on perspex plates and were covered, carefully avoiding forming air bubbles, with wetted glass plates. A heavy weight was applied to the gels until they were set. Gels were then stored overnight in a moist atmosphere at $4^{o}C$ before use.

2. Staining Mixtures for Starch Gels

2.1 Early gels were stained using the following enzyme mixture (Beutler, 1969).

15 ml 0.5 M Tris hydroxymethyl aminomethane hydrochloride buffer pH 8.0 (Sigma no. T-3253)
32 mg 3-phosphoglyceric acid (Sigma no. P-8877)
61 mg adenosine triphosphate (Sigma no. A-3127)
15 mg magnesium chloride (May and Baker Ltd)
57 mg EDTA - disodium salt (Ethylenediaminetetra - acetic acid - disodium salt, BDH Chemicals, Ltd)
12 mg β-nicotinamide adenine dinucleotide (β-NAD, reduced form - Sigma no. N-8129)
20 ul glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Sigma no. G-5162)

To this was added 15 ml 2% Noble agar at 56°C.
Bands of NAD^+ activity (PGK-1 activity) could be viewed under long wavelength ultraviolet light.

2.2 Histochemical Overlay for Starch Gels

15 ml 0.5 M Tris - HCl buffer pH 8.0
15 mg MTT (3 - 4,5 - Dimethylthiazol - 2 - yl - 2,5 - diphenyl tetrazolium bromide: Thiozolyl blue. Sigma no. M-2128)
6 mg PMS (Phenazine methosulphate, Sigma P-9625)
15 ml 2% Noble agar at 56°C

This stain allowed bands of PGK-1 activity to be observed as dark blue bands against a green/blue background.

For later starch gels a modification of the cellogel staining method

(Bücher et al, 1980) with an MTT/PMS overlay was used. The recipe for this stain can be found later in this appendix (page 175).

3. Buffers and Staining Mixtures for Cellogels and Helena Gels

(unless otherwise stated all ingredients supplied by Sigma)

3.1 Sample (DTE - BSA) Buffer

for 200 ml, in distilled water, 50 mM, pH 7.6

Ingredient	molarity/concentration	quantity
tri-ethanolamine - HCl (T-eth HCl)	0.01 M	1.856 g
Dithioerythritol (DTE)	0.3 mg/ml	60 mg
Bovine serum albumin (BSA)	0.5 mg/ml	100 mg
digitonin	2.0 mg/ml	400 mg

adjust to pH 7.6 before adding DTE, BSA and digitonin.

3.2 Freezing Buffer

To 25 ml of sample buffer add 30 mg DTE
50 mg BSA
50 ml glycerol

3.3 Electrophoresis Buffer

for 1 litre, in distilled water, pH 8.8

Ingredient	molarity	quantity
EDTA	2 mM	0.744 g
Sodium citrate	10 mM	2.94 g
Magnesium sulphate	5 mM	1.23 g
Sodium barbital	20 mM	4.12 g

Before electrophoresis add 0.1 mg/ml DTE to buffer and adenosine-5-monophosphate (AMP - free acid) to cathodal buffer.

3.4 Indicator Stock

for 10 ml, in distilled water, 0.1 M pH 7.6

Ingredient	molarity	quantity
T-eth HCl	100 mM	185.6 mg
Magnesium sulphate	130 mM	320 mg
Glucose	150 mM	270 mg
ADP	25 mM	112.5 mg
NADP	40 mM	306 mg

Adjust to pH 7.6 with approximately 50 ul 2N NaOH/ml, then adjust in 0.1 ml quantities and store at -20°C.

3.5 PGK Assay Stock

in 40 ml of electrophoresis buffer add:

Ingredient	molarity	quantity
NAD	1.2 mM	32 mg
Na_3 - F - 1,6 - PD	40.0 mM	650 mg
K_2HPO_4	40.0 mM	279 mg

Aliquot in 0.4 ml quantities and store at -20°C.

3.6 Stock solutions of MTT and PMS

for 100 ml in distilled H_2O

MTT 10 mg/ml

PMS 2.5 mg/ml

store in dark brown bottles.

3.7 Enzyme Staining Mixture

Ingredient	Quantity		
	^{14}C-Cellogel	^{14}C-Helena	MTT-Helena and Starch
Electrophoresis buffer	800 µl	600 µl	800 µl
Sample buffer	30 µl	30 µl	75 µl
G-6-PDH	10 µl	10 µl	10 µl
Hexokinase	5 µl	5 µl	5 µl
Aldolase	10 µl	10 µl	10 µl
GDH	5 µl	5 µl	5 µl
GAPDH	5 µl	5 µl	5 µl
^{14}C-glucose	15 µl (15 uCi)	15 µl (15 µCi)	-
Assay stock	400 µl	400 µl	400 µl
Indicator stock	100 µl	100 µl	100 µl
MTT	-	-	780 µl
PMS	-	-	620 µl
1.2 % agar (37°C)	-	-	2400 µl
Staining time	15 minutes	15 minutes	variable

The volumes of the 5 enzymes used are based on the following unit activities:

G-6-PDH	340 units/mg
Hexokinase	320 "
Aldolase	12 "
GAPDH	44 "
GDH	180 "

As the unit activity of new batches of enzymes may change significantly the volumes may have to be altered accordingly.

^{14}C-staining: the staining mixture is spread over a PEI sheet and overlaid by the gel.

MTT-staining: the staining mixture, in agar, is poured carefully onto the gel.

3.8 To Fix and Preserve Histochemically Stained Cellogels and Helena Gels

Gels were fixed using one of the following two methods:

(a) immerse gel in a solution of 15% glycerol and 3% acetic acid in distilled water for about one hour.

(b) immerse gel in formalin for about two minutes.

Following fixation cellogels were preserved by whitening or transparentising the gel.

To whiten cellogels:

- submerge fixed gels in 2 baths of 7% glycerol for 3 minutes. Lay gel on a glass plate and eliminate excess glycerol solution with filter paper. Warm plate at 90°C, for about five minutes, until the gel has become completely white. Remove gel from plate while still hot (Del Campo, 1968).

To transparentise cellogels:

- immerse the fixed gel in a solution of water:acetic acid:glycerol (69:30:1) for five minutes. Place on a glass plate with porous surface to the glass, remove excess liquid and warm at 70°C, for about five minutes, until transparent.

APPENDIX 2 TABLES OF EXPERIMENTAL DATA

APPENDIX 2 TABLE 1

Serial Bleeds of Normal Heterozygotes

Bleeds marked with an asterisk (*) were taken at 14 day intervals. All other bleeds were taken at 28 day intervals. Values shown are % PGK-1A present in blood samples.

Bleed no.	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6
1*	67	73	89	63	81	64
2*	72	91	82	67	86	63
3*	73	90	87	82	81	72
4*	71	91	92	72[c]	84	65
5*	65	87	87	73[c]	84	73
6*	58	88	90	65	86	67
7*	55	88	89	71	90	64
8*	65	87	92	68	88	60
9*	75	81	87	65	90	64
10	74	79	79	69	90	68
11	77	79	84	74	89	65
12	80	78	80	72	81	65
13	67	78	86	73	88	78
14	63	81	86	76	88	88
15	78	79	89	73	84	81
16	79	90	92	68	83	79
17	81	78	86	68	86	84
18	67	87	84	65	88	68
19	77	82	94	68	90	80
20		84			88	80
21		82			85	74
22		81			88	61
23		89			91	69
24		86			88	74
25		73			88	75
26		47[c]			77	79
27					86	78
28					93	77
29					88	73
Mean %A	71	83	87	70	86	72

APPENDIX 2 TABLE 1 continued

Bleed no.	Mouse 7	Mouse 8	Mouse 9	Mouse 10
1*	86	82	53	53
2*	83	73[c]	60	51
3*	83	88	63	47
4*	83	93	64	53
5*	81	86	68	51
6*	76	74	60	55
7*	78	87[c]	57	53
8*	73	88	60	53
9*	67	77	58	53
10	71	74	58	47
11	67	78	55	45
12	72	78	61	47
13	69	72	75	39
14	71	67	69	49
15	75	69	61	45
16	78	71	67	43
17	77	75	69	39
18	69	78	63	47
19	75	74	63	49
20	75	74	73	
21	76	71	65	
22	82	74	73[c]	
23	71	76	72	
24	74	69	80	
25	67	74	80	
26	64	78	84[c]	
27	75	84	63	
28	63	86	81	
29		82	79[c]	
Mean %A	74	78	67	48

[c] = verified by cellogel electrophoresis only.

APPENDIX 2 TABLE 2

Serial Bleeds of Normal Heterozygotes of Known Pedigree

All bleeds at 28 day intervals. Mice within groups are siblings.

	Percent PGK-1A				
Bleed no.	GROUP A			GROUP B	
	1	2	3	1	2
1	74	60	81	74	57
2	72	85	60	63	58
3	65	61	67	65	72
4	76	68	68	73	74
5	73	57	60	57	69
6	81	78	63	61	71
7	79	60	65	58	63
8	80	63	74	72	71
9	63	91	87	75	79
10	75	53	63	69	71
11	84	55	71	73	78
12	67	50	82	67	60
13	73	60	82	73	77
Mean % A	74	65	71	68	69

APPENDIX 2 TABLE 2 continued

Bleed no.	Percent PGK-1A				
	GROUP C		GROUP D		
	1	2	1	2	3
1	68	84	93	100	88
2	61	80	81	95	74
3	74	80	79	96	85
4	78	80	71	91	93
5	58	79	76	96	79
6	68	77	91	96	83
7	83	65	80	90	82
8	71	78	79	89	81
9	79	75	75	82	93
10	69	83	87	97	92
11	73	79	81	95	81
12	NT	74	85	93	98
13	60	80	78	90	84
Mean % A	70	78	81	93	86

APPENDIX 2 TABLE 3

Bone Marrow Repopulation Studies using Marrow from Donors of Known PGK-1 Phenotypes

B mice were lethally irradiated (1050 rads) and then repopulated using 10^7 AB bone marrow cells per recipient.

Donor mice for each group were as follows:

Group A - Known heterozygotes mouse 1 (mean % A = 71)
Group B - " " " 3 (mean % A = 87)
Group C - " " " 4 (mean % A = 70)
Group D - " " " 10 (mean % A = 48)

All bleeds at 28 day intervals.

Bleed no.	GROUP A % PGK-1A		
	1	2	3
1	71	61	85
2	79	75	82
3	57	76	80
4	53	64 - 75	68
5	68	69	58
6		75	81
7			75
8			
9			
10			
Mean % A	66	71 72	76

APPENDIX 2 TABLE 3 Continued

Bleed no.	. GROUP B % PGK-1A			
	1	2	3	4
1	~100	84	85	90
2	89	92	86	87
3	86	94	97	94
4	78	89	86	83
5	82	95	86	85
6	90	90	88	81
7	96	94	86	94
8		92	91	86
9		92	88	89
10		94	~100	81
Mean % A	89	92	89	87

APPENDIX 2 TABLE 3 Continued

Bleed no.	GROUP C % PGK-1A				
	1	2	3	4	5
1	63	65	63	72	75
2	67	71	76	69	73
3	55	78	82	75	79
4	55	67 - 73	69	72	81
5	69 - 76	61	80	74 - 79	87
6	72	61	69	75	93
7	68	60	82	82	89
8	58	60	69	63	65
9	65		65		63 - 73
10	72		60		68
Mean % A	64 65	65 66	73	73 73	77 78

Bleed no.	GROUP D % PGK-1A				
	1	2	3	4	5
1	43	43	50	51	43
2	55	22	23	47	31
3	55	27	21	47	47
4	51	22	42	47	53
5	31	21	53	42	37
6	37	36	50	34	29
7	31		30	43	34
8	18		26	53	22
9	15		50	36	29
10	22		21		
Mean % A	36	28	37	44	36

APPENDIX 2 TABLE 4 PGK-1A Repopulation Studies

PGK-1B mice lethally irradiated (1050 rads) and repopulated with 2.5×10^6 PGK-1A cells (bone marrow).

