PERTURBATION OF CELL RENEWAL IN THE HAEMOPOETIC TISSUES OF DRUG-TREATED AND LEUKAEMIC MICE

Rosalind A. Meldrum

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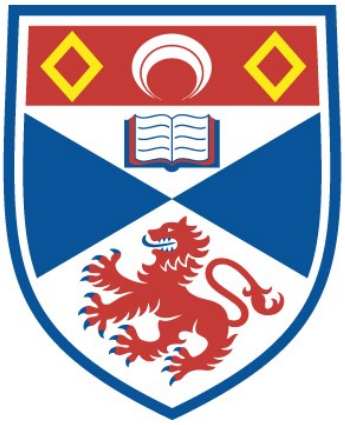

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PERTURBATION OF CELL RENEWAL IN THE HAEMOPOETIC TISSUES OF DRUG-TREATED AND LEUKAEMIC MICE

ROSALIND A MELDRUM

PH.D THESIS 1982 UNIVERSITY OF ST. ANDREWS

DECLARATION

This is to certify that the thesis I have submitted in fulfilment of the requirements governing candidate for the degree of Doctor of Philosophy, in the University of St. Andrews , entitled '' Perturbation of cell renewal in the haemopoietic tissues of drug - treated and leukaemic mice '' is my own composition and is the result of work done mainly by me during the period of matriculation for the above degree. No part of this has been previously submitted for a higher degree.

The research was conducted in the Dept. of Anatomy and Experimental Pathology, United College of St. Salvator and St. Leonard, University of St. Andrews and the MRC Radiobiology Unit , Harwell, under the supervision of Professor T. Brynmor Thomas and Dr. A.C. Riches.

CERTIFICATE

We hereby certify that Rosalind Anne Meldrum has spent nine terms engaged in research work under our supervision, and that she has fufilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1 , 1967), and that she is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

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ABSTRACT

The murine lympho-myeloid complex is depleted by a large dose of nitrogen mustard and the patterns of recovery of the haemopoietic tissues and cells followed to establish the relationships of the precursor and mature cells.

Culture of bone marrow in agar and stathmokinetic techniques are used to examine the controlled proliferation fo the granulocyte elements in the bone marrow recovering from nitrogen mustard and the influence of the spleen is also considered.

The control of the proliferation of the granulocyte cells is lost in myeloid leukaemia. Stathmokinetic methods and spleen colony assays are used to asses cell proliferation in irradiation-induced myeloid leukaemias in mice and the relevence of these parameters so measured to those demonstrated by normal bone marrow is discussed.

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Perturbation of Cell Renewal in the Haemopoietic Tissues of Drug-treated and Leukaemic Mice

1. INTRODUCTION

1.1 The Search for a Haemopoietic Stem Cell

The term kinetics is currently used to embrace the origin of living cells, their migration and movement, their destination, rate of proliferation and differentiation and their relationship to each other.

New living cells are produced by division of pre-existing cells. An adequate number of cell division is necessary to ensure the growth of expanding cell population and the maintenance of cell populations from which cells are being lost (Thomas, 1974).

The blood cell population from which cells are continuously lost, clearly required a source of maintenance, and the evolution of this concept led to a search for a haemopoietic stem cell, ie. one which is capable of providing an output to more differentiated compartments, whilst maintaining its own number.

Before the introduction of transplantation and tissue culture techniques, efforts were concentrated on finding a morphological identity for the haemopoietic stem cell.

There were two schools of thought on the origin of the haemopoietic stem cell, the monophyletic, maintaining that all types of blood cells were derived from a single type of cell and the polyphyletic which maintained that there were two or more stem cells involved in maintaining the blood cell lineages.

In embryonic life and early stages blood cells are derived from multipotent primitive mesenchymal cells first outside the embryo in the vasculosa of the yolk sac and later within the embryo, in the liver, spleen and thymus.

From the fifth month onwards haemopoiesis is concentrated in bone marrow and lymphatic tissue. After birth it is restricted to these sites for the remainder of life except in pathological circumstances.

Twenty years ago most haemoatologists agreed that blood cells are originally derived from fixed reticular cells attached to a fine network of reticulum fibres which extends as a delicate mesh between arteries, veins and sinusoids.

However this group of cells termed the reticulum cells was somewhat ill-defined and under the classification of some workers included several cell types.

Yoffey (1960) confined the origin of blood cells to a compartment of free cells which he termed the lymphocyte-transitional compartment. These cells have a morphology similar to lymphocytes and a high labelling index (Everett et al, 1960), a property required of haemopoietic stem cells. Yoffey has described in detail the morphology of the spectrum of cells belonging to this compartment (Yoffey, 1973).

The historical controversies of 30 or 40 years ago between monophyleticists , polyphyleticists were largely concerned with the differentiating potentialities of the earliest free haemopoietic cells and less with their ability to multiply without differentiating so as to function as a self-maintaining stem cell pool. The quantitative and dynamic aspects of haemopoiesis were poorly explored at the time and detailed morphological study, much of it with tissue sections, formed the basis of numerous theories put forward.

The multiplicity of these theories, the confusion of nomenclature, the reliance on simple morphological similarities as criteria of relationships between cells and the general absence of a quantitative approach is apparent.

A considerable advance in overcoming the problems of identifying the stem cell compartment and determining the potentialities of the least mature haemic cells, was made in 1961 when Till and McCulloch (1961) introduced the spleen colony technique. Further significant contributions were made by culturing bone marrow in agar - the technique of Pluznik and Sachs (1965) and Bradley and Metcalf (1966), which gave rise to granulocyte colonies, Stephenson et al (1971) and Iscove and Sieber (1975) to erythroid colonies, Metcalf et al (1975) to lymphocyte colonies and Metcalf et al (1975) to megakaryocyte colonies.

The complexity of the haemopoietic system, however, seems to have increased in direct relation to the assiduity with which investigators have explored it. It is important though to unravel the mechanisms of homeostatic control of the formed elements of the blood in health and the ways in which these mechanisms function or fail to function in states of disease.

1.2 Properties of the Stem Cell

Evidence that the stem cell belongs to the transitional compartment has been produced by experiments which examined the haemopoietic recovery after sublethal irradiation and experiments on colony formation.

The sequence of events in guinea pigs recovering from 150 R fit well with a stem cell role for LT cells (Harris, 1960). Lajtha et al (1964) using the erythropoietin response in mice observed in the marrow a stem cell overshoot with the same kind of time relationship as the LT overshoot in guinea pigs (Harris, 1956).

Marrow which contained increased numbers of LT cells protected very effectively against lethal irradiation (Harris and Kugler, 1963, 1957 ; Morrison and Toepfer, 1967; Osmond and Yoshida, 1970).

The order of proliferative variation of the spleen colony forming cell, as measured by tritiated thymidine suicide studies, is well exemplified by the LT compartment (Yoffey, 1973).

Hurst et al (1969) found that an increase in the number of CFU-S during marrow rebounds paralleled the increase in transitional cells described by Turner et al (1967).

The effects of irradiation of in vitro colony-forming cells (CFU-C) parallel the effects on transitional cells (Yoffey, 1973). Moore et al (1972) expressed the view that 'in vitro' colony-forming cells are in fact transitional cells.

Niewisch et al (1957) fractionated the cells from spleen colonies and concluded that the stem cell was a large cell with a leptochromatic nucleus and intensely basophilic cytoplasm.

Orlic et al (1968) observed thymidine labelling of cells with the general configuration of transitional cells after erythropoietic stimulation. Van Bekkum et al (1971) enriched CFU content of mouse bone marrow by a factor of up to 30 and identified a 'candidate stem cell' whose ultrastructure is almost identical with that of a medium transitional cell (Bainton and Yoffey, 1970).

Van Bekkum et al (1971) described the morphology of this 'candidate stem cell' as seen in electron microscope sections. It is about 7-10 μm^2 in size, with a round indented nucleus containing one or two large nucleoli and a finely dispersed chromatin pattern with small aggregates at the nuclear margins. The nuclear- cytoplasm ratio is large and the cytoplasm contains abundant free ribosomes and several small mitochondria. Other organelles are not observed.

It differs from the small lymphocyte whose nucleus shows large areas of densely aggregated granular chromatin and smaller light regions whose cytoplasm contains dense bodies and many vesicles of different sizes. Unlike the stem cell Golgi apparatus is present in the small lymphocyte although poorly developed and the mitochondria are large and the ribosomes clustered.

Evidence that the stem cell may belong to the lymphocyte-transitional compartment is also given by Thomas (1973). In the bone marrow of mice recovering from treatment with mustine hydrochloride there is an increase in the proportion of CFU-S on the fourth day (Sharp and Thomas, 1971). This is paralled by

a corresponding increase in only one morphological group of bone marrow cells - the transitional cells (Sharp et al, 1971).

Osmond (1973) summarised certain characteristics of the lymphocyte population. Small lymphocytes (< 8.0 μm in nuclear diameter) constituted 55% of the entire population of marrow lymphoid cells. Only 2% incorporated tritiated-thymidine and therefore they could be regarded as an essentially non-proliferating compartment.

Large lymphocytes (> 8.0 μm in nuclear diameter) included most of the proliferating cells, 28% being in DNA synthesis at any given time. Most large lymphocytes had a relatively leptochromatic nucleus which corresponds with the morphological description of transitional cells.

The kinetics of the large lymphocytes were investigated using the isotopes ^{3}HTdR and ^{14}C. They showed striking differences when classified by nuclear diameter. The following table shows the duration of S phase and labelling index of the cells as related to their nuclear diameter.

Nuclear diameter (μ)	Duration of S phase (hours)	Labelling Index (%)
8-9	10-12	12-16
9-10	8-10	28
10-11	6-8	40-44
11	3-4	52-56

As the nuclear diameter increases the duration of S phase is shorter and the proportion of cells synthesising DNA is greater. The cells with smaller nuclear diameter have pachychromatic nuclear chromatin and pale staining cytoplasm. As the nuclear diameter increases, the cells'nuclear chromatin changes from an intermediate to a leptochromatic structure and their cytoplasmic staining from intermediate to basophilic.

1.21 Clonal nature of CFU-S

The curve relating the number of nucleated marrow cells that are transplanted to the number of colonies that develop in the spleen of lethally irradiated mice is linear and shows no threshold (Till and McCulloch, 1961; McCulloch, E.A. and Till, J.E., 1962). The radiation survival curve of CFU-S closely resembles the survival curves obtained for single cells in culture (Becker et al, 1963).

When recognisable marker chromosomes are present in the cells of a colony, an overwhelming majority of the cells contain the same marker (Becher et al, 1963; Barnes et al, 1959). These observations support the concept that the spleen colony originates from one cell.

The spleen colonies may consist of either myeloid, erythroid, megakaryocytic or mixed cell types. When a discrete colony containing only one differentiated cell type was dissected out and transplanted into a second irradiated recipient, the colonies which subsequently developed in the spleen of the secondary recipient, rather than being limited to the cell type of the transplant are

distributed among the various cell types in much the same fashion as with the primary transplant. This indicates the pluripotent nature of cells giving rise to spleen colonies and that the differentiated cells of the bone marrow have a common cell origin (Becher et al, 1963). Further evidence to indicate that the myeloid elements of the bone marrow share a common precursor with the erythroid and megakaryocytic lines comes from the fact that the Philadelphia chromosome seen in chronic myelocytic leukaemia is common to all three cell types (Whang-Peng et al, 1963).

1.22 Clonal nature of CFU-C

There is a linear relationship between the numbers of colonies which develop in agar and the number of marrow cells plated (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). Evaluation of CFU-C 48 hours after varying doses of irradiation showed a dose-response relation between the dose of irradiation and the numbers of surviving CFU-C (Robinson et al, 1972). These observations are compatible with, but not conclusive evidence that colonies originate from a single cell origin. Paran and Sachs (1969) demonstrated using a mapping technique that granulocyte and macrophage colonies arise from a single cell.

To resolve the question of whether mixed macrophage and granulocyte colonies arose from two different cells, Metcalf (1971) using a single cell transfer from an 'in vitro' colony, demonstrated that mixed colonies could be derived from a single cell.

Moore et al (1972) fractionated monkey marrow by centrifugation in continuous albumin gradients. Individual colony-forming cells, from an enriched fraction containing up to 25% of CFU-C, were transferred by micromanipulation to agar culture and a proportion of transferred cells gave rise to typical colonies. A cloning efficiency of 25-100% was demonstrated and it was proved that individual cells could generate colonies which were a mixture of granulocytes and macrophages. This confirmed that granulocytes and monocyte-macrophages were closely related populations sharing a common ancestor - the in vitro CFU-C.

The close ancestral relationship could explain why leukaemia frequently develops in which both cell populations are involved in neoplastic process (eg. myelomonocytic leukaemia). Karyotypic and microdensitometry studies in a murine myelomonocytic leukaemia which exhibited a colony growth in agar indicated that the leukaemic stem cells in the population generated both granulocytes and monocytes (Warner et al, 1969; Metcalf et al, 1969; Metcalf and Moore, 1970).

Work on another murine myeloid leukaemia has also shown that leukaemic granulocytic cells can generate macrophages when cloned in agar (Ichihawa , 1969; Ichihawa, 1970).

1.3 Colony Stimulating Factor

Colony formating in agar is dependent on stimulation by a specific glycoprotein, colony-stimulating factor (CSF). This appears to be a regulator of both granulopoiesis and monocyte formation.

The serum and urine of mice and humans exhibit detectable colony-stimulating activity when assayed in test cultures of mouse bone marrow cells (Robinson et al, 1967; Chan et al, 1971).

Studies on normal human urine have demonstrated that CSF is a neuraminic acid containing glycoprotein of molecular weight 45000 and that it constitutes less than 0.001% of the total urinary protein. (Stanley and Metcalf, 1969;1972).

Digestion of CSF with neuraminidase reduces the charge of the molecule, but does not destroy its biological activity 'in vitro'. CSF is active in vitro at extremely low concentrations (10^{-11} or 10^{-12}M). This implies that the primary action of CSF may be restricted to specific membrane receptor sites on target CFC and colony cells 'in vitro'.

CSF can now be radioiodinated, however, autoradiographic studies might be confused by the capacity of granulocyte cells to bind many proteins, eg. labelled bacterial antigens (Metcalf and Moore, 1973). CSF may be extracted from cell tissues in the mouse, some tissues yielding higher amounts per milligram weight than others. Large amounts of CSF are produced or released by cells in culture. Outstanding in this regard are fibroblasts, whole embryo cells, L-cells, peritoneal macrophages and the stromal cells that adhere to the wall of the bone marrow

cavity (Pluznik and Sachs, 1966; Bradley and Sumner, 1968; Austin et al, 1971; Chan and Metcalf, 1972; Bradley et al, 1971a).

Human urine CSF is antigenic in rabbits (Stanley et al, 1970) and in most cases the antibodies inactivate CSF 'in vitro' from a variety of human materials but do not inactivate CSF from mouse sources.

Mouse CFU-C 'in vitro' responded well to the stimulation of CSF from most mammalian species, eg. human, monkey, dog, rabbit, guinea pig and rat. 'In vitro' CFU-C from other species tended to respond best to stimulation by homologous CSF and were sometimes quite unresponsive to heterologous CSF eg. human CFU-C 'in vitro' responded to stimulation by medium conditioned by human and monkey cells but less well to medium by pig, mouse, guinea pig or rabbit cells (Metcalf and Moore, 1973).

CSF from different sources has been identified as having different molecular weight, ie. 15000 or in the range 45000-60000.

Endotoxin and other bacterial products induce a rise in the concentration of CSF in the serum and tissues. It is, however, considered that biologically important CSF is produced locally in the haemopoietic organs. The production of this local CSF is discussed in the next chapter on Regulation of Myelopoiesis.

1.4 Regulation of Myelopoiesis

"Stimulate the phagocytes"

The Doctors' Dilemna (1906) Act I

Bernard Shaw

CSF is produced by monocytes and macrophages (Moore and Williams, 1972) and it acts to promote increased monocyte production and macrophage proliferation (Metcalf and Moore, 1971).

'Virgin' macrophages generated in continuous marrow culture are not constitutive producers of CSF; however exposure of these cells to macrophage activating agents such as lipopolysaccharide (LPS) or BCG rapidly induces CSF synthesis and secretion (Moore, 1978). An exogenous source of stimulation such as endotoxaemia due to Gram-negative bacterial infection lead to increased CSF production from monocytes and macrophages.

Proliferation of mature macrophages is dependent upon a factor which is similar if not identical to CSF (Stanley et al, 1976). In mouse three major subpopulations of cells, with respect to buoyant density, can be stimulated to colony formation (Williams and Jackson, 1977). After six days in culture all density subpopulations give rise to granulocyte, mixed and macrophage colonies and no one progenitor cell population can specifically give rise to granulocytes or macrophages (Moore, 1978).

Different kinds of CSF-containing preparations in presence or absence of certain enhancing activities such as erythrocyte lysate or serum factors suggest different activities influence in vitro cloning of various subpopulations (Metcalf and MacDonald, 1975; Williams and Van Den Engh, 1975; Williams and Jackson, 1977).

Two subpopulations of CFU-C have been detected thus. One subpopulation responds to lung conditioned medium and another to human urine CSF. The density of the lung conditioned medium responsive cell is 1.070 g cm^{-3} and that of the human urine responsive cell is 1.081 g cm^{-3}. Different subpopulations of CFU-C have been detected in human marrow.

Cells forming neutrophil granulocyte colonies in fibrin clot diffusion chamber (CFU-D) in peritoneum of mice have a peak sedimentation rate of 5.2-5.4 mmh^{-1}. Rapidly sedimenting CFU-C population (7.0-7.2 mmh^{-1}) forms colonies in agar after one week of incubation (Moore, 1978). CFU-C of intermediate sedimentation velocity form colonies after 14 days (Jacobsen et al, 1977).

Human CFU-D and CFU-C have different cell cycle characteristics. $8 \pm 16\%$ of CFU-D are in S-phase whereas on day 7 of culture $33 \pm 8\%$ of CFU-C are in S-phase (Moore, 1978). It may be likely that the CFU-D are more immature cells than CFU-C.

The spectrum of CFU-C in mouse can be defined by biophysical heterogeneity with respect to cell size and buoyant density. Heterogeneity is also reflected in dose response of CFU-C to different species of CSF and morphology of the colonies.

Human urinary and human macrophage CSF stimulate predominantly macrophage colony formation in mouse marrow culture and appear to act on a more differentiated CFU-C subset than do other species of CSF which stimulate predominantly granulocytic colony formation.

High molecular weight species of CSF produced by human monocytes and macrophages stimulates macrophage cluster formation in human bone marrow culture. Human-active CSF is produced early in monocyte cultures but production ceases in 1-2 weeks whereas mouse active CSF is produced continuously for many weeks (Moore, 1978). Neoplastic monocyte or macrophage cell lines also retain the capacity to produce CSF; however in some cases the leukaemia cell lines are constitutive producers and in other cases CSF production only occurs after lipopolysaccharide stimulation suggesting retention of a degree of normal responsiveness by transformed cells (Ralph et al, 1977).

Medium conditioned by human monocytes contains two species of CSF, one with a molecular weight of 30,000. This is a human CSF in that it stimulates human granulocyte-macrophage colony formation. The other has a molecular weight of 150,000 and stimulates mouse marrow colony formation but not human (Shah et al, 1977).

1.41 Feedback Control

Negative feedback control of granulopoiesis has been reported in various systems and the concept of a granulocyte chalone specifically inhibitory to CFU-C (Aardal et al, 1977) or to more differentiated myeloid cells has been suggested (Lord et al, 1974).

Broxmeyer, Mendelsohn and Moore (1977) and Broxmeyer, Moore and Ralph (1977) indicated a more indirect mechanism of granulocyte negative feedback.

'Spontaneous' colony formation in the absence of an exogenous source of CSF is observed in marrow cultures of all species so far investigated when the cells are cultured at a sufficiently high concentration (Moore and Williams, 1972). This is brought about by endogenous elaboration of CSF by marrow monocytes and macrophages and is considerably enhanced by removal of mature granulocytes from the cultured cell population. Addition of mature granulocytes, granulocyte extracts or medium conditioned by incubation with granulocytes markedly inhibits spontaneous colony formation (Broxmeyer, Moore and Ralph, 1977).

Granulocyte-derived inhibitory activity (GIA) acts in a non-species specific manner to suppress CSF production by monocytes and macrophages. GIA is not inhibitory to monocyte-macrophage proliferation and is distinct from granulocyte chalone since no inhibition of granulocyte colony formation is observed in the presence of exogenous source of CSF (Moore, 1978).

CSF producing cells in chronic myeloid leukaemia patients were less sensitive than normal to GIA derived from normal mature granulocytes (Broxmeyer, Mendelsohn and Moore, 1977). This may explain granulocytic hyperplasia associated with chronic myeloid leukaemia.

The macrophage has been implicated in the elaboration of a spectrum of molecules which alter or modulate the proliferation and differentiation of lymphoid cells. These include lymphocyte activating factor (LAF) which potentiates the mitogenic response of T-lymphocytes to lectin and histocompatibility antigens (Gery and Waksman, 1972), and factors which increase the helper function

of T-lymphocytes (Wood and Gaul, 1974), and promotion of soluble mediator production by lymphocytes (Nelson and Leu, 1975).

Macrophages are required for humoral immune responses 'in vitro' (Hoffman and Dalton, 1971) yet mitogen-induced B-lymphocyte proliferative responses are suppressed in the presence of macrophages (Lipsky and Rosenthal, 1976). A functional sub-population of murine B-lymphocytes proliferate in semi-solid agar culture to form colonies (Metcalf, 1976; Kincade, Ralph and Moore, 1976). The process is dependent upon 2-mercaptoethanol and B-cell mitogens native to laboratory grade agar.

Kurland, Kincade and Moore (1977) using a two-layer culture system which prevented macrophage-lymphocyte contact and permitted B-cell activation. Adherent peritoneal macrophages potentiated both number and size of developing B-cell colonies particularly when low numbers of spleen or lymph node cells or macrophages depleted lymphoid cell suspensions were used. Macrophage-depleted lymph node cells gave virtually no colonies but colony formation was restored by the presence of an optimal number of macrophages. When the number of macrophages exceeded that required for optimal stimulation, colony formation was suppressed. This effect was largely prevented by indomethacin. Similar stimulatory and inhibitory activities were also present in media conditioned by varying numbers of peritoneal macrophages.

The inhibitory activity was identified as prostaglandins-E, which suppress B-lymphocyte cloning at concentrations as low as 10^{-8}-10^{-10}M

(Kurland and Moore, 1977). Stimulatory factor may be similar to 5×10^{-4} molecular weight factor described by Namba and Hanaoka (1974) produced by adherent phagocytic cells and stimulating normal and neoplastic B-cell proliferation. Conditioned medium from murine myelomonocytic cell line (WEHI-3) which elaborates CSF and T-lymphocyte activating factor, can not substitute for macrophages in initiation of B-lymphocyte colony formation suggesting non-identity of B-lymphocyte stimulatory activity with other known factors which alter haemopoiesis and lymphoid function (Moore, 1978).

Lipopolysaccharide (LPS) and sheep red blood cells (SRBC) also modulate elaboration of immunoregulatory factors by the macrophage. In presence of low numbers of macrophages SRBC and LPS facilitate the production of B-lymphocyte stimulatory activity but when added to high concentrations of macrophages, suppressor activity is generated due to induction of prostaglandin synthesis (Kurland, Kincade and Moore, 1977).

The phagocytic mononuclear cell population and specifically the resident macrophages within the haemopoietic tissues as of central importance in controlling the proliferation and differentiation of granulocyte-macrophage committed stem cells and B-lymphocytes (Moore, 1978).

A committed unipotential megakaryocytic stem cell (CFU-M) with a frequency of 10-20 per 10^5 nucleated bone marrow cells can proliferate in agar culture in the presence of an appropriate stimulatory factor to produce colonies of polyploid, platelet producing megakaryocytes (Metcalf et al, 1970; Williams et al, 1978).

Megakaryocyte colony stimulating activity can be obtained from similar sources as granulocyte CSF.

As in other haemopoietic cloning systems megakaryocyte colony formation is inhibited by prostaglandin elaborated by high numbers of macrophages.

The intimate interrelationships of macrophages with proliferating myeloid and lymphoid cell populations in marrow, spleen and lymph nodes suggests that such cells may be uniquely situated to stimulate cell differentiation and modulate proliferation by elaboration of cell-line specific stimulatory macromolecules and an opposing non-specific activity, prostaglandin E (Moore, 1978).

Prostaglandin synthesised by phagocytic mononuclear cells may be of central importance in modulation of haemopoiesis. Prostaglandin E (PGE) and other agents capable of elevating intracellular levels of cAMP inhibit granulopoiesis and macrophage proliferation 'in vitro' (Kurland and Moore, 1977a, b).

Radioimmunoassay has shown a linear relationship between the number of phagocytic mononuclear cells and the concentration of PGE in the conditioned medium (Kurland et al, 1977).

Titration of varying numbers of adherent macrophages or blood monocytes as a source of stimulus for human or murine marrow CFU-C has shown that colony formation is stimulated by low numbers of phagocytic mononuclear cells (0.05×10^5-2×10^5) and inhibited if higher concentrations are used. Parallel studies using indomethacin, a potent inhibitor of prostaglandin synthesis revealed a linear relationship between the number of colonies stimulated and the number of phagocytic mononuclear cells used as the source of CSF (Kurland et al, 1977). Observations point

to the unique ability of the macrophage to control the proliferation of its own progenitor cell by the elaboration of opposing regulatory influences.

LPS stimulates macrophage production of both CSF and PGE. This occurs in a temporal sequence. LPS in vivo and in vitro induces increased macrophage CSF production very rapidly with significant changes observed within minutes, whereas increased prostaglandin synthesis is delayed for 18-24 hours.

Stimulus for increased PGE production is not directly due to LPS but to LPS stimulated CSF production which in turn activates macrophage prostaglandin synthetase.

Incubation of non-activated macrophages with increasing concentrations of CSF in absence of LPS leads to a proportional increase in prostaglandin synthesis. This illustrates a very direct relationship between CSF levels and induction of an opposing activity (Kurland et al, 1977).

The evidence points to the macrophage as a surveillance cell, which under steady state conditions is elaborating basal levels of CSF and prostaglandin E. The process is self-limiting since a progressive increase in CSF beyond a critical concentration within a local milieu of the macrophage is ultimately sensed and stimulates the coincident production and release of PGE which opposes stimulatory action of CSF.

Granulocyte-derived CIA does not inhibit CSF production by endotoxin-stimulated monocytes and macrophages nor by mitogen-stimulated lymphocytes (Broxmeyer, et al, 1977a, b). Activation of normal or neoplastic B and T-lymphocytes by an appropriate mitogenic or antigenic stimulus leads to induction of CSF production (Parker and Metcalf, 1974; Ruscelti and Chervenick, 1975).

Similar activation protocols also lead to increased production of interferon which is inhibitory to human and murine granulocyte-macrophage colony formation in vitro (Greenberg and Mosny, 1977). This interferon may play a physiological role in counteracting stimulatory activity of lymphocyte-derived CSF.

Polymorphonuclear cell-derived lactoferrin, in its iron saturated form, inhibits murine macrophage and human monocyte release and/or production of CSF, thereby limiting CFU-GM proliferation (Broxmeyer et al, 1978; Broxmeyer, 1979; Pelus et al, 1979).

Specificity analysis indicates that prostaglandins of the E series (PGE_1, PGE_2) are the most active naturally occurring prostanoate compounds inhibiting CFU-GM proliferation (Pelus et al, 1980).

Another potential regulator of the formation of granulocyte-monocyte-macrophage colonies is the humoral factor 'neutrophil-releasing activity', which mediates the release of mature granulocytes from the marrow-granulocyte reserve pool (Boggs et al, 1968). This activity and CSF evolve in many of the same situations, but they are separate entities (Broxmeyer et al, 1974).

1.5 The Relationships of the Haemopoietic Precursors

Although a great deal is now known about haemopoietic precursor cells they could still be said to retain a certain enigmatic quality.

It became obvious that several blood cell lines were derived from one cell when Till and McCulloch (1961) observed the formation of several different haemopoietic cell types in spleen colonies in irradiated mice. This gave credence to the ideas of those of the monophyletic school of thought.

The spleen colony forming cell (CFU-S) however may not represent the most primitive of the haemopoietic stem cells since Loutit (1980) found that mouse bone marrow deficient in CFU-S repopulated successfully, lethally irradiated recipients.

Moreover when Yoffey (1960) was able to confine the origin of the haemopoietic stem cell to a compartment of cells, it was to cells associated by name and of close morphological identity with the multifaceted lymphocyte. Yoffey"s view that the haemopoietic stem cells belong to the lymphocyte transitional compartment was well supported by numerous studies, as mentioned previously.

Thomas et al (1977) extended these studies on morphology of the stem cell. The transitional cells, measuring between 9-11 μm in diameter, have pale blue cytoplasm and exhibit high nuclear:cytoplasmic ratio. The uniform distribution of chromitin (leptochromatic) distinguishes them from the uneven (pachychromatic) distribution of the small lymphocyte.

It is apparent that the stem cell compartment encompasses a spectrum of cells which may be distinguished from each other by

refined physical and functional characteristics.

A larger fraction of bone marrow derived CFU-S than of spleen derived CFU-S is capable of producing daughter CFU-S.(Lahiri and van Putten, 1969). This implies that spleen derived CFU-S are more mature than marrow derived CFU-S and are committed to earlier differentiation.

Splenic CFU-S showed a shorter doubling time than marrow CFU-S both in primary and secondary grafts. Primary grafts of bone marrow and spleen were estimated to have a doubling time of 25 and 19 hours respectively. Secondary grafts of bone marrow and spleen produced respective doubling times of 33 and 26 hours (Schofield, 1970). This may reflect the existence of definite populations of different maturity of splenic and bone-marrow CFU-S.

Vassort et al (1971) using hydroxyurea estimated the average cell cycle of CFU-S to be 12 hours.

Bol and Williams (1976) examined the cellular composition of CFU-C and observed that mature cells first appear in colonies from high density CFU-C (1.080 g/cm^3) followed by colonies from the mid density population (1.074 g/cm^3) while colonies derived from low density CFU-C (1.070 g/cm^3) contain undifferentiated cells for the longest period. This identifies three subpopulations of CFU-C.

Three subpopulations of erythroid colony forming cells - CFU-E (colony forming units - erythroid) cells forming small erythroid colonies after 2 days culture, BFU-E (burst forming units - erythroid) cells forming small early appearing bursts by day 3 of culture and BFU-E cells forming larger and later appearing burst by day 8 of culture.(Gregory and Henhelman, 1976; Wagemaker et al, 1976;

Testa and Dexter, 1977).

These large erythroid colonies have been given the term "burst" forming units because of their dispersed appearance and explosive growth .

Each of the three sequential maturation stages of CFU-C respond to stimulating factors from different sources.

CFU-C with density 1070 g cm^{-3} and sedimentation rate 4.3 mmh^{-1} (G_1 cells) respond to CSF from postendotoxin serum. CFU-C of density 1.075 g cm^{-3} and sedimentation rate of 4.8 mmh^{-1} (G_1 cells) respond to CSF from pregnant mouse uteri. CFU-C with density 1.080 g cm^{-3} and sedimentation rate 5.3 mmh^{-1} (G_1 cells) are the cells which proliferate when CSF is enhanced by erythrocyte lysate. The diameters of these subpopulations of CFU-C range from 7.4 to 7.6 μm (Bol et al, 1979).

Kriegler et al (1981) concluded that the enhancing factor from erythrocyte lysates was in fact haemoglobin, itself.

Ben-Ishay et al (1978) describe a BFU-E formed in agar in peritoneal diffusion chambers which appears to belong to a class of cells which are predominantly in the resting place in normal marrow. The ancestral relationship of the CFU-S to the CFU-C has been well established with respect to their cell cycle status and sequential recovery after the bone marrow has been depleted by cytotoxic agents.

The normal rate of CFU-S proliferation as shown by tritiated thymidine suicide studies is less than 10% in DNA synthesis (Becher et al,

1965; Lord et al, 1974) whereas thymidine suicide reduces the incidence of 'in vitro' CFU-C in mouse marrow cell populations by 30-45% in the normal state (Lajtha et al, 1969; Iscove et al, 1970; Metcalf, 1969).

Two doses of 1 mg/g body weight of hydroxyurea 7 hours apart killed 30% of CFU-S and 92% of CFU-C in normal mice (Hodgson et al, 1975). Cytosine arabinoside killed 7% CFU-S and 23% CFU-C when bone marrow was incubated by one hour with a dose greater than 16 μg/ml of the drug. This dose was the plateau value (Millard and Okell, 1974).

Similar results were produced by thymidine suicide 'in vitro' (Blackett et al, 1974).

Seidel and Opitz (1979) found regeneration of CFU-C and CFU-E in the marrow after 4 x 500 mg/kg of hydroxyurea at 6 hour intervals, which eliminated most CFU-C and CFU-E and 80% of CFU-S, started 2 to 3 days after treatment. An overshoot in CFU-C and CFU-E per femur was seen at day 4.

The sequential recovery from cytotoxic stress and different cell cycle status of the more primitive bone marrow cells illustrates their relationships to each other.

A primitive stem cell not identified by any colony-formation technique (Loutit, 1980) may give rise to the spleen colony forming cell (CFU-S).

The burst forming unit (BFU-E) which has a low proportion of its population in S-phase must be closely related to the CFU-S and in turn give rise to the subpopulations of BFU-E and CFU-E already

described. These in turn give rise to the morphologically recognisable erythroid precursors.

Origingating at a similar stage as the more mature BFU-E or less mature CFU-E are the CFU-C and its subpopulations which give rise to morphologically identified granulocytic cells and monocytes and macrophages.

1.6 The Spleen - "Plenum mysterii organon"

- Galen

The spleen has always presented a puzzle. Historically it has been assigned a variety of functions - an anatomical counterbalance to the liver, a digestive organ, the source of emotions or a ductless gland.

It may be said to have a glandular function like the thymus in that in small mammals such as the mouse, in conditions of stress, haemopoietic cells migrate to it to find appropriate stimulus for proliferation and differentiation.

Some of the functions that philosophers have subscribed to the spleen today seem quite amusing.

Erasistratus claimed that aside from maintaining the symmetry of the abdomen the spleen had no function at all.
Plato said that its function was to keep the liver bright and shining.
Hippocrates postulated that the spleen maintained a vital balance of four essential humours - blood, phlegm, golden bile and black bile.
Galen proposed that it was responsible for the withdrawal from the stomach of the water part of the food . He, however, concluded that it was an organ filled with mystery (Crosby, 1980).

Splenectomy became a procedure for investigative curiosity. Sir Christopher Wren while Professor of Astronomy at Oxford performed a splenectomy on a dog (Major, 1954). Splenectomy became popular whenever enlarged by leukaemia and malaria.
It was found to be a cure for haemolytic anaemia.

Although normally weighing about 120 g in an adult, the

spleen's size in chronic infection and hereditary haemolytic anaemias not uncommonly increases by 10 to 20 times. Neoplastic spleens may be even larger- weights of almost 10 Kg have been reported (Crosby, 1980). The normal human spleen receives about 5% of the cardiac output. With massive splenomegaly, the spleen may receive more than 50% (Garnett et al, 1969). This places a heavy burden upon the circulatory system, not only upon the heart but upon the portal circulation, into which the splenic venous blood must flow.

Splenic sequestration of red cells from the circulating blood intensifies anaemia in patients with large spleens. Splenomegaly also causes an expansion of circulating plasma volume.

"Anaemia" is a consequence of dilution by the expanded plasma volume as well as of sequestration of red cells in the spleen. The concentration of albumin in the expanded plasma is normal which means the total amount of albumin is increased: albumin production by the liver must therefore be increased. This suggests massive splenomegaly provokes the liver to produce more albumin. After splenectomy expanded plasma volume subsides very slowly. It is six months or a year before the normal volume is restored. Production of albumin decreases only gradually during this time (Hess et al, 1976).

In some animals the spleen is an important adjunct to the marrow in the production of blood cells but this is not true in the human.

Red cell production is a normal function of the human spleen only around the time of the fifth fetal month. Then the marrow takes over this function. In tiny animals the marrow cavity of bone

is relatively small and provides insufficient room for blood formation. The spleen shares in this function. In lower vertibrates with cartilagenous bones the spleen does the entire job (Crosby, 1980).

In the absence of the spleen the mean surface of mature red cells may be larger than normal. The surface of the red cell is composed for the most part of lipid. How the spleen controls the loss of surface lipid remains a topic of conjecture (Crosby, 1977). Removal of the spleen results in a multitude of subtle changes in the blood cells, in blood volume and in the immune system. Some of these changes differ from species to species and may vary according to the age and health of the animal.

Hypersplenism was a point of contention between Dameshek and Doan (Crosby, 1980). Dameshek believed that hypersplenism results from inhibition of the bone marrow by humoral factors from the spleen and Doan argued that hypersplenism results from sequestration and destruction of blood cells on the spleen.

In Dameshek"s laboratories a platelet transfusion was given to a girl with idiopathic thrombocytopenia pupura undergoing splenectomy. During the transfusion blood was taken simultaneously from her other arm and from her splenic vein. There were many platelets in the arm blood, few in the splenic blood, as though the transfused platelets had been lost in the spleen. There was good clot retraction in the arm blood but none in the splenic blood. Dameshek acceded that Doan was right.

Gunale et al (1976) claimed that the spleen exerted an inhibitory effect on the bone marrow. This was assessed by an increase in

the numbers of CFUc after splenectomy. Meldrum et al (1979), however, reported a decrease in bone marrow CFUc in splenectomised mice. Gunale also found that a splenic extract decreased the numbers of CFUc, but this extract was somewhat crude which makes the results uninformative since no extract from a tissue with non-specific relevance to haemopoiesis was compared with the splenic extract.

It was suggested by Meldrum et al that there was an increase in the rate of that red cell production in the bone marrow of splenectomised mice since there was an increase in blood reticulocytes. It was proposed that stem cells to the granulocyte compartment were redirected to the erythroid so depressing the number of CFUc. The increase in reticulocytes however could be due to the absence of the "pitting action" of the spleen.

Nishioka et al (1973) show a splenic humoral factor which affects leukocyte function but not proliferation. They isolated a splenic-dependent tetrapeptide "tuftsin" which regulates leukocyte function. This substance acts to increase neutrophil phagocytosis and chemotaxis; it has not, however, been shown to have an effect on circulating leukocyte numbers.

Lepault et al (1980) discussed the influence of the spleen on the blood cells and their precursors. Splenectomy increased the number of white blood cells in CBA/01a female mice. However splenectomy did not modify the number of nucleated cells nor the number of CFU-C in the bone marrow.

CFU-S are quiescent and enter the cell cycle after T-dependent antigenic stimulation in normal mice but not in adult thymectomised mice (Frindel et al, 1976). Antigenic stimulation in normal mice

increases considerably the pool of CFU-C in the spleen (12 fold) but has no effect on the CFU-C number in the bone marrow. An identical increase of splenic CFU-C has been found in adult thymectomised mice (20 fold) (Lepault et al, 1980).

T-dependent antigenic stimulation is brought about by painting the skin with oxazolone which has an inflammatory effect and stimulates myelopoiesis.

Adult splenectomy has no influence on the number of CFU-S and CFU-C in unstimulated mice (Lepault et al, 1980). Lahiri and van Palten (1969) suggested that splenic CFU-S are more mature than bone marrow CFU-S. A larger proportion of injected bone marrow CFU-S than of spleen derived CFU-S could be recovered from the recipients spleen and femur. They concluded that a larger fraction of bone marrow derived CFU-S than of spleen-derived CFU-S was capable of producing daughter CFU-S. They postulated that there was a commitment to early differentiation of many spleen CFU-S.

Adler and Trobaugh (1978) investigated the role of the spleen in the spleen in myelogenous leukaemia in RFM mice. They found that splenectomised mice which were given transplanted leukaemic cells had a longer mean survival than non-splenectomised mice and that 30% of splenectomised mice were spared from developing leukaemia. There has been some cytogenetic evidence showing independent clonal evolution of human chronic granulocytic leukaemia in the spleen as compared with bone marrow (Gomez et al, 1975).

There were no gross differences in the karyotypes in the spleen cells as compared with those of the bone marrow cells although the chance of finding Ph1-negative myeloid mitoses is greater with spleen

than with bone marrow cells (Lawler, 1977). [The Ph^1 chromosome is found characteristically in adult chronic granulocytic leukaemia.]

Elective splenectomy during the course of the chronic granulocyclic leukaemia (CGL) is now being assessed as a possible means of delaying the advent of metamorphosis in CGL. However, independence of the bone marrow and the spleen illustrates that splenectomy could not be expected to prolong the chronic phase in all cases.

There are a number of CGL patients who experience prolonged pancytopenia following chemotherapy. These patients may show improvement within one month of splenectomy (Canellos et al, 1972). Evidence to date suggests that neither the survival nor the tolerance or response to chemotherapy in the blastic phase of those patients who have prophylactic splenectomy is improved (Ihde et al, 1976).

1.7 Nitrogen Mustard

During the first world war mustard gas exposure in man was found to be associated with leukopenia. This was believed to be due to superadded infection; it was not recognised that it was due to a chemical effect on the bone marrow.

Mustard gas was first discovered by Ritchie in 1854 and prepared for manufacture by Meyer (1886). It was first used by the Germans as a war gas at Ypres in 1915. The clinical course of 400,000 victims of mustard gas was described by Marshall (1919) and Mandell and Gibson (1917). Lynch et al (1919) studied the effects of mustard gas upon experimental animals. These studies led to the application of the nitrogen mustards and sulphur mustards in clinical therapy.

The historical background as well as the chemical, pharmacological, toxicological and experimental aspects have been reviewed by Gilman and Phillips (1947). Initial clinical results were described by Jacobsen (1946) and Goodman et al (1946).

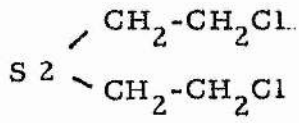
Nitrogen mustard appeared to be most useful in reticulum cell proliferations including Hodgkin's disease, reticulum cell sarcoma and reticulosis (Dameshek, 1949) but results with leukaemia were not favourable (Dameshek et al, 1949).

The chemical structure of the sulphur and nitrogen mustards are shown on the next page.

The sulphur mustards are soluble only in oils whereas the nitrogen mustards are readily soluble in water.

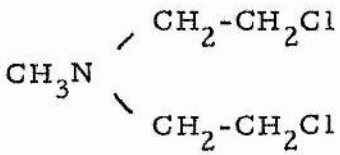
In aqueous solutions, the nitrogen mustards undergo "intramolecular cyclisation (Gilman and Philips, 1946) have shown that the imino ring

SULPHUR MUSTARD



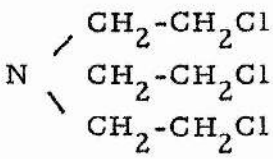
Bis (β-Chloroethyl) Sulphide
(Mustard Gas)

NITROGEN MUSTARD



Methyl Bis (β-Chloroethyl)
Amine

HN_2



Tris (β-Chloroethyl)
Amine

HN_3

HN_2 is most widely used.

possesses an unusual reactivity. It reacts with a great variety of biologically important groups ie. alpha amino, sulfhydryl, phenolic, carboxyl, imidazole, imino, inorganic phosphates, chick pepsin peptodase, choline oxidase etc.

In the presence of the chloride ion, the reaction tends to revêrse itself with reformation of the parent amine (Anslow et al, 1947). This probably occurs in extracellular fluids where the concentration of chloride ion is high. Entrance of the parent amine into the cell

where there is little, if any, chloride ion to compete with water, results in a rapid transformation with intramolecular cyclisation and alkylation of labile groups. The speed of this reaction was demonstrated by Karnofsky et al (1948). By occluding the circulation to the femoral bone marrow and the small intestine for periods ranging from 2 to 5 minutes these organs were completely protected from the generalised leukotoxic action of the nitrogen mustards.

The alkylating agents bind covalently to DNA. Bifunctional substances can make cross link between complementary DNA strands. DNA cross-links are thought to be responsible for the relatively high toxicity of the bifunctional alkylating agents (Lawley, 1966). Part of the damage resulting from alkylating agents can be repaired like radiation induced lesions in the DNA (Waring, 1970).

Alkylating agents such as the nitrogen mustards can act as radiosensitisers (Streffer, 1975). These agents can induce malignacies (Schmähl,1970) although mutagent effects they show are apparently weak (Russell, 1972).

As a radiosensitising agent with respect to cell killing these substances generally show an additive effect. Nitrogen mustard appears not to interfere with the recovery from sublethal damage (Elkind and Sahomoto, 1970).

Suppression of the erythroid activity was noted within twenty-four hours after initiation of HN_2 therapy. No immediate reflection of this depression was noted in the peripheral erythrocyte and haemoglobin levels, in all probability because of the normal red cell survival time of 120 days.

Bloom and Bloom (1948) showed that the chick erythroblast was the most sensitive cell in the marrow following the administration of x-ray therapy. Dameshek et al (1949) noted the suppression of granulopoiesis within two to four days. The fall in the peripheral leukocyte level occurred shortly thereafter reaching a maximal leukopenia of the 25th day. This prompt reflection of an effect on the marrow is due to the short survival time of the leukocyte in the peripheral blood.

Megakaryocytes proved to be the most resistant of all marrow elements and platelet reduction occurred in only 20.2 per cent of cases.

Kindred (1947) studied the reaction of the femoral bone marrow of the albino rat to sulphur and 3 nitrogen mustard preparations. A marked suppression of erythroid and granulocytic elements was noted 2 days after injection. Mitotic activity was diminished. Megakaryocytes showed some signs of injury but no reduction in number. Reticulum cells and plasma cells were unaffected. Similar results were obtained in dogs, rabbits (Cameron et al, 1947) and in mice (Graef et al, 1948).

Barron et al (1948) showed that the addition of choline, dimethyl amino ethanol and methionine to bone marrow in vitro protected it from the inhibition of respiration by the nitrogen mustards. Kindred (1947) showed marked lymphoid atrophy and lymphocytic degeneration in the albino rat on the second postinjection day. Similar changes were present in the thymus and spleen. Peripheral lymphocytopenia coincided with decreased production within the lymphoid organs rather than a direct effect upon the

peripheral lymphocyte. Mice and rabbits showed essentially the same changes (Graef et al, 1948).

Daneshek et al (1949) found that nitrogen mustard appeared to exert a karyolytic effect upon liver cells. Polymorphonuclear infiltration is present within 24 hrs. Resolution of the necrotic foci probably takes place between 19 and 54 days after therapy. Boursnell et al (1946) demonstrated the ability of rat liver to concentrate as much as 50% of the injected radioactive sulphur mustard within the bile within one hour. It is probably during this time that the hepatotoxic effect occurs. Zimmerman (as quoted in Graef et al, 1948) reported focal necrosis in liver of cats after oral administration of nitrogen mustard.

Dameshek et al (1949) obtained the impression that HN_2 is more specific against reticulo-endothelial cellular proliferations than against those of any other cell type.

Sharp et al (1973, 1975) described detailed quantitative studies on the effects and recovery from HN_2 on the murine haemopoietic and lymphoid tissues. The LD_{50} for young adult female albino mice was approximately 100 μg. There was a maximum depression in the stem cell numbers one day after treatment with 100 μg HN_2 the CFU-S femoral content being about 10% of control values. This was followed by an increase which restores the CFU-S content of femoral diphysis to normal levels between the 4th and 8th day after treatment. On the 4th day the bone marrow was very hypocellular and sinusoids were dilated and filled with blood. By the 8th day cellularity was restored and the histological appearance was essentially normal with possible exception of megakaryocytes which appeared to

be very prominent in this regenerating tissues. On day 8 the cellular composition of the bone marrow was 94 ± 1% granulocytes compared to 60% in controls. Lymphocytes and erythrocytes were virtually absent. The phase of granulocytosis was replaced by erythroid hyperplasia lasting from day 12 after treatment to day 30, when the cellular composition returned to normal. The marrow was lightly hypercellular 40 days post-injection.

In a review on cancer chemotherapeutic agents Marsh (1976) summarises the effects of HN_2 on normal haemopoietic precursor cells. Dunn (1972) reports an LD_{50} in mice of 1.0 mg HN_2/kg body weight, whereas Valeriote and Tolen (1972) estimate an LD_{50} of 6.8 mg/kg. Brown and Carbone (1971) reports an LD_{10} of 0.5 mg/kg. A comparison of the dose-response effects of HN_2 on mouse CFU-S, 16-24 hours after administration of the drug is summarised in the following table.

Strain	Time of assay (hr)	Dose Range (mg/kg)	Route	D_{10} (mg/kg)	Shape of curve
C57BL, C57BL x CBA	16	1-5	8 C	1.5	Exp to 10^{-3}
C57BL, C57BL x CBA	16	1-12	1 P	7	Exp to 10^{-2}
AKR	24	4.5-18	1 P	4.5	Exp to 10^{-4}
AKR	24	4.5-18	1 P	3.2	Exp to 10^{-4}
CSI/ASH	24	4.5	1 V	4.5	1 dose only
CBA	24	0.25-2	1 V	1.0	Exp to 10^{-2}
C57BL	24	0.5 - 3	1 V	1.8	Exp to 10^{-2}

Most workers have found exponential dose-response curves when normal CFU-S are assayed 16 to 24 hours after HN_2 administration. The drug injected subcutaneously is more effective than given

intraperitoneally (van Putten et al, 1972). Fractionation of the dose did not change the survival curve (Bruce et al, 1966).

Rapidly proliferating cells were 1.6 to 2.7 times more sensitive than normal CFU, depending on the route of administration (IP or SC), according to the results of van Putten and Lelieveld (1971) and van Putten et al (1972), but Dunn (1972) using the IV route found no differences.

Marrow and spleen CFU-S were equally sensitive (van Putten and Lelieveld, 1971). When normal marrow was incubated with varying concentrations of HN_2 for 1 hour 'in vitro' exponential survival was found with a C_{10} of 0.25 M. The survival of rapidly proliferating CFU-S was similar (Ogawa et al, 1973).

The CFU-C survival curve of C57BL mice 24 hours after IP administration of HN_2 was exponenetial with a D_{10} of 10 mg/kg (Brown and Carbone, 1971). A direct comparison with CFU-S was not made, but this is more than CFU-S D_{10} after IP drug in this strain reported by Van Putten et al (1972) suggesting that the CFU-C are less sensitive. However, the survival of normal and rapidly proliferating (marrow post-transplant) CFU-C incubated with HN_2 'in vitro' was identical to that of CFU-S (Ogawa et al, 1973).

The erythroid repopulating ability (ERA) is measured by incorporation of ^{59}Fe into peripheral blood erythrocytes by an irradiated, transplanted host and calculated as a percentage of the administered dose (Hodgson, 1962).

The survival of the ERA 24 hours after IV HN_2 was exponential with a D_{10} of 1.0 to 1.8 mg/kg (Dunn, 1972; Blackett and Adams, 1972; Dunn and Constable, 1973). The ERA of rapidly proliferating marrow in C1 mice was much more sensitive (Blackett and Adams, 1972).

Three hours after HN_2 the ERA survival after 2.25 mg/kg was 1.2% (Hellman and Grate, 1971) - more sensitive than the CFU-S and much more sensitive than the granulocyte repopulating ability (GRA) assayed simultaneously.

The granulocyte repopulation ability (GRA) is measured by the granulocyte response to endotoxin. GRA survival 24 hours after HN_2 showed a plateau at 20% at doses above 1 mg/kg (Dunn and Constable, 1973) and an average of 65% survival 3 hours after HN_2, 2 mg/kg (Hellman and Grate, 1971). In both studies CFU-S and ERA assayed simultaneously were more sensitive.

The incorporation of ^{59}Fe into peripheral red blood cells after administration of cytotoxic agents was measured by Twentyman and Blackett (1970). The incorporation of ^{59}Fe, 48 hours after HN_2, was exponential with a D_{10} of 1.5 mg/kg (Hellman and Grate, 1971). The sensitivity of marrow of C1 mice was normal.

Human marrow CFU-C incubated for 1 hour in vitro were killed exponentially with a C_{37} (in vitro concentration) of 0.5 M; this is somewhat less sensitive than that for mouse marrow (Ogawa et al, 1973).

1.8 Vincristine

Vincristine is one of a group of structurally related alkaloids derived from the periwinkle (Vinca rosea). Extracts of this plant were found to possess anti-tumour activity, after initially attracting attention as possible hypoglycaemic agents (Johnson et al, 1963).

The most characteristic biological effect of the vinca alkaloids, vinblastine (VBL) and vincristine, differing only by an N-methyl group,

is the arrest of mitosis at the metaphase stage (Cardinate et al, 1963; Frei et al, 1964).

In low concentrations these compounds bind to soluble microtubular proteins and inhibit polymerisation of spindle microtubules, finally resulting in a lack of the mitotic spindle apparatus. In cells exposed to high concentrations large eosinophilic crystals and ribosomal complexes are observed (Owellen et al, 1974; Krisham and Frei, 1975). Vincristine is an unstable compounds in solution. It has a short half life (Owellen et al, 1977). Vinblastine and vincristine produced enhanced transport of methotrexate in cells (Krishan and Frei, 1975).

Vincristine inhibits RNA and protein synthesis in isolated human leukaemic leukocytes long before an effect on mitosis is demonstrable. Furthermore, there are indications that DNA synthesis is also reduced by vinca alkaloids (Cline, 1967, 1968). The effects on RNA and DNA are dose-dependent and have tissue specificity (Johnson et al, 1963; Richards et al, 1966; Sartorelli and Creasey, 1969).

In a review Camplejohn (1979) criticises the use of vincristine to synchronise cell populations in tumour chemotherapy. He claims that the lack of success in these ventures is due to the irreversible reaction of vincristine on mitosis. He reports that the action of colcemid on mitosis appears to be reversible. However Klein (1979) reports that mitotic arrest of cultured cells continuously exposed to VCR is seen at a dose ten times lower than that needed to cause lethality (Madock-Jones and Mauro, 1975).

Creasey (1968) reports that there are two separate sites of action of vinca alkaloids: reversible mitotic arrest through effects on the

cytoplasmic precursors of the spindle and inhibition of RNA synthesis through effects on the DNA-dependent RNA polymerase system. Reversible temperature-dependent binding of [^{3}H]VLB by low molecular weight protein in the soluble fraction of human and murine cells was demonstrated by gel filtration and dialysis.

The most sensitive segment of the cell cycle to VLB seems to be the S/G_2-boundary. Hela cells exposed at this time to the drug (30 min at 0.01 μg/ml) develop multinuclei because of partial spindle disintegration (Chirife and Studyinski, 1975). Cells from the human cell line NHIK 3025 exposed for 6 hr to 0.002 g VCR/ml, were arrested at metaphase with a peak at 9 hours. Twenty-four hours after treatment the metaphase index decreased and some anaphases and telophases as well as several multinucleate cells were observed. Many cells had obviously completed mitoses, some in an irregular way (Dahl et al, 1976). Hartenstein et al (1973) observed recovery after VCR 'in vivo'.

^{125}IudR uptake by human tumour cells, normal human fibroblasts and hela cells after VCR treatment often exceeds control levels (own observations - unpublished - Med. Physics, Aberdeen).

The duration of VCR induced metaphase block may be of variable length in normal and neoplastic cells (Wagner et al, 1977). Ernst (1973) observed in human basophilic erythroblasts after 0.04-0.075 mg VCR/kg an increase in abnormal cells (polynucleated cells, cells with Howell-Jolly bodies). These cells began to appear at the time the mitotic index was decreasing. Ernst suggested that the abnormal cells have escaped the metaphase block but incomplete spindle

restoration led to these polynucleate erythroblasts. Some of these cells incorporated [^{3}H]TdR which indicates that although the nuclear architecture is disturbed some potential for DNA synthesis is retained. Ernst excluded the possibility that DNA repair was the cause of the DNA precursor incorporation.

Multinucleate cells induced by a sublethal dose of VCR are able to proliferate. They can pass through DNA synthesis and mitosis with their small nuclei as observed by George et al (1965) in Hela cells 'in vitro' and by Klein et al (1980) in Ehrlich ascites tumour cells 'in vivo' by means of Feulgen and flow cytophotometry and autoradiography.

In human tumours of several types a mitotic peak was observed 12 hours after administration of vincristine. A second peak could be detected nearly 50 hours after VCR (Klein and Lennarty, 1974). The mitotic peaks in 5 human tumours were relatively small [maxima between 7 and 12%,(Klein et al, 1976)].

Hydroxyurea

Hydroxyurea (HU) inhibits the incorporation of tritiated thymidine into DNA in vitro and in vivo but does not affect the incorporation of tritiated uridine into RNA nor the incorporation of tritiated leucine into proteins (Young and Kamofsky, 1966; Schwartz et al, 1967). HU also inhibits tumour growth, as well as cell proliferation of the bone marrow and of intestinal and epithelial cells (Thuman et al, 1963; Thuman, 1964).

1.9 Leukaemia-Abnormal Haemopoietic Cell Production

The techniques for identifying the normal haemopoietic stem cell and of the mechanisms which produce and regulate its progeny have led to methods of studying the disturbed and aberrant cell production in leukaemia.

Comparisons of the numbers of stem cells, their rate of proliferation and studies of normal cell interaction with leukaemia cells help to elucidate the disturbances in normal blood cell production which occurs in leukaemia.

Although on the whole leukaemia remains an idiopathic disease, and it cannot be said exactly what causes it, several factors that could and some that definitely do contribute to its causation in human beings, are known (Gunz, 1980).

A good deal is known about the cause of leukaemia in animals, through laboratory work. The outcome of these studies is that leukaemia never arises from a single cause. The disease comes about when several factors act together.

The genetic constitution, the animals innate resistance, the activity of virus and the influence of a large variety of physical and chemical agents are known to affect the initiation of leukaemia in the animal.

Detailed reports of human leukaemia which has apparently been induced by irradiation have been made (Stewart and Kneale, 1970; Court Brown and Doll, 1957; Iskimaru et al, 1971; Hirashima et al, 1980). Radiation can induce leukaemia in the laboratory mouse (Duplan, 1976; Upton, 1958, 1964, 1970; Major and Mole, 1978).

HU selectivity kills cells in S phase. Sinclair (1967) suggests that HU stops the progression of cells into the S phase which results in the accumulation of cells at the end of G_1. In vivo HU slows down the progression of tumour cells into S phase (Mauro and Madoc-Jones, 1969; Rajewsky, 1970).

The block of DNA synthesis produced by HU in bone marrow cells is followed by a disturbance of the kinetics of stem cell proliferation. There is a recruitment of quiescent marrow stem cells into the cell-cycle (Vassort, 1968).

About two-thirds of the excess leukaemia in adult Japanese survivors of the atomic bombs was myeloid type (acute plus chronic). The thymic leukaemia (Duplan, 1958) of the laboratory mouse may have no human counterpart (Major and Mole, 1978).

Myeloid leukaemia was not found to develop spontaneously in CBA/H mice (Major and Mole, 1978) thus making radiation induced myeloid leukaemia in CBA/H mice a suitable model for the study of the induction process. This leukaemia does not seem to display immunogenic properties. This makes it a suitable model for studying growth properties of the leukaemic cells, since no other variables are introduced by immunogenicity and growth of the cells appear to be autonomous (Meldrum and Mole, 1981; Meldrum and Mole, 1982).

1.91 Irradiation induced leukaemia

It became evident that radiation induced leukaemia when an excess frequency of leukaemia was found in many human populations exposed to ionising radiation, medically, occupationally or in warfare.

Antenatal diagnostic radiography provides direct evidence that small dosebrief exposures are leukaemogenic (Stewart and Kneale, 1970). In man leukaemia frequencies cover the range from 10^{-4} to a few per cent whereas in experimental mice values range up to virtually 100 per cent down to a few per cent.

Sometimes it is believed that leukaemia is more easily induced in man by ionising radiation than other kinds of malignant disease and that bone marrow is more sensitive to malignant transformation than any other tissue. There have now been as many deaths from breast

cancer as from leukaemia in Japanese bomb survivors who were young adult females at the time of irradiation, when breast tissue was physiologically active (Mole, 1977).

X-Ray exposure of the whole spine and the sacroiliac joints and often of other joints was practised extensively in UK for treatment of ankylosing spondylitis. In a single course of treatment individual doses of up to a few hundred R were given several times a week for a number of weeks, each exposure at a dose rate in the range 10-100 R/min (Court Brown and Doll, 1957). Excess leukaemia followed and it was found that leukaemia incidence in males could well be linearly dependent on mean radiation dose in the irradiated bone marrow.

The leukaemia rate per irradiated subject was also shown to be linearly dependent on age at first X-ray treatment. The extent of the increase is about 4-5 x from 20-60 years of age. An important age factor to consider is the progressive centripetal retreat of red bone marrow from the peripheral to the axial skeleton with increasing age.

From six dose-groups in the irradiation spondylitics it is clear that the incidence per man-year increased markedly for marrow doses exceeding 2000R.

Leukaemia has occurred in excess following pelvic irradiated for premenopausal bleeding but not following the larger dosage used in treatment of carcinoma of the cervix.

An extensive survey of practising radiologists in USA showed a death rate from leukaemia progressively increasing with calendar age for those who practised over the period 1920-1939, but virtually no excess leukaemia in those beginning practice in later years.

The difference is in line with the tightening up of radiological protection standards (Ulrich, 1946; U.N. S.C.E.A.R. 1964).

In the currently available information on the bomb survivors from Hiroshima and Nagasaki, there is a difference between the two cities in the spectrum of cytologically defined types of leukaemia in those irradiated as young or older adults aged 15 years or over but not those irradiated as juveniles at 0-14 years of age (Ishimaru et al, 1971). The differences between cities in the numbers of leukaemias in the unirradiated is as expected from the relative numbers of subjects.

The overall difference between the cities is due to the differences for chronic granulocytic and acute "other" leukaemias but there is no significant residual heterogeneity when only chronic granulocytic leukaemia is excluded. Acute lymphocytic and acute "other" leukaemias were more characteristic of those exposed as juveniles and acute granulocytic and chronic granulocytic leukaemias more characteristic of exposure at adult ages. The immediate relevant difference between the two cities is that a substantial fraction of the dose at Hiroshima was contributed by fission neutrons but not at Nagasaki.

Mole (1975) claims that the types of leukaemia found from much higher frequency at Hiroshima than at Nagasaki, chronic granulocytic and acute "other", are neutron dependent in the sense that they are much more easily induced by fission neutrons than by γ-rays. The dose response relationship for these two types combined for all ages against neutron dose is not linear but can be well fitted by the expression $aDe^{-\lambda D}$ which is appropriate for linear induction by

high LET (linear energy transfer) radiation modified by cellular inactivation. Other types of leukaemia found with similar frequency in each city, acute granulocytic and acute lymphocytic are neutron-independent in the sense that there is no evidence that neutrons are more effective than γ-rays in inducing them.

The distribution of leukaemia cases in the Japanese cities is as follows.

	Hiroshima	Nagasaki
acute granulocytic	14	7
acute lymphocytic	9	6
acute 'other'	11	1
chronic granulocytic	25	3
Total	59	17

The number of cases of leukaemia in Nagasaki is somewhat low for assessment. Therefore statistical analysis of these figures will lead to spurious significance. It cannot be said that if the whole human population was irradiated with the same type of irradiation as the victim's in Nagasaki received the same spectrum of leukaemia types would appear in the same proportions. The case for claiming that different types of irradiation induce different types of leukaemia is not substantiated.

Upton (1977) reports that the reticular tissue neoplasm which has been most thoroughly studied is lymphoma of the thymus which characteristically predominates in whole-body irradiated mice. In the mouse irradiation induced non-thymic lymphoid reticular tissue

neoplasms occur later in life than do thymic lymphomas. They occur predominantly during the second year of life and represent a complex mixture of different neoplasms, varying in morphology and anatomical distribution.

Granulocyte (myeloid) leukaemia has received less study than induction of thymic lymphoma, principally because it is less common. In those strains of mice examined for myeloid leukaemia, radiation has been observed to increase incidence. In few strains other than RF, SJL/J and CBA/H has maximum incidence exceeded 10% after irradiation.

The neoplasm presumably arises in the marrow or spleen but neoplastic cells are widely disseminated by the time the disease is detectable clinically. The thymus is usually grossly atrophic although often infiltrated microscopically. In some cases, infiltrations are of a greenish colour (chloromyeloid leukaemia). The disease usually runs a rapid course, terminating with leukaemic leukocytosis and anaemia (Upton, 1977).

RF mice a single exposure to 200-400 rads of whole-body radiation early in adult life may increase the incidence to 30-40 per cent at a level at least ten times the control incidence of 1-4%. In CBA/H mice the incidence of leukaemia in irradiated mice is about 20% at the optimum leukaemogenic X-ray doses of 150-300 rads (Major and Mole, 1978). No myeloid leukaemia was detected in unirradiated CBA/H mice. The myeloid leukaemia frequency in CBA mice given 300 rads was markedly reduced by a further dose of 600 rads 1, 4, 16 or 32 weeks later, presumably by inactivation of the transformed cells. At these times the number of normal

haemopoietic stem cells must still outnumber the transformed cells. The dose-response relationship was curvilinear, leukaemia incidence decreasing in the range 400-600 rads.

Upton (1977) reports that as in induction of thymic lymphomas the effectiveness per rad of radiation delivered in a single brief exposure is maximal at intermediate dose levels (100-400 rads). Latency varies inversely in relation to the dose after exposure to 100-400 rads the age-specific incidence rising to a peak within one year and remaining elevated for the rest of the lifespan.

In general a given dose of X-rays or gamma-rays is less than leukaemogenic when protracted over an interval of days or weeks than when administered in a single brief exposure, whereas leukaemogenic effectiveness of fast neutrons appears relatively dose-rate independent, with the result that RBE increases with decreasing dose and dose rate. Irradiation at low dose rates from internally deposited strontium-89, strontium-90, or colloidal gold 198 is relatively ineffective in inducing the disease, as compared with whole-body irradiation at high dose rates. The rate of induction of the neoplasm per integral dose of x-rays is inhibited relatively little by partial body shielding.

Susceptibility of RF mice to induction of granulocyte leukaemia is low during the neonatal period increases during maturation and remains relatively constant thereafter, at least until the 6th month of life. Radiation-induced granulocyte leukaemias are less common in females than in males. Castration reduces the incidence but not to a level as low as is characteristic of females.

Ovariectomy affects the incidence in females relatively little, the suppressive action of oestrogen on the cell-free transmission of the disease suggests that an inhibitory effect of oestrogen may account for the sex difference in intact mice (Upton and Cosgrove, 1968). Adrenal corticoids given after irradiation do not materially affect the induction of the disease.

Thymectomy, which prevents the induction of thymus-dependent lymphomas, does not inhibit the induction of myeloid leukaemia or of lymphomas arising outside the thymus. Splenectomy materially inhibits the induction of myeloid leukaemia. Spontaneous and induced incidence of the disease is reduced in germ-free and specific-pathogen-free mice.

Chronic inflammation, caused by intramuscular injection of turpentine or repeated painting of the skin with Croton oil increases the yield of myeloid leukaemia in irradiated RF mice.

Myeloid leukaemia as well as thymic lymphomas are reduced in frequency if mercaptoethyl-guanidine is given before irradiation (Upton et al, 1959). 5-Metoxytryptamine has been reported to inhibit only the induction of lymphomas, not myeloid leukaemias (Svyatukhim et al, 1966).

Myeloid leukaemias in irradiated RF mice have been found to be tranmissable by cell-free filtrates or ultracentrifugates and to contain type-C virus particles (Upton, 1959).

Non-thymic lymphosarcomas and lymphoid leukaemias occur later in life than thymic lymphomas and granulocytic leukaemias.

Incidence has generally been decreased by single whole-body irradiation, except in thymectomised mice. Fractionated whole-body irradiation at higher cumulative dose levels has been observed to increase their incidence in CBA and LAF mice (Upton, 1977).

Reticulum cell sarcoma is manifested by nodular as well as diffuse proliferation of reticulum cells and causes infiltrations which may vary from a sarcomatous pattern to a granulomatous or Hodgkin's disease-like pattern from site to site within the same animal.

In a minority of cases abnormal monocytoid cells appear in the blood. It occurs later in life and is more common than other haemopoietic neoplasms in non-irradiated aging mice of most strains. The incidence is characteristically reduced by whole-body irradiation, but not in all strains (Upton and Cosgrove, 1968; Mellali et al, 1974).

1.10 Haemopoietic Stem Cells in Murine Leukaemias

Virally induced leukaemias

Some RNA tumour viruses are the apparent cause of much 'natural' cancer or leukaemia in chickens and mice.

Friend virus leukaemia Anaemia-inducing Friend virus (FVA) was isolated from a Swiss mouse. The virus induces a dose dependent enlargement of the spleen and liver after a latency period of only a few weeks in DBA/2, Swiss, BALB/C, C_3H and CBA mice.

Thymus tumours are never observed with FVA virus in sharp contrast to the great majority of spontaneous forms of mouse leukaemias and peripheral or internal lymph nodes are not enlarged. Induction by Friend virus is not influenced by the presence or absence of the thymus (Metcalf et al, 1959).

In blood smears stained with Giemsa, the predominant cells are hyperbasophilic cells, related morphologically to the proerythroblast. These are known as 'Friend' cells. Anaemia and thrombocytopenia are partly due to a shortened life span of red blood cells and platelets on which the virus is budding (Tambourin, 1978). Friend virus is more potent in the female mouse.

The Rauscher leukaemia virus of which the H origin of the strain is not clear was able to induce a two-step type of disease. Mice developed a disease comparable to FVA-induced syndrome with minor be reproducible differences. This virus is more potent in male than in female mice.

Viruses can change and give rise to two or more different diseases and this can lead to different results in different labs. Friend and Rauscher viruses are rather complex mixtures in which at least two viral entities exist. One of these viruses the Spleen Focus-Forming virus (SFFV) induces macroscopic foci of primitive erythroid cells in the splenic red pulp (Pluznik, D.H. and Sachs, L., 1964; Axelrad and Steeves, 1964; Ikawa et al, 1967 and Mirand et al, 1968). The second is the murine lymphoid leukaemia virus (MuLV). The SFFV is defective and depends for its replication on the presence of its associated MuLV helper. McGarry et al (1974) isolated a myelogenous leukaemia-inducing virus (MgLV) from a chloroleukaemic $C_{57}Bl$ mouse injected neonatally with the MuLV-containing helper component of the Friend virus complex.

After large doses of the polycythaemia-inducing Friend virus, 30 hours later, the hyperbasophilic cells, the so-called Friend cells, appeared massively in the spleen red pulp only, not in the lymphoid areas and outside the granulopoietic and megakaryocytic foci (Tambourin, 1978).

The spleen colony assay of Till and McCulloch (1961) using irradiated recipients has been widely used to quantify or demonstrate the existence of a tumour cell population (Bruce and Van der Gaag, 1963; Wadinsky et al, 1967; Bergsagel et al, 1968; Tanaka and Lajtha, 1969).

In virus producing murine leukaemias the spleen colonies do not appear to come solely from the transplanted leukaemia cells. The viruses, themselves also produce tumour CFU's (TCFU) as mentioned before. Thomson et al (1974) and McGarry and Mirand

(1977) demonstrated that TCFU and haemopoietic CFU do not constitute identical populations. The TCFU must be an infected cell with a radiation survival curve comparable to the typical radiation survival curve of haemopoietic cells (D_o = 90 rads; Thomson and Axelrad, 1968). They are present in a proportion of about 3-4 TCFU per 10^5 cells in the spleen (Thomson and Axelrad, 1968; Bentvelzen, 1972), and 20 TCFU per 10^5 cells in the bone marrow (Tambourin, 1978). Matioli (1974) compared Friend tumour cells with normal and 'leukaemic' stem cells. There was high metastatic activity, lack of differentiation and deterministic growth and independence from the spleen micro-environment experienced by tumour cells in contrast to the normal and Friend-leukaemia-derived stem cells.

The spleen colony assay has been used to quantify lymphoma (Bruce and Van Der Gaag, 1963) plasma (Bergsagel and Valeriote, 1968) myeloid (Tanaka and Lajtha, 1969), L1210 (Wodinsky, Swiniarski and Kensler, 1967) tumour cell populations which produce spleen colonies in irradiated or non-irradiated syngeneic hosts.

In Friend and Rauscher leukaemia cytological and histological investigations of the colonies developing in the spleen of irradiated recipients grafted with spleen or bone marrow cells from leukaemic donors, indicated that the pattern of differentiation along erythrocytic, myelocytic (either neutrophil or eosinophilic) and megakaryocytic lines appeared comparable with the pattern for differentiation observed in spleen colonies derived from grafts of normal haemopoietic cells (Wendling et al, 1972; Bromner and Bentvelzen, 1974).

Spleen colonies originating from a leukaemic cell graft showed only a slightly greater percentage of erythroid colonies and a slight decrease in the percentage of megakaryocytic colonies below control values. No tumour colony was found in most cases except in one experiment out of 100 in which a donor infected 27 days previously was used. In that particular case the number of colonies observed on the recipient spleens was at least ten-fold greater than usual. These colonies were macroscopically larger and whiter than the typical haemopoietic colonies. Histologically large cells with faintly basophilic cytoplasm and a few cells morphologically related to maturing erythroblasts could be seen. These colonies were indefinitely transplantable in either irradiated or non-irradiated hosts by subcutaneous or intravenous method (Tambourin, 1978).

The CFU-S from leukaemic animals were able to repopulate their own compartment with a doubling time of 20 to 25 hours (Tambourin, 1978) which is quite similar to the values obtained with normal cells (Simonovitch et al, 1963; McCulloch and Till, 1964).

Wendling et al (1972) and Ohunewitch et al (1972) found that the relationships between the number of colonies counted in the spleen and the number of cells injected remained linear after infection.

After injection with Friend or Rauscher leukaemia viruses there was an exponential increase in the spleen weight (Wendling et al, 1972; Marhoe et al, 1973; Bromner and Bentvelzen, 1974; O'kunewick et al, 1972; Siedel, 1973).

The number of CFU-S per 10^5 spleen cells remained nearly the same whether grafted cells came from normal or leukaemic spleens.

Consequently, the total number of CFU-S in infected spleens was increased by a factor (15-30 fold) depending on spleen weight.

After infection with Rauscher or Friend virus, CFU-S in femoral bone marrow remained nearly constant (about 3000 CFU-S per femur) despite a more or less pronounced cytopenia (Wendling et al, 1972; Seidel, 1973; Iturrizza and Seidel, 1974).

Okunewick and Phillips (1973) observed a pronounced fall in the number of bone marrow CFU-S of Rauscher-infected SJL/J mice but a slight increase occurred when the same mouse strain was infected with FVP.

Van Griensven et al (1974) found that the total number of bone marrow CFU-S was increased by a factor of 2.7 in Rauscher infected BALB/C mice.

The mean number of circulating CFU-S was increased 500-1000 fold after FVP virus infection and total number of circulating CFU-S detected in the blood of well-developed murine leukaemia has been assessed at 40,000 CFU-S per mouse as compared to 45 in normal mice (Wendling et al, 1972; Seidel, 1973b). Seidel reported that the increase in CFU-S occurred at about the time of increase in number of peripheral lymphoid cells.

Wendling et al (1972) reported an increase by a factor of only 10 in the number of CFU-S in the mouse after infection with Friend or Rauscher virus.

In normal animals the spleen represents only 6-10% of haemopoietic tissue. A large increase in the size of the CFU compartment in blood or the spleen does not represent a large increase when the haemopoietic

system is considered in total. The size of the haemopoietic stem cell compartment however is greatly modified by virus-induced leukaemias.

A 'specific' antiserum against a mixture of murine viral leukaemia cells and virus was effective against the haemopoietic stem cells of infected animals (Tambourin, 1978). Infection with virus affects the number of stem cells in cycle.

In vitro suicide tests showed that 4 hours after infection with Rauscher virus the number of spleen CFU-S killed by tritiated thymidine exposure increased to 20% from a base line of 10% in controls. In the next 2 weeks the proportion of CFU-S in DNA synthesis remained at approximately 20%. In the 3rd week after virus infection the proportion of CFU-S killed by ^{3}HTdR decreased to a point of being no longer detectable even though the total number of CFU-S was increased several fold over normal (Okunewick et al, 1976).

In FVP leukaemia splenic CFU-S were about 8-fold less efficient at repopulating lethally irradiated mice than CFU-S from normal mice. The efficiency of the bone marrow was only slightly altered. In Rauscher leukaemia the radioprotective ability of the spleen cells was at least equivalent to normal ($LD_{50/30}$ = 17 CFU-S) (Tambourin, 1978). These results suggest that haemopoietic stem cells in leukaemic spleens maintain their relative numbers but also retain their differentiation efficiency.

CFU-S derived from Friend leukaemic spleens were able to provide reticulocytes, leukocytes and platelets in peripheral blood. However, they had poor ability to produce thrombocyte progeny and erythroid production was lowered 7.5 fold. Normal numbers of granulocytes were produced (Wendling et al, 1973a). Conversely CFU-S from

FVP-infected mice behaved like their normal counterparts as regards erythropoietic and granulopoietic functions but production of peripheral platelets was quantitatively reduced although the time of platelet emergence was not delayed (Wendling et al, 1973b).

From ^{59}Fe studies Okunewick et al (1973) found that erythropoietic activity of the RLV progeny was much lower than normal.

The CFU-C compartment size from CBA/J mice with Rauscher leukaemia initially decreased 2 days after virus infection (as did CFU-S) but thereafter they increased rapidly up to 30-fold normal values 2-6 weeks after initiation of the disease. Iturizza and Seidel (1974) found that CFU-C paralleled the evolution of CFU-S.

Van Griensven et al (1973, 1975) found that in the bone marrow of BALB/C mice infected by Rauscher leukaemic viruses a 2.6 fold increase in CFU (CFU-S, CFU-C) occurred on day 12 after infection; thereafter this number returned to normal values.

Golde et al (1976) found that colony formation dependent on CSA and cloning efficiency of Friend leukaemic spleen cells found to be approximately 10 times the normal values. Colonies appeared morphologically normal. The total CFU-C per leukaemic spleen was 300-fold normal but cells elaborating CSA were decreased.

When 10^5 leukaemic spleen cells from a spontaneous myeloid leukaemia in an RF mouse were injected into syngeneic mice 6 to 7 days later there was a decline of all normal parameters involving erythropoiesis, pluripotent stem-cells and spleen CFU-C. The CFU-C however showed a significant rise between days 3 and 6 (Lajlha et al, 1972). The CFU-S were decreased during the development of this transplanted myeloid leukaemia.

Seidel (1973c) found a transitory decrease of CFU-S in the bone marrow and spleen followed by an increase in the blood. L1210 lymphatic leukaemia showed no modification in CFU-S number in the marrow (Chevalier C and Frindel, E., 1973).

In AKR-spontaneous primary T-lymphoid leukaemia there was a decrease in the number of CFU-C in bone marrow and an increase in the spleen when compared to the number of CFU-C's in non-leukaemic mice of the same age (Chevalier et al, 1974).

When anti-Θ serum was used to eliminate lymphoid cells in the leukaemic bone marrow there was a decrease in the number of CFU-S in the bone marrow in the terminal stage of spontaneous AKR leukaemia (Frindel and Chevalier C, 1975)

In transplanted AKR leukaemia the decrease in CFU-S was correlated with the progress of the disease, the spleen weight and with an increase in the proportion of CFU-S in DNA synthesis phase (Sainteny et al, 1978).

When the AKR leukaemic cells were destroyed with arabinosyl-cytosine (Ara-C) the number of medullary CFU increased rapidly after a transitory decrease, surpassing normal control values (Sainteny et al, 1977).

The number of bone marrow CFU-S decreases at the terminal stage of spontaneous AKR leukaemia. This decrease is compensated by an increase of CFU-S in the spleen, lymph nodes, liver and thymus. Thus overall CFU-S number is basically equivalent to that found in normal mice. This implies that there is a spatial redistribution of CFU-S in AKR leukaemia (Sainteny and Frindel, 1978).

Hagenbeek et al (1977) found an increase in spleen and blood CFU-S in a chemically induced myeloid leukaemia in BN rats, with a decrease of CFU-S in the bone marrow.

Most studies , however, of spleen colony formation by murine leukaemic cells have used virally induced leukaemias.

Sainteny and Frindel (1978) reported that Charbord et al had found it impossible to assess CFU-C in leukaemic bone marrow (human) in which fibrosis was predominant. In patients with myelofibrosis CFU-C numbers in the blood were increased as compared to the controls.

2. MATERIALS AND METHODS

2. Materials and Methods

2.1 Mice These investigations were carried out using CDI mice.

Specific pathogen free random bred CDI females, weighing between 20 and 25 grams were supplied by Charles River UK Ltd, Margate, Kent.

The mice were housed in the Animal House of the Bute Medical Buildings in a room which was artificially lit and kept at a temperature of 20°C.

The mice were kept in small sawdust lined cages. Usually each cage held no more than 5 mice.

Feeding:- The mice were given unlimited quantities of dry food (41B Rat Cake, North Eastern Farmers Ltd, Aberdeen) and tap water.

Fifteen day old foetuses were used for the embryo cultures.

2.2 Autopsy Procedures

Mice were anaesthetised by exposure to ether vapour and killed by cervical dislocation.

The spleen, thymus, inguinal lymph nodes and brachial lymph nodes were dissected and weighed immediately.

A femur was excised and the medullary cavity was opened at the proximal end. A 23 guage needle [Everett Medical Products, Ltd, Mitcham, Surrey] was inserted into the femoral medullary cavity and the contents were washed out and gently aspirated in single strength Fischers medium.

2.3 Cell Counts

The suspension of femoral bone marrow cells in single strength Fischers medium was diluted in 'Isoton' (Coulter Electrons Ltd,

Harpenden, Herts), 'Zaponin' (Coulter Electrons Ltd, Harpenden, Herts) was added to lyse erythrocytes. The nucleated cell content of the suspension was determined using a model D Coulter particle counter. The attenuation was set at 1, the aperture current at 0.017, the manometer volume selector at 0.5 ml and the threshold at 7 μm. The following formula was used to correct cell counts for particle coincidence:

for 100 μ aperture

$$n = n_1 + \frac{n_1^2}{1000} \times 2.5$$

where n_1 = number of cells counted

2.4 Cytology

Cytological preparations were made on clean glass slides using a Shandon-Elliot cytospin. Femoral bone-marrow cells were suspended in single strength Fischers medium supplemented with 20% horse serum. About 10^6 cells suspended in 0.5 ml of medium were introduced into each cytospin container.

The smears were stained by the Jenner-Giemsa method described by Dacie and Lewis (1963).

2.5 Staining for reticulocytes

Blood was removed from the retio-orbital sinus of mice using microhaematocrit tubes containing heparin.

Blood from two of these tubes was emptied into a small test-tube and equal volumes of new Methylene blue stain (Brechner, G, 1949) were added. The test-tube was stoppered and placed in a water bath

at 37°C for 20-25 mins. Films of this solution were made on glass slides and allowed to dry before counting.

2.6 Histology

Tissues were fixed in Bouin's fluid, dehydrated and embedded in Paraplast (Sherwood Medical Industries, St. Louis, Missouri). Sections of 5 μ thickness were cut and these were stained with Mayer's Haematoxylin and Eosin.

2.7 Preparation of Trypsin

Trypsin powder ('Bacto-trypsin', Difco Lab, Detroit) was dissolved in a 10 ml of sterile distilled water to give a 5% solution. This was diluted with single strength Fischers medium to give a final concentration of 0.25%. The pH was adjusted to 7.8 using 4.4% sodium bicarbonate solution (Wellcome, Beckenham). Trypsin solution remains active for only one week if stored at -20°C so the solution was prepared just before use.

2.8 Preparation of Fischers Medium

100 ml of Fischers 10 x concentrated medium with L-glutamine (Gibco-bio-cult, Glasgow) was mixed aseptically with 900 ml of sterile double distilled water.

Streptomycin solution (50 mg in 0.5 m) (Streptomycin sulphate B.P. Dista, Liverpool) and 5 x 10^5 units of penicillin (Sulupox-Benzypen Dista, Liverpool) was added to the medium. The solution was stored in aliquots of 96.85 ml at 4°C. Just prior to use 3.15 ml

of 4.4% sodium bicarbonate (Wellcome Ltd) was added to adjust the pH to 7.4 . Horse serum (Flow Lab, Irvine) was added to the medium in the appropriate concentration just prior to use.

2.9 Preparation of 5% Agar Gel

75 g of agar powder ('Agar Noble', Difco Lab, Michigan) was dissolved in 1500 ml double distilled water in a 2 litre conical flask and heated in a water bath at 100°C for 1 hour. Aliquots of 10 ml were dispersed into McCartney bottles and autoclaved at 15 lb pressure for 15 minutes. The agar gel was stored at 4°C.

2.10 Conditioned medium

Conditioned medium containing the growth factor essential for colony formation in culture was prepared using fifteen day old mouse foetuses.

The foetuses were delivered by abdominal hysterectomy and killed by decapitation. After being washed in saline, they were minced using sterile scissors with curved points. Mincing was continued until the smallest possible fragments were obtained. 0.25% trypsin solution (5 ml per embryo) was added to the homogenate which was incubated for 1 hour at 37°C.

The cell suspension was then centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and the cells were washed twice in single strength Fischers medium supplemented with 20% horse serum.

The cell concentration was determined using a model D Coulter Counter and the suspension diluted to a concentration of 4 or 5 x 10^6 cells per ml, using Fischers medium and horse serum.

Subsequently the solution was divided into 20 ml aliquots in plastic tissue culture bottles [75 cm^2, Corning/Jobling, New York]. The bottles were gassed with 5% CO_2 in air, sealed and incubated at 37°C.

A monolayer of cells adhering to the plastic, was formed after 3 or 4 days. On the 7th day of incubation the supernatant was harvested and fresh medium with 20% horse serum was pipetted over the layer of cells. The bottles were regassed and incubated for a further 7 days. This process of harvesting and refeeding was repeated as long as the monolayers survived.

2.11 Culture of Haemopoietic Cell Colonies in Agar

The method used was that described by Testa, 1972.

The following constituents were mixed together

a ml of single strength Fischers medium supplemented with 40% horse serum

b ml of mouse cell suspension (to give the appropriate final cell dilution)

2.7 ml of conditioned medium (to give a final concentration of 15%)

The total volume would be equal to 18 ml. Usually the required number of cells were suspended in 1 ml of Fischers medium so the amount of medium with horse serum added would be 13.2 ml.

The agar was added to the cells, medium and horse serum at a fairly high temperature. The heat from the agar was quickly dissipated when it was pipetted into the mixture and did not harm the cells. Adding the molten agar at a high temperature prevented it

gelling before it was thoroughly mixed with the media and spread evenly over the petri dishes.

Aliquots of 3 ml of the mixture were plated in 50 mm plastic petri dishes (Nunclon, Gibco-bio-cult, Glasgow) and spread evenly over the dishes by gentle aggition of the plates.

The agar mixture was allowed to gel at room temperature, before being placed in a sealed, humidified box.

The cultures were gassed with 5% CO_2 in air and incubated for 7 days at 37°C.

2.12 Bone marrow cell suspensions

Mice were killed by cervical disslocation and the femora removed asceptically. The muscle tissue was cleaned off using a gauze swab. The proximal ends of the femora were removed and the marrow was washed out into Fischers medium using a syringe with a 23 G needle. A single cell suspension was obtained by gentle aspiration of the solution.

2.13 Spleen cell suspensions

The mice were killed and the spleen dissected out, free of surrounding tissues. Very small pieces were removed from each end of the spleen and the splenic pulp was gently forced out from the capsule by stroking the spleen surface with the blunt edge of a sterile scalpel. The cell free splenic capsule was then discarded and a single cell suspension in Fischers medium was obtained from the pulp by gentle aspiration with a syringe and needle.

2.14 Bleeding of Mice

Mice were anesthetised by 20 secs exposure to ether and blood removed by insertion of a microhaematocrit tube into the retro-orbital

sinus. For most experiments the maximum amount removed was 0.3 ml. To measure the PCV (packed cell volume) the haematocrit tubes were sealed at one end by heating. The tubes were spun in a microhaematocrit centrifuge (Hawksley, London) for 5 minutes. The PCV was measured using a microhaematocrit reader (Hawksley, London).

2.15 Splenectomy

Mice were anaesthetised with Sagatal Pentobarbitone Sodium Solution (35 mg/kg body weight).

The skin overlying the spleen was shaved and cleaned with alcohol. An incision was made on the lateral aspect of the skin where the spleen could be viewed through the abdominal wall. The abdominal cavity was opened by a small incision and the spleen was gently lifted out. The splenic vessels were tied with surgical thread (Ethicon sutures, Edinburgh) and the spleen excised distal to the ligature. The pedicle of the spleen and the pancreas were replaced within the abdominal cavity and the abdominal incision was stitched with surgical thread. The skin incision was closed with 2 or 3 'Michel' clips.

In sham operated controls, the spleen was exteriorised and then replaced in the abdominal cavity without interrupting the blood supply.

2.16 Radioiron (^{59}Fe) Uptake Studies

Each mouse was injected intraperitoneally with 1μCi ^{59}Fe (Riches, 1970) (ferric citrate, specific activity 10 Ci/g Fe, Radiochemical Centre, Amersham). 24 Hours after administration

the appropriate tissues were removed, weighed and placed in test-tubes. The activities were measured by an Intertechnique CG30 Gamma Counter. Standards were prepared from Fe^{59} solution, to enable the gross uptake in each tissue to be calculated.

2.17 Mice

CBA/H male mice of about 100 days old were used for irradiations. This strain has been inbred for almost 25 years in the M.R.C. Radiobiology Unit, Harwell.

The animals were provided with food pellets and chlorinated water 'ad libitum'.

2.18 X-irradiation

A filter consisting of concentric copper sheets was used to give a uniform field with a half-value layer of 1.1 mm of Cu. Constant voltage (250 kV) was maintained by automatic compensation. Measurements were made in the actual boxes used to contain the animals in which phantom mice made of Lincolnshire bolas in polythene bags were placed on a sawdust bed to simulate experimental conditions. Exposure was dorsal ventral and the dose rates were 4.2, 57.3 and 552.0 rad/min and the appropriate S.S.D.'s allowed constant dosage through the mice to within 1-2%.

2.19 Metaphase arrest studies in irradiation induced murine myeloid leukaemias

Vincristine ("Oncovin", Vincristine sulphate, B.P. Eli-Lilly) or colcemid(Demecolcine Ciba Lab, Horsham) was administered by intraperitoneal injection to CBA/H male mice. These mice had

received different doses of irradiation when approximately 100 days old. Leukaemia was diagnosed at various intervals after irradiation (Major, 1979).

2.20 Culture of leukaemia cells

Spleen cells from mice with primary of passaged leukaemias were suspended in Eagles medium (Eagles MEM with Earles salts without L-glutamine - Gibco-BioCult, Glasgow). Single cell suspensions were obtained by passing the suspension through progressively smaller bore needles until the solution passed easily through a 25 G needle.

Cells were cultured, with the appropriate concentration of vincristine or colcemid, for 2 hours in sterile universal tubes, kept mobile on a rotary mixer.

Cells were cultured at a concentration of 10^6/ml.

Smears of cultured cells were made using a cytospin (Shandon-Elliott). Cell suspensions were spun at 800-100 rpm for 10 minutes.

Smears of leukaemic tissue were made by teasing the tissue in small amounts of foetal calf serum. Small drops of the cell suspension obtained were spread on glass slides air dried and fixed in methanol for 10 minutes. The smear preparations were stained with May-Grünwald Giemsa stain.

2.21 Counting of metaphases

One thousand leukaemia cells were counted from each smear and the number in metaphase recorded. Several slides of each preparation were counted. The means and errors of the binomial proportions, obtained from these counts, were calculated.

3. RESULTS

3.1 The Effects of Concentration of Horse Serum on Colony Yield

Aliquots of femoral bone marrow cell suspension from one CDI female mouse were cultured with various amounts of horse serum present in the medium.

Fischers medium containing 20, 30, 40, 50 and 60 per cent horse serum was made up. The bone marrow cells and agar were added to these mixtures subsequently, so the above percentages do not represent the final concentration of horse serum in the agar medium

Results

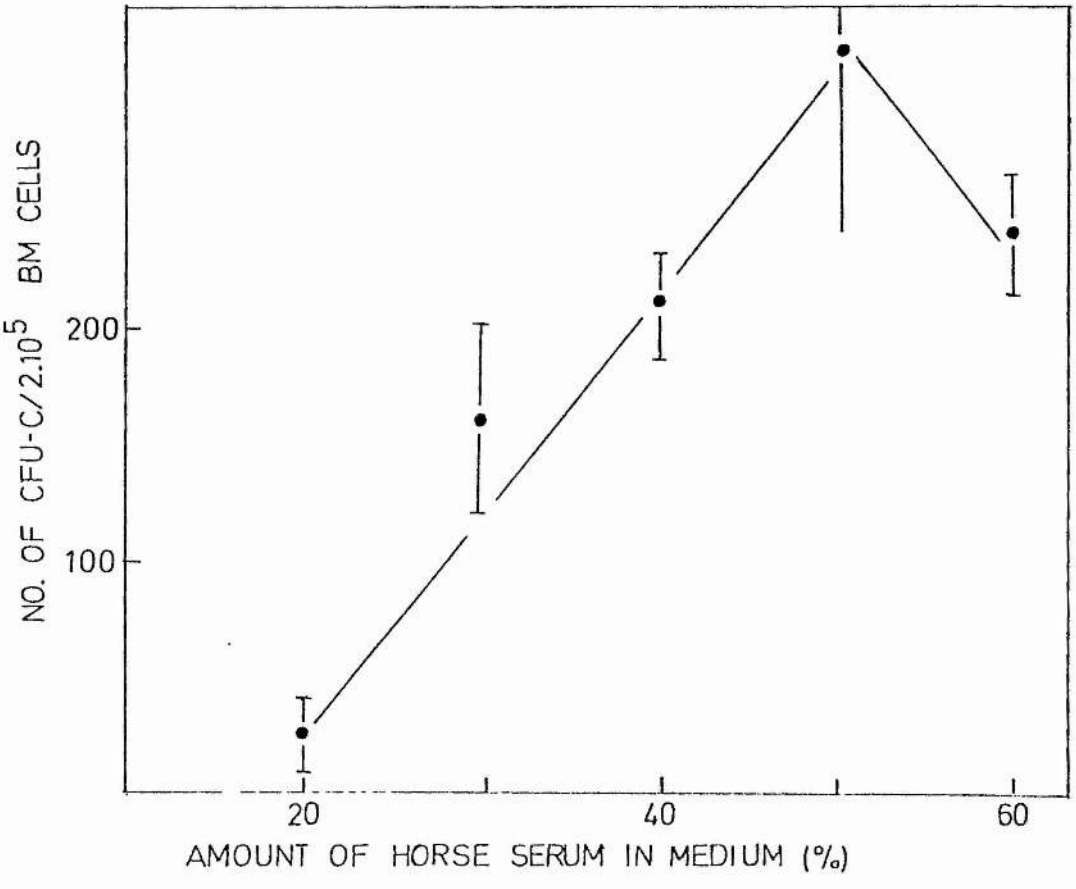
Fig. I shows the number of $CFU_c/2 \times 10^5$ nucleated cells, formed with various concentrations of horse serum in the medium.

There is a steady increase in the numbers of CFU_c with the increase in the amount of horse serum up to 50%. At 60% the numbers of CFUc fall, suggesting there is some inhibition of colony formation in cultures containing this high amount of horse serum, although this decrease in CFUc could be due to the fact that other nutrients in the medium have been diluted by this amount of serum.

This experiment illustrates the dependency of colony formation on the active principal in serum and the concentration of that active principal present in the medium.

The same batch of conditioned medium was used in each assay.

Figure 1



The number of CFU-C obtaining x 2.10^{-5} femoral bone marrow cells when different amounts of horse serum were plated.

3.2 Effects of amount of Conditioned Medium on colony yield

In this experiment normal mouse bone marrow was cultured in the usual manner, but with different concentrations of the same conditioned medium.

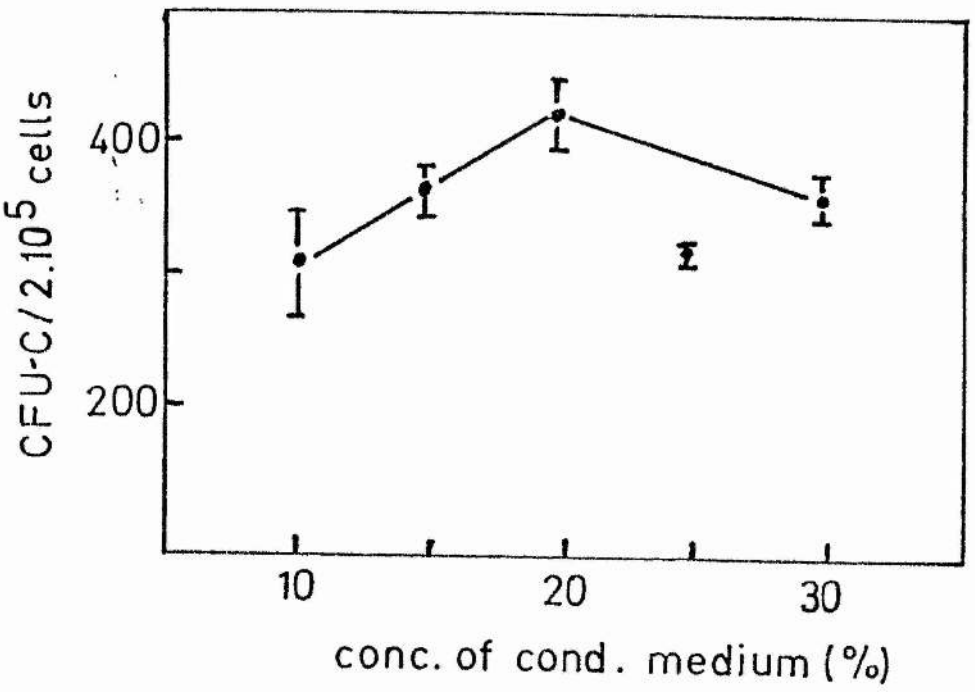
Five groups of five plates each contained 10, 15, 20, 25 and 30 per cent conditioned medium respectively. These percentages represent the final concentration of conditioned medium in the agar medium.

Results

The colony numbers are seen to plateau at the values of 15 and 20 per cent [Fig. 2]. This result is consistent with the sigmoid close relationship reported by other workers (Metcalf and Moore, 1971).

There is an increase in the proportions of mixed and granulocyte colonies, when higher concentrations of CSF are present, but this increase is not highly significant.

Figure 2



The number of CFU-C obtained × 2.10^{-5} cells femoral bone marrow cells with different concentrations of conditioned medium plated.

Table 1

Amount of conditioned medium present %	Total number of CFUc $\bar{x} \pm$ SE	Type of colony			Proportion of each type (per cent of total)			Clusters $\bar{x} \pm$ SE
		Mac	Mix	Gran	Mac	Mix	Gran	
10	305.6 ± 40.9	218.6 ± 24.7	52.6 ± 9.5	34.3 ± 7.5	71.5 ± 8.0	17.2 ± 3.1	11.2 ± 2.4	158.3 ± 22.0
15	360.6 ± 18.8	237.0 ± 11.0	72.0 ± 7.5	51.6 ± 11.6	65.7 ± 3.0	19.9 ± 2.0	14.3 ± 3.2	235.0 ± 70.5
20	370.0 ± 26.2	238.3 ± 18.8	92.6 ± 4.2	39.0 ± 8.5	64.4 ± 5.0	25.0 ± 1.1	10.4 ± 2.2	256.6 ± 20.4
25	312.6 ± 4.3	177.0 ± 4.7	90.6 ± 1.6	45.0 ± 3.0	56.6 ± 1.5	28.9 ± 0.5	14.4 ± 0.9	183.3 ± 16.6
30	357.3 ± 16.5	181.6 ± 2.6	112.0 ± 8.0	63.6 ± 10.3	50.8 ± 0.7	31.3 ± 2.2	17.8 ± 2.8	265.0 ± 20.8

3.3 Variation in numbers and types of CFU-C produced by different batches of conditioned medium

Cultures of normal bone marrow from the femurs of CDI female mice were set up using three different batches of conditioned medium.

The following results were obtained

Conditioned Medium	Total No. of Colonies	Types of Colonies % of total ± SE		
		Macrophage	Mixed	Granulocyte
11 ii a	188.00 ± 15.50	51.9 ± 0.10	26.9 ± 0.10	21.06 ± 0.09
12 ii a	88.66 ± 14.53	35.33 ± 0.29	19.54 ± 0.30	45.11 ± 0.25
14 ii	196.67 ± 16.80	55.25 ± 0.10	24.40 ± 0.20	18.64 ± 0.10

Two batches of medium gave very similar results namely 11iia and 14ii. These batches which gave a higher yield than the third also produced similar proportions of types of colonies, macrophage colonies making up the largest proportion followed, in order, by mixed and granulocyte colonies.

Granulocyte colonies, however, made up the highest proportion in the cultures total, while granulocyte made up 64%. This distribution of colony type corresponds to the situation where a weak conditioned medium was used. Although colony numbers were low the proportions of mixed and granulocyte colonies were higher than the proportion of macrophage colonies which is the reverse of the situation when an active conditioned medium was used.

Since the concentration of CFU-C on the femur in marrow regenerating 3 days after HN_2 is the same as normal marrow the same

number of colonies should form per million cells.

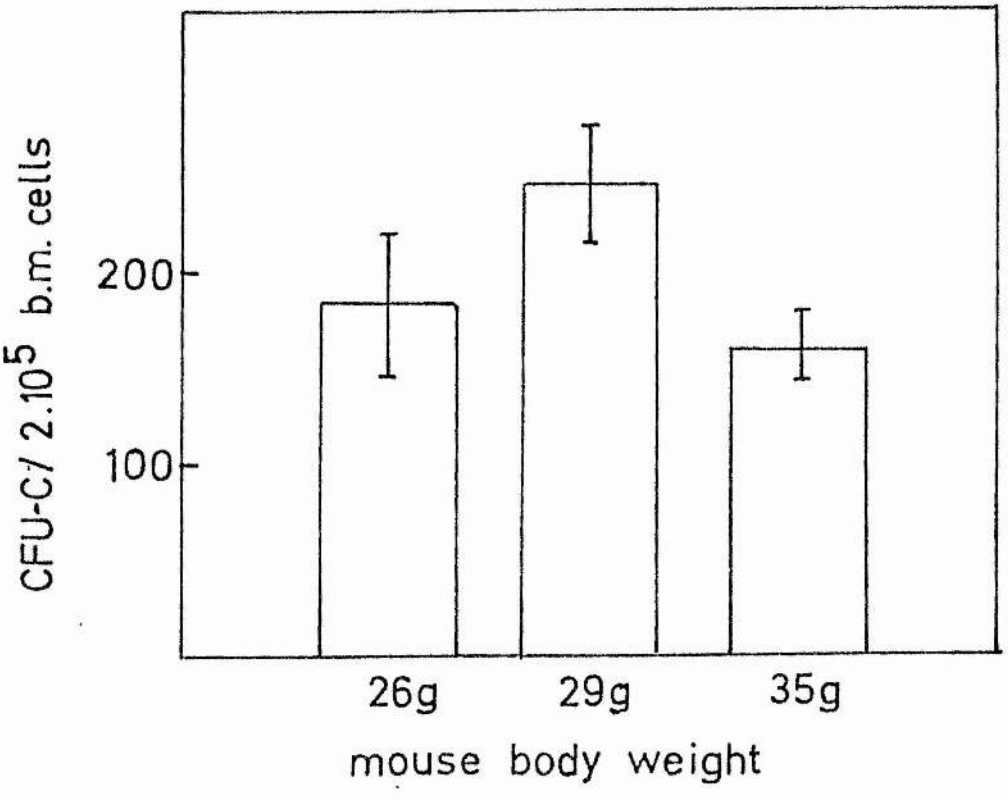
The colony formation in the regenerating marrow cultured with no CSF must be due to the presence of a higher proportion of marrow cells releasing CSF.

3.4 Variance of CFUc content among individual mice

The femoral content of individual CDI mice was measured. The number of CFUc per 2×10^5 cells was recorded since this was the form of measurement adopted in most experiments.

The number of CFUc is not related to the body weight of the animal. The CFUc/2×10^5 cells in an animal weighing 29 grams differed significantly from the number of CFUc/2×10^5 cell in two animals weighing 26 grams and 35 frams respectively ($p < 0.05$). This level of biological variance was considered acceptable for most experiments and might be overcome by pooling the marrow from 5 identically treated mice.

Figure 3



The number of CFU-C obtained x 2.10^{-5} femoral bone marrow cells of mice of different body weights.

Table 2

Number of CFU-C/2 x 10^5 femoral bone marrow cells in normal CDI female mice of different body weights

Mouse Weight in Grams		Total number of CFU-C/2 x 10^5 cells	Macrophage Colonies	Mixed Colonies	Granulocyte Colonies	Clusters
26	$\bar{x}$	172.50	105.25	41.00	26.25	127.50
	SD	36.64	29.71	6.68	5.37	54.39
	SE	18.32	14.85	3.34	2.68	27.19
29	$\bar{x}$	238.75	130.50	66.25	42.00	180.00
	SD	29.81	7.85	10.72	18.22	40.00
	SE	14.90	3.92	5.36	9.11	20.00
35	$\bar{x}$	158.00	116.00	24.75	17.25	117.50
	SD	15.12	13.44	6.13	2.06	29.86
	SE	7.56	6.72	3.06	1.03	14.93

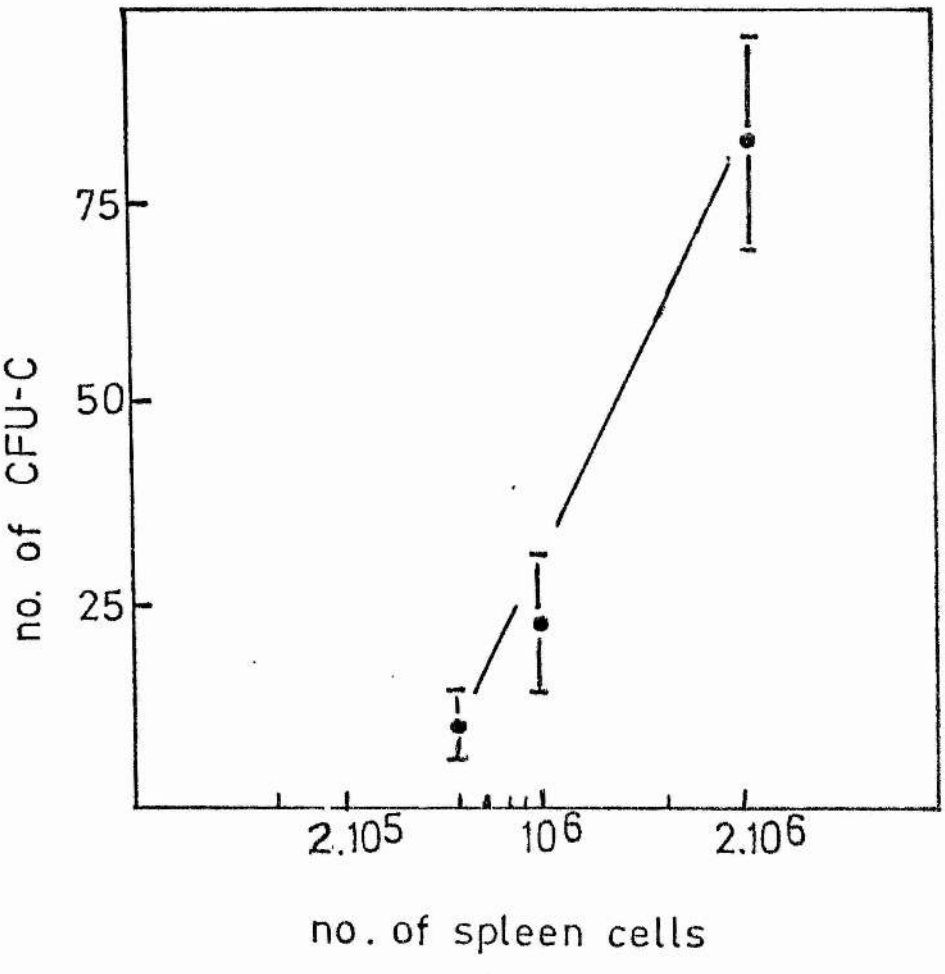
3.5 Splenic CFUc

An estimate of the number of CFUc per number of spleen cells plated was made with a view to finding a suitable number of spleen cells to seed in each culture dish. Spleen cells were suspended in medium then seeded at different concentrations in agar medium and cultured for formation of colonies.

Results

Although the number of CFUc formed varies greatly with the batch of conditioned medium used, it required about 10^6 normal spleen cells to produce as many colonies as 10^5 normal bone marrow cells produced with the same batch of conditioned medium.

Figure 4



The number of CFU-C obtained from plating different numbers of spleen cells.

Table 3

Number of CFU-C in plates containing different numbers of spleen cells

Numbers of Spleen Cells		Macrophage Colonies	Mixed Colonies	Granulocyte Colonies	Total Colonies	Clusters
5×10^5	$\bar{x}$	3.00	2.40	3.80	9.20	8.8
	SD	2.00	1.94	1.64	2.77	2.28
	SE	0.894	0.87	0.73	1.24	1.02
10^6	$\bar{x}$	8.20	8.60	7.00	23.80	26.60
	SD	5.07	2.70	3.32	8.71	5.41
	SE	2.27	1.21	1.48	3.96	2.42
2×10^6	$\bar{x}$	36.80	16.60	29.80	83.20	39.60
	SD	11.30	8.44	7.39	13.36	12.83
	SE	5.05	3.77	3.30	5.90	5.74

3.6 Effects of 100 μg HN_2 on Haemopoietic and Lymphoid tissues in the 12 days after treatment

Groups of 5 CDI female mice were autopsied at regular intervals during a 12 day period of lymphomyeloid tissue regeneration following the hypocellularity which occurs after treatment with 100 μg HN_2. The mice were weighed before killing. The thymus, lymph nodes and spleens were dissected out. The femurs were dissected out, cleaned and the bone marrow washed out into Fischers medium for cell counts.

Figure 5 shows that the mouse body weight falls slightly and remains around 20 grams.

Figure 6 shows that the thymus falls to a minimum weight between days 3 and 8 after 100 μg HN_2 then rises again towards normal values.

Figure 7 shows that the pooled lymph node weights (auxillary and inguinal) fall to a minimum between days 2 and 10 after 100 μg HN_2 and rise after 10 days.

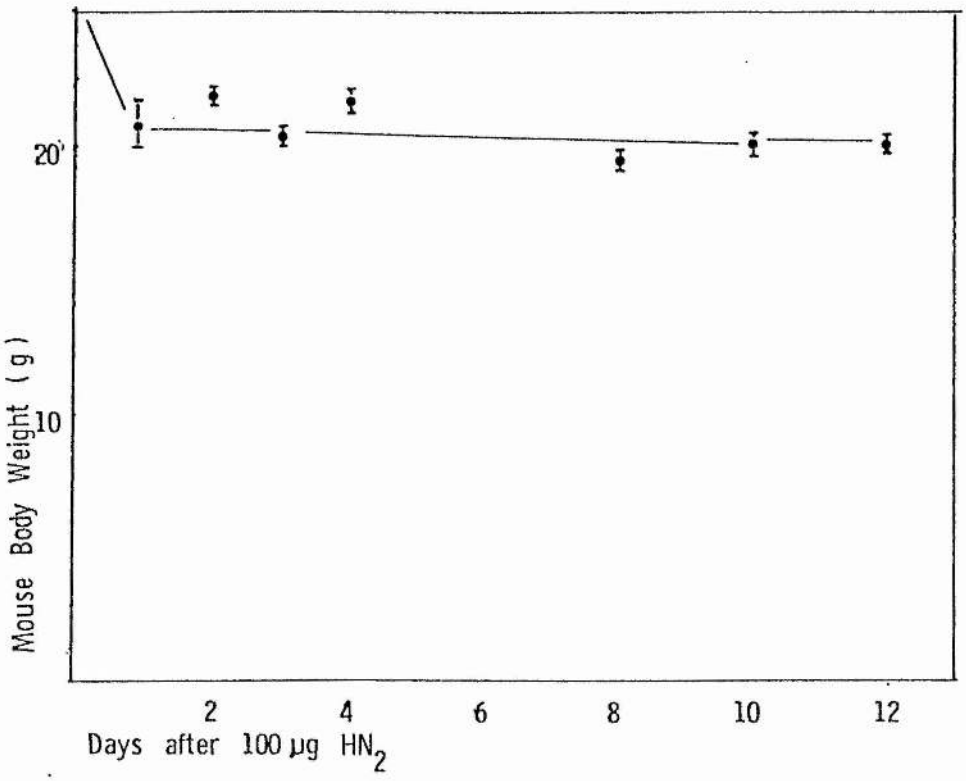
Figure 8. The mesenteric lymph node weight falls to a minimum between days 3 and 8 then rises between days 8 and 12.

Figure 9. The spleen weight falls between days 3 and 8 and rises between days 8 and 12.

Figure 10. The femoral cellularity falls to a minimum between days 2 and 6 then rises to normal values by day 10 and then begins to fluctuate around the normal value.

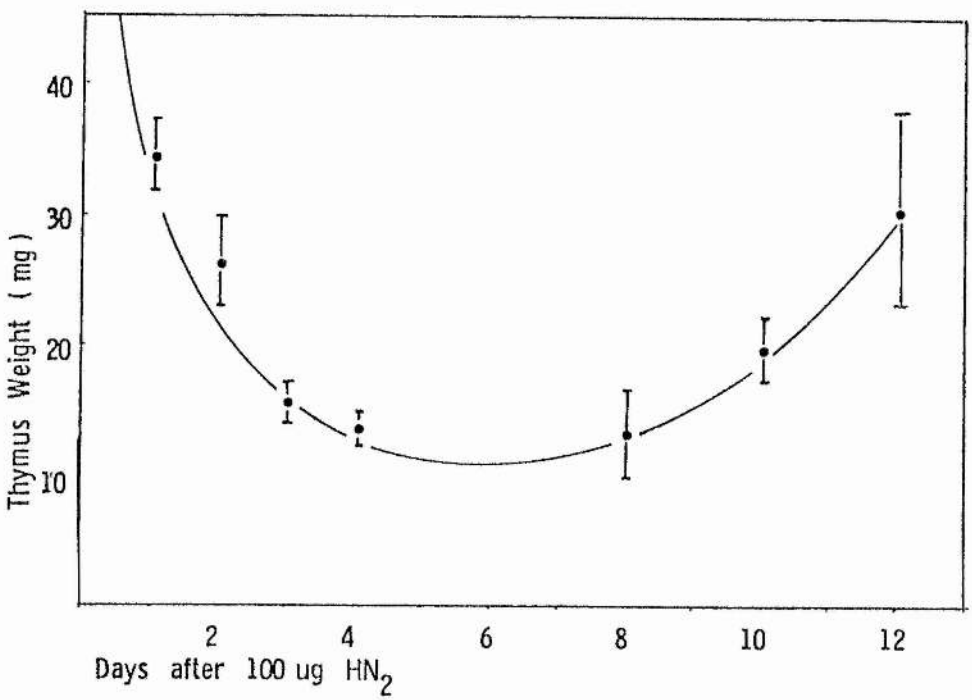
These results are similar to those obtained by Sharp et al (1973) on CSI/ASH female mice.

Figure 5



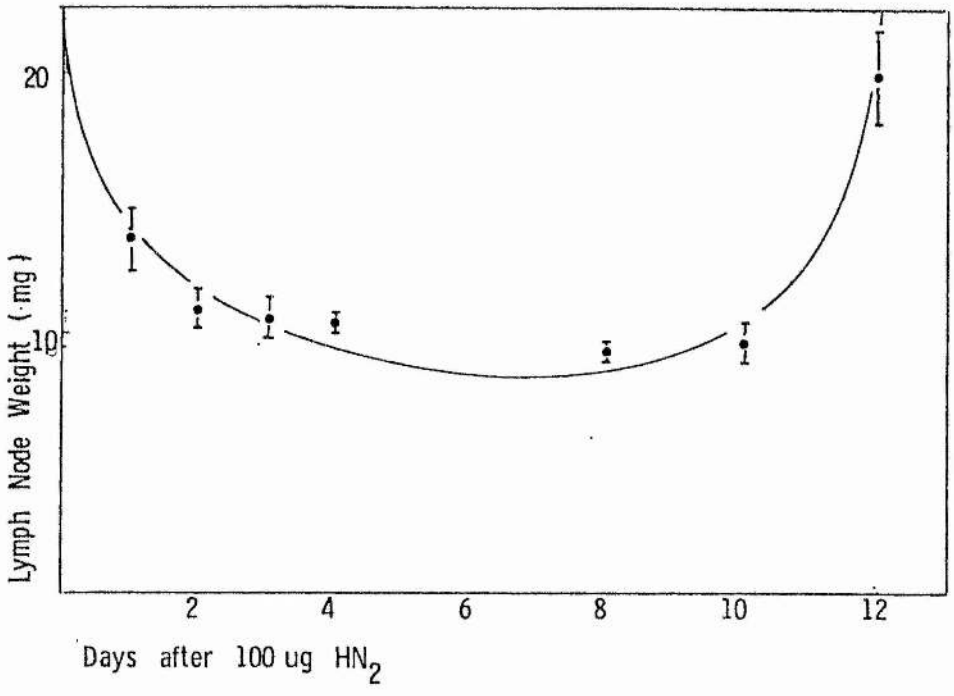
Mouse body weight on days following treatment with 100 μg HN_2

Figure 6



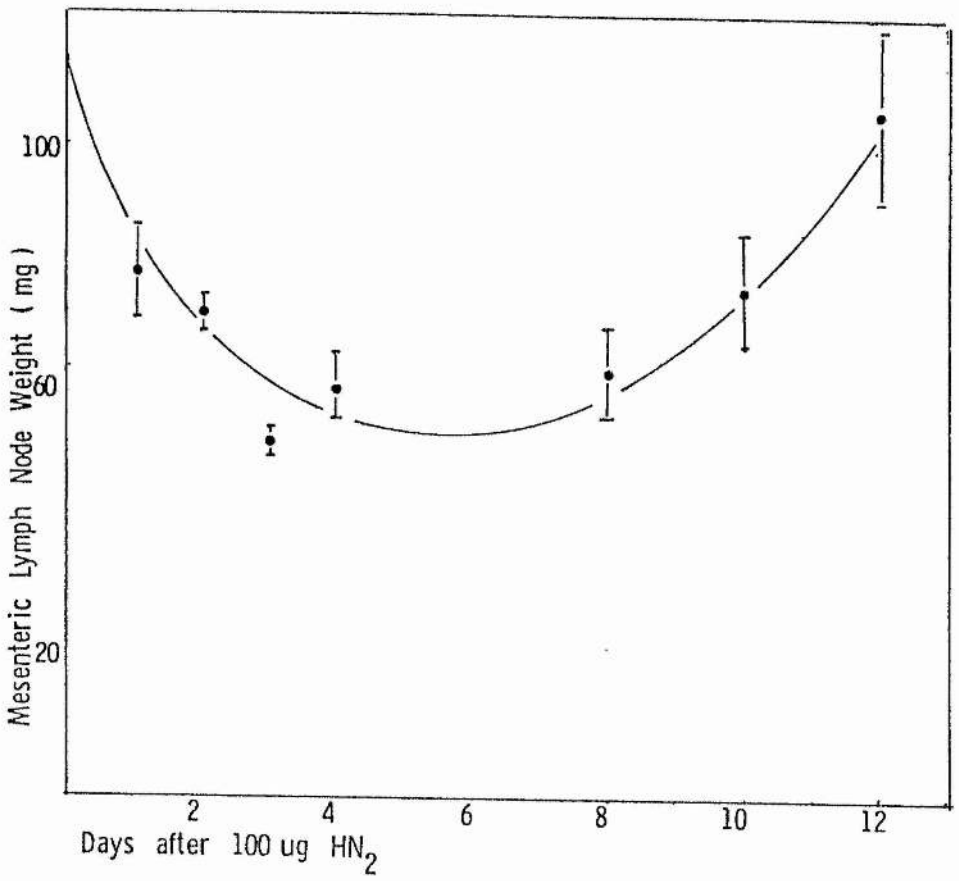
Thymus weight (mg) on days following treatment with 100 μg HN_2

Figure 7



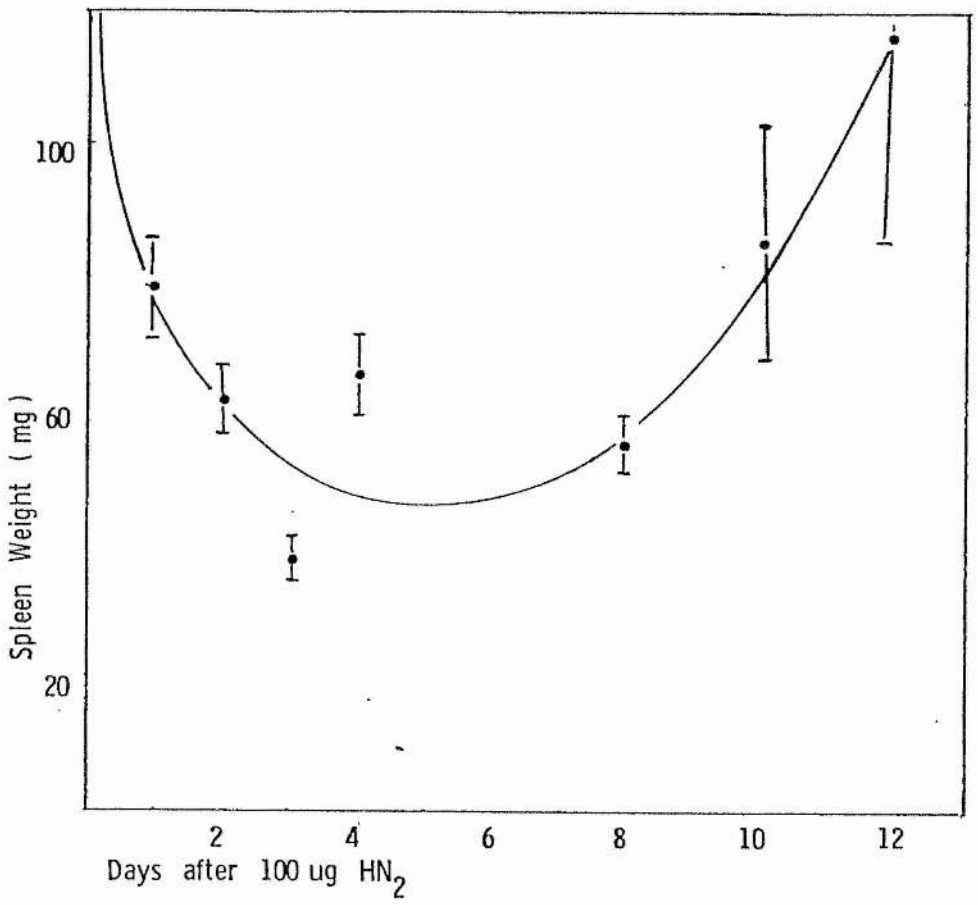
Pooled lymph node (auxillary and inguinal) weight (mg) on days following treatment with 100 μg HN_2

Figure 8



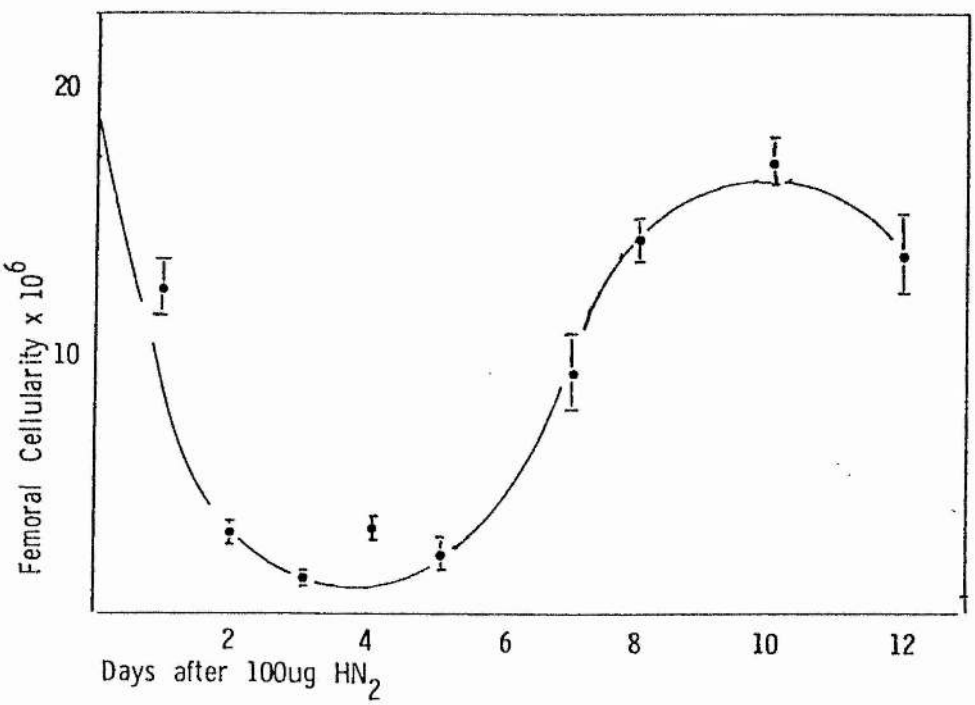
Mesenteric lymph node weight (mg) on days following treatment with 100 μg HN_2

Figure 9



Spleen weight (mg) on days following treatment with 100 μg HN_2

Figure 10



The femoral cellularity x 10^6 on days following treatment with 100 μg HN_2

Table 4

Weights of mice, pooled lymph nodes, mesenteric lymph nodes, thymus and spleen and femoral bone-marrow cellularity on days following administration of 100 μg of mustine hydrochloride to female CDI mice.

Day	Mouse Body Weight (g)	Pooled lymph node weight (mg)	Mesenteric L.N L.N. weight (mg)	Thymus weight (mg)	Spleen weight (mg)	Femoral BM Cellularity (millions)
Control	26.76 ± 0.39	21.99 ± 1.25	112.56 ± 9.52	74.53 ± 7.28	122.23 ± 9.56	17.11 ± 1.28
1	21.52 ± 1.11	17.29 ± 2.40	80.27 ± 7.55	34.29 ± 2.80	78.21 ± 7.98	12.24 ± 1.11
2	23.62 ± 0.48	11.63 ± 1.36	74.36 ± 3.40	26.19 ± 3.52	60.89 ± 5.08	3.05 ± 0.42
3	20.68 ± 0.76	10.99 ± 1.68	54.98 ± 2.33	15.25 ± 1.70	47.69 ± 3.46	1.46 ± 0.17
4	23.40 ± 0.98	11.27 ± 0.78	63.58 ± 5.36	13.68 ± 1.27	65.50 ± 6.19	3.46 ± 0.19
5	-	-	-	-	-	2.94 ± 0.70
7	-	-	-	-	-	11.51 ± 1.41
8	18.84 ± 0.61	9.23 ± 0.82	65.80 ± 7.18	13.24 ± 3.35	55.21 ± 4.09	14.23 ± 0.82
10	20.93 ± 0.94	10.66 ± 1.63	78.17 ± 9.14	18.99 ± 2.54	85.76 ± 17.82	17.03 ± 0.90

3.7 Recovery of CFU-C after 100 μg HN_2

CDI female mice weighing 20-25 g, were given 100 μg mustine hydrochloride intravenously. On the days after treatment which are tabulated, CFU-C assays were set up. Bone marrow from the femurs of 5 mice was pooled for each assay.

The number of colonies present after treatment with HN_2 is expressed as a percentage of the number of colonies from normal marrow cultured at the same time and under the same conditions. This obviates the need to consider the influence of the variance in the effectiveness of different condtioned media on the results.

The total CFU-C femoral content after 100 g HN_2 is very low on days 1 and 2. It rises to half normal levels by day 5 then rises sharply to almost twice normal levels by day 6. It then falls again to half normal levels by day 10 (Figure 11). There is possibly considerable oscillation around normal values for some time after this.

The concentration of CFU-C in the femur after HN_2 is very low on days 1 and 2 but rises sharply to three times normal levels by day 6, falling again to almost half normal levels by day 10 (Figure 12).

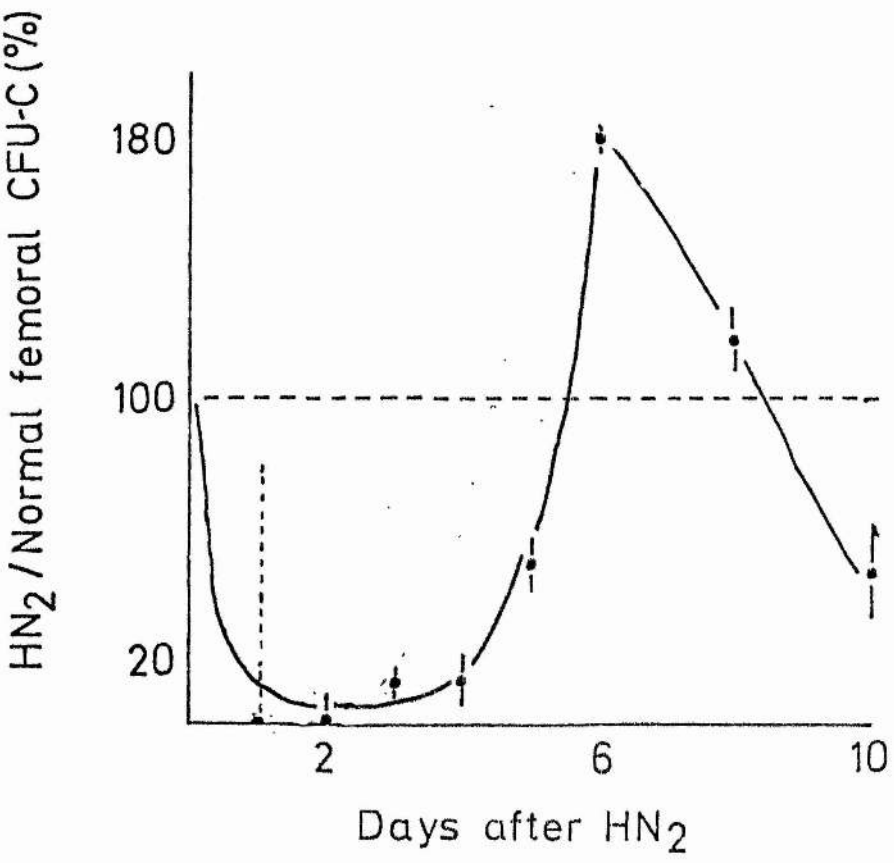
The massive errors on the early values (ie. days 1 and 2) are produced by the formula used to calculate errors of proportions,

ie. $$SE(r) = \left[\frac{SE(a)}{a}\right]^2 + \left[\frac{SE(b)}{b}\right]^2$$

where $r = {}^{a}/_{b}$

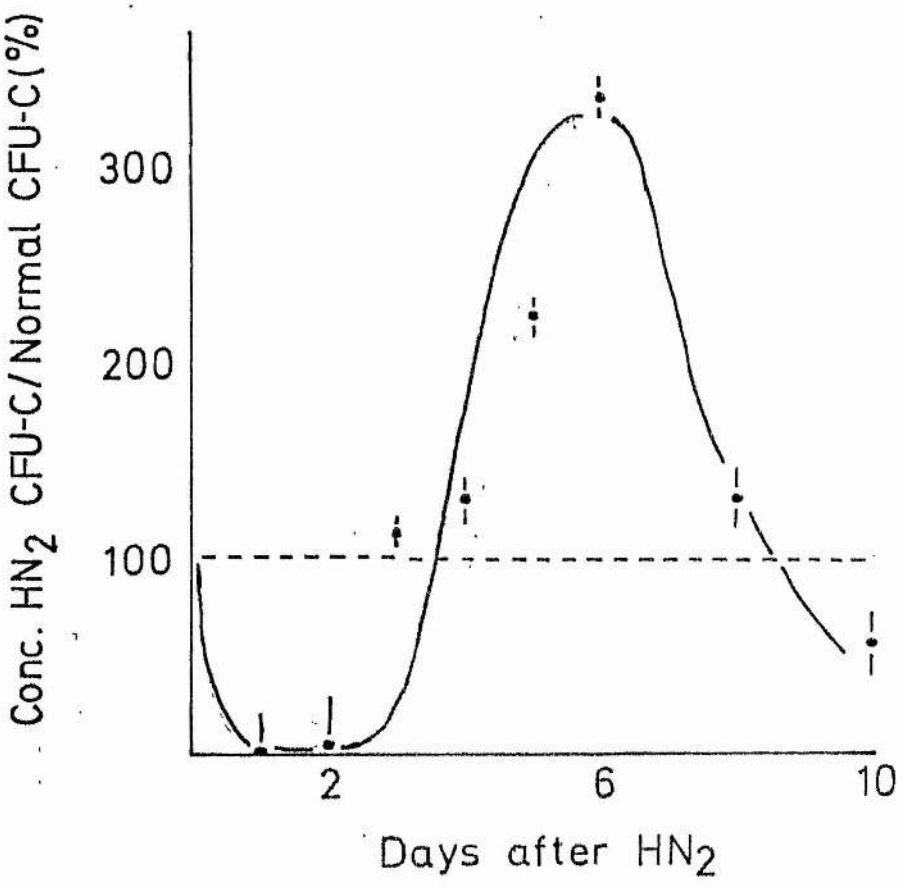
They should not be considered to have any biological implications.

Figure 11



The percentage of normal femoral CFU-C content of bone marrow on days following treatment with 100 μg HN_2

Figure 12



The concentration of CFU-C in femoral bone marrow following days after treatment with 100 μg HN_2 expressed as a percentage of normal femoral CFU-C concentration.

Table 5

Recovery of CFU-C after 100 μg HN_2

Days after HN_2	CFU-C/femur		% HN_2 CFU-C/Control CFU-C
	Control	HN_2	CFU-C (per femur)
1	32293.18 ± 480.0	103.10 ± 81.8	0.32 ± 78.0
2	14754.00 ± 1522	52.94 ± 11.10	1.88 ± 7.07
3	21766 ± 454	2931.96 ± 102.6	13.47 ± 3.74
4	21240 ± 586	3256.5 ± 289.8	15.33 ± 8.70
5	25860 ± 1134	14347.2 ± 818.6	55.48 ± 7.14
6	25928 ± 516	46680 ± 259	180.03 ± 1.81
8	20758.4 ± 1930	24825.6 ± 907	119.59 ± 9.64
10	18080 ± 2254	8416 ± 289	46.54 ± 12.6

Table 6

Concentration of CFU-C during recovery from 100 μg HN_2

Days after HN_2	CFU-C/10^5 femoral cells	
	Control	HN_2
1	144.16 ± 8.1	1.074 ± 0.2
2	73.77 ± 7.61	1.764 ± 0.37
3	108.83 ± 2.72	122.16 ± 5.13
4	88.50 ± 2.93	108.55 ± 9.66
5	107.75 ± 5.67	239.12 ± 13.81
6	92.6 ± 2.58	311.2 ± 25.9
8	99.80 ± 9.65	129.30 ± 9.07
10	90.4 ± 11.27	52.6 ± 2.89

	% Conc HN_2 / Control
1	0.74 ± 18.16
2	2.39 ± 22.30
3	112.24 ± 4.79
4	122.65 ± 8.94
5	221.92 ± 7.07
6	336.06 ± 8.71
8	129.55 ± 11.40
10	58.18 ± 13.41

3.8 Cell Production in Bone Marrow Regenerating after HN_2

Mice were given vincristine (2 mg/kg body weight) [Oncovin, vincristine sulphate, BP, Eli Lilly and Co Ltd, Basingstoke, England] intraperitoneally on days 1, 2, 4 and 6 after 100 μg HN_2. On these days mice were killed at 2, 4 and 6 hour intervals after receiving vincristine.

Femurs from 2 mice were pooled at each point. The contents of the femurs were suspended in Fischers medium with 20% horse serum. Smears were then prepared using a cytospin.

The means and errors of the binomial proportions were calculated at each point. Six thousand cells were counted for each value. A linear regression was fitted to the points using an iterative procedure based on the method of maximum likelihood. The goodness of fit of the linear regression is tested by the x^2 value. The slope of the line gives the production rate of the cells (Figure 13.

Metaphases/1000 femoral nucleated cells $\bar{x} \pm SE$

Days after HN_2 treatment	Hours after vincristine 2	4	6
1	0.83 ± 0.11	0.86 ± 0.11	0.71 ± 0.10
2	1.00 ± 0.40	6.60 ± 1.10	8.60 ± 1.10
4	60.00 ± 3.00	78.50 ± 3.40	136.00 ± 4.40
6	60.50 ± 3.00	114.00 ± 4.10	134.00 ± 4.40

Regression line $y = ax + b$

Day 1	$y = 9.24 - 3.02x$	$\chi^2_2 = 4.05$ $s^2 = 4.05$	1 df
2	$y = 2.08x - 3.07$	$\chi^2_2 = 1.82$ $s^2 = 1.82$	1 df
4	$y = 18.2x + 18.4$	$\chi^2_2 = 2.23$ $s^2 = 2.23$	1 df
6	$y = 19.3x + 25.1$	$\chi^2_2 = 1.21$ $s^2 = 1.21$	1 df

On days 1 and 2 the cell production is negligible. On days 4 and 6 the cell production rate is 18.2 and 19.3 cells per 1000 cells per hour. This would give a turnover time of 54.9 hrs on day 4 and 51.8 hrs on day 6 which is more or less constant.

It might be said, although that the age distribution of the cells in the regenerating marrow is exponential rather than rectangular. In this case it would be more appropriate to apply the collection function of Puck and Steffen (1963)

$$\log_2 [1 + M(t)] = \frac{t_m + t}{T_c}$$

where M(t) is the mitotic index, t_m the duration of mitosis, t the time of metaphase accumulation and T_c the cell cycle time.

However the regression of the binomial proportions on x as estimated by maximum likelihood methods, where $\log [1 + M(t)] = a + bx$ gives the following results.

Day

4 $\log [1 + M(t)] = 0.0290 + 0.0245x$

$a = 0.290 \pm 0.293$

$b = 0.0245 \pm 0.0075$

$\chi^2_1 = 19.61 \quad (p = 0.0595)$

6 $\log [1 + M(t)] = 0.0396 + 0.0253x$

$a = 0.0396 \pm 0.0259$

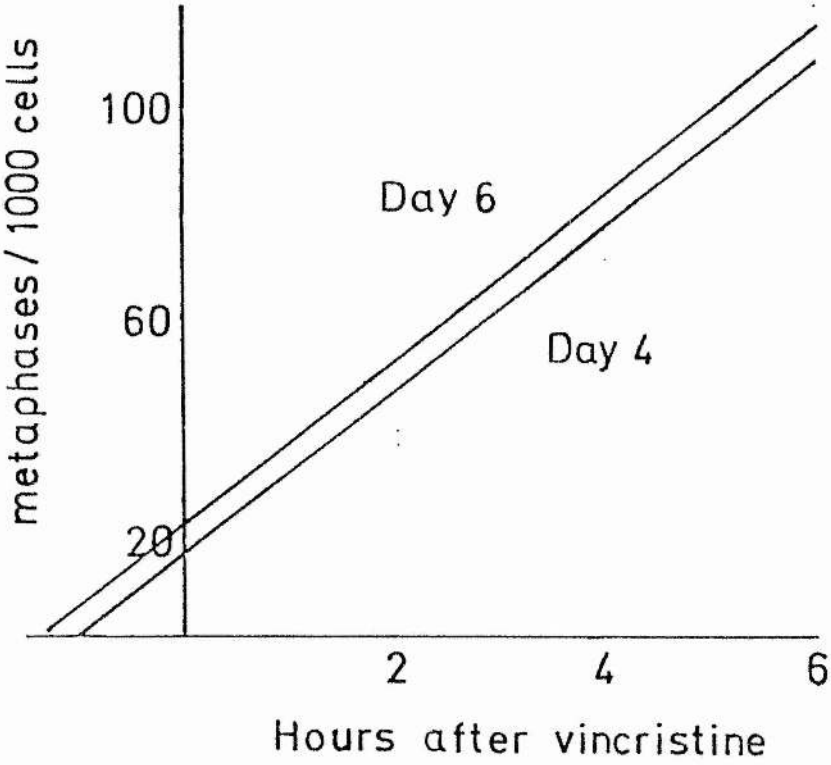
$b = 0.0253 \pm 0.0065$

$\chi^2_1 = 15.76 \quad (p = 0.00021)$

χ^2 is highly significant therefore the regression is a poor fit.

It would seem therefore that the regenerating marrow does not have an exponential age distribution.

Figure 13



Number of cells arrested in metaphase x 10^{-3} femoral bone marrow cells on days 4 and 6 following treatment with 100 μg HN_2

3.9 CFU-C in S-phase during bone marrow regeneration after treatment with HN_2

20 mice were given 100 µg HN_2 IV. On the 3rd and 6th day after treatment 5 mice were given 100 mg hydroxyurea which is specifically toxic to cells in S-phase. 40 minutes later the mice were killed along with 5 mice which had received 100 µg HN_2 3 or 6 days previously.

The number of nucleated cells in the femurs of each set of mice was measured. The number or CFU-C/2 x 10^5 bone marrow cells plated was recorded. The number of CFU-C in the femur after the described treatments was then calculated.

CFU-C per femur
$\bar{x} \pm SE$

	After 100 µg HN_2	After 100 µg HN_2 + 100 mg HU
Day 3	859.8 ± 105.6	182.4 ± 31.2
Day 6	18270.0 ± 65.1	16128.0 ± 140.4

Proportion of CFU-C in S-phase

Day 3	Day 6
78.8%	11.8%

In mice not treated with nitrogen mustard the CFU-C content before and after HU was as on the next page.

Femoral Cellularity

Normal	100 mg HU
2.8×10^7	1.6×10^7

CFU-C/2 x 10^5 femoral cells

Normal	HU
191.6 ± 8.45	96.00 ± 11.14

CFU-C/femur

Normal	HU
53648	24960

Proportion in S-phase 50.10%

3.10 Erythropoietic Activity in the Spleen and Femur after nitrogen mustard and bleeding

Erythropoiesis was measured in the spleen and femur by measuring the uptake of Fe^{59} in these tissues.

10 CDI female mice were used for each point. The mice were given 1 μCi Fe^{59} each and the percentage uptake calculated. A standard vial containing 0.01 μCi Fe^{59} was always counted along with vials containing the tissues. The activity in 1 μCi was then estimated from this and counts from the tissues expressed as a percentage of this activity after background counts had been subtracted.

In each of the categories, normal, day 1, 4 and 6 after nitrogen mustard mice were bled one day before the Fe^{59} uptake was measured.

Uptake Fe^{59} %

Days after HN_2	Unbled $\bar{x} \pm$ SE %		Bled $\bar{x} \pm$ SE %	
	Spleen	Femur	Spleen	Femur
No drug	2.96 $\pm$ 0.05	1.10 $\pm$ 0.10	4.71 $\pm$ 0.52	0.52 $\pm$ 0.05
1	0.78 $\pm$ 0.05	0.12 $\pm$ 0.005	0.89 $\pm$ 0.09	0.13 $\pm$ 0.011
4	2.009 $\pm$ 0.317	0.263 $\pm$ 0.068	1.979 $\pm$ 0.866	0.415 $\pm$ 0.119
6	1.367 $\pm$ 0.678	0.386 $\pm$ 0.137	2.643 $\pm$ 1.61	0.428 $\pm$ 0.145

With no drug the Fe^{59} uptake in the spleen and femur is significantly greater in mice which have had 0.3 ml of blood removed the previous day. However, in the spleen and marrow which are regenerating from HN_2 the difference in bled and unbled animals on days 1, 4 and 6 after the drug, is not statistically very significant, but shows a trend of higher uptake in the bled animals. On day 3 after 100ug. HN_2 the femoral CFU-C content is reduced by bleeding. This response of regenerating marrow to a requirement for erythroid production is reflected by a slightly raised uptake of Fe^{59}. (Table 8)

Table 8

	Haematocrit PVC(%)		Femur Cellularity $\times 10^6$		Spleen Weight (mg)	
	NB	B	NB	B	B	B
	$\bar{x} \pm$ SE					
Normal	45 ±0.45	41.6±0.98	18.6±0.05	21.9±0.13	109.69±3.73	161.74±13.33
					t sig p = 0.01	
DAY 3 HN_2	46.7±2.86	36.37±0.96	4.5±0.08	5.5±0.10	48.4 ±5.59	50.75± 6.16
DAY 4 HN_2	45.8±2.07	34.87±1.32	4.89±1.06	3.30±0.37	82.99±5.95	67.65± 5.24
DAY 6 HN_2	46.5±0.64	34.24±0.59	12.56±2.31	14.10±3.05	69.35±7.2	67.2 ± 4.9

NB = not bled

B bled

Nos CFUc on days after HN_2 may not be directly compared to each other since different conditioned media had to be used in each colony assay.

Femoral Fe^{59} uptake (% of 1 μCi)		Splenic Fe^{59} uptake (% of 1 μCi)		No. of CFUc x 2 x 10^{-5} cells	
NB	B	NB	B	NB	B
1.10±0.10	0.52±0.05	2.96±0.05	4.71±0.52	159.2± 9.48	151.25±36.9
t sig	p = 0.05	t sig	p = 0.05		
0.12+0.05	0.13+0.01	0.78+0.05	0.89+0.09	568.2+19.04	316.6+22.24
				t_8 = 8.61	
0.26±0.02	0.42±0.03	2.01±0.10	1.98±0.29		
0.38±0.04	0.42±0.04	1.36±0.22	2.64±0.50	235.8±30.51	242.6±17.00

3.11 Splenic CFU-C Content after 100 μg HN_2

Splenic cellularity was measured by estimating the number of cells per mg weight of spleen and multiplied by the corresponding weight of the spleen on the appropriate days after nitrogen mustard treatment.

The concentration of CFU-C in the spleen on these days was assayed. These concentrations were then converted to total splenic CFU-C content by multiplying by the corresponding spleen cellularity.

The values for splenic CFU-C content on days 1, 3 and 5 after 100μg HN_2 were compared to the normal splenic CFU-C content. On day 1 after HN_2 the splenic CFU-C are four per cent of normal, The spleen weight at this time is about 60% of normal. On day 3 when the spleen weight is at its lowest after HN_2 the CFU-C content is almost negligible. On day 5 when the spleen weight is increasing the CFU-C content has rebounded to half normal levels.

Bone marrow CFU-C reach this proportion 3 days after 100μg HN_2.

Spleen weights

Day after HN_2	Normal mg ($\bar{x} \pm$ SE)	After 100μg HN_2 mg
1	90.66 $\pm$ 3.69	42.83 $\pm$ 4.08
3	110.00 $\pm$ 4.32	50.00 $\pm$ 4.49
5	128.00 $\pm$ 5.31	40.50 $\pm$ 7.77
7	111.1 $\pm$ 6.40	39.0 $\pm$ 7.07

Spleen Cellularity

Day after HN_2	Normal	After 100μg HN_2
1	8×10^7	3.5×10^7
3	19.2×10^7	5.3×10^7
5	19.2×10^7	6.0×10^7
7	24.0×10^7	6.0×10^7

CFU-C/2 x 10^6 spleen cells

Day after HN_2	Normal	After 100μg HN_2
1	196.66 ± 10.81	2.0 ± 0.71
3	416.6 ± 5.56	1.2 ± 0.84
5	15.4 ± 2.54 (weak conditioned medium)	18.4 ± 1.36
7	Colony assays failed	

HN_2 spleen CFU-C content/normal spleen CFU-C content

Day after HN_2	
1	0.0433 ± 0.06
3	$0.8 \times 10^{-3} \pm 0.6$
5	0.44 ± 0.17

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No drug	2.96 $\pm$ 0.05	1.10 $\pm$ 0.10	4.71 $\pm$ 0.52	0.52 $\pm$ 0.05
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7	111.1 $\pm$ 6.40	39.0 $\pm$ 7.07

Spleen Cellularity

Day after HN_2	Normal	After 100μg HN_2
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3	19.2×10^7	5.3×10^7
5	19.2×10^7	6.0×10^7
7	24.0×10^7	6.0×10^7

CFU-C/2×10^6 spleen cells

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5	15.4 ± 2.54 (weak conditioned medium)	18.4 ± 1.36
7	Colony assays failed	

HN_2 spleen CFU-C content/normal spleen CFU-C content

Day after HN_2	
1	0.0432 ± 0.06
3	$0.8 \times 10^{-3} \pm 0.6$
5	0.44 ± 0.17

3.12 Spontaneous Colony Formation

Bone marrow of female CDI mice was cultured in agar without colony stimulating factor. The volume of CSF usually added was replaced by Fischers medium with serum.

One million cells from normal bone marrow and one million cells from marrow from mice which had received 100 g HN_2 three days earlier were seeded in each culture plate.

The total CFU-C content in the femur 3 days after HN_2 is 20% of normal but the concentration of CFU-C is 100% of normal.

Results

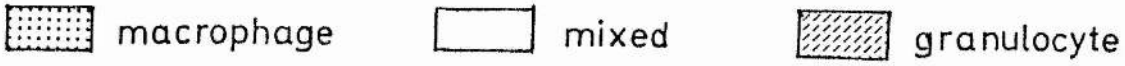
In plates containing normal marrow no colonies formed. In plates containing marrow regenerating from HN_2 the mean number of colonies $\bar{x} \pm SE = 9.4 \pm 1.60$. These colonies consisted entirely of the mixed or granulocyte type. Mixed colonies made up 36% of the with the conditioned medium which yielded the lowest number of colonies.

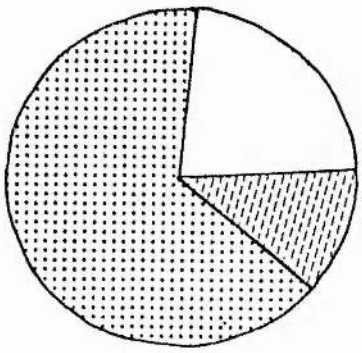
For all experiments an active conditioned medium was used ie. one which yielded 100-200 + colonies per 2×10^5 normal bone marrow cells. With such media macrophage colonies always formed the largest proportion, followed by mixed and then granulocyte. This ratio held true for nitrogen mustard treated marrow even when a very low number of colonies formed despite the presence of an active conditioned medium. The low number of colonies of course was due to depletion of CFU-C by the drug.

It is clear, however, that the ratio of the types of colonies formed depends on the nature of the stimulating factor and not the status of the bone marrow colony forming cells.

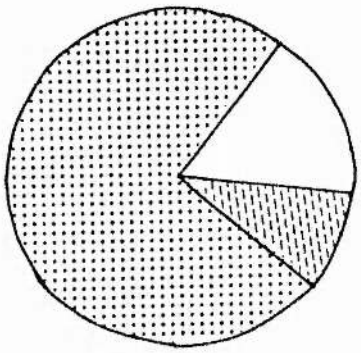
		Day 3 after HN_2	Control
%	Macrophage	72.63	63.14
Colony	Mixed	15.86	23.22
Types	Granulocytes	11.51	13.62

Figure 14 The proportions of colonies types in agar cultures obtained from normal marrow, and marrow 3 days after treatment with 100 μg HN_2 plated with an 'active' conditioned and the proportions of colony types from normal marrow plated with a weak conditioned medium and from marrow which formed 'spontaneous' colonies 3 days after treatment with 100 g HN_2.



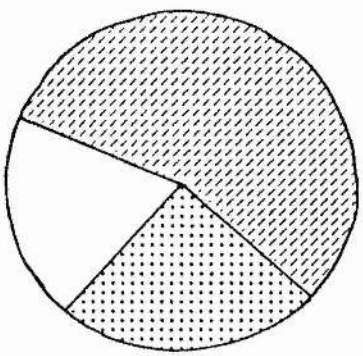


Normal marrow



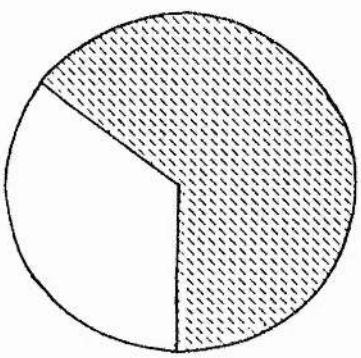
Day 3 HN_2

Active conditioned medium



Normal marrow

Weak conditioned medium



Day 3 HN_2

Spontaneous colony formation

Plate 1 Colonies in Agar.

A) Mixed colony

B) Granulocyte colony

C) Macrophage colony

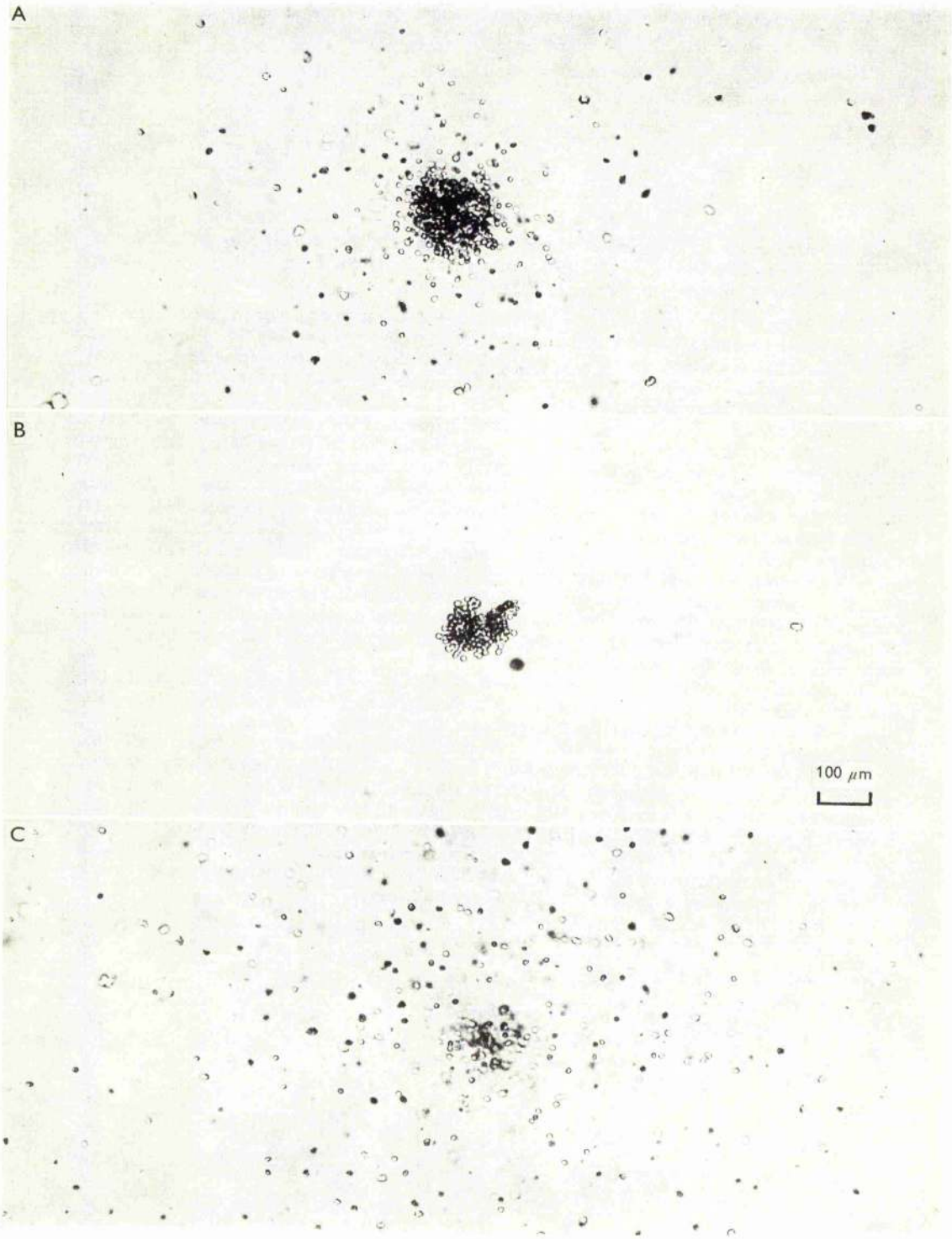


Fig. 2

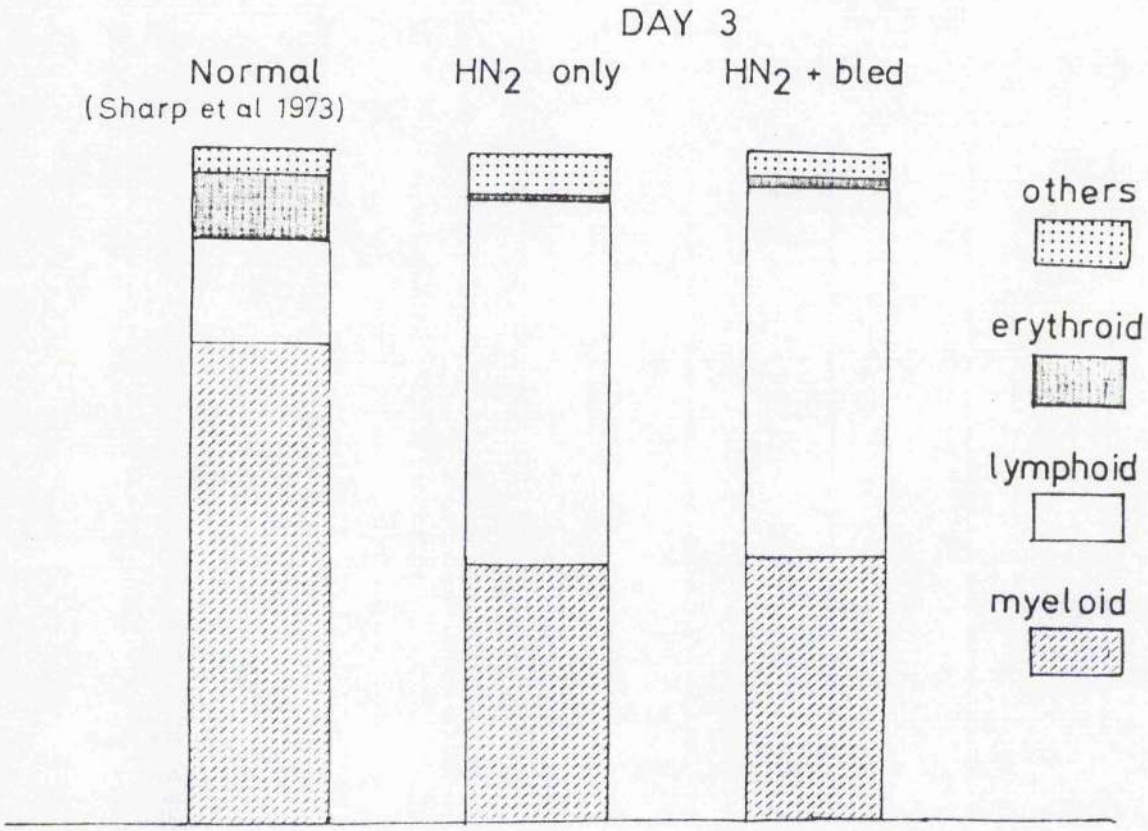
3.13 Differential counts of bone marrow cells 3 days after 100μg HN_2 and bleeding

Differential counts of 500 bone marrow cells were performed on mice which had received 100μg HN_2 intravenously 3 days previously and on mice which had received 100μg HN_2 three days previously and had 0.3 ml of blood removed from the retro-orbital sinus 1 day after treatment with nitrogen mustard. No significant difference is very apparent in any of the cell classes.

The transitional lymphocytes show a slight but not highly significant decrease. It may be suggested therefore that precursors of erythroid cells come from the transitional lymphocyte compartment. On day 3 after 100μg HN_2 the lymphocytes constitute the highest proportion of cells in the bone marrow. In normal marrow granulocytes make up the highest proportion of nucleated cells and lymphocytes the second highest proportion.

This is exaggerated in marrow 8 days after 100μg HN_2 [Sharp et al, 1973].

Figure 15



The distribution of cell types in normal marrow and marrow 3 days after 100μg HN_2 and marrow from mice which had been bled after treatment with 100μg HN_2

Table 9

Distribution of cell types in marrow recovering from nitrogen mustard (% of nucleated cells)

Day after 100 μg HN_2	Myeloid	Monocytes	Erythroid	Lymphocytes	Others
1	20.64	1.002	0.40	35.80	41.15
3	10.22	1.20	0.20	39.67	48.71
4	8.60	1.20	0.00	41.00	49.20
5	40.00	1.20	0.00	23.20	35.60

"Others" include damaged, unclassified, plasma cells, macrophages, maked nuclei and tissue cells.

Table 10

Distribution of cell types in marrow of mice bled 1 day after 100 μg HN_2

Day after 100 μg HN_2 (bled day 1)	Myeloid	Monocytes	Erythroid	Lymphocytes	Others
2	22.2	2.80	2.00	30.80	42.20
3	27.0	2.20	0.80	34.00	36.00
4	18.0	1.40	0.20	43.20	37.20
5	25.4	1.80	1.20	27.80	43.80

3.14 CFU-C in bone marrow of splenectomised and non-splenectomised mice

Five CDI female mice were splenectomised and five were sham-operated. A sham operation was carried out by cutting open the skin and muscle wall above the spleen, pulling out the spleen slightly and then replacing it. The muscle wall and skin were then closed in the same manner described for the operation of splenectomy. The mice were allowed to recover from the operation for three weeks.

The animals were then killed and the bone marrow femurs of the 5 mice pooled for each group. The bone-marrow was then assayed for CFU-C content.

The cellularity in both splenectomised and non-splenectomised for 2.6×10^7 cells per femur.

The results were as follows

Total number of CFU-C $\bar{x} \pm SE$	
Non-splenectomised	Splenectomised
124.4 ± 8.29	148.2 ± 3.31

$t = 1.86$ at 8 degrees of freedom

This is significant at the 5% level. This significant increase of CFU-C in splenectomised mice, although not great, may reflect a proliferative response of CFU-C to operative stress.

Femoral CFU-C content in splenectomised and sham splenectomised mice

Three CDI female mice were splenectomised and three CDI female mice were sham operated. These mice were given 100 μg HN_2 IV after seven days recovery from the operation.

On day 3 when the concentration of CFU-C in the femur is about 100% of normal the bone marrow was assayed for CFU-C's. The total femoral content on day 3 after nitrogen mustard is about 20%

of normal.

The cellularity in the femurs of splenectomised and sham splenectomised mice were the same. The femoral bone marrow from the 3 mice was pooled in each group.

Number of CFU-C/2 x 10^5 bone marrow cells
$\bar{x} \pm SE$

Sham Splenectomised	Splenectomised
51.6 ± 21.38	50.4 ± 6.42

The difference between CFU-C content in the bone marrow of splenectomised and sham splenectomised mice treated with HN_2 is not significant.

3.16 Effect of Bleeding on CFU-C content in splenectomised and non-splenectomised mice

Five female CDI mice were splenectomised and five were sham operated.

After seven days recovery from the operation 0.3 ml of blood was removed from the retro-orbital sinus of these mice. The following day the bone marrow was assayed for CFU-C content.

The femoral cellularity in both groups of mice was the same.

CFU-C
$\bar{x} \pm SE$ / 2 x 10^5 bone marrow cells

Sham operated and bled	Splenectomised and bled
58.6 ± 2.89	82.6 ± 2.71

These numbers are significantly different $p < 0.025$.

It would appear that the presence of the spleen modifies the bone marrow response to bleeding.

3.17 Effects of Splenectomy and Bleeding

Three groups of CDI female mice were given the following treatments.

10 mice were left intact

10 mice were sham-splenectomised

10 mice were splenectomised.

After a post-operative recovery period of seven days, 0.3 ml of blood was removed from the retro-orbital sinus of five mice from each group.

The packed cell volumes, femoral cellularities, blood reticulocytes and femoral CFU-C content of each subgroup of mice was measured.

Results

Femoral Cellularity

$\bar{x} \pm SE \times 10^6$

	Unbled	Bled
Intact	12.04 ± 0.92	15.15 ± 0.83
Sham-splenectomised	14.14 ± 1.67	11.82 ± 3.47
Splenectomised	14.97 ± 0.48	16.30 ± 1.44

Packed Cell Volume

$\bar{x} \pm SE$ %

	Unbled	Bled
Intact	45.0 ± 0.45	41.6 ± 0.98
Sham-splenectomised	44.4 ± 1.20	39.75 ± 0.85
Splenectomised	44.4 ± 2.14	39.8 ± 0.58

Reticulocyte Counts

$\bar{x} \pm SE \quad 10^{-3}$ cells

	Unbled	Bled
Intact	4.23 ± 1.53	12.37 ± 2.66
Sham-splénectomised	2.50 ± 0.27	7.80 ± 3.57
Splenectomised	9.07 ± 1.50	11.32 ± 0.76

CFU-C

$\bar{x} \pm SE \quad 2.10^{-5}$ bone marrow cells

	Unbled	Bled
Intact	159.2 ± 9.48	151.25 ± 36.93
Sham-splenectomised	120.8 ± 1.15	111.20 ± 4.74
Splenectomised	86.47 ± 19.25	118.56 ± 10.76

Splenectomy or sham-splenectomies did not affect the packed cell volume. In bled animals the packed cell volume, as expected, was decreased.

Splenectomy appeared to increase the blood reticulocyte counts in animals which had not been bled. Bleeding raised the reticulocyte counts in all three groups of animals.

Splenectomy and sham splenectomy depressed the number of femoral CFU-C in unbled mice. Bleeding only raised the number of femoral CFU-C in the splenectomised mice.

3.18 The effect of bleeding on CFU-C recovering from 100 μg HN_2

0.3 ml of blood was taken from mice which had received 100 μg HN_2 one day earlier. The mice were anesthetised with ether and the blood removed by inserting a haematocrit tube into the retro-orbital sinus

and allowing the blood to flow through the haematocrit into a graduated test tube.

Measurements were taken of the packed cell volumes, spleen weights, femoral cellularity and CFU-C on days 3 and 6 after HN_2.

Day after HN_2	Unbled	Bled 1 day after HN_2
	Packed Cell Volume	
1	48.48 ± 3.29	
3	46.70 ± 6.38	36.37 ± 1.92
6	46.50 ± 1.73	34.24 ± 1.67
	Spleen Weights	
3	48.40 ± 12.47	50.75 ± 12.32
6	69.35 ± 14.41	67.20 ± 9.80
	Femur Cellularity	
3	3.2×10^6	4.8×10^6
6	18.4×10^6	12.0×10^6
	CFU-C/2 x 10^5 bone marrow cells $\bar{x} \pm SD$	
3	568.2 ± 42.48	316.6 ± 49.60
6	235.8 ± 68.04	242.6 ± 37.92
	CFU-C/femur $\bar{x} \pm SD$	
3	9091.20 ± 679.68	7598.40 ± 1190.4
		$t_8 = 4.38$ sig $p < 0.01$
6	21694.0 ± 6259.7	14556.0 ± 2275.2
		$t_8 = 2.4$ sig $p < 0.05$

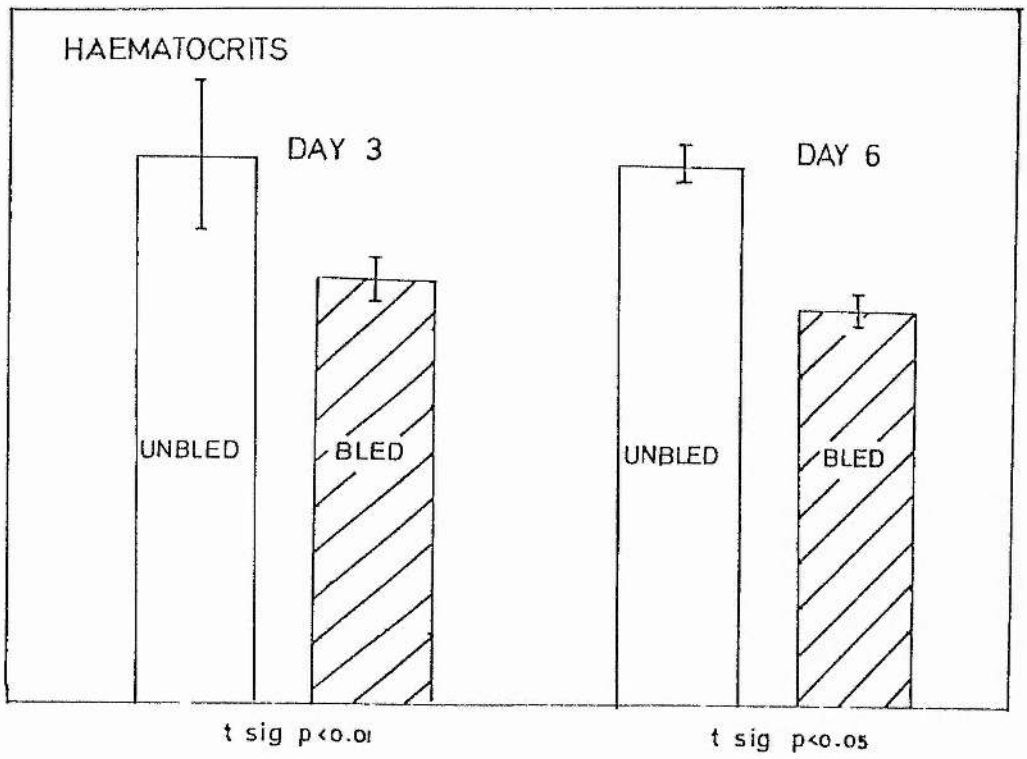
During the early days of recovery from HN_2 the packed cell volume was reduced significantly 2 days after bleeding and remained so for a further 2 days.

The CFU-C in the femur, although continuing to regenerate after depletion by HN_2 were reduced in number by bleeding 2 days previously and remained so 4 days after bleeding although the reduction was less significance.

The spleen weights and femoral cellularity was unaltered by bleeding.

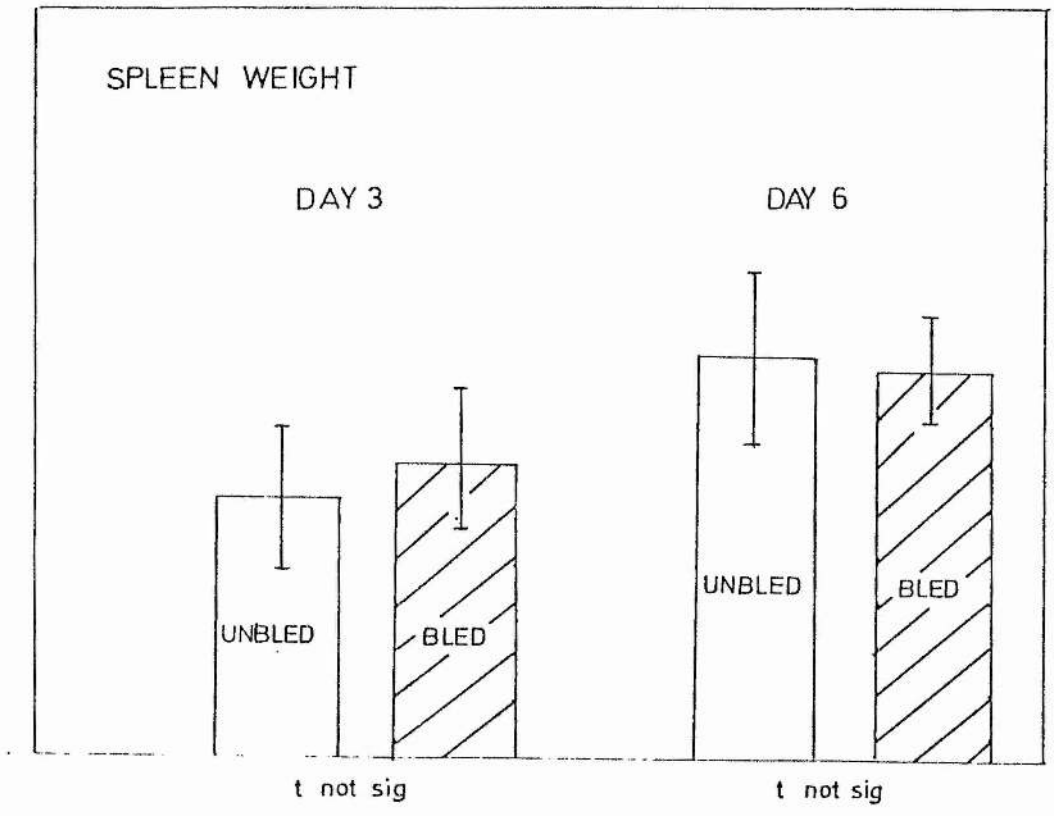
Since CFU-C numbers in normal mice are reduced by bleeding, marrow regenerating from HN_2 therefore appears to be capable of responding normally to a requirement for erythropoiesis.

Figure 16



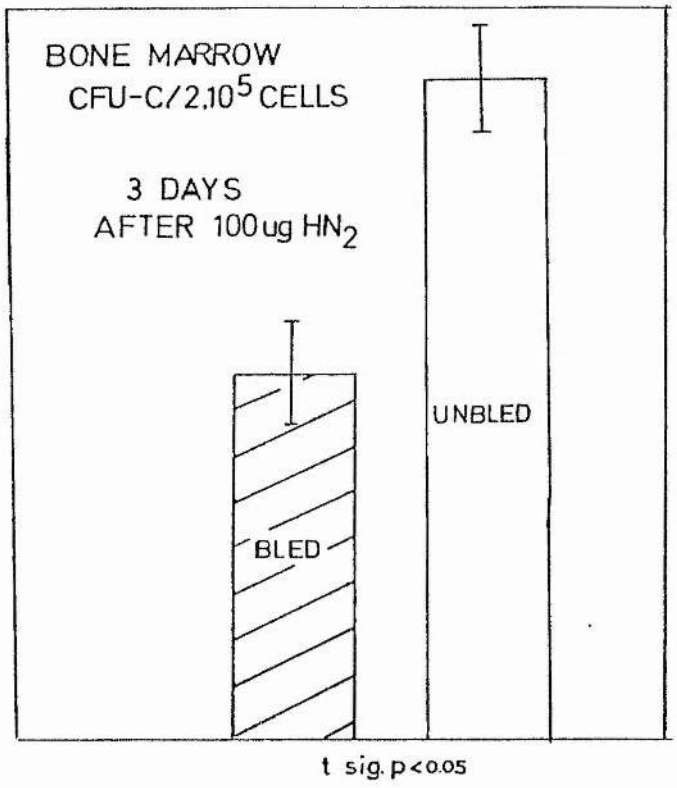
The effect of bleeding, on day 1 after treatment with 100 μg HN_2, on the haematocrits on days 3 and 6 following nitrogen mustard treatment.

Figure 17



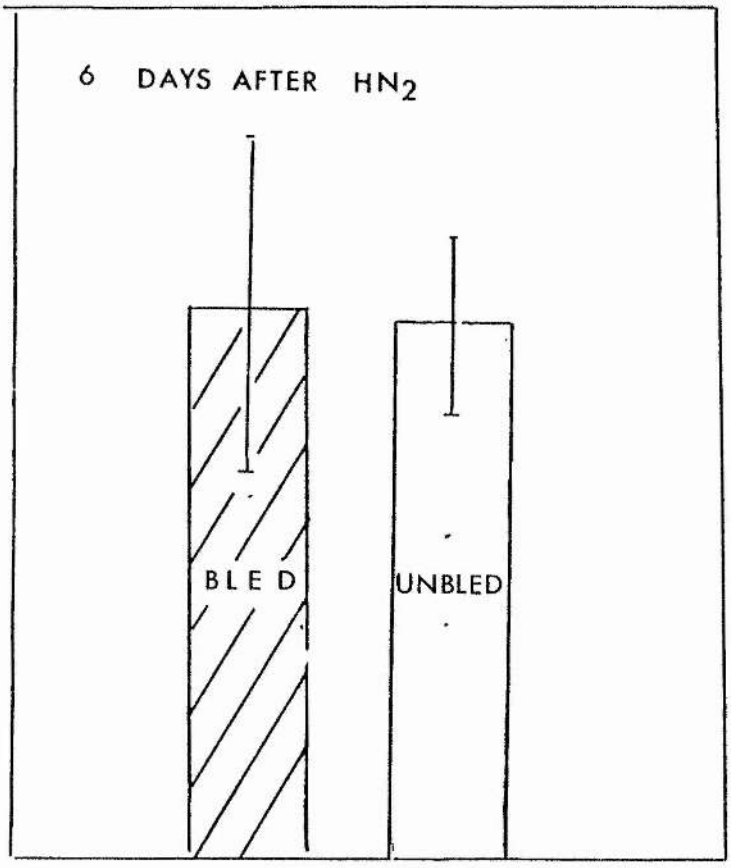
Spleen weights of mice on days 3 and 6 after 100 μg HN_2 compared with the spleen weights of mice which had been bled on day 1 following treatment with HN_2

Figure 18



CFU-C in the bone marrow in mice 3 days after treatment with 100 μg HN_2 compared with CFU-C in the bone marrow of mice bled one day after HN_2

Figure 19



CFU-C in the bone marrow in mice 6 days after treatment with 100 µg HN_2 compared with CFU-C in the bone marrow of mice bled one day after HN_2

3.19 Pathology of myeloid leukaemias of CBA/H mice

When the mice develop leukaemia this may be detected in life by a noticeable pallor of the feet and rapid loss of weight.

If blood is taken from the tail vein it shows a greatly increased white blood cell count, 10^3 to $10^5/mm^3$, and a greatly reduced packed cell volume 10-20%. Many abnormal and juvenile forms of myeloid cells are seen in smears prepared from the blood. Particularly distinctive are large forms of the ringed-nucleus metamyelocyte of the rodent. Also characteristic of murine myeloid leukaemia are elongated cells, possibly forms of the promyelocyte. At this stage the mouse may survive for a further 2-3 weeks.

On post-mortem examination there is usually splenomegaly. The size of the spleen may be increased 2 to 15 times. Enlargement of the spleen is most pronounced on the early passages of myeloid leukaemia. After 30 or so transplantations the splenic enlargement decreases and by the 50 to 60th passage the spleen is only twice its normal size. The colour and texture of the spleen varies from pale uniform pink to variegated and fairly lobulated with distinct white or cream areas. In some cases the spleen is a greenish brown colour.

The liver is sometimes enlarged but by no means in every case. In most cases on examination of histological sections of the liver infiltration can be seen with leukaemic cells situated around the portal vessels.

In some cases the kidneys are enlarged with leukaemic infiltration.

Occasionally the lymph nodes are involved and show a greenish colour. Enlargement of the lymph nodes however is usually confined to 2 or 3 times the normal size. In a few primary and transplanted

myeloid leukaemias the bone marrow is almost acellular, showing a fibrous-fatty change.

3.20 Pathology of lymphoid leukaemias in CBA/H mice

Lymphoid leukaemias may be detected in the same way as myeloid leukaemias. The white blood cell count is usually in the higher ranges 10^4-10^5/mm^3 and blood smears show large lymphoid type cells with pachychromatic chromatin.

Splenic enlargement is usually very considerable and the spleen may weigh between 1000-1500 mg. The enlarged spleen is usually dark red in colour.

Lymph nodes may be heavily involved, reaching 10 times their normal size. Lymph node involvement usually decreases with increasing passage of transplanted leukaemias.

As with myeloid leukaemias splenic enlargement in transplanted lymphoid leukaemias decreases when the 50-60th passage is reached.

Plate 2

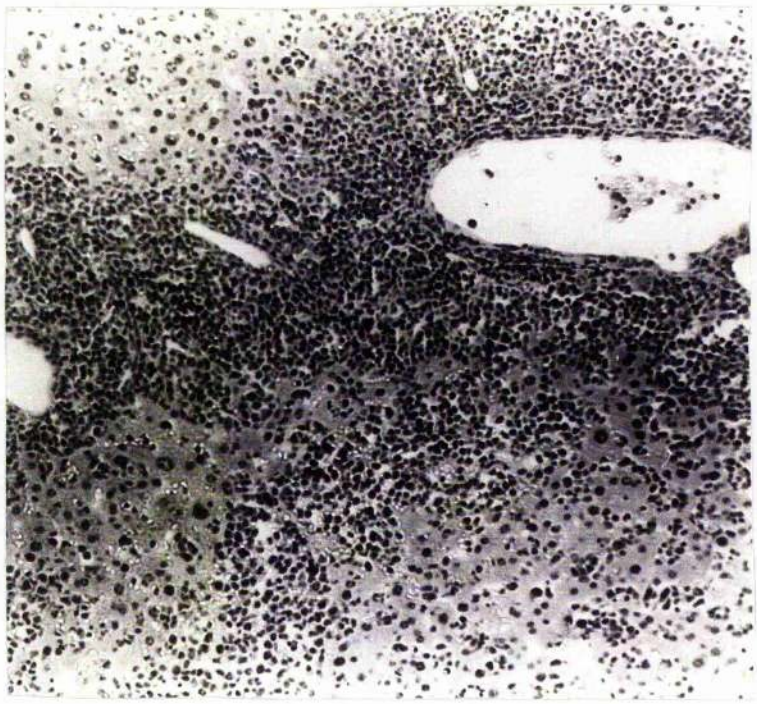
Myeloid leukaemic (primary)

infiltration in liver.

Plate 3

Lymphoid leukaemic (primary)

infiltration in liver.



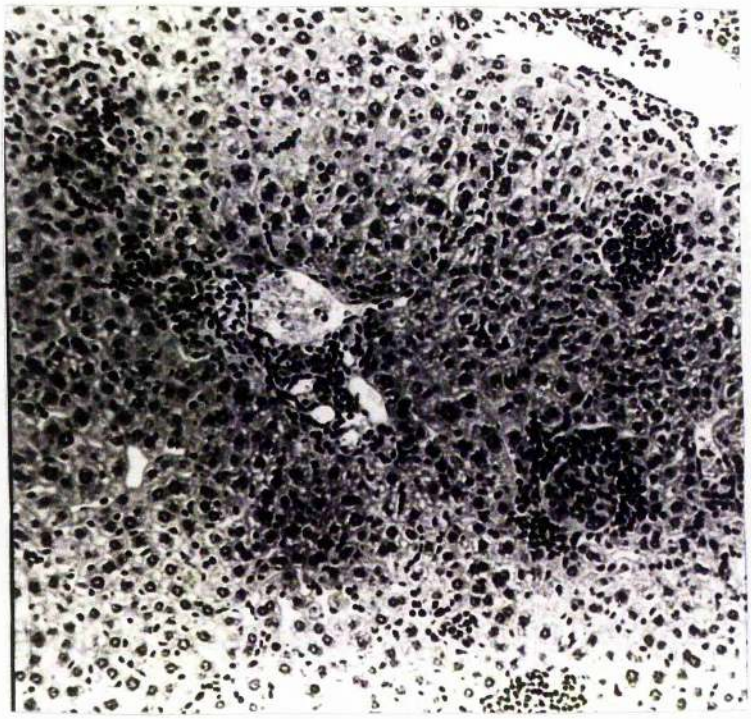


Plate 4

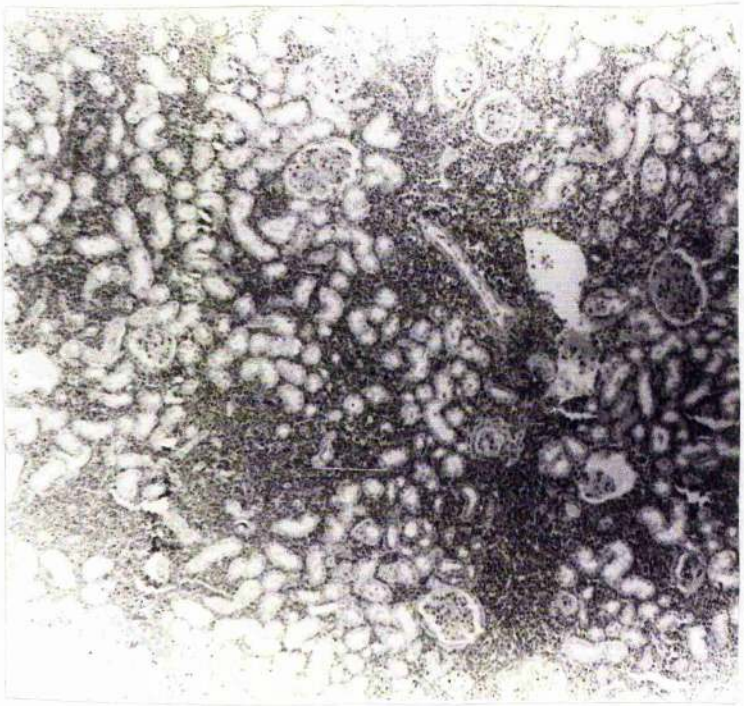
Myeloid leukaemic (primary)

infiltration in kidney.

Plate 5

Lymphoid leukaemic infiltration

in kidney.



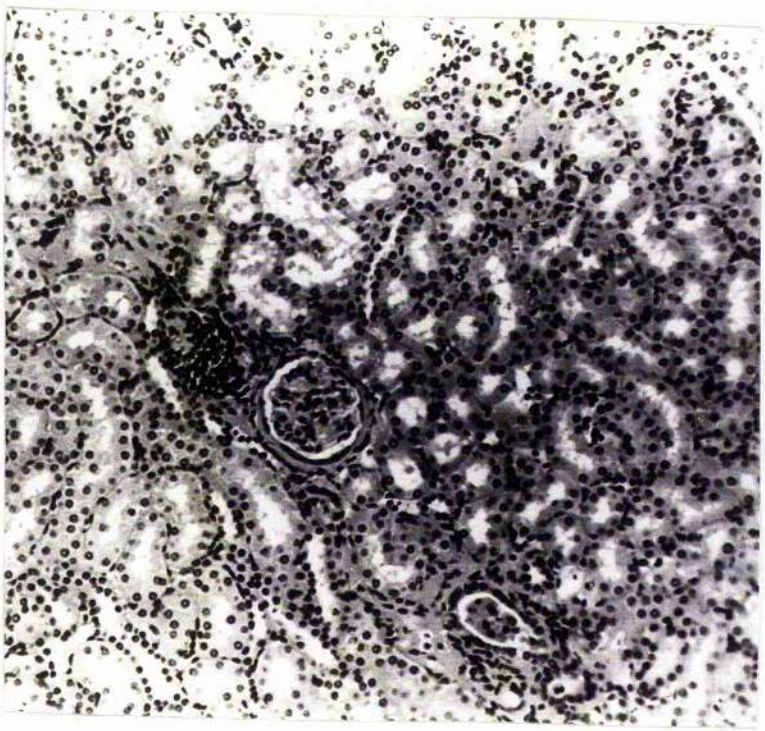
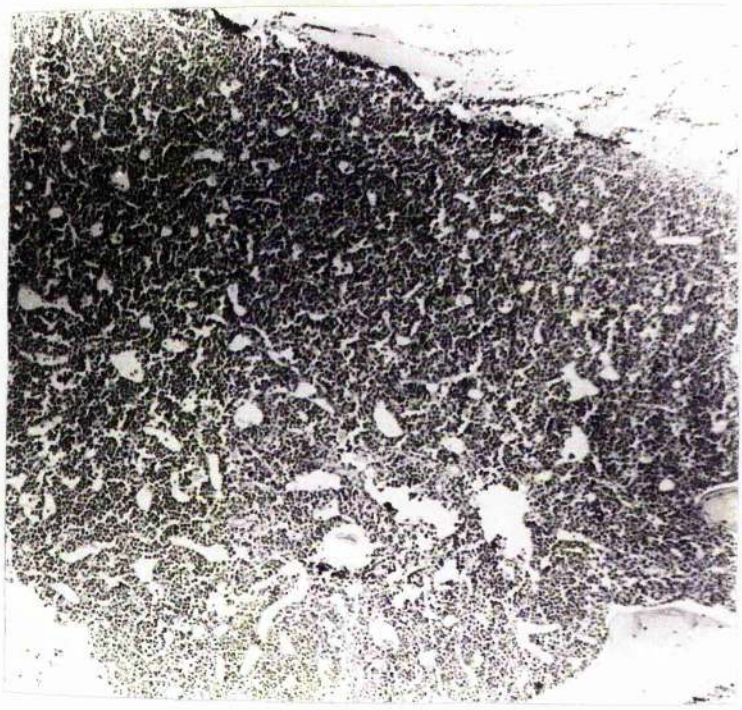


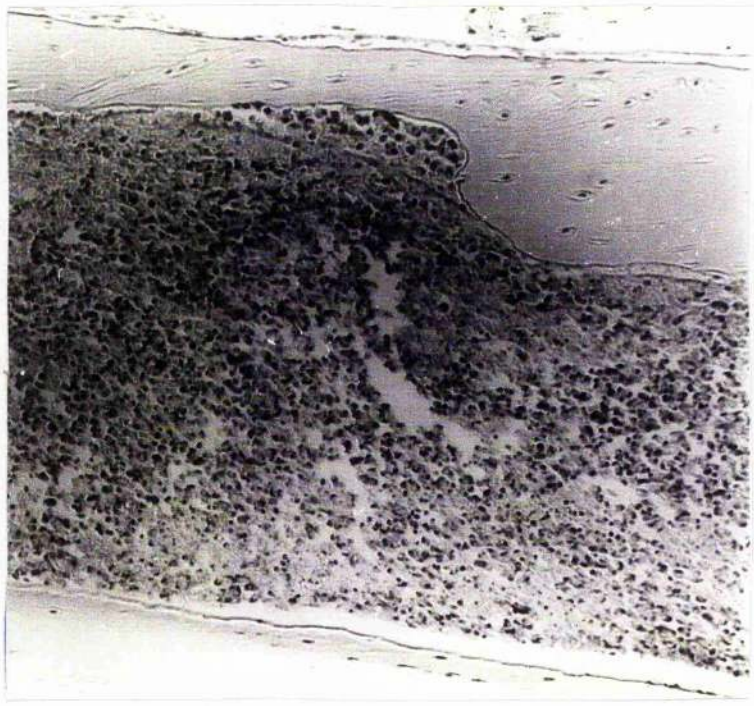
Plate 6

Femoral marrow from primary myeloid leukaemia.

Plate 7

Femoral marrow from primary myeloid leukaemia showing fibrous, fatty change.





3.21 Metaphase arrest of myeloid leukaemia cells by colcemid and vincristine

Colcemid is the most commonly used mitotic arrest agent in chromosome studies. In radiation induced murine myeloid leukaemia the mitotic arrest by colcemid had proved to be rather low.

Myeloid leukaemia can be induced regularly in CBA/H mice by single brief exposures of X-rays, rays and fission neutrons over a wide range of doses (Major and Mole, 1978; Mole and Davids, 1980; Major, 1979). The leukaemias are readily transplanted.

Smith et al (1974) reported vincristine sulphate to be a more useful metaphase arrest agent than colcemid, when studying metaphase accumulation in CBA mammary adenocarcinomas. Metaphase accumulation was said to be linear over 10 hours with vincristine but fell after 2 hours with colcemid. Metaphase arrest was independent of drug dose in the range 1-4 mg/kg body weight with both drugs. The effectiveness of vincristine and colcemid in arresting primary or passaged myeloid leukaemia cells was tested in vivo and in culture.

Results

Dose response curves of vincristine and colcemid on two different myeloid leukaemia lines were examined (Figures 20, 21).

Line A was about the sixtieth intraperitoneal passage of a myeloid leukaemia. Mice survived for about 15 days after an injection of 10^6 leukaemic cells.

Line B was about the sixteenth passage of a myeloid leukaemia where mice survived about 30 days after intraperitoneal injection of 10^6 leukaemic cells.

In the 'faster growing' leukaemia line A the percentage of cells arrested in metaphase by both drugs was very much greater than the percentage arrested in the 'slower growing' line B.

The difference in number of cells arrested by each drug is very much more pronounced in line A than in line B, vincristine arresting a very much greater proportion of cells in line A than colcemid.

With both lines A and B the dose response after colcemid is fairly flat, this also being true of vincristine with line B. In both lines the peak arrest by vincristine is at about a concentration of 0.05 μg/ml.

A dose at which peak arrest by colemid occurs is more difficult to establish, but peak arrest lies between 0.01 and 0.1 μg/ml in both lines studied. The dose at which both drugs show maximum arrest may therefore be taken to be 0.05 μg/ml. This dose may be said to be equivalent for each drug.

In line B the proportion of cells in metaphase before culture with the drug was 0.02 $\pm$ 0.02%.

Spleen cells from 3 primary leukaemias and 3 multipassaged leukaemias were cultured with 0.05 μg/ml of colcemid or vincristine. Table11 shows significantly higher arrest by vincristine in 2 of the 3 primary leukaemias although in leukaemia y the difference is not very significant.

Only one of the passaged leukaemias showed higher metaphase arrest by vincristine, although the difference is very highly significant.

Line A was 'faster growing' than the other two. Mice survived 15 to 20 days after intraperitoneal injection of 10^6 leukaemic spleen cells. With the other two lines B and C mice survived 30 to 40 days

after an injection of 10^6 leukaemic cells. These leukaemias would appear to be slower growing assuming it takes the same number of leukaemic cells and cell divisions to kill an animal.

The number of cells arrested 'in vivo' by the two drugs was examined. Mice were injected intraperitoneally with 3-4 mg/kg body weight of colcemid or vincristine. Table 13 shows that the proportion of cells arrested by vincristine is very much higher than the proportion arrested by colcemid in 2 out of the 3 multipassaged leukaemias. Leukaemias A and B are the same leukaemia lines as those tested 'in vitro'.

Three mice with primary myeloid leukaemia were given 2 mg/kg body weight of colcemid and three mice with primary myeloid leukaemia were given the same dose of vincristine. The mice were killed after 2 hours and the percentage of cells in metaphase assessed.

None of these leukaemias are of course directly comparable to each other.

An analysis of variance between the two groups ie. mice receiving colcemid and mice receiving vincristine was carried out.

A χ^2 test showed there was considerable heterogeneity within each group. This heterogeneity masks any difference between the two groups.

No strict conclusion can be drawn about the relative efficiency of vincristine to colcedmid in arresting cells in metaphase in primary murine leukaemias.

Very high doses of colcemid ie. 16 mg/kg body weight or very low doses ie. 0.25 mg/kg did not achieve higher metaphase arrest 'in vivo' than 3 mg/kg.

An assessment of the effect of dose of vincristine on metaphase arrest in primary myeloid leukaemias showed that a dose of 1-3 mg/kg gave the optimum mitotic arrest.

Table 11

Primary Leukaemia (in culture)	Percentage of cells arrested in metaphase $\bar{x} \pm SE$ Colcemid	Vincristine	χ^2
x	1.40 ± 0.17	1.57 ± 0.20	p = 0.49
y	0.78 ± 0.12	1.16 ± 0.15	p = 0.053
z	0.70 ± 0.12	1.15 ± 0.14	p = 0.015

χ^2 - 2 x 2 contingency test for homogeneity

Table 12

Passaged Leukaemia (in culture)	Percentage of cells arrested in metaphase $\bar{x} \pm SE$ Colcemid	Vincristine	χ^2
B	1.10 ± 0.16	1.40 ± 0.19	p = 0.23
C	1.20 ± 0.15	1.20 ± 0.15	p = 1.0
A	0.83 ± 0.17	5.55 ± 0.51	$p = 9.7 \times 10^{;24}$

Table 13

Passaged Leukaemia (in vivo)	Percentage of cells arrested in metaphse $\bar{x} \pm SE$ Colcemid	Vincristine	χ^2_1
B	0.95 ± 0.22	1.06 ± 0.18	p = 0.69
D	0.73 ± 0.15	2.03 ± 0.25	$p = 1.6 \times 10^{-5}$
A	0.97 ± 0.16	4.00 ± 0.31	$p = 3.7 \times 10^{-19}$

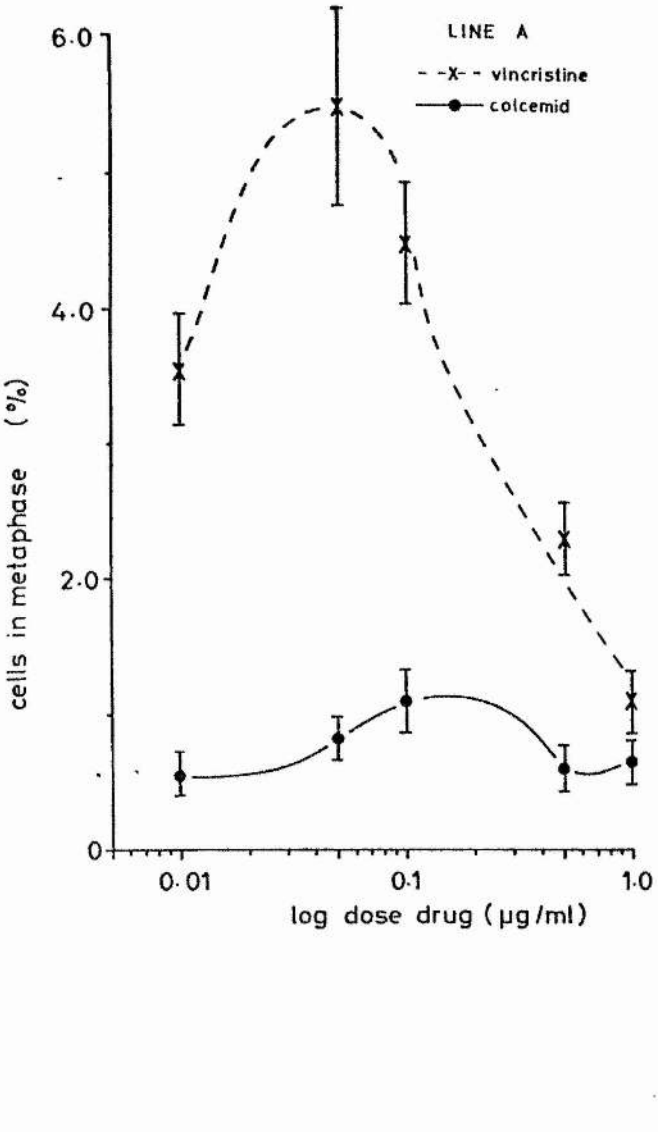
Table 14	Primary Leukaemia	Percentage of cells arrested in metaphase $\bar{x} \pm SE$	
	r	2.25 ± 0.17	χ^2_2
Colcemid	s	0.30 ± 0.12	$p = 1.6 \times 10^{-9}$
	t	1.15 ± 0.24	
	d	3.06 ± 0.31	χ^2_2
Vincristine	e	2.35 ± 0.33	p = 0.00048
	f	1.35 ± 0.25	

$\chi^4 = 55.74$ $p = 2.3 \times 10^{-11}$

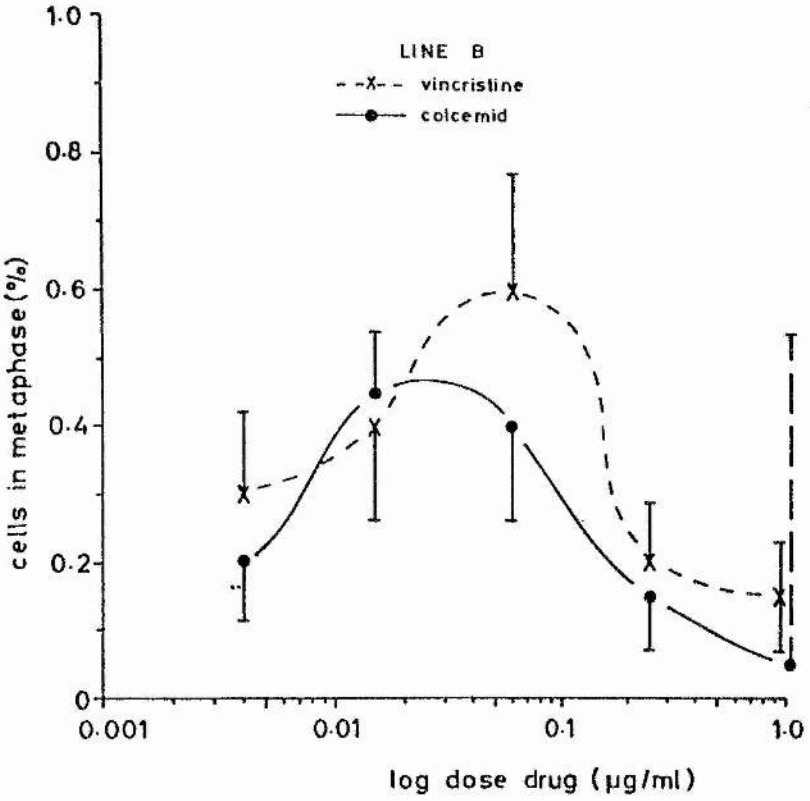
difference between groups $F(1,4) = 0.650$ $p = 0.47$

Dose responses 'in vitro'

	% of cells arrested in metaphase	$\bar{x} \pm SE$	
Leukaemia line Z	Dose (µg/ml)	Colcemid	Vincristine
	0.01	0.55 ± 0.16	3.55 ± 0.41
	0.05	0.83 ± 0.16	5.55 ± 0.72
	0.1	1.10 ± 0.23	4.5 ± 0.45
	0.5	0.60 ± 0.17	2.3 ± 0.27
	1.0	0.65 ± 0.17	1.10 ± 0.23
Leukaemia line X	0.004	0.2 ± 0.09	0.3 ± 0.12
	0.015	0.45 ± 0.14	0.4 ± 0.14
	0.06	0.4 ± 0.14	0.6 ± 0.17
	0.25	0.15 ± 0.08	0.2 ± 0.09
	1.00	0.05 ± 0.49	0.15 ± 0.08



Dose-response curves of vincristine and colcemid on leukaemia line A



Dose response curves of vincristine and colcemid on leukaemia line B

3.22 Cells entering metaphase in murine myeloid leukaemia

Since administration of vincristine to mice in the terminal stages of leukaemia often caused early death of the animal low doses of the drug were given to try to extend the time of metaphase arrest.

Tables 15 and 16 show the number of leukaemic cells in metaphase per 1000 cells after the given dose and time in primary and passed myeloid leukaemias. It was assessed, however, that a dose of at least 20 μg was required to give maximum metaphase accumulation.

Although the results are very variable it appears that a maximum number of cells entering metaphase in 1 hour is about 2.10^{-2} assuming that metaphase accumulation is linear. This production rate of about 20 cells per thousand per hour is very similar to that in marrow regenerating from nitrogen mustard.

The proportions of cells in metaphase in the spleen and marrow were compared by a two-way analysis of variance using a logit transformation of the binomial proportions.

In the primary myeloids the proportions of cells in metaphase are lower, on the whole, for bone marrow than for the corresponding spleen, but the differences are by no means consistent in magnitude or direction [$x^2_{20} = 446$; P very small]. When this interaction is taken into account, the difference between bone marrow and spleen is not statistically significant [$t_{20} = 1.39$; $P = 0.18$]. Figure 22 shows a scatter diagram of the proportions of cells in metaphase in the primary myeloid leukaemic spleens and bone marrow.

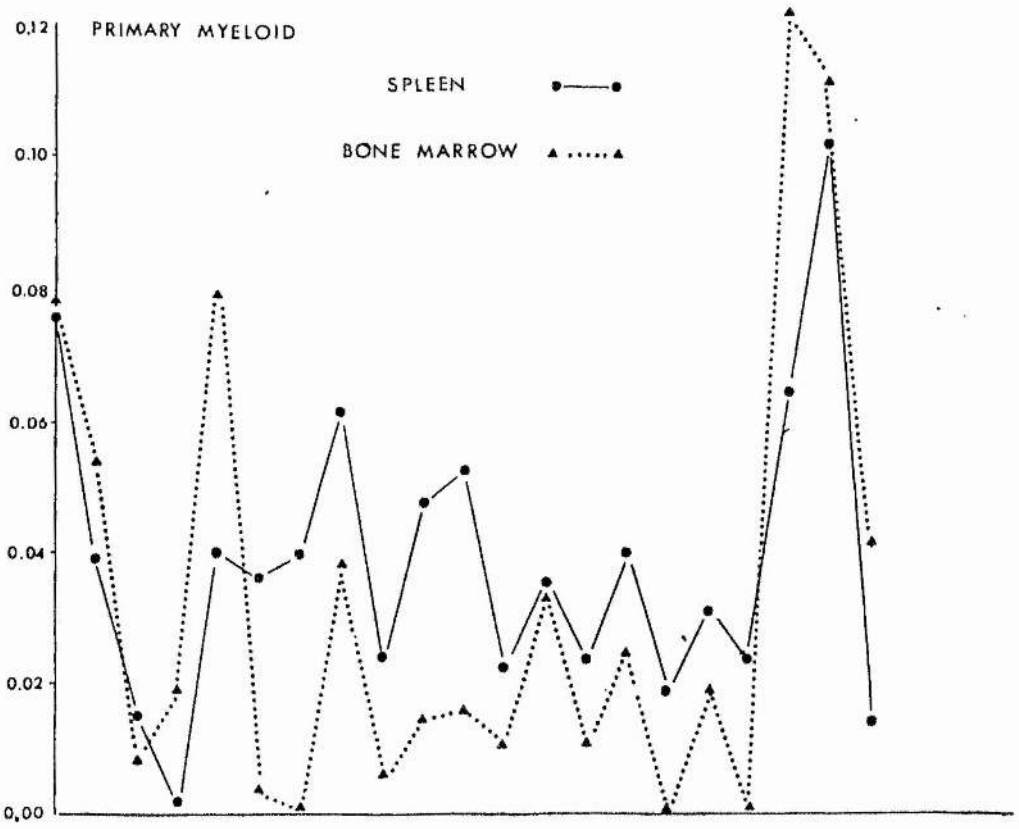


Figure 22

Scatter diagram of percentage of cells arrested in metaphase in the bone marrow and spleen of primary myeloid leukaemias

In passaged myeloids the proportions of cells in metaphase are lower, on the whole, in the bone marrow than for the corresponding spleen, but the differences are not consistent in magnitude or direction [$x^2_{10} = 128$; P very small]. In spite of this significant interaction, however, the overall difference between bone marrow and spleen is statistically significant [$t_{10} = 2.21$; $P = 0.051$].

Figure 23 shows a scatter diagram of proportions of cells in metaphase in passaged myeloid leukaemias.

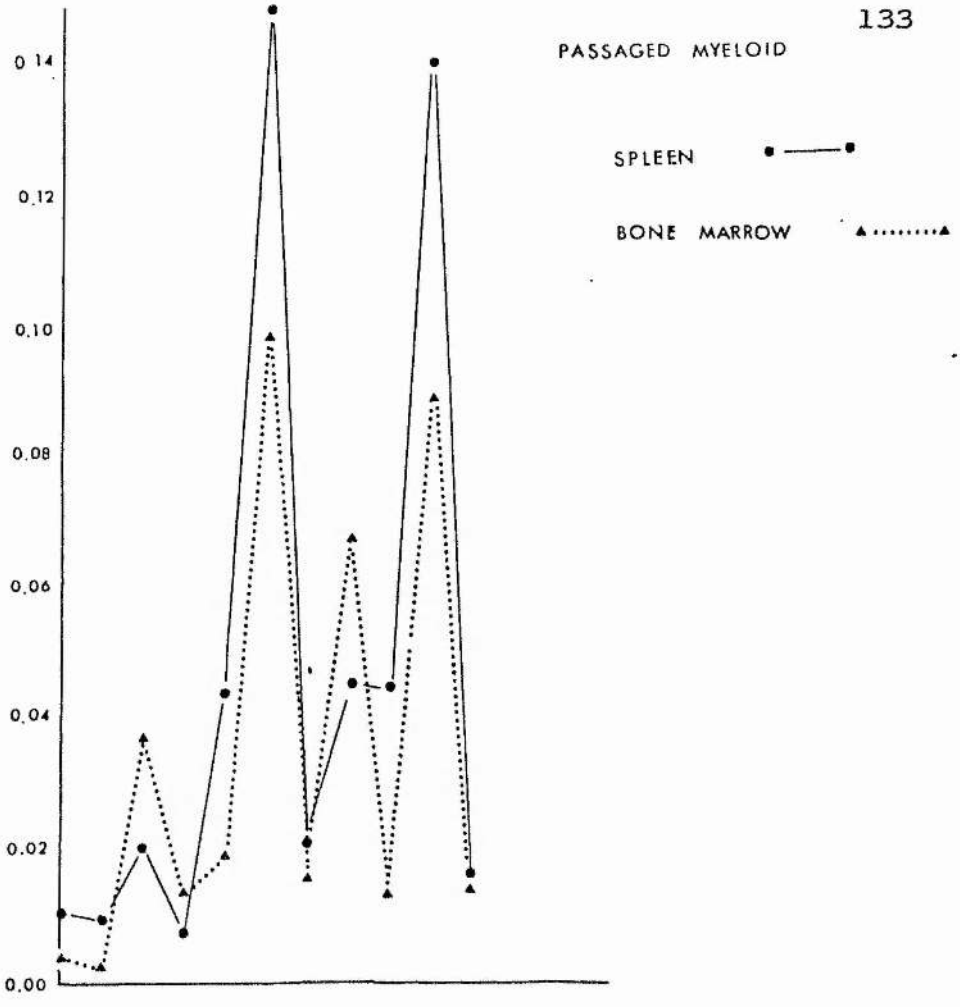


Figure 23

Scatter diagram of percentage of cells arrested in metaphase in the bone marrow and spleen of passaged leukaemias

Leukaemia cells arrested in metaphase in the tissues of CBA/H male mice with radation-induced myeloid leukaemia.

Table 15

Primary Myeloids

Mouse	Dose Vincristine (μg)	Time (hrs)	Spleen	Liver	Bone Marrow	Lymph Node	Heart Blood
SPR 5.4	0	-	18.53 ± 3.02	5.00 ± 1.50	0	-	
DFN10.2	1	3 (killed)	23.32 ± 2.70	11.33 ± 1.94	5.30 ± 1.31	1.00 ± 0.90	-
DRY16.2	1 3 1=2	6½(killed)	39.62 ± 3.54	33.00 ± 3.20	0	16.64 ± 2.33	0
DFN16.3	1 3 1=2	7 (killed)	22.02 ± 2.64	14.62 ± 2.13	9.00 ± 1.73	10.00 ± 1.80	-
LDR 8.6	1 3 1 3 1=3	9½(killed)	39.64 ± 3.52	30.01 ± 3.14	24.00 ± 2.73	10.00 ± 0.90	-
LDR13.3	5	4½ (dead)	39.63 ± 3.52	53.34 ± 4.10	79.05 ± 8.52	-	0.52 ± 0.43
SPR14.4	5	3 (killed)	61.03 ± 5.32	17.01 ± 2.83	37.62 ± 3.43	-	0
DRY 7.2	5	3 (killed)	47.02 ± 3.83	18.63 ± 2.44	14.03 ± 2.12	7.34 ± 1.56	2.50 ± 1.17
DFN 2.2	5	5½(killed)	55.02 ± 3.63	22.24 ± 2.36	15.33 ± 2.24	4.64 ± 1.23	6.00 ± 2.40
SPR11.6	2 3 1 3 2=6	9½(killed)	35.00 ± 3.31	13.06 ± 2.50	32.05 ± 3.93	11.00 ± 1.90	1.51 ± 0.84
DRY 1.4	2½ 3½ 2½ 2¾ 2½=7½	6¾(killed)	23.21 ± 2.34	18.00 ± 2.94	10.00 ± 1.86	23.04 ± 3.32	1.04 ± 0.50
SPR 1.3	10	5 (dead)	1.54 ± 0.83	2.06 ± 1.43	19.04 ± 4.33	-	9.02 ± 2.15
DEN20.5	10	6½ (dead)	35.52 ± 4.10	37.03 ± 4.24	3.00 ± 1.74	-	4.04 ± 1.41
DRY18.5	15	4 (dead)	14.53 ± 2.62	9.00 ± 2.90	7.53 ± 1.94	-	4.53 ± 1.44
DRY 4.2	20	4½ (dead)	38.51 ± 4.32	47.56 ± 4.73	53.51 ± 5.25	-	-
DFN13.5	40	5½(killed)	64.00 ± 5.42	12.00 ± 1.00	117.02 ± 7.15	23.01 ± 3.32	9.00 ± 2.10
SPR11.5	40	4½ (dead)	101.50 ± 6.73	91.00 ± 6.41	111.13 ± 7.00	2.00 ± 1.43	7.05 ± 2.63
DFN15.2	80	3 (dead)	30.62 ± 3.12	25.34 ± 2.83	18.32 ± 2.42	-	0
LDR10.1	80	2¾ (dead)	23.52 ± 3.33	21.61 ± 2.64	u/s	75.00 ± 1.93	0
MST 4.3	90	2	13.52 ± 2.54	48.50 ± 4.81	40.53 ± 4.42	-	-

Table 16

Passaged Myeloid Leukaemia

Line	Dose Vincristine (μg)	Time p (hrs)	Spleen	Liver	Bone Marrow	Lymph Node	Heart Blood
XVI p30	2	$2\frac{1}{4}$ (dead)	21.00 ± 2.63	20.32 ± 2.56	15.53 ± 2.72	-	0.50 ± 0.40
XVIII p21	2	$6\frac{1}{2}$	45.00 ± 3.73	23.62 ± 2.72	13.34 ± 2.02	-	-
XV p27	2.5 x 3=7.5	8	44.00 ± 4.53	20.02 ± 3.14	19.00 ± 2.44	-	-
XVI p38	40	5	45.50 ± 4.62	26.63 ± 2.91	67.52 ± 5.64	-	0
XVIII p26	80	6	141.00 ± 7.70	80.03 ± 6.02	89.34 ± 5.21	-	0
XV p31		8	148.32 6.41	70.02 4.64	97.51 6.63	10.80 5.62	0

3.23 Colony formation in Irradiation Induced Murine Leukaemias

A range of doses of normal or leukaemic spleen or bone marrow cells were injected intravenously into lethally irradiated CBA/H male mice of about 100 days old. If stocks were low female CBA mice of the same age were used. Assays carried out with normal and leukaemic cells showed that the same numbers of colonies formed in male and female mice corresponding to the cell dose injected.

The LD_{50} for male CBA/H mice is about 900 rads at which it was found no endogenous colonies formed. The LD_{59} for female mice is about 850 rad. No endogenous colonies form in the spleens of female mice at this dose.

Single cell suspensions were made in Eagles medium. Four or five mice were injected with each cell dose. Ten days later the mice were killed, the spleens exised and fixed in Bouin's fluid. After 24 hours fixation the colonies were counted.

Results

CFU-S formed from normal CBA/H male mouse bone marrow in male and female CBA/H mice

	No. of colonies $\bar{x} \pm SE$				
No. of cells injected	5×10^3	10^4	5×10^4	10^5	5×10^5
male mice	2.00 ± 1.75	2.66 ± 1.00	7.33 ± 0.50	9.33 ± 1.33	confl.
female mice	2.33 ± 1.81	2.14 ± 1.13	5.36 ± 0.70	10.52 ± 1.14	confl.

CFU-S formed from a primary myeloid leukaemia spleen cells in male and female CBA/H mice

	No. of colonies $\bar{x} \pm SE$		
No. of cells Injected	10^5	5×10^5	10^6
male mice	12.75 ± 1.78	19.50 ± 0.74	confl. sp+
female	13.50 ± 1.20	19.00 ± 1.24	confl. sp+

Normal CFU-S in CBA/H mice

	No. of colonies $\bar{x} \pm SE$					
No. of cells injected	5×10^3	10^4	5×10^4	10^5	5×10^5	10^6
bone marrow	2.00 ± 1.75	2.66 ± 1.00	7.33 ± 0.50	9.33 ± 1.33	confl.	
spleen	0	0	0	0.75 ± 0.54	-	7.00 ± 3.56

Normal CBA/H bone marrow contains over 10 times as many CFU-S as normal CBA/H spleen, assuming the seeding efficiency is about the same.

Primary myeloid leukaemia

	No. of colonies formed				
No. of cells injected	10^3	10^4	10^5	5×10^5	10^6
Leukaemia					
2.1			12.75 ± 1.78	19.00 ± 0.74	confluent sp+
3.1	0	0	0.25 ± 0.28	-	9.50 ± 1.45
6.1	0.50 ± 0.57	1.00 ± 0.00	0.75 ± 0.28	-	13.50 ± 2.02
11.5	0	0	0.60 ± 0.27	-	9.00 ± 1.50

In three out of four primary myeloid leukaemias colony numbers formed corresponded to the numbers formed by normal spleen cells at the higher cell doses. However, in the fourth leukaemia (ie. 2.1) colony numbers were at least 17 times greater than those formed by normal spleen cells. In leukaemia 6.1 the relationship between colony number and cell dose is exponential. This may appear to be true of normal spleen cells although only two doses are available from this study.

Passaged myeloid leukaemias

Passaged myeloid leukaemias invariably did give rise to surface spleen colonies.

Line XIX	passage 50	No. of colonies		
No. of cells injected	10^3	10^4	10^5	10^6
spleen	0	18.33 ±	confluent sp+2	confluent sp+3
bone marrow	5.5 ±	22.75 ±	confluent sp+4	confluent sp+4

Line XIX	passage 67	No. of colonies				
No. of cells injected	10^3	5×10^3	10^4	5×10^4	10^5	5×10^5
bone marrow	0	0.25 ± 0.28	2.00 ± 0.28	2.50 ± 0.90	19.50 ± 1.52	confl. sp+2

Again the relationship between colony number and cell dose is not linear but of an exponential nature indicating a complex of influences on leukaemic cell colony formation.

In histological section the colonies from line XIX, appeared to be of purely myeloid leukaemic cells (plate 10)

Line XXVII passage 7

No. of spleen cells injected	No. of colonies					
	10^3	10^4	2×10^4	10^5	2×10^5	10^6
	0	0	0.75 ± 0.54	1.40 ± 0.70	4.75 ± 0.98	15.40 ± 7.70

Colony number is again exponentially related to cell dose although colony numbers are lower than those formed by line XIX.

Line XXX passage 10

No. of spleen cells injected	No. of colonies			
	10^3	10^4	10^5	10^6
	0.40 ± 0.44	1.00 ± 0.46	3.00 ± 1.00	14.75 ± 1.72

This line also shows an exponential relationship between colony numbers and cell dose.

Line XXXIII passage 5

No. of spleen cells injected	No. of colonies			
	10^3	10^4	10^5	10^6
	0	0	confl. sp+2	confl. sp+2

In the cell dose range examined colony numbers could not be assessed because of the extreme exponential nature of the relation of colony formation to cell dose. Splenic enlargement also obscured colony formation at the high cell doses indicating a rapid spread proliferation of the leukaemic cells throughout the splenic pulp.

Colony formation in lymphoid leukaemia

Lymphoid leukaemia on the whole did not give rise to colonies in the spleens of irradiated mice.

Two primary leukaemias gave the following results.

Primary lymphoid leukaemia 2.6

	No. of spleen cells injected			
	10^3	10^4	10^5	10^6
No. of colonies ($\bar{x} \pm SE$)	0	0	0.5 ± 0.57	3.75 ± 0.98
Splenic enlargement	-	-	-	+

Ten days after injection of leukaemic cells at a dose of 10^6 cells the spleen was double the size of spleens in lethally irradiated mice which had received no cells.

In histological section the visible surface colonies which had formed appeared to be of normal haemopoietic cells as in plate 8

Primary lymphoid leukaemia 1.3

	No. of spleen cells injected	
	10^4	10^5
No. of colonies ($\bar{x} \pm SE$)	0	0
Splenic enlargement	+	+

In histological section this leukaemia showed small foci of cell proliferation in the periarteriolar region. Mitotic figures can be seen in this region (plate 13)

Multipassaged lymphoid leukaemias almost invariably did not produce spleen colonies which were macroscopically visible.

Lymphoid leukaemia line XX

		No. of spleen cells injected			
	Passage	10^3	10^4	10^5	10^6
No. colonies ($\bar{x} \pm SE$)	30	0	0	0	0
	40	0	0	0.25 ± 0.28	1.00 ± 0.66
Splenic Enlargement	30	-	0	+ 2	+ 7
	40	-	0	+	+

Lymphoid leukaemia line XXIV

		No. of spleen cells injected			
	Passage	10^3	10^4	10^5	10^6
No. of colonies ($\bar{x} \pm SE$)	5	0	0	0	0
	8	-	0	surface lumpy	0
Splenic Enlargement	5	-	-	-	-
	8	-	-	sp+	sp+5

This lymphoid line changed its spleen colony forming characteristics between passages 5 and 8. No colonies or splenic enlargement was detected in passage 5. Splenic enlargement did however take place

with passage 8 and when spleens were exised on day 9 some showed rough, undulating surfaces. These elevations were however much flatter than those produced by normal haemopoietic colonies. They may be due to the projection of expanding lymphoid follicles on the surface of the radiation reduced spleens.

Plate 12 shows a section of a spleen 10 days after irradiation and injection of cells from this lymphoid leukaemia line. The lymphoid follicles are enlarging and a few mitotic figures can be seen. A few dark areas of normal haemopoiesis are present.

Lymphoid leukaemia line XXV passage 20

	No. of spleen cells injected				
	10^2	10^3	10^4	10^5	10^6
No. of colonies $\bar{x} \pm SE$	0	0	0	0	0
Splenic enlargement	-	-	-	+4	+6

Lymphoid leukaemia line XXIX passage 15

	No. of spleen cells injected			
	10^3	10^4	10^5	10^6
No. of colonies $\bar{x} \pm SE$	0	0.25 ± 0.28	0.25 ± 0.28	0.25 ± 0.28
Splenic enlargement	-	-	sp+	sp+2

This lymphoid leukaemia again produced spleens with rough undulated surfaces at high cell doses.

Sections from spleens exised from lethally irradiated mice which had received 10^6 leukaemic cells from line XXIX were studied on successive days after treatment.

Without taking into consideration the plane of section of the spleen the lymphoid follicles seemed to increase in number and size

during the days following irradiation and injection of cells. The follicles reached very much greater sizes and were very much more defined than those of the normal spleen or spleens recovering from radiation after an injection of normal haemopoietic cells.

From a crude count, the average number of mitotic figures per follicle increased during successive days in an almost linear fashion. The areas of normal haemopoiesis in the splenic red pulp decreased in number a size during this time.

Lymphoid leukaemia line XXIX

Day after irradiation	No. of follicles	Total no. mitotic figures in follicles	Average no. mitotic per follicle
2	31	10	0.32
5	23	42	2
7	40	600	15
9	48	908	19

These mitotic figures were distributed randomly throughout the follicle.

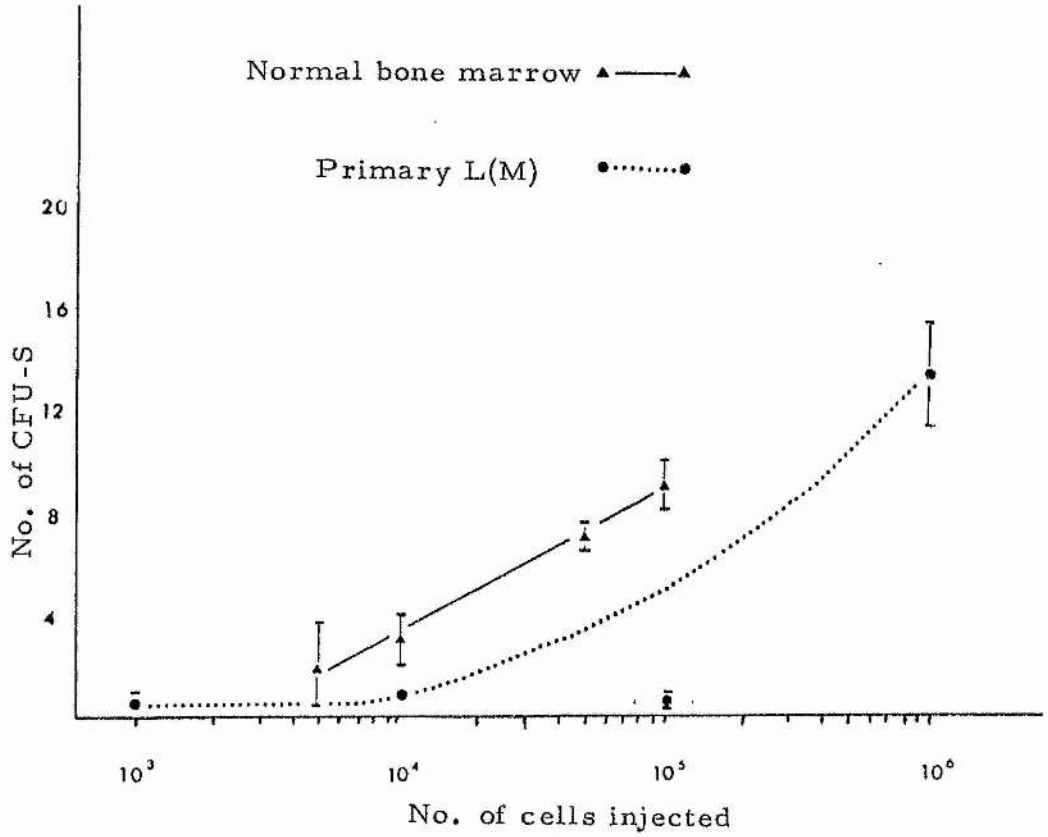


Figure 24

CFU-S in normal bone marrow and CFU-S in spleen of primary myeloid leukaemia 6.1

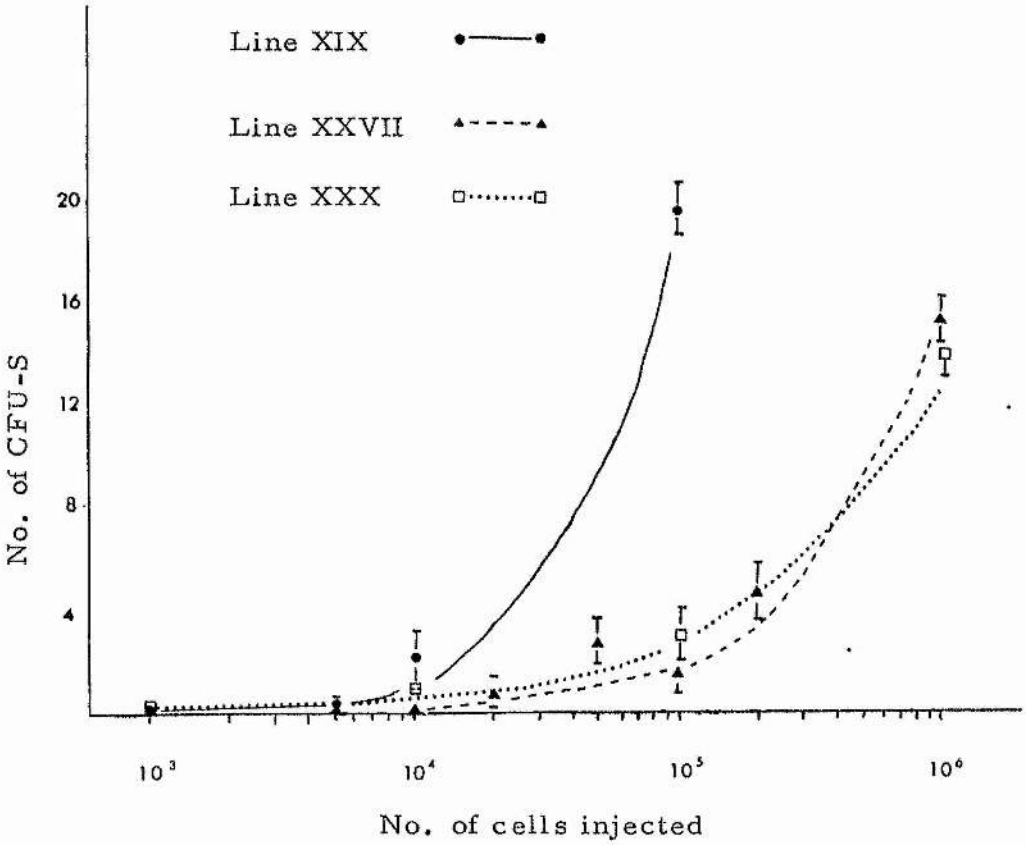


Figure 25

CFU-S formed from spleens cells of three multipassaged myeloid leukaemias

4. DISCUSSION

4.1 Recovery of the lympho-myeloid complex from nitrogen mustard

The alkylating agents were at one time termed radiomimetic drugs because they caused gene mutation, chromosome breakage or translocation as did radiation. However the mechanisms of action and the biological effects of radiation and alkylating agents are clearly different, so the term is no longer used.

Bone marrow transplantation is required after a lethal dose of irradiation if the animal is to survive, but it is not necessary after a highly destructive dose of nitrogen mustard.

The haemopoietic cells recover rapidly from nitrogen mustard but not from radiation. The stem cell would appear to be relatively resistant to nitrogen mustard.

X-rays break the main polynucleated chains of DNA by direct action, nitrogen mustard damages DNA by causing interstrand cross-linking. The stem cells relative resistance to nitrogen mustard may be because resting cells do not incorporate the drug so easily in DNA formation.

In this study 4 mg/kg body weight of HN_2 produced in CDI female mice a drop in body weight, weight of lymph nodes, spleen and thymus and a fall in bone marrow cellularity. The nadir of the thymus, lymph nodes and spleen weight occurred around day 6 after treatment. At this point they were 25-50% of their normal weights. They then began to recover, attaining near normal levels by day 12 (apart from the thymus). The femoral cellularity fell to a nadir on day 3 after treatment. At this time it was about 1% of normal. It then recovered

to normal values on days 10-12 when it appeared to proceed to oscillate around this level (Figures 5 - 10). These patterns of damage and regeneration are similar to those reported by Sharp et al (1973).

The femoral CFU-C content fell to a nadir of less than 1% of normal on day 1 after HN_2 treatment. They immediately began to recover on day 2 reaching 50% of normal values by day 5. The CFU-S recovered to this value by day 4 in CSI mice studied by Sharp (1970). There was a considerable overshoot of CFU-C numbers on day 6 reaching 180% of control values. Levels began to fall again returning to less than 50% of normal by day 10. During this time there would be an outflow from this compartment to the more differentiated compartments of the haemopoietic system, particularly the granulocyte compartment which makes up 94% of the recognisable haemopoietic cells on day 8 after 100 μg HN_2 (Sharp et al, 1973).

The sequential recovery of CFU-S and CFU-C and the cellularity of the bone marrow from 100 μg HN_2 reflects the ancestral relationship of the CFU-S to the CFU-C and that of the CFU-C to the myeloid elements of bone marrow. The CFU-S as estimated by Sharp et al (1970) in CSI mice reached 50% of normal levels on day 4 after 100 μg of HN_2. The CFU-C was found from this study to reach 50% of normal levels on day 5 after HN_2 and the femoral cellularity had recovered to 50% of normal by day 7 (Fig. 26). Myeloblasts from a high proportion of the identifiable cells at this time.

During the early stages of recovery from nitrogen mustard the

proportions of recognisable myeloid cells amongst nucleated cells in the marrow decrease and then begin to recover by day 5. Monocytes and erythroid cells form a very low proportion of the nucleated marrow cells and remain fairly constant in the first 5 days after nitrogen mustard treatment (Tables 9, 10).

The lymphoid cells show an increase in proportion until day 4. These cells include the transitional cells, the origin of the haemopoietic cells. This compartment shows an increase concommitant with the increase of CFU-S following marrow depletion by nitrogen mustard as previously described by Sharp et al (1971).

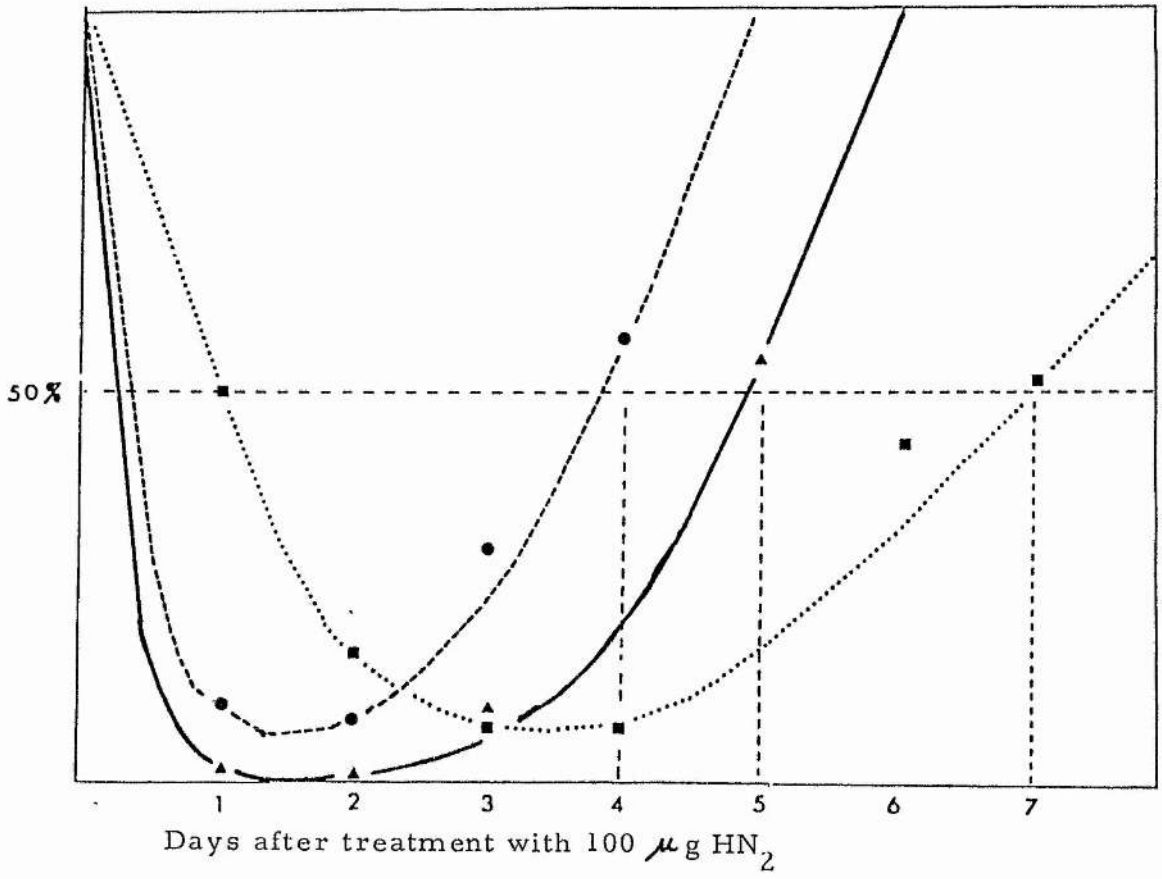
Although the CFU-S and CFU-C continue to increase in number beyond day 4 after 100 μg HN_2 the lymphoid cells decrease in proportion as the cellularity of the bone marrow and more mature elements especially the myeloid, increase.

On days 4 and 6 after nitrogen mustard the turnover time of cells in the bone marrow is just over 50 hours when assessed by metaphase accumulation with vincristine.

On day 3 when femoral CFU-C content was about 20% of normal but the concentration of CFU-C was equal to normal values, the proportion of CFU-C in S-phase as assessed by hydroxyurea killing was 78.8%. The proportion on day 6, when femoral CFU-C were 180% of normal and the concentration over 300% of normal, was only 11.8%.

The proportion of CFU-C in S-phase in normal marrow was estimated to be 50%. This is in good agreement with the values obtained by Lajtha et al (1969), Iscove et al (1970) and Metcalf (1972).

Figure ' 26



CFU-S (Sharp et al, 1973)

CFU-C

Femoral Cellularity

On day 3 after nitrogen mustard the marrow was able to form spontaneous colonies when plated in high numbers, whereas normal marrow did not form colonies spontaneously at the same numbers plated.

The results imply a presence in greater concentration, in marrow regenerating from HN_2, of cells which release colony stimulating activity, ie. those cells which form an adherent layer in 'Dexter' long term cultures (Dexter et al, 1977).

These cells consisting of phagocytic mononuclear cells, 'endothelial' cells and giant fat-containing cells provide an environment 'in vitro' which promotes proliferation, differentiation and maturation of haemopoietic cells (Allen and Dexter, 1976). The phagocyte mononuclear cells are possibly the same as the macrophages which release CSF described by Moore (1978).

Lord et al (1977) detected a stimulator of CFU-S when CFU-S were proliferating in regenerating bone marrow. This stimulator was present in the 30-50 K dalton fraction.

It follows that stimulators of CFU-C from the local environment should be present in higher proportion in regenerating marrow. Gidali and Lajtha (1972) demonstrated the existence and importance of local stem cell proliferation in the bone marrow. One million cells from marrow regenerating from nitrogen mustard at a stage where marrow cellularity is low, as on day 3, will no doubt contain a higher proportion of stromal cells which promote development of colony forming cells.

One million cells from normal marrow will contain a greater number of mature blood cells and much fewer stromal elements. The number of cells which would have to be plated from normal marrow to induce spontaneous colony formation would have to be very much higher.

The proportion of mature granulocytes in marrow 3 days after nitrogen mustard treatment is very low. Rytömaa and Kiviniemi (1968) reported an inhibitory factor, a chalone, released by mature granulocytes which inhibited the production of myeloid cells. Normal marrow if plated at sufficiently high concentration gives rise to spontaneous colonies, which are enhanced by the removal of mature granulocytes or inhibited by addition of mature granulocyte extract (Broxmeyer et al, 1977).

4.2 Regulation of Colony Formation in Agar

There are several variables amongst the culture constituents that might affect the formation of colonies in agar.

0.3% Agar has shown to be a critical concentration for colony formation (Metcalf and Moore, 1971).

In agar cultures the colony yield is altered by the amount of horse serum present or the concentration and quality of colony stimulating factor. In both cases there is an optimum concentration at which the maximum number of colonies is formed when one or other and the number of cells plated is kept constant.

In both cases the depression of colonies formed when large amounts of the substance are present may be due to dilution of other essential nutrients in the culture medium. This, however, in the case of colony stimulating factor is less likely to be true since the amounts of medium containing the colony stimulating activity form a relatively small proportion of the final mixture plated, ie. colony formation was depressed when conditioned medium containing CSA represented only 30% of the mixture. Other workers report a plateau of colony formation at high concentrations of CSF (Metcalf and Moore, 1971). These results as far as they have been taken possibly represent a plateau of values at higher concentrations.

High concentrations of conditioned medium showed a tendency to produce a higher proportion of granulocyte colonies and a lower proportion of macrophage colonies.

Strangely, this result was obtained when a weak conditioned medium was used (ie. one producing a low total number of colonies at the standard concentration of 15%).

This distribution of colony type was even more pronounced when colonies formed, in the absence of added CSF, from marrow regenerating from nitrogen mustard.

Macrophage colonies predominated at all cell concentrations when the cell number plated was varied either with bone marrow or spleen cells.

The identity and source of CSF has clearly an effect on the distribution of colony type obtained in this culture system.

These results may reflect the presence of different subpopulations of CFU-C which respond to CSF at different molecular weights.

Metcalf and Moore (1973) however report no discrepancies in the distribution of colony types formed in the presence of different colony-stimulating factors ie. L-cell conditioned medium yielding a CSF of molecular weight 15,000 and human urine yielding a CSF of molecular weight 40,000-60,000.

Moore (1978) states that no one progenitor cell population can specificially give rise to granulocytes or macrophages.

High concentrations of CSF may stimulate the production of macrophages producing PGE, described earlier, which in turn may inhibit the production of macrophages producing CSF and enhancing colony-formation initiated by exogenous CSF.

In contrast a weak source of CSF may not stimulate colony formation and differentiation to the point where macrophages which produce CSF are formed and add to the effect of the exogenous CSF.

4.3 The response of regenerating marrow to a requirement for erythropoiesis

The recovery of the mature granulocyte elements in the bone marrow precedes the recovery of the recognisable erythroid elements, after a lethal dose of nitrogen mustard. Granulocytes make up the largest proportion of differentiated cells in the marrow by day 8 after treatment with HN_2. The erythroid recovery takes place from day 12 onwards (Sharp et al, 1975).

The response of marrow regenerating from nitrogen mustard to a stimulus for erythropoiesis is shown by a depression in the number of CFU-C's in bled animals treated with HN_2 compared with unbled animals treated with HN_2 three days previously. This possible diversion of the CFU-C to the erythroid compartment may be reflected by a slightly higher uptake, on day 4 after HN_2, of ^{59}Fe by the femurs of mice which had been bled on day 1 after HN_2.

Although the marrow precursors at this time are preferentially differentiating towards the granulocyte compartment an added stimulus for erythropoiesis creates what appears to be a normal response.

The spleen weight and splenic uptake of ^{59}Fe are increased significantly in normal mice 3 to 5 days after bleeding. Spleen weight and splenic ^{59}Fe uptake increases on the 5th day of hypoxia (Turner et al, 1967). The femoral marrow however shows a decrease in ^{59}Fe uptake in 'normal' bled mice whilst it shows an increase in uptake in hypoxic mice.

No very significant differences were noted in these parameters in mice that had been bled during recovery from nitrogen mustard.

The response to bleeding on day 1 after nitrogen mustard is reflected only slightly by the erythroid cells present in the bone marrow on the first 5 days following drug treatment (Tables 9, 10)

4.4 The Role of the Spleen in Haemopoiesis

The adult spleen is generally regarded as predominantly a lymphoid organ composed of cells involved in the immune reactions and as a reticuloendothelial organ whose mononuclear phagocytes are involved in trapping and destruction of microorganisms and senescent blood cells.

The haemopoietic potential of the spleen is usually expressed in fetal and perinatal life (Metcalf and Moore, 1971; Metcalf and Stevens, 1972) and during times of increased need for blood cells (haemorrhage or infection).

The adult murine spleen is composed predominantly of lymphoid cells with some macrophages and granulocyte elements present. In the adult mouse the spleen contains pluripotent stem cells (CFU-S) (Metcalf and Moore, 1971; Blazi-Poljak and Boranic, 1975) as well as progenitors of erythroid cells (Gregory et al, 1973) and granulocytes and monocytes. CFU-C are present in relatively low numbers to the bone marrow CFU-C and appear to be resting (Metcalf and Stevens, 1972).

In contrast the bone marrow contains a heterogeneous population of granulocytic precursors with concatenated proliferative and maturational activity.

In this study spelenectomy in one trial increased the number of femoral CFU-C but not very significantly. In other trials sham splenectomy and splenectomy depressed the femoral CFU-C content. This may be a result of operative stress. Bleeding lowered the femoral CFU-C content slightly in intact and sham splenectomised animals but in splenectomised

mice the femoral CFU-C in the bled mice was significantly higher. The absence of the spleen modifies the response of the femoral CFU-C to bleeding.

When the spleen was present there was a slight reduction in femoral CFU-C content in the bled animals.

When the spleen is absent a stimulus for erythropoiesis brings femoral CFU-C into cycle and proliferative status thus increasing in number relative to unbled splenectomised animals.

Other workers (see introduction) have not reported a depression in femoral CFU-C in splenectomised animals.

Richard et al (1971) found that in response to erythropoietic stress, when red cell production appeared to be at a maximum the CFU-C were reduced in the bone marrow and there was usually a reciprocal rise in the spleen.

Metcalf (1969) reported that bleeding 0.3ml daily for 3 days reduced the total number of CFU-C in the bone marrow to less than 50% of that in control mice and caused a significant increase of

erythropoietic cells in the bone marrow. Bled mice developed spleen enlargement with some increase in the total number of in vitro colony forming cells in the spleen but this did not compensate for the reduced number of such cells in the bone marrow.

Although the rise in reticulocytes in the blood was previously interpreted as an indication of erythroid production which would depress CFU-C numbers in the femur, this raised reticulocyte count may be due to the absence of the pitting action of the spleen alone (Crosby, 1980).

As mentioned before, Gunale 's claim that the spleen exerted an inhibitory effect on marrow CFU-C proliferation in unstimulated mice is not supported by the results (Gunale et al, 1976).

Dons et al (1974) in fact detected a spleen extract (supernatant from cell homogenate spun at 34,000 g for 37 minutes) which like fetiun (an alpha globulin protein fraction extracted from fetal calf serum) enhanced the rate of regeneration of CFU-S, the predecessors of CFU-C, in the bone marrow. The spleen extract and fetiun caused a transient decrease in marrow cellularity and particularly number of mature myeloid cells in the marrow. The actions described here suggest a destructive rather than inhibitory effect of spleen extract on marrow myeloid elements.

Hereditary asplenic mice and normal littermates were transplanted subcutaneously with 2×10^7 congeneic spleens at birth. They developed subcutaneous nodules closely resembling the structure of normal murine spleen. Growth of the graft was

associated with an increased number of stem cells (CFU-S) and agar colony forming cells (CFU-C) per femoral shaft in both asplenic and normal mice. There was hyperplasia of the granulocyte series. The presence of the graft did not modify the erythrocytic series and leukocytes of asplenic mice but reduced average platelet count. This indicates humoral or cellular influences of spleen on release of and production of marrow cells (Lezzio and Machado, 1975).

The splenic CFU-C increased initially after a lethal dose of HN_2 but rapidly fell. Their recovery is preceded by the CFU-C of the bone marrow. The splenic pool of CFU-C may respond rapidly to depletion, thus exhausting itself in a short time.

Silini et al (1976) assessed that the number of CFU-S per femur in intact and splenectomised C57Bl/Cne x C3H/Cne mice was 6280 $\pm$470 respectively. They found that the presence of the spleen affects marrow repopulation only at early post-irradiation stages. Expansion of the marrow progenitor pool proceeds, rather independently of the spleen and the marrow CFU-S remain eventually as the main source of haemopoietic cells. Thus, the reaction of the spleen may be envisaged as fast, important, but making transient contribution to the overall haemopoietic function of heavily irradiated animals.

The spleen was the major haemopoietic organ in mice whose bone marrow had been ablated with the bone-seeking radionuclide ^{89}Sr (Jacobsen, Simmons and Block, 1949). There was a marked increase in the CFU-S compartment of the spleen in such mice (Teried, Gurney and Swatek, 1966). There was also a marked increase in the numbers of CFU-S in the blood of intact ^{89}Sr-treated mice which dramatically declined after splenectomy (Adler, Knospe

and Trobaugh, 1977).

The spleen supplies an important microenvironment for erythropoiesis in mice.

In the genetically anaemic Sl/Sl^{d} mice in which the haemopoietic microenvironment is defective, the anaemia could be alleviated by transplants of normal intact spleen tissue but injection of spleen cell suspension had no beneficial effect (Bernstein, 1970).

Responses of plethorised, splenectomised mice to erythropoietin were very much smaller than those of intact mice ranging from 1.4-12.0% when measured by ^{59}Fe incorporation in RBC (Bozzini et al, 1976).

The environmental influences of the spleen would appear to be outlined by the results with the different leukaemias when assayed for CFU-S.

About half the spleen colonies formed from a transplant of normal bone marrow are located on the surface of the spleen, and 80% of these colonies are erythroid or of mixed composition (Lewis et al, 1968). Erythroid colonies are also present in the red pulp but not in empty lymphoid follicles, whereas granulocytic colonies grow along the trabecula of the spleen or in subcapsular sheets. Megakaryocytic colonies usually grow beneath the capsule (Curry and Trentin, 1967). Curry and Trentin called this environmental influence the "hematopoietic inductive microenvironment" and concluded that the location of the colony-forming cells within the spleen determines differentiation.

Myeloid leukaemia cells formed colonies in the red pulp of the spleen as do their normal myeloid counterparts. These colonies did form projections on the surface of the spleen although these were somewhat flatter than those formed by normal bone marrow cells (plates 8 -11). Colonies from lymphoid leukaemia cells formed around the periarteriolar regions of the spleen, thus not creating visible surface colonies.

B cells proliferate in the lymphatic nodules formed within the periarteriolar sheaths of the spleen. T cells migrate from the circulation to the marginal zone (the transition between white and red pulp. The T cells then mix and interact with the B cells in the lymphoid nodules (Ham, 1974).

Mitotic figures were seen distributed throughout the lymphatic nodules formed by lymphoid leukaemia cells in irradiated mouse spleens. None of the leukaemias examined in the spleen colony assay had shown thymic involvement at any time.

The leukaemias seem to illustrate that the different types of cells select the specific environment in the spleen which stimulates their proliferation.

Although the spleen colony technique provides clear distinction between murine radiation-induced myeloid leukaemia and murine lymphoid leukaemia, in that myeloid leukaemia produces colonies in the splenic red pulp (plate 10) and lymphoid leukaemias do not form visible surface colonies but produce highly developed lymphoid

nodules (plate 12), it may provide no distinction for T and B lymphoid leukaemia. A leukaemia that showed thymic involvement was not available for testing. The lymphoid leukaemia cells tested were in fact later shown to have Thy 1,1 and Thy 1,2 antigens.

Ford (1969) showed that labelled lymphocytes from the thoracic duct, migrated to the spleen and formed a ring around the peri-arteriolar lymphoid sheath into which only a few cells penetrated.

Tanaka and Lajtha (1969) described colonies formed by the transplanted lymphoma in AKR mice. These cells are seen to be proliferating around the periarteriolar regions forming surface colonies. This leukaemia showed thymic involvement (Bruce and van der Gaag, 1963).

The several mechanisms described here and previously in the introduction make it possible to speculate on the effects of splenectomy on leukaemia.

It is clear that the homeostasis of the bone marrow is grossly upset in a leukaemic state. The presence of the spleen may prolong a degree of normal homeostasis in the haemopoietic system, delaying fatality.

The presence of the spleen enhances regeneration of stem cells in the bone marrow, but could equally well enhance the growth of transformed leukaemic stem cells. Leukaemic cells seem to retain a considerable ability to behave like their normal counterparts.

Adler and Trobaugh (1978) reported that splenectomy delays and lowers the incidence of transplanted myelogenous leukaemia in mice and Upton (1977) reported that splenectomy inhibits radiation induction of myeloid leukaemia in mice.

However, once the leukaemia has reached a stage of development, as it will have done in clinical cases, splenectomy may be of no benefit unless the spleen is so grossly enlarged and placing a burden upon the circulation.

Priesler et al (1979) reported that splenectomy had no influence on the survival of animals after transplantation of myelogeneous leukaemia in RFM/UN mice.

The murine spleen, of course, also plays a greater role in normal haemopoiesis than the human spleen.

4.5 Perturbation of normal haemopoiesis in leukaemias

If the techniques for measuring and investigating normal haemopoietic cell production and regulation are used in assessing these mechanisms in conditions of leukaemia it must be established if these parameters so demonstrated relate to those expressed during normal haemopoiesis. The degree of imbalance of normal haemopoiesis created by the leukaemia condition might be assessed if assays for stem cells are applied to leukaemias.

Cell production rates, in leukaemia, do not seem to greatly exceed the production rates of normal haemopoietic cells responding to depletion by drugs, however the production or growth of the leukaemia cells is not arrested by the mechanisms which act when populations of normal haemopoietic cells reach a certain number. In addition the growth of normal haemopoietic cells is inhibited by the overgrowth and production of certain substances by the leukaemia cells.

Leukaemia cells form colonies in the spleens of irradiated mice but these are not identical to those formed by normal haemopoietic stem cells. If colonies formed by normal cells or leukaemic cells could be distinguished with certainty these parameters would indicate the ability of the marrow to regenerate normal haemopoietic cells, by assessment of the normal stem cell content, if leukaemic cells were removed.

The pluripotent haemopoietic stem cell, however, appears to be the cell in which a leukaemic transformation is initiated in radiation induced myeloid leukaemia.

4.6 Cell production in irradiation-induced myeloid leukaemias in CBA/H mice

Colcemid is the most commonly used drug for arresting cell in metaphase for chromosome preparation. Smith et al (1974) however reported that vincristine sulphate was a more efficient drug to use for metaphase accumulation and cell production studies.

The abilities of vincristine and colcemid to arrest cells in metaphase were compared.

Within the limits imposed by the availability of the material used for the study, it may be assessed that vincristine is a more efficient mitotic arrest agent in murine myeloid leukaemias than colcemid.

In a study carried out on transplanted rat mammary tumour and rat small intestine, Tannock (1967) found that vincristine and colcemid gave sharply peaked dose response curves for the tumour whereas for the intestine, vincristine gave a more broadly peaked dose response and colcemid showed no peak on the dose response curve.

The dose response curves achieved with colcemid for a 'fast growing' and a 'slow growing' transplanted murine myeloid leukaemia were similarly flat, whereas vincristine showed a sharp peak in the dose response curve for the 'fast growing' leukaemia.

Tannock (1967) mentions that metaphase degeneration was often evident when colcemid was used. Metaphase degeneration was evident in leukaemic cells cultured with colcemid. Cells arrested

in metaphase by vincristine appeared intact. The degeneration could be responsible for loss of metaphase arrested cells and so lower the proportion counted. The viability of the cells after culture with colcemid or vincristine was similar.

The difference in the results with primary and passaged leukaemias reflects the difference in the behaviour of transplanted cell lines to that of cells in the primary disease.

Repeated transplantation of myeloid leukaemia cells in the rat (Hagenbeek et al, 1977) and the mouse (Meldrum and Mole, 1981) has shown an increase in the growth rate of the leukaemia cells with increasing number of passages.

Since the greater efficiency of vincristine, when compared to colcemid in arresting metaphase in murine leukaemias, is more pronounced in transplanted leukaemias. This may be evidence to suggest that caution should be exercised when using transplanted cells to assess the effect of drugs on primary cancers.

Since vincristine shows an optimum dose at which it arrests metaphases in murine myeloid leukaemia and these metaphases do not degenerate this drug would appear to fulfil the criteria stated by Tannock (1967) for an effective statmokinetic agent to use in this leukaemia cell system.

4.7 Cell production in myeloid leukaemia

Irradiation induced myeloid leukaemia in CBA/H mice most resembles acute myeloid leukaemia in humans. However in the absence of cytogenetic studies a distinction between chronic myeloid leukaemia and acute myeloid leukaemia cannot always be clearly drawn (Gunz, 1977). In childhood the majority of cases are acute.

Chronic myeloid leukaemia usually terminates in a 'blast crisis'. There is an accumulation of myeloblasts and a loss of more differentiated cells. The similarity between this situation and the kinetics of acute leukaemia is obvious.

Myeloblasts are the youngest cells with distinct morphology and these have been shown to be less proliferative than normal myeloblasts (Gavosto, 1974). The labelling idices decrease with the percentage increase in myeloblasts. It is probable that during blast crisis an increasing number fail to divide. Analysis of the kinetic parameters of the chronic myeloid leukaemia proliferating compartment and their comparison with normal granulopoietic tissue, usually shows that the labelling index and proliferation rate are lower, whereas cell cycle times and intracompartmental transit times are longer in CML and that leukaemic granulocyte proliferation is partly ineffective (Gavosto, 1974).

Maloney et al (1971) however conclude that there is ineffective granulopoiesis in the normal state. Flash-labelled granulocyte to erythroid progenitors was unity (1.02 ± 0.05) but the net production as revealed by peripheral blood turnover is greater for erythrocytes than granulocytes.

In human acute myeloid leukaemia measurements of the labelling index range from 3-6% and the mitotic index from 0.4-0.8% (Gavosto and Pileri, 1971).

In the irradiation induced myeloid leukaemias in CBA/H mice the highest number of cells accumulating in metaphase was about 20-25 per 1000 per hour if metaphase accumulation is assumed to be linear over time. This is true for both primary and passaged leukaemias. This rate of metaphase accumulation however is not very much greater than the rate of metaphase accumulation in marrow regenerating from nitrogen mustard at a time when myeloblasts make up a large proportion of the cell population.

The spleen shows a slightly higher proportion of cells in metaphase than the bone marrow in passaged myeloid leukaemias, but this is not highly significant.

The proportions, on the whole, were lower in the bone marrow in primary myeloid leukaemias but this was not statistically significant. Lower proportions in the bone marrow were mostly due to cases where the bone marrow was accellular, following a fibrous fatty change. The proportions of cells in metaphase in the blood were very low, not exceeding 10% of the proportions in the spleen.

Since the proportions of cells in metaphases a similar in the tissues examined (spleen, bone marrow and liver) this suggests that these tissues are supporting the growth of a similar or identical clones of leukaemia cells, whose growth rates are similar.

Transplantation of the leukaemia cells has shown that clones of leukaemia cells of different growth rates exist. Slower growing clones are revealed when low numbers of cells are transplanted (Mole and Meldrum, to be published).

The low proportions of cells in metaphase in the blood may suggest that these cells are not representative of the clones of leukaemia cells in the tissues.

4.8 Spleen colony formation in murine leukaemias

When normal bone marrow or spleen cells are injected in suitable doses into lethally irradiation mice, the surface colonies formed in their spleens (CFU-S) reflect primarily erythropoiesis and not total haemopoiesis (Lewis et al, 1968). Plethora reduces the number of CFU-S formed (Curry et al, 1964) but this number may be restored by exogenous erythropoietin (Curry et al, 1964, and Bleiberg et al, 1965).

The murine spleen provides a major contribution to erythropoiesis (Thomas, 1973).

Granulocyte colonies are not sharply circumscribed and because they invade the splenic tissue they do not produce a significant displacement and elevation of the splenic capsule (Lewis et al, 1968).

A spleen colony formed from normal spleen cells in a lethally irradiated CBA/H mouse (plate 8) shows sharper elevation than spleen colonies formed from a passaged myeloid leukaemia (plate 10).

Cells from leukaemias often formed mixed colonies of normal and leukaemic cells

Cells from lymphoid leukaemias on the whole did not produce surface colonies but the lymphoid follicles of the spleen were highly developed (plate 12) and showed many mitotic figures (plate 13). Normal haemopoiesis could be seen in the splenic red pulp.

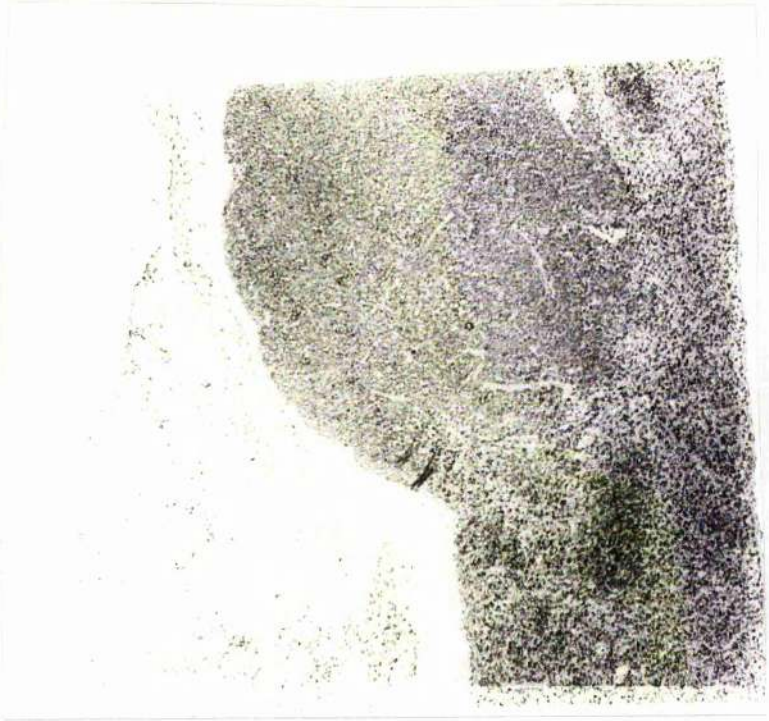
A linear relation between the mean number of colonies per spleen and cell dose injected was not detected over the range of cell dose examined. Tanaka and Lajtha (1969) observed a linear relationship between the number of colonies formed from RFM/Un myeloid

Plate 8

Spleen colony formed from normal bone marrow.

Plate 9

Cells from normal spleen colony.



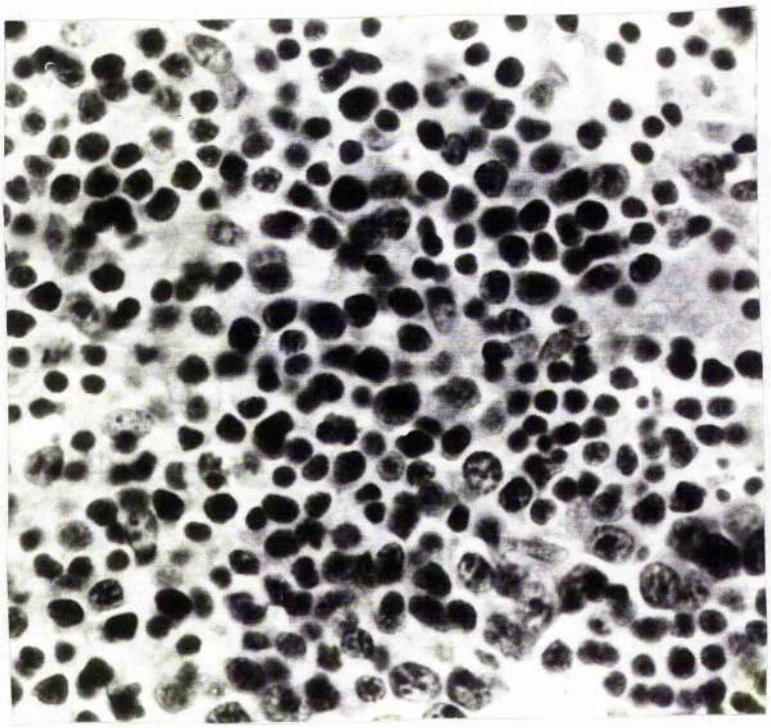
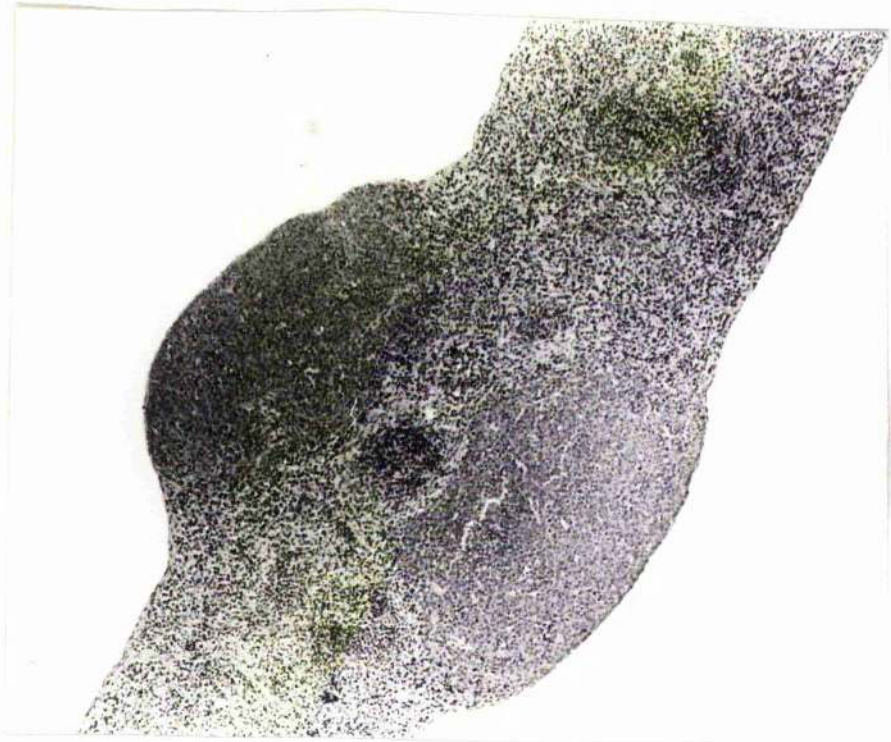


Plate 10

Spleen colonies formed by cells from multipassaged myeloid leukaemia.

Plate 11

Cells of myeloid leukaemia spleen colonies.



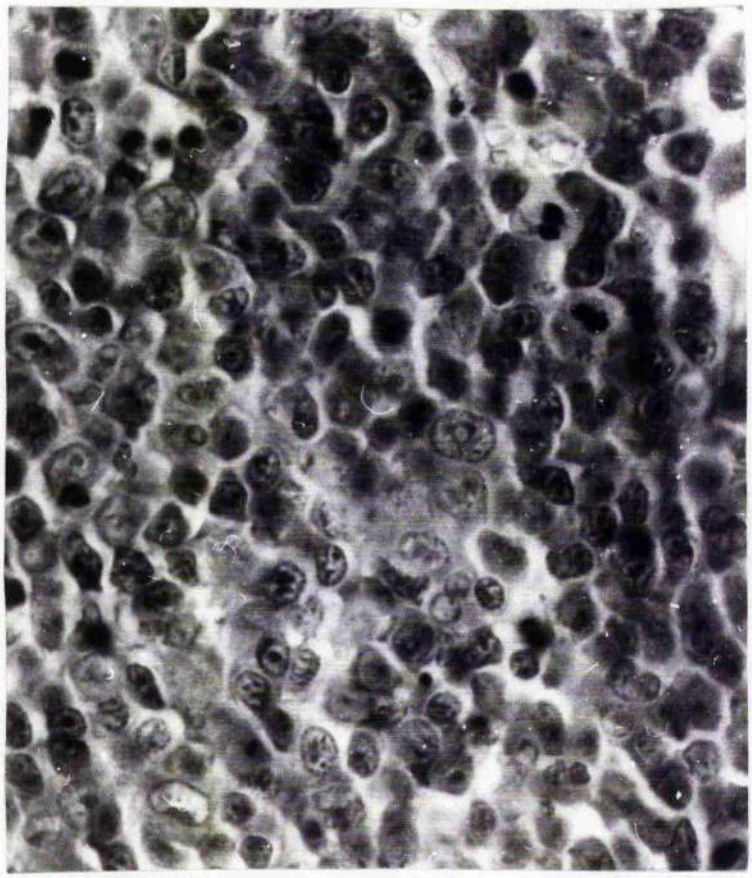
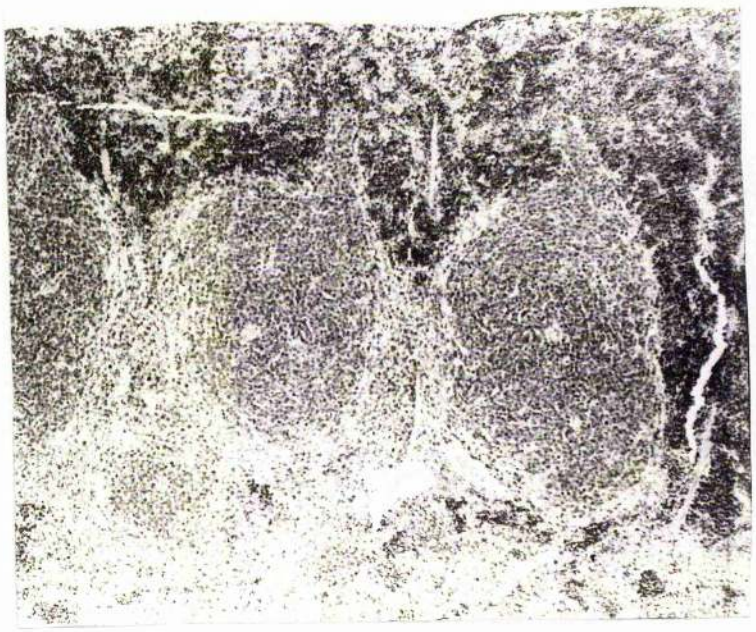


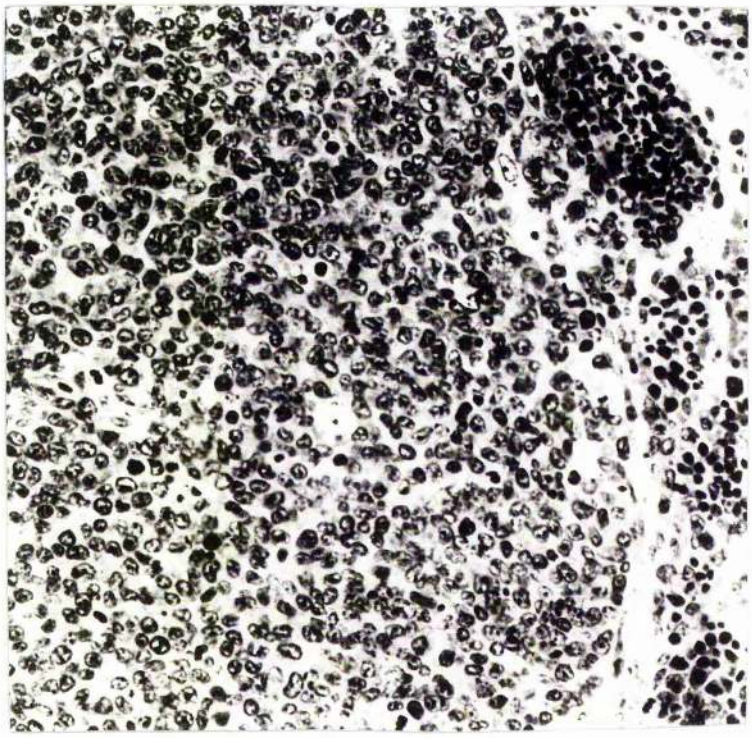
Plate 12

Spleen of lethally irradiated mouse given 10^6 multipassaged lymphoid leukaemic cells ten days previously.

Plate 13

Cells of enlarged lymphoid follicles showing mitotic figures and the splenic red pulp on the right.





leukaemic bone marrow and cell dose in the range of 2×10^3 to 1.2×10^5 leukaemic cell inoculum.

In line XIX the relationship of number of colonies to number of bone marrow cells injected might be linear over the range 10^4 to 10^5. In the other leukaemia lines and the primary leukaemia examined the relationips might be linear over the range 10^5 to 10^6 cells injected. In CBA/H mice normal bone marrow gives a linear relationship between number of colonies formed and number of cells injected over the range 5×10^3 to 10^5 cells

Line XIX was known to give a 'take' if only ten cells were injected (Meldrum and Mole , 1982). Since at least one in ten cells injected is clonogenic and only 2 colonies per ten thousand cells injected form in the spleen the seeding efficiency in the spleen is very low, ie.0.02%.

The histology and shape of the colonies formed from line XIX , which were purely myeloid, were similar to the colonies from the RFM/Un myeloid leukaemia described by Tanaka and Lajtha (1969).

The leukaemia cells which form colonies do not seem to express a multipotentiality as do the cells that form spleen colonies from normal marrow. The leukaemic spleen colonies appear to consist of blast cells of the series of haemopoietic compartments in which they originated. The myeloid leukaemia cells invade the splenic red pulp and form flatter surface colonies than the erythroid type formed by normal haemopoietic cells. The lymphoid leukaemia cells grow around the periarteriolar regions of the spleen. Such development of the lymphoid follicles in the spleen may be seen in the non-irradiated spleens in primary lymphoid murine leukaemias.

4.9 Interaction of leukaemic cells with normal cells

Cure or control of leukaemia could be made more effective by an understanding of the interaction of the leukaemic cells with the normal stem cells during the progression of the disease.

Kinetic studies of the stem cell pool following depletion with cytotoxic drugs have revealed the relationships of the precursors to each other and the more differentiated forms of haemopoietic cells and the capacity of the stem cell pool for recovery. However the presence of leukaemic cells will impair this capacity for recovery and production of normal differentiated cells.

Leukaemic cell suspensions form colonies in the spleens of irradiated mice. These colonies consists of different compositions of cell types as described previously. The presence or absence of erythroid cells which predominate in normal spleen colonies might be confirmed by autoradiograph with uptake of ^{55}Fe.

Transplanted leukaemic cells exert a suppressive effect on normal haemopoiesis. Injection of 10^5 spleen cells from RFM mice with myeloid leukaemia produces a decline of all normal haemopoietic parameters between the sixth and seventh days after injection of leukaemic cells (Lajtha et al, 1969). Erythropoiesis, measured by ^{59}Fe incorporation is lowered as are the normal stem cells, measured by repopulating capacity of the marrow and the numbers of CFU-C also fall.

Lajtha, Tanaka and Testa, 1972) conclude that the reduction in normal haemopoiesis cannot be attributed solely to overcrowding by the leukaemic cells, but that it is the result of humoral interaction between the leukaemic and normal stem cells.

In RMF leukaemia colonies grown from normal and leukaemic mouse cells are not distinguishable by morphology or growth pattern in culture. Transplantation of CFU-C cultured from leukaemia cells failed to produce leukaemia in recipient mice whereas high numbers of leukaemia cells plated in agar produced leukaemia in recipient mice (Gordon and Coggle, 1974). Gordon and Coggle found there was a rise and fall in the numbers of normal CFU-C from leukaemia spleens. Lajtha, Tanaka and Testa (1972) detected the same rise and fall and suggested the effect may be due to some form of interaction between normal and leukaemic cell populations. Metcalf, Moore and Warner (1969) reported a stimulating effect on the formation of granulocytic colonies by cells from murine myelomonocytic leukaemia. Lymphoid leukaemia, erythroleukaemia or plasma cell tumours did not have a stimulatory effect. The stimulatory effect of myelomonocytic leukaemia comes from CSF releasing monocytic leukaemic cells.

On the other hand it has been found that cells from acute leukaemias (mostly myeloid leukaemias) (Chiyoda et al, 1975) or extracts from leukaemic cells (Chiyoda et al, 1976) depress colony formation by normal CFU-C.

Leukaemia inhibitory activity (LIA) is found associated with acute and chronic myeloid and lymphoid leukaemia, although much greater quantities are found during acute leukaemia than during

chronic leukaemia or acute leukaemia during remission (Broxmeyer et al, 1978a, Broxmeyer et al, 1978b).

RNA and protein synthesis are required for LIA production. Protein synthesis is evident in cells of irradiated induced CBA/H leukaemia by the deep basophilic cytoplasm of the blast-like cells.

LIA inhibited CFU-C during S phase but did not appear to affect cells in suspension culture generating CFU-C or immature recognisable granulocytes in the colonies developing from CFU-C (Broxmeyer et al, 1978b). The LIA producing cell population is not synonymous with the blast cell population as a whole. Only non-adherent, low density and slowly sedimenting cells contained and released LIA.

These cells have been characterised as belonging to a lymphoid-like cell population but are neither T or B but have Fc receptors. They are non-phagocytic, and belong to the sheep red blood cell rosetting populations which are E^-, EAC^-, Ig^-, EA^+ and Ia^- as determined by cytotoxicity tests.

Certain immunoregulatory agents suppress the production of LIA. LIA was not detected in normal human tissue and evidence for its specificity of action was obtained since LIA did not inhibit BFU-e or CFU-e proliferation (Broxmeyer et al, 1979).

Greater concentrations of LIA are found during acute leukaemia than during chronic leukaemia (Broxmeyer et al, 1978a, 1978b) and remission of acute leukaemia is associated with low levels of LIA (Broxmeyer et al, 1979a). LIA has also been detected in mouse tissue infected with Abelson and Friend virus (Broxmeyer et al, 1980,

and Marcelletti and Furmanski, 1980). LIA is not effective in suppressing growth of CFU-C from many patients with leukaemia or from virus infected mice.

LIA was identified as acidic isoferritins (Broxmeyer et al, 1981) whose role as a normal inhibitory regulator of myelopoiesis was distinct from that of lactoferrin, a polymorphonuclear cell-derived lactoferrin, which in its iron saturated form, inhibits murine macrophage and human monocyte release and production of CSF (Broxmeyer, 1979; Pelus et al, 1979).

Prostaglandins are selective and highly specific regulators of human bone marrow colony-forming cells committed to monocytoid differentiation (CFU-M) but not those which give rise to neutrophils or eosinophils. CFU-C which give rise to neutrophils (CFU-GM) and pre-CFU-GM (cells giving rise to CFU-GM in suspension culture) are heterogeneous in their sensitivity to PGE.

Acid isoferritins which produce the inhibitory effect of LIA have been found in tissues from normal healthy individuals at a very low concentration, but their presence is markedly elevated in individuals with leukaemia and lymphoma (Broxmeyer et al, 1981).

The transplanted myeloid leukaemia line XIX, which originated in a primary host given two doses of 120 rads was known to have at least one in ten clonogenic cells (Meldrum and Mole, 1982). This leukaemia, after several passages, was estimated to have 25 cells entering metaphase each hour. This would produce a doubling time of about 40 hours, assuming no cell loss. If 10^7-10^8 leukaemia cells were fatal to a mouse the survival times of mice given between

one and ten cells might be expected to fall between 40 and 50 days. Even if only 10^6 cells were fatal to a mouse the survival time would be over 35 days.

The actual survival times of mice given a low number of the multipassaged leukaemia cells was lower than 35 days (Meldrum and Mole, 1982 and see addendum). This suggests that some other influence such as a humoral suppression of normal haemopoiesis contributed to the rapid death of the animal.

Hagenbeek and Martens (1981), on the other hand, concluded that humoral factors of short-range cell-to-cell interaction did not play an essential role in the redistribution and depression of CFU-S during the progression of transplanted rat (BNML) myeloid leukaemia. Their conclusions were based on diffusion chamber experiments and 'in vitro' coculturing of leukaemic and normal bone marrow cells. In the late stages of leukaemia the majority of the remaining CFU-S is found in the spleen and in the blood.

Excessive production of normal cell proteins in tumour cells is discussed by Klein (1981). It is suggested that this overproduction of protein causes transformation and it is hypothesised that due to the integration of the oncogene in the wrong place or in the wrong way, a certain normal cellular product is synthesised in excessive quantity or at the wrong time.

It is apparent that leukaemic cells retain a considerable degree of normal behaviour patterns but the fatal state co-incides with loss of mature cells which produce inhibitory material that acts on precursor cells.

This loss of normal inhibition is possibly not only accelerated by the inhibition by leukaemic cell product, of normal precursor cells which produce these end cells but also by the destruction or loss of the bone marrow stromal elements on which regulatory cells depend, as reflected by the adherent layer in Dexter cultures.

The growth of the bone marrow stroma will be physically limited by the boundaries of the bone cavity and will not be able to expand to produce enough regulatory control which might have some influence on leukaemic cells or enough stimulus for the normal cells to overcome any inhibitory action produced by the overproduction of proteins by the leukaemic cells.

When Sir Ralph, in Bernard Shaw's play 'The Doctors Dilemna' referred to 'stimulation of the phagocytes' as the natural remedy for all diseases, he was referring to the destructive immunological effect in which macrophages are involved "The phagocytes are stimulated; they devour the disease; and the patient recovers - unless, of course, he is too far gone." He could not, of course, envisaged the regulatory role played by macrophages, and its possible manipulation for control of leukaemia.

It is difficult to assess though which mechanism or mechanisms should be interferred with to produce to most effective control of leukaemia. Stimulation of normal cell proliferation to compete with leukaemic cell proliferation may stimulate potential leukaemic stem cell proliferation, since it is most likely that myeloid and other leukaemias are stem cell diseases, as will be discussed next.

4.10 Radiation-induced leukaemia - A stem cell disorder?

Evidence that a number of haematological disorders represent neoplastic transformation of pluripotent stem cells has come from chromosome markers. The Philadelphia chromosome, an abnormality specific for CML, was found to be present in erythroid, granulocytic and megakaryocytic cells lines, indicating that the disease was primarily at the pluripotent stem-cell level (Wang-Peng et al, 1963).

The glucose-6-phosphate dehydrogenase (G-6-PD) marker which is X-chromosome linked demonstrated that clones produced erythrocytes, granulocytes, platelets, monocyte/macrophages and B lymphocytes indicating a stem cell which gives rise to all these lines (Fialkow et al, 1978).

Abramson et al (1977) showed that by injecting cells with radiationed-induced chromosome markers into sublethally irradiated mice of the W, W^v strain, which have a genetic malfunction of their own haemopoietic stem cells, that the myeloid and lymphoid stem cells were partly independent. On the other hand Prchal et al (1978) reported the single marker, G-6-PD, in all haemopoietic and lymphoid cell types in a case of sideroblastic anaemia.

With radiation induced leukaemias the time of the initial leukaemogenic insult is known. Most quantitative data arise from the Japanese survivors of the atomic bombs at Hiroshima and Nagasaki.

Leukaemia in man would begin to be diagnosed when about 10^{10} to 10^{11} leukaemia cells are present (Cronkite, 1981). If there was no leukaemic cell death the time to grow out to 10^{10}-10^{11}

leukaemic cells from one or more similar leukemic mutants with a doubling-time of 48 hours is 68-75 day. In Hiroshima and Nagasaki the first clear cut increase in the evidence of leukaemia was observed 18 months after exposure and peaked about 5-6 years after exposure. Incidence of all sorts of leukaemia was higher and peaked sooner in younger age groups, eg. those under 15 at the time of bombing had peak incidence 6 years after exposure, 15-29 years at 9 years, 30-44 years at 14 years, and 45 and over at 22 years after exposure (Ichimaru and Ichimaru, 1975). In those under 15 (at the time of bombing) the increased incidence seemed to disappear by 17 years after exposure and is still slightly elevated for all older age groups 36 years after exposure.

Radiation is clearly an initiating agent. Modan (1974) suggests that radiation suppression of the immune processes may act as a promotor. However, the detectable suppression of immunity by radiation in animals is short-lived, lasting only a few weeks to a few months and the duration of suppression is a direct function of radiation dose (Cronkite, 1981). Irradiation of the hosts of first passage primary murine myeloid leukaemia cells did not shorten the survival time of the recipients of the transplanted leukaemia cells, indicating the suppression of the immune system had no influence on the growth of leukaemic cells (Meldrum and Mole, 1981). The optimum leukaemogenic dose of 300R, in male CBA/H mice (Major and Mole, 1978) is not a dose which has a great effect on the immune system.

Lajtha (1981) points out how the long latent period between the leukaemogenic insult and detection of the disease is consistent with time required for amplification of the pluripotent stem cell compartment to produce recognisable blood cell precursors.

Since radiation causes breaks in strands of DNA and abberations during the repair of the damaged DNA that takes place is most likely to initiate the transplantation that leads to carcinogenesis. Indications are that cells which are in S-phase at the time of irradiation are lost. Lajtha et al (1969) report that a daily regime of 50 rads acute or 70 rads continuous irradiation depresses the femoral CFU-S content to less than 1%. Each successive dose of irradiation will destroy cells in S-phase, so promoting further CFU-S to enter the cell cycle.

Necas and Neuwirt (1976) claimed that cells which are synthesising DNA in the S-phase of the cell cycle inhibit the entry of non-proliferating G_o cells into the cell cycle.

A greater proportion of cells were promoted into cycle by the agents hydroxyurea and cytosine arabinoside which are toxic to the S-phase cells than by the agents toxic in other parts of the cell cycle (ie. colchicine which kills cells in mitoses).

Millar et al (1978) postulated that cellular damage releasing free DNA nucleosides enhances the regrowth of surviving stem cells. Although the effect is weaker with radiation, a non-specific cytotoxic agent, continuous or successive doses of irradiation will destroy cells and successively bring more CFU-S into cycle, explaining the effect observed by Lajtha et al (1969).

The CFU-C does not have the extensive self-maintenance of the CFU-S as indicated by the ineffectiveness of WW^{v} marrow, deficient in CFU-S but normal with respect to CFU-C, to maintain granulopoiesis when grafted into irradiated recipients. A high proportion of CFU-C are normally in S-phase - about 50%. A high number of CFU-C would be likely to be lost during a leukaemogenic dose of irradiation.

After 300 rads repopulation of the CFU-C compartment is not complete until 220 days, whereas repopulation of the CFU-S compartment is complete within a month from time of irradiation (Hirashima et al, 1980). The incidence of leukaemia was very rare after complete repopulation of CFU-C compartment. The period of 220 days is consistent with the shorter latency periods for leukaemia in irradiated mice.

The transit times for morphologically recognisable granulocyte precursors are only a few hours. The metamyelocyte transit time was estimated to be 16 hours and the band cell transit time 31 hours (Constable and Blackett, 1972). The cell cycle time as estimated by flash labelling by ^{3}HTdR for mouse granulocytes and mononuclear cells was 15 hours (Testa and Lord, 1973). The total transit time of 10-15 days of the 'post-stem' differentiated maturing cells would seem to exclude them as the origin of the leukaemic cells, for if they were so, the latency period of leukaemia would be much shorter (Lajtha, 1981).

The parameters for the transit times in the mouse granulocyte compartment, as given previously, are too short to account for a latency period of about 200 days for leukaemia which might originate

in cells of this compartment. There are only 2 to 3 days between recovery of CFU-C population and the recovery of mature granulocyte elements in the bone marrow after a lethal dose of nitrogen mustard.

It is unlikely that cells which do not divide ie. mature blood cells, whose chromosomes are damaged by radiation, would contribute to carcinogenesis.

The CFU-S has normally the lowest proportion of cells in S-phase of all identifiable blood cell precursors. All larger number of these pluripotent stem cells are more likely to survive radiation damage and have abberations in DNA from repair of the radiation damage which consequently leads to leukemogenesis.

Although it has been claimed that different types of radiation induce different types of leukaemia (Mole, 1975; Gunz, 1977) the leukaemia types in irradiation induced leukaemias appear to follow a similar distribution to that observed according to ages of leukaemia sufferers. In childhood acute lymphocytic leukaemia is most common, in adulthood and middle age granulocytic (chronic) is most common, in the irradiated populations.

The distributions of leukaemia types in the Japanese bomb survivors are as follows

Mole (1975) and Ishimaru (1971)

Type leukaemia	All ages		0-14 years		15+ years	
	H	N	H	N	H	N
Acute granulocytic	14	7	0	3	14	4
Acute lymphocytic	9	6	6	2	3	4
Acute "other"	11	1	5	1	6	0
Chronic granulocytic	25	3	4	2	21	1
Total	59	17	15	8	44	9

The total number of cases in Nagasaki are somewhat low to make any assessment on the distribution of leukaemia cases.

In old age although the incidence of chronic granulocytic leukaemia still rises, chronic lymphocytic leukaemia is even more common (Gunz, 1977).

The distribution of types of leukaemia reported in all the populations - irradiated and non-irradiated would seem to be influenced by the physiological status of the population.

In childhood the lymphoid system is developing and the thymus is active until the age of puberty. The strongest physiological influence on haemopoietic stem cells for differentiation may be along the lymphoid pathway. In adulthood the dominating physiological stimulus may be for differentiation along the granulocyte pathway. The polymorphonuclear cells are short lived in the blood. The would be granulocyte proliferation in response to infection. After depletion by cytotoxic agents, as with nitrogen mustard, granulocyte elements of the bone marrow are often the first mature cells to recover.

In childhood the majority of leukaemias are acute, and the acute leukaemias for the majority at all ages up to 70. Chronic granulocytic leukaemia occurs rarely but constantly in childhood and then increases in proportion with a broad peak in middle age. Chronic lymphocytic leukaemia is very rare before the age of 40 and increases steadily thereafter (Gunz, 1977).

From data from 11 Cancer Registries chronic granulocytic leukaemia was found to be greater than chronic lymphocytic leukaemia in middle age and chronic lymphocytic leukaemia greater in old age in both male and females (Doll, Muir and Waterhouse, 1970).

Given that different types of leukaemia occur and that one type predominates in different physiological states and the long latency period, radiation induced leukaemia seems most likely to originate in an uncommitted stem cell.

The initial lesion in the stem cell may go through a series of complimentary changes on each cell division which leads to progressive loss of responsiveness to regulatory controls and produces a leukaemic state. Lajtha (1981) postulates a decreased sensitivity of the stem cell to specific inhibitor Lajtha and Wright, 1978; Lord, Wright and Lajtha, 1978; Lord et al, 1976, Riches et al, 1980; Wright and Lord, 1979) or an increased sensitivity to specific stimulator (Lord, Mori and Wright, 1977) gives the transformed stem cell a proliferative advantage over normal stem cells.

However injection of 5 million normal syngeneic bone marrow cells during the lag phase in recovery of CFU-S after 450 R (Fig. 27) (ie. 24 hours after irradiation) markedly reduces the incidence of myeloid leukaemia in CBA/H mice induced by this dose of X-rays.

This effect is not observed when the bone marrow is injected when the CFU-S are recovering (ie. on days 3, 7 and 28 after irradiation - see addendum). If irradiated stem cells had acquired the change in the first 24 hours after irradiation that lead to loss of control by inhibitors and stimulators it might be expected that these cells would have a proliferative advantage over injected normal stem cells and the incidence of myeloid leukaemia would not be altered at any time. If bone marrow is injected later than day one after irradiation, irradiated stem cells already have a proliferative advantage by the virtue of the fact they have been brought into cycle by the depletion of their pool by the irradiation. The loss of proliferative control in leukaemia is not apparent until the cells have reached a committed stage in the haemopoietic system.

Injection of bone marrow does not influence the incidence of non-myeloid leukaemia in CBA/H mice. The incidence of other leukaemias is similar in each group given 450 rads X-rays with or without bone marrow injection on various days after irradiation. These leukaemias were of lymphoid type. There is a small percentage of spontaneous lymphomas in CBA/H mice.

These results are an illustration of a certain independence of lymphoid cells from the pluripotent stem cell which gives rise to erythroid, granulocyte, monocyte and megakaryocyte cell lineages.

The lymphomas in CBA/H mice may indeed involve a different mechanism of induction ie. viral. Injection of marrow as late as 4 days after irradiation confers substantial protection against lymphoma development (Kaplan et al, 1955). The lymphoid complex shows a different pattern of recovery from the bone marrow

after irradiation. There are two phases of recovery in tissues of the lymphoid complex after whole-body irradiation: one occurring at about 10-14 days and the other at 24 days (Sharp and Watkins, 1980). Injection of marrow would therefore protect from radiaton-induced lymphomas for some days after radiation insult. The first phase of recovery would appear to be associated with a radioresistant progenitor while the second depends on repopulation from progenitors generated from the multipotential stem cell (Sharp and Crouse, 1980).

Injection of marrow does not however affect the incidence of lymphoid leukaemias in CBA/H mice suggesting that the induction of these lymphomas is brought about by a different mechanism. Cells from these leukaemias expressed Thy 1-1 and Thy 1-2 antigens (L. Cobb - personal communication) suggesting they were T-cell leukaemias. Trisomy of chromosome 15 was detected in one of these leukaemias (G. Breckon - personal communication). Trisomy of chromosome 15 is the dominating and often the only change in virally and non-virally induced murine T-cell leukaemias (Klein, 1981).

There is a requirement for whole body irradiation rather than local X-irradiation of the thymus to induce lymphoid tumours in C_{57} black mice (Kaplan, 1949; Kaplan and Brown, 1952). This implies that pre-thymic cells in the bone marrow must be irradiated to produce the lymphomas in these mice.

The importance of the physiological status on leukaemia type is illustrated in murine irradiation or virally-induced leukaemias. If thymuses are grafted into thymectomised irradiated mice, the

incidence of lymphomas is appreciably higher in mice bearing either C57B1 or F1 hybrid thymus grafts, than those bearing C3H grafts. The thymuses of C57 black mice may be more 'active' than those of C3H mice.

Gross (1951) found that cell free extracts prepared from spontaneous lymphomas of AKR mice could induce similar lymphatic leukaemias after injection into C3H/Bi mice. He had more success with these experiments than other workers because the inoculated the filtrates and extracts into newborn, rather than adult mice (Kaplan, 1967). The Gross virus induces myeloid leukaemia in thymectomised mice (Gross, 1960) indicating that when the strongest physiological stimulus for differentiation of the transformed cells along a specific pathway is removed the second strongest physiological influence takes precedence.

Leukaemias induced by viruses in mice appear only after a long latency period, in middle-aged or old mice (Klein, 1981). The exception to this is the Friend virus induced erythroleukaemia which has a short latency period. In this case a different mechanism not involving initially, the multipotent stem cell may be involved.

There is a higher incidence of chronic lymphoid leukaemia in old age. This may relate to the long-life span of lymphoid cells, which have long periods in non-cycling states. Chronic lymphocytic leukaemia is absent from all irradiation-induced leukaemias in the irradiated populations studied (Mole, 1975; Gunz, 1977). These populations, however, do not include a large number of aged subjects.

Evidence from radiation induced myeloid leukaemias and virally induced murine leukaemias suggests that the leukaemia is initiated in a cell that is long-lived and normally capable of producing a considerable number of progeny.

The recognition of the structure and controlling mechanisms of the haemopoietic stem cell compartment and confirmation of a stem cell origin of leukaemia may assist to centre searches on a specific lesion or lesions which iniate leukaemic transformation. Progression of the disease, however, may be further complicated by excessive production of normal proteins (Klein, 1981) by the progeny of the transformed stem cell, which, in turn, may induce transformations.

SUMMARY

Depletion of the haemopoietic cells in mice by nitrogen mustard produces a situation where the recovery patterns of the cells reveal their relationships to each other.

The spleen colony forming cells (CFU-S), at present the most primitive pluripotent stem cell detected in mice, preceeds the recovery of cells which form colonies of granulocytes and monocytes/macrophages in agar under suitable culture conditions indicating the ancestral relationship of the CFU-S to the CFU-C.

Depletion by nitrogen mustard of the haemopoietic cells, however, does not create an inability of the system to respond in the normal way to extra stimulus to provide for a specific cell compartment as indicated by the effect of bleeding and erythroid cell proliferation.

The influence of the spleen on the granulocyte precursors (CFU-C) is examined. It's role appears transitory and minor during the recovery from depletion by nitrogen mustard. It's absence exaggerates the response of the femoral CFU-C to bleeding and it plays a greater role in the recovery of erythropoiesis from nitrogen mustard and bleeding than the femoral marrow as indicated by the higher Fe^{59} uptake in the spleen.

The controlled production of granulocytes in normal marrow recovering from nitrogen mustard is illustrated by the large proportion of CFU-C in S-phase at the nadir of the recovery curve and the small proportion of CFU-C in S-phase at the peak of the

recovery curve.

In the leukaemic condition this controlled proliferation is lost.

The rate of division of leukaemic cells in vivo or in vitro does not seem to greatly exceed the rate of division of bone marrow cells recovering from nitrogen mustard when assessed by metaphase accummulation by stathmokinectic agents. Vincristine was assessed to be a more reliable stathmokinetic agent than colcemid for murine myeloid leukaemias.

The nature of the spleen colony forming cell in leukaemia is examined and is not consistently identical to that in normal bone marrow.

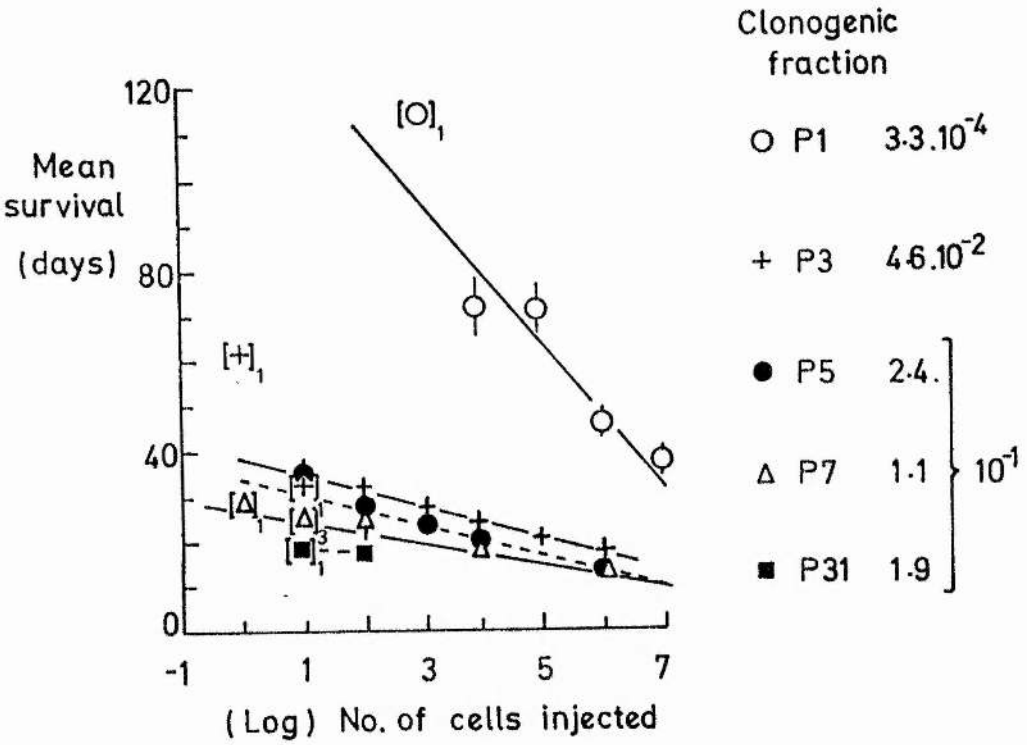
Radiation-induced myeloid leukaemia does however appear to be iniated in a stem cell of at least as primitive a level as the CFU-S. The proliferation of the CFU-S during recovery from radiation is discussed with relevence to prevention of induction of leukaemia by injection of normal bone marrow one day after a leukaemogenic dose of radation and the lack of prevention by bone marrow given on later days.

Although it has been postulated that the transformed stem cell 'slips away' from the control of inhibiting factors released by bone marrow cells, giving it a proliferative advantage over normal stem cells, the lesion may not be effectively leukaemogenic and unresponsive to controlling mechanisms until the cell has gone through a number of divisions and series of complimentary changes in it's genetic coding and entered a specific differentiation compartment in the

haemopoietic system.

In view of the different kinetics of the lymphoid system from the myeloid and erythroid ,it is possible some lymphoid leukaemias may originate in the lymphoid cells.

ADDENDUM

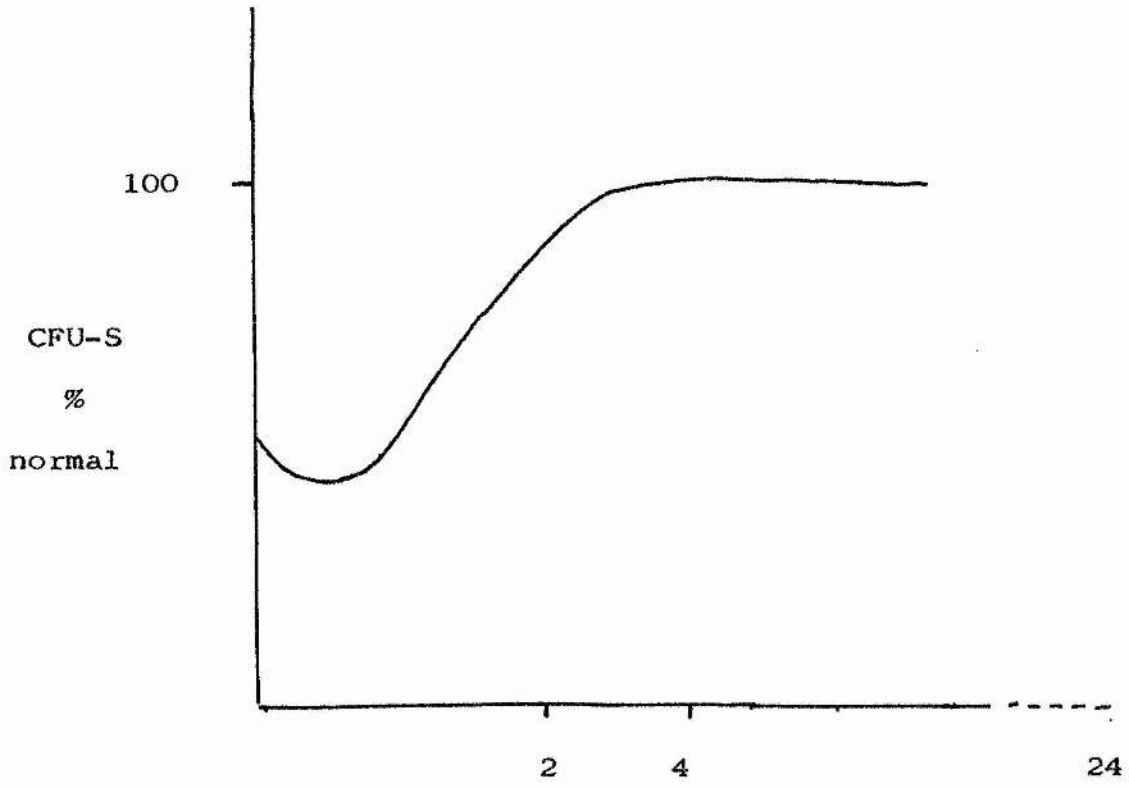


This diagram shows the shortening survival times of mice with successive passages of transplanted myeloid leukaemic cells from line XIX. The survival time of mice is lower than 35 days when the time for the leukaemic cells to grow out to 10^6 cells would be longer if estimated from the number of cells entering metaphase. (ref. pages 176-177)

The clongenic fraction is estimated from a regression on the poisson proportions of 'takes' (i.e. proportions of mice killed) by the transplanted leukaemic cells.

Effect of injection of normal bone marrow cells after irradiation on the incidence of leukaemia.

The following table shows the incidence of leukaemia in irradiated mice which were given an injection of 5.10^6 normal bone marrow cells on days 1, 3, 7 and 28 after irradiation. It can be seen that the bone marrow is only effective in reducing the incidence of myeloid leukaemia when administered during the lag phase of recovery of the CFU-S from the given dose of irradiation as seen in figure 27.



Recovery of CFU-S after 450 rads. X- irradation.
(Hendry J.H. BIR meeting . London, 1979)

STATISTICAL PROCEDURES

Linear regression for binomial proportions.

(pages 94-96)

Program description

This program fits the straight line

$y = a + bx$.

to a set of observations (x_1,y_1), (x_2,y_2) ---(x_m,y_m),

where the y's are **binomial** proportions , say $y_i = \frac{r_i}{n_i}$

The values of a and b are computed iteratively by the maximum likelihood.

At each iteration, the program uses the current approximations a_o,b_o to a and b in order to calculate:-

1. improved approximations a_1,b_1 and their estimated standard errors;
2. the variance-covariance matrix (V) of a_1,b_1 ;
3. the weighed sum of squares of deviations of the current line, its degrees of freedom , and the corresponding mean-square (this information is used to test the goodness of fit of the line.)

Formula used :-

$$b_1 = \frac{[x,y]}{[x,x]} \qquad a_1 = \frac{\Sigma wy - b\Sigma wx}{\Sigma w}$$

sum of squares of deviations from line (SSR)

$$(SSR) = [y,y] - b_1[x,y]$$

degrees of freedom = m-2

mean-square devation from line $(s^2) = \frac{SSR}{m-2}$

$$V = \begin{bmatrix} \frac{\Sigma wx^2}{(\Sigma w)[x,x]} & -\frac{\Sigma wx}{(\Sigma w)[x,x]} \\ -\frac{\Sigma wx}{(\Sigma w)[x,x]} & \frac{1}{[x,x]} \end{bmatrix}$$

$$\text{estimated S.E(a)} = \begin{cases} \sqrt{V_{11}} & \text{if the fit is satisfactory} \\ \sqrt{s^2 V_{11}} & \text{otherwise} \end{cases}$$

$$\text{estimated S.E(b)} = \begin{cases} \sqrt{V_{22}} & \text{if the fit is satisfactory} \\ \sqrt{s^2 V_{22}} & \text{otherwise} \end{cases}$$

where

$$[x,x] = \Sigma wx^2 - \frac{(\Sigma wx)^2}{w}$$

$$[x,y] = \Sigma wxy - \frac{(\Sigma wx)(\Sigma wy)}{\Sigma w}$$

The weighting factors in the above equations are given by:-

first iteration	second iteration
$w_i = \frac{n_i}{f_i(1-y_i)}$	$w_i = \frac{n_i}{f_i(1-f_i)}$

when $f_i = a_0 + b_0 x_i$.

Thus it is not necessary to provide first approximations to a and b to start the iterative procedure.

Goodness of fit is ordinarily tested by referring SSR to tables of the chi-squared distribution with m-2 degrees of freedom. However , this test needs to be modified if the y's are the pooled results for several samples and there is significant heterogeneity, overall, between samples.

In this case, if s_1^2 is the pooled heteroçeneity factor between samples, with ν degrees of freedom, goodness of fit is tested by the variance- ratio.

$$F = \frac{s_2^2}{s_1^2} \quad \text{on } (m-2, \nu) \text{ degrees of freedom.}$$

The estimated standard errors of a_1 and b_1 are then computed according to the following rule:-

$$\text{estimated S.E.}(a_1) = \begin{cases} \sqrt{s_1^2 V_{11}} & \text{if the fit is satisfactory} \\ \sqrt{s^2 V_{11}} & \text{otherwise} \end{cases}$$

$$\text{estimated S.E.}(b_1) = \begin{cases} \sqrt{s_1^2 V_{22}} & \text{if the fit is satis factory} \\ \sqrt{s^2 V_{22}} & \text{otherwise} \end{cases}$$

Paired Comparison of Binomial Proportions.

Program Description.

The program performs a paired comparison of two treatments in randomized blocks. In the case of comparing the proportions of cells in metaphase in the tissues of mice with myeloid leukaemia the bone marrow and the spleen enviroments are regarded as treatments.

The design is as follows :

	Treatment 1		Treatment 2	
Block	Sample response	Sample size	Sample response	Sample size
1	r_{11}	n_{11}	r_{21}	n_{21}
2	r_{12}	n_{12}	r_{22}	n_{22}
3	r_{13}	n_{13}	r_{23}	n_{23}
--	--	--	--	--
--	--	--	--	--
m	r_{1m}	n_{1m}	r_{2m}	n_{2m}

The test statistic μ is given by

$$\mu = \frac{\sum_{i=1}^{m} w_i d_i}{\sqrt{\sum_{i=1}^{m} w_i \hat{p}_i \hat{q}_i}}$$

where $w_i = \dfrac{n_{1i} n_{2i}}{n_{1i} + n_{2i}}$

$$d_i = \frac{r_{1i}}{n_{1i}} - \frac{r_{2i}}{n_{2i}}$$

cont.

$$\hat{p}_i = \frac{r_{1i} + r_{2i}}{n_{1i} + n_{2i}} \quad , \qquad \hat{q}_i = 1 - \hat{p}_i$$

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Dose of radiation	Day of bone marrow injection	Percentage of Leukaemias	Percentage of myeloid leukaemia	Mean Survival Time	Percentage non-myeloid leukaemia	Mean Surival Time
				$\bar{x} \pm SD$		$\bar{x} \pm SD$
450	none	24.44	17.77	434.25 ± 178.19	6.67	340.66 ± 126.77
450	1	11.66	5.00	559.33 ± 145.77	6.66	459.75 ± 131.57
450	3	23.25	18.60	538.75 ± 95.23	4.60	528.50 ± 67.18
450	7	17.77	15.55	565.85 ± 127.33	2.22	396.00
450	28	28.88	24.44	449.81 ± 151.40	4.44	438.50 ± 62.93

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