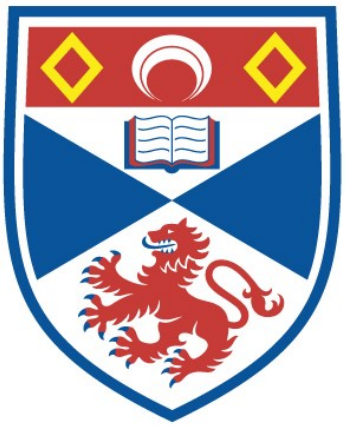
# CELLULAR RELATIONSHIPS IN THE DEVELOPING MURINE LIVER

Allison Blair

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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# Cellular Relationships In The Developing Murine Liver

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Thesis submitted for the degree of Doctor of Philosophy

School of Biological and Medical Sciences

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## Dedication

To my Aunt, Margaret Gordon for all her care, support and encouragement over the past 25 years.

## Abstract

Haematopoietic activity in the murine fetal liver was observed to increase from the 12th day of gestation to reach a peak on the 15th day of gestation. Hepatic haematopoiesis is predominantly erythroid. As maturation proceeds the numbers of myelopoietic cells increase. Haematopoietic activity declines after the 15th day of gestation, though haematopoietic cells were still obvious in the liver of the 10 day neonate.

Cellular interactions were studied in vivo with the electron microscope and in vitro using long term cultures. Haematopoietic cell clusters were abundant in fetal liver both in vivo and in vitro. Macrophages and hepatocytes act as the central supporting cells of these clusters. There was evidence of communication between the central supporting cells and the blood cells precursors, which may be essential for the maintenance of haematopoiesis both in vivo and in vitro.

A high proportion of early HPP-CFC derived from fetal liver are synthesising DNA, while HPP-CFC derived from adult bone marrow are relatively quiescent. The kinetic properties of the HPP-CFC population are very similar to those of the CFU-S population both in fetal liver and in adult bone marrow. A high proportion of GM-CFC which are a more mature class of progenitor cells were cycling in both fetal liver and adult bone marrow.

Fetal liver extract was shown to have an overall inhibitory effect on haematopoiesis in normal bone marrow and irradiated bone marrow and on colony formation by leukaemic cells. Detrimental effects on normal bone marrow cells were observed only with high concentrations of fetal liver extract.

Fetal liver blood cell precursors were found to adhere preferentially to bone marrow stromal layers, which could not be attributed to the kinetic properties of these cells. This preferential adhesion may promote the in vivo accumulation of haematopoietic stem cells derived from fetal liver in the medullary cavities.

I, Allison Blair, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree of professional qualification.

Signed Date 20/1/94

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No. 12 on the 1st October 1990 and as a candidate for the degree of Ph.D. on the 1st October 1990.

Signed Date 20/1/94

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor Date 20. 1. 1994.

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Finally to Pete, for the occasional meal, very rare truffle eggs and for being gorsey on my behalf, while I was writing this thesis.

# Contents

# Abbreviations

| | |
|---|---|
| AcSDKP | Acetyl-N-Ser-Asp-Lys-Pro |
| ADA | Adenosine Deaminase |
| AML | Acute Myeloid Leukaemia |
| Ara-C | Cytosine Arabinoside |
| bc | Band Cells |
| Bcp | Blood Cell Precursors |
| BFU-E | Burst Forming Unit-Erythroid |
| Bl-CFC | Blast Colony Forming Cells |
| BM | Bone Marrow |
| BMT | Bone Marrow Transplantation |
| BPF | Burst Promoting Factor |
| BSA | Bovine Serum Albumin |
| CAM | Cell Adhesion Molecule |
| CFC-mix | Mixed Colony Forming Cells |
| CFU-E | Colony Forming Unit-Erythroid |
| CFU-GM | Colony Forming Unit-Granulocyte Macrophage |
| CFU-Meg | Megakaryocyte Colony Forming Units |
| CFU-S | Colony Forming Unit-Spleen |
| CML | Chronic Myeloid Leukaemia |
| CMV | Cytomegalovirus |
| $CO_2$ | Carbon Dioxide |
| CSA | Colony Stimulating Activity |
| CSFs | Colony Stimulating Factors |
| DNA | Deoxyribonucleic Acid |
| Eb | Erythroblast |
| ECM | Extracellular Matrix |
| EGF | Epidermal Growth Factor |
| EGM-CSF | Erythroid Granulocyte Macrophage Colony Stimulating Factor |
| Eos-CFC | Eosinophil Colony Forming Cells |
| Epo | Erythropoietin |
| ES cells | Embryonic Stem Cells |
| FL | Fetal Liver |
| FLE | Fetal Liver Extract |
| FLT | Fetal Liver Transplantation |

| | |
|---|---|
| FU | Fluorouracil |
| G | Granulocyte |
| GAG | Glucosaminoglycan |
| G-CSF | Granulocyte Colony Stimulating Factor |
| GM-CFC | Granulocyte-Macrophage Colony Forming Cells |
| GM-CSF | Granulocyte-Macrophage Colony Stimulating Factor |
| GvHD | Graft Versus Host Disease |
| H | Hepatocyte |
| HbA | Adult Haemoglobin |
| HbE | Embryonic Haemoglobin |
| HGF | Haematopoietic Growth Factors |
| HIM | Haematopoietic Inductive Microenvironment |
| HLA | Human Leukocyte Antigen |
| HP5b | (pEEDCK) |
| HPP-CFC | High Proliferative Potential Colony Forming Cells |
| HSC | Haematopoietic Stem Cells |
| HS-PG | Heparan Sulphate Proteoglycan |
| HUC | Human Umbilical Cord Cells |
| IL | Interleukin |
| INT | 2(4 iodophenyl)-3(4 nitrophenyl)-5 phenyltetrazolium chloride |
| L | Lymphocyte |
| LTBMC | Long Term Bone Marrow Cultures |
| LTFLC | Long Term Fetal Liver Cultures |
| M | Macrophage |
| mb | Myeloblasts |
| M-CSF | Macrophage Colony Stimulating Factor |
| Meg-CFC | Megakaryocyte Colony Forming Cells |
| Mg | Megakaryocyte |
| MIP-1α | Macrophage Inflammatory Protein-1α |
| Mn | Monocyte |
| MP | Methylprednisolone |
| mt | Metamyelocytes |
| N | Neutrophil |
| NaOH | Sodium Hydroxide |
| NBM | Normal Bone Marrow |
| NK | Natural Killer |
| PBSC | Peripheral Blood Stem Cells |

| | |
|---|---|
| pEb | Proerythroblast |
| pm | Promyelocytes |
| PTX | Pentoxyfylline |
| RBM | Regenerating Bone Marrow |
| RER | Rough Endoplasmic Reticulum |
| Rh 123 | Rhodamine 123 |
| s | Segmented Nuclei |
| SCID | Severe Combined Immune Deficiency |
| SE | Standard Error of Mean |
| TNF | Tumour Necrosis Factor |
| TGF | Transforming Growth Factor |

# Chapter 1

# Introduction

## 1.1 Haematopoiesis

The bone marrow has been designated the seedbed of the blood (Tavassoli 1980), which provides an ideal microenvironment for the proliferation and the differentiation of blood cell precursors. The stem cell population remains relatively quiescent (Lord et al 1976), while their differentiating progenies mature into various blood cells that are delivered into the circulation on demand. This entire process is known as haematopoiesis. Although it is appropriate to designate the bone marrow as the seedbed of the blood in adult mammals, blood cell production in the bone marrow is preceeded by blood cell production in the yolk sac, in the liver and in other extra-medullary sites.

## 1.2 Haematopoietic Development During Ontogeny

The earliest blood cells appear outside the body of the embryo in blood islands which form in the wall of the yolk sac at about the third week of gestation in the human embryo. In these blood islands cells which are usually considered to be of mesenchymal origin (Sadler 1985), but which have been attributed to endodermal origin (Gladstone & Hamilton 1942), are arranged in small groups. The peripheral elements of which give rise to endothelium, while the central elements give rise to the first blood cells. These haemocytoblasts are initially devoid of haemoglobin but gradually their nuclei become smaller, their cytoplasm becomes more dense and embryonic haemoglobin (HbE) begins to appear. The haemocytoblast has become a primordial erythroblast (Bessis 1973).

The liver begins to function as a haematopoietic organ around the third month of gestation and continues to do so until the time of birth. Erythropoiesis is the predominant form of haematopoiesis in the fetal liver (Thomas, Russell & Yoffey 1960). The first granulocytes appear shortly thereafter and steadily increase in number, megakaryocytes are also seen. Electron microscopical techniques have demonstrated the existence of ferritin and rhopheocytotic vesicles (Sorenson 1960). Haematopoiesis is entirely extravascular at this stage.

Haematopoiesis is initiated in the thymus and the first primitive lymph nodes appear in embryos at approximately 3 months of gestation (Keleman et al 1979). Haematopoiesis is often said to begin in the spleen during the 5th month of gestation and to persist for a month or so, although the role of the spleen as a preferential haematopoietic site has been questioned. The haematopoietic function of the liver decreases from the 5th month and ceases shortly before birth, during this period medullary, splenic and nodal haematopoiesis are initiated (Fukuda 1974).

The pattern of haematopoiesis is different in the liver than that of the bone marrow. The liver is particularly erythropoietic whilst the bone marrow is particularly granulopoietic (Thomas et al 1960). The preponderance of erythropoiesis and paucity of myelopoiesis, lymphopoiesis and megakaryocytopoiesis during intrauterine development may be due to decreased demand for such cells during this period on account of a protected germ-free environment. Unlike myeloid precursors, there is a tendency for lymphoid cells to increase in number as the fetal age advances (Sharma, Bhargava & Kochupillai 1985).

As hepatic haematopoiesis declines, the bone marrow, spleen, thymus and lymph nodes assume their adult specialisations. The mesenchymal cells, which

gave rise to the primitive erythroblasts, are transformed into quiescent fixed cells which retain their potential to give rise to new haematopoietic islands (Bessis 1973).

Theories of the development of mammalian haematopoiesis have focused around two viewpoints:- One hypothesis proposes a model whereby a single stem cell pool originates in the yolk sac and migrates in the bloodstream to colonise the liver parenchyma (Moore & Metcalf 1970; Johnson & Moore 1975). During this course of time the stem cells undergo a gradual change both morphologically and in haemoglobin synthesis. The primordial erythroblasts are succeeded by a second generation of smaller cells which appear in the embryo of 10-12mm in length, multiply actively and have completely replaced primordial erythroblasts in a 50mm embryo. These definitive erythroblasts contain only adult haemoglobin (HbA) (Brotherton et al 1979). The other model suggests that hepatic haematopoietic tissue arises directly from the transformation of liver mesenchymal cells independently of vitelline haematopoiesis (Cole & Paul 1966; Rifkind, Chui & Epler 1969; Tarbutt & Cole 1970; Samoylina, Gan & Medvinski 1990) or from endodermal cells (Thomas et al 1960; Riches & Hoyes 1972), which transform into lymphoid elements and eventually give rise to haemocytoblasts (Hoyes, Riches & Martin 1973).

Pre-haematopoietic fetal liver has been shown to be capable of supporting blood cell production if it was supplied with an exogenous source of haematopoietic stem cells. However, pre-haematopoietic fetal liver did not become a site of active blood cell production when grafted into an irradiated adult host (Johnson & Moore 1975). Long term repopulation by embryonic donor cells was reported following injection of yolk sac cells into anaemic mice, when the liver was the main site of haematopoiesis (Toles et al 1989). A dramatic depletion of the yolk sac BFU-E pool in human embryos occurs at the

5th week of gestation, when BFU-E become detectable in the blood stream and their number in the liver parenchyma increases considerably. The depletion of yolk sac BFU-E could not be attributed to differentiation as the number of yolk sac CFU-E did not increase (Migliaccio et al 1986). These results were interpreted as supporting the cell seeding hypothesis and invalidating the alternative theory of induction (Rifkind et al 1969). However other reports do not support the predominant role of the yolk sac.

Dieterlen-Lievre (1992), demonstrated two distinct waves of haematopoiesis in the avian system using quail-chick chimeras. The first wave is produced in the yolk sac and the progeny of this wave are transient. The second wave of haematopoiesis was of intra-embryonic origin, thought to be produced in the aortic region, the progeny of this wave are not transient. Data on the murine system also point to a succession of stem cells during ontogeny. CFU-S are initially detected in the body of the murine embryo, then in the yolk sac and blood, and finally in the liver (Samoilina et al 1990). The doubling time of CFU-S was more rapid at the initiation of hepatic haematopoiesis. It was concluded that yolk sac haematopoietic stem cells were incapable of differentiating into CFU-S, instead the CFU-S were derived from intra-embryonic stem cells, which are distributed to the yolk sac and liver via the circulation. The earliest T-cells detected in the fetal thymus are characterised by the Vγ3 gene transcript (Ikuta et al 1990). It has been shown that only haematopoietic stem cells derived from fetal liver could give rise to T cells with this gene arrangement, adult bone marrow derived stem cells could not give rise to these characteristic early T-cells. Furthermore differentiation of early fetal stem cells into Vγ3 T-cells only takes place in the thymic microenvironment of the fetus (Ikuta et al 1990). These observations suggest the existence of successive populations of stem cells in mammalian embryos.

To date the arguments over the two hypotheses have not been conclusively

resolved. The question remains whether the successive populations of stem cells emerge independently, like the avian systems, hepatic haematopoiesis may arise *de novo* in a similar manner to that of the yolk sac. Alternatively, the successive stem cell populations may be the result of microenvironmental influences on seeded yolk sac derived stem cells.

## 1.3 Renewal and Commitment of Stem Cells

A structured hierarchy of multipotential haematopoietic stem cells (HSC), assayable in vitro and in vivo exists. The earliest stem cells have a high self-renewal capacity and are the most important cells in the establishment of a graft. There are two separate stem cell compartments in lymphohaematopoietic organs - lymphoid and haematopoietic, both of which are derived from a totipotent stem cell compartment. Populations of haematopoietic stem cells are defined by two characteristics:- they have a self-renewal capacity, in order to maintain the size of the compartment at a fixed level, secondly they are multipotent and can thereby differentiate to produce specific cell lineages which ultimately form mature blood cells (Lajtha 1979). Several models have been proposed to explain the nature of commitment of HSC's. The presence of several models reflects the uncertainty of this subject which is of great theoretical importance. If a stem cell vacates its compartment to differentiate, haematopoiesis cannot be maintained, however if the stem cell only undergoes self-renewal it cannot respond to the body's demands for blood cell formation - both processes must take place in parallel. Each model focuses on a different aspect of stem cell function however, they are not necessarily exclusive of each other.

The stochastic model proposed by Till, McCulloch & Siminovitch (1964) suggests that the decision to remain in the stem cell compartment or to yield progeny committed to differentiation is governed by a probablistic rule. The

control of the stem cell compartment may be achieved by changes in a distributional parameter, the probability of self-renewal of stem cells.

The haematopoietic inductive microenvironment (HIM) model proposed by Trentin and his associates (1970, 1989) proposes that the commitment of pluripotent HSC to progenitor cells is determined by a specific inductive microenvironment surrounding individual stem cells. The microenvironment is not thought to be obligatory for stem cell commitment (Ogawa, Porter & Nakahata 1983), instead it may play a supportive role in the self-renewal of stem cells as observed in colony forming unit-spleen (CFU-S) maintenance in Dexter Cultures (Dexter, Allen & Lajtha 1977).

The third model is that of "stem cell competition", which proposes that humoral factors such as erythropoietin (Epo) and colony stimulating factors (CSFs) play an active role in stem cell commitment (Ogawa et al 1983). However it is generally believed that development of receptors for these growth factors occurs post hoc to the commitment.

The most viable model proposes that stem cell commitment is governed by progressive and stochastic restriction in the differentiation potentials of HSC. This model is supported by documentation of oligopotent haematopoietic progenitors which show differentiating potential for various combinations of 2 or 3 lineages (Humphries, Eaves & Eaves 1979; Ogawa et al 1983; Allen 1992).

## 1.4 Haematopoietic Regulation

In view of the extent of the proliferative activity which is required simply to maintain the steady state levels of mature cell production, it is not surprising that occasional mistakes are made during replication resulting in the emergence of tumour cells. Obviously many controls are built into the system

to maintain their integrity both intrinsic - genetic housekeeping of cells and extrinsic influences in the form of environmental components (Dexter 1989).

Many environmental components have been described which can regulate both proliferation and differentiation of HSC. These include interaction of cells with growth regulatory factors; direct cell-cell contact and interaction of cells with extracellular matrix molecules known to be important in the development of embryonic and adult tissue. One of the most intriguing problems is to determine how these factors combine to regulate homeostasis.

1.4.1-1 Growth Factors

Blood cell formation is controlled by a group of specific glycoprotein growth factors which initiate a mitogenic response by binding to a specific receptor usually located at the cell surface. The application of recombinant DNA technology allows molecular cloning of genes for these substances and production of synthetic material. A substantial number of factors have been characterised this way and named according to the lineage stimulated in vitro (Devalia & Linch 1991). In other cases haematopoietic growth factors have been recognised as lymphocyte secretory products and thus included in a list of factors known as interleukins (IL-1 - IL-7) or lymphokines (Plumb & Pragnell 1988; Nicola 1989). The first of these growth factors to be identified was erythropoietin (Epo), which is produced in the kidney and stimulates erythropoiesis in vivo in haematopoietic sites and also in vitro in culture systems (Devalia & Linch 1991).

Classes of growth factors are recognised: Class I are not lineage specific, they act mostly on pluripotent or oligopotent cells. Interleukin 3 (IL-3) or multi-CSF for example can recruit clonal proliferation and development of mixed colony forming cells (CFC-mix), burst forming unit-erythroid (BFU-E), granulocyte-macrophage colony forming cells (GM-CFC), eosinophil colony forming cells

(Eos-CFC) and megakaryocyte colony forming cells (Meg-CFC) in addition to facilitating growth and survival of CFU-S. Granulocyte macrophage colony stimulating factor (GM-CSF) is a potent stimulus for growth and development of GM-CFC, Eos-CFC and in high concentrations can also recruit CFC-mix into proliferation (Dexter 1989).

Class II CSF's are more restricted in their target cells, they act on more mature cells and are required later on in the course of development. Granulocyte colony stimulating factor (G-CSF) has a direct effect only upon neutrophil specific restricted precursor cells (G-CFC), while macrophage-CSF (M-CSF) stimulates both GM-CFC and macrophage colony forming cells (M-CFC) to undergo growth and development into macrophages (Stanley & Heard 1977).

There is also evidence for a third class of factors, those with a synergistic activity for IL-3 or M-CSF. Acting alone these factors have no colony stimulating activity, however when added to appropriate growth factors of the other two classes their action is considerably enhanced (Stanley et al 1986).

The site of production of these haematopoietic growth factors has been the subject of intensive investigation. It appears that all organs can potentially synthesise CSF, however certain cell types in particular are known to produce these factors:- T-lymphocytes, monocytes, endothelial cells and macrophages (Devalia & Linch 1991).

1.4.1-2 Inhibitory Factors

Negative regulators of haematopoiesis are also considered to play an important role in the maintenance of a quiescent stem cell population. They are regarded to be responsible for the low number of stem cells engaged in DNA synthesis (Zipori & Hanigwachs-Sha'anani 1992). Inhibitory factors are also regarded to oppose the actions of stimulatory factors on the same cells and the

balance of these two opposing factors determines whether or not a cell synthesises DNA (Axelrad 1990). Furthermore, such an arrangement has the advantage of permitting rapid proliferative responses to changes in haematopoietic demand. There is now increasing awareness that regulation of haematopoiesis, under normal conditions, involves inhibitory and stimulatory molecules (see Axelrad 1990; Wright & Pragnell 1992; Zipori & Hanigwach-Sha'anani 1992 for review).

Transforming growth factor β (TGF-β) was the first negative regulator of haematopoiesis to be cloned (Keller et al 1990). It is a potent inhibitor of haematopoietic stem cell and progenitor cell proliferation, however it has reduced effects on mature cells (Wright & Pragnell 1992). HP5b (pEEDCK) is a pentapeptide purified from extracts of rat bone marrow, which inhibits colony formation by GM-CFC and CFU-S (Axelrad 1990). A tetrapeptide inhibitor (Ac-N-Ser-Asp-Lys-Pro or AcSDKP), was first reported by Frindel and colleagues in 1977 (Lenfant et al 1989). This inhibitor prevents recruitment of CFU-S into S-phase, thereby protecting cells from S-phase cytotoxic agents, it has no effect on actively proliferating cells (Lenfant et al 1989).

A negative regulatory protein has been detected in murine bone marrow, which also inhibits DNA synthesis in the CFU-S population (Lord et al 1976). The active molecule of this inhibitor is thought to be macrophage inflammatory protein (MIP-1α) (Plumb et al 1991; Wright & Pragnell 1992), which is produced by macrophages and has no inhibitory effect on mature progenitor cells (Broxmyer et al 1990).

### 1.4.1-3 Clinical Applications of Regulatory Factors

Erythropoietin, G-CSF, GM-CSF, IL-1, IL-3 and IL-4 have all reached the clinical arena. Epo has been successfully used in the treatment of anaemia in

end stage renal diseases. The combination of G-CSF and GM-CSF has been used to reduce the period of neutropenia following conventional chemotherapy, autologous bone marrow transplantation and myelodysplastic syndromes (Devereux & Linch 1989). It has also more recently been used to enhance granulopoiesis and to reduce the rate of infection in patients with AIDS (Dexter 1989).

IL-1 together with GM-CSF have been shown to have a radioprotective effect by promoting granulocyte recovery following chemotherapy. IL-1 given intraperitoneally in mice can enhance harvesting of peripheral blood stem cells. One of the most exciting potential applications is the use of HGF's to allow dose escalation enabling high doses of drugs or radiotherapy to be given over a shorter period of time. HGF's may enable more efficacious treatment rather than just reduced toxicity. It is likely that combinations of HGF's will be more effective than single growth factors alone (Devereux & Linch 1989).

Negative regulatory factors also have important clinical applications. MIP-1α, as a potent inhibitor of CFU-S proliferation should have a therapeutic potential to protect stem cells from damage during cancer chemotherapy (Broxmyer et al 1990). AcSDKP can protect mice from lethal doses of cytosine arabinoside (Ara-C), without protecting malignant cells from its cytotoxic effects (Lenfant et al 1989). It may be possible to use stem cell inhibitors in the treatment of leukaemia. Chronic myeloid leukaemia (CML) may be caused by loss of sensitivity to inhibitory factors leading to unregulated proliferation of leukaemic cells. It may be possible to use drug purging regimens to selectively eliminate leukaemic cells (Eaves et al 1986).

The role of haematopoietic regulatory factors in sustaining normal haematopoiesis is not well defined. Under normal conditions, quiescence in the

the stem cell compartment is essential - haematopoietic regulatory factors are not solely responsible for the maintenance of a quiescent state.

### 1.4.2 Haematopoietic Inductive Microenvironment

The term haematopoietic inductive microenvironment (HIM) is usually restricted to a localised environment in the stroma of haematopoietic organs which directly influences the haematopoietic cells and events governing haematopoiesis (Wolf 1979). Trentin (1970) was the first to approach this modern concept proposing the action to be that of a stromal cell line inducing differentiation of HSC in erythroid, granuloid or megakaryocytic lineages. The stromal cells were responsible for inducing commitment of a stem cell, hence the term haematopoietic inductive microenvironment was proposed.

Effective haematopoiesis is the product of interplay between HSC and it's supporting stroma, the latter providing a favourable environment for sustained and regulated proliferation and differentiation. A major function of the microenvironment is to protect the stem cell pool from excess differentiation by creating conditions which favour self-renewal. The stem cell niche hypothesis proposed by Schofield (1978), suggests that certain microenvironmental cells of the marrow stroma can maintain the stem cells in a primitive state, thus preventing differentiation. The cellular architecture of the niche is unknown, however several observations support the idea of microenvironments which facilitate either granulocyte or erythroid cell development in vivo (Trentin 1970; Wolf 1979) and in vitro (Dexter et al 1977; Allen & Dexter 1984).

Haematopoietic stromal tissues may thus control the organisation of haematopoietic organs by restricting growth and differentiation of particular cell types and by inducing self-renewal of stem cells. The most compelling evidence for this derives from the localisation of haematopoiesis to specific

sites within the body (Trentin 1989). It is well documented that in normal animals HSC circulate freely in the peripheral blood (Loutit 1968; Tavassoli 1980; Bentley 1982), however Till & M$^{c}$Culloch (1961) reported haematopoietic colonies developed only in the bone marrow and spleen in irradiated animals which had been reconstituted with donor bone marrow cells. HSC will only proliferate and differentiate in the haematopoietic tissue. These observations suggest that certain organs provide microenvironments which are unique in their capacity to support haematopoiesis (Bentley 1982). Other functions include influencing megakaryocyte differentiation and initiation of DNA synthesis which occurs in precursor cells resident in the adherent microenvironment (Choudhury & Sparks 1989).

1.4.2-1 Evidence for the Role of Microenvironment in Haematopoietic Regulation

In the adult haematopoiesis is restricted to the marrow and, to a lesser extent other haematopoietic organs. This is evidenced by the "homing" of transfused marrow cells to the marrow and spleen of irradiated mice (Dexter et al 1977; Aizawa & Tavassoli 1988). In the embryo haematopoiesis begins in the yolk sac and finally settles in the bone marrow. As the stroma of one organ becomes unsuitable, HSC can move in the circulation and home to a more favourable microenvironment (Tavassoli et al 1990).

After high-dose radiation a biphasic aplasia is observed, the early phase is attributed to destruction of HSC, the late aplasia is the result of injury to the stroma. Subsequent recovery from insult by irradiation is invariably preceded by development of a stromal architecture, only when this has been functionally established does haematopoiesis resume (Bentley 1982).

Mutant anaemic Sl/Sl$^{d}$ mice have defective microenvironments while W/W$^{v}$ mice have defective stem cells. The two systems are cross-correcting both in

vivo and in vitro (Bernstein 1970).

The most direct evidence comes from the observation that cultured adherent cells, derived from primary explants of bone marrow cells, apparently stromal in origin, provide a microenvironment which supports haematopoiesis in vitro. This evidence comes from the continuous bone marrow culture system, described by Dexter et al (1977), who were able to reconstitute the interactions of stromal cells and HSC in liquid culture. A layer of adherent non-haematopoietic cells develops on the floor of the culture flask which can support HSC seeded onto it for extensive periods of time.

1.4.2-2 Bone Marrow Stromal Composition

(i) In Vivo

Haematopoiesis does not occur in vivo in the absence of site specific structures which both support and modulate stem cell expression. Reticular cells and macrophages are the two major stromal components in the bone marrow and other haematopoietic organs. Additional cellular components include endothelial sinusoids, which are coated by adventitial reticular cells, and adipocytes. Maturation of haematopoietic cells takes place within the intersinusoidal spaces closely associated with reticular cell processes (Singer, Keating & Wright 1985).

Reticular cells are histogenetically recognisable from haematopoietic cells, there are two types of reticular cells of the haematopoietic cord: adventitial and fibroblastic. Adventitial cells are located around the venous sinuses, partially covering the endothelial abluminal side (Shaklai 1989). They have slender cytoplasmic processes which extend deep into the haematopoietic compartment and form a continuous anastomosing bridgework between sinuses. Fibroblastic reticular cells are characterised by their long thin

cytoplasmic extensions which envelop maturing blood cells.

Macrophages are found topographically in 3 sites, as a central macrophage in the erythroblastic island, perisinal macrophages on the abluminal side of the sinus endothelium and also dispersed between developing myeloid cells of the haematopoietic cord (Shaklai 1989). The central macrophage is an important constituent of in vivo erythropoiesis. It is possible that the central macrophage elaborates certain factors which are transferred to developing erythroid cells. The rhopheocytotic theory first proposed by Policard & Bessis (1962), for the transfer of ferritin, has not been conclusively proven by other investigators. However the assumption that the macrophage has a trophic function is plausible and is generally accepted.

(ii) In Vitro

Long term Dexter cultures have provided an invaluable model to study the in vitro microenvironment. In vitro the stroma also consists of several cell types. Stromal cells have the common characteristic of adhering to the substratum. The major component is a fibroblastic or epithelial cell which is capable of producing collagen types I and III in addition to fibronectin (Bentley 1982; Tavassoli & Takahashi 1982). This cell may be related to the in vivo reticular cells. These cells, together with macrophages endothelial cels and adipocytes, spread out over the surface of the culture dish, providing a "blanketing" floormat for haematopoiesis.

In murine long term bone marrow cultures, macrophages constitute a major component of the stroma. Adipocytes have also been described in continuous marrow culture (Dexter et al 1977). Both macrophages and epithelioid cells can accumulate fat under the conditions of culture, therefore fat cells could be considered as a culture related phenomenon, however other groups have demonstrated that fat cells do not derive from lipid-laden macrophages (Allen

& Dexter 1984). The presence of endothelial cells in long term culture has been debated (Bentley 1982), however Zuckerman & Wicha (1983) provided evidence of their existence by the demonstration of factor VIII antigen and other endothelial cell by-products such as collagen type IV and laminin.

1.4.2-3 Hepatic, Splenic & Thymic Microenvironments

It is postulated that haematopoietic inductive microenvironments of different kinds exist in fetal liver, spleen, bone marrow and thymus (Trentin 1989). These individual HIM's determine the differentiation of pluripotent stem cells into each of the haematopoietic lineages. Thomas (1971) compared differentiation of transplanted haematopoietic cells from murine bone marrow, fetal liver and spleen. The cell populations established in the haematopoietic sites were all of similar composition and did not reflect the nature of haematopoiesis in the donor cells. The microenvironments of the host, not the donor cells, were responsible for determining the pattern of haematopoiesis. Erythropoietin may therefore induce erythroid differentiation of only those stem cells under the influence of an erythroid HIM, like the fetal liver or the spleen. Alternatively Epo may only act as a growth and function stimulator of those stem cells which have been induced by an erythroid HIM into a state of Epo responsiveness (Trentin 1989).

A cellular network of developing hepatocytes is present in the fetal liver and haematopoietic cells are scattered within the network. Ultrastructurally haematopoietic cells have many broad areas of cellular association with fetal hepatocytes such as the rosette formation observed by Medlock & Haar (1983).Their findings were evidence that when hepatic endodermal rudiments were cultured without mesenchyme interactions neither hepatocytes nor haematopoietic cells developed. Desmosome-like structures between erythroblasts and fetal hepatocytes have been observed (Fukuda 1974; Emura, Sekiya & Ohnishi 1984; Emura et al 1985). Emura and his associates (1985)

reported that hepatocytes which were closely associated with immature haematopoietic cells showed marked differentiation compared to those in areas where haematopoiesis had not appeared. These observations together with those of Hata et al (1993) strongly suggest that hepatocytes together with phagocytic cells lining the sinusoids serve as stromal cells in the haematopoietic microenvironment of the fetal liver.

Two main roles of the macrophage associated with haematopoietic cells have been proposed. Yoffey & Yaffe (1980) proposed a scavenger role for ingesting extruded nuclei of erythroblasts, while Policard & Bessis (1962) proposed the transfer of ferritin with some form of information which could influence the development of erythroblasts with which the macrophage was associated. Emura et al (1984) suggest that the erythroblastic islet with its central macrophage in itself is a HIM, controlling maturation and differentiation of erythroblasts.

In the splenic microenvironment mesenchymal and reticular cells have been described as the stromal cells which support haematopoiesis (Medlock & Haar 1983). While in the thymus reticuloendothelial cells are considered responsible for haematopoietic support. The fact that interaction between HSC and a niche occurs, of necessity at close range, might suggest that components of the cell surface and/or local extracellular matrix are involved.

### 1.4.3 Extracellular Matrix (ECM)

The extracellular matrix of BM and stromal cells has recently been a subject of investigation from two aspects: actual involvement of the ECM proteins in attachment and growth of the haematopoietic cells and secondly the role of the matrix proteins in holding CSF's which then act on tightly bound stem cells (Gordon et al 1987). Matrix proteins produced by stromal cells include haemonectin, fibronectin, collagen, laminin and proteoglycans. Collagen

appears to be the major structural protein of bone and bone marrow. Some of the other matrix proteins are thought to play an important role in the promotion of cell-cell adherence and interactions of cells with other components of the organ stroma (Zuckerman, Prince & Gay 1989). The importance of the ECM was demonstrated by a study in which inhibition of the production of ECM components by stromal cells in vitro resulted in the abolition of haematopoiesis (Zuckerman et al 1985).

There is interest as to whether a receptor ligand complex can function as a homing system for haematopoietic and stromal cells. Anklessaria et al (1990) have shown that bone marrow stromal cells expressing a recombinant gene for transforming growth factor α (TGFα), which is known to compete for the same receptor as epidermal growth factor (EGF), stimulate homing of haematopoietic cells which contain the EGF receptor gene. In vitro mixing experiments with EGF sensitised cells, which proliferate in response to EGF, were cocultured with stromal cells producing TGFα. The EGF cells formed connections with the TGFα stroma which were indistinguishable from cobblestone islands in normal long term bone marrow cultures (Anklessaria et al 1987; 1989). Thus the first evidence was provided for juxtacrine stimulation of growth, which is thought to occur during bone marrow stem cell attachment to stromal cells in situ. This was also suggested by Gordon et al (1987) who reported the effect of ECM on the concentration of GM-CSF and other colony stimulating factors in the haematopoietic microenvironment. Components of the membrane associated ECM, in particular heparan sulphate proteoglycans, appear to be important molecules for binding IL-3 and GM-CSF and presenting these to the haematopoietic cells (Gordon et al 1987, 1990; Roberts et al 1988). Erythroid maturation has been shown to be associated with a developmentally regulated change in fibronectin receptors (Vuillet-Gaugler et al 1990), which strongly suggests the fibronectin component of the matrix may regulate the maturation of erythroid cells. Dexter et al (1990) have suggested

that in the bone marrow microenvironment the stromal cells and the ECM molecules secrete microenvironments which preferentially promote development of different cell lineages.

The mechanism by which the majority of stem cells are held in growth arrest is not known. In vitro experimental models cannot explain this mechanism as in almost every case, attachment, proliferation and differentiation follow in independent fashion (Greenberger 1991). The model of attachment without proliferation is the one which may be most relevant to in vivo haematopoiesis. It is more likely that a combination of GF's, ECM and crucial stem cell-stromal cell interactions combine to yield precise regulation of proliferation in the haematopoietic compartment.

## 1.5 Intercellular Relationships

Proliferation and differentiation of stem cells in vivo and in vitro are dependent on close interactions with stromal cells of that particular microenvironment.

### 1.5.1 In vivo

Morphological aspects of human haematopoiesis during embryonic and fetal life have been described extensively (Bessis 1973; Fukuda et al 1974; Emura et al 1984). The erythropoietic cell lineage is the first haematopoietic lineage observed in human embryonic liver at the 5th week of gestation and remains the largest of the haematopoietic cell populations during development. Large mononuclear cells morphologically of the myelomonocytic lineage appear at the 6th week of gestation. These cells are mainly located in the connective tissue of portal spaces (Emura et al 1984). At 5-6 weeks of gestation macrophages and Kupffer cells appear, both cell types increase in number with

age (Timens et al 1990).

Electron microscopical studies have revealed that immature erythroblasts are commonly arranged around a central reticular cell. These "erythroblastic islets" were first described by Bessis in 1959, (Bessis & Breton-Gorius 1962). Erythroblastic islands have been observed in all haematopoietic organs during ontogeny from the yolk sac to the bone marrow, they are particularly prevalent during erythropoietic stimulation. Erythroblastic islands consist of a central reticular cell, usually a macrophage, surrounded by a ring of erythroblasts. Occasionally more than one ring of erythroblasts are observed surrounding the central cell, the more mature cells being always located on the periphery (Bessis 1973). The central macrophage has long stellate cytoplasmic processes which extend in all directions to embrace the maturing erythroblasts, and some cytoplasmic extensions completely surround developing erythroblasts. These cytoplasmic processes frequently invaginate the cytoplasm of the developing erythroid cells. The macrophage contains numerous micropynocytotic and endocytic vesicles, some of which contain ferritin, the end product of haemoglobin digestion. Iron also appears in the central macrophage in the form of haemosiderin which is derived from the breakdown of red cells and the rim of cytoplasm surrounding extruded nuclei (Bessis 1973).

Examination of the islets in fetal liver has revealed that there are two types of central cell: the macrophage and the hepatocyte (Emura et al 1984). Hepatocytes, like the macrophages, are considered to play an important role in the differentiation of erythroblasts. The haematopoietic component of the blood island is not only restricted to erythroblasts, cells of the granulocytic and megakaryocytic lineages have also been observed in close association with the central cells. The islands occupy considerable space in the liver parenchyma, altering the architecture of the cords and inducing irregularities in the profiles of cells. The bloodstream is reached through existent spaces in the sinusoidal

walls or as suggested by Zamboni (1965) through interruptions induced by the pressure of underlying cells.

Two main roles have been assigned to the central cells of the islands; one is that of removal of extruded red cell nuclei-its so called "clearance function" (Yoffey & Yaffe 1980). The second role is the transfer of digestion products, such as ferritin, to the developing erythroblasts. This function was initially described by Pollicard & Bessis (1962) which they termed "rhopheocytosis". This latter role may be regarded as only one aspect of the trophic function of the central cell, other possible trophic actions include production of monokines to promote or inhibit erythroid growth and differentiation (Nathan 1987). Desmosome-like attachments have been observed between liver cells and proerythroblasts and between yolk sac endoderm and erythroid precursors. These points of contact are considered to play a role in the differentiation of erythroblasts (Fukuda 1974; Keleman & Balaugh 1976).

It is generally accepted that the central macrophage is an important constituent of in vivo erythropoiesis. The concept of a trophic function is supported by close apposition of the membrane of the central macrophage with the membranes of the surrounding erythroid cells which can lead to the transfer of hormones such as Epo. Further evidence supporting this functional relationship was provided by modifying erythropoiesis (Ben-Ishay & Yoffey 1972; Yoffey & Yaffe 1980). By creating a hypoxic state, which stimulates red cell production, they observed an increase in the size of the central macrophage and enhancement of its phagocytic ability. Conversely when erythroid production is suppressed by hypertransfusion, the central macrophage is destroyed (Shaklai 1989). The proposed role for transporting ferritin to erythroblasts for haemoglobin synthesis remains unproven and controversial. It has not yet been possible to determine the direction in which the ferritin travels, whether it is transported from macrophage to erythroblast

or vice versa.

### 1.5.2 In vitro

Long term bone marrow (LTBM) cultures have made a significant contribution to the understanding of the interrelationships between haematopoietic and stromal cells. These studies have helped to confirm Trentin's original hypothesis on the role of the haematopoietic microenvironment. A complex relationship is established between the stroma and haematopoietic cells which involves both production of growth-regulatory molecules and creation of an appropriate microenvironment. Granulocytes are normally produced in murine LTBM cultures, this production involves interactions with a macrophage subpopulation in addition to a stromal blanket cell population (Allen & Testa 1991).

Haematopoiesis is focal in long term cultures, the adherent layer showing variation over the growing surface. It can range from dense multi-layered regions of high proliferative activity to very sparse regions containing only a few macrophages and fibroblasts (Allen & Dexter 1984). Four or five days after plating the initial marrow inocculum, blanket cells appear to attract macrophages which migrate and proliferate underneath the blanket cells where they become fixed and are observed interspersed with fibroblasts (Allen & Dexter 1982). The blanket cells secrete both fibronectin and laminin both of which have been shown to promote cellular migration and may therefore play a role in the attraction of macrophages and granulocytes in their migration under the blanket cells, thus forming the "cobblestone" areas of haematopoiesis. These areas are the closest equivalent to the in vivo 'niches' for granulocyte development and maturation (Allen & Dexter 1984).

Concomitant with cell production there is proliferation of pluripotent stem

cells into a variety of precursor cells. This occurs in the absence of detectable colony stimulating activity. It has been postulated that the adherent layer is acting as an in vitro microenvironment in which the stem cells become lodged and here they are capable of extensive and prolonged self-renewal (Dexter et al 1977). Granulocyte precursors undergo rounds of division in close association with the central macrophages before migrating out from underneath the blanket cells to form aggregates with developing fat cells. Megakaryocytes are also evident in the long term cultures, though in fewer numbers than granulocytes. With extended periods of culture, granulocytes progressively disappear and phagocytic cells predominate (Dexter 1979; Radley et al 1991).

Erythropoiesis has normally ceased in bone marrow cultures by the end of the first week, although it has been known for some time that erythroid precursors persist in the cultures. These precursors are blocked at the BFU-E stage. Erythropoiesis can be manipulated however by addition of Epo with anaemic mouse serum (Allen & Testa 1991). Erythroid differentiation in culture is also focal in nature and is observed to occur in distinct foci directly associated with cells of the adherent layer. The stromal cells with which they associated were always macrophages, forming in vitro erythroblastic islands (Allen & Dexter 1982). Cytoplasmic extensions of macrophages were observed in close association with erythroblasts similar to those in vivo.

Long term fetal liver cultures have been established under the same conditions as those of bone marrow, with very similar results (Vogt, Noe & Rich 1991). Fat cells are not observed in fetal liver cultures. Like bone marrow cultures, erythropoiesis lasts approximately 1 week, then is replaced entirely by granulopoiesis and megakaryocytopoiesis. However unlike bone marrow cultures, Epo has been detected in the erythroblastic islands. The source is presumed to be the central macrophage (Zucali et al 1977; Rich 1986).