		Percent PGK-1A per tissue						
Days post irradiation	Mouse	Blood	Spleen	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph Nodes
Day 10	1	43	92	95	94	79	*	81
	2	37	93	96	93	45		77
	3	40	93	97	100	43		47
	4	24	89	96	97	80		68
	5	22	83	92	94	42		57
Day 14	1	63	95	100	100	14	11	34
	2	34	88	100	100	16	11	22
	3	24	89	100	100	60	24	21
	4	36	86	97	100	13	22	28
	5	42	92	100	97	61	100	60
Day 18	1	40	71	100	100	100	77	81
	2	24	90	100	100	100	88	65
	3	50	75	100	86	100	100	20
	4	25	94	88	100	NT	NT	63
	5	31	75	96	100	100	100	39
Day 21	1	40	90	100	97	100	100	84
	2	55	89	100	100	89	100	47
	3	33	93	100	100	100	100	51
	4	NT	94	100	100	100	100	68
	5	40	91	100	100	65	100	74

* On Day 10 post irradiation the thymus was assayed as a whole organ.

NT = not tested.

APPENDIX 2 TABLE 4 Continued

NT = not tested

Days post irradiation	Mouse	Percent PGK-1A per tissue						
		Blood	Spleen	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph Nodes
Day 24	1	79	81	100	100	100	100	42
	2	57	88	100	100	100	100	51
	3	45	96	100	100	100	100	63
	4	61	78	97	100	100	92	51
	5	53	94	100	100	100	100	82
Day 28	1	49	81	100	100	100	100	78
	2	57	83	100	100	81	100	69
	3	51	85	100	100	100	100	68
	4	63	87	100	96	100	100	74
	5	50	NT	100	91	100	100	76
Day 35	1	39	94	100	100	83	100	87
	2	77	86	100	100	100	100	77
	3	63	80	100	100	100	100	75
	4	69	87	100	100	100	100	81
	5	47	87	100	100	100	100	84
Day 42	1	81	91	100	93	100	100	83
	2	77	98	100	100	100	100	93
	3	82	96	100	100	100	100	75
	4	85	93	100	100	100	100	NT
	5	76	94	100	100	100	95	78
Day 56	1	100	94	100	100	100	100	NT
	2	100	93	100	100	100	100	89
	3	100	95	100	100	100	100	93
	4	100	87	100	100	100	100	81
	5	100	94	100	100	100	100	91
Day 70	1	100	94	100	100	100	100	92
	2	100	96	100	100	100	100	96
	3	100	97	100	100	100	100	100
	4	100	94	100	100	100	100	89
	5	100	92	100	100	100	100	86

APPENDIX 2 TABLE 5 PGK-1AB Repopulation Studies

PGK-1B mice lethally irradiated (1050 rads) and repopulated with 2.5×10^6 PGK-1AB bone marrow cells.

		Percent PGK-1A per tissue						
Days post irradiation	Mouse	Blood	Spleen	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph nodes
Day 10	1	25	67	71	93	0	*	61
	2	25	84	95	91	0		63
	3	29	79	47	73	0		42
	4	4	80	79	79	0		43
	5	12	76	89	73	0		79
	6	23	61	87	47	0		37
	7	13	64	81	78	0		11
	8	21	94	76	61	0		50
	9	26	80	69	74	0		10
	10	21	78	53	67	0		9
Day 14	1	37	51	80	88	50	*	33
	2	51	74	89	69	28		47
	3	30	65	93	75	45		72
	4	50	60	89	68	14		81
	5	47	80	89	83	16		42
	6	28	79	87	68	84		67
	7	51	68	86	88	30		55
	8	37	68	73	64	50		50
	9	31	83	75	71	50		42
	10	40	75	83	89	34		73

* On days 10 and 14 the thymus was assayed as a whole organ.

APPENDIX 2 TABLE 5 Continued

Days post irradiation	Mouse	Percent PGK-1A per tissue						
		Blood	Spleen	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph nodes
Day 18	1	34	74	89	71	78	24	82
	2	49	85	92	57	60	61	88
	3	31	78	93	83	NT	64	82
	4	53	89	84	90	58	55	65
	5	24	74	64	84	55	57	67
	6	43	77	87	93	53	68	72
	7	63	71	85	42	31	73	84
	8	60	89	61	43	14	24	82
	9	42	60	73	81	23	43	89
	10	49	91	90	64	71	75	82
Day 21	1	43	43	74	63	75	100	78
	2	36	68	80	93	89	49	79
	3	57	65	84	78	72	64	80
	4	53	69	53	87	84	98	80
	5	58	51	71	71	45	51	67
	6	39	73	81	73	89	NT	85
	7	42	80	96	76	91	72	53
	8	53	68	71	30	58	93	81
	9	45	65	93	43	72	30	88
	10	37	36	82	49	71	36	73
Day 24	1	63	71	93	97	94	90	60
	2	49	61	89	96	78	81	57
	3	36	65	84	87	80	73	65
	4	43	57	79	73	71	63	75
	5	60	68	45	63	40	65	77
	6	51	68	82	67	75	81	57
	7	63	60	93	92	94	73	58
	8	64	79	60	91	71	55	67
	9	51	45	67	91	78	79	85
	10	63	51	94	89	89	67	86

APPENDIX 2 TABLE 5 Continued

		Percent PGK-1A per tissue						
Days post irradiation	Mouse	Blood	Spleen	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph nodes
Day 28	1	28	60	65	45	64	67	64
	2	58	58	84	84	82	71	67
	3	50	58	94	87	73	77	87
	4	67	71	89	94	81	68	65
	5	47	65	83	91	61	42	61
	6	51	75	91	73	81	64	74
	7	65	67	97	81	75	72	68
	8	53	69	85	84	60	73	65
	9	53	72	89	78	76	73	61
	10	64	68	82	89	47	49	55
Day 35	1	78	87	75	51	31	77	63
	2	65	72	86	73	51	80	60
	3	65	75	78	91	92	63	83
	4	47	64	81	87	89	94	77
	5	72	45	55	72	84	67	64
	6	50	80	71	77	75	87	49
	7	85	77	81	75	89	64	50
	8	61	60	81	84	69	75	60
	9	64	69	73	79	94	75	53
	10	63	65	64	84	83	90	71
Day 42	1	65	63	69	80	69	85	68
	2	71	63	88	78	67	68	55
	3	89	67	85	90	89	76	68
	4	77	61	89	81	55	67	55
	5	74	57	89	84	63	67	53
	6	69	77	82	67	45	79	65
	7	55	55	90	85	77	90	49
	8	55	74	75	45	67	82	55
	9	61	60	68	65	78	53	43

APPENDIX 2 TABLE 5 Continued

Days post irradiation	Mouse	Percent PGK-1A per tissue: Blood	Spleen	Left Femur	Right Femur	Left Thymus	Right Thymus	Lymph nodes
Day 56	1	71	65	43	57	94	93	77
	2	82	79	74	90	89	60	73
	3	81	65	80	78	63	71	67
	4	79	70	82	90	81	69	77
	5	81	68	92	97	79	96	79
	6	82	49	84	73	89	64	77
	7	83	68	89	90	91	85	57
	8	89	78	94	93	90	90	65
	9	84	67	80	81	68	84	79
	10	81	74	78	68	72	80	75
Day 70	1	74	65	93	55	75	69	45
	2	85	87	94	89	84	71	69
	3	79	60	60	98	81	93	82
	4	82	82	87	84	87	93	69
	5	78	76	93	73	69	53	53
	6	94	88	93	58	82	61	69
	7	90	68	65	93	91	93	85
	8	72	69	97	97	98	86	92
	9	88	92	89	92	78	73	45
	10	92	71	71	93	89	77	39*

* Only one estimate - very faint therefore not included in calculation of mean.

NT = not tested.

APPENDIX 2 TABLE 6

PHENYLHYDRAZINE EXPERIMENT 1

Repeated PHZ :- mice were given 3 daily injections of 1.2 mg PHZ in 0.24 ml distilled water. This regime was repeated in 28 day cycles. Results are expressed as % PGK-1A present in the blood at each sample time. Days marked with an arrow are those on which the mice were bled prior to being injected with the first of 3 daily doses of PHZ.

Day of bleed	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7
→ 0	100	83	75	90	88	90	81
7	100	86	72	93	90	94	78
14	100	74	78	95	88	92	76
21	NT	NT	NT	NT	NT	NT	NT
→ 28	100	92	57	89	89	88	86
35	98	82	66	91	94	92	76
42	91	83	69	83	91	91	69
49	NT	NT	NT	NT	NT	NT	NT
→ 56	100	88	76	94	92	78	78
63	100	86	69	96	83	94	76
70	100	Mouse	64	84	70	73	61
77	NT	dead	NT	NT	NT	NT	NT
→ 84	100		76	81	90	90	71
91	100		68	92	89	89	73
98	100		72	84	83	88	74
105	NT		NT	NT	NT	NT	NT
→ 112	100		70	79	90	90	69
119	100		74	79	87	85	72
126	100		71	80	76	93	70
133	100		70	98	89	97	75
→ 140	100		73	94	87	93	77
147			70		80		70
154			69		83		82
161			70		86		71
168					85		74
Mean % A		84	70	88	86	89	74

Appendix 2 Table 7

PHENYLHYDRAZINE EXPERIMENT 2

Single PHZ:- The same dose of PHZ was given on days 0, 1 and 2 as in PHZ experiment 1. No further injections were given.

Day of bleed	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	Mouse 8
→ 0	66	64	83	92	96	94	68	77
7	67	62	78	84	89	86	53	84
14	58	67	66	84	91	93	66	89
21	68	76	72	88	90	93	70	84
28	60	80	85	86	94	91	58	86
35	55	74	79	86	86	89	65	83
42	55	68	83	89	93	88	73	82
49	62	68	74	80	86	81	72	90
56	70	72	76	89	92	95	71	87
63	52	71	NT	NT	NT	NT	NT	NT
70	60	77	87	76	93	87	67	90
77	66	63	78	78	90	91	72	91
84	62	58	77	89	95	92	63	89
91	63	63	79	88	93	82	66	87
98	NT	NT	82	80	90	96	66	77
105	65	80	85	83	87	89	71	86
112	63	71	73	82	79	93	71	88
119	58	73	76	80	82	78	66	84
126	66	75	74	83	82	84	66	86
133	50	74	74	88	80	71	66	80
140	54	75	72	84	81	91	NT	88
Mean % A	61	71	78	84	88	88	67	85

Appendix 2 Table 8

PHENYLHYDRAZINE EXPERIMENT 3

PHZ control :- mice bled at 7 day intervals. No PHZ injections given.

Day of bleed	Mouse 1	Mouse 2	Mouse 3	Mouse 4	Mouse 5	Mouse 6	Mouse 7	Mouse 8
0	76	78	66	80	74	61	85	95
7	82	74	71	82	76	67	90	88
14	91	74	56	86	70	64	88	90
21	86	81	61	85	61	69	85	96
28	NT	76	57	NT	57	66	82	88
35	87	71	54	85	73	64	82	90
42	92	82	60	67	68	68	76	98
49	92	70	57	75	66	64	85	91
56	81	70	64	77	56	54	80	94
63	90	67	60	76	73	57	91	95
70	93	NT	62	69	69	52	90	95
Mean % A	87	74	61	78	68	62	85	93

Appendix 2 Table 9 HYDROXYUREA STUDIES

Days post HU treatment	Mouse	PERCENT PGK-1A PER TISSUE						
		Left Femur	Right Femur	Left Humerus	Right Humerus	Left Thymus	Right Thymus	Spleen
Day 2	H1	37	40	87	84	NT	45	40
	H2	91	94	69	72	86	81	83
	H3	90	83	89	84	81	89	68
	H4	74	65	84	81	89	68	50
	H5	97	38	71	81	90	42	76
	C1	30	11	68	53	65	58	45
	C2	82	58	78	77	77	97	78
Day 5	H1	94	91	72	63	NT	NT	60
	H2	82	86	74	89	NT	NT	83
	H3	89	87	67	83	NT	NT	94
	H4	98	91	93	71	72	77	72
	H5	99	65	75	74	NT	NT	76
	C1	80	81	80	93	75	61	50
	C2	78	77	57	75	90	82	79
Day 8	H1	71	76	86	86	NT	NT	79
	H2	80	57	73	77	NT	NT	61
	H3	60	74	NT	71	NT	NT	79
	H4	76	63	88	87	NT	NT	58
	H5	81	65	89	91	NT	NT	78
	C1	82	81	75	91	76	79	47
	C2	69	73	68	51	73	68	63
Day 14	H1	86	76	90	86	73	88	68
	H2	89	77	83	85	51	83	73
	H3	87	64	87	63	93	NT	100
	H4	89	91	82	89	87	88	88
	H5	89	86	93	60	77	77	81
	C1	81	63	54	82	85	58	57
	C2	79	91	88	81	93	83	91

Appendix 2 Table 9 Continued

Days post HU treatment	Mouse	PERCENT PGK-1A PER TISSUE						
		Left Femur	Right Femur	Left Humerus	Right Humerus	Left Thymus	Right Thymus	Spleen
Day 21	H1	73	86	80	50	86	89	68
	H3	94	85	79	92	75	76	79
	H4	78	86	94	90	79	81	73
	H5	79	67	57	71	69	53	71
	C1	61	75	NT	83	81	81	64
	C2	89	86	92	91	75	92	79
Day 28	H1	65	72	51	57	50	69	76
	H2	100	67	96	NT	96	100	77
	H3	57	93	83	91	90	95	72
	H4	89	86	87	89	82	92	75
	H5	91	93	NT	82	91	81	81
	C1	58	69	89	NT	95	96	68
	C2	89	69	50	NT	61	93	65
Day 35	H1	83	94	94	93	96	81	86
	H2	79	76	91	89	94	75	81
	H3	75	86	77	81	89	89	71
	H4	63	55	51	47	47	68	55
	H5	80	78	81	85	88	79	NT
	C1	88	81	94	78	95	93	81
	C2	78	80	86	89	65	88	71

H = hydroxyurea-treated

C = control

NT = not tested.

APPENDIX 3 PUBLISHED PAPERS

Clonal Organization of Murine Haematopoiesis: Studies with an X-linked Enzyme Marker

H. S. Micklem, D. I. Burton, J. D. Ansell & R. A. Gray

INTRODUCTION

It is virtually self-evident that haematopoiesis is maintained by a succession of cell clones. Clonal succession is an essential part of any scheme where stem cells (by definition self-renewing) differentiate into non-self-renewing daughters. The size of a clone defined in this way – i.e. derived from a cell at the point where it ceases to have 'stem' characteristics – will depend on the number of divisions that occur along the differentiation pathway before the mature, non-dividing end-cell is reached. This number is difficult to establish exactly, but it is estimated to be about 12–14 in the erythron (Testa 1979). Such a clone would yield some 10^4 cells and obviously at that level there would have to be clonal succession, since each clone, being incapable of self-renewal, would be doomed to extinction. Assuming 2×10^{10} erythrocytes in a mouse, each with a lifespan of 45 days (Russell & Bernstein 1966), the daily steady-state output would have to be $4–5\times 10^8$. Thus, $4–5\times 10^4$ clones, or perhaps one might say clonelets, have to be expended each day to maintain erythropoiesis. From another standpoint, the haematopoietic system can be viewed as a subline of a single clone initiated by the fertilized ovum. What is largely unknown is how the haematopoietic population is structured between those extremes. To start at the beginning, cells must be set aside in the early embryonic mesoderm as founders for the haematopoietic system. This founder population must henceforth not only differentiate, but also self-renew so as to provide a (probably expanded) stock of stem cells for the adult. Two specific and, as we shall show, potentially

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becomes irreversibly inactivated. Subsequently, any somatic cell and its descendants fail to express genes carried on the inactivated chromosome (Lyon 1974, Monk & Harper 1979). X-linked genetic variants can therefore be used as markers for cell population studies; an electrophoretic variant of glucose-6-phosphate dehydrogenase has indeed been so used in attempts to estimate embryonic founder cell numbers for the haematopoietic system in man (or, more precisely, woman) (Gandini & Gartler 1969, Fialkow 1973). More recently, with the discovery in feral mice (*Mus musculus*) of an electrophoretic variant of X-encoded phosphoglycerate kinase (PGK-1) (Nielsen & Chapman 1977) and the development of improved methods for measuring PGK activity in electrophoresis strips (Bücher *et al.* 1980), a convenient marker has become available in the mouse. We received variant enzyme-carrying mice from Dr. J. D. West (Oxford University) after nine generations of back-crossing onto the C3H genetic background. In our laboratory we have back-crossed, for 15 generations so far, onto the CBA/Ca background; CBA was chosen because we already have chromosome (T6) and immunoglobulin allotype (Igh^b) markers on this strain.

EXPERIMENTS

Variations in alloenzyme ratio in normal heterozygous mice

Our first experiment with this system was the simplest we could think of. A group of 10 normal heterozygous mice, 3–7 months old at the outset, was bled repeatedly. For 16 weeks the blood samples were taken at 14 day intervals and thereafter at 28 day intervals. The experiment continues, but data are available for the first 68 weeks.

The percentages of A and B alloenzymes (A + B = 100) were determined electrophoretically from haemolysates of blood cells using the following modification of the technique of Bücher *et al.* (1980). After electrophoresis, the Cellogel electrophoresis strips were applied to a polyethylene imine (PEI) thin layer chromatography sheet on which had been spread a solution of the necessary enzymes, substrates and cofactors for the visualization of PGK activity, with the addition of ^{14}C-labelled D-glucose. The radioactive final products of the linked enzyme reactions (glucose-6-phosphate and 6-phosphogluconolactone) were adsorbed onto the PEI sheet (Chasin & Urlaub 1976), which was then autoradiographed on Kodak XS X-ray film. The autoradiographs were scanned with a double-beam recording densitometer and the area under each peak was integrated. The figures obtained were corrected to

answerable questions can be formulated. (1) How large is the haematopoietic founder pool in the embryonic mesoderm? (2) How many stem cells contribute to the differentiating population in any unit of time?

Stem cells

In the mouse, the concept of stem cells has become intertwined with the phenomenon of spleen colony-forming cells (CFC). Unfortunately, it has long been clear that there is no simple equation between the two. CFC are undoubtedly heterogeneous by a number of criteria: most importantly, they differ in their capacity for self-renewal. Thus, even if for a given category of cells the decision between self-renewal (birth) and differentiation (death) is a stochastic process, with certain probability values for the two outcomes, the probablility is not uniform for the whole CFC pool. This conclusion is supported by, for example, comparisons between the long-term repopulating ability of a cell suspension and the number of CFC in it (Micklem *et al.* 1972, Ogden & Micklem 1976, Ross *et al.* 1982) and by the observation that CFC from some sources (e.g. bone marrow) are more likely to generate daughter CFC in a spleen colony than are others (e.g. from blood) (Schofield 1970, Micklem *et al.* 1975, Vos, 1981). Relationships have been postulated between the probability of self-renewal, the cycling status of CFC and the length of a CFC's mitotic history (Micklem & Ogden 1976, Rosendaal *et al.* 1976, 1979, Hellman *et al.* 1978). However, just as not all CFC may be stem cells, so not all stem cells may show up as CFC. Indeed, it is already clear that some highly self-renewing CFC may be missed in a standard 8-day spleen assay, appearing only by day 10–12 (Hodgson & Bradley 1979, Magli *et al.* 1982). It is very possible that others do not form colonies at all, either because they are reluctant to divide or for purely technical reasons (difficulty of obtaining in cell suspension, failure to locate in spleen, for example).

These technical considerations limit the interpretation of all existing *in vivo* assays for stem cells. All this is not to decry assays of stem cell function. They have yielded many insights into the workings of the haematopoietic system. But it does underline the need to view the system from some new angle, if possible without the drawbacks of experimental interference. The present paper represents an attempt to do that.

X-linked enzyme markers

Early in the embryogenesis of female mammals, one of the two X chromosomes

present in any blood sample, the greater will be the variation in the representation of the two alloenzymes between samples. A cursory inspection of the data suggests not only that few clones are present, but also that their identity is capable of changing rapidly. With the simplifying assumption that all erythropoietic clones are of equal size, the number of clones n can be estimated by the binomial equation

$$n=p(1+p)/s^2$$

In this equation, p is the best available estimate of the overall proportion of A alloenzyme in the haematopoietic system, calculated as the mean from several blood samples. $1-p$ is the proportion of B alloenzyme and s^2 is the variance of individual and independent samples around p. Blood samples taken at 14 or 28 day intervals cannot be regarded as independent since individual erythrocytes can be expected to survive for up to 45 days (Russell & Bernstein 1966). Hence, n was calculated from the samples taken at day 0 and at 56 day intervals thereafter. The mean value of n was calculated as 10 and the range 2–30. This wide estimated range can be very largely attributed to statistical error since only eight–nine independent samples have so far been studied.

If there are 10 clones represented in a blood sample and erythrocytes survive for 45 days, it follows that each clone must be large enough, on average, to sustain steady-state erythrocyte production single-handed for 4.5 days: 3 clones could provide for 14 days. The next question is how rapidly in fact do clones come and go: the large changes that can occur in A:B ratios within 14 days suggest that they may do so rapidly. Computer simulations of the data support this notion. Fig. 2(a–h) shows an unselected series of simulations based on the assumption that 3 clones sustain the entire production of erythrocytes for 14 days and then disappear. These simulations correspond quite closely to the actual data, though generating, if anything, slightly less variability. Simulations assuming any substantially larger number of clones – for example 50 in a blood sample, corresponding to 15 over a 14-day period Fig. 2(i–j) – clearly generate too little variability to fit the experimental data.

The production of a clone of 2×10^9 erythrocytes requires 31 doublings from the clonogenic cell – far more than the 12–14 doublings calculated for the production of erythrocytes from a CFC. If this is so, it follows that CFC themselves, at the stage when they lose the capacity to self-renew, are members of substantial clones, separated by up to 19 doublings from the clonogenic cell, and that these clones are used sequentially to maintain erythropoiesis.

compensate for the log-linear response of the X-ray film to exposure, using a calibration curve obtained from known alloenzyme ratios, and the proportions of the A and B alloenzymes in each experimental sample were calculated.

The results are shown in Fig. 1, which plots the percentage of A alloenzyme against time for each individual. Two points stand out. (1) Large, and often sudden, variations in the relative amounts of A and B alloenzyme are evident in the blood of every individual. (2) Half of the individuals show a marked preponderance of A alloenzyme.

Interpretation of alloenzyme changes: haematopoiesis is effected by a succession of large clones

From simple binomial considerations it is plain that the fewer clones that are

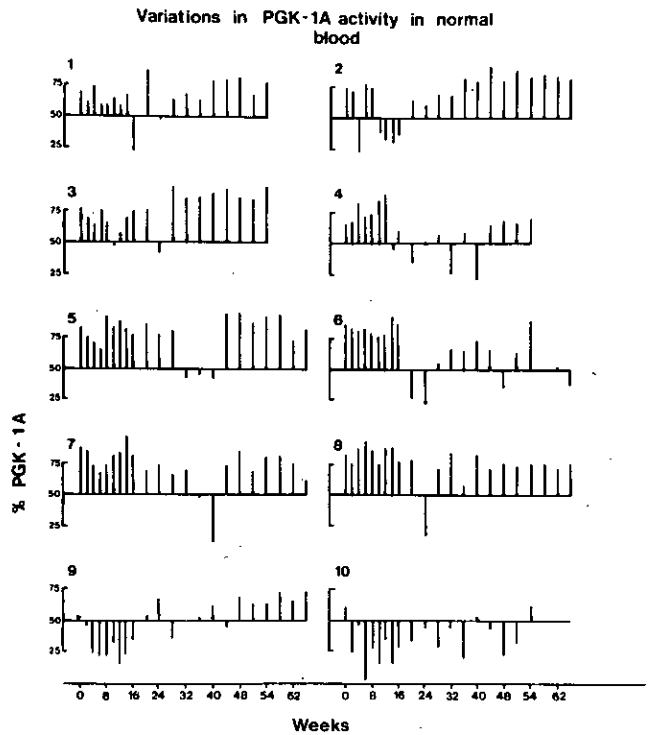

Fig. 1. Percentages of PGK activity attributable to the A alloenzyme in serial blood samples from 10 PGK-1AB heterozygous female mice.

undiminished throughout an erythrocyte's lifespan. CBA (PGK-1BB) mice were lethally gamma-irradiated (10.5 Gy) and restored with CBA-PGK-1AA bone marrow. Figure 3 shows that host-type PGK disappeared at a rate consistent with the expected disappearance of host erythrocytes and their replacement from donor marrow. The large variations between individuals are presumably attributable to variable erythrocyte loss through post-radiation haemorrhage and/or a variable rate of replacement by donor cells.

The overall predominance of A alloenzyme

It is often assumed that X-chromosome inactivation is a purely random event in somatic tissues and that the expected ratio of allelic products of the two X-chromosomes in a tissue would be 1:1. However, this has proved not to be so in the mouse, where an X locus (Xce) has been found to control the probability of a given X-chromosome escaping inactivation. There appear to be at least 3 alleles at this locus, which is closely linked to PGK-1 (Johnston & Cattanach 1981). Evidently the Xce allele linked to PGK-1A in the original feral donor was different from that present in most inbred laboratory mouse strains, including CBA. The linkage has persisted through many generations of back-crossing, resulting in an approximately 7:3 ratio of A:B (Johnston & Cattanach 1981).

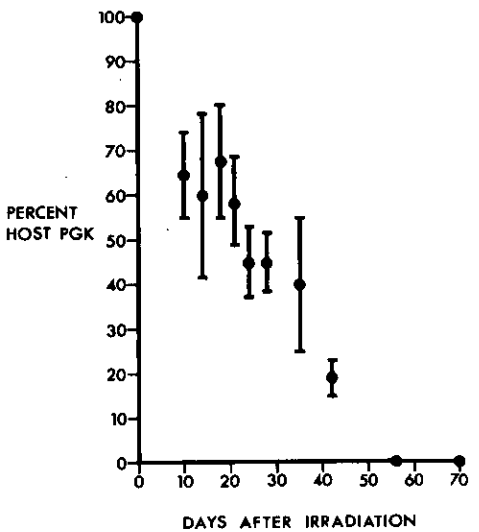

Fig. 3. Disappearance of host-type PGK from the blood of CBA mice after lethal irradiation and injection of CBA-PGK-1AA bone marrow.

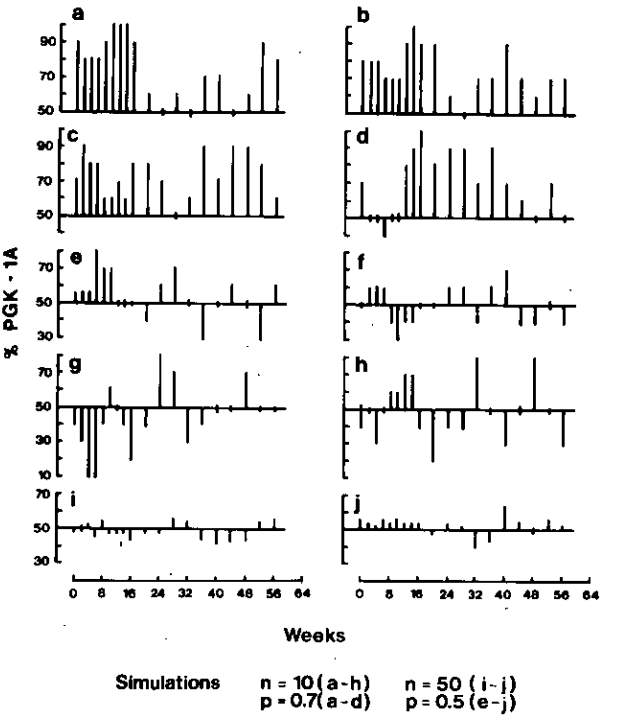

Fig. 2. Computer simulations of PGK phenotypes in serial blood samples, generated according to the assumptions that (1) erythrocyte lifespan=45 days, (2) PGK-1A- and 1B-expressing cells are selected at random, and (3) each clone produces erythrocytes for only a limited period before becoming extinct or quiescent: 3 clones (a–h) or 15 clones (i–j) productive over any 14-day period. The simulations are unselected – i.e. the first of series that were generated with p set to 0.70 (a–d) or 0.50 (e–j).

One technical factor could render these calculations invalid: if PGK were to disappear, or its relative concentration diminish sharply, as erythrocytes aged, then the older erythrocytes in a blood sample would be poorly represented in our enzyme measurements. Suppose, to take an extreme case, that PGK disappeared rapidly from erythrocytes more than 1-day-old, then our figure of 10 clones would refer to erythropoiesis during 1 day, not 45 days. That would bring the average clone size down to $4\text{–}5\times10^7$ – still, incidentally, 1000 times or 10 doublings larger than the clonelets derived from a differentiating CFC. However, we have evidence that PGK activity in fact persists more or less

Phenylhydrazine-stimulated erythropoiesis

In order to see whether clonal succession, such as we have postulated in normal mice, occurs under conditions of erythropoietic stress, we have treated PGK-1AB heterozygotes with phenylhydrazine at 4-weekly intervals, resulting in 70–80% reticulocytosis after each treatment. The A:B ratio was found to vary widely between samples (Fig. 4), again suggesting the activity of a rapid succession of large clones.

RELEVANCE OF RESULTS TO OTHER STUDIES

These data offer an unusual perspective on the haematopoietic system and thus are relevant to the interpretation of several kinds of investigation. Three are discussed below.

Chimaeric ('tetraparental') mice

By constructing chimaeras, it is possible to use autosomally encoded enzyme markers to analyze the clonal organization of haematopoiesis in the same way that we have used PGK in normal mice. As far as we are aware, nobody has attempted this, although several workers have analyzed short series of blood samples to see whether chimaeric drift occurs. Chimaeras followed over 4 months were classified as being either stable or unstable by Stephens & Warner (1980). Our data (Fig. 1) suggest that there may have been no real difference between these categories: it is possible for animals to appear 'stable' for many weeks, but still be highly unstable over longer periods.

Cellular ageing

The failure of bone marrow cells to self-renew after serial passage through lethally irradiated mice led several authors to postulate that haematopoietic stem cells are subject to senescence, perhaps as a result of passing through many cell divisions. More recently, it has become clear that this population decline is largely an artifact caused by the repeated disruption and reconstruction of the bone marrow (Harrison *et al.* 1978, Ross *et al.* 1982). Work in our laboratory (Ross *et al.* 1982) has shown that oft-repeated *in-situ* depletion of the bone marrow with hydroxyurea (up to 37 pairs of injections at 21-day intervals) does not affect the numbers or self-renewal capacity of CFC or the ability of bone marrow cells to repopulate irradiated recipients. Does this imply that the stem cell pool has been forced to pass through many extra divisions, emerging from

This ratio agrees quite well with the overall haematopoietic ratios calculated for eight of our initial series of 10 mice, but poorly with the other two where the ratio is closer to 1:1 or even 3:7. In the light of more recent results, these two mice (together with two in the phenylhydrazine series below appear to be exceptional and may possibly result from a rare cross-over between the PGK-1 and Xce loci. This possibility is unfortunate because, in principle, the data for the series should allow a binomial estimate to be made of the number of mesodermal founder cells for the haematopoietic system. For this calculation one would use the best estimates of the A:B ratio in each individual mouse's haematopoietic system. In the binomial equation s^2 would be the variance of the individual estimates around the mean for the whole series of animals. Plainly, the likelihood of a cross-over having occurred invalidates any such calculation, which will therefore have to await further data. Fialkow (1973) calculated 16 founder cells in man, using the X-linked G6PD variant, but, as our present data show, such an estimate is of dubious validity unless based on multiple blood samples taken from each individual over a long period.

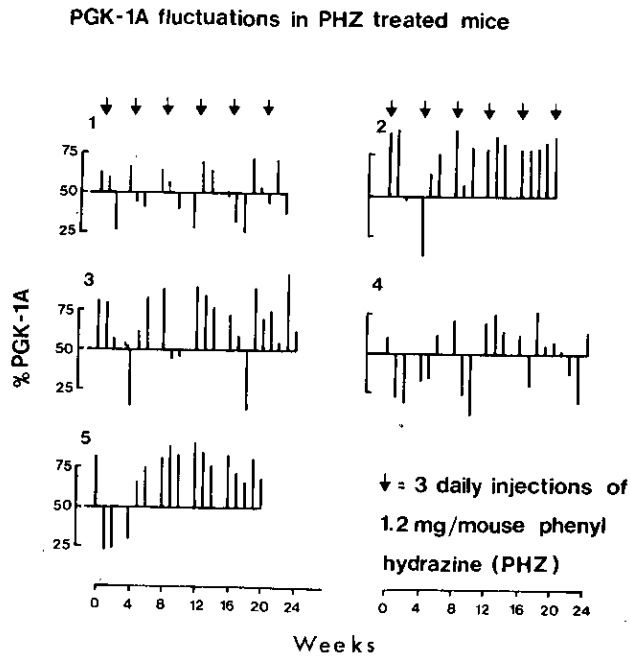

Fig. 4. Percentages of PGK-1A alloenzyme activity in serial blood samples from PGK-1AB heterozygous females serially exposed to phenylhydrazine.

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the ordeal in good health? No: the data also showed (in confirmation of Rosendaal *et al.* 1976) that stem cells with a high capacity for self-renewal were very difficult to coax into division. This is, of course, what would be expected if haematopoiesis is maintained by a succession of clones. Clonal succession may be seen as biologically desirable in that long-term effects of any somatic mutations or DNA copying errors are minimised (Micklem & Ogden 1976). Our present data indicate that the clones are very large and the needful number of clonogenic cells correspondingly small: about 250 would provide a mouse's lifetime requirements for erythrocytes. Whether a stock of clonogenic cells is built up during ontogeny and is then gradually used up through differentiation, or whether they self-renew during adult life is a matter for conjecture.

A marker for transplantation studies

Irrespective of its X-linkage, the PGK variant provides a useful marker for transplantation studies, easier to use than chromosome markers and applicable to all tissues and cells both dividing and non-dividing. Our PGK-1A subline of CBA/Ca is already sufficiently histocompatible to allow successful transplants to normal non-irradiated recipients (Brecher *et al.* 1982).

CONCLUSIONS

The data presented here, obtained with the X-linked PGK enzyme marker, provide the beginnings of a quantitative basis for the concept of clonal succession, which was suggested long ago by Kay (1965), but has hitherto remained rather vaguely formulated. The data strongly suggest that steady-state erythropoiesis is maintained by very large ($>10^9$ cells) clones which arise and disappear in rapid succession, and hence that the CFC compartment itself has a constantly changing oligoclonal composition. However, this remains to be confirmed by direct observations on purified reticulocytes and on spleen colonies derived from PGK-heterozygous donors. Work on these, and on other isolated blood cells and their precursors, is in progress.

ACKNOWLEDGMENTS

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DISCUSSION

METCALF: I am a little confused as to how big these clones are. I gather that the sample of blood you take is only a minute proportion of the circulating blood volume. Your estimates of clone numbers is 2-30 for the whole animal. If so, what number of cells in the sample do you expect to be able to detect? What was the sample volume?

MICKLEM: The volume of blood from which lysates were made was 0.2 ml.

METCALF: How many cells of each type are needed to be detectable by this method? Doesn't this influence the calculation?

MICKLEM: I don't think so in practice. There are two main factors. One is the size of the samples, which, as I said, was 0.2 ml or more than 10^9 erythrocytes; so the error due to limited sample size is negligible. The second is the sensitivity of detection. We lyse the cell sample, with thorough mixing, and have then routinely applied 1 μl (equivalent to 7×10^6 erythrocytes) to the gel. Under these conditions we can measure down to 1% of a minority enzyme, but usually we are dealing with less-extreme ratios.

METCALF: Are you able to estimate the amount of enzyme in one burst colony grown *in vitro* – a colony containing, say, 10,000 cells? If so, you could put your clonal succession theory to a direct test by determining whether the genotype of the BFU-E population changes some days before comparable changes in mature circulating red cells.

MICKLEM: It might be possible, but there are two problems. One is timing which, if there is rapid clonal succession, becomes critical. The other is sampling enough BFU-E and analyzing enough colonies.

VAN DEN ENGH: What are the limits of sensitivity?

MICKLEM: We haven't explored the limits of sensitivity, but they can certainly be pushed back further than we have attempted in this series of experiments. For example, only 0.4% of the glucose in the enzyme-substrate mixture was labeled with ^{14}C the disintegration of which, after phosphorylation of glucose, is our

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gestation period in the mouse, but that does not absolutely rule out the persistence of a few 2X-active stem cells.

DEXTER: I presume that if both X-chromosomes are active in stem cells this would not invalidate your model?

MICKLEM: No. Look at it this way. If none of the hematopoietic system were X-inactivated, then one would get a constant, probably 1:1, ratio between the two alloenzymes in heterozygotes. Our estimates of clone size and number of doublings relate to the descendants of *one X-inactivated cell.* They suggest that X-inactivation occurred at least 31 cell generations ancestral to the reticulocyte; that seems to mean 17 generations or more ancestral to a late CFU-S. Probably it is reasonable to call such a cell a stem cell. Its own immediate ancestor may or may not have been X-inactivated: we have no way of telling.

measure of PGK activity. One can reduce the amount of cold glucose up to 100-fold and this gives an equivalent increase in sensitivity. Modifications of the electrophoresis conditions have given some further increase. Finally, nucleated cells are often richer in PGK than erythrocytes; for example, single granulocyte-macrophage colonies can easily be analyzed, even using a less-sensitive (tetrazolium dye-based) assay for PGK activity. So experiments of the kind Dr. Metcalf mentioned just now are perfectly feasible as far as sensitivity is concerned.

VAN DEN ENGH: The variation in your assay method must also contribute to the variation you are observing. Is it that much smaller than the 10–30% variation that you can neglect it?

MICKLEM: The variation between repeated assays of the sample is small, normally less than 2%.

VAN BEKKUM: You commented that this marker had more universal applicability than the hemoglobin markers. Can you explain on what this statement is based?

MICKLEM: The hemoglobin marker is useful only for red cells. Since PGK is an essential component of the glycolysis pathway, this marker can be used for all metabolizing cells.

VAN BEKKUM: Including granulocytes?

MICKLEM: Yes, so one of the things we want to do, obviously, is to isolate granulocytes and see whether they behave in the same way.

DEXTER: Are you certain that the X-chromosome is inactivated in stem cells? Or could it be that in the stem cells, both X-chromosomes are active and the random inactivation occurs only at the committed progenitor cell level?

MICKLEM: There is no *direct* evidence that hematopoietic stem cells of the adult are X-inactivated. Many studies have shown that no detectable population of somatic cells with two X-chromosomes persists beyond the first half of the

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may be masked by a dual effect of αMn. Blocking of binding to mucus may facilitate the ascent of bacteria to the kidney, as well as increase the elimination of bacteria from the urinary tract. The inhibition of experimental infection by globotetraose, on the other hand, illustrates a new approach to prophylaxis and treatment of infections occurring via mucous membranes. Compounds mimicking the host receptors may competitively bind to bacterial surface ligands[20,22]. This might decrease the number of bacteria attaching to the mucosa sufficiently to alter the delicate balance of host–parasite interaction in favour of the host.

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A stem cell for stem cells in murine haematopoiesis

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Mature erythrocytes and granulocytes have limited lifespans, do not replicate and must therefore be replenished constantly. They are derived from pluripotent stem cells (PSCs) which are capable of self-renewal[1]. The numbers and properties of PSCs can be inferred in part from studies of their progeny. Such studies have depended largely on highly artificial experimental systems, involving such procedures as X-ray irradiation, bone marrow transplantation and parabiosis[2]. We now describe a method for studying the behaviour of haematopoietic cell populations in normal mice, and show that erythropoiesis is maintained by the products of a very small number of clones which, as predicted by Kay[3], arise and decline in succession. These results suggest that spleen colony-forming cells (CFC), usually regarded as stem cells, are themselves members of substantial clones which differentiate in sequence.

In female mammals, one of the two X chromosomes becomes irreversibly inactivated early in embryogenesis; subsequently any somatic cell and its descendants fail to express genes carried on the inactivated chromosome[4,5]. X-linked genetic variants can therefore provide markers for cell population studies, and have been used to estimate embryonic founder cell numbers for the haematopoietic system[6,7]. Recently, the discovery of an electrophoretic variant of the X chromosome-encoded enzyme phosphoglycerate kinase (PGK-1)[8], and the development of improved methods for measuring PGK activity in electrophoresis strips[9], have provided a convenient marker system in the mouse.

Female mice heterozygous at the PGK-1 locus were bled at 14-day intervals for 16 weeks and then at 28-day intervals for a further 40 weeks. The enzyme phenotypes were determined electrophoretically from haemolysates using a modification of the technique of Bücher *et al.*[9].

The percentages of PGK-1A alloenzyme in serial blood samples from 10 normal heterozygous mice are shown in Fig. 1. Large, and frequently sudden, variations in the relative amounts of A and B alloenzymes are evident in every individual

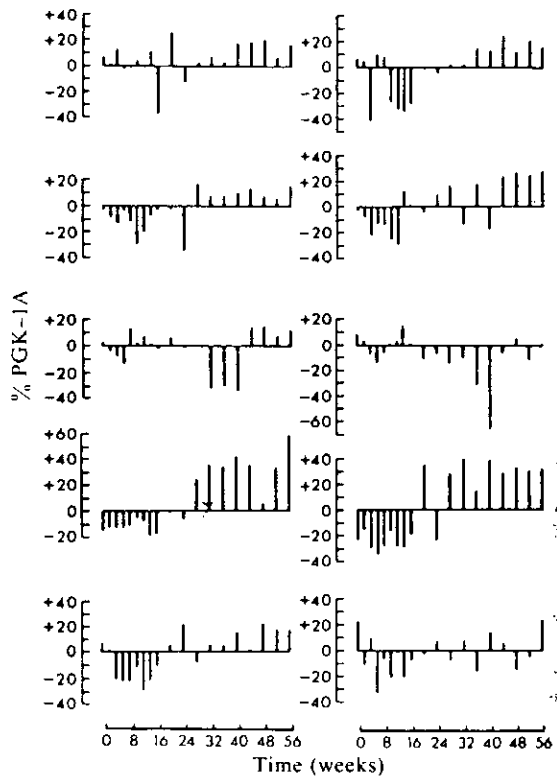

Fig. 1 Percentages of PGK activity attributable to the A alloenzyme in serial blood samples from 10 PGK-1A/B heterozygous female mice. The data for each mouse are plotted as the deviation of individual samples from the overall percentage of A alloenzyme in the mouse; this was estimated as the mean of the readings obtained on day 0 and at 56-day intervals thereafter (see text). The percentages of A and B alloenzymes were determined electrophoretically from haemolysates as described by Bücher *et al.*[9] with the following modification. After electrophoresis, the Cellogel electrophoresis strips were applied to a polyethylene imine (PEI) TLC sheet on which had been spread a solution of the enzymes, substrates and co-factors necessary for the visualization of PGK activity[9], with added ^{14}C-labelled D-glucose. The radioactive final products of the linked enzyme reactions (glucose-6-phosphate and 6-phosphogluconolactone) were adsorbed onto the PEI sheet[14], which was then autoradiographed on Kodak XS X-ray film. The autoradiographs were scanned with a double-beam recording densitometer and the area under each peak integrated. The figures obtained were corrected to compensate for the log-linear response of the X-ray film to exposure, as determined by calibration with a series of known alloenzyme mixtures, and the proportions of the A and B alloenzymes were calculated. In most animals the overall A:B ratio was ~7:3, a value consistent with the expected linkage of *xce* alleles (controlling the probability of a given X chromosome escaping inactivation) to the PGK-1A and 1B alleles[15]. The mice used were from the eighth generation back-cross of PGK-1A onto the CBA/Ca (PGK-1B) strain.

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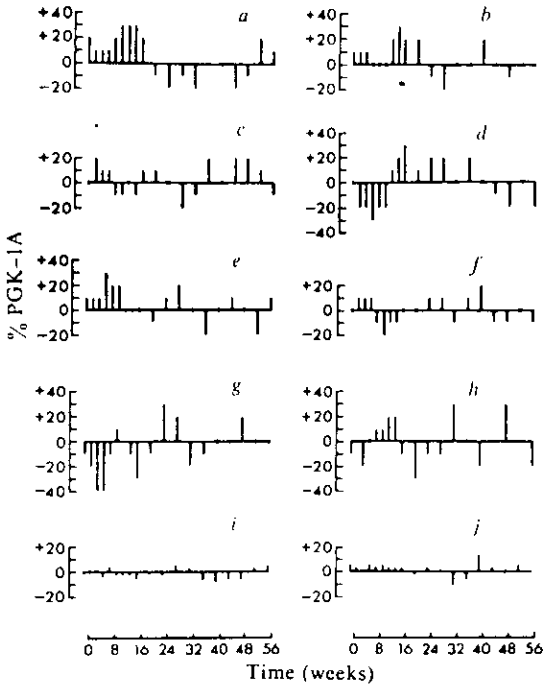

Fig. 2 Computer simulations of PGK phenotypes in serial blood samples, generated on the assumptions that (1) erythrocyte lifespan = 45 days; (2) PGK-1A and 1B-expressing clonogenic cells are selected at random; and (3) each clone produces erythrocytes for only a limited period before becoming extinct or quiescent. Three (*a–h*) or 15 (*i, j*) clones productive over any 14-day period. The simulations shown are unselected, i.e. first series generated.

suggesting that only a few clones of cells are represented in each blood sample and that the identity of these can change rapidly. On the simplifying assumption that all erythropoietic clones are of equal size, the number of clones (n) can be estimated by the binomial equation $n = p(1-p)/s^2$ (refs 10, 11) where p is the best estimate of the overall proportion of A alloenzyme in the haematopoietic system, calculated as the mean from several blood samples; $1-p$ is the proportion of B alloenzyme; and s^2 is the variance of individual and independent samples around p. As the lifespan of erythrocytes in the mouse is ~40–45 days[12], blood samples taken at 14- and 28-day intervals are not independent and therefore cannot all be used to estimate p and s^2. Accordingly, n was calculated from the samples taken at day 0 and at 56-day intervals thereafter. The mean value of n was 10 and the range 2–30. This wide estimated range is largely due to statistical error, as only eight independent blood samples were obtainable over the 56-week period of the study. Using the value 10, and assuming 10^{10} erythrocytes per ml of blood with a blood volume of 2 ml, each clone must average 2×10^9 erythrocytes and is therefore large enough alone to provide the total steady-state erythrocyte requirements for 4.5 days; three clones could provide for 14 days. The large changes that can occur in A : B ratios within 14 days suggest that clones do indeed arise and decline in quite rapid succession. Computer simulations of our data, assuming that three clones maintain the whole burden of erythropoiesis for 14 days and then cease production, are shown in Fig. 2(*a–h*). These simulations correspond quite closely to the actual data, while simulations using any substantially larger number of clones—for example 50 in a blood sample, corresponding to 15 over a 14-day period—do not generate sufficient variability (Fig. 2*i–j*).

To make a clone of 2×10^9 cells requires a production line of 31 doublings, which is far longer than has been estimated for the production of erythrocytes from CFC—about 12–14 doublings[13]. CFC have long been regarded as haematopoietic stem cells and some of them at least have the stem cell properties of self-renewal and pluripotency. Our data show that those CFC which undergo erythroid differentiation are themselves substantial clones, separated by 17–19 doublings from the clonogenic cell, and that these clones are used sequentially to maintain erythropoiesis.

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Analysis of fibroblast proteins from patients with Duchenne muscular dystrophy by two-dimensional gel electrophoresis

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Duchenne muscular dystrophy (DMD), the most common and severe form of the muscular dystrophies, is an X-linked inborn error of metabolism with multiple tissue involvement. Although the major pathological changes are observed in skeletal muscle, abnormalities have also been detected in the heart[1], nervous system[2], red blood cells[3], lymphocytes[4] and cultured skin fibroblasts[5–7]. For many reasons, such as readily available tissue material, fewer secondary changes and the potential for prenatal diagnosis, cultured skin fibroblasts should be the tissue of choice to search for the primary defect. Several abnormalities have been reported in DMD fibroblasts, suggesting that the genetic abnormality is expressed in these cells[5–7]. To search for potentially mutant protein(s) we have compared the protein composition of normal and DMD fibroblasts by two-dimensional gel electrophoresis and have now found one protein spot consistently missing in DMD cells. The nature of this protein and its relation to the DMD gene are unknown.

Genetic alterations responsible for a disease are generally reflected in specific proteins which could be structurally altered, synthesized in abnormal quantities or not made at all. We have searched previously for mutant proteins in DMD fibroblasts using one-dimensional polyacrylamide gel electrophoresis in combination with a dual labelling technique and found no consistent abnormality[8]. This indicated that the most abundant proteins in fibroblasts are not affected by the DMD gene. With the development of two-dimensional polyacrylamide gel electrophoresis[9], the power of resolution for protein composition was greatly improved. However, using the O'Farrell technique[9] we encountered problems in obtaining absolute consistency between the protein maps of separate experiments. We therefore used a dual labelling approach, in which normal and DMD cells are labelled with two different isotopes (^{14}C- and ^{3}H-leucine), mixed and then co-electrophoresed on the same gel.

Clonal interaction in tumours

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The development of cancer is contingent on the emergence of at least one clone of transformed cells. One method used to investigate whether human tumours are monoclonal depends on the mosaicism in the normal tissues of women heterozygous for the two forms of the enzyme glucose-6-phosphate dehydrogenase (G-6-PD)[1]. This mosaicism results from the inactivation of one X chromosome in all somatic cells and should not exist in a monoclonal population. Following the discovery[2] in feral mice of an electrophoretic variant (A) of the X-coded enzyme phosphoglycerate kinase (PGK-1) which differs from the form (B) found in common laboratory mouse strains it was reported[3] that fibrosarcomas induced chemically in hybrids of feral and laboratory-bred mice expressed both enzyme phenotypes, but the conclusion that both were expressed by neoplastic cells was based solely on morphological evidence. The development of histocompatible substrains of mice homozygous for one or other alloenzyme has made it possible to study the clonal composition of tumours under experimental conditions in which the neoplastic status of subpopulations of cells can be verified by transplantation. The experiments we now report, while confirming that murine fibrosarcomas are often pleoclonal, show that the clonal composition may change markedly during tissue culture and on transplantation to congenic hosts. These changes presumably reflect changes in the growth kinetics of differentiating subpopulations of the tumour. Cloned sublines are less readily transplantable than uncloned tumour cell populations, and some sublines are less readily transplantable than others; this suggests that sublines resistant to a host's attack are selected on transplantation or that some sublines require the cooperation of others to survive. We postulate that changes in clonal composition occur also during tumour development, metastasis and recurrence.

Fibrosarcomas were induced in adult female CBA backcross mice heterozygous at the PGK locus[4] (*Pgk-1*^{a}/*Pgk-1*^{b}, abbreviated to AB) by subcutaneous (s.c.) injection of methylcholanthrene (MC) dissolved in tricaprilin (S tumours) or s.c.

Table 1 PGK-1 alloenzyme assays of uncloned cultures

No. of tumours	Reference numbers of tumours	Results	Interpretation (types of neoplastic clones)
19	S1, 6, 7, 8, 9, 11, 15; D6, 7, 9, 12, 16, 17	A in all cultures; B in none	A only
	D1, 5, 8, 18	A in all cultures, B trace to 10% in some primary cultures	
	S3; D2	A in all cultures; B trace to 10% in some primary and 2nd generation cultures	
1	S5	B in all cultures; A 50% falling to 20% in primary cultures only	B only
2	S12, 14	A in all cultures; B increased from trace in primary to 20% in 3rd generation cultures, then disappeared	A only or A and B
2	D4, 14	B in all cultures; A increased from trace in early primary to 20% in 3rd gen. cultures, then disappeared	B only or A and B
10	S13; D15	A in all cultures; B 30–50% in primary and 2nd generation cultures, then declined and disappeared	A and B
	S4	A in all cultures; B 80% in primary culture, then declined and disappeared	
	S2; D10	A in all cultures, B 50% in early primary cultures, persisted to 4th to 6th culture generation in amounts which varied from trace to 50% and then disappeared	
	D13	A with trace B in primary and early 2nd generation cultures; B only, or with trace to 20% A in all subsequent cultures (to 8th generation)	
	D3	B in all cultures; A increased during primary cultures from trace to 50%, persisted to 5th culture generation in amounts which varied from 10–60% and then disappeared	
	S10	B in all cultures; A absent in primary but increased in 2nd generation to 50% and persisted in all subsequent cultures in amounts which varied from 20–50%	
	D11	A and B in substantial amounts in all cultures, A being the larger component in some and B in others	
	D19	A and B in equal amounts in primary culture; subcultures did not grow	

Fibrosarcomas were induced by s.c. injection of 0.5 mg methylcholanthrene (MC) dissolved in tricaprilin (S tumours) or s.c. implantation of a disk (6 mm diameter) of Millipore membrane (0.22 μm pore diam.) impregnated with 0.1 mg MC (D tumours). Sarcomas developed within 9 months in 35 out of 38 mice and were numbered in accordance with the time they took to develop. Cell suspensions were prepared with Dispase from tumours collected 113–211 days after carcinogen administration, washed, and resuspended in Ham's F10 medium with 10% fetal calf serum (FCS). As a rule the hosts were killed, but in a few animals the tumour-bearing hind limb was removed surgically and in two of these the tumour recurred at the site of amputation. Tissue culture flasks (Falcon 75 cm^2) were seeded with 10^7 viable cells and incubated at 37 °C in an atmosphere containing 5% CO_2. After culture for 18 h to 20 days, non-adherent cells were discarded and adherent tumour cells detached by brief exposure to trypsin (0.07%) and EDTA (0.027%) were used to set up subcultures. This procedure eliminates nearly all the leucocytes (which are non-adherent) and macrophages (which are strongly adherent), but not fibroblasts. Samples from primary cultures and subcultures, and usually also of suspensions prepared from the whole tumour and from host liver, were frozen and thawed in lytic buffer, and the approximate proportions of the two alloenzymes were determined by gel electrophoresis, using a modification of the linked enzyme assay developed by Buchet *et al.*[8], the production of NADPH being visualized by the reduction of a tetrazolium dye, thiazolyl blue, to its formazan derivative.

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implantation of a disk of Millipore membrane impregnated with MC[5] (D tumours). The relative amounts of the two alloenzymes were determined in whole tumour suspensions[6] and uncloned cultures after gel electrophoresis (Table 1). As expected, both A and B components were found more often in whole tumour suspensions (17 out of 24 studied) than in primary cultures of the same tumour (11 out of 24), which should contain fewer normal cells. With many tumours the A/B ratio fluctuated greatly during culture. Contrary to the earlier finding of Reddy and Fialkow[3], there was a marked preponderance of tumours with an A component; this is not surprising, however, because the *Pgk-1* locus is closely linked to another locus, *Xce*, which determines the probability of the X-chromosome being inactivated[7]; the resulting ratio of A cells to B cells in the normal tissues of AB mice of our CBA stock is about 70:30.

Clones were isolated from 10 tumours, and samples from cloned and uncloned cultures were injected into homozygous females ($Pgk\text{-}1^b/Pgk\text{-}1^b$, abbreviated to BB) or hemizygous males ($Pgk\text{-}1^a/Y$, abbreviated to AY); when tumours developed these were assayed in the same way as the primary tumours (Table 2). Other samples were used for karyotyping and determination of the cellular DNA content by automated scanning[9] of Feulgen-stained preparations (1,500–4,000 cells). With four tumours (D11, D13, D15, S10), we have formal proof of the existence of both A and B neoplastic components, since on transplantation to BB mice each gave rise to some tumours with only an A component and others with no detectable A component. In the case of D11 and S10, many A clones and B clones have been isolated and shown to be polyploid. Two A clones from D11 (Nos 6 and 10) had a modal chromosome number of 98, and automated scanning of one of these showed a mean DNA content 2.6 times that of normal diploid cells; one B clone (no. 12) had a modal chromosome number of 79 and mean DNA content 1.9 times that of normal cells. Clones 6 and 10 gave rise to A tumours, and clone 12 to a B tumour, when transplanted to BB mice. With tumour D15 the B neoplastic component disappeared in the course of tissue culture. Tumour S10 is unusual in that we have isolated A clones, B clones, and 'clones' expressing both A and B. Six of the latter were harvested from 192 wells seeded with, on average, 1 cell; and since no colonies developed in 139 wells the mean number of clonogenic cells seeded (assuming a Poisson distribution) was probably about 0.3 per well. It seemed just possible that these supposed clones were mixtures, but on recloning at the same and more extreme dilution we have obtained 19 'subclones' which still express both A and B, 20 which express only B and none which express only A. While the possibility of mixed populations of cells growing in symbiosis is still not formally excluded, it seems likely that AB clones have arisen from hybrid cells formed by fusion, either in the mouse or in tissue culture, of an A cell and a B cell, at least one of which had undergone transformation, and that disappearance of the A component in some cases on recloning was due to chromosome loss. An alternative possibility is that there has been reactivation of the inactive X chromosome in a cell which prior to transformation expressed only A or B. Preliminary observations based on flow cytometry of cells stained with the dye Hoechst 33342 have shown a higher proportion of diploid cells in suspensions from the original tumour and primary cultures than from later cultures. This may be due entirely to selection, but raises the question of whether some of the tetraploid and hypertetraploid clones we have found in other tumours may also have developed from hybrid cells formed during tissue culture. Karyotypic analysis and studies with new tumours may resolve some of these uncertainties.

Clones from five tumours (D11, D13, D14, S2, S10) which transplanted readily in the form of uncloned cultures were transplanted to normal BB mice, and in some cases also to thymectomized, irradiated BB mice protected with cytosine arabinoside[10], by s.c. injection of 1.5×10^6 viable cells. Only 4 out of 21 clones grew in the normal mice, whereas 6 out of 6 which had failed to grow in the normal mice grew in the thymectomized mice. After passage in thymectomized mice, 5 of the 6 tumours grew when retransplanted to normal mice. These preliminary findings suggest that clones with a high capacity for survival are selected when pleoclonal populations are transplanted; in addition, some clones may require the cooperation of others to survive. The successful transplantation of five clones to normal mice after passage in thymectomized, irradiated mice suggests further that cells resistant to surveillance may emerge when tumours grow under conditions in which surveillance is impaired. The features which favour survival of a clone have not been elucidated, but possibilities to be considered include lack of strong rejection-inducing antigens; failure, despite the presence of such antigens, to evoke an effective immune response; and relative insensitivity to surveillance mediated by NK cells and macrophages.

Our results highlight the problems involved in assessing the clonality of tumours with X-linked markers, whether in man or mouse, and lend support to the view that tumours of pleoclonal origin may appear, at a particular time, to be monoclonal because all clones except one have been competitively eliminated or reduced to the point of being undetectable.

It seems likely[12,13] that clonal interaction plays a significant role in the life history of pleoclonal tumours in general. At

Table 2 Characterization of clones, transplants and recurrent tumours

No. of tumour	No. and type of clones isolated	Transplants: Material transplanted	Transplants: Neoplastic components proved	Recurrence of primary tumour
D17	6A			
S3	12A			
S5		Primary tumour	B*	B
D4	60B	Primary tumour	B*†	
D14	16B	Primary tumour	B*	
		B clone	B*†	
S13				A
D15		Primary tumour	A†B*	
		1st generation transplant	A*†	
		2nd generation transplant	A†	
S4		Primary tumour	A†	
		1st generation transplant	A*	
		2nd generation transplant	A*†	
S2	20A‡	A clone	A†	
D10	15A			
D13	1A, 6B	Uncloned culture	B*†	
		A clone‖	A†	
D3	78B	Primary tumour	B*	
S10	A, B, AB§	A clone‖	A†	
		B clone‖	B*	
		AB clone	A†B*†	
D11	21A, 36B	Primary tumour	B*	
		A clone	A†	
		A clone	A†	
		B clone‖	B*	

Clones were isolated either in wells of microtest plates (Falcon 30401) seeded with 0.2 ml of a suspension containing tumour cells and irradiated (60 Gy) human fibroblasts which yielded on average 1, 2 or 5 tumour cells and 2,000 fibroblasts per well, or by ring cloning in small (25 cm^2) flasks.

* Proof based on development of tumours with A component *only* in AY mice or B component *only* in BB mice.

† Proof based on development of tumours with A component in BB mice or B component in AY mice.

‡ One apparent clone of type B was isolated but on transplantation this yielded only A tumours in AY mice. On retesting, the 'clone' was found to contain a trace of A in addition to the main B component.

§ See text.

‖ These clones grew only in, or after passage in, thymectomized, irradiated mice.

present we do not have sufficient data to construct models of the cell population kinetics of such tumours, but it is possible to suggest some general principles on which these might be based: (1) The number of clones present at any given time will depend on the number of cells originally transformed and the growth rate of each clone. The number of cells transformed will depend *inter alia* on the nature and dose of the carcinogen. The growth rate of each clone may be influenced by inherent properties of the transformed cells, the host reaction, and interaction between the clone in question and other clones of transformed or initiated cells. (2) If growth rates remain constant the clone with the greatest growth rate will eventually outgrow all others, but this does not necessarily exclude the persistence of small numbers of very slowly growing or dormant cells. (3) Growth rates may change suddenly, and dormant cells may resume cycling. Such changes may be influenced by treatment but may also occur for no apparent reason. Clones which grow slowly or remain dormant in primary tumours may grow rapidly in metastases or locally recurrent tumours. (4) The symbiotic relationship between different clones may be antagonistic, neutral or mutualistic.

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Dihydroouabain is an antagonist of ouabain inotropic action

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The Na^+, K^+-pump controls a wide variety of cellular systems and its inhibition by cardiac glycosides modifies important physiological functions and evokes several pharmacological effects (refs 1, 2 and refs therein). However, not all the actions of cardiac glycosides can be attributed to Na^+, K^+-pump inhibition and several observations show that, at low doses, cardiac glycosides stimulate the pump[3–5]. It has been proposed that their positive inotropic effect could be the sum of two processes: the inhibition of the pump and a still unknown additional inotropic mechanism[6]. In guinea pig heart, low doses of ouabain interact with high-affinity binding sites, which differ from the lower-affinity sites responsible for Na^+, K^+-pump inhibition[3,7–9]. It has been suggested that ouabain interaction with these high-affinity sites could be responsible for the additional inotropic mechanism[6]. The existence of two classes of ouabain-binding sites has been documented not only in guinea pig heart, but also in dog[10], rat[11,12] and human heart[13]. Dihydroouabain, a derivative of ouabain in which the lactone ring is saturated, is about 50-fold less potent than ouabain as an inhibitor of Na^+, K^+-pump and does not stimulate the pump at low doses[7]. Its inotropic effect can be entirely accounted for by the inhibition of the pump[6,14]. We have examined the pharmacological action of ouabain in the presence of dihydroouabain and report here that dihydroouabain reduces ouabain inotropic action but not Na^+, K^+-pump inhibition.

Figure 1 shows the inhibition of $(Na^+ + K^+)$ATPase activity (Fig. 1*a*) and of specific ^{3}H-ouabain binding (Fig. 1*b*) in guinea pig heart microsomes as a function of ouabain or dihydroouabain concentration. With both glycosides, the slope of the Hill plot was equal to 1 for $(Na^+ + K^+)$ATPase inhibition. In contrast, the slope was 0.64 ± 0.001 for the displacement of ^{3}H-ouabain. This confirms the previously reported heterogeneity of ouabain binding sites[4,15] and shows that dihydroouabain competed with ^{3}H-ouabain at both high- and low-affinity sites. In view of this finding, we have examined whether dihydroouabain could interact with the action of ouabain.

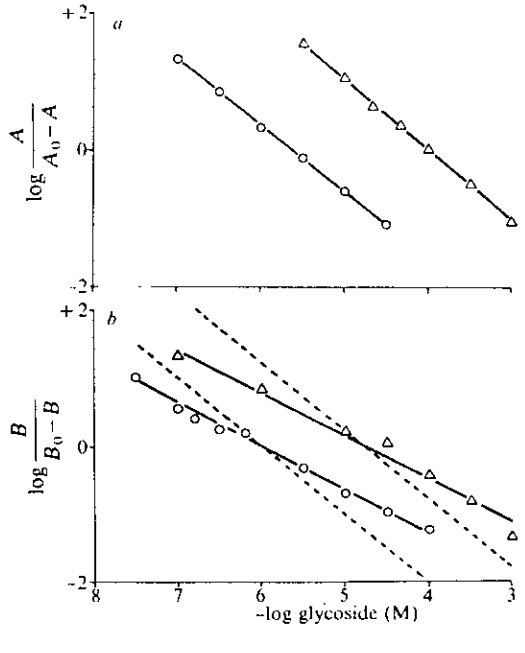

Fig. 1 Interaction of ouabain and dihydroouabain with guinea pig heart microsomes. The methods for microsomal $(Na^+ + K^+)$ATPase preparation and assay[20], and for ^{3}H-ouabain assay[13] have been described elsewhere. *a*, $(Na^+ + K^+)$ATPase inhibition by cardiac glycosides. About 0.01 mg protein was incubated for 120 min in 2 ml medium containing 100 mM NaCl, 10 mM KCl, 3 mM $MgCl_2$, 3 mM ATP, 1 mM EGTA, 20 mM Tris-maleate (*p*H 7.4, 37 °C) and various concentrations of glycoside. The released inorganic phosphate was measured by colorimetry[20]. ATP hydrolysis was <15%. Ordinate: log $(A/A_0 - A)$ where A_0 is the control $(Na^+ + K^+)$ATPase activity and A the activity in the presence of ouabain (○) or dihydroouabain (△). Abscissa: −log glycoside concentration *b*, Displacement by ouabain or dihydroouabain of the specific ^{3}H-ouabain binding. About 0.24 mg protein was incubated in the conditions described above. The incubation medium was supplemented with 10^{-8} M ^{3}H-ouabain (19 Ci mmol^{-1}; Amersham), and with 0.01 mM $NaVO_3$, 5 mM inorganic phosphate, 3 mM creatine phosphate and 0.01 mg creatine kinase (25 U per mg) to limit ATP hydrolysis and keep the conditions constant[8]. The reaction was stopped by filtration on Whatman GF/F glass fibre filters. The filters were washed with 10 ml of chilled 0.25 M sucrose solution. The radioactivity trapped by the filters was measured as described previously[13]. The nonspecific binding was estimated in the presence of 1 mM ouabain and subtracted from the total. Ordinate: log $(B/(B_0 - B)$ where B_0 is the control binding and B the binding in the presence of ouabain (○) or dihydroouabain (△). Abscissa: −log glycoside concentration. The dashed lines represent theoretical curves with slope equal to 1. The points are means from three experiments. The departure from parallelism between experimental and theoretical curves was checked by a comparison of difference in slopes between the two curves (for ouabain, $t = 5.2$, $P < 0.001$; for dihydroouabain, $t = 8.30$, $P < 0.001$).

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Sources of Inter-tissue Variation in PGK-1 Heterozygotes

In female mammals heterozygous for X-chromosome linked genes, functional mosaicism for the products of such genes occurs early in embryonic development when one X-chromosome is inactivated. As X-chromosome inactivation is cell autonomous and irreversible in somatic cells, clones of cells arise with either the maternal or the paternal X-chromosome active.

Assuming that X-chromosome inactivation occurs randomly within the embryo, variation in mosaic composition will occur between individuals heterozygous for X-linked genes. The number of cells in the embryo at the time of X-inactivation will affect the degree of variation between embryos. If X-inactivation occurs when the embryo if composed of few cells, as appears to be the case, large variations between embryos are more likely to occur than if X-inactivation occurs later in embryonic life when the embryo is made up of many cells. Variation between embryos will also depend on the subsequent growth of clones of cells which arise as a result of X-inactivation. Coherent clonal growth may occur with little cell mixing or there may be extensive cell mingling during growth. In addition to cell growth and mixing, inter-embryonic variation will also be affected by the sampling of cells for different cell lineages. Both the number of samplings for different tissues and the timing of formation of different tissues will affect the overall phenotype of each individual.

It is likely that at some stage, early in embryonic life, a series of departures of different cell lineages from a pluripotent stem cell occurs to produce distinct and discrete tissue types. This step is probably caused by a restriction of the genotype causing differentiation (Monk, 1981). From the resultant stem cell pools the various tissues of the body are formed. Assuming that the allocations of cells to different lineages and tissues are random sampling events, variation in mosaic composition among different tissues within mosaic individuals

may occur.

Variation between tissues within individuals may be enhanced by the effects of the precursor pool sizes for individual tissues and also by patch size at the time of tissue formation. If there is a lot of coherent clonal growth after X-inactivation occurs but before tissue formation commences, then the importance of patch size is greatly enhanced. If small numbers of cells form individual tissues, large variations in mosaic composition among tissues will occur. If, however, tissue formation begins soon after X-inactivation with relatively little increase in the number of cells, less variation in tissue composition due to patch size is likely to occur. Cell mixing occurring in the period between the time of X-inactivation and tissue differentiation will also reduce inter-tissue variation.

McMahon et al (1983) reported that the broad similarities generally found among various tissues in 12½ day p.c. embryos suggest that the tissues examined were derived from the same pool of X-inactivated cells. Considerable growth and cell mixing must occur before sampling for the various lineages to explain the similarity in the mosaic composition of tissues within an embryo. McMahon and his colleagues suggested that as all the pairs of tissues they measured showed high levels of correlations in mosaic composition, it was unlikely that any individual tissue was formed from a much larger, or smaller, number of cells than the mean value of n_2 (the number of tissue seeding events per tissue), which they calculated, of 193 cells. McMahon et al also derived the number of cells at X-inactivation, n_1, which they estimated to be 47. In the animals studied in the present study n_1 may be the same as McMahon et al's number. However, n_2 for bone marrow will be a composite of the mesodermal precursor number with sampling to the chorio-allantoic membrane, the foetal liver and the bone marrow.

In the haematopoietic tissues of adult mice (as shown in the present study) PGK-1A:1B ratios can vary quite considerably over small periods

of time, which indicates that clones of cells maintaining this tissue may be succeeding each other. Independent variation within individual tissues could give rise to increased or decreased variation between tissues at different sample times.

The effect of clonal succession on the variation between tissues will not be constant - in some, the blood forming tissues, for example, the high rate of cell turnover may lead to rapid changes in tissue phenotype, whereas in others where cell turnover is very much less rapid (e.g. kidney, liver) the tissue phenotype will only change very gradually, if at all. The contribution of experimental technique to observed variance must also be included when considering inter-tissue variations in phenotype.

Enumeration of Stem Cell Populations

In using genetic mosaics to estimate the number of cells present in the embryo at X-inactivation and tissue formation, a number of very important assumptions are made. First, X-inactivation is assumed to occur synchronously within the embryo, or at least within the embryonic mesoderm. A second assumption is that there is no cell selection favouring either of the two distinct cell types arising after X-inactivation. Thirdly, it is assumed that at the time immediately preceeding the sampling event, cells are randomly distributed and the cells sampled are withdrawn from a pool at random. Fourthly, individual precursor cells are assumed to give rise to clones of equal size and longevity. McMahon et al (1983) add two further assumptions to this list; these are (1) that all tissues are derived from precursor pools of equal size and (2) that variations between tissues in the embryo are non-significant. Variations between tissues in the McMahon study were shown to be non-significant, but only in certain tissues and at certain developmental times. For the reasons outlined earlier this statement may not apply to the adult haematopoietic system. It is unlikely that all, if any, of these assumptions are entirely correct;

however, they form an essential basis for any statistical calculations on X-inactivation mosaics.

In order to estimate the clone numbers at the time of X-inactivation and tissue formation the binomial approach has been used. Both Nesbitt (1971) and McMahon et al (1983) were forced, by a lack of repeat sampling of individual tissues, to use the covariance between pairs of tissues to derive estimates of n_1, the number of embryo precursor cells at the time of X-inactivation. Nesbitt derived many independent estimates of n_1, while McMahon and his colleagues suggested that it is more appropriate to consider all tissues as replicate measurements and calculate a single estimate, $\hat{n}_1$ of n_1.

For the experimental approach used in the present study Nesbitt's statistical analysis is not entirely appropriate: she was observing chromosomes in individual cells and, as a result, knew the number of cells, k, scored per organ. Obviously, sampling of cells for scoring will contribute to the observed variance for any tissue type. The harmonic mean of k, $\hat{k}$ was calculated for each tissue type and was used to estimate the number of embryonic precursor cells.

In the present study, and in that of McMahon et al (1983), the proportions of PGK-1A and -1B are estimated from a cell lysate of the tissue being tested. The sample size, and hence k, is unknown. By using cell lysates of tissue samples, one should be able to measure a mixture of all the cells making up the tissue, thus allowing analysis of much larger samples than can be analysed using chromosome markers. By using lysates of large numbers of cells sampling errors due to small sample size are eliminated. In McMahon et al's study, samples were taken from whole tissue preparations, whereas in the present study, particularly on blood, tests were made on random samples taken from the whole tissue at various times. These random samples were so large (10^8 cells) that the contribution of this sampling error to the total variance can reasonably be ignored.

McMahon et al (1983) and Stone (1983) adapted Nesbitt's approach to suit the PGK-1 system. Using the equation $s^2 = \frac{pq}{n_1}$, where s^2 = embryo variance, p = probability that X-inactivation will yield a PGK-1A cell type and q = 1 - p, they calculated a single estimate of n_1, $\hat{n}_1$, for all embryos studied. They estimated this figure to be 47 cells. As only one tissue was measured in the sequential blood samples taken in the experiments reported in Chapter 3, there is insufficient data to derive an estimate of n_1. A previously published value of n_1 can, however, be used to derive n_2, the precursor pool size for the haematopoietic system.

In studying repeated blood samplings from a series of individuals, there are two sampling events and the resulting variance is a mixture of the variance between individuals (s_e^2) and the tissue variance within individuals (s_t^2). From McMahon et al's analysis

$$s_e^2 + s_t^2 = \frac{pq}{n_1} + \frac{1}{n_2}\left(1 - \frac{1}{n_1}\right) pq ,$$

hence by substituting into the above equation McMahon et al's value of n_1 of 47, and our own values for the other known parameters values of n_2, the number of cells seeding the haematopoietic system in tables 3.2 and 3.6, can be derived. These values are shown for individual sampling times in the revised tables 3.2a and 3.6a. In addition, single estimates $\hat{n}_1$ were calculated for (a) all bleeds to bleed 19, (b) 56 day bleeds to bleed 19 and (c) 56 day bleeds to bleed 29 in table 3.2, and (d) all bleeds to bleed 13 and (e) 56 day bleeds to bleed 13 in table 3.6. These values are shown below, the bracketed figures are the previously estimated values of N using Nesbitt's equation $N = \frac{pq}{s^2}$

(a) $\hat{n}_2 = 15$ (11)
(b) $\hat{n}_2 = 20$ (14)
(c) $\hat{n}_2 = 20$ (14)
(d) $\hat{n}_2 = 46$ (23)
(e) $\hat{n}_2 = 53$ (25)

These values for $\hat{n}_2$ are very much smaller than the value of 193 calculated by McMahon et al. The usefulness of $\hat{n}_2$ in the present case is questionable however as one cannot tell whether is applies to original tissue seeding or to other seedings up to bone marrow. In the experiments described in Chapter 3, the main aim was to calculate cell numbers involved in clonal succession. If the variance attributable to those numbers was much larger than for n_1 or n_2, which it was in the present data, it is convenient to combine n_1 and n_2 and hence calculate N. It is unfortunate that insufficient data was obtained in the present study to derive a value of $\hat{n}_1$, however, even using McMahon et al's value of $\hat{n}_1$ the two sets of figures are generally highly comparable.

In table 6.2 in the text it may appear that the calculation previously used to calculate N is now being used to calculate a different cell number. This is not the case, however; as explained in the text (pages 128 and 134), the clones of cells repopulating the haematopoietic system following hydroxyurea treatment may or may not reflect accurately the original stem cell pool in each animal. It must be remembered that the action of hydroxyurea will wipe out most haematopoietic cells, with only those most resistant to hydroxyurea treatment surviving. If one assumes that there are 3×10^5 CFC-S per mouse, and that 97% of CFC-S are killed by 5-HU injections then of the order of 500 CFC-S per bone (or about 10^4 per mouse) will survive hydroxyurea treatment. Therefore, after hydroxyurea treatment, cell samples are being derived from the descendants of a limited pool of stem cells. In normal animals, with a much greater pool of stem cells, clonal succession appears to act in the maintenance of haematopoiesis. In hydroxyurea-treated animals

variation due to clonal succession may be greater due to the very much smaller stem cell pool from which cells are drawn.

It would be possible to apply the equation used by McMahon et al (1983) to estimate n_2, the number of cells sampled for each tissue considered, to the hydroxyurea data,

$$s^2 = \frac{1}{n_2}\left(1 - \frac{1}{n_1}\right) pq$$

where s^2 = random deviation for a particular tissue from the mean for all tissues. McMahon et al, however, consider all tissues as replicate measurements. They are able to do this because they measured tissues with very little variation. In the present experiments, however, large variations between tissues within individuals were found (see appendix 2, table 9) and so all tissues cannot be considered as replicate measurements, hence the composite number N (the sampling of stem cells from which the cells actively maintaining haematopoiesis are drawn - shortened to clone numbers maintaining haematopoiesis in the table title) was calculated for each tissue. In addition, because of the great stress placed on the haematopoietic system following hydroxyurea treatment McMahon's assumption that n_2 would be the same for all tissues is probably untrue. In this experiment it is impossible to gain information concerning embryonic tissue founder numbers, because it is impossible to take sequential samples from individual tissues as one can with blood in order to determine how much of observed variance is due to clonal succession.

The only valid conclusion which could be drawn from the hydroxyurea study is that if the variance in experimental animals is consistently greater than in controls then the number of stem cells responsible for haematopoietic regeneration is smaller than the number normally maintaining steady state haematopoiesis. No consistent trend was, however, found and so the conclusion must be drawn, as discussed in the text, that haematopoiesis following hydroxyurea treatment is not

maintained by a smaller number of stem cells with a greater repopulating ability, but that a more complex control mechanism is operating.

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Table 3.2a Precursor Pool Sizes for Haematopoietic Tissues

n_2 was calculated from the equation $s_e^2 + s_t^2 = \frac{pq}{n_1} + \frac{1}{n_2}(1 - \frac{1}{n_1})\,pq$, using McMahon's published value of n_1. Previously calculated values of N are shown in backets for comparison.

Bleed Number	Mean % PGK-1A $\pm$ s.d.	Number of Precursor Cells
1	71.1 $\pm$ 13.1	16 (12)
2	72.8 $\pm$ 12.7	16 (12)
3	76.6 $\pm$ 13.3	13 (10)
4	76.8 $\pm$ 13.8	11 (9)
5	75.5 $\pm$ 11.8	18 (13)
6	71.9 $\pm$ 12.9	16 (12)
7	73.2 $\pm$ 15.1	10 (9)
8	73.4 $\pm$ 14.3	12 (10)
9	71.7 $\pm$ 12.3	18 (13)
10	70.9 $\pm$ 11.9	21 (15)
11	71.3 $\pm$ 13.5	14 (11)
12	71.4 $\pm$ 10.9	26 (17)
13	72.5 $\pm$ 13.6	14 (11)
14	73.8 $\pm$ 12.6	16 (12)
15	73.4 $\pm$ 12.7	16 (12)
16	75.0 $\pm$ 14.0	12 (10)
17	74.3 $\pm$ 13.9	11 (9)
18	71.6 $\pm$ 12.8	16 (12)
19	75.0 $\pm$ 13.1	14 (11)

$\hat{n}_2$ for all bleeds to bleed 19 = 15 (11)

$\hat{n}_2$ for 56 day bleeds to bleed 19 = 20 (14)

$\hat{n}_2$ for 56 day bleeds to bleed 29 = 20 (14)

Table 3.6a Precursor Pool Number for Haematopoietic Tissues

n_2 was calculated from the equation $s_e^2 + s_t^2 = \frac{pq}{n_1} + \frac{1}{n_2}\left(1 - \frac{1}{n_1}\right) pq$,

using McMahon's published value of n_1. Previously calculated values of N are shown in brackets for comparison.

Bleed Number	Mean % PGK-1A $\pm$ s.d.	Number of Precursor Cells
1	77.9 $\pm$ 14.0	11 (9)
2	72.9 $\pm$ 12.4	17 (13)
3	74.4 $\pm$ 10.8	24 (16)
4	77.2 $\pm$ 8.7	45 (23)
5	67.9 $\pm$ 11.9	22 (15)
6	76.9 $\pm$ 11.4	19 (14)
7	72.5 $\pm$ 11.4	22 (15)
8	75.8 $\pm$ 7.2	141 (35)
9	79.9 $\pm$ 8.9	35 (20)
10	75.9 $\pm$ 13.7	12 (10)
11	77.0 $\pm$ 10.3	25 (17)
12	75.1 $\pm$ 15.7	9 (8)
13	75.7 $\pm$ 9.7	33 (20)

$\hat{n}_2$ for all bleeds = 46 (23)

$\hat{n}_2$ for 56 day bleeds = 53 (25)