Gap junctions have been observed between macrophages, between macrophages and erythroblasts and between macrophages and blanket cells in long term bone marrow culture (Allen & Dexter 1982; Hasthorpe et al 1991). In freeze fracture specimens of erythroblastic islands, structures resembling intercellular bridges between membranes of macrophages and erythroid cells have been reported (Shaklai et al 1989). Rosendaal et al (1991) used a dye transfer technique to provide evidence for the existence of direct cell-cell communication between haematopoietic cells and stromal cells. Freeze fracture electron microscopy confirmed that the communication was via gap junctions.

The identification of proteins integrins and cell adhesion molecules involved in cell-cell adhesion, cell-cell interaction mediated by proteins such as fibronectin, vitronectin and fibrinogen in addition to the ECM molecules has enabled more detailed characterisation of the molecular basis for cellular interactions. The growth of blast cell colonies on stromal feeder layers requires adhesion to the stroma by the blast cells. Gordon, Riley & Clarke (1988) suggested heparan sulphate proteoglycan proteins are ideally suited to regulate specific intercellular interactions. Crocker, Morris & Gordon (1991) have defined two receptors involved in the binding of haematopoietic cells to murine macrophages. A divalent cation-dependent receptor which can mediate reversible attachment of erythroblasts in vitro, is present in both fetal liver and bone marrow. The second, exclusive to adult bone marrow, is a divalent cation-independent receptor which can mediate binding of erythroid and myeloid cells.

The observations that both erythroid and granulocytic proliferation and differentiation takes place in intimate contact with macrophage cell processes indicates a potential role for the macrophage in these interactions. This may be relevant to the evidence showing that macrophages can produce a variety of soluble regulatory factors (Quesenberry et al 1990). The close membrane

oppositions of these cells, other stromal cells and haematopoietic cells together form a complex regulatory network (Allen & Dexter 1984).

## 1.6 Haematopoietic Colony Forming Cells

Exposure of Dexter cultures to lithium, stimulates virtually all haematopoietic stem cells in the culture system, these include colony forming unit spleen (CFU-S), high proliferative potential colony forming cells (HPP-CFC), granulocyte-macrophage colony forming cells (GM-CFC) and megakaryocyte colony forming units (CFU-Meg) (Quesenberry et al 1990). This stimulation is thought to be due to increased activity of CSF-1, GM-CSF, G-CSF and IL-3 in the long term cultures.

### 1.6.1 Colony Forming Unit Spleen

Till & M$^{c}$Culloch (1961) pioneered the in vitro experiments on early stem cells. They found that when irradiated mice were injected with fresh bone marrow cells, macroscopic nodules of proliferating cells appeared in the spleen. Erythroid, granuloid, and megakaryocytic cell nodules were detected. There was a linear relationship between the number of cells injected and the number of colonies formed in the spleen. These findings were compatible with the assumption that single viable cells from the bone marrow could give rise to colonies in the spleen. However since the marrow injections contained many different cell types the identity of the cells giving rise to the colonies was unknown and hence they were referred to as colony forming units spleen (CFU-S).

Moore & Metcalf (1970) demonstrated that the yolk sac was the first organ to contain detectable in vivo CFU-S. These cells were observed in murine yolk sac

on the 8th day of gestation. By the 10th day of gestation CFU-S were found in the blood stream and in the liver. However other investigators have failed to find CFU-S in the 8 day embryo (Samoylina, Gan & Medvinski 1990). Instead they found CFU-S in the body of a murine embryo before they appeared in the yolk sac or liver. Samoylina et al, suggested that there are two sites of localisation of CFU-S, in the yolk sac and in the embryo body, from which HSC can migrate to the developing liver. Recently Medvinski et al (1993), have reported finding CFU-S activity in the aorta, gonad and mesonephros at a higher activity than in the yolk sac and appearing earlier in these regions than in fetal liver.

In the normal steady state the majority of CFU-S are non-proliferating, they can be stimulated into cell cycle by treatments which cause a decrease in cell number. The proportion of CFU-S which are synthesising DNA in normal murine bone marrow is 10-15% (Riches, Cork & Thomas 1981). Following treatment with cytotoxic drugs or X-irradiation the number of CFU-S in S-phase increases markedly (40-60%). There is a marked difference in the proliferative characteristics of normal bone marrow and those of fetal liver. In contrast to the quiescent state of CFU-S in bone marrow, the proportion of CFU-S which are engaged in DNA synthesis in fetal liver is 30-40% (Thomas, Cork & Riches 1981).

There is evidence that the control of the proliferative status of CFU-S is local. Proliferation of CFU-S in bone marrow, shielded from irradiation, is normal while CFU-S in the rest of the animal were depleted and undergoing intense proliferation (Wright & Lord 1977). More recently proliferation control by stem cell specific regulatory factors has been proposed. Maintenance of CFU-S in a quiescent state is thought to be brought about by an inhibitor of CFU-S proliferation (Lord et al 1976; Riches, Cork & Thomas 1981). Similarly, maintenance of an increased proportion of proliferating CFU-S in fetal,

regenerating or physiologically stressed tissue is brought about by a stem cell specific stimulator (Thomas, Cork & Riches 1981; Dawood et al 1990).

### 1.6.2 High Proliferative Potential Colony Forming Cells

Bradley & Hodgson (1979) described a previously undetected population of colony forming cells which they demonstrated in post fluorouracil (5FU) treated mice and in normal mice treated with a combination of colony stimulating factors. These cells were characterised by their survival after treatment with 5FU and by their high proliferative capacity in vitro, colonies contained an average of $5x10^4$ macrophage cells. Hence they were named high proliferative potential colony forming cells (HPP-CFC). Unlike CFU-S, the HPP-CFC were not severely depleted following treatment with 5FU, suggesting they are a slow cycling population. Humphries, Eaves & Eaves (1979) described a similar cell in normal mouse bone marrow which was responsive to Epo and spleen cell conditioned medium. Acting alone the CSF's could not stimulate HPP-CFC proliferation, a combination of growth factors is required.

HPP-CFC are among the most primitive progenitors yet identified in vitro. They have many characteristics of stem cells with haematopoietic-reconstituting ability in vivo. They are highly quiescent, multipotential and generate transplantable haematopoietic cells and progenitor cells of various lineages in vitro (McNeice et al 1988; Muench & Moore 1992).

Three HPP-CFC populations have so far been defined. The early population detected by Bradley & Hodgson is termed HPP-$CFC_1$. A second population, HPP-$CFC_2$, is stimulated by IL-3 together with CSF-1/M-CSF (Falk & Vogel 1988; McNeice et al 1988). These growth factors do not stimulate HPP-$CFC_1$ proliferation and HPP-$CFC_2$ are more severely depleted with 5FU treatment. In vitro experiments have confirmed the existence of two populations of HPP-

CFC and that HPP-$CFC_1$ differentiate to form HPP-$CFC_2$ and progenitor cells for the megakaryocyte, granulocyte, and macrophage lineages (McNeice et al 1992). HPP-$CFC_2$ in turn differentiate to produce macrophage colony forming cells (M-CFC), which further differentiate to produce macrophages.

The HPP-$CFC_3$ population is also severely depleted with 5FU treatment and is present in normal mouse marrow at a higher incidence than the other two populations suggesting it is a more mature population. This population is stimulated by haemolymphopoietic growth factor and CSF-1. The precise relationship of HPP-$CFC_3$ with the other subpopulations has not been clearly defined, it has been proposed that they are an intermediate population situated between HPP-$CFC_2$ and M-CFC (McNeice et al 1990). All three populations are inhibited by transforming growth factor β (TGFβ) (Keller et al 1990).

The kinetic properties of HPP-CFC are similar to the in vivo CFU-S. In normal bone marrow 9% of HPP-CFC are in S-phase, while in irradiated bone marrow this increases to 51% (Robinson & Riches 1991). There is also evidence for self-renewal and sensitivity to the CFU-S stimulator and inhibitor (Pragnell et al 1988; Eckmann et al 1988). However the effects of both stimulator and inhibitor are greater on CFU-S than on HPP-CFC, suggesting HPP-CFC is a relatively early component of the stem cell compartment (Robinson & Riches 1991).

### 1.6.3 Granulocyte-Macrophage Colony Forming Cells

Granulocyte-macrophage colony forming cells (GM-CFC) form colonies in vitro provided a suitable source of colony stimulating activity (CSA) is available, usually in the form of IL-3. Factors such as endotoxin, bacterial and viral infections can increase production of CSA but increase in number of GM-CFC does not necessarily correlate with this. In GM-CFC assays, macrophage, granulocyte-macrophage and granulocyte colonies are produced (Heyworth & Spooncer 1993).

In normal murine bone marrow 40% of GM-CFC are engaged in DNA synthesis, while in human fetal liver < 14 weeks gestation only 5% of GM-CFC are in S-phase. Incubation of bone marrow cells with human fetal liver at this gestational age will decrease the proportion of GM-CFC in S-phase to 5%. In contrast the proportion of GM-CFC in S-phase in human fetal liver >14 weeks gestation is 30%. Incubating late fetal liver with bone marrow has no effect on the proliferative status of the bone marrow GM-CFC, it can however stimulate non-cycling GM-CFC into cycle (Cork, Wright & Riches 1982).

## 1.7 Adhesion of Haematopoietic Cells to Stromal Layers in Vitro

The growth of haematopoietic cells in tissue culture conditions is a well established practise. Haematopoietic stem cells require direct interaction with the components of a supportive microenvironment for optimal maintenance, proliferation and differentiation. The mechanisms underlying the homing and lodging of stem cells in the bone marrow and other haematopoietic organs are mediated by membrane interactions of stem cells with stromal cells and with components of the extracellular matrix. Fibronectin is involved in diverse adhesive interactions with a variety of cell types including haematopoietic progenitor and stem cells (Aizawa et al 1991).

Erythroid progenitor cells have been demonstrated to adhere to fibronectin-coated substrate, but this adhesion is lost upon differentiation (Coulombel 1991, 1992; Vuilet-Gaugler et al 1990). Restriction of this expression to colony forming units-erythroid (CFU-E) and the first subsequent cell divisions strikingly correlates with the migratory capacity of these cells. These findings emphasise the necessity of direct interactions between haematopoietic cells and stromal cells, however it has not been possible to accurately quantify the specific attachment of haematopoietic cells to stroma.

Liesveld et al (1989) showed that $CD34^+$ haematopoietic cells could bind to fibroblasts just as effectively as they bound to stromal cells, although the stromal cells were better at supporting haematopoiesis. Thus specific binding of haematopoietic cells to stromal cells may be required in haematopoiesis. This could explain why haematopoietic cells home to specific sites.

Lineage and stage specific adhesion of haematopoietic cells to stroma or ECM has been reported. Gordon et al (1985), found blast progenitors would adhere to cells grown in the presence of methylcellulose but not to cells grown in its absence. More mature cells showed no preference to adhere to either layer. Bearpark & Gordon (1989) reported that CFU-S would readily adhere to stromal layers, while GM-CFC populations would not. Neuraminidase has no effect on the adhesion of blast cells, it can however increase the adhesion of GM-CFC. This suggests that the cell adhesion molecules alter with lineage and maturation. Gordon et al (1990) found that heparan sulphate proteoglycan (HS-PG) was essential for binding of blast-CFC to stroma. They suggested that haematopoietic cells of different lineages at different stages in development recognise and occupy specific microenvironments in the bone marrow. AcSDKP, which is a known inhibitor of entry into cell cycle, increased adhesion of CFU-S to stromal layers but had no effect on adhesion of GM-CFC to the layers (Lenfant et al 1989). Aizawa et al (1992), have suggested that AcSDKP improves binding through activation of stromal cells. Verfaillie et al (1990, 1992) found that early progenitor cells had the capacity to adhere to pre-established irradiated stroma while more committed progenitor cells lacked this capacity.

Aizawa & Tavassoli (1988) reported that homing of intravenously transplanted haematopoietic progenitor cells to the bone marrow occurs through a recognition system with galactosyl and mannosyl specificities. Both galactosyl and mannosyl BSA restricted homing of haematopoietic cells to bone marrow

but had no effect on the homing of stem cells to the spleen. This may reflect a difference in the surface characteristics of stromal cells in the bone marrow and spleen and different molecular bases for the binding of progenitor cells in the two sites.

Houssaint & Hallet (1988) grafted a precolonised liver rudiment under the kidney capsule of an adult mouse. Hepatocytes developed as normal, however the rudiment was never colonised by HSC. These results are in accordance with those of Johnson & Moore (1975), who suggested a host with normal haematopoiesis had no requirement for extra-medullary haematopoietic sites. Houssaint & Hallet proposed that ontogenic maturation of HSC occurs around the time of birth, which would alter their homing capacity. The grafted liver rudiment would be receptive to embryonic HSC only. In contrast Fleischman, Custer & Mintz (1982), injected normal bone marrow cells into the placental circulation of an 11 day murine fetus. The adult HSC were able to seed the fetal liver environment, proliferate and subsequently colonise the bone marrow, indicating that adult HSC capable of colonising fetal liver, in the appropriate host environment, are present in adult bone marrow (Houssaint & Hallet 1988).

Riley & Gordon (1987) and Gordon et al (1990), reported selective adhesion of Bl-CFC to adherent stroma. They found that marrow derived Bl-CFC would adhere to adult derived marrow stromal layers, but not to fetal liver or fetal marrow derived layers. Fetal derived HSC would not produce blast colonies when seeded onto adult derived stromal layers.

In vitro studies by Zanjani, Ascensao & Tavassoli (1993), suggest a hierarchy of homing sites, in which the bone marrow has the highest affinity for homing HSC. Before the bone marrow is haematopoietically active, transplanted HSC home to the liver and the spleen. Once the marrow has developed, its homing potential superseeds that of the other organs, despite the liver and spleen

remaining the major haematopoietic sites till birth. From the initial stage of its development, the bone marrow is capable of engrafting circulating stem cells, but does not support their differentiation and proliferation. Thus while HSC can pool in the fetal bone marrow, the liver remains the main haematopoietic organ till birth. Recent information has emphasised the adhesive capacity as an important property of haematopoietic cells and of their microenvironment. The adhesive properties can determine the distribution of cells and influence their exposure and response to growth factors.

## 1.8 Transplantation

### 1.8.1 Bone Marrow Transplantation

Bone marrow grafting is currently applied to a wide variety of malignant, non-malignant and genetically determined diseases, particularly leukaemia. Infusion of bone marrow cells is usually performed following total body irradiation or cytotoxic therapy to destroy pre-existing diseased marrow.

The potential application of marrow grafting to protect the individual from radiation exposure and chemotherapy in the treatment of leukaemia was first reported in the early 1950's when it was a highly experimental procedure. It soon became clear that a successful allogeneic graft depended on close histocompatibility matching between the donor and the recipient if the occurrence of fatal graft-versus-host disease (GvHD) was to be minimised (Thomas 1991). Marrow transplants are most commonly performed using an HLA genotypically identical sibling donor. However such a donor is only available for 30% of patients who require a bone marrow transplant, if an HLA non-identical donor is used then graft failure, infection and acute GvHD are more common.

Several procedures have been used in an attempt to eliminate GvHD causing cells, with a limited degree of success. It is generally considered that the T-cell population present in the donor graft causes or promotes GvHD. Marrow grafts have been treated with antilymphocytic serum, anti-T cell sera and anti-T cell antibody to eliminate T cells from the donor marrow (Vallera et al 1982). However T cell depletion leads to an increased risk of graft failure and recurrence of malignancy (Thomas 1991).

Recent findings that the stroma of patients with aplastic anaemia becomes more effective at binding Bl-CFC than stroma from healthy individuals, in response to marrow failure, suggests that engraftment may be achieved more easily in these patients (Gordon 1994).

### 1.8.2 Reduction of Relapse Following Bone Marrow Transplantation

Improved preparative regimens are essential if graft failure is likely to occur. Intensifying the dose or schedule of high-dose chemoradiotherapy increases the incidence of graft take unfortunately it also causes increased morbidity and mortality from damage to other organs especially the lung, liver and heart (Touraine et al 1987; Sullivan et al 1989). Monoclonal antibodies are being used in vitro for selective removal of subsets of T-cells in an effort to prevent GvHD without increasing the possibility of graft rejection or recurrence of infection (Thomas 1991). Following an allogeneic marrow graft with immunosuppresive treatment of GvHD, the patients are profoundly immunoincompetent and therefore at greater risk from infection. A leading cause of death is cytomegalovirus (CMV) pneumonia. If both donor and recipient are CMV negative, then infection can be prevented by the use of CMV negative blood products (Reusser et al 1990).

Recent attention has been focused upon administration of haematopoietic

growth factors to reduce the time taken for engraftment. Clinical trials in patients with acute myeloid leukaemia (AML) indicate that administration of rhGM-CSF and rhG-CSF was effective in elevating blood levels of granulocytes, monocytes and eosinophils and hence significantly reducing the duration of post-transplant leukopenia (Metcalf 1989). Pentoxyfylline (PTX) can partially reduce transplant related morbidity by suppressing circulating tumour necrosis factor (TNF) levels. TNF is thought to play an etiological role in venoocclusive disease of the liver, idiopathic pneumonia and GvHD (Nemunaitis & Singer 1992).

It has been reported that a stimulator and inhibitor of CFU-S are found in fetal liver (Cork et al 1982; Guigon & Wdzieczak-Bakala 1985). Wu et al (1989) have demonstrated the presence of a natural tumour suppressor in human fetal liver extract. Addition of this extract to culture systems had a suppressive effect on leukaemic cell lines. A suppressive effect was also observed on GM-CFU but not to the same extent as that observed on the leukaemic cells. This suggests selective suppression of leukaemic cells rather than normal bone marrow cells by the fetal liver extract. This selective suppression could be adopted as an effective method for the removal of leukaemic cells prior to autologous bone marrow transplantation for the treatment of AML (Pei & Wu 1990).

### 1.8.3 Fetal Liver Transplantation

It is of great interest that fetal liver stem cells can reconstitute the haematopoietic and lymphopoietic systems of both animals and humans without causing GvHD, even when a mismatched donor is used. The immature human fetal liver stem cells, when confronted by the allogeneic host antigens, acquire immunological tolerance to these antigens (Barnes, Ibery & Loutit 1958; Uphoff 1958; Lengerova 1959), this accounts for the absence of GvHD (Billingham, Brent & Medawar 1956; Gale 1985).

Infusion of fetal liver cells in patients infected with severe combined immune deficiency (SCID) syndrome, severe aplastic anaemia and acute leukaemia results in sustained engraftment and a possible cure even when the infused fetal liver cells were from a mismatched donor (O'Reilly 1985; Touraine 1991). Fetal liver transplantation (FLT) has also been used to treat a variety of inborn errors of metabolism, however the benefits in these patients are usually partial and transient (Touraine et al 1987).

Evidence for engraftment of donor cells following FLT is not abundant (Gale 1985; Izzi et al 1985). It is thought that the immunogenetic fetal liver cells are rejected following transplantation into immune competent hosts. Immunologic recovery is incomplete despite sustained lymphoid engraftment. In many patients with acute leukaemia, graft failure and leukaemic relapse frequently occur (Champlin et al 1985).

Immune reconstitution following fetal liver transplantation has been enhanced by simultaneous transplantation of the thymus from the same donor. This provides a syngeneic environment for differentiation of the transplanted T-lymphocyte precursors (Touraine et al 1985). Roncarolo et al (1991) have reported a SCID patient reconstituted with non-compatible fetal liver and thymus cells. The patient's T cells are of donor origin while the B cells and natural killer (NK) cells are host derived. Sanhadji (1992) reported the cure of leukaemic mice following FLT. The absence of post-transplant leukaemic development or relapse was attributed to a graft versus leukaemic effect of the FLT. Increasing the number of transplanted fetal liver cells, when mismatched donors are used, together with higher doses of irradiation/ chemotherapy has considerably improved engraftment (Royo et al 1987).

Transplants of fetal liver cells have been successfully used for reconstitution of defective cell lines in fetal recipients by injection via the placental circulation.

This method takes advantage of the immunological tolerance of the fetal recipients together with the optimal environment for differentiation of the transplanted fetal stem cells and the isolation of the fetus in the uterus. For optimal results the transplant should be performed as soon as possible after a diagnosis has been made (Flake, Harrison & Zanjani 1991).

Erythroid replacement in genetically anaemic $W/W^v$ mice using in utero fetal liver transplantation has been demonstrated (Fleischman et al 1982). Long term analysis revealed sustained and co-ordinate production of various lineages together with progressive substitution of host cells with those of the donor. Harrison et al (1989) successfully engrafted fetal liver cells into fetal monkeys of the opposite sex, without occurrence of GvHD. Touraine et al (1991, 1992) have successfully treated human fetuses which had been diagnosed as having SCID, bare lymphocyte syndrome or thalassaemia major by in utero infusion of fetal liver cells. Fetus-fetus haematopoietic stem cells transplants which can be performed without the need for cytoablative therapy, immunosuppression and GvHD may offer the earliest effective therapy of a genetic disorder diagnosed in utero (Zanjani et al 1991).

In the future bone marrow transplants and fetal liver transplants, as they are presently performed, may be replaced by purified stem cell transplants. Stem cell transplants may be combined with growth and differentiation factors to improve engraftment. Gene therapy is another potential method of correcting defective genetic diseases.

## 1.9 Prospects for Transplantation

### 1.9.1 Peripheral Blood Stem Cell Transplantation

The use of peripheral blood stem cells (PBSC) as an alternative to bone marrow

stem cells following high dose myeloablative chemotherapy, in selected cases, was first reported in man in 1985 (Juttner et al 1989). The advantages of PBSC include decreased likelihood of contamination with malignant cells, infusion of immunocompetent cells which hasten the immune recovery and the ability to collect PBSC from the donor without general anaesthesia. An extremely rapid haematopoietic recovery was observed due to the high numbers of GM-CFU present in PBSC collections.

PBSC infusion after high dose irradiation/chemotherapy has been successfully used to treat both chronic and acute myeloid leukaemias, lymphoma and multiple myeloma. Kessinger et al (1991) used PBSC to successfully treat patients with bone marrow metastases and relapsed lymphoma. In most cases haematopoietic recovery was more rapid than that observed in patients treated with an autologous bone marrow transplant (Juttner et al 1989; Williams et al 1990; Zander et al 1991).

Platelet recovery however is not so rapid, this may be due to infusion of insufficient numbers of PBSC or the nature of PBSC may be more committed to the granulocyte lineage rather than the megakaryocyte lineage (Williams et al 1990). Further investigations are required to answer these questions and to extend the use of PBSC to more patients for whom a compatible bone marrow donor is not available.

### 1.9.2 Umbilical Cord Transplantation

The use of human umbilical cord cells (HUC) for therapeutic reconstitution was proposed by Boyse, who observed successful haematopoietic reconstitution in lethally irradiated mice which were infused with syngeneic neonatal blood (Broxmyer et al 1989). The use of HUC has several advantages namely, disposing of the need for bone marrow donors, providing a disease-free source of haematopoietic stem cells, a matched donor is not essential and

as yet occurrence of GvHD has been minimal (Broxmyer et al 1991).

Cryopreserved HUC has been used as a source of HSC for HLA-identical transplantation of children with leukaemia or Fanconi's anaemia (Gluckman et al 1989; Hows et al 1992). HUC is a rich source of primitive haematopoietic progenitor cells and the T cells which it contains are immature, hence potential for inducing GvHD is reduced. Cell production in cultured HUC is greater than in normal bone marrow cultures. It may therefore be possible to treat adults with infusions of HUC (Hows et al 1992). To date haematopoietic engraftment has been successful following umbilical cord transplantation, however many questions remain unanswered. The use of the relatively limited number of cord blood progenitors in larger and mismatched recipients may amplify engraftment problems. The possibility exists that HUC may be contaminated with maternal cells. Transfer of mature T cells which are incompletely matched with the histocompatibility antigens of the recipient would almost definitely contribute to GvHD (Linch & Brent 1989). The hypothesis proposed by Boyse that use of cord blood as a source of stem cells, rather than bone marrow, might reduce the incidence and severity of GvHD is far from proven.

### 1.9.3 Gene Therapy

Gene transfer for the correction of genetic diseases is currently under intense investigation. Insertion of functioning genes into deficient cells is becoming a valid alternative to allogeneic cell transplantation in some inborn errors of metabolism. Methods have been devised for transferring specific genes with high efficiency into various cell types which exploit the use of replication-defective retroviruses as vectors. Murine studies have shown that genes can be incorporated into HSC which then reconstitute the marrow of syngeneic recipients (Lehn 1987).

The adenosine deaminase (ADA) deficiency which results in SCID has been successfully corrected by this method. The defective gene in the T-lymphocytes was corrected and the cells were then reinfused into the patient (Steinberg 1991). A clinical protocol for ADA gene therapy has now been provisionally accepted (Moen 1991).

In vitro studies have shown that haematopoietic progenitors of dogs, cats monkeys and humans can be transduced, however at a lower rate than transduction in murine progenitors. Prestimulation with appropriate haematopoietic growth factors may enhance the rate of retroviral gene transduction in human progenitor cells (Lehn 1990). Tumour infiltrating cells can be transduced with retroviral vectors. The integrated genome directs a high level of antibiotic resistance promoting enzyme. When the tumour infiltrating cells are re-infused into the patient they home to the tumour sites. Some patients have had a remarkable tumour regression, however the results are very unpredictable (Moen 1991). In the future it may be possible to cure thalassaemias and haemoglobinopathies using gene therapy, unfortunately the barriers currently facing investigators are formidable and much research remains to be done (Steinberg 1991).

## 1.10 Aims of Research

The aims of this research were to :- (i) examine the relationships between fetal liver derived blood cell precursors and environmental cells using morphological criteria and to compare these relationships in fetal, and neonatal liver, (ii) compare the in vivo relationships between blood cell precursors and environmental cells with those observed in vitro using long term cultures of both adult bone marrow and fetal liver, (iii) investigate the

proliferative status of early blood cells precursors derived from fetal liver and adult bone marrow, (iv) determine the effects of fetal liver extract on colony production by normal bone marrow and leukaemic cells and (v) investigate the ability of adherent stromal layers, derived from fetal liver and adult bone marrow, to retain and support early precursors of blood cells.

# Chapter 2

# Materials and Methods

## 2.1 Transmission Electron Microscopy

### 2.1.1 Mice

CBA/H mice were used throughout, they were maintained on a 12 hour light/dark cycle and fed R & M No. 3 pellets and water containing 15ppm chloride free ions, ad libitum. Mice were mated and checked each morning for the appearance of a vaginal plug, this was taken to be Day 0 of gestation. Fetal and neonatal mice were studied on the 12th, 13th, 14th, 15th, 18th days of gestation, on the day of birth, and on the 10th day of the neonatal period. The progeny of 3 adult females, each containing an average of 8 fetuses, were used for each gestational age examined.

### 2.1.2 Fixation

Mice were killed by cervical dislocation and their livers were excised. Livers were immediately transferred into 5% glutaraldehyde/4% paraformaldehyde fixative (Emscope Biorad) buffered with phosphate (BDH Chemicals Ltd) at pH 7.3 (Karnovsky 1965). Specimens were sliced in the fixative and left for one hour then washed 3 times in 0.08M Na cacodylate buffer (BDH Chemicals Ltd) for 15 minutes each. Specimens were then post-fixed in 1% osmium tetroxide for one hour and washed in cacodylate buffer as before, then dehydrated through a graded series of alcohols. Specimens spent 10 minutes in each of 50%, 70%, 80% and 90% ethanol then a further hour in 3 changes of 100% ethanol to ensure dehydration.

Specimens were then passed through 3 changes of propylene oxide for 20 minutes each to remove all traces of alcohol. Araldite resin was made up from 27ml araldite CY212 (Emscope Biorad), 23ml hardener DDSA (Emscope Biorad), 0.60ml dibutyl phthalate and 1.2ml accelerator BDMA (Emscope Biorad). Specimens were infiltrated in a mixture of 2 parts propylene oxide to 1 part resin for one hour, then in 1 part propylene oxide to 2 parts resin for a further hour. Specimens were finally embedded in resin and polymerised for 48 hours at $60^0$C.

2.1.3 Sectioning

Individual pieces of liver were cut out from the resin and mounted on araldite blocks for sectioning with a Reichart OM-U2 microtome using glass knives. Thick sections (1μm) were cut and mounted on glass slides then stained with Methylene blue for analysis with a Zeiss WL light microscope.

Ultrathin sections (50-70 nm), silver/grey interference colours were cut for electron microscope analysis. Sections were stretched out with chloroform before being mounted on copper grids.

2.1.4 Staining

10ml of 70% ethanol was added to 0.2g uranyl acetate and the solution left in darkness for 30 minutes. 1.76g trisodium citrate and 1.33g lead nitrate were dissolved in 30 ml distilled water and left for 30 minutes. 8ml 1N NaOH was added to clear the solution, which was then made up to a total volume of 50ml with distilled water. Both solutions were centrifuged for 15 minutes prior to use, (Reynolds 1963).

Drops of uranyl acetate solution were added to a piece of dental wax containing NaOH pellets Grids were transferred to the acetate drops, matt surface downwards and left to stain in the dark for 20 minutes. The grids were rinsed

in three successive beakers of distilled water then transferred to drops of lead citrate solution on dental wax to stain for 5 minutes. Grids were initially washed in 0.2M NaOH then rinsed three times in distilled water (Reynolds 1963). The grids were left to air-dry before analysis using a Philips 301 Electron Microscope at 60kV.

## 2.2 Confocal Laser Scanning Microscopy

### 2.2.1 Mice

Livers were excised from fetal CBA/H mice on the 15th day of gestation and from neonatal mice on the 8th day post partum.

### 2.2.2 Fixation, Dehydration and Embedding.

Segments of liver were fixed in neutral 4% formaldehyde buffer (BDH) for 4-6 days. Liver segments were placed in 75% alcohol overnight to commence dehydration, this was completed with two changes in each of 96% alcohol and absolute alcohol. The tissue was then cleaned in two changes of chloroform (BDH) and impregnated with paraffin wax in a vacuum embedding bath at 60°C.

### 2.2.3 Sectioning and Staining.

Liver segments were mounted on blocks. 7µm sections were cut, floated out in a water bath at 46°C, before mounting onto Subbed slides and left to dry overnight at 45°C. (Subbed slides-acid cleaned slides washed in running tap water and dipped in a 1% gelatine (Biorad), 0.1% chrome alum mix (Biorad) at 45°C and dried). Sections were de-waxed in xylene (BDH) and rehydrated through an alcohol series:- absolute, 96% and 75% then placed in distilled water.

Ferric salt, present in the liver samples, was treated with Perls mixture of equal parts 2% potassium ferrocyanide (Sigma) and 2% hydrochloric acid (Sigma) for 30 minutes. The samples were then washed several times with distilled water before treatment with 0.1% nuclear fast red (BDH) in 5% aluminium sulphate (BDH) for 90 seconds. Following this treatment nuclear material had a pale red appearance whilst cytoplasmic material appeared pale pink. Sections were washed in running tap water, dehydrated as before, then cleaned in xylene and mounted in DePeX (BDH).

## 2.3 Long Term Bone Marrow and Fetal Liver Cultures

### 2.3.1 Mice

CBA/H x C57BL10 crossbred mice were used throughout. Fetal livers were removed on the 15th, 17th and 19th days of gestation. Bone marrow was removed from the femurs of young adult mice, at least 8 weeks post-partum.

### 2.3.2 Long Term Liquid Cultures.

(i) Bone Marrow

Bone marrow cultures were established as detailed by Dexter et al (1973, 1974). The contents of a single femur were flushed through a 23G needle into a 25cm$^2$ tissue culture flask (Corning), containing 10ml Fischers growth medium (Gibco) supplemented with 20% horse serum (Gibco), 50 i.u./ml penicillin, 50mg/ml streptomycin (Sigma), 2mM glutamine (Flow Labs.) with $10^{-3}$ M hydrocortisone (Sigma).

(ii) Fetal Liver

An intact fetal liver was added to a tissue culture flask containing 10ml growth medium and gently disaggregated by aspiration through a 1ml syringe.

Cultures were gased with 5% $CO_2$ in air and incubated at 33°C. After 7 days and subsequently at weekly intervals, the cultures were demidepopulated and half of the supernatant medium was replaced with fresh growth medium. Suspension cells recovered after feeding were used for cytospin preparations, using a Shandon Cytospin 2, fixed and stained with Jenner/Giemsa (BDH). The establishment and appearance of a stromal cell layer in the culture flasks was monitored weekly by examination under an inverted microscope.

### 2.3.3 Flash Labelling of Long Term Layers

The supernatant was removed from flasks to be labelled. 10μCi $^3H$ (Amersham International) was added to the flasks which were then left to incubate for 24 hours at 37°C. Flasks were rinsed a few times with PBS buffer and allowed to air dry for 24 hours. The stromal layers were rinsed and fixed in methanol for 15 minutes The base of the flasks were cut off and dipped in K2 emulsion (Ilford Nuclear Research) with 1% glycerol solution (1:1 dilution) for 1 second and left to dry for 6 hours. Once dry the flasks were put into boxes containing silica gel, taped closed and inserted into 2 black bags which were also taped closed, then stored in the fridge for 4 days exposure.

### 2.3.4 Developing

Samples were removed from boxes and submersed in Kodak D19b developer for 3.5 minutes, then rinsed with distilled water fo 30 seconds before fixation in Kodak acid fix (Hypo) for 5 minutes and a final rinse in tap water for 15 minutes. Samples were allowed to dry before staining with Jenner/Giemsa and mounted with euparal (Raymond Lamb).

### 2.3.5 Irradiated Stromal Layers

To establish irradiated stromal cell layers, long-term bone marrow and fetal liver cell cultures were set up as described. Once a confluent stromal layer was established, after approximately 3 weeks for bone marrow culture and 6-8

weeks for fetal liver, the cultures were irradiated with 15Gy γ irradiation from a Cis IBL 437C source with a $^{137}Cs$ radionuclide at a dose-rate of 4.6 Gy/min. This was followed by a rinse and total change of medium 6 hours later (Spooncer, Lord and Dexter, 1985). This dose kills all haematopoietic cells, quiescent stromal cells however remain intact and provide a nude inductive stroma which can function to promote the growth of haematopoietic cells for several weeks. 6-10 days later the layers were reseeded with fresh bone marrow or fetal liver cells to re-establish haematopoiesis, the flasks were fed weekly as before.

## 2.4 High Proliferative Potential-Colony Forming Cell Assay.

### 2.4.1 Mice

The livers of CBA/H mice were used on the 13th, 15th, 17th and 19th days of gestation, on the day of birth and on the 3rd and 8th days of the neonatal period, bone marrow was obtained from the femurs of young adult mice. The livers of at least 4 mice were pooled for each experiment.

### 2.4.2 Haematopoietic Tissue Preparation

Pregnant mice and neonatal mice at appropriate stages in gestation were killed by cervical dislocation. The livers were excised, pooled and placed into a known volume of Dulbecco's medium supplemented with 50 i.u/ml benzylpenicillin (Gibco), 50μg/ml streptomycin sulphate (Sigma), 2mM L-glutamine (FlowLabs) and 20% horse serum (Sigma). Normal adult mice were also killed by cervical dislocation and their femora removed. The contents of a single femur were flushed through a 23G needle into a known volume of Dulbecco's medium. A single cell suspension of haematopoietic tissue was produced, by aspirating fragments of liver/bone marrow through progressively finer gauge needles (No. 23G to 25G) Cellularities were determined using a coulter counter ZM and adjusted to $5x10^6$ cell/ml.

2.4.3 Determination of the Proportion of Cells in S-Phase (Fig. 2.4-1)

Two 1ml samples of $5x10^6$ haematopoietic cells were produced in centrifuge tubes and incubated at 37°C in either 100µl medium alone, as a control, or in the presence of 100µl Cytosine arabinoside (Ara-C) (Sigma) at a concentration of 250 µg/ml ($10^{-4}$ M). Ara-C is an S-phase specific cytotoxic agent which kills all cells engaged in DNA synthesis. By comparing the number of colonies formed by cells treated with medium alone and those treated with Ara-C, a measure of the proportion of colony forming cells which are synthesising DNA can be determined. Samples were incubated for one hour with frequent vortex mixing.

After one hour the cells were put through a series of two washes:-7ml fresh medium was added to each sample and the cell suspension centrifuged at 1000 r.p.m. for 15 minutes. The supernatant was removed to waste, the cell pellet resuspended in 8ml fresh medium and centrifuged for 15 minutes at 1000 r.p.m. Supernatants were removed and pellets were resuspended as a single cell suspension in 1ml medium. Cellularities were determined and adjusted to $2x10^5$ cells/ml.

Two millilitres of $2x10^4$ FL or BM cells/ml or 2ml $4x10^4$ neonatal liver cells/ml in supplemented Dulbeccos medium containing 0.3% (w/v) agar (Difco Bacto Agar) were aliquoted in 50mm, triple vent, tissue culture grade, plastic petri dishes (Cell Cult) over 2ml aliquots of Dulbeccos medium containing 0.5% (w/v) agar and 10% (v/v) each of medium conditioned by the WEHI 3B myelomonocytic leukaemia cell line, a source of Multi-CSF/Interleukin 3(IL-3) (Ihle et al, 1982); and medium conditioned by the L929 fibroblast cell line, a source of M-CSF/CSF-1 (Stanley & Heard, 1977). Cultures were incubated in a fully humidified, 5% $CO_2$ in air atmosphere for 14 days at 37°C (Fig. 2.4-2).

**Fig 2.4-1**

## Determination of the Proportion of Cells in S-phase

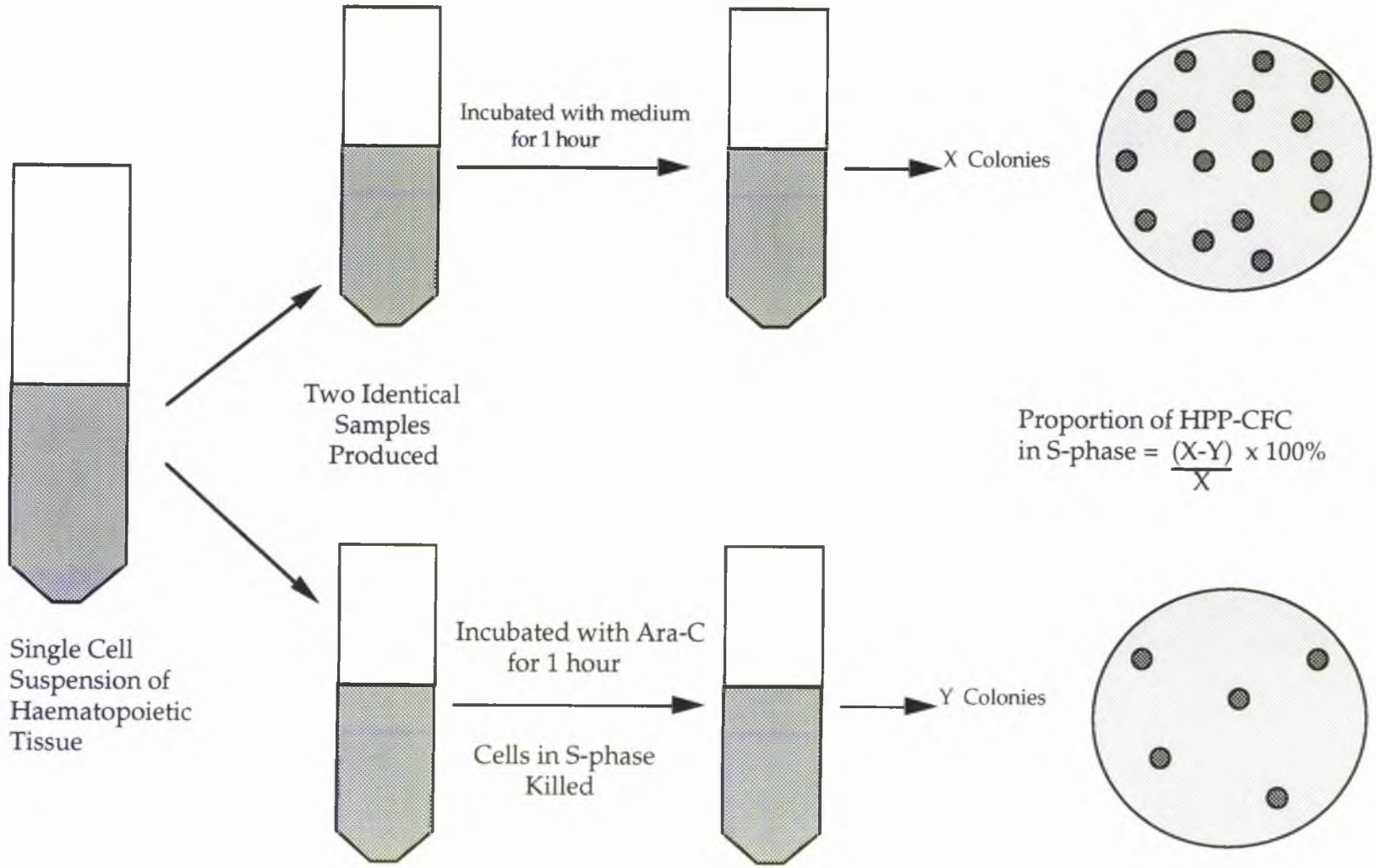
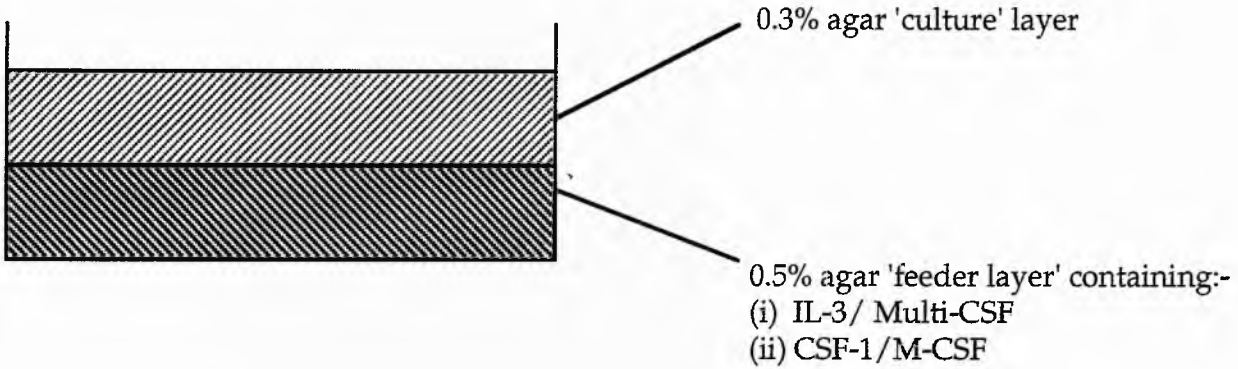


**Fig.2.4-2 The in-vitro High Proliferative Potential Colony Forming Cell (HPP-CFC) Assay.**




Colonies are assayed after 14 days at 37 °C in a 5% CO2, fully humidified atmosphere.
Colonies in exces of 2mm are considered to be derived from the HPP-CFC population of interest.

Twenty-four hours prior to assay, 1ml of an autoclaved solution of 1mg INT/ml 0.9% saline was added to each culture INT- 2 (4 iodophenyl)-3 (4 nitrophenyl)-5 phenyltetrazolium chloride (BDH). Colourless INT is metabolised by viable cells and converted to a red tetrazolium salt which precipitates in the cells allowing macroscopic assay of colony numbers. Colonies in excess of 2mm diameter were considered to be derived from the HPP-CFC population (Falk & Vogel 1988; Pragnell et al 1988). The difference in colony numbers in the absence and in the presence of Ara-C treatment gives a measure of the proportion of HPP-CFC in S-phase.

## 2.5 CFU-GM Assay

Haematopoietic tissue was obtained from the livers of 15 day fetal and newborn mice and from the bone marrow of young adult mice. This was added to a known volume of supplemented Dulbecco's medium and a single cell suspension produced. The cells were counted and diluted to $5x10^6$ cells/ml.

1ml cell suspension was incubated in the presence of 100µl Ara-C, or 100µl Dulbecco's at 37°C for one hour. Cells were washed twice, resuspended in 1ml medium and diluted to $5x10^5$ cells/ml. 1ml aliquots of $5x10^4$ cells/ml in Dulbeccos medium containing 0.3% (w/v) agar and 10% (v/v) medium conditioned by the WEHI 3B cell line, as a source of colony stimulating activity, were plated in 30mm triple vent plastic petri dishes. The cultures were incubated in a humidified 5% $CO_2$ in air atmosphere for 7 days and colonies (>50 cells) were counted using dark field illumination.

## 2.6 Effect of Fetal Liver Extract on the Formation of HPP-CFC by Normal and Irrariated Bone Marrow.

### 2.6.1 Mice

Adult CBA/H mice given 10Gy, 7.5Gy, 5Gy, 2.5Gy or 0Gy γ irradiation were sacrificed on the fourth day post-irradiation (Sharp & Thomas 1971). Femora were dissected free and their contents flushed into a teklab containing 3mls Dulbeccos medium supplemented with penicillin, streptomycin , glutamine and 20% horse serum. A single cell suspension was produced, cellularity was determined using a coulter counter ZM and adjusted to $2x10^5$ cells/ml.

### 2.6.2 Fetal Liver Extract

CBA/H mice were used for the preparation of fetal liver extract (FLE). On the 15th day of gestation mice were killed by cervical dislocation and the fetuses removed. The fetal livers were excised and each suspended in 1ml Fischers medium supplemented with penicillin, streptomycin, glutamine and 10% horse serum. Single cell suspensions of fetal liver were produced and incubated at 37°C for 24 hours. Suspensions were centrifuged for 15 minutes at 1200 rpm in a chillspin MSE. Supernatants were removed and stored at -10°C.

### 2.6.3 Assay

1ml of $2x10^5$ BM cells/ml was added to the standard HPP-CFC culture layer together with 1ml of FLE and 1ml of 3% agar, FLE was not added to the control group. 2mls of this solution were then plated over the standard HPP-CFC feeder layer, containing Wehi 3B, L929 and 0.5% agar. The colonies were incubated for 14 days in a fully humidified $CO_2$ in air atmosphere. 1ml of INT was added to each dish one day prior to assay. The difference in the number of

colonies produced in the absence and in the presence of FLE gives an indication as to whether FLE influences the production of HPP-CFC by irradiated bone marrow.

### 2.6.4 Effect of Diluted FLE on NBM.

These experiments were performed to establish the optimal concentration of FLE which would influence the formation of HPP-CFC by normal bone marrow. Experimental protocol was identical to the original experiments, however the volume of FLE added was diluted to 0.5, 0.25, 0.125, 0.0625 and 0.03125 of the original. Supplemented Fischers medium was added to the FLE to make the final volume up to 1ml. This solution was then added to the culture layer together with NBM cells and 3% agar. 2ml of the culture solution was plated over the standard HPP-CFC feeder layer and incubated for 14 days as before.

## 2.7 Effect of FLE on Leukaemic Cell Colony Formation

### 2.7.1 SA2 Leukaemic Cell Line

The SA2 cell line used was cloned from the SA2 T38 leukaemic cell line in liquid form. SA2 myeloid leukaemia was induced into CBA/H male mice following exposure to 3 Gy whole body X-irradiation (Hepburn et al 1987) and passaged routinely at 2-3 week intervals in CBA/H recipients (Hepburn et al 1987). SA2 cells are removed from the medullary cavities of the 38th passage (SA2 FT38), grown in liquid culture and cloned (Riches et al 1991). The cloned SA2 cells are stored frozen in liquid $N_2$ until required for use. The cells are thawed out rapidly in a water bath at 37°C then transferred to a centrifuge

tube. 9ml Fischers medium supplemented with penicillin, streptomycin sulphate, glutamine and 10% horse serum is added dropwise whilst gently agitating the centrifuge tube to prevent osmotic rupture of the cells. The suspension is centrifuged at 1000 rpm for 10 minutes and the supernatant removed. The pellet is then resuspended in 10ml Fischers medium and transferred to a tissue culture flask. The flasks are stored at 37°C and passaged at regular intervals (Riches et al 1991).

A cell viability test must be performed prior to use. A single cell suspension is obtained by pipetting the cells through a 10ml pipette a few times. 1ml of the single cell suspension is removed, placed in a teklab with 50µl fast green and incubated in a water bath at 37°C for 15 minutes. The sample is removed, placed on a slide and a cell count is performed, non-viable cells are stained by fast green and appear green under the microscope. The percentage of viable cells is calculated (viability >90%).

### 2.7.2 Leukaemic Cell Assay

1ml of $2x10^3$ SA2 cells together with 0.25, 0.5 or 1ml FLE were added to supplemented Dulbeccos medium and 1ml of 3% agar (standard GM-CFC layer), FLE was not added to the control groups. 1ml of this solution was added to 30mm triple vent non- tissue culture grade petri dishes and incubated for 7 days in a 5% $CO_2$ in air atmosphere. Colonies containing >50 cells were counted using a dark field illumination microscope. The difference in colony number between experimental and control groups was considered to be a result of the presence of FLE.

## 2.8 Adhesion of HPP-CFC to Irradiated Stromal Layers (Fig. 2.8-1)

### 2.8.1 Mice

CBA/H x C57BL10 mice were used throughout, regenerating bone marrow was obtained from mice which had been given 2Gy γ irradiation 3 days prior to use. Mice were killed by cervical dislocation and experimental tissue was obtained from femora or fetal liver as appropriate, and flushed into 5mls Dulbeccos medium, supplemented with 50i.u./ml penicillin, 50μg/ml streptomycin, 2mM glutamine and 20% horse serum. Single cell suspensions of haematopoietic tissue were produced by aspiration through progressively finer gauge needles. Cellularity was determined using a coulter counter ZM and adjusted as required.

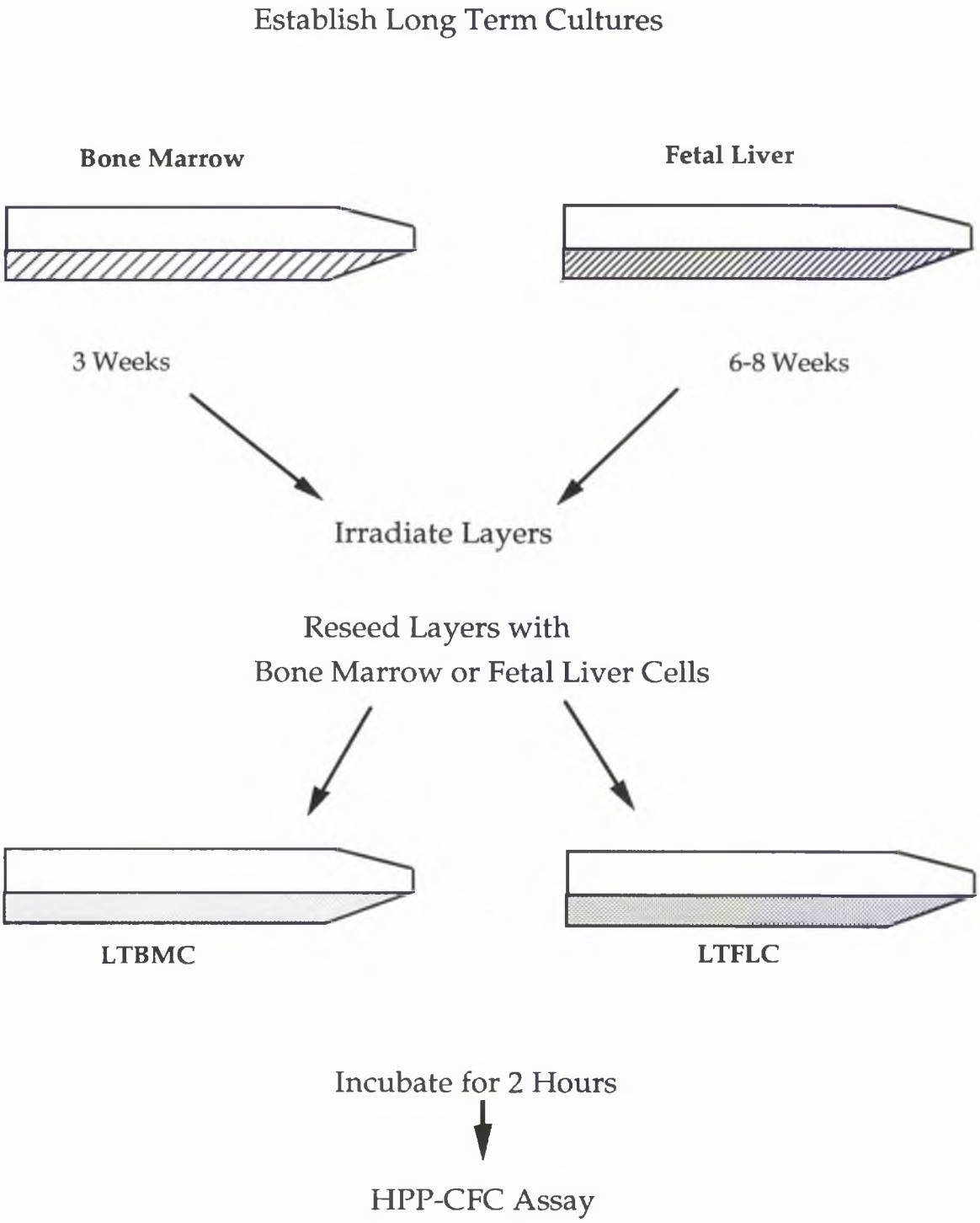
### 2.8.2 Incubation of Stromal Layers with BM or FL cells.

Supernatants were removed from irradiated stromal layers 6-10 days after irradiation (Spooncer, Lord & Dexter, 1985), and replaced with 1ml of a known concentration of either normal bone marrow (NBM), regenerating bone marrow (RBM) or fetal liver (FL) cells in 6mls Fischers medium.The flasks were gased and incubated for 2 hours at 33°C (Gordon et al, 1987).

### 2.8.3 HPP-CFC Assay.

2mls of $2x10^4$ control NBM, RBM or FL cells/ml in supplemented Dulbeccos medium containing 0.3% (w/v) agar were aliquoted in 50mm triple vent plastic petri dishes over 2ml aliquots of Dulbeccos medium containing 0.5% (w/v) agar and 10% (v/v) each of Wehi 3B and L929. After 2 hours incubation the supernatant was removed and the stromal layer rinsed with 3ml Fischers

**Fig. 2.8-1** **Adhesion of HPP-CFC to Irradiated Stromal Layers**



medium. This was then removed, added to the supernatant and centrifuged at 1000rpm for 10 minutes. 10 ml of fresh supernatant was added to the flasks, which were gased, returned to the incubator and maintained as before for several weeks. After centrifugation, the pellet was resuspended as a single cell suspension in 1ml Dulbeccos medium, the cellularity was determined and adjusted to $2x10^5$ cells/ml. As with the controls, 2ml aliquots of $2x10^4$ cells/ml in Dulbeccos medium containing 0.3% agar were plated over 2ml aliquots of medium containing 0.5% agar with Wehi 3B and L929.

Cultures were incubated for 14 days at 37°C in a fully humidified 5% $CO_2$ in air atmosphere. Twenty four hours prior to assay, 1ml of INT was added to each culture. The difference in colony numbers between the control and experimental group gives a measure of the proportion of cells which had adhered to the stromal layer.

All results were statistically analysed, using the Students t-test and analysis of variance (ANOVA).

# Chapter 3

# Inter-Cellular Relationships

During the 21 day period of embryonic and fetal development in the mouse, there is a sequential change in the site of haematopoiesis together with changes in haemoglobin type and production of annucleate erythrocytes. Haematopoiesis is initiated in the fetal liver at around the 10th day of gestation and continues almost until birth, when it is supplanted by the spleen and the bone marrow.

## 3.1 In Vivo

The relationships between the cells and morphological changes which occur in fetal and neonatal liver during development have been investigated by electron microscopy.
Confocal laser scanning microscopy was used to examine fetal liver and neonatal liver which had been stained for iron with Perl's Prussian Blue.

## Results

### 3.1-1 Fetal Liver

The earliest liver sample examined was that of the 12 day fetus. At this stage the hepatic parenchyma had not fully developed. The hepatic tissue formed a loose mass of cells. Hepatocytes (figs. 3.1-1 & 3.1-2) were relatively

low in number, they exhibited a number of lipid inclusions and had characteristic pyramidal outlines, in the absence of large numbers of haematopoietic cells. Haematopoietic cells were not obvious at this early stage. A few erythroblasts were observed randomly situated within the hepatic parenchyma. Occasional monocytes were observed (fig. 3.1-3), which was unusual in such an early stage.

In the 13 day fetus, with the development of the hepatic cords, the hepatocytes appeared to be much more cohesive. Some fenestrations remained in the parenchyma, however it was considerably more compact than that of the 12 day fetus (fig. 3.1-4). Haematopoietic cells were observed extravascularly, arranged in distinct clusters around a central hepatocyte (fig. 3.1-5 & 3.1-6) with resulting irregularities in the oulines of the hepatocytes.

In the 14 day fetus, the hepatic stroma is very cohesive due to increasing numbers of cells, which are very closely packed. Endothelial cells which line the liver sinusoids were apparent, with their characteristic long flattened outline. The number of haematopoietic cell clusters or "erythroblastic islands" had markedly increased. Developing erythroblasts were seen to form cell clusters around a central macrophage (fig. 3.1-7) in addition to those arranged around a central hepatocyte (fig. 3.1-8).

The number of constituent cells in each cluster was relatively high at this stage. Cytoplasmic extensions of the central cell were seen to be intimately associated with the adjacent blood cell precursors. Gap junctions and desmosome-like attachments between the blood cell precursors and the cytoplasmic processes were numerous (fig. 3.1-7). In addition, cytoplasmic processes of the blood cell precursors were observed in close proximity to the processes of the central cells (fig. 3.1-8). Gradients of differentiation were

apparent in the clusters. The immature erythroblasts were situated in close proximity to the central macrophage or hepatocyte whilst more mature elements were located peripherally. Nuclear expulsion by erythroid cells was observed (fig. 3.1-9) and macrophages were observed with ingested red cell nuclei in their cytoplasm (fig. 3.1-7).

Other haematopoietic lineages became apparent at this stage in development. In addition to monocytes, occasional granulocytes were seen, as part of an erythroblast cluster (fig. 3.1-10). Megakaryocytes were also observed at this stage in development, however they did not form an integral part of the mixed-lineage cell clusters, they remained as a single entity in close proximity to hepatocytes (fig. 3.1-11).

12 Day Fetal Liver

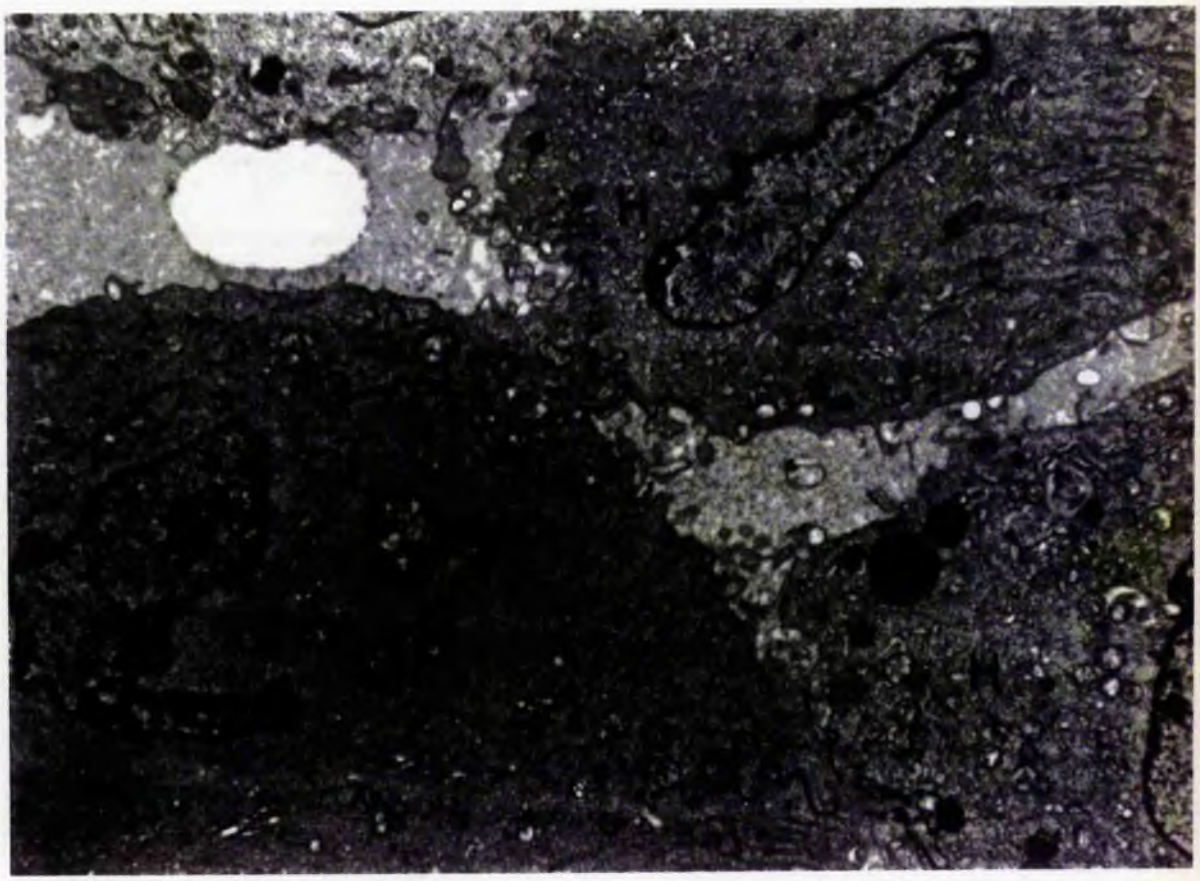



Fig. 3.1-1 x 5 265

Cluster of three early hepatocytes (H), forming a loose network of cells. Mitochondria and RER are not abundant in these early cells

12 Day Fetal liver

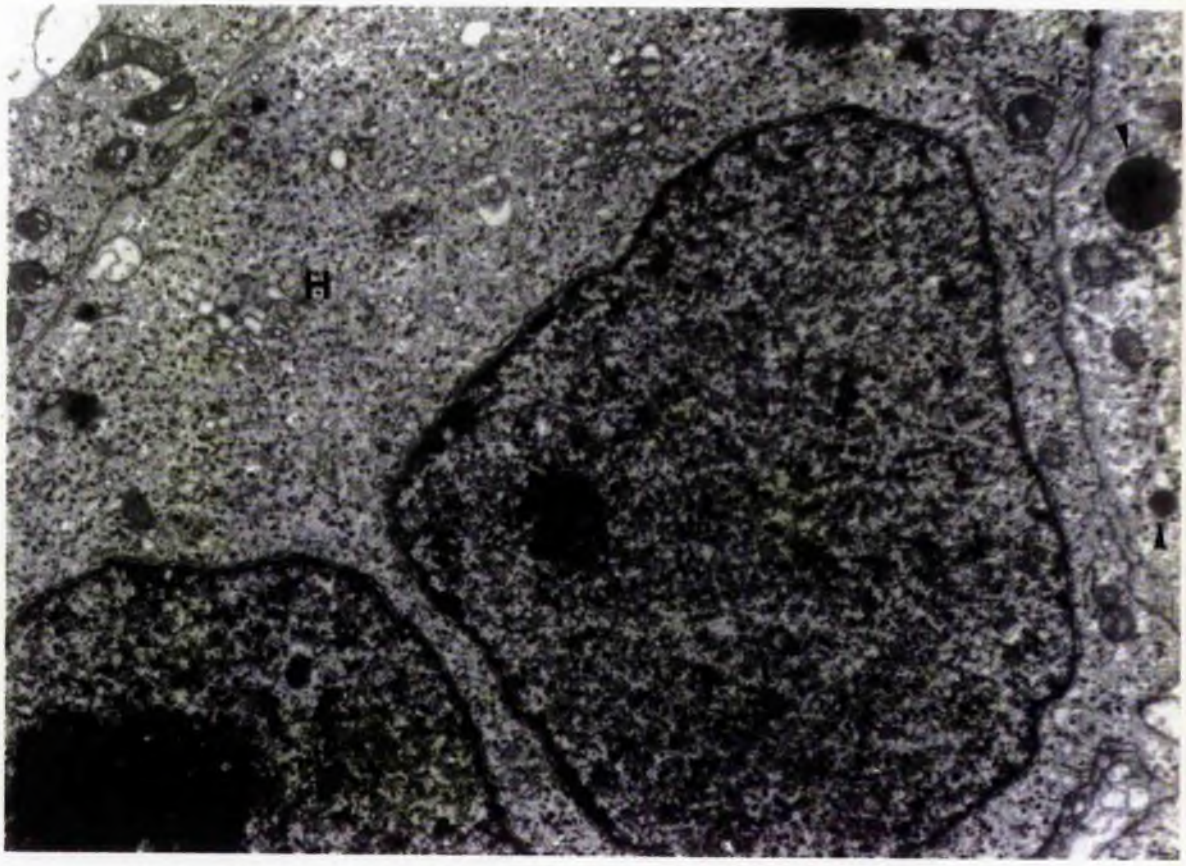



Fig. 3.1-2 x 14 250

Early hepatocyte (H) closely associated with adjacent hepaocytes. Only a few organelles are visible within the cytoplasm. A few lipid vacuoles (arrows) are present in the neighbouring hepatocytes.

12 Day Fetal Liver

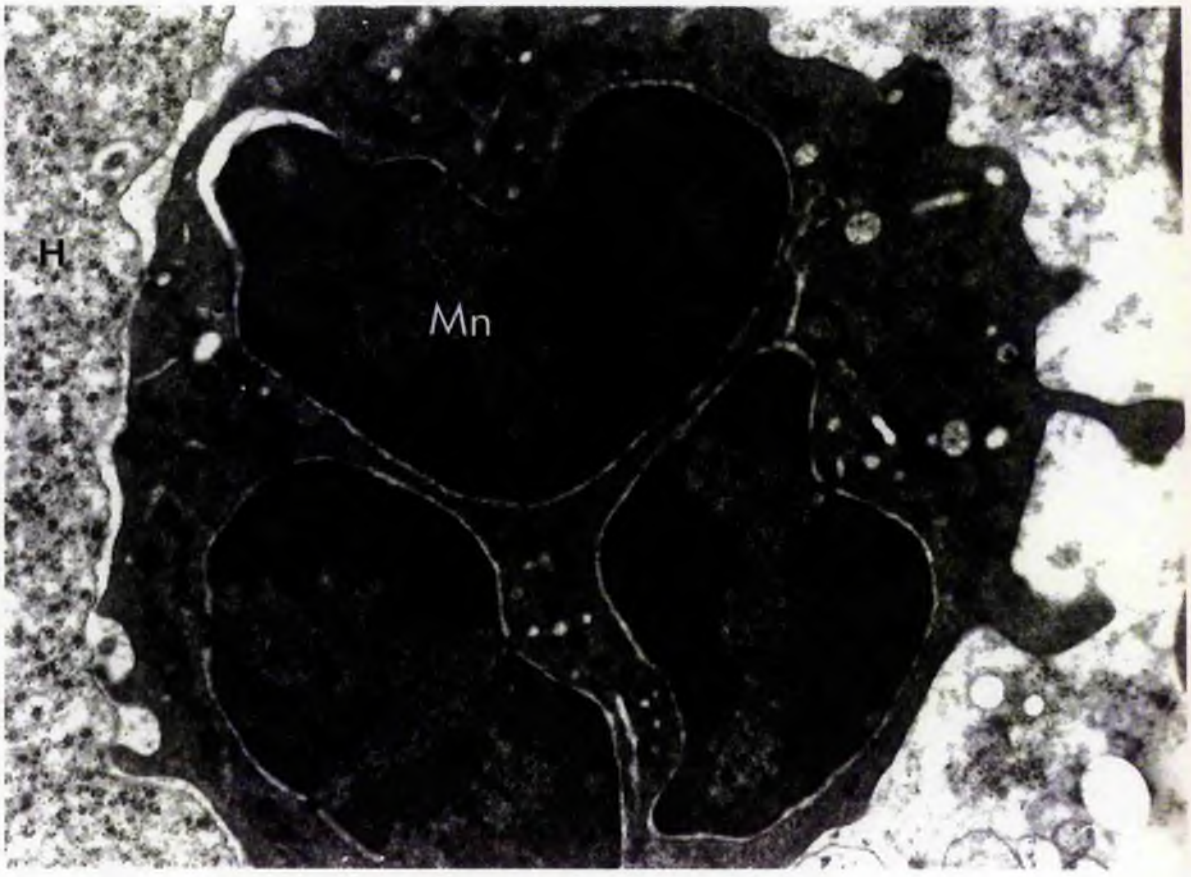



Fig. 3.1-3 x 3 000

Early monocyte (Mn), closely associated with a hepatocyte (H). The monocyte extends cytoplasmic processes towards the hepatocyte.

13 Day Fetal

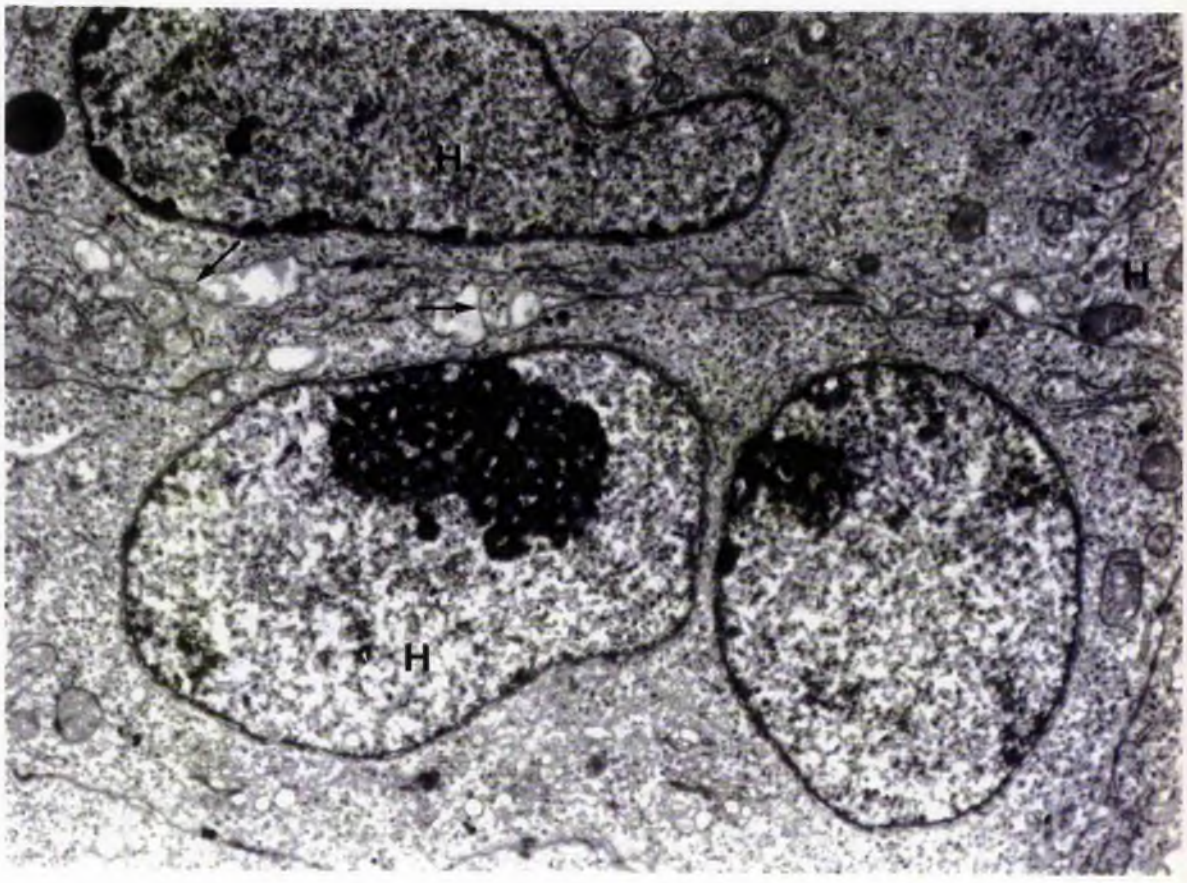



Fig. 3.1-4 x 11 171

Cluster of hepatocytes (H) forming a compact cell network. Mitochondria and RER are more predominant in the cytoplasm. In addition to close apposition of the cell mebranes, cytoplasmic processes can be seen extending towards adjacent cells (arrows).

13 Day Fetal

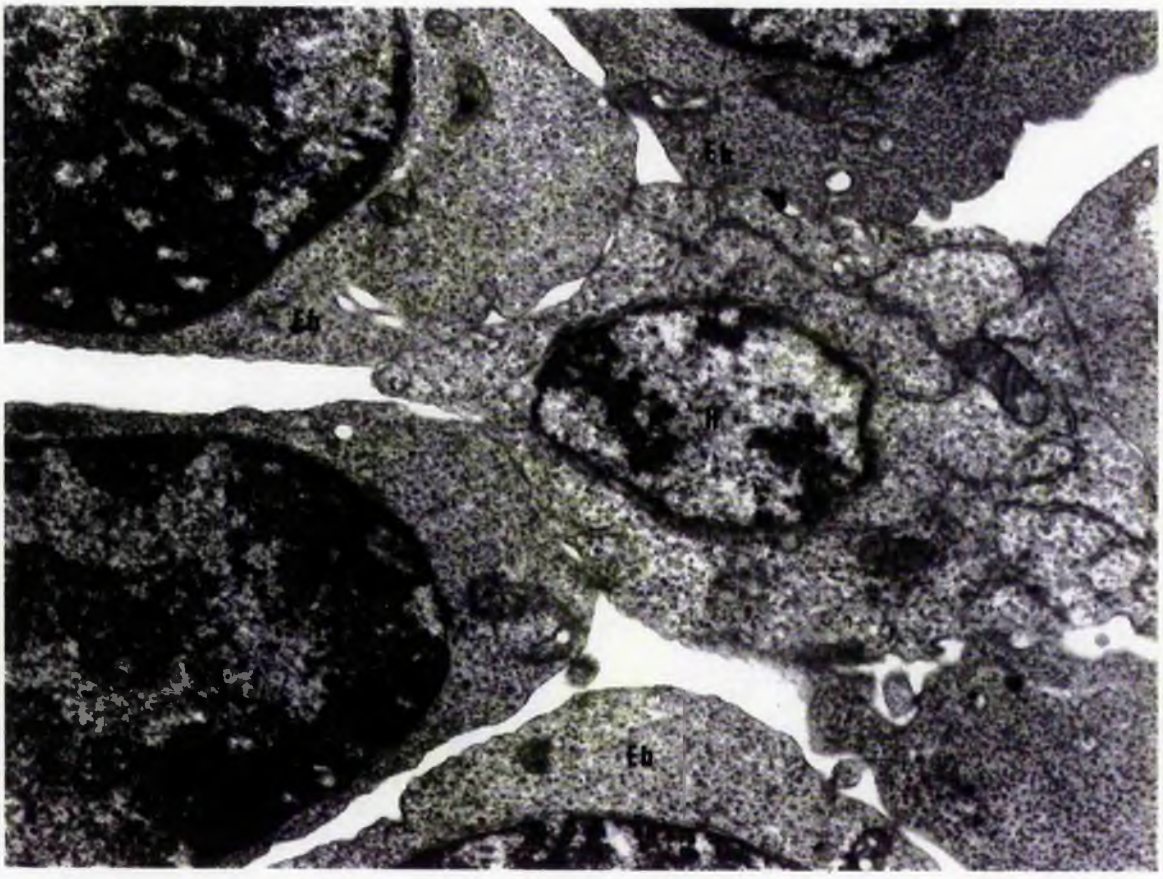



Fig. 3.1-5 x 14 200

Cluster of immature erythroblasts (Eb) around a hepatocyte (H). The cell membranes of the erythroblasts are closely apposed to that of the hepatocyte, desmosomes and gap junctions can be observed in these regions.

13 Day Fetal

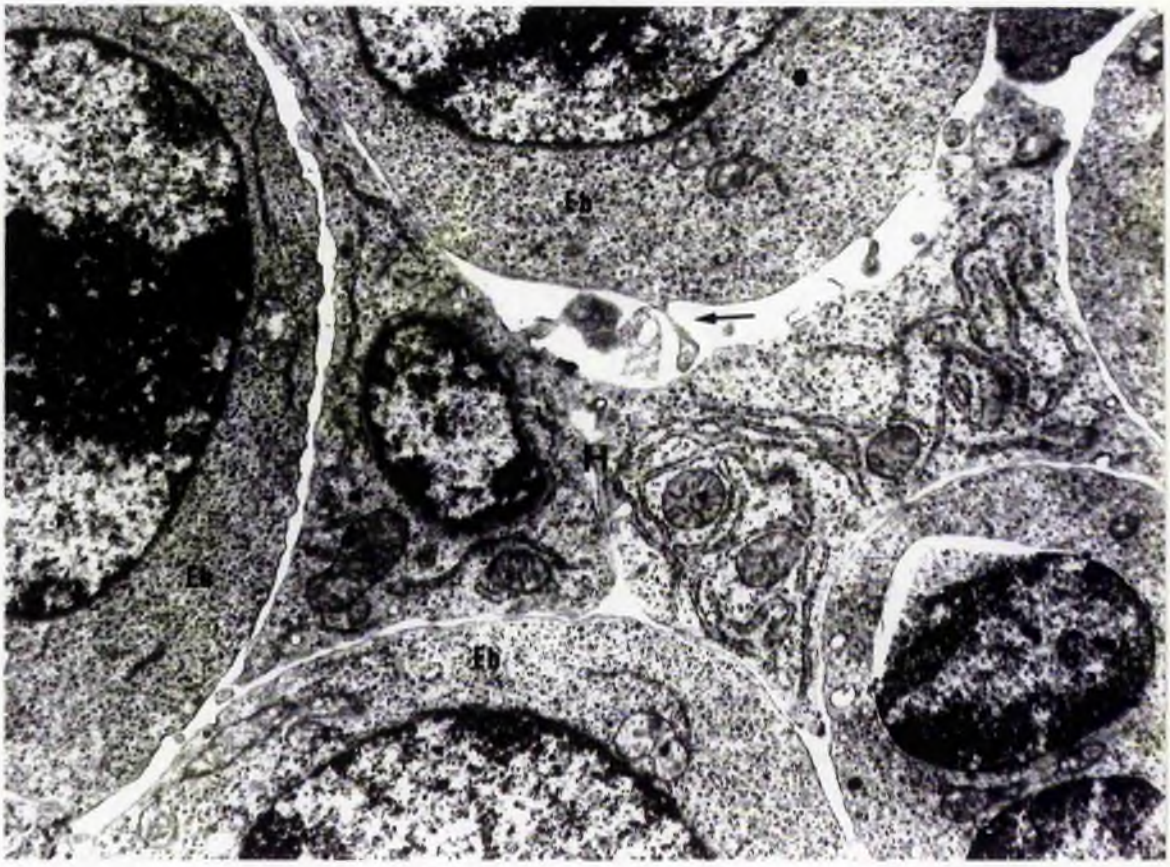



Fig. 3.1-6 x 18 525

Cluster of erythroblasts (Eb) around a central hepatocyte (H). Cytoplasmic processes of the hepatocyte can be seen passing between and in close apposition to the adjacent blood cell precursors. Cytoplasmic processes of the erythroblasts can be seen extending towards the central hepatocyte (arrow).

14 Day Fetal Liver

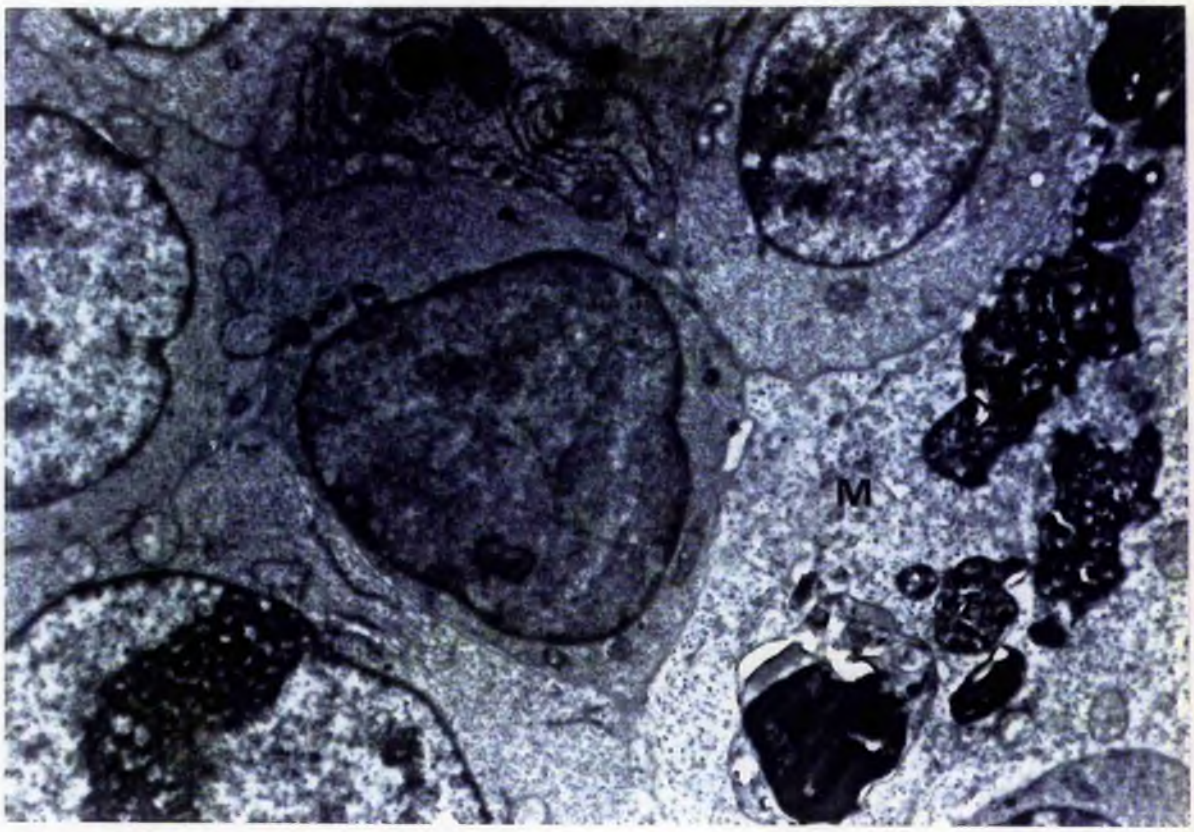



Fig. 3.1-7 x 9 000

Central macrophage (M) surrounded by a large number of immature erythroblasts. There is close apposition of the macrophage cell membrane with those of the surrounding cells. Ingested debris can be seen in the cytoplasm of the macrophage.

14 Day Fetal Liver

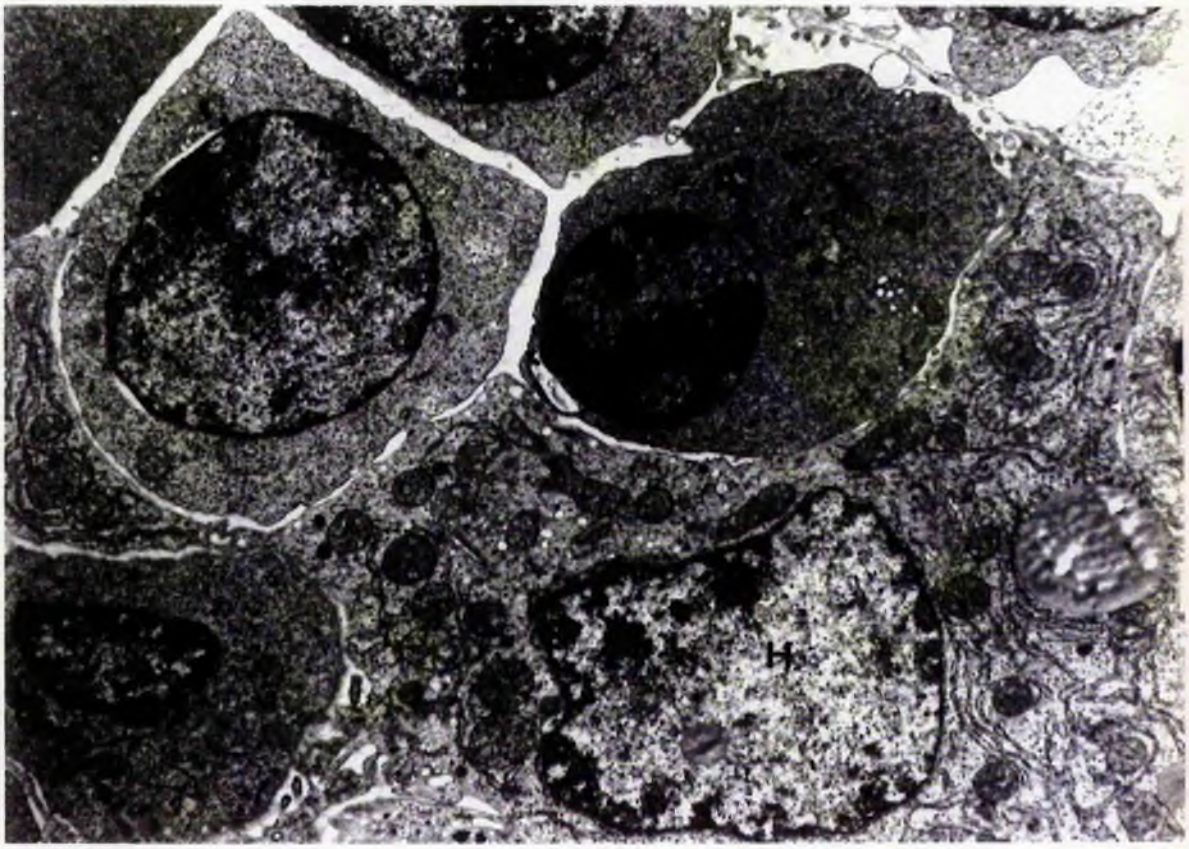



Fig. 3.1-8 x 9 000

Developing erythroblasts arranged around a central hepatocyte (H). The blood cell precursors are intimately associated with the cytoplasmic processes of the hepatocyte through desmosomes, gap junctions and their own cytoplasmic projections.

14 Day Fetal Liver

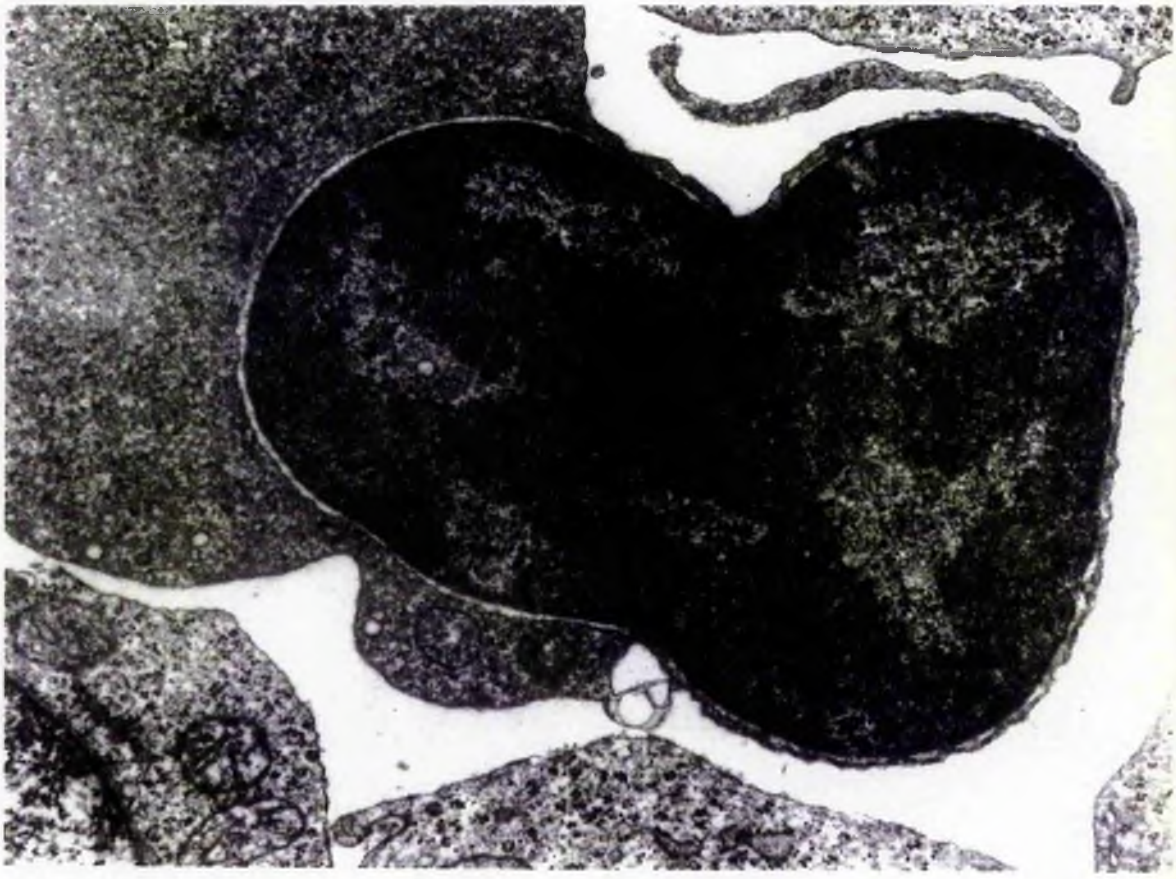


Fig. 3.1-9 x 2 500

Nuclear expulsion by an erythroblast. A narrow rim of cytoplasm remains around the expelled nucleus. This cell was situated at the periphery of an erythroid cluster.

14 Day Fetal Liver

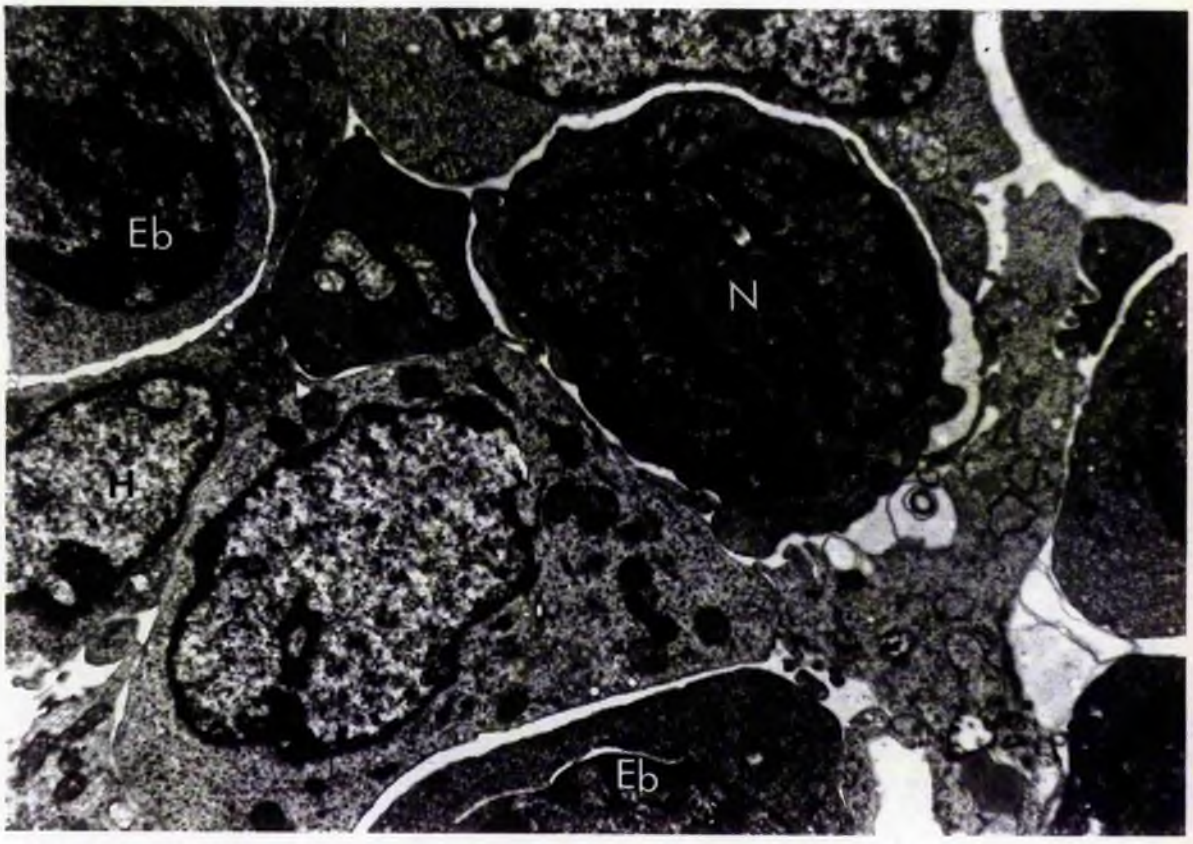



Fig. 3.1-10 x 10 400

Mixed-lineage cluster around a hepatocyte (H). An early neutrophil (N) forms an integral part of the cluster together with the developing erythroblasts (Eb). Granules are apparent in the cytoplasm of the neutrophil.

14 Day Fetal Liver

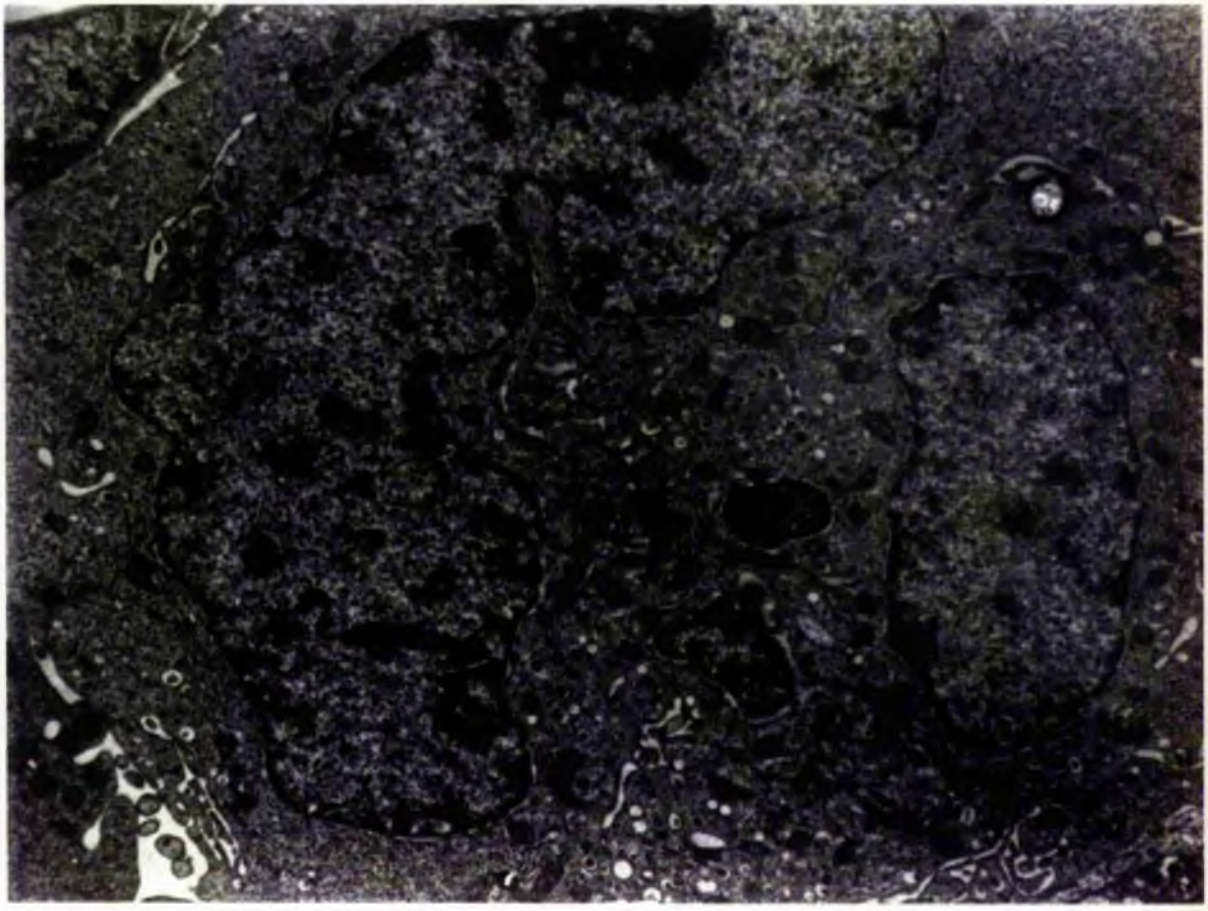


Fig. 3.1-11 x 9 500

Early megakaryocyte in close apposition to the cell membrane of a hepatocyte (H). Demarcation membranes can be seen within the cytoplasm of the megakaryocyte.

Haematopoiesis, in particular erythropoiesis, was maximal on the 15th day of gestation. The hepatic parenchyma was congested with cells of different haematopoietic lineages, erythroid cells however were by far the dominant lineage.

The number of haematopoietic cell clusters had increased, however the number of clusters with a central hepatocyte (fig. 3.1-12), remained higher than those with a central macrophage (fig. 3.1-13). The blood cell precursors remained intimately associated with the cytoplasmic extensions of the central cells. Some early blood cell precursors were completely enveloped in the extensions of central cells (fig. 3.1-14). Finger-like cytoplasmic projections of the central cells were frequently associated with the adjacent blood cell precursors. Evidence of cell-cell contact in such a manner was abundant. Granules which may contain glycogen were dispersed throughout the cytoplasm of both central cells and blood cell precursors. Siderosomes, which are considered to be aggregates of ferritin (Bessis 1977), were present in the cytoplasm of erythroblasts (fig. 3.1-13) and small granules which may also contain ferritin were present in all other blood cell precursors and the central cells. These granules were present in rhopheocytic vesicles of cytoplasm which appeared to be passing between the central cells and the adjacent blood cell precursors (figs. 3.1-15 & 3.1-16). These rhopheocytic vesicles were numerous where the cell membrane of the central cells was closely apposed to that of the blood cell precursors (fig. 3.1-17). It is plausible that the central cell may be furnishing the surrounding blood cell precursors with substances to promote their proliferation and differentiation.

Desmosomes connecting the cytoplasm of early blood cells and that of the central cells were numerous (fig. 3.1-18). Such attachments were also

observed between the cytoplasmic processes of adjacent hepatocytes. Gap junctions through which substances could pass from one cell to another were observed between blood cell precursors and central cells. Some peripheral blood cell precursors were connected to each other via cytoplasmic processes.

The number of granulocytes, monocytes and megakaryocytes had steadily increased. Both granulocytes (fig. 3.1-19) and monocytes (fig.3.1-20) were associated with cell clusters and displayed cytoplasmic processes which were closely apposed to neighbouring cells and the processes of the central cell. Cell clusters consisting of myelomonocytic cells only were not observed. Cells of the erythroid (fig. 3.1-21), granuloid and monocytic lineages were observed undergoing mitosis at this stage. Megakaryocytes remained isolated from the clusters, however close membrane associations with hepatocytes were retained. A number of megakaryocytes, closely situated to sinusoids, displayed organelle-free pseudopdia of their cytoplasm (fig. 3.1-22).

15 Day Fetal Liver

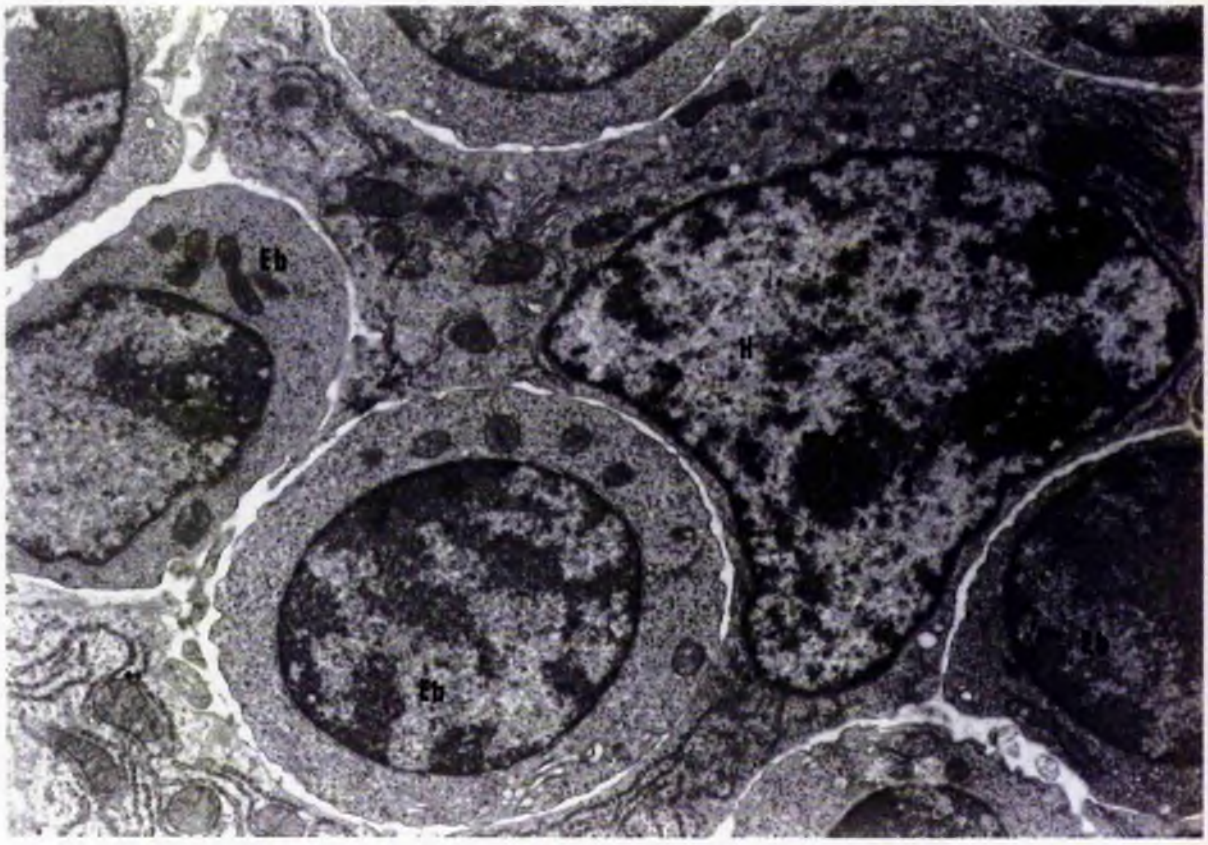



Fig. 3.1-12 x 7 500

Large number of developing erythroblasts (Eb) arranged around a central hepatocyte (H). Cytoplasmic associations between the central hepatocyte and the erythroblasts are numerous. Some erythroblasts are almost entirely encircled in the cytoplasmic processes of the hepatocyte.

15 Day Fetal Liver

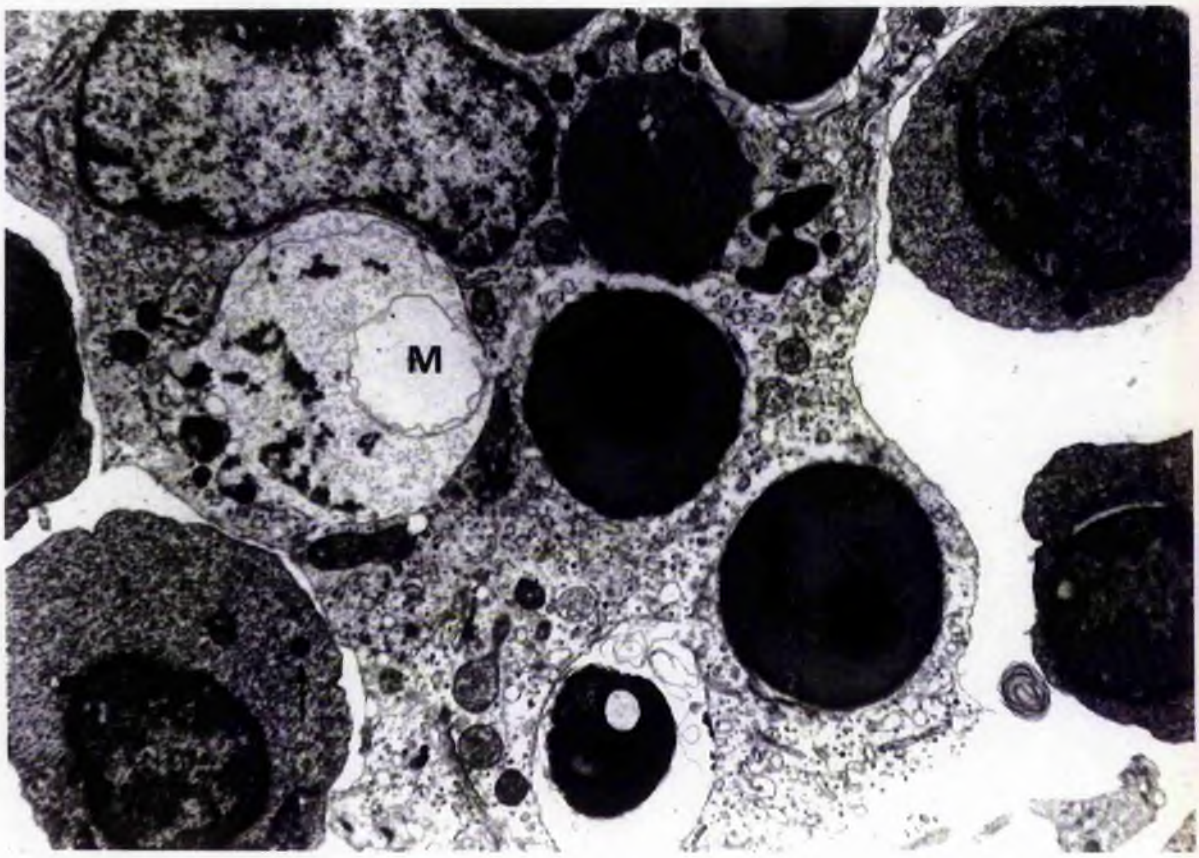



Fig. 3.1-13 x 9 000

Erythroblasts of various stages in maturation clustered around a central macrophage (M). The central macrophage contains a considerable amount of ingested debris in its cytoplasm, including recently extruded nuclei. A siderosome can be seen in the cytoplasm of an erythroblast (arrow).

15 Day Fetal Liver

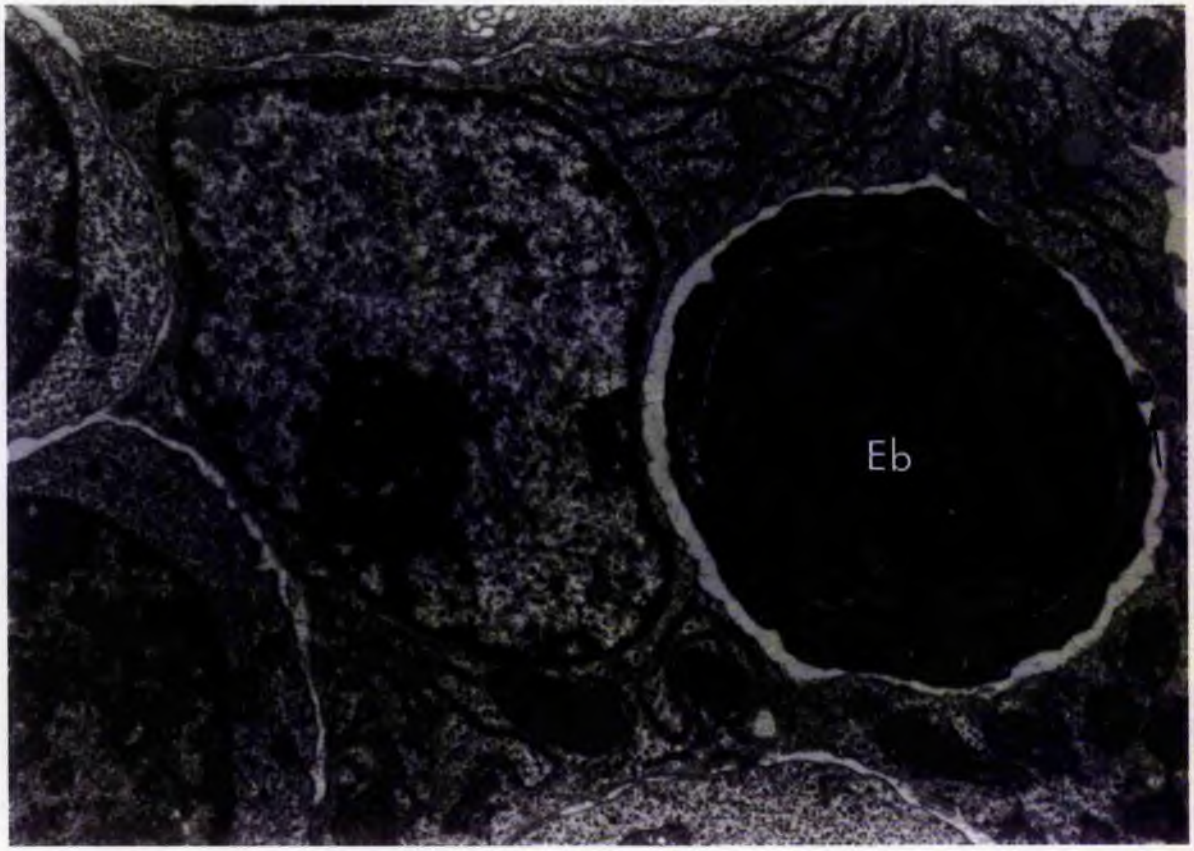
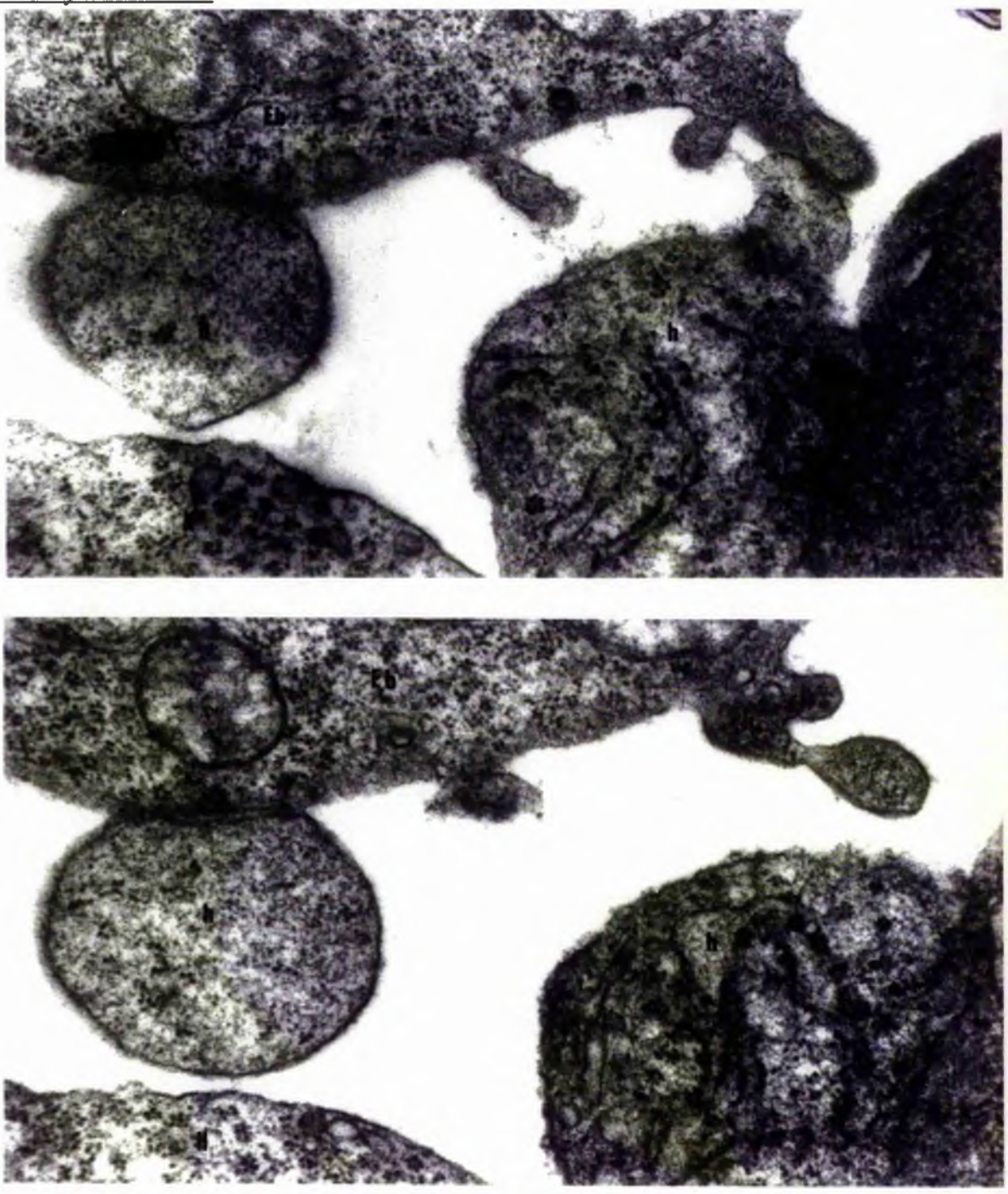



Fig. 3.1-14 x 14 200

An erythroblast (Eb) completely surrounded by hepatocyte cytoplasmic processes (H). There is close apposition of the cell membranes in places. A small piece of cytoplasm appears to be passing between the two cells (arrow).

15 Day Fetal Liver




Figs. 3.1-15 & 3.1-16 x 45 000

Movement of rhopheocytic vesicles between a hepatocyte (H) and an erythroblast (Eb). Fragments of hepatocyte cytoplasm (h) appear to attach to the cell membrane of the erythroblast and to the cytoplasmic extrusion of the erythroblast. The cytoplasmic vesicles may contain ferritin.

15 Day Fetal Liver

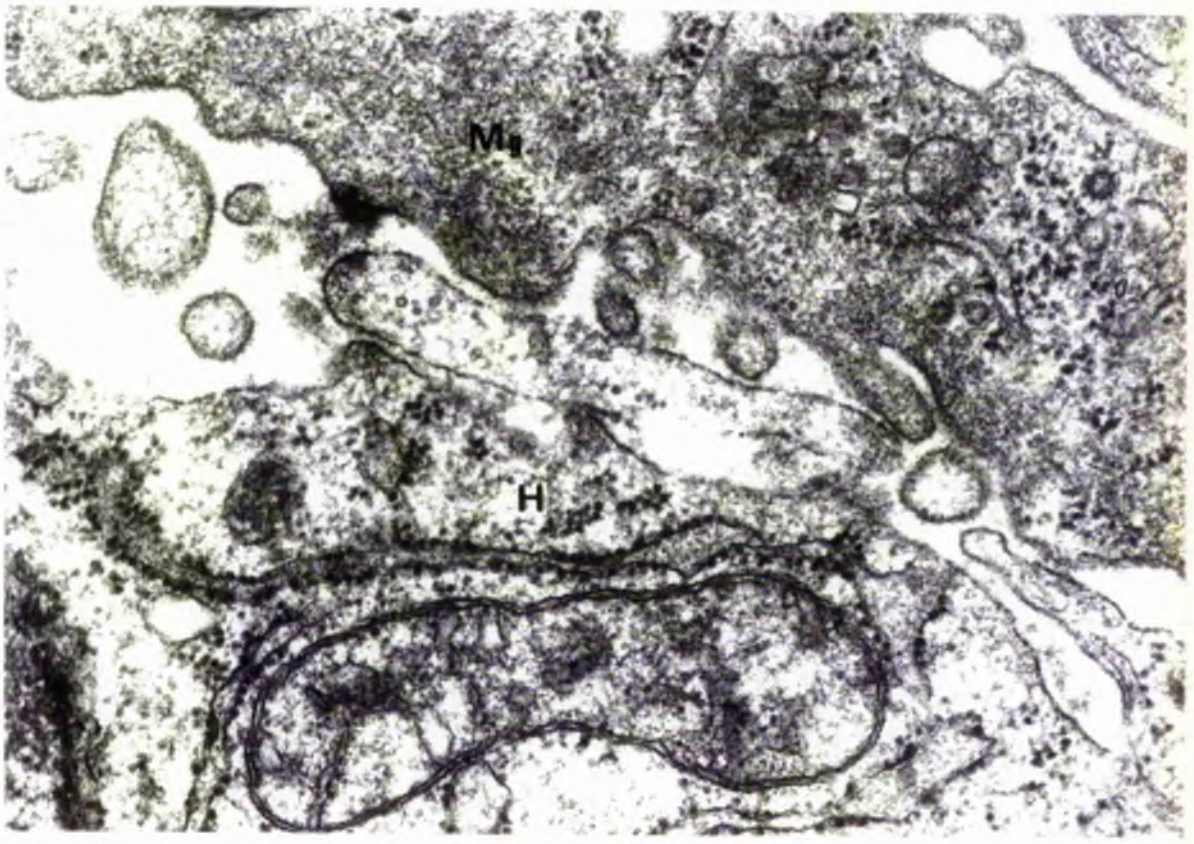



Fig. 3.1-17 x 68 000

Cytoplasmic vesicles, which may contain ferritin, in an area of close association of cell membranes of a hepatocye (H) and a megakaryocyte (Mg). Fragments of cytoplasm appear to be breaking off from both cells and could be moving between the cells.

15 Day Fetal Liver

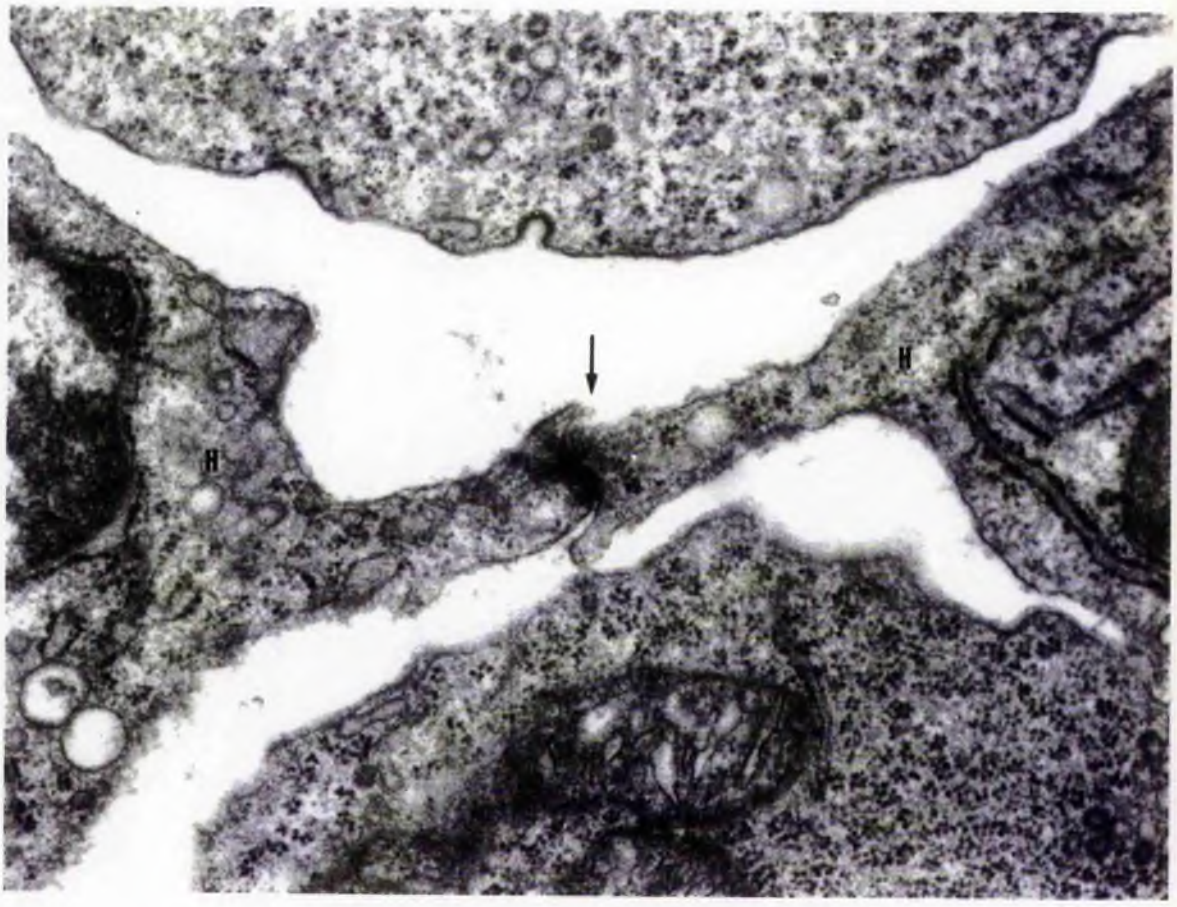



Fig. 3.1-18 x 50 000

Desmosome-like attachment at the junction of the cytoplasmic processes of two hepatocytes (arrow). A dense plaque which may contain intercellular proteins can be observed at the junction of the two processes.

15 Day Fetal Liver

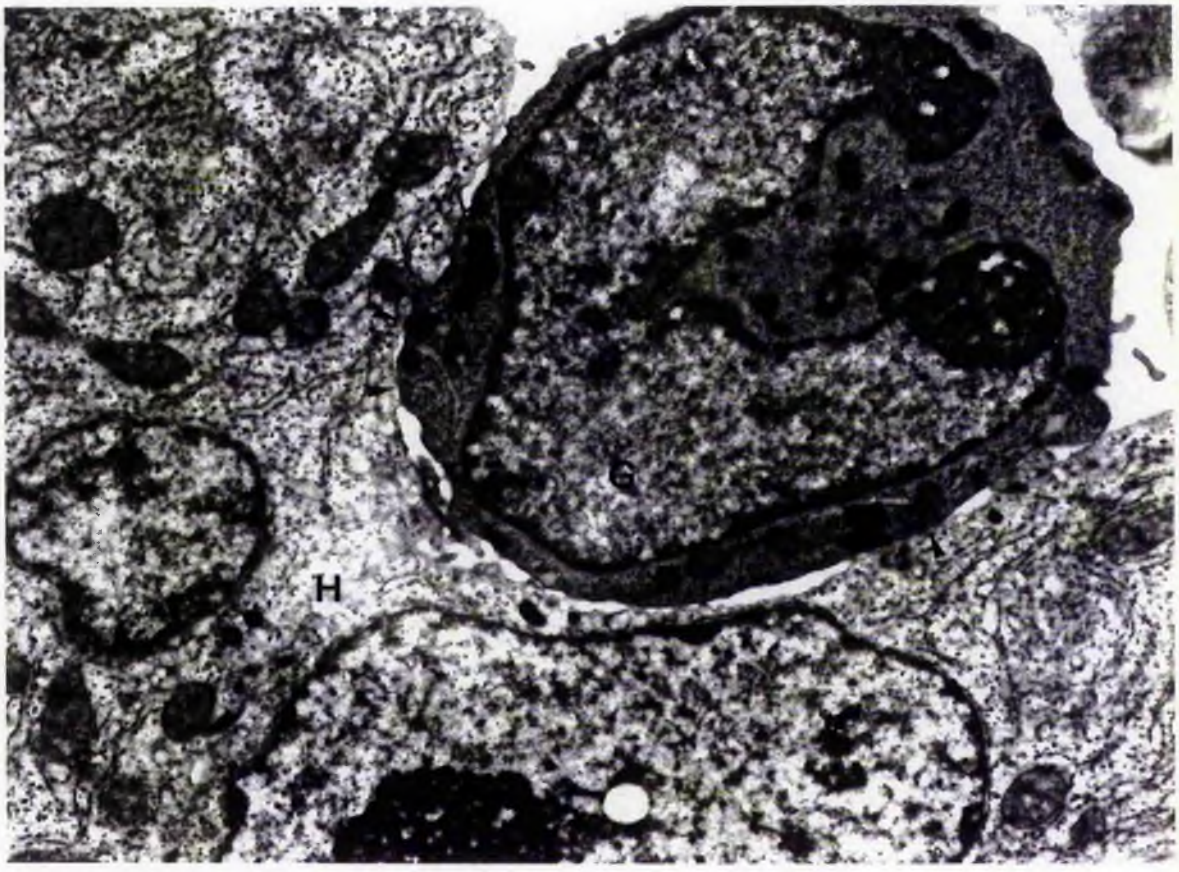



Fig. 3.1-19 x 14 200

Early granulocyte (G) in close association with a hepatocyte (H). There is very close apposition of the membranes of the two cells in many places and gap junctions are observed (arrows), vessicles of cytoplasm are also apparent. A centriole can be seen in the cytoplasm of the neutrophil

15 Day Fetal Liver

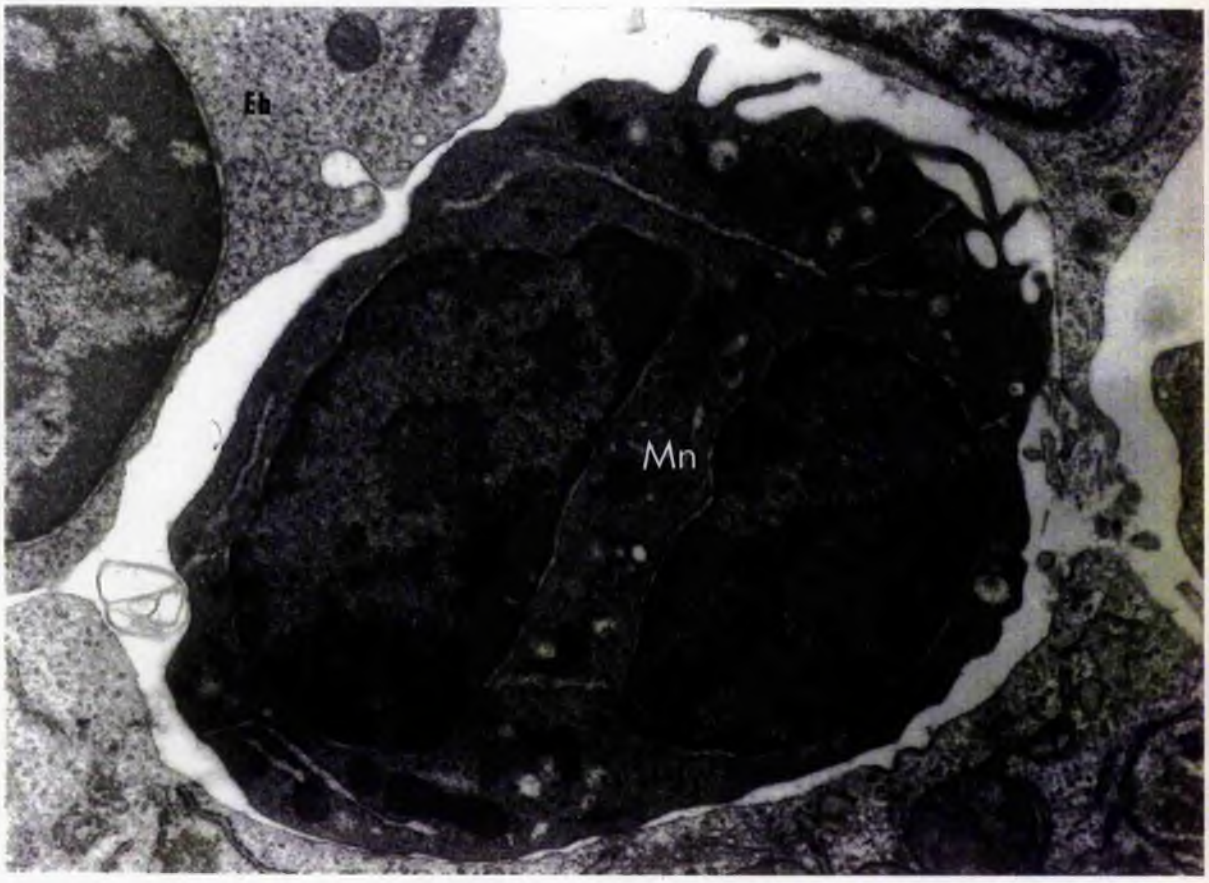



Fig. 3.1-20 x 22 000

Monocyte (Mn) closely associated with a hepatocyte (H) and an adjacent erythroblast (Eb).Cytoplasmic processes of the monocyte extend towards the hepatocyte. Cytoplasmic vessicles of the hepatocyte are also observed in close association with the monocyte cell membrane.

15 Day Fetal Liver

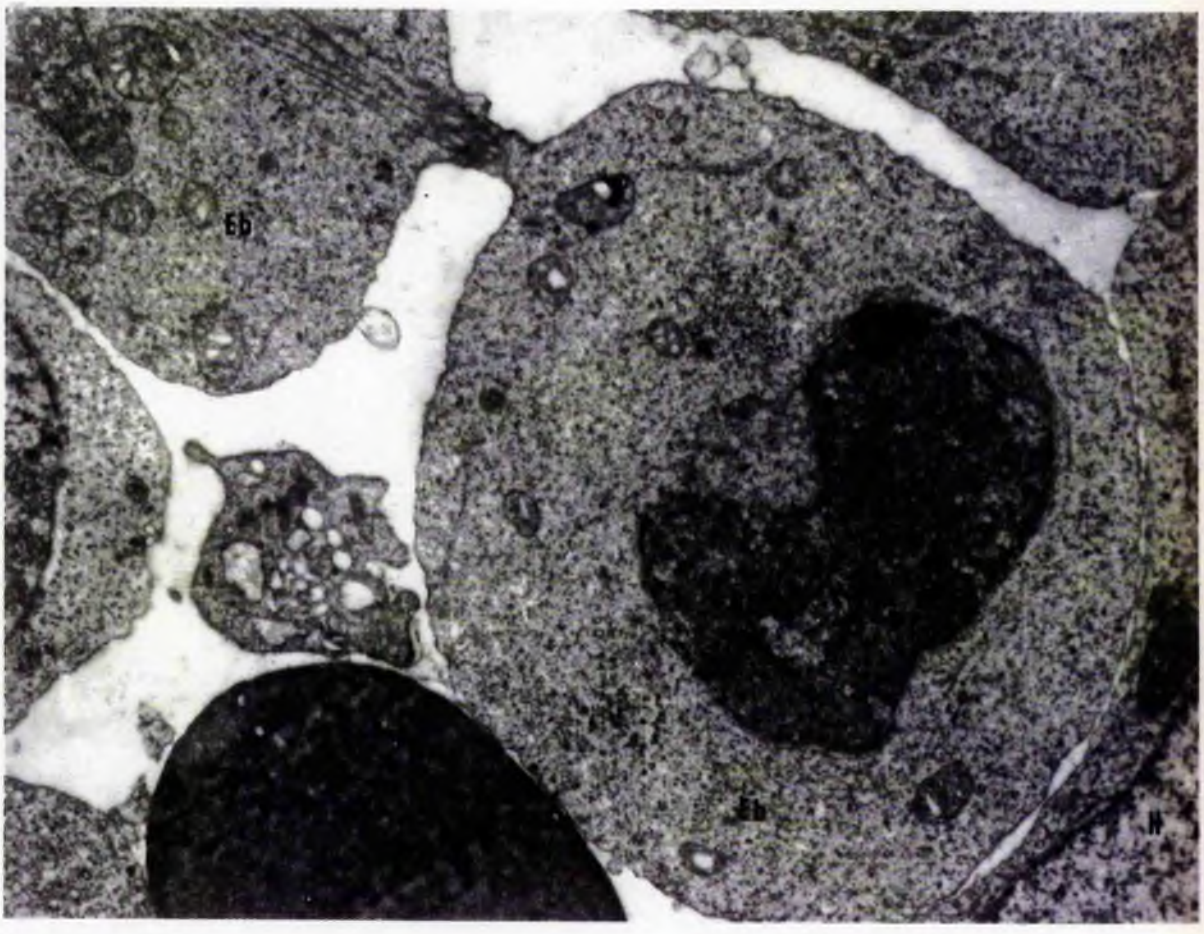



Fig. 3.1-21 x 24 267

Erythroblasts (Eb) undergoing cytokinesis in close proximity to a hepatocyte (H). Microtubules are evident in the midbody.

15 Day Fetal Liver

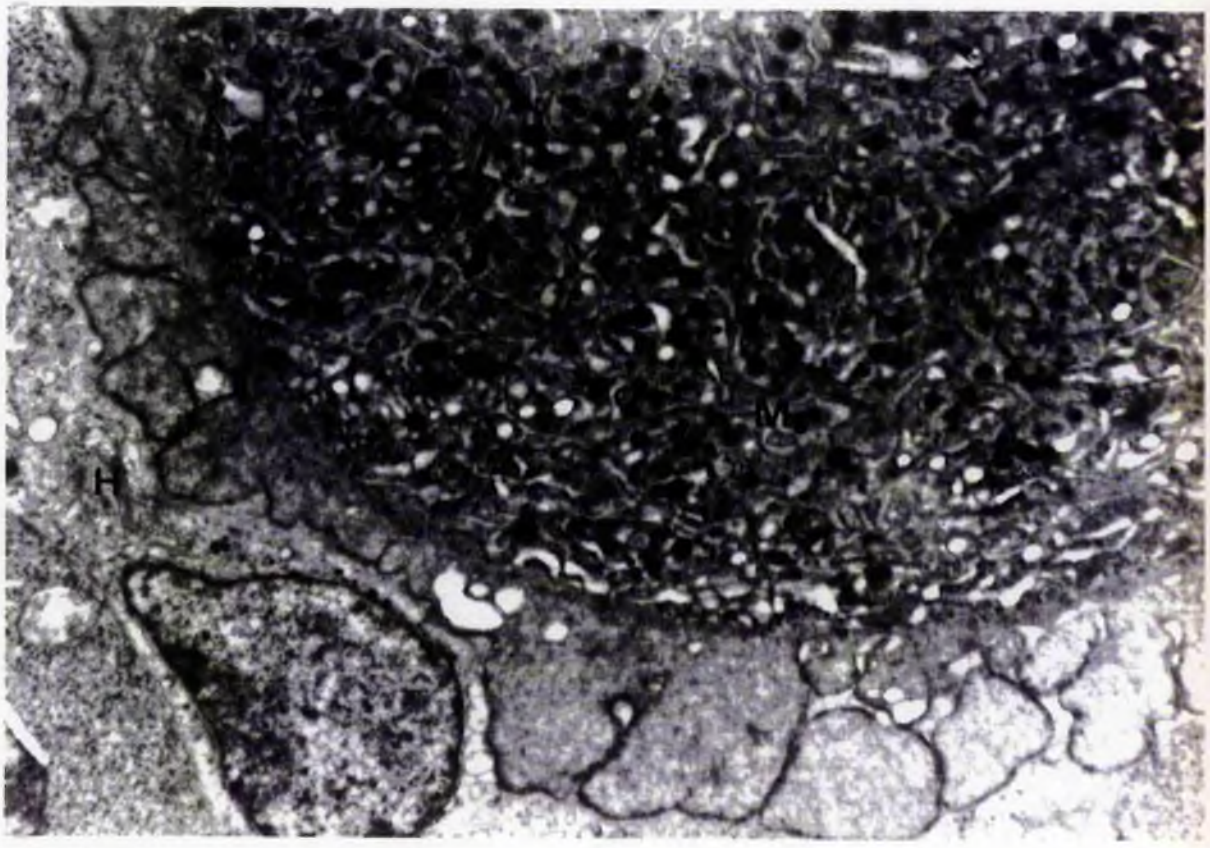



Fig. 3.1-22 x 15 200

Megakaryocyte (Mg) which is positioned close to a sinusoid and intimately associated with a hepatocyte (H). The megakaryocyte displays pseudopodia which do not contain any organelles. This phenemenon appears to be common in hepatocytes which are situated close to sinusoids.

The liver of the 18 day fetus was very different from that of the 15 day fetus. There was a reduced number of cell clusters and the number of cells within each cluster was also reduced. The number of blood cell precursors of the erythroblast lineage had decreased together with the number of megakaryocytes present. However undifferentiated stem cells were still observed to form distinctive cells clusters around macrophages (fig. 3.1-23) and hepatocytes (fig.3.1-25), while megakaryocytes remained associated with hepatocytes only.

In contrast to the reduction in the number of erythroblasts and megakaryocytes, the number of granulocytes and monocytes had increased. Early monocytes were observed completely enveloped in the cytoplasmic processes of hepatocytes. Rhopheocytic vesicles (fig. 3.1-24), desmosomes and gap junctions between the cytoplasm of central cells and the surrounding precursors remained numerous. Cytoplasmic processes of blood cell precursors were observed closely associated with the processes of the central cells (fig. 3.1-25).

The number of hepatocytes was also markedly increased and those not associated with haematopoietic cells were arranged in compact groups (fig. 3.1-26). Morphological differences were apparent in hepatocytes which had not been involved in haematopoiesis when compared to those hepatocytes which had. The hepatocytes which were closely associated with haematopoietic cells exhibited a distinct cytoplasmic arrangement to form close associations with haematopoietic cells, an increase in glycogen and ferritin deposits and a lamellar stacking arrangement of the endoplasmic reticulum. These charactersistics were not observed in hepatocytes which had not been associated with haematopoiesis, nor did they display

cytoplasmic processes, however the cell membranes of adjacent hepatocytes were closely associated and connected via gap junctions.

18 Day Fetal Liver

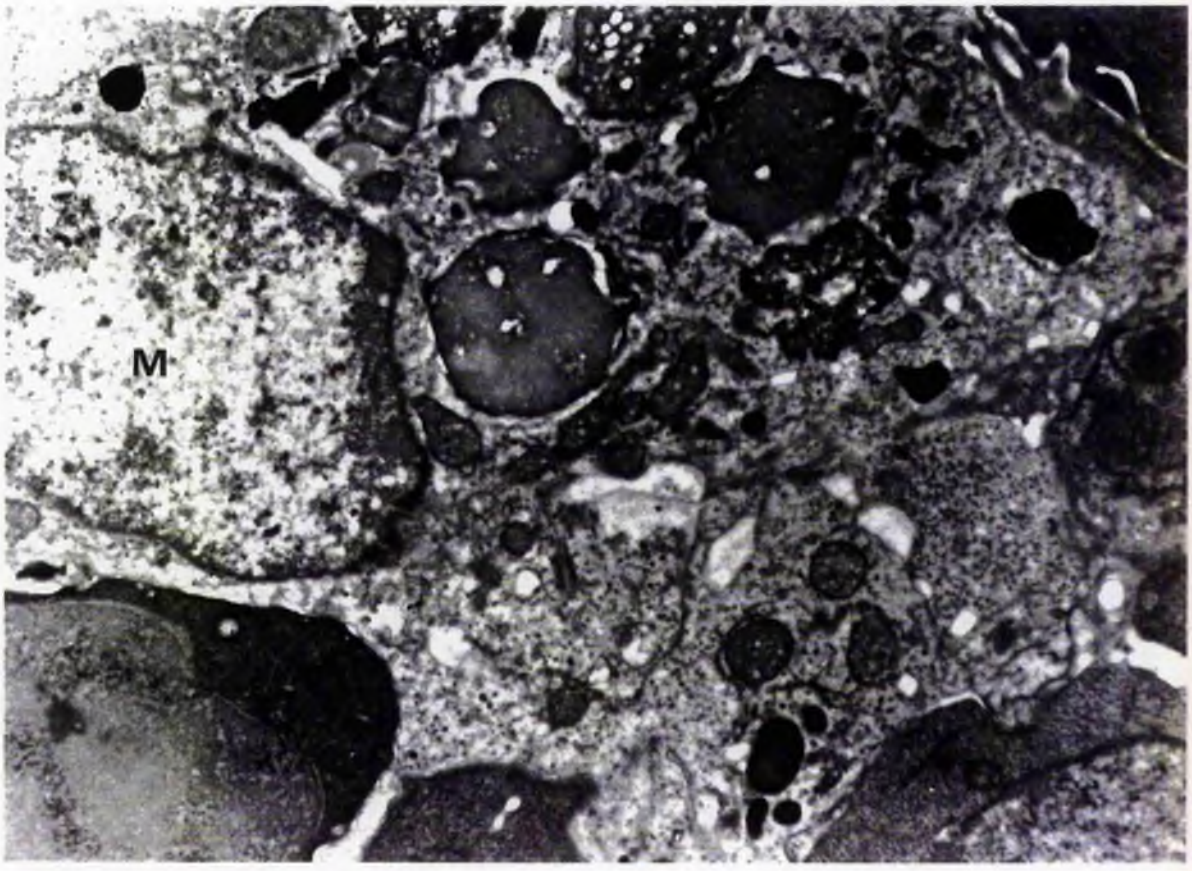



Fig. 3.1-23 x 11 400

Central macrophage (M) of an erythroblastic island. The macrophage contains a large amount of degredation products within its cytoplasm, including a few extruded nuclei. Intimate associations with the macrophage and the surrounding erythroblasts are observed.

18 Day Fetal Liver

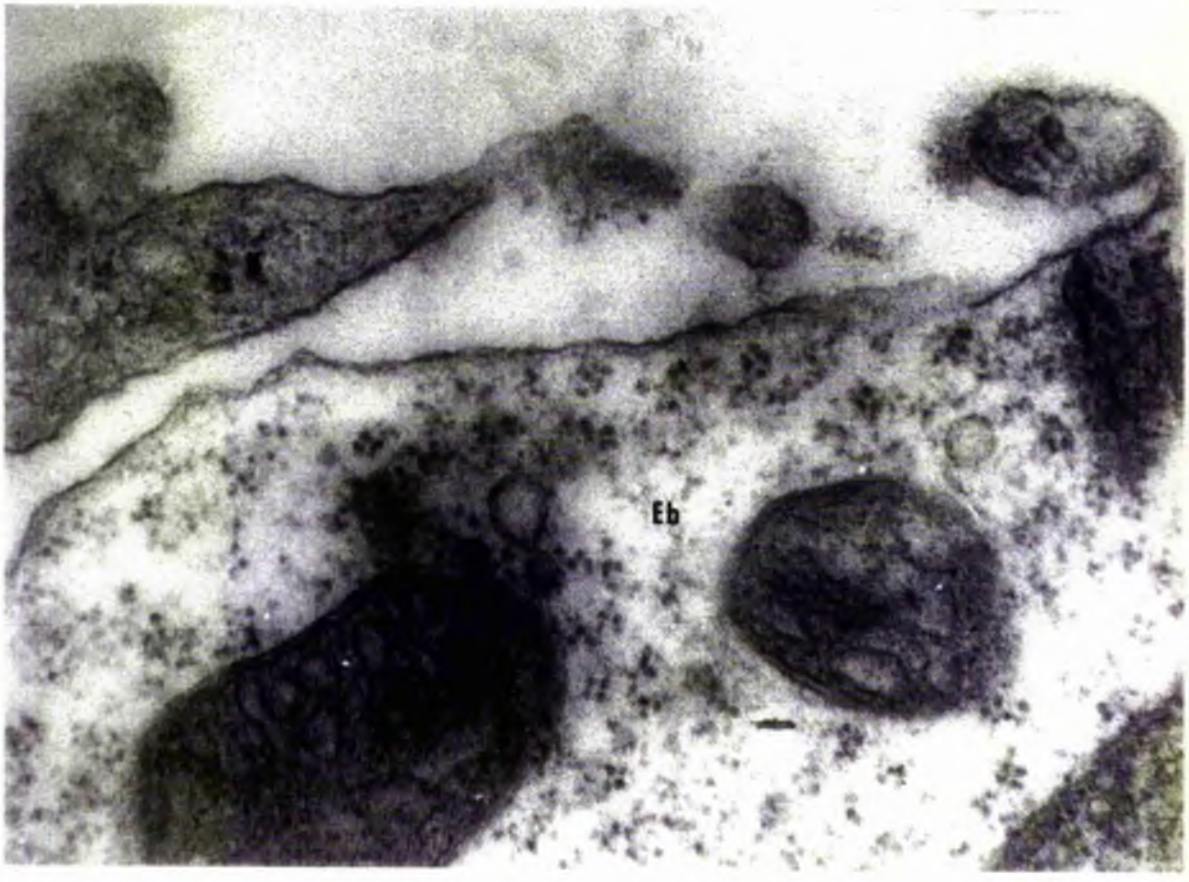



Fig. 3.1-24 x 85 000

Cytoplasmic vesicles derived from a hepatocyte process (H). The vesicles are observed closely associated with the cell membrane of an erythroblast (Eb) and may form an attachment to the erythroblast.

18 Day Fetal Liver

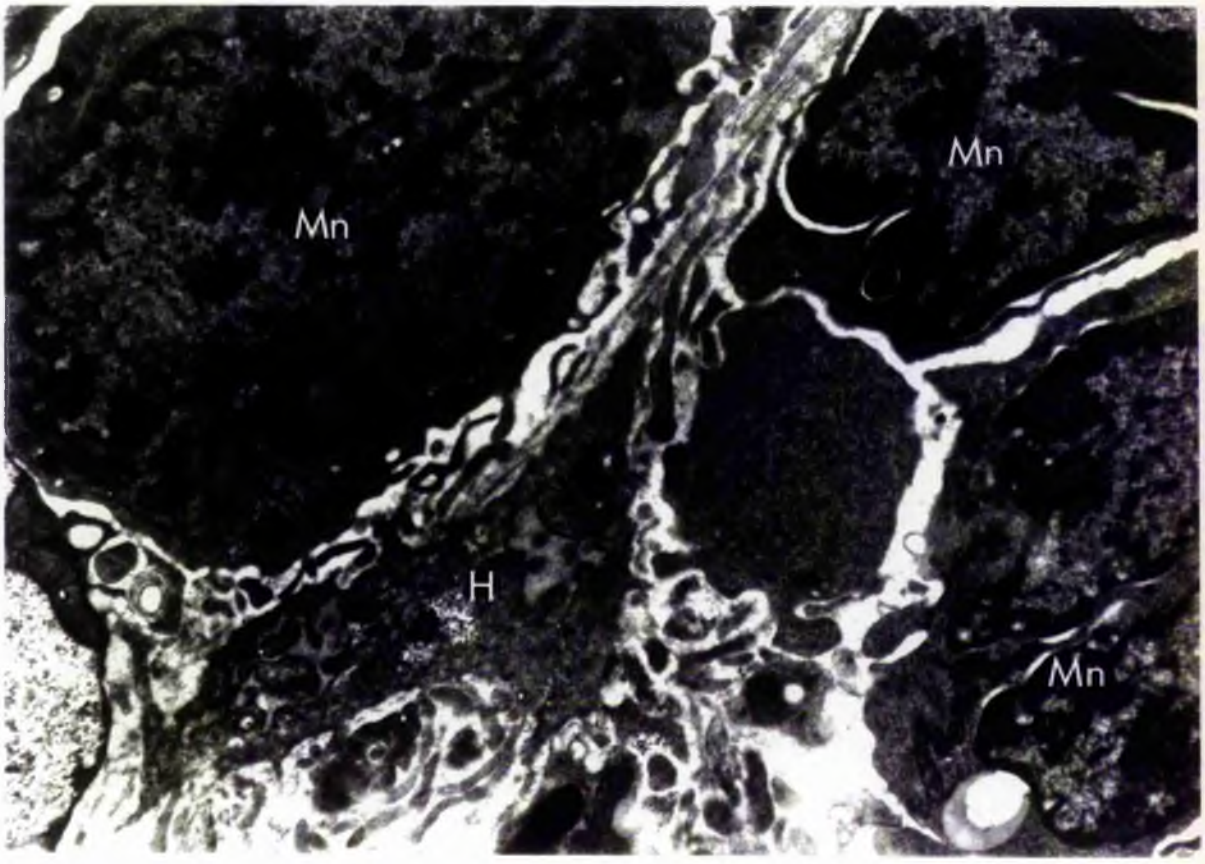



Fig. 3.1-25 x 12 780

Monocytic cells (Mn) around a central hepatocyte (H). The monocytes extend numerous proceses and rhopheocytic vesicles are abundant between the cell membranes of the hepatocyte and the monocytes.

18 Day Fetal Liver

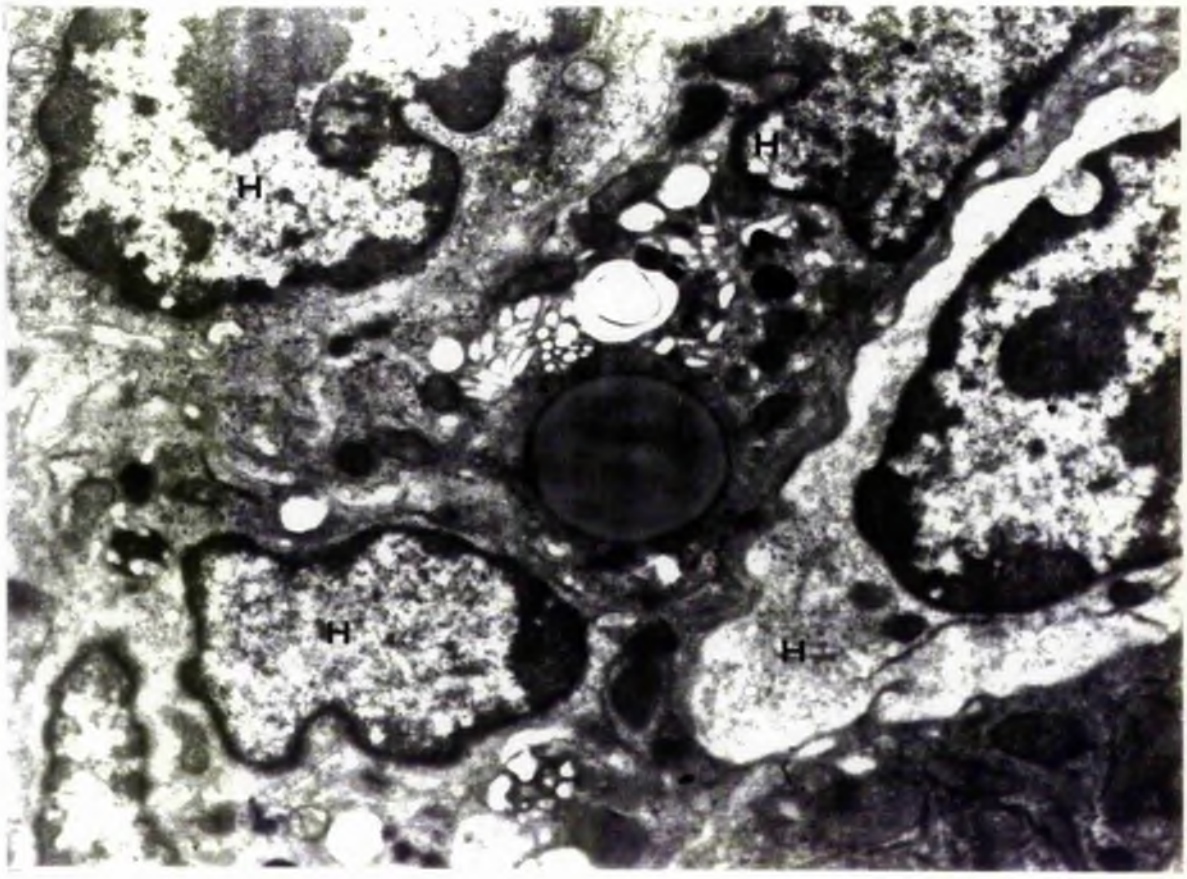



Fig. 3.1-26 x 31 240

Cluster of hepatocytes which have not been associated with haematopoietic cells. These hepatocytes did not display a stacking arrangement of their RER nor an increase in ferritin deposists. The cell mebranes of the adjacent hepatocytes are closely apposed but cytoplasmic processes and rhopheocytic vesicles are not observed.

### 3.1.2 Neonatal Liver

The pattern of haematopoiesis in the newborn liver showed further changes from those observed in the 18 day fetus.

The number of granulocytes and monocytes had further increased, erythroid cells however remained the dominant lineage. Definitive lymphoid cells were apparent (fig. 3.1-27). These cells could be distinguished from both erythroid and granuloid cells, in that they were non-granular and had uniform ultrastructural features such as irregular nuclei and their pattern of nucleochromatin was different to that of erythroblasts.

Distinct cell clusters were still obvious, however they appeared to consist of more mature elements rather than very early precursors. Clusters consisted of mixed lineage cells, although the more central positions remained mostly occupied by erythroid cells (fig. 3.1-28). The number of clusters with a central hepatocyte far outnumbered those with a central macrophage, which appeared to have decreased in number. The configuration of most of the clusters had changed, the blood cell precursors were often surrounded by hepatocytes (fig. 3.1-27), rather than one central cell surrounded by blood cell precursors. Intimate associations with the central cells of the cluster and the blood cell precursors remained obvious (fig. 3.1-29), thus the potential for transfer of substances between the cells remained high. Cytoplasmic processes of blood cell precursors and rhopeocytic vesicles (fig. 3.1-30) remained abundant and bridges connecting adjacent blood cell precursors had increased in number (figs. 3.1-28 & 3.1-31).

The number of hepatocytes had further increased and many were seen to be associated with adjacent hepatocytes through cytoplasmic projections (fig.

3.1-32). The hepatocytes had a more regular outline, contained a number of lipid vacuoles and a considerably higher number of mitochondria, with well defined cristae, than those previously observed.

The most dramatic changes were observed in the neonatal liver on the 10th day after birth. The number of hepatocytes had further increased and they were organised into cords. This organisation restricted haematopoietic cells to anatomically isolated foci, close to the sinusoids, rather than the diffuse distribution of haematopoietic cells observed at other developmental stages. The mitochondria, with well defined cristae, and endoplasmic reticulum of the hepatocytes were prominent (fig. 3.1-33), suggesting a high degree of cellular activity and numerous lipid vacuoles were observed in the cytoplasm. Cell membranes of adjacent hepatocytes were very closely apposed to each other or linked via interdigitating cytoplasmic projections forming a dense network of cells.

The number of haematopoietic cell clusters was markedly reduced and their composition was restricted. Single-lineage clusters of erythroid, granuloid or lymphoid cells were observed. The number of cells in each cluster was lower than those observed in fetal liver, however intimate associations with hepatocytes remained abundant (fig. 3.1-34). Macrophages were present, however they were not associated with haematopoietic cells. Isolated monocytes were commonly observed closely associated with hepatocytes (fig. 3.1-35). Megakaryocytes were present in lower numbers than previous liver samples. They remained closely associated with hepatocytes and did not form single-lineage clusters.

Newborn Liver

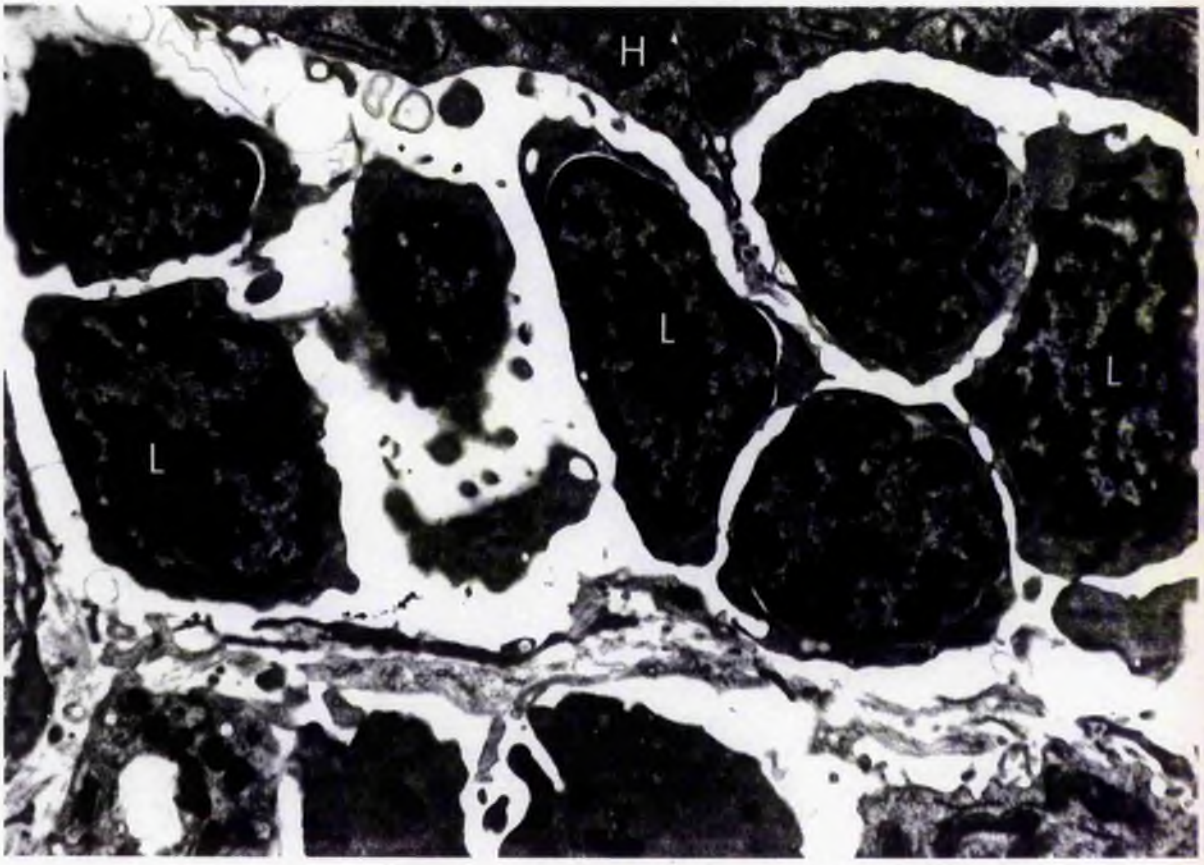



Fig. 3.1-27 x 9 000

Cluster of lymphocytes (L) surounded by hepatocytes. This arrangement is more common, than haematopoietic cells forming clusters around a central cell, in the newborn liver. Intimate associations of the cytoplasmic processes of the hepatocytes with the lymphocytes are observed.

Newborn Liver

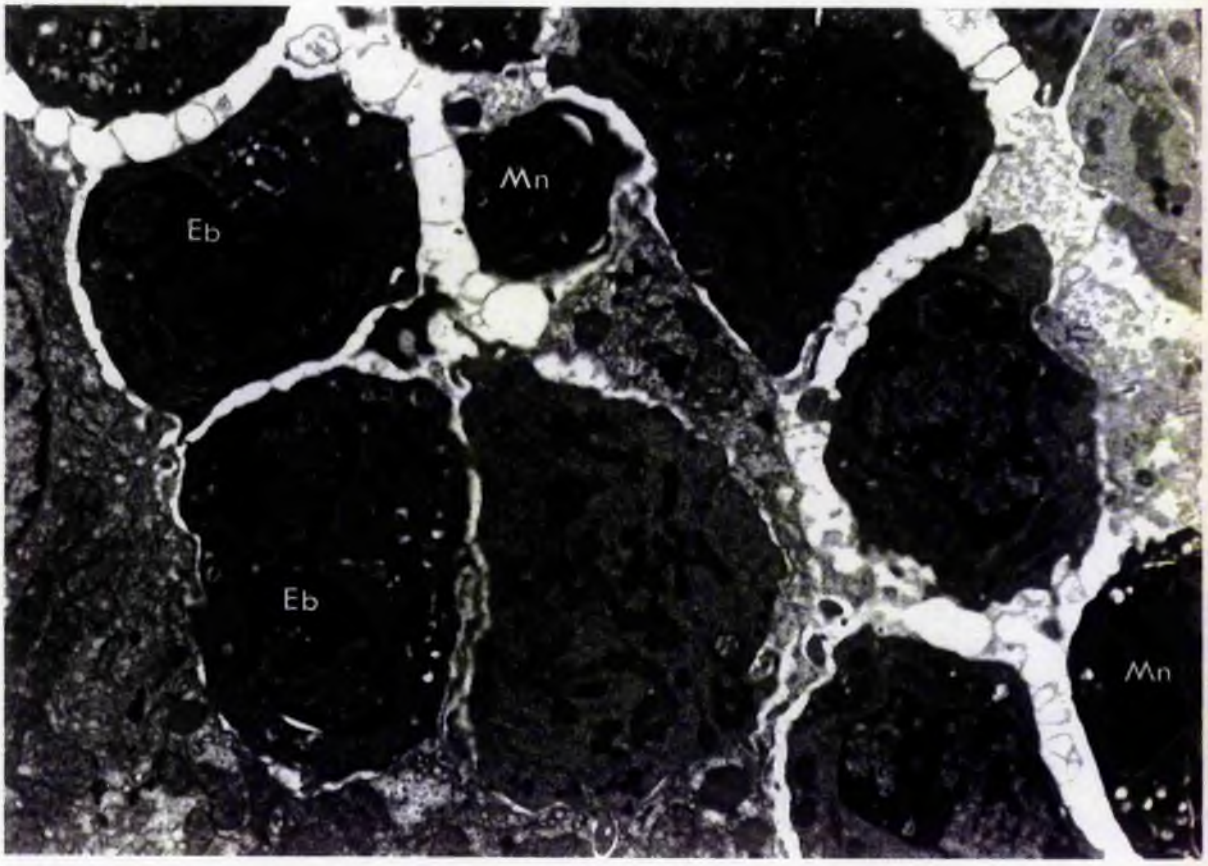



Fig. 3.1-28 x 9 000

Mixed-lineage cluster of erythroblasts (Eb) and monocytes (Mn) arranged around a central hepatocyte (H). The erythroblasts remain in central positions, closer to the hepatocyte, while the monocytes are situated on the periphery. Intimate associations of the hepatocyte cytoplasm with the blood cell precursors remain abundant.

Newborn Liver

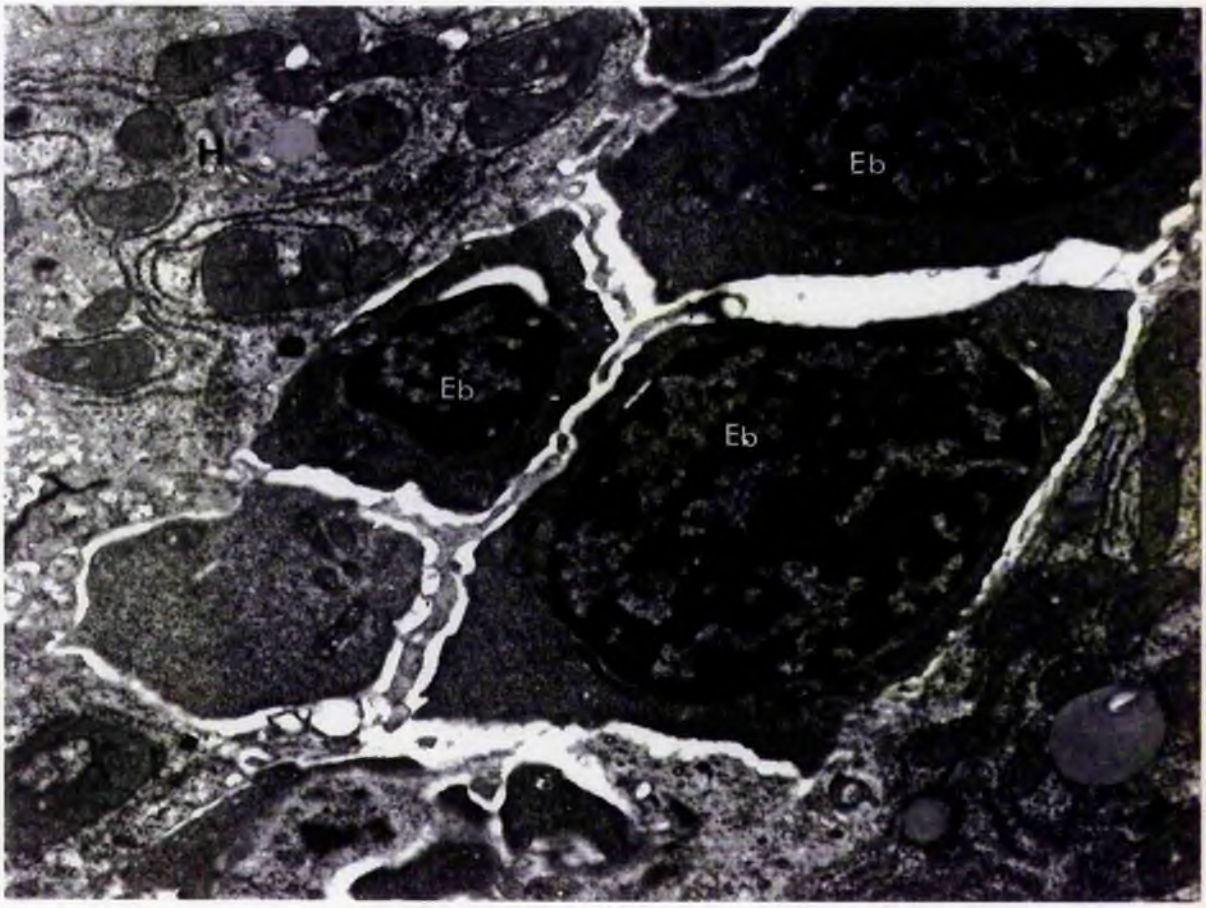



Fig. 3.1-29 x 7 100

Erythroblasts (Eb) at various stages in development surrounded on either side by two hepatocytes (H). Cytoplasmic processes of the hepatocytes pass between the erythroblasts and in places the cell membranes of the hepatocytes are closely apposed to those of the erythroblasts.

Newborn Liver

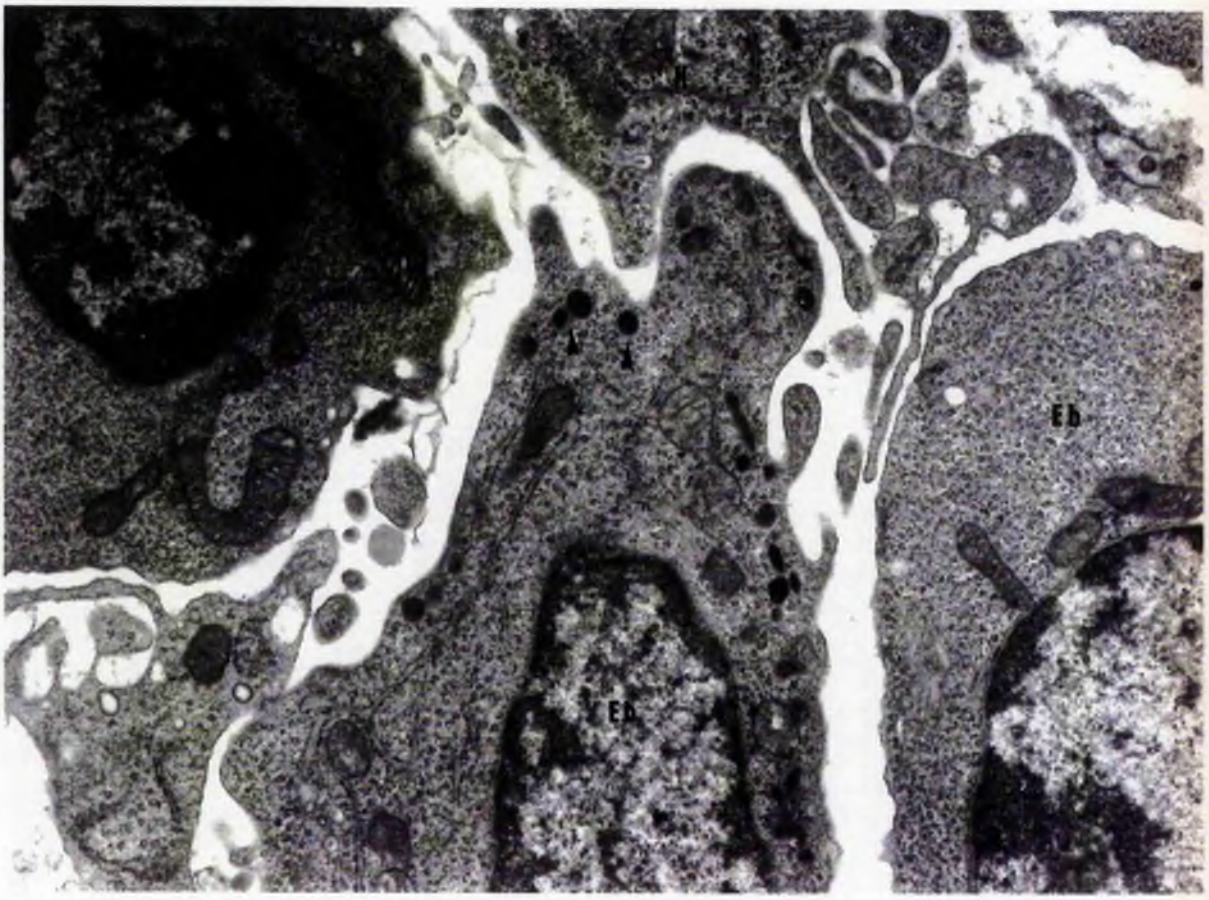



Fig. 3.1-30 x 18 333

Cytoplasmic vessicles and processes between the cell membranes of a hepatocyte (H) and three erythroblasts (Eb). A hepatocytic process can be seen investing into the cytoplasm of an erythroblast. Siderosomes are present in the cytoplasm of an erythroblast (arrows).

Newborn Liver

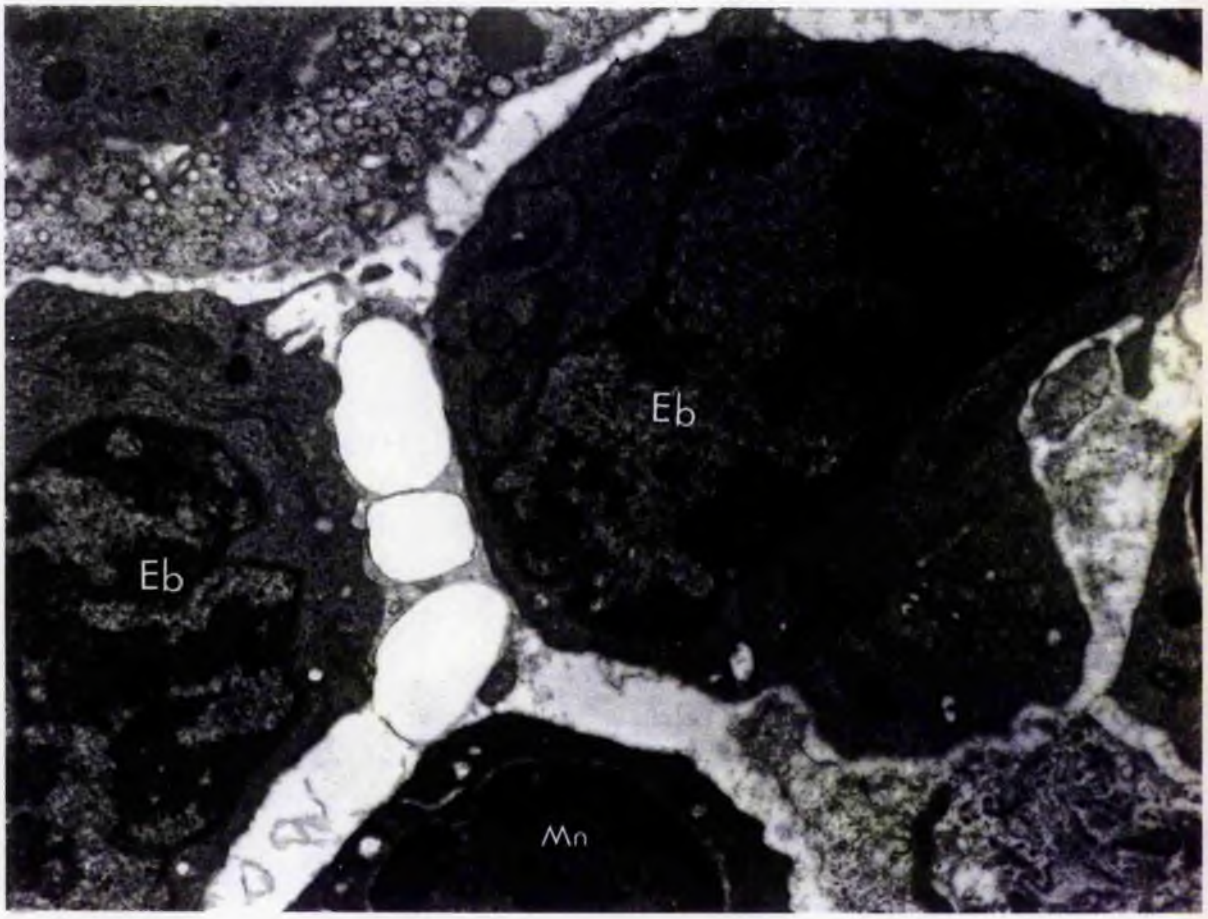



Fig. 3.1-31 x 18 200

Erythroblasts (Eb) and a monocyte (Mn) which are linked to each other by bridges. Such linking of adjacent blood cell precursors was never seen at earlier stages in development. In newborn liver however, it was a common occurence.

Newborn Liver

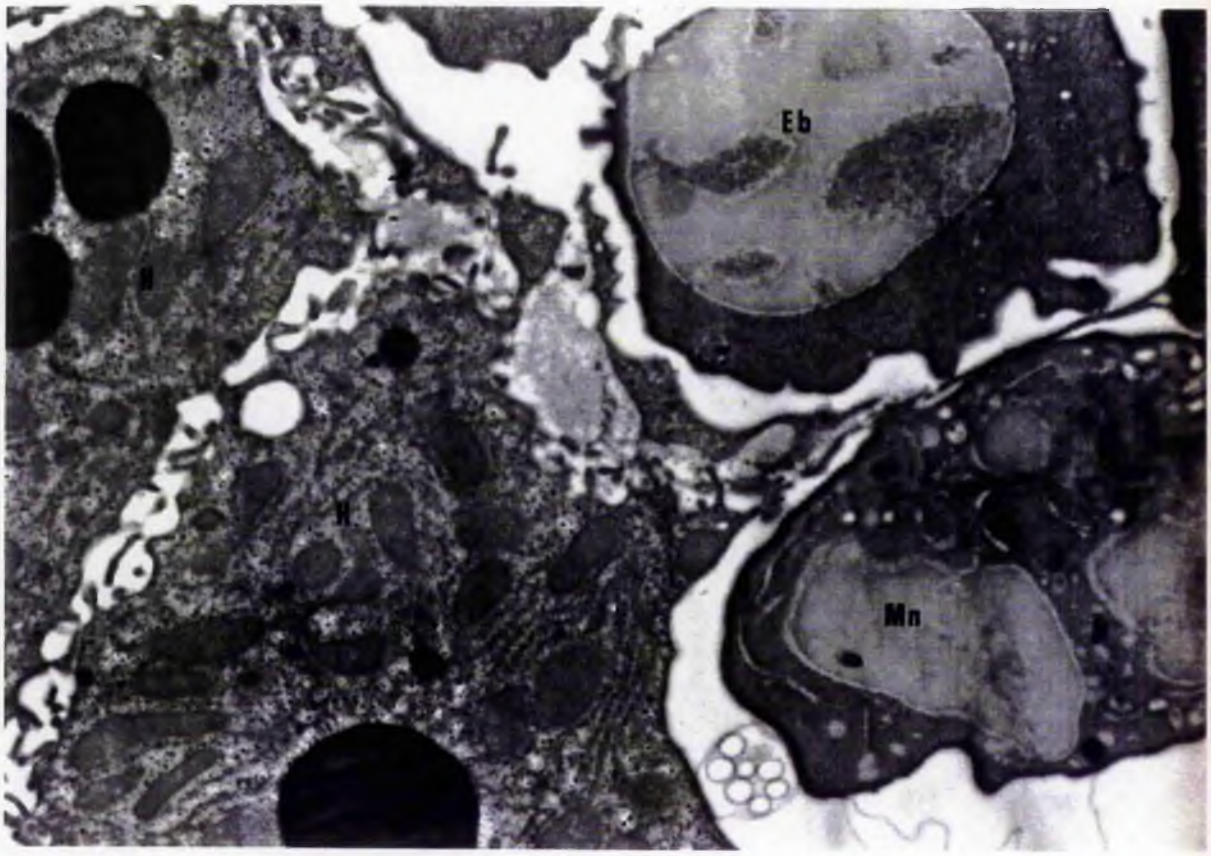



Fig. 3.1-32 x 17 100

Erythroblasts (Eb) and a monocyte (Mn) clustering around a group of hepatocytes (H). The erythroblasts are associated with cytoplasmic processes of the hepatocyte. The number of mitochondria and lipid vacuoles within the hepatocytes has increased and adjacent hepatocytes are closely associated via cytoplasmic processes.

10 Day Neonatal Liver


Fig. 3.1-33 x 18 200

Three hepatocytes of neonatal liver. The membranes of the hepatocytes are closely apposed to form a tight network of cells. The number of mitochondria has increased and the cristae are well defined. There is also an increase in the number of lipid vacuoles. These observations suggest a high degree of hepatocytic activity.

10 Day Neonatal Liver

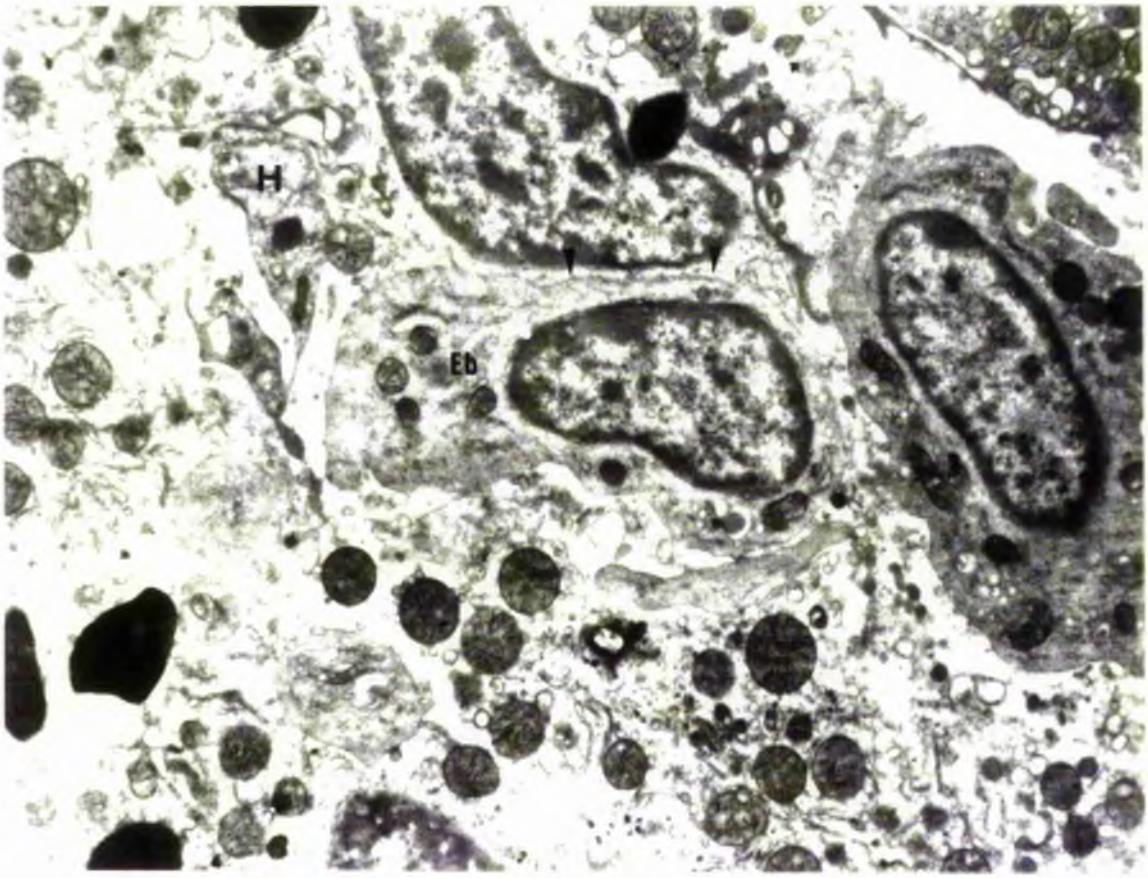



Fig. 3.1-34 x 11 400

Erythroblasts (Eb) completely surounded by the cytoplasm of a hepatocyte (H). Gap junctions are apparent between the erythroblasts and the hepatocyte cytoplasm (arrows) and cytoplasmic vesicles, in close proximity to the erythroblasts are evident.

10 Day Neonatal Liver

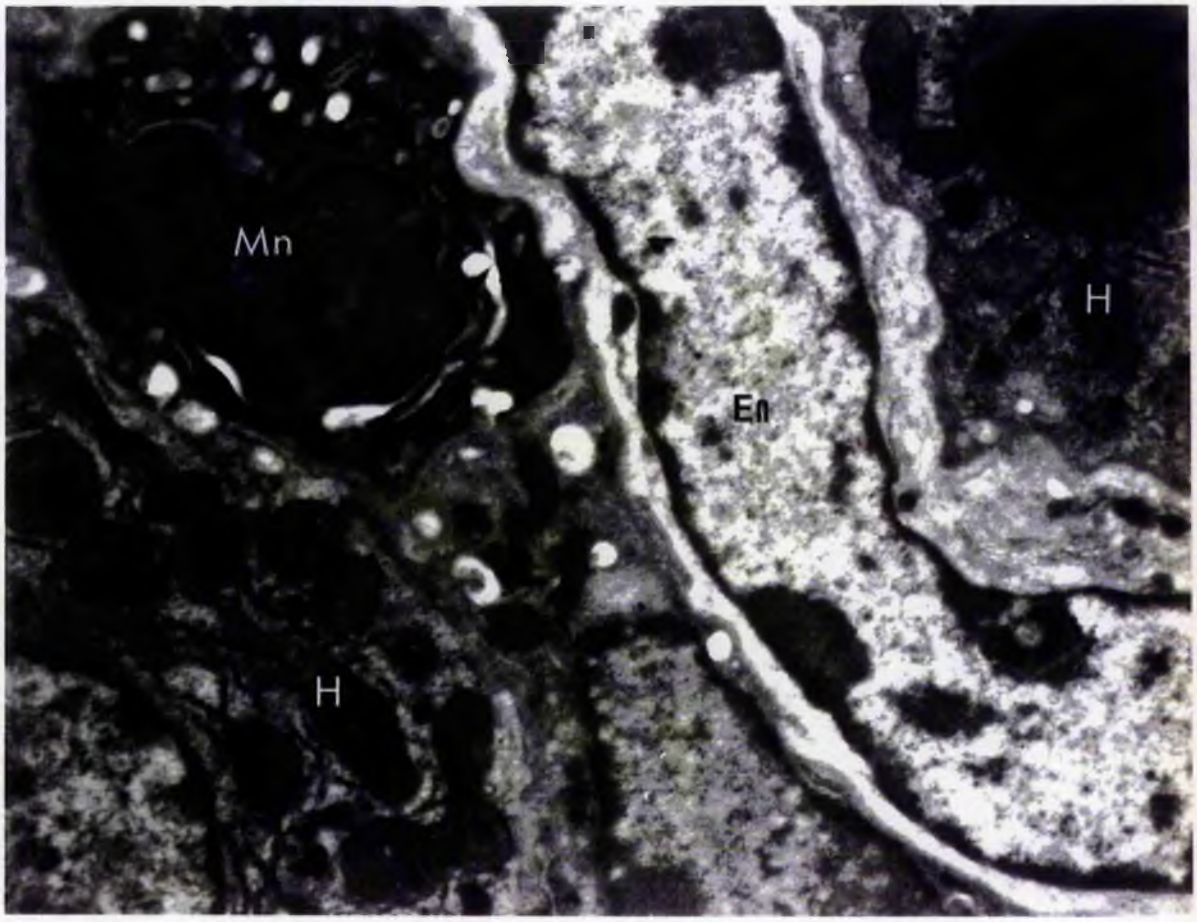



Fig. 3.1-35 x 15 620

Isolated monocyte (Mn) observed surounded by the cytoplasm of hepatocytes (H) and an endothelial cell (En). The cell membrane of the hepatocyte is very closely apposed to that of the monocyte.

## 3.2 Confocal Laser Scanning Microscopy of Fetal and Neonatal Liver

Fetal and neonatal liver tissue were stained with Perl's Prussian Blue which is specific for iron. The iron within the cells can be easily recognised by its diffuse blue colour when examined by light microscopy. Examination with the confocal laser scanning microscope, permits the addition of colour to the field of view. The ferritin containing cells are easily recognised as the brightest and most intensely stained cells, regardless of the colour applied to the field of view.

### 3.2.1 Day 15 Fetal Liver

Cell clusters were abundant at this stage in development, randomly dispersed throughout the sections examined (fig. 3.2-1). The blood cell precursors were observed arranged around central macrophages (fig. 3.2-2) and hepatocytes (fig. 3.2-3). Gradients of differentiation, as previously described, were apparent in the clusters. The least mature cells, which were the most intensely stained, were situated close to the central cell (fig. 3.2-2). The more mature blood cell precursors were situated in peripheral positions and did not display the intense colour to the same degree as the immature precursors.

Cytoplasmic processes of the central cells were observed in close association with the adjacent blood cell precursors, ferritin particles were observed within the cytoplasmic processes. Some immature cells were observed completely enveloped in the cytoplasmic processes of the central cells (fig. 3.2-4). A high degree of ferritin was present in both the enveloped cell and within the cytoplasmic processes.

Megakaryocytes were observed, (figs. 3.2-3 & 3.2-5), dispersed throughout the parenchyma, usually closely associated with adjacent hepatocytes. The

megakaryocytes also displayed positive staining for ferritin, though not to the same extent as the immature erythroblasts.

15 Day Fetal Liver

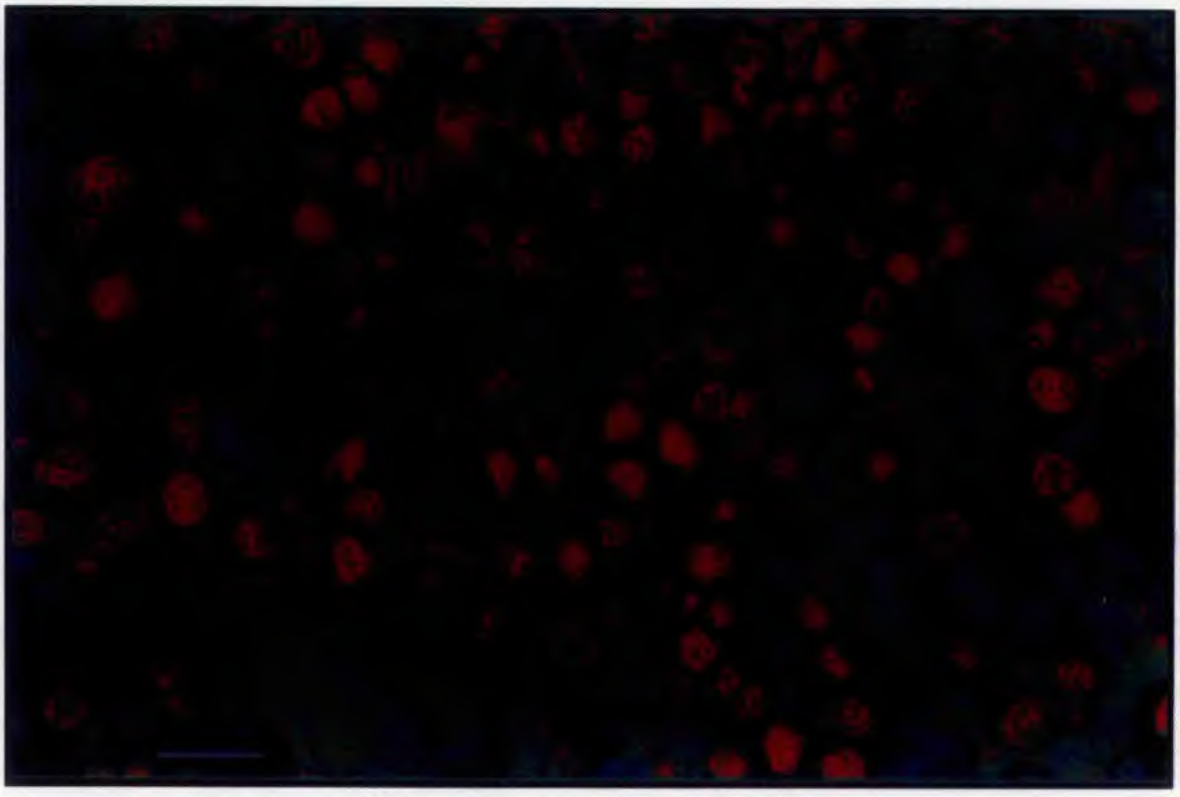


Fig. 3.2-1 Scale bar 10μ

Random distribution of haematopoietic cells throughout the hepatic parenchyma in the 15 day fetus. The bright, red cells are the immature precursors which contain a considerable amount of ferritin.

15 Day Fetal Liver

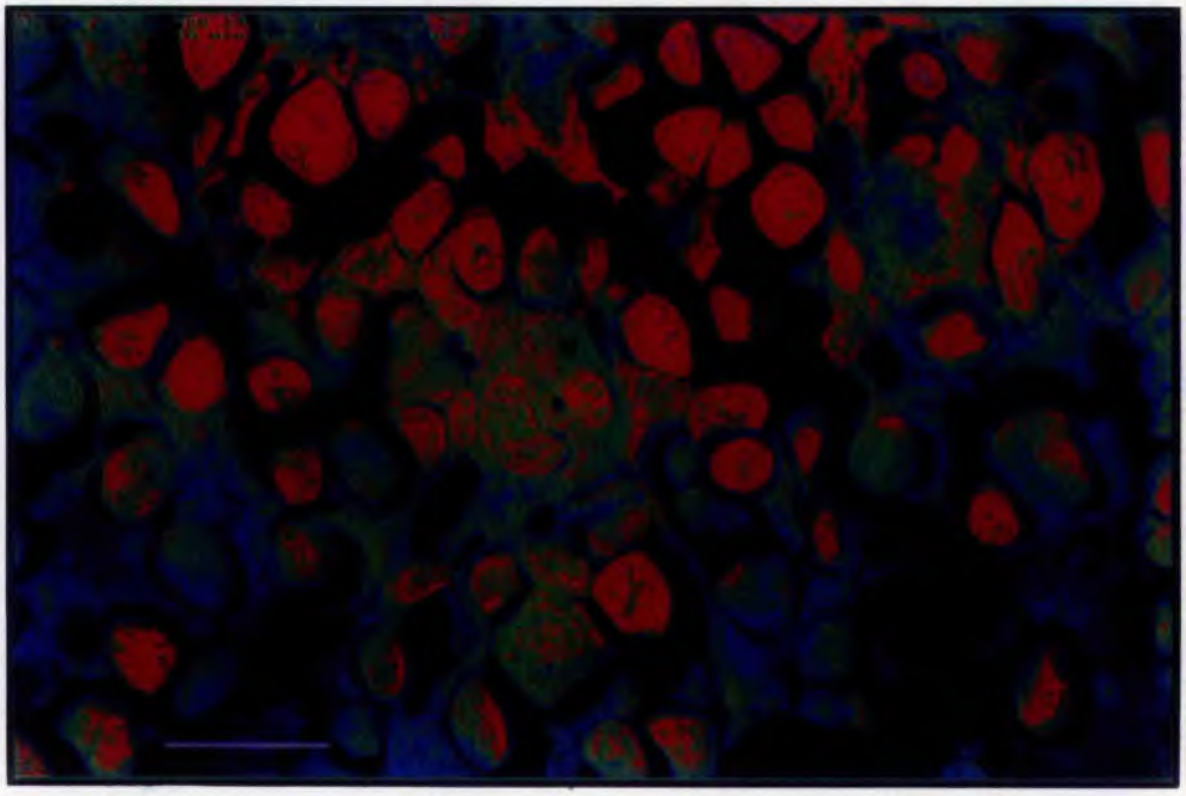


Fig. 3.2-2 Scale bar 10μ

Blood cell precursors of various maturation states clustered around a central macrophage (M). The most immature, brightly stained cells are centrally located while, the more mature, less intensely stained cells are peripherally situated.

15 Day Fetal Liver

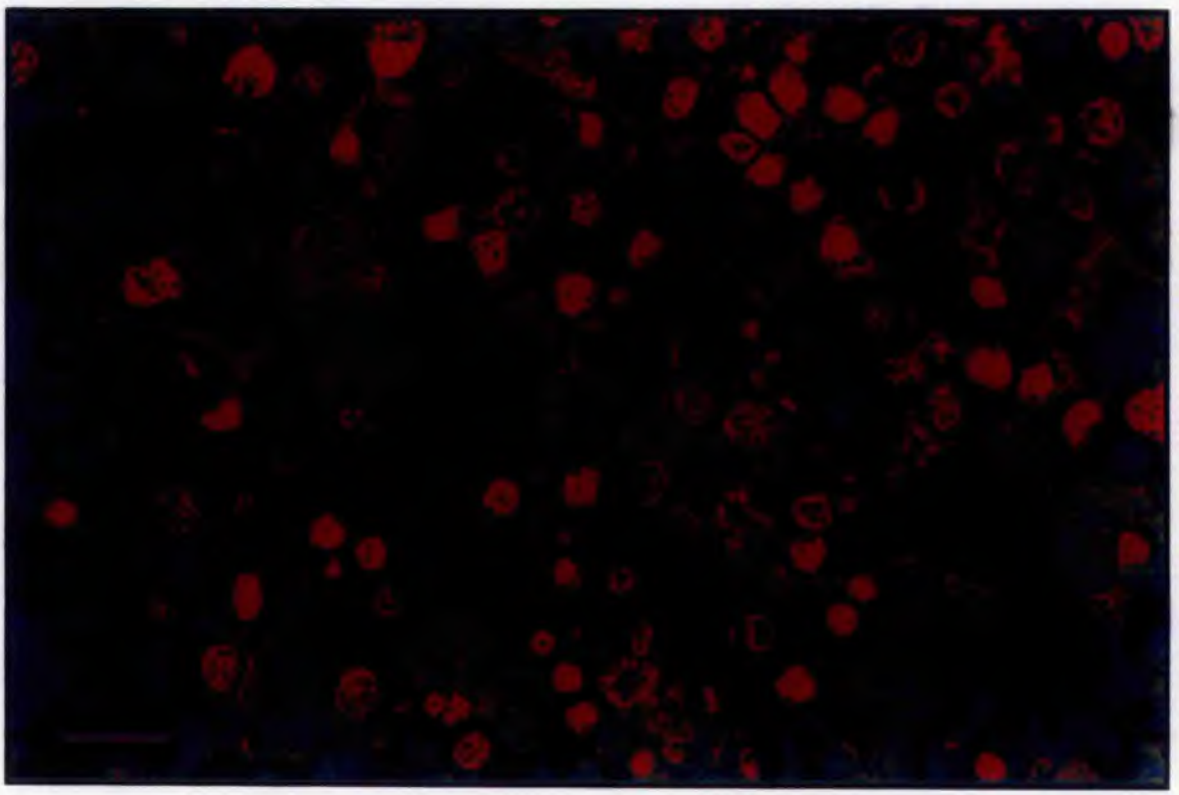


Fig. 3.2-3 Scale bar 10μ

Blood cell precursors arranged around a central hepatocyte (H). Ferritin particles can be seen dispersed throughout the cytoplasm of the hepatocyte and in particular, in the cytoplasmic proceses which are associated with early precursors.

15 Day Fetal Liver

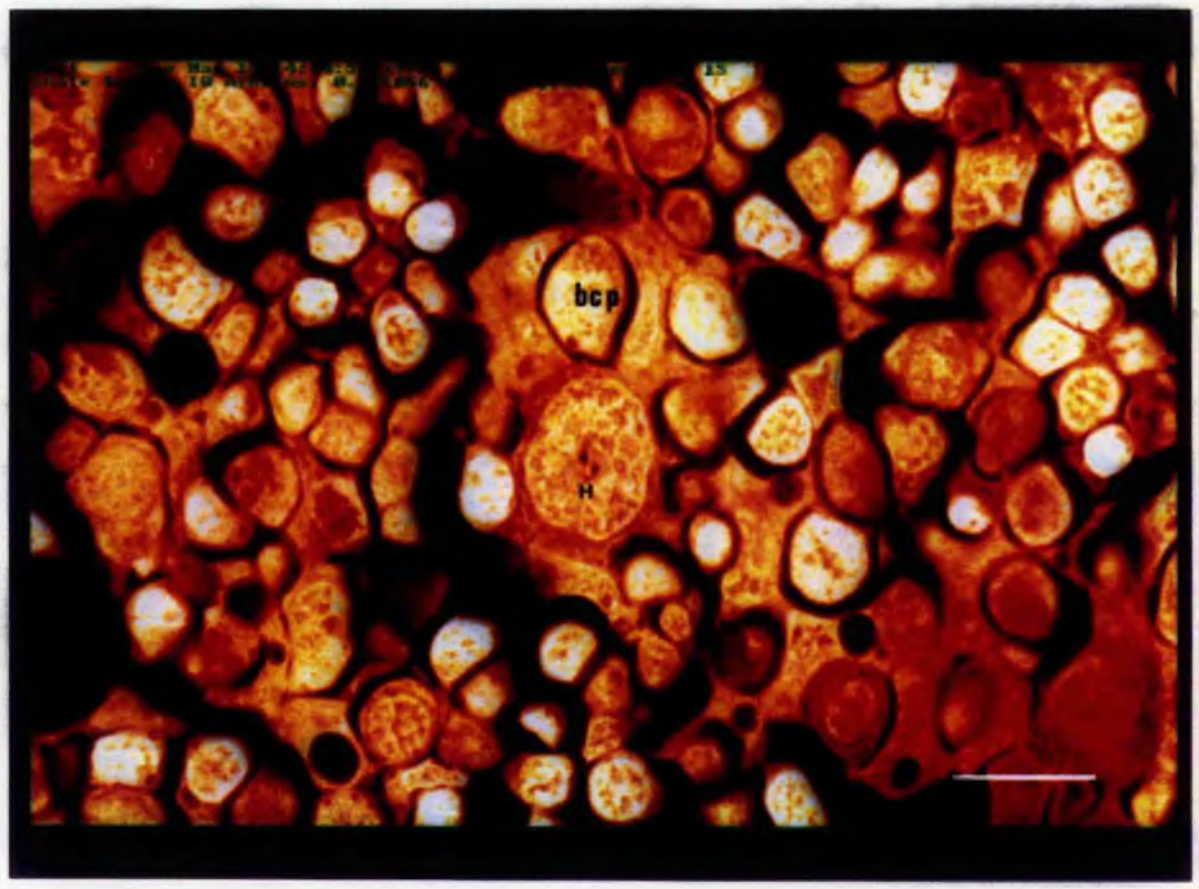



Fig. 3.2-4 Scale bar 10μ

Immature blood cell precursor (bcp) enveloped in the cytoplasmic extensions of a hepatocyte (H). The precursor cell displays intense staining for ferritin, as do the cytoplasmic processes which surround it.

15 Day Fetal Liver

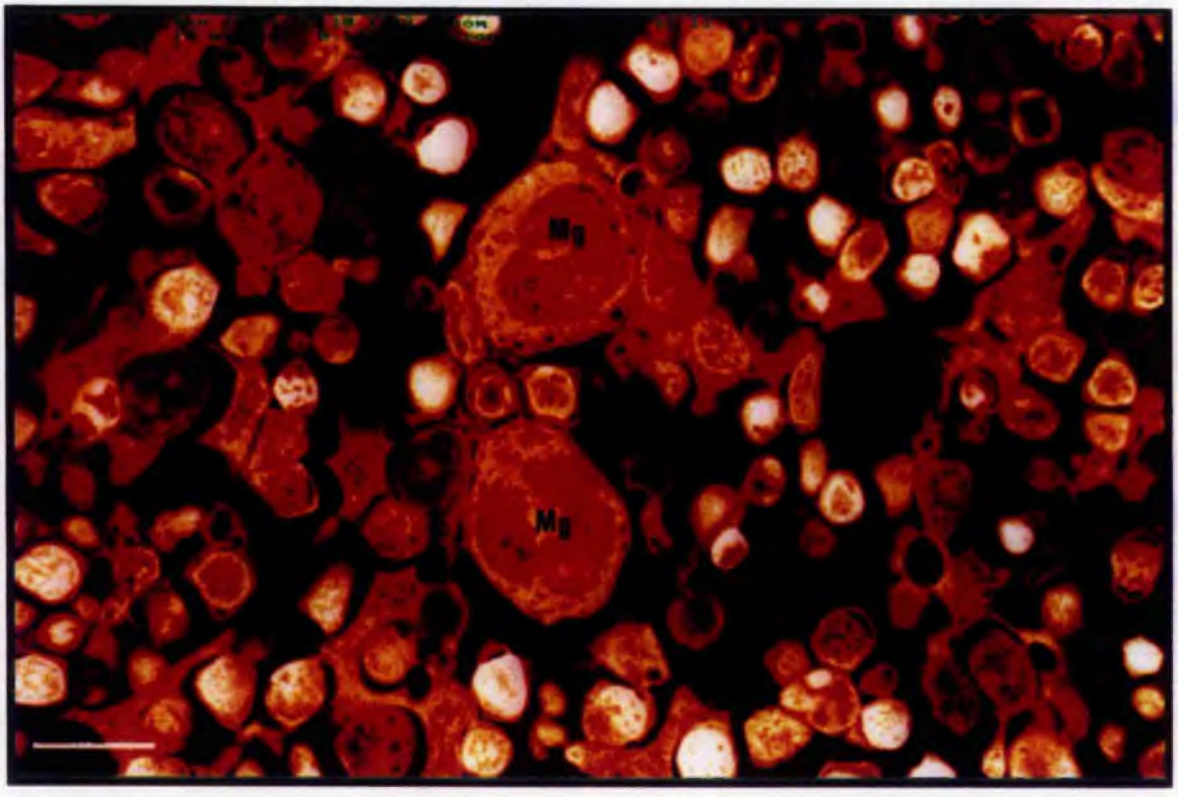



Fig. 3.2-5 Scale bar 10μ

Megakaryocytes (Mg), which were observed randomly situated in the parenchyma, usually closely associated with hepatocytes. Megakaryocytes were observed to contain a considerable amount of ferritin in their cytoplasm.

### 3.2.2 Day 8 Neonatal Liver

The 8 day old neonate, like the 10 day neonate, showed a very different pattern of haematopoiesis in comparison to the fetal liver.

Haematopoiesis was minimal (fig. 3.2-6) and restricted to isolated foci. Hepatocytes were the predominant cell lineage and at this stage displayed very little reaction to the ferritin stain. Ferritin granules were usually only observed within their nuclei.

The haematopoietic cells were easily identified as they retained a high ferritin content in comparison to the hepatocytes. They were observed in isolated clusters surrounded by hepatocytes (figs. 3.2-7 & 3.2-8).

A few megakaryocytes were observed (fig. 3.2-9). Ferritin was also observed within their lobar nuclei but also, to a lesser extent, dispersed throughout the cytoplasm.

Reticulocytes were observed in the sinusoids (fig. 3.2-10). The majority of these cells contained a little peripheral ferritin, however some cells retained a very intense degree of stain, like that observed in the immature blood cell precursors.

8 Day Neonatal Liver

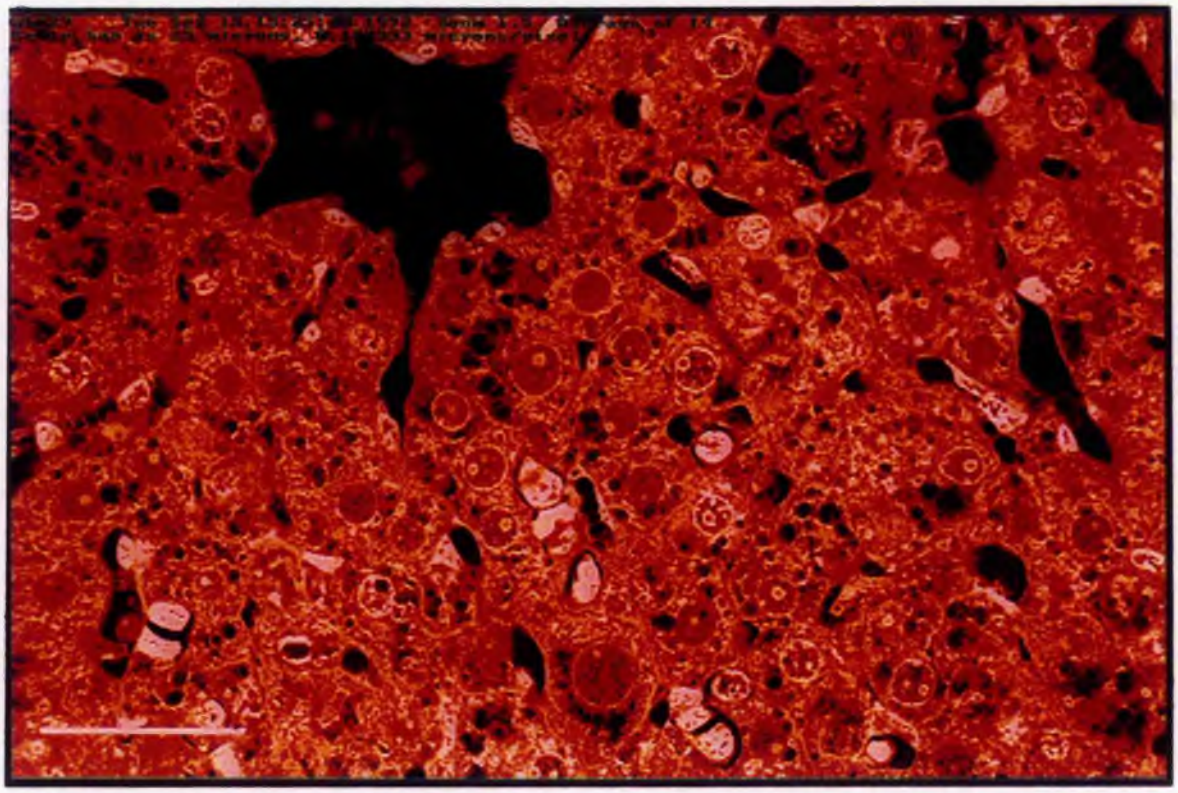


Fig. 3.2-6 Scale bar 10μ

Hepatic parenchyma of the 8 day neonate. At this stage the parenchyma consists mainly of hepatocytes, only a few brightly stained precursor cells are observed.

8 Day Neonatal Liver

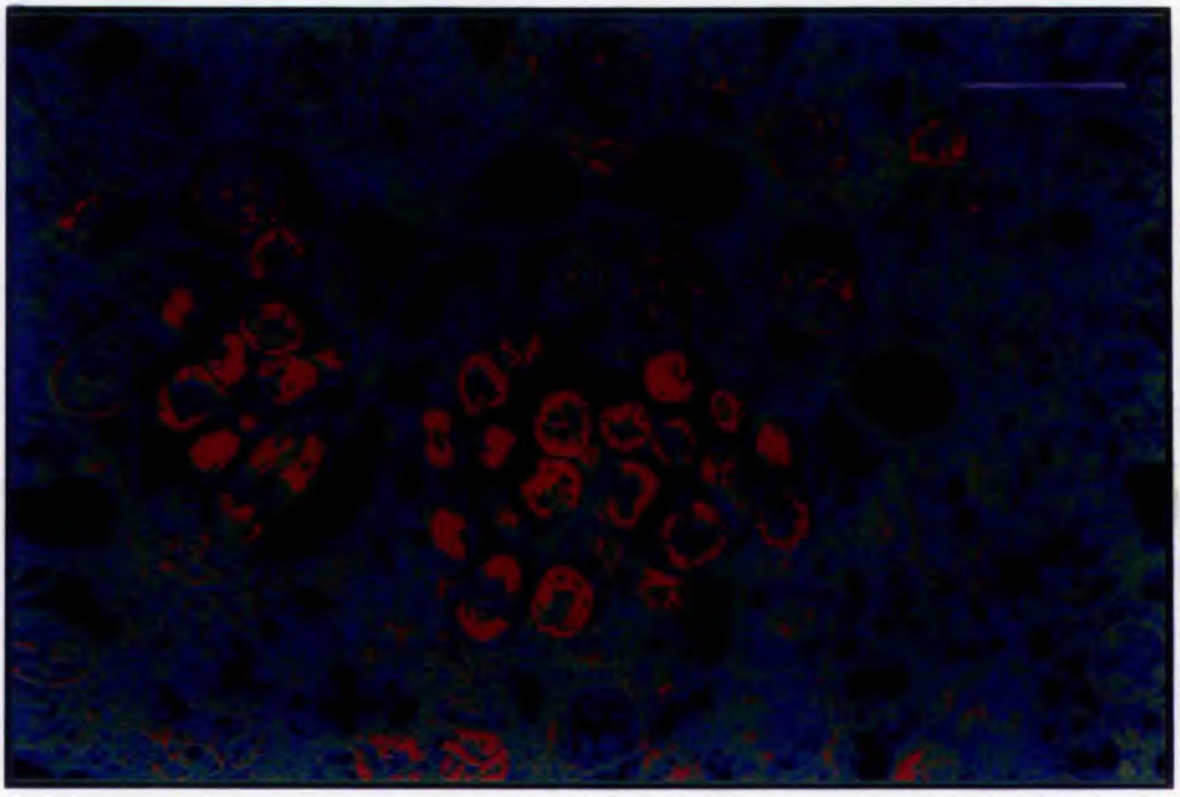


Fig. 3.2-7 Scale bar 10μ

Clusters of blood cell precursors surrounded by hepatocytes. The haematopoietic cells retain a high ferritin content in comparison with the hepatocytes. The hepatocytes display a few ferritn particles both within their nuclei and cytoplasmic processes associated with the haematopoietic cells.

8 Day Neonatal Liver

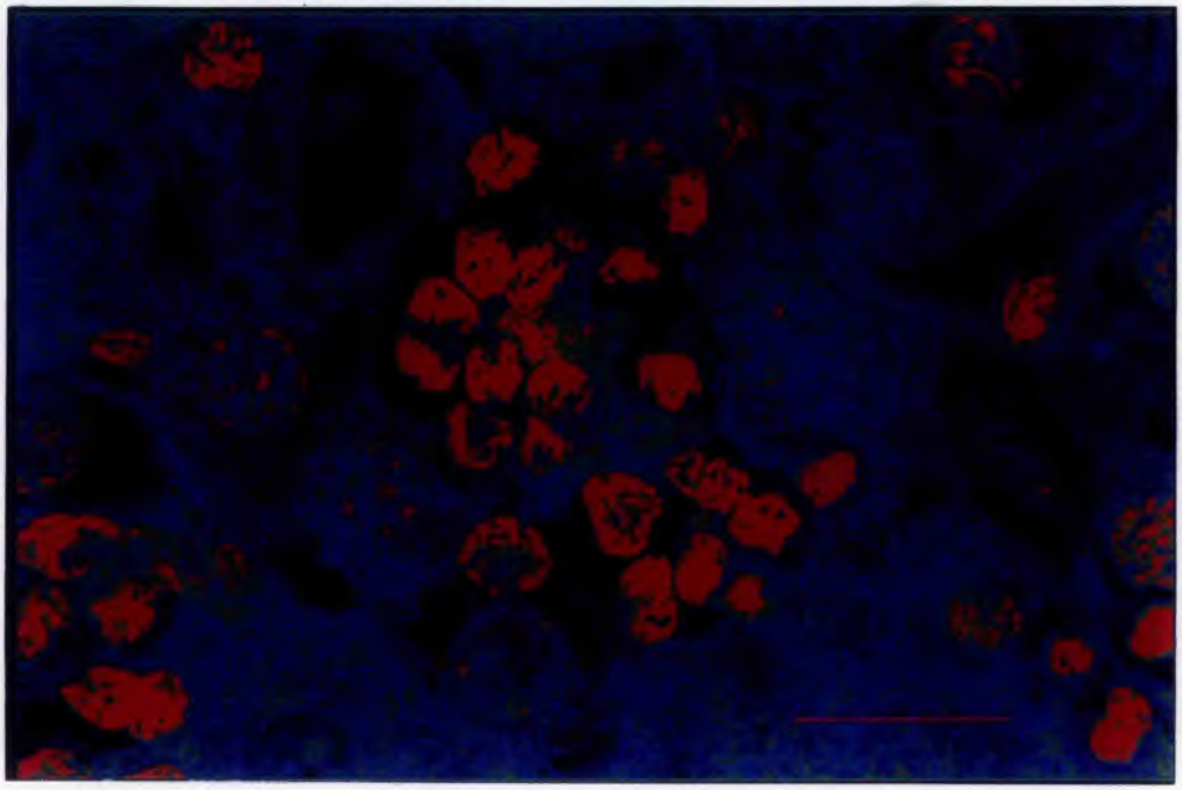


Fig. 3.2-8 Scale bar 10μ

Isolated foci of erythroblasts surrounded by hepatocytes. The hepatocytes contain a very small amount of ferritin compared to the erythroblasts.

8 Day Neonatal Liver

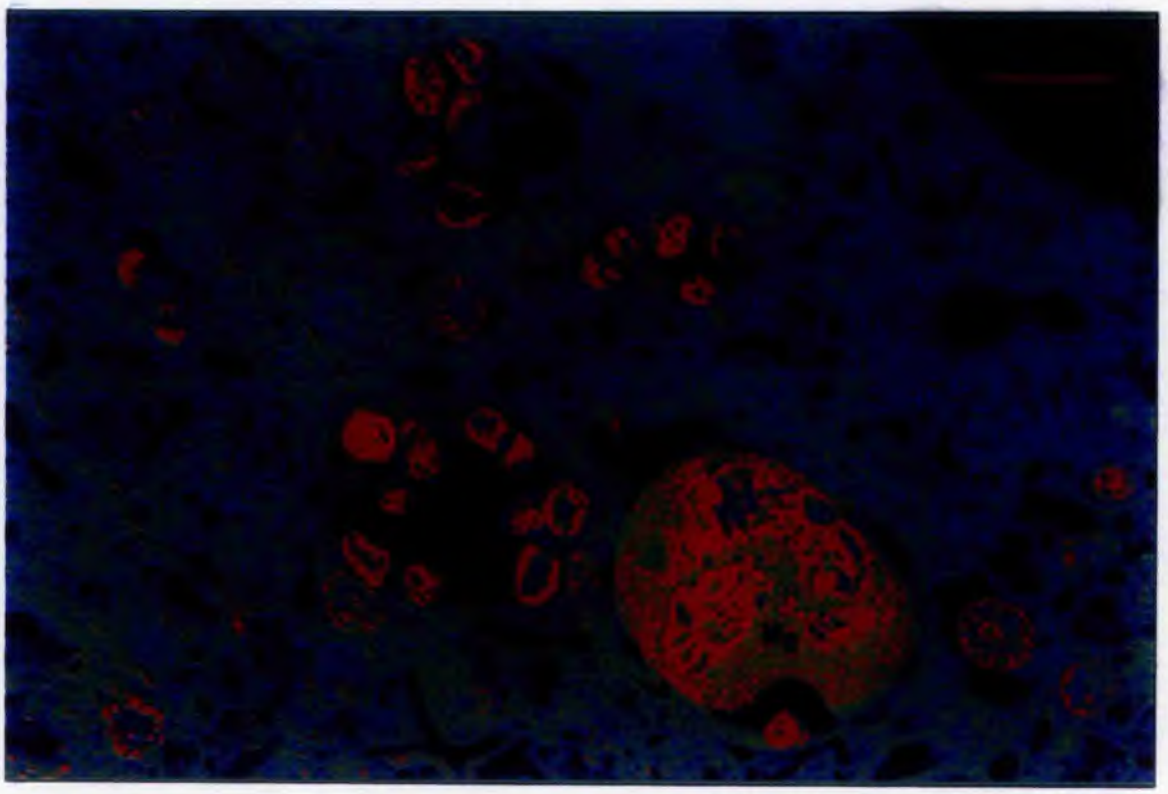


Fig. 3.2-9 Scale bar 10μ

Clusters of haematopoietic cells and a megakaryocyte (Mg), surrounded by hepatocytes. Like the other haematopoietic cells, the megakaryocyte retains a high concentration of ferritin both in its nucleus and in its cytoplasm.

8 Day Neonatal Liver

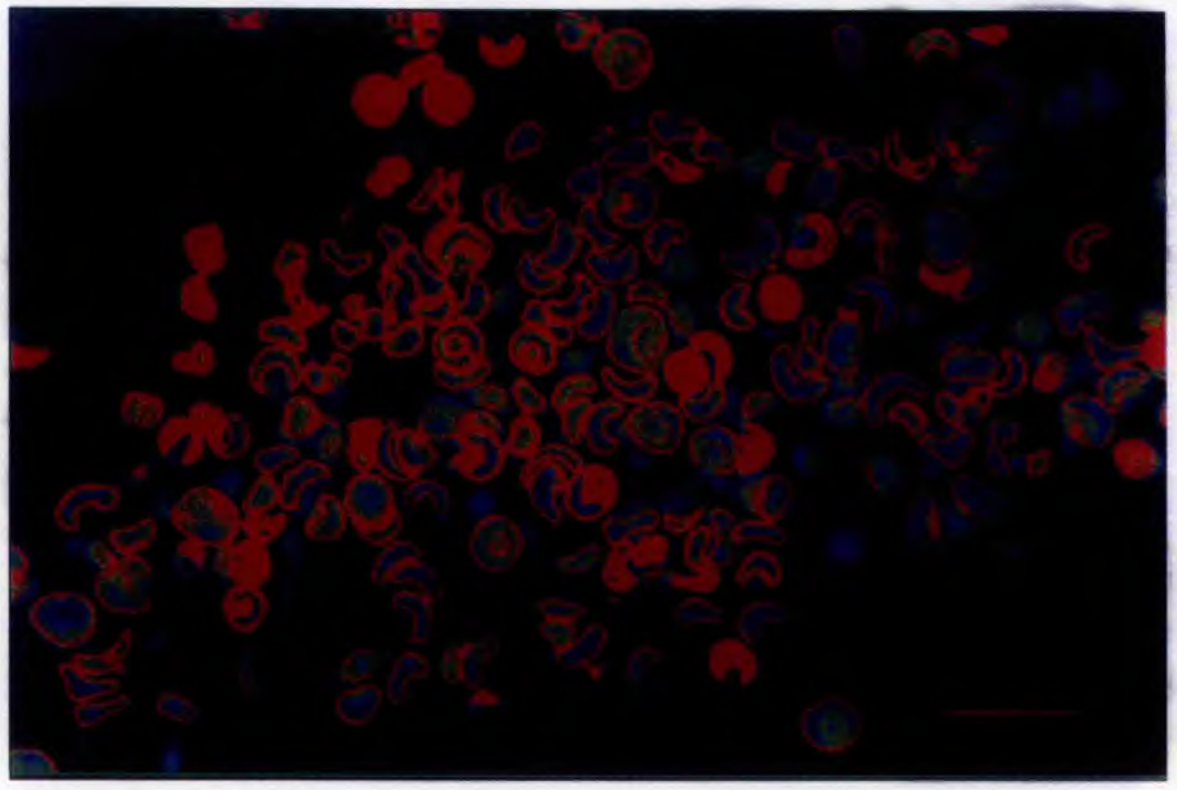


Fig. 3.2-10 Scale bar 10μ

Large number of reticulocytes in a sinusoid. Most of the reticulocytes only retain a small amount of peripheral ferritin, however some cells appear to retain more than others.

## 3.3 Haematopoietic Activity of Fetal Liver and Adult Bone Marrow in Vitro

Long term Dexter cultures of adult bone marrow (BM) and fetal liver (FL) were established as described previously. Once a confluent stromal layer had been established, some flasks were re-seeded with either BM or FL cells and monitored over a period of time. Cytospin preparations of the culture supernatants were performed regularly.

### 3.3.1 Long Term Bone Marrow Cultures (LTBMC)

A confluent stromal layer was established in cultures 3 weeks after the infusion of normal bone marrow cells (fig. 3.3-1). The stromal layer consisted mainly of macrophages and fibroblasts, which were distributed throughout the entire culture, and adipocytes also formed a substantial component of the supportive stromal layer. "Blanket cells" with long cytoplasmic processes were also observed throughout the layer. Confocal microscopy of the intact layers revealed structures similar to gap junctions between cytoplasmic processes of adjacent blanket cells. The blanket cells are considered to be derived from fibrobasts, however an endothelial origin has also been suggested (Hasthorpe et al 1992).

Following infusion of BM or FL cells, the cultures became haematopoietically active (figs. 3.3-2 & 3.3-3). LTBMC are predominantly myeloid and foci of haematopoiesis were observed on top of and within the stroma, resulting in the "cobblestone appearance" of the stroma. The haematopoietic foci were usually associated with one or more macrophages. Erythrobasts and erythrocytes were present, up to the end of the first week in culture (fig. 3.3-1).

Loosely adherent groups of maturing metamyelocytes were observed on top of the adherent stromal layer (fig. 3.3-4). These cells eventually detach from the layer and are released into the supernatant. Metamyelocytes were easily identified in cytospin preparations of supernatants by their distinctive nuclei (fig. 3.3-2).

Proliferation of cells was observed to occur over periods of several months. The majority of cells present in the supernatants were granulocytes at all stages of maturation (figs. 3.3-2 & 3.3-3). Megakaryocytes and mast cells were also present in the cytospin preparations.

Bone marrow layers which had been re-seeded with fetal liver cells (figs. 3.3-3 & 3.3-4), demonstrated active granulopoiesis which was not morphologically distinct from that observed in bone marrow layers re-seeded with BM cells (fig. 3.3-2). With extensive periods of time in culture, 5-6 months, the granulocytes progressively disappeared and macrophages predominated.

LTBMC which had not been re-seeded with haematopoietic cells were also observed to be haematopoietically active. However granulopoiesis persisted for a shorter period of time, around 16 weeks, before the phagocytic cells took over, than cultures which had been re-seeded with haematopoietic cells.

Bone Marrow Culture

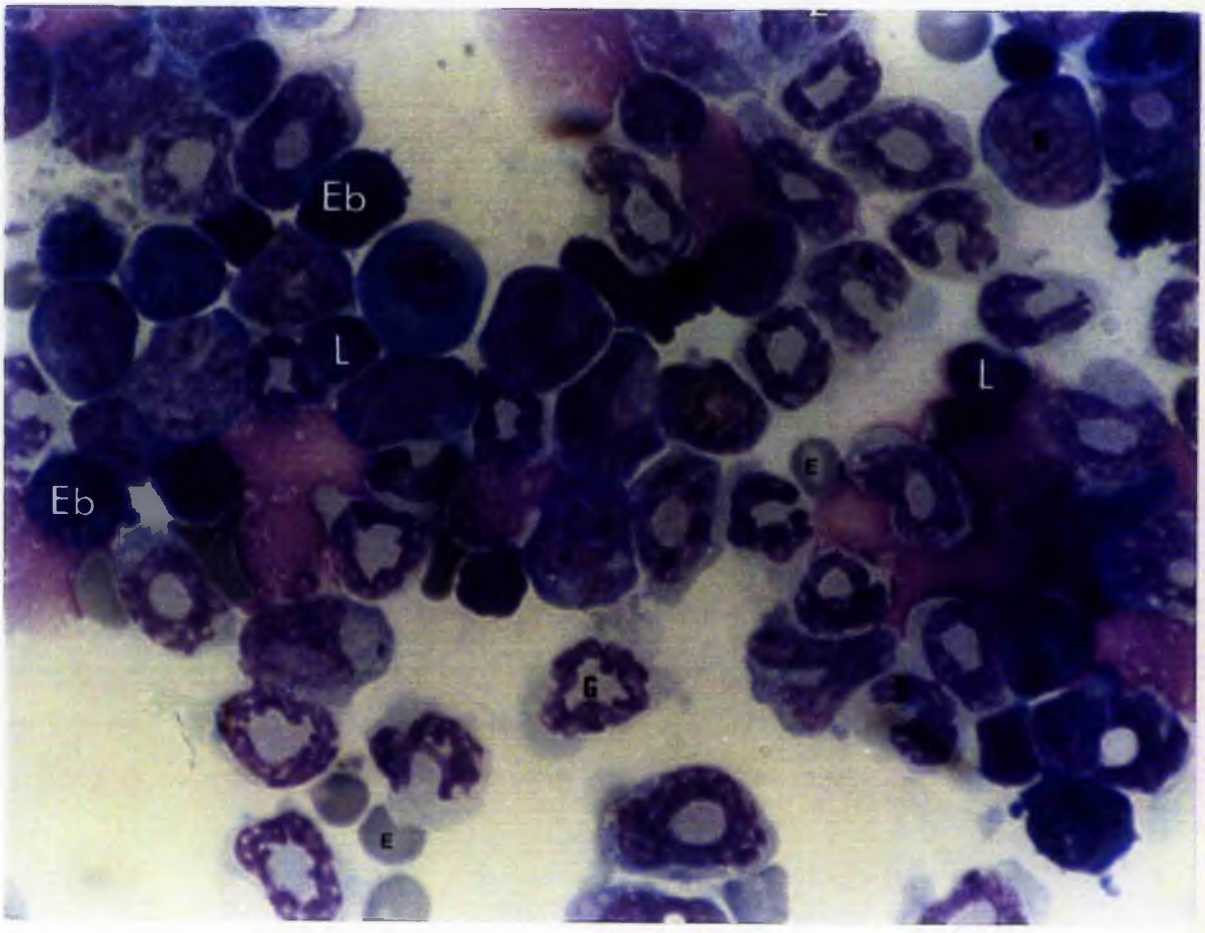



Fig. 3.3-1 x 1 750

Range of haematopoietic cells in normal bone marrow - erythroblasts (Eb), erythrocytes (E), early and late granulocytes (G) and lymphocytes (L).

Bone Marrow Culture

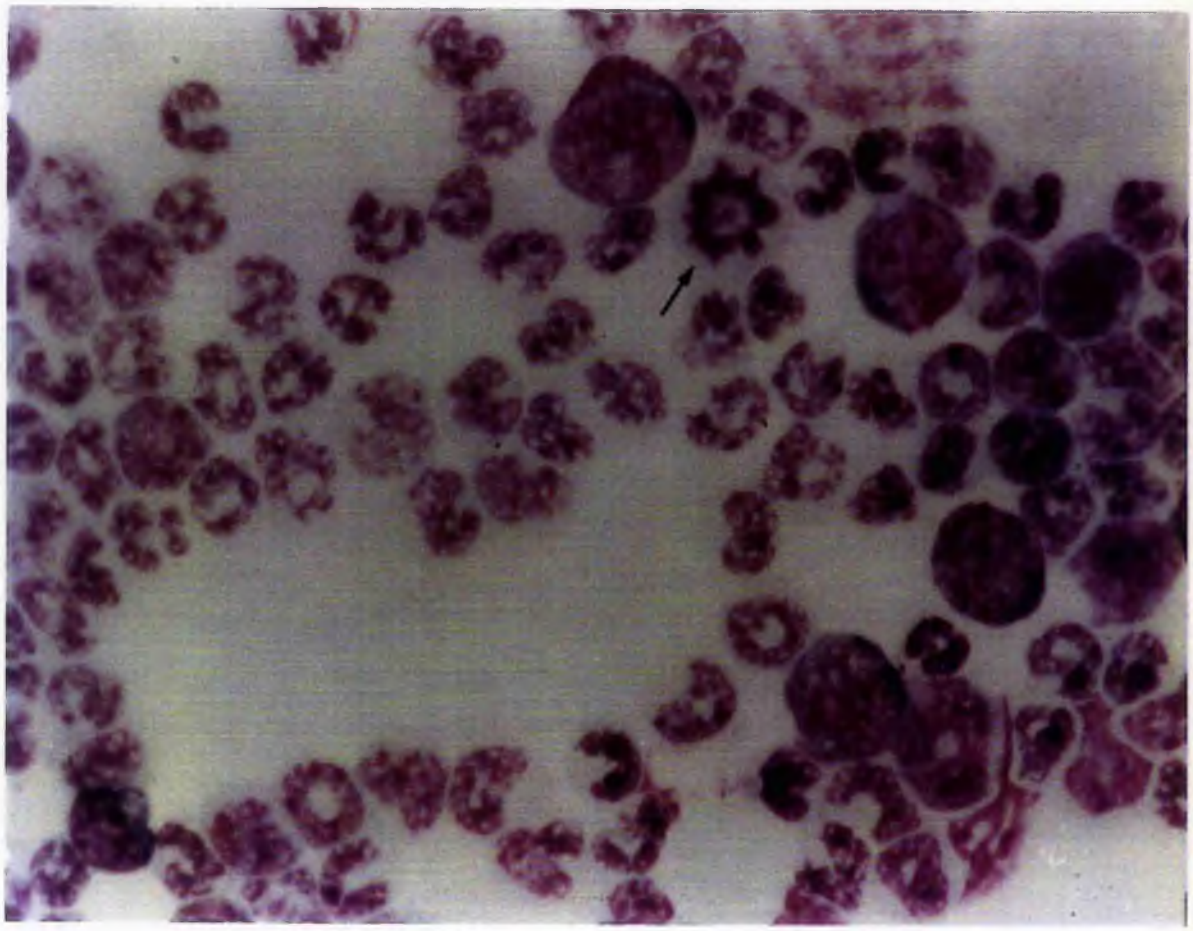


Fig. 3.3-2 x 484

Cytospin of BM stromal layer re-infused with bone marrow cells. Clusters of granulocytes at all stages in maturation are observed, together with a cell in metaphase (arrow).

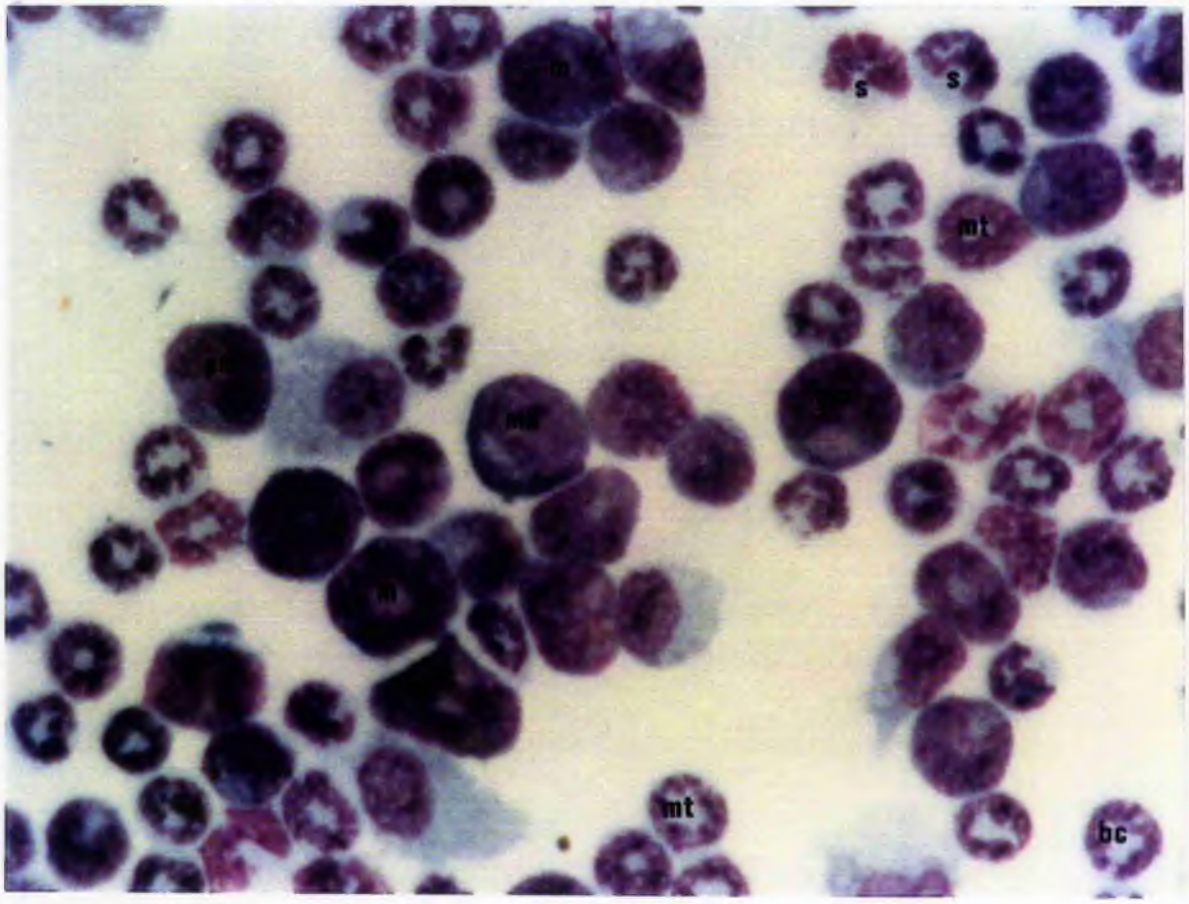



Fig. 3.3-3 x 1 750

Cytospin preparation of BM stromal layer infused with FL haematopoietic cells. A cluster of granulocytes of various maturation states is observed, from myeloblasts (mb), promyelocytes (pm), myelocytes (m), metamyelocytes (mt) band cells (bc) to the end stage cells with segmented nuclei (s).

Bone Marrow Culture

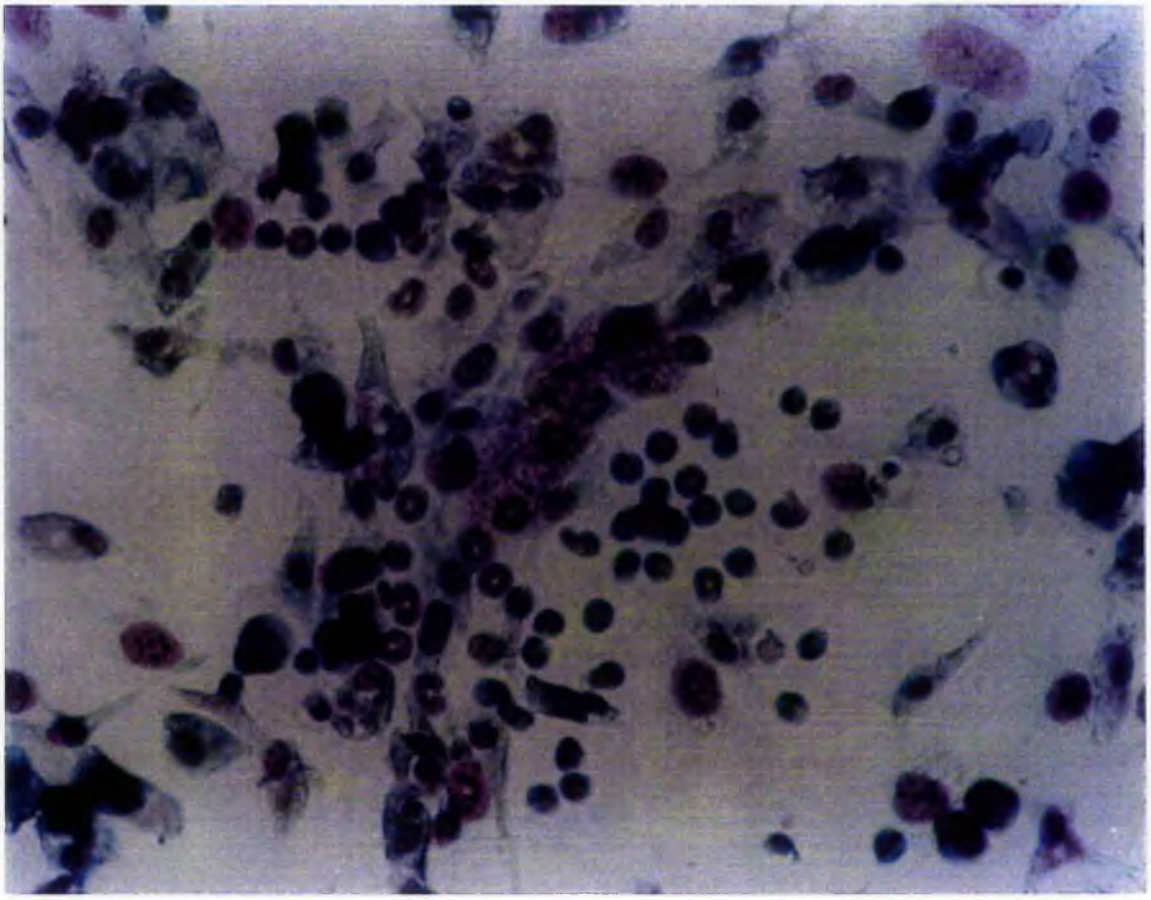


Fig. 3.3-4 x 625

Cluster of fetal liver derived granulopoietic cells at various stages in maturation growing upon a bone marrow stromal layer. Metamyelocytes are observed growing on top of stromal cells.

### 3.3.2 Long Term Fetal Liver Cultures (LTFLC)

Cultures of fetal liver cells formed confluent stromal layers in a manner similar to that observed in the bone marrow cultures. This however took a longer period of time, usually 4-6 weeks, before a confluent layer was established.

The stromal layers, like those of BM origin, consisted predominantly of macrophages and fibroblasts. Fat cells were only rarely observed in FL cultures (Fig. 3.3-5). Cells which closely resemble hepatocytes were observed in the stroma of the FL cultures. These cells were more numerous in the cultures of day 17 (fig. 3.3-6) and day 19 FL cells (fig. 3.3-7) than in the cultures of fetal liver on the 15th day of gestation. The hepatocytes were evident in culture of Day 17 and Day 19 FL four days after their initiation and were observed to increase in number with time in culture. Channels appeared between groups of the hepatocytes which resembled the bile cannaliculi of mature liver (figs. 3.3-6 & 3.3-8).

Fetal liver haematopoiesis in vivo is predominantly erythropoietic (fig. 3.3-9), however like the BM cultures, erythroblasts and erythrocytes were not evident in cytospin preparations of the supernatant after the 9th day in culture. At this time the fetal liver cultures became predominantly granulopoietic (Fig. 3.3-10), metamyelocytes were observed in the supernatants together with megakaryocytes and monocytes. Autoradiographs were performed on the adherent layers of Day 17 and Day 19 FL cultures (Fig. 3.3-11). A large number of cells were very heavily labelled and therefore close to cell division, providing evidence that the cultures were haematopoietically active. Granulocytes and megakaryocytes persisted for up to 10 weeks in FL cultures which had not been re-seeded with haematopoietic cells (Fig. 3.3-12), then decreased with time and

macrophages predominated thereafter. In the corresponding BM cultures large numbers of granulocytes and megakaryocytes persisted for up to 16 weeks of culture, after this time their numbers progressively decreased.

Established fetal liver stroma supported haematopoietic cells derived from both FL (fig. 3.3-13) and BM (fig. 3.3-14). Haematopoiesis in these cultures was evident for longer periods of time, up to 5 months, compared to the cultures which had not been re-seeded with haematopoietic cells. However stroma derived from fetal liver on the 15th day of gestation appeared to support haematopoiesis more effectively than stroma from day 17 or day 19 FL. Granulocytes and megakaryocytes were evident in cultures with a day 15 FL derived stroma (fig. 3.3-14) one month after it had ceased in the day 17 and day 19 cultures.

Fetal Liver Culture

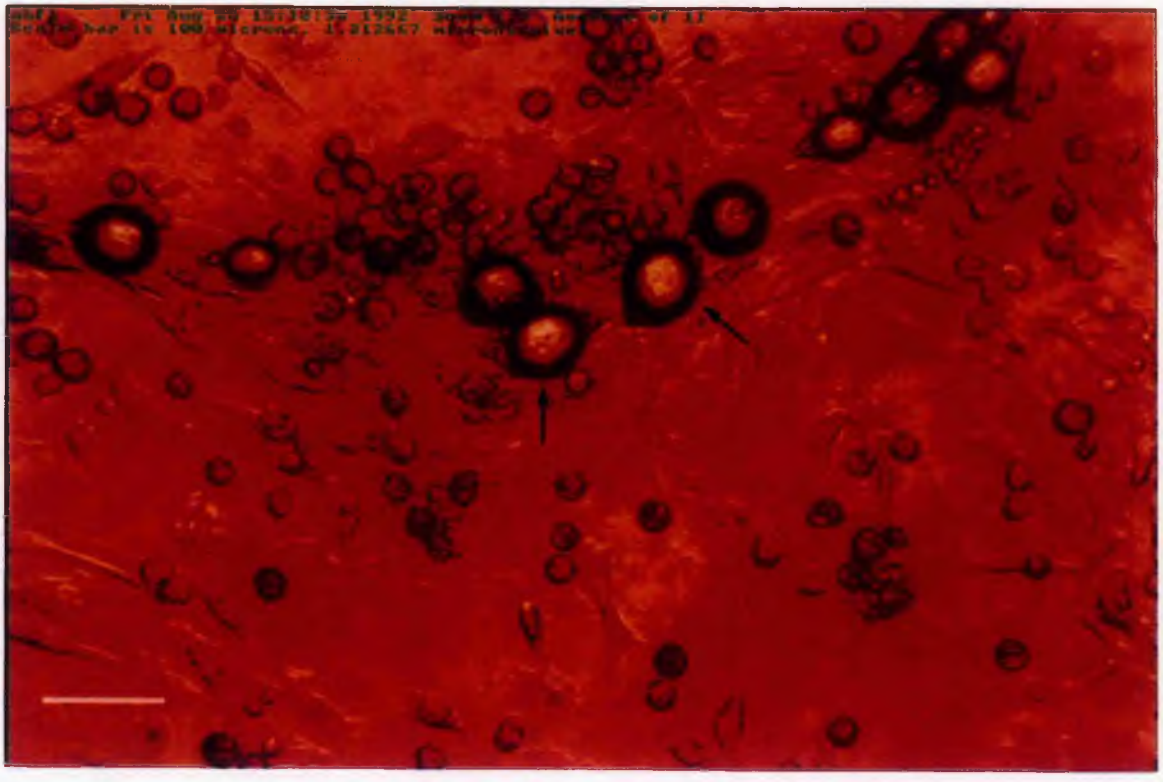


Fig. 3.3-5 Scale bar 100μ

Intact fetal liver stromal layer observed with a confocal laser scanning microscope, demonstrating the presence of fat cells (arrows) in fetal liver stroma.

Fetal Liver Culture

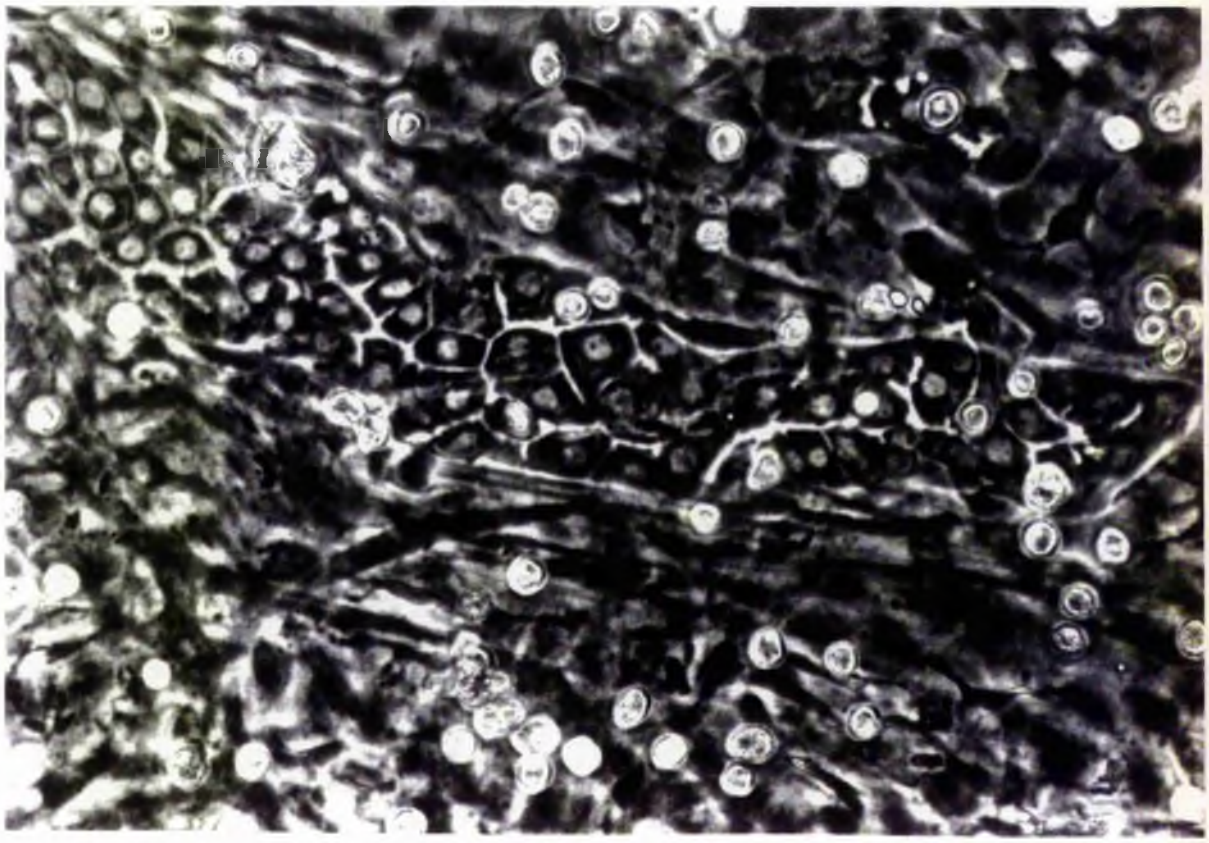


Fig. 3.3-6 x 960

17 day fetal liver cell culture at 10 weeks. A band of hepatocytes is observed to form an integral component of the stromal layer. Distinct channels are observed between adjacent hepatocytes.

Fetal Liver Culture

Fig. 3.3-7 x 1 580

Day 19 fetal liver stromal layer after 6 weeks in culture. The stromal layer consists of large numbers of hepatocytes, on top of which refractile cells are observed.

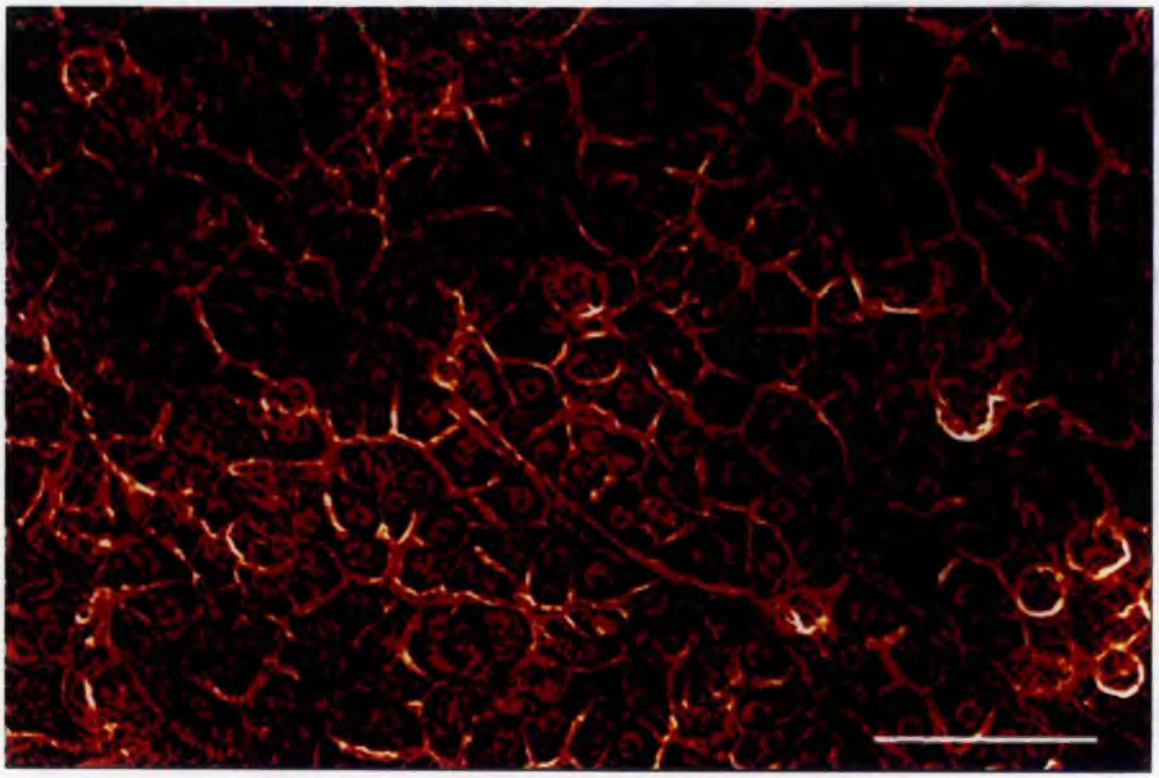


Fig. 3.3-8 Scale bar 100μ

Confocal micrograph of day 19 fetal liver stromal layer, demonstrating the large expanse of hepatocytes and the distinct channels between adjacent cells.

Fetal Liver Culture

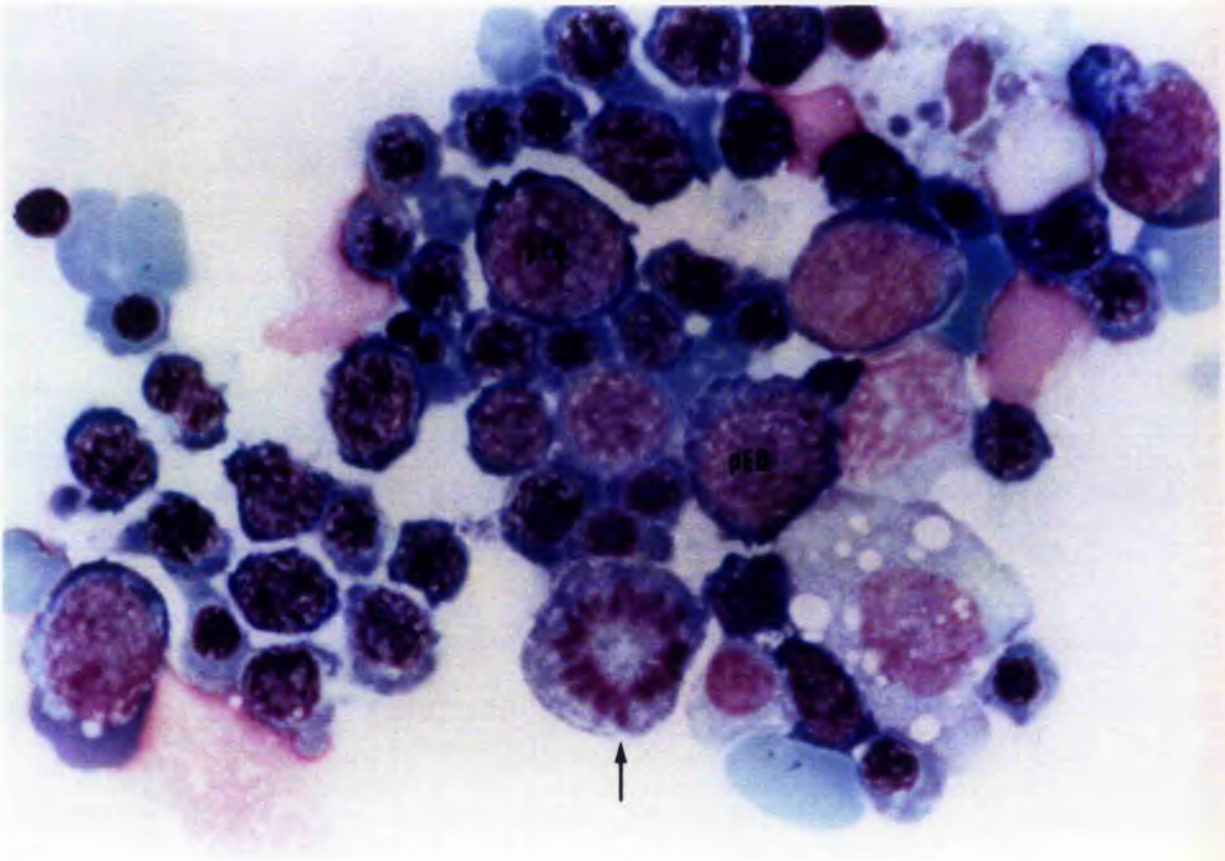


Fig. 3.3-9 x 1 750

Full range of erythroid cells in normal fetal liver, from early proerythroblasts (pEB) to red blood cells. A mitotic cell is observed (arrow).

Fetal Liver Culture

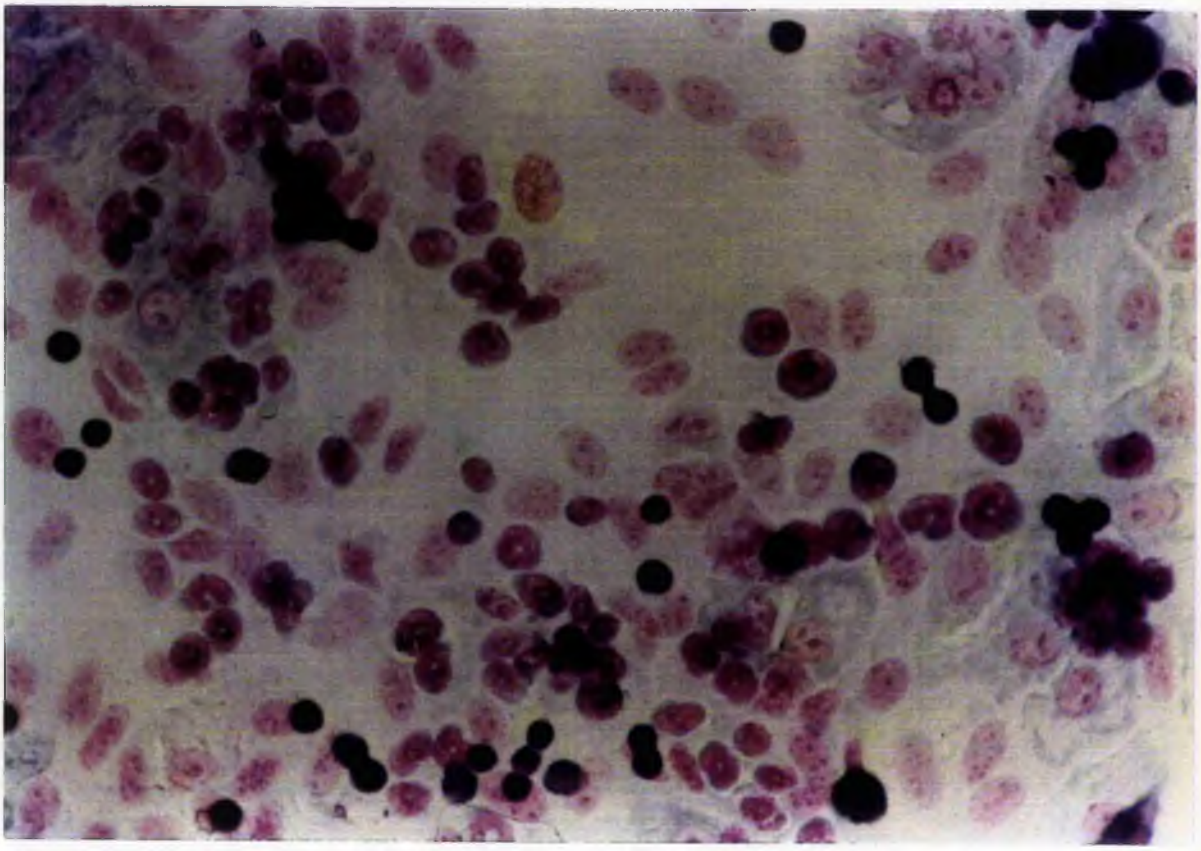


Fig. 3.3-10 x 781

Haematopoietically active day 19 fetal liver layer, after 6 weeks in culture, demonstrating, early granulocytes (g), metamyelocytes (mt) and stromal cells.

Fetal Liver Culture

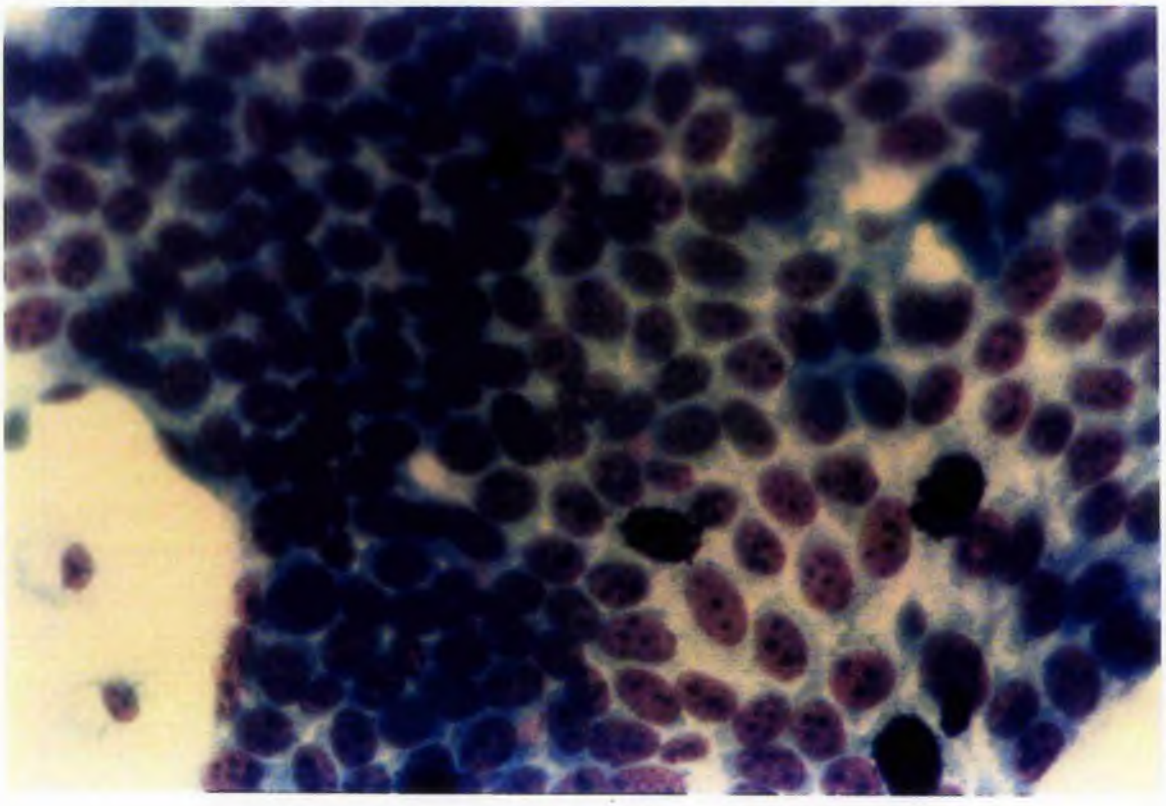


Fig. 3.3-11 x 688

Autoradiograph of the adherent layer of a day 17 fetal liver culture. Labelled hepatocytes are observed, some more heavily than others, indicating the cells are actively synthesising DNA.

Fetal Liver Culture

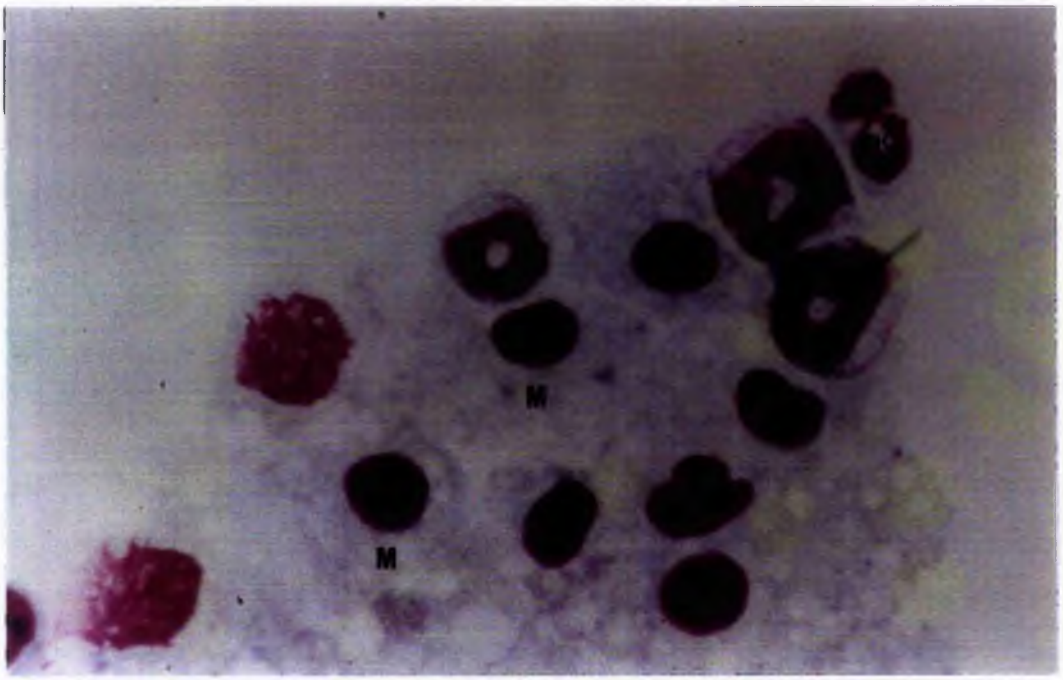



Fig. 3.3-12 x 2 083

19 day fetal liver stromal layer after 10 weeks in culture. Metamyelocytes (mt), band cells (bc) and increasing numbers of macrophages (M) are observed.

Fetal Liver Culture

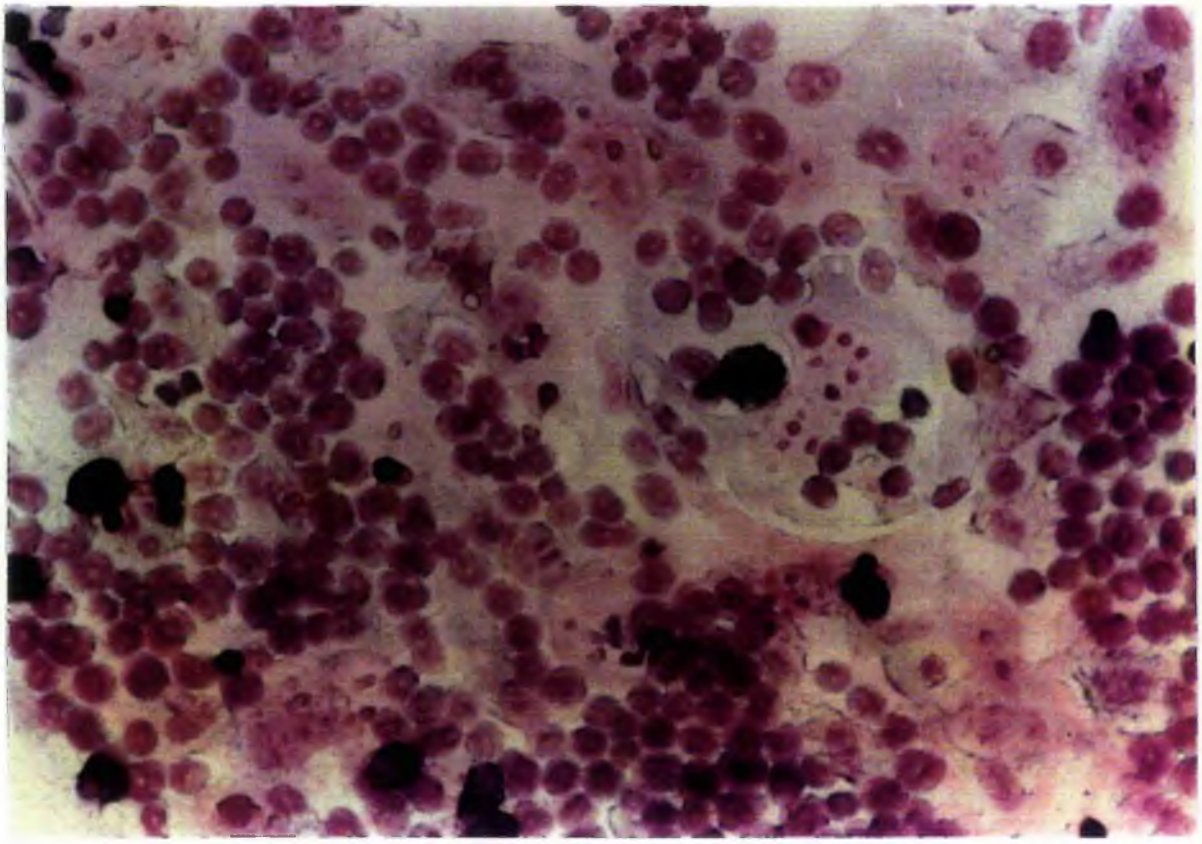


Fig. 3.3-13 x 890

Fetal liver stromal layer re-infused with fetal liver cells. Granulopoiesis is active in the culture, indicated by the presence of early and later metamyelocytes.

Fetal Liver Culture

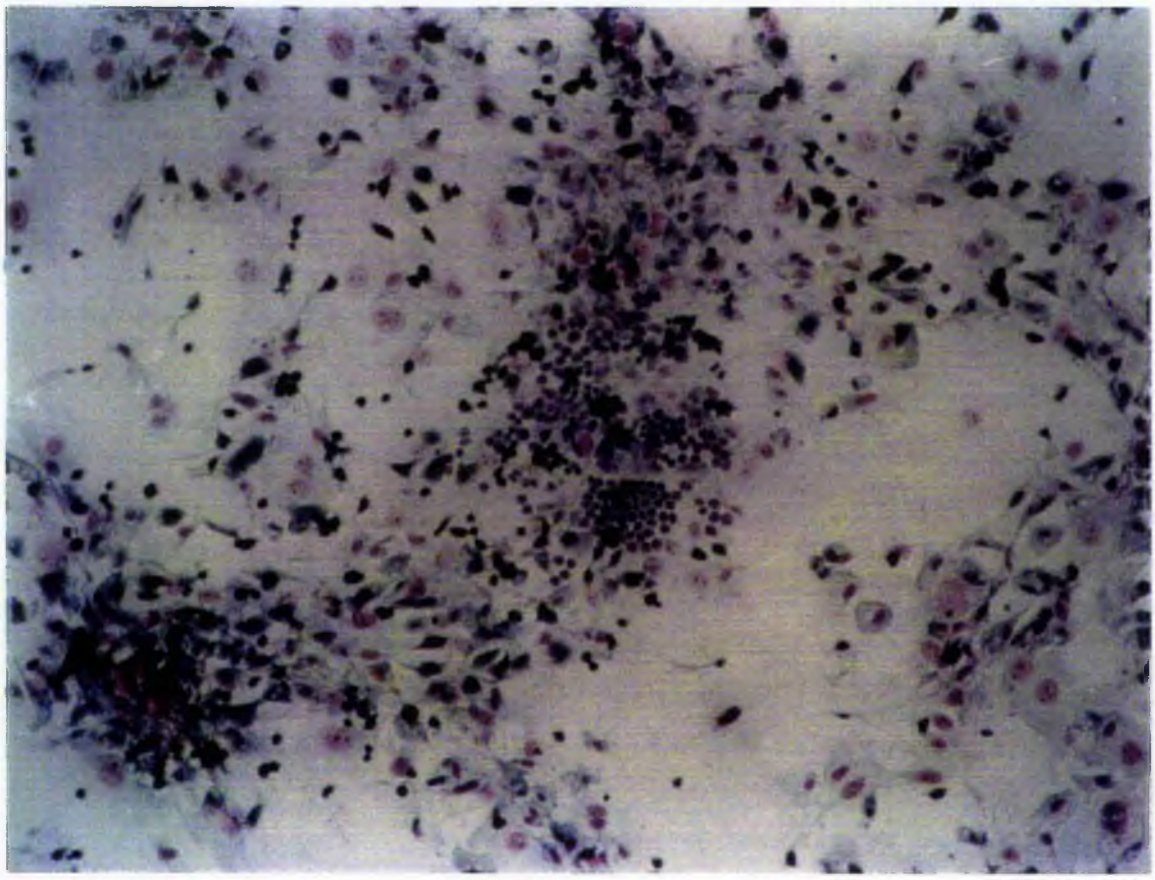


Fig. 3.3-14 x 146

Fetal liver stromal layer re-infused with bone marrow cells after 20 weeks in culture. Clusters of granulocytes are observed closely associated with the fibroblasts of the stromal layer.

## 3.4 Discussion

### 3.4.1 Haematopoietic Activity of Fetal and Neonatal Liver in Vivo

This present study confirms the preponderance of erythropoiesis and paucity of myelopoiesis, megakaryocytopoiesis and lymphopoiesis throughout the development of the fetal and neonatal liver reported by Thomas et al (1960). It also demonstrates the reciprocal relationships between immature blood cell precursors of erythroid, granuloid and lymphoid lineages with macrophages and hepatocytes, in similar formations to Bessis' "Erythroblastic Island" (Bessis & Breton-Gorius 1962).

It has been postulated that on the 10th day of gestation the fetal liver assumes active haematopoietic activity, taking over from the yolk sac (Rifkind, Chui & Epler 1969). The earliest developmental stage examined in the present investigation was the fetal liver on the 12th day of gestation. At this stage the hepatic tissue consisted of a loose mass of cells, hepatocytes had a regular outline and were not compressed or distorted by large numbers of haematopoietic cells. Haematopoietic cells were present, though not in high numbers, this finding is not surprising as haematopoiesis has just been initiated in the liver at this stage (Hata et al 1993). The haematopoietic cells were observed to be randomly distributed throughout the hepatic parenchyma in accordance with previous reports (Medlock & Haar 1983; Grossi, Velardi & Cooper 1985). The haematopoietic cells were predominantly erythroid, however a few isolated monocytes were observed. This was unusual as haematopoiesis did not appear to be fully underway at this stage in gestation. It may be possible that these monocytes are the precursors of the central macrophages observed in

haematopoietic islands in the liver on the fourteenth day of gestation (Crocker et al 1991).

Hepatic cords had developed by the 13th day of gestation and the hepatic parenchyma was much more cohesive than that observed on the 12th day of gestation. An overall increase in cell number was observed, which would contribute to the compact the structure of the parenchyma, only a few fenestrations remained. Haematopoiesis now appeared to be fully underway, the number of haematopoietic cells had increased and they formed distinct clusters around a central hepatocyte, throughout the parenchyma. The cell clusters were similar to the erythroblastic island described by Bessis. The erythroid cells were relatively immature and very little maturation had taken place. Haematopoiesis was entirely extravascular.

In the liver of the 14 day fetus the parenchyma had further developed and haematopoietic activity was high. Blood cell precursors formed clusters around central macrophages in addition to those with a central hepatocyte. Cytoplasmic processes from the central cells were intimately associated with the adjacent blood cell precursors. The presence of desmosome-like attachments and gap junctions between the cells strongly suggests that the central cell may be acting as a "nurse cell" which furnishes homeostatic substances to the erythroblasts (Bessis 1973; Emura 1984; Shaklai 1991). This was further substantiated by the presence of rhopheocytic vesicles between the central cells and the blood cell precursors. The observed gradients of differentiation within the cluster have been reported previously (Jones 1970). The peripheral positions of the more mature elements suggests they are less dependent on the central cell. At this stage the cell clusters no longer consisted entirely of erythroid cells, granulocytes and monocytes were observed on the periphery of the clusters. These mixed-lineage

clusters remained throughout development and their constituent elements were further expanded when lymphoid cells appeared in the newborn liver.

The haematopoietic activity was observed to peak on the 15th day of gestation. The hepatic parenchyma was densely packed with cells of various haematopoietic lineages, which were observed to be undergoing mitosis and hence further distorting the hepatocytes. The number of clusters was greater than in the 14 day fetal liver and the number of cells within each cluster had increased, granuloid and monocytic cells were more evident. Central macrophages contained expelled red cell nuclei and other cell debris. Granules which may contain ferritin were present in the cytoplasm of the central cells and the blood cell precursors in addition to the siderosomes (Bessis 1977) present in the cytoplasm of erythroblasts. These granules were also present in the cytoplasmic processes of the central cells and haematopoietic cells. It is possible that ferritin was being transferred from the central cell to the developing cells in the abundant rhopheocytic vessicles.

Haematopoiesis was declining in the 18 day fetus, this gradual decline and change from a haematopoietic site to a metabolically active organ was progressively observed in all other developmental stages examined. The number of cell clusters was reduced and there was a reduction in the number of erythroblasts, in particular. This was mirrored by a reduction in the number of megakaryocytes. In contrast the number of granulocytes and monocytes increased indicating a wave of myelopoiesis which occurs later than that of erythropoiesis. The hepatocyte population increased with development and cytological differences were apparent between hepatocytes which had been involved in haematopoiesis and those which had not (Emura et al 1984). Adjacent hepatocytes were closely associated via

interdigitating projections of cytoplasm or close apposition of the cell membranes.

In the newborn liver early precursors of erythropoiesis were few in number. Cell clusters remained obvious, however the number of constituent cells of each cluster had decreased. Monocytes, granulocytes and now lymphoid cells most commonly occupied peripheral positions within the clusters. Intimate associations of the central cells with the surrounding haematopoietic cells remained numerous.

Further changes were observed in the hepatic environment in later stages of the neonatal period. In both confocal microscopic examination of 8 day neonatal liver and electron microscopic examination of 10 day neonatal liver, the vastly diminished population of haematopoietic cells was restricted to anatomically isolated foci, in contrast to the fetal liver environment where haematopoietic cells were randomly dispersed throughout the hepatic parenchyma. This focal arrangement is probably due to the increased number of hepatocytes and their structural organisation into cords (Grossi et al 1985). The number of cell clusters was further reduced and they no longer consisted of cells of mixed lineages. Macrophages were no longer a component of cell clusters. The blood cell precursors of the 8 day neonatal liver displayed intense staining for ferritin in comparison to the extensive hepatic stroma which showed a minimal response to the stain. Hepatic production of haematopoietic cells has almost entirely ceased by the end of the second post-natal week, during this time the bone marrow becomes the main site of haematopoiesis and thus remains in the adult (Grossi et al 1985).

The erythropoietic cell lineage was the first haematopoietic cell lineage observed in murine fetal liver and remained the largest haematopoietic

cell population throughout all developmental stages examined. The cells were observed in circumscribed clusters randomly dispersed throughout the hepatic parenchyma as previously described (Jones 1970; Fukuda et al 1974; Keleman et al 1979; Emura et al 1984; Migliaccio et al 1986). The classical features of erythropoiesis were apparent from the 14th day of gestation, these included; decrease in cell size, disappearance of the nucleoli, increased nuclear density and peripheralisation of the nucleus. The nucleus is finally expelled and removed by macrophages. The enucleate cells were frequently observed alongside the sinusoidal wall, when they have extruded their nuclei the cells traverse the sinusoidal wall by diapedesis and then ultimately pass into the circulatory system.

Myelomonocytic cells were present in the fetal liver from the fourteenth day of gestation and progressively increased with development to reach a peak in the newborn liver. This peak occurs at a later stage than that of erythropoiesis. Thus there may be two waves of haematopoiesis in the fetal liver, the first is that of erythropoiesis and the second occurring later in development is that of granulopoiesis which is shorter and less pronounced than that of erythropoiesis. Granulocytes and monocytes were observed extravascularly in close association with hepatocytes and erythroid cells (Jones 1970; Fukuda et al 1974; Timens 1990). They were commonly found impinging on the periphery of erythroid clusters. As the number of erythroid cells decreased, the myelomonocytic cells occupied more central locations within the clusters. The milieu of the fetal liver is primarily inductive of erythropoiesis, however unlike the yolk sac, it does permit differentiation of other cell lineages.

Megakaryocytes were observed in fetal liver from the 14th day of gestation and persisted throughout development. Concomitant with erythropoiesis, the number of megakaryocytes was high in fetal liver and was observed to

decline in neonatal liver. These cells were usually observed as single cells in close apposition to hepatocytes. At most two megakaryocytes were observed closely associated with each other and with the surrounding hepatocytes. Megakaryocytes were never observed associated with cell clusters (Jones 1970). Megakaryocytes displayed positive reactions for ferritin staining in both the 15 day fetal liver and the 8 day neonate, their close associations with hepatocytes may permit the transfer of ferritin between the cells.

At birth megakaryocytes were observed to be fully differentiated, when the tubules were arranged in long chains throughout the cytoplasm. The presence of demarcation membranes in the cells suggests they are actively producing thrombocytes (Thomas 1974). In the 15 day fetal liver, some megakaryocytes displayed organelle-free membrane pseudopodia. This was also reported by Jones (1970) and Fukuda (1974) who suggested that this type of budding occurs prior to the megakaryocyte passing into the sinusoids.

Lymphoid cells were the last lineage to appear. In the newborn liver they were randomly distributed within the parenchyma, in fewer numbers than all other haematopoietic cells. Lymphoid cells were observed in cell clusters of mixed lineages with a central macrophage or a hepatocyte. In the 10 day neonate their distribution was restricted like that of the other lineages. They were observed as lymphoid foci closely associated with hepatocytes. These lymphocytes are most probably B-cells (Jones 1970; Timens 1990).

Hepatocytes which were involved in the haematopoietic activity of the liver underwent a marked differentiation in comparison to hepatocytes which were not involved. In the 12 day fetal liver, hepatocytes formed a loose mass of tissue and exhibited a typical pyramidal shape. Their size was variable and the number of mitochondria and lipid vacuoles was small.

When haematopoietic activity was high, 14th & 15th days of gestation, the hepatocytes formed a cohesive stroma. Prominent nucleoli were observed and chromatin was finely dispersed. The hepatocytes exhibited a distinct polarity of cytoplasm to form close associations with haematopoietic cells and their rough endoplasmic reticula were arranged in parallel stacks. There was an increase in the number of mitochondria, glycogen and ferritin particles. This difference appears to be dependent on their intimate relations with erythroblasts (Emura et al 1984). The hepatocytes exhibited various shapes due to compression by the large numbers of haematopoietic cells. These observations suggest that the hepatocytes serve as a stromal cell in the haematopoietic environment of the fetal liver.

In neonatal liver, while the number of hepatocytes had increased in both size and number, the number of haematopoietic cells had decreased. The hepatocytes acquired a more regular outline and contained a few lipid vacuoles. The number of mitochondria had increased and well defined cristae were observed indicating a high degree of activity. Many hepatocytes were closely associated with each other through cytoplasmic processes or through close apposition of cell membranes. This close association may reflect a change in functional status from a stromal element in a haematopoietic environment to that of a parenchymal component of a metabolically active liver (Medlock & Haar 1983).

### 3.4.2 Haematopoietic Activity of Fetal Liver and Adult Bone Marrow in Vitro

Proliferation and differentiation of murine haematopoietic cells can occur for several months in culture in the presence of adherent stromal layers. The production and maintenance of particular cell lineages depends on the composition of the cultures. Primitive haematopoietic progenitor cells can

be maintained in the absence of exogenously added growth factors. Under these conditions the stromal cells are responsible for both the maintenance and subsequent proliferation of the haematopoietic cells (Sutherland et al 1991).

Stromal cell production of a number of growth factors, including IL-6, G-CSF, GM-CSF and IL-1 (Quesenberry et al 1992), can be enhanced by the addition of fresh culture medium. Stromal cells have been genetically engineered to produce high levels of a specific growth factor which can enhance the maintenance and differentiation of the cells, responsive to the growth factor, without reducing their proliferative potential (Coutinho et al 1990; Sutherland et al 1991).

Stromal cells can also play a role in returning primitive haematopoietic cells to a quiescent state. TGF-β and MIP-1α are produced in the adherent layer of LTBMCs, these inhibitors are specific for primitive haematopoietic progenitors (Eaves et al 1992). Thus stromal cells can control haematopoiesis by enhancing and inhibiting its different stages. Gordon et al (1983) showed that fibroblasts, fat cells and macrophages were essential stromal elements for maintenance of haematopoiesis in LTBMC. They suggested that fat cells and fibroblasts could trap progenitors and sustain the proliferation of primitive cells added to the cultures. Cilia have been reported on blanket cells, in long term cultures, which may be able to trap and transport progenitors into the haematopoietic compartment (Tavassoli 1989).

Endothelial cells have been proposed as an important constituent of the murine stromal layer (Zuckerman & Wicha 1983). Adjacent endothelial cells show intercellular contacts in the form of desmosomes. These cells have also been reported in human LTBMC (Keating et al 1982). Recently,

Hasthorpe et al (1992) demonstrated the presence of endothelial cells in LTBMC using cell specific markers. The endothelial cells were observed to cover the differentiating cells with a thin layer of cytoplasm. From these observations, Hasthorpe et al suggested that "blanket cells" are in fact endothelial cells. Other groups remain unconvinced and believe the blanket cells are derived from fibroblasts (Allen & Dexter 1984; Tavassoli 1989). Blanket cells cover the adherent layer, separating it from the supernatant, thus compartmentalising the culture system. This is analogous to bone marrow haematopoiesis in vivo which is compartmentalised both intra and extravascularly by the marrow sinus endothelium (Tavassoli 1989).

Endothelial cells can secrete GM-CSF and G-CSF, together with the fact that numerous gap junctions between endothelial cells and macrophages have been reported, it is possible that endothelial cells may play an important role in the regulation of both granulocyte and macrophage proliferation and differentiation in long term cultures. In this investigation it was not possible to identify endothelial cells using an inverted microscope. It is assumed however that these cells form an important part of the stromal layer.

The stromal cells formed a confluent layer on the floor of the flasks by the end of the 3rd week in bone marrow cultures, this took a little longer in FL cultures, usually 4-6 weeks. The stromal layers of both LTBM and LTFL cultures were essentially of similar composition. One difference being the very rare occurence of fat cells in the FL cultures compared to the numbers of these cells in the BM cultures, stroma of both origins contained macrophages and fibroblasts. Furthermore an additional stromal element was observed in FL cultures. These cells which are believed to be hepatocytes were not observed in BM cultures. The hepatocytes were fewer

in number in cultures of 15 day FL compared to those in the 17 day and 19 day FL, however this is consistent with the increase in hepatocyte numbers observed as development advanced in vivo. The numbers of hepatocytes were observed to increase with the length of time in culture. The hepatocytes had polygonal shapes and were observed to grow in a mosaic-like arrangement. Intercellular cannalicular structures were observed, which had a similar appearance to bile cannaliculi. Refractile cells were often observed on the surface of the hepatocytes or closely associated with them. These observations suggest that the hepatocytes are supporting the dividing cells and may strengthen the in vivo evidence for the supportive role of hepatocytes in haematopoiesis. Cells of a similar nature have been recently reported (Hata et al 1993). They suggested that the cells are epithelial, derived from a hepatic origin. Hata et al observed tight junctions and desmosomes between the cells and demonstrated that these cells could produce GM-CSF and M-CSF. Thus the hepatocytes may be the fetal liver equivalent of the endothelial cells in LTBMC.

Fetal liver cultures were observed to be less cellular than those of the bone marrow, and granulocyte production was observed to fall off earlier in the FL cultures. This earlier decline in colony numbers in FL cultures compared to those of BM origin has bee previously reported (Hassan et al 1979). The temporary nature of haematopoiesis in the fetal liver in vivo could be an explanation for this phenomenon.

The stromal layer of LTBMC maintained beyond the stage when haematopoietic cell production has ceased, (16-20) weeks, remains intact and is capable of supporting renewed haematopoiesis when re-infused with a source of blood cells (Cappellini et al 1984). When BM or FL cells were re-infused onto a BM layer, which was no longer haematopoietically active, there was a rapid infiltration of the layer by the haematopoietic cells and

foci of haematopoiesis were observed, after one week. Haematopoietic cells were produced in the layer and released in to the supernatant for up to 6 months, after which time haematopoiesis was observed to decline. Similar observations were made when BM or FL cells were re-infused onto "burnt-out" FL stromal layers. Refractile cells were observed on the surface of the hepatocyte-like cells, suggesting these cells can support haematopoiesis, even from cells of an alternative origin. Haematopoietic activity lasted for around 5 months in these cultures before phagocytic cells became predominant in the supernatant.

Erythroblasts and erythrocytes were observed in both FL and BM cultures up to the end of the first week in culture. After this time erythroblasts were never observed again and granulopoiesis became dominant in the BM cultures and in the FL cultures of all three developmental stages. Granulocytes were observed in all stages of maturation after 3 weeks in culture. They were observed to form cobblestone foci of haematopoiesis within the cultures closely associated with macrophages and fat cells, these observations have also been reported (Allen & Dexter 1976). The granulocytes proliferate in these foci and extensive interactions have been reported between granulocytes and macrophages (Allen 1992). Thus it is not unreasonable to suggest that stimulatory factors may be passed from the macrophage to the granulocyte. Once the granulocytes mature they move out of the adherent layer and undergo no further proliferation only differentiation. Some mature granulocytes are observed to form islands with isolated macrophages not associated with stromal cells (Allen 1992). As the cells mature they are released in to the supernatant, thus mature cells form the major component of cytospin preparations.

There have been many reports of the disappearance of erythroid cells after 1 week in culture. This is especially surprising in FL cultures, as

erythropoiesis is the predominant form of in situ FL haematopoiesis. The disappearance of erythroid cells is thought to be due to a lack of suitable culture conditions. Dexter et al (1977) demonstrated that the standard culture conditions, used in this investigation, clearly favour granulopoiesis however, they could not detect humoral inhibitors of erythropoiesis in the culture medium.

Gordon, Kearney & Hibbin (1983) demonstrated that marrow derived stromal fibroblasts inhibited burst formation by peripheral blood BFU-E. The BFU-E survive in long term culture but cannot proliferate or differentiate unless they are specifically stimulated by the addition of anaemic mouse serum and/or Epo (Allen & Testa 1991). However Cappellini et al (1984) could not significantly stimulate erythropoiesis by addition of anaemic mouse serum and Epo. This could be explained by the fact that they were unable to recover BFU-E from FL cultures, one week after initiation. Other investigators have reported the formation of erythroid colonies from fetal liver cells, following addition of Epo (Johnson & Barker 1985), and in the absence of added Epo (Rich & Kubanek 1980; Zucali et al 1980). They were also able to demonstrate the presence of both CFU-E and BFU-E. Zucali et al (1980) reported that trypsin digestion of FL cultures produced an erythroid stimulating activity which increased with the length of time in culture. They concluded that this stimulatory activity was produced by the cultured fetal hepatic cells.

Megakaryocytes were also observed closely associated with macrophages in the stromal layer. The numbers of these cells were small in comparison to the number of granulocytes. More mature megakaryocytes migrated from the adherent macrophages and were released into the supernatant, where they were detected in cytospin preparations.

Concomitant with cell production there is proliferation of pluripotent stem cells. CFU-S can be maintained in the cultures for several months and function as normal. They can produce erythroid, granuloid, megakaryocytic and spleen colonies and can protect mice from potentially lethal irradiation (Dexter et al 1977). Erythroid precursors are also known to exist however, these are blocked at the BFU-E stage, their more mature progeny, CFU-E, have not been detected (Dexter 1982). CFU-C produced in the cultures undergo maturation into granulocytes and macrophages, this occurs in the absence of detectable colony stimulating activity. Tavassoli & Takahashi (1982) reported that CFU-C numbers were related to the accumulation of fat cells and suggested that the fat cells served as an energy source for stem cell proliferation. Direct cellular interactions between the stromal cells and precursor cells may be a mechanism of regulation of haematopoiesis in culture.

## 3.5 Cellular Interactions

### 3.5.1 Erythroblastic Islands in Vivo

A central macrophage surrounded by maturing red blood cells, to form a distinct anatomical unit is commonly referred to as an erythroblastic island. These islands have been extensively reported in human and murine bone marrow (Bessis 1958, 1973), in the yolk sac of guinea-pigs (Sorenson 1961) and embryonic liver (Rifkind et al 1969).

Erythroblastic islands were initially observed in the 13 day fetal liver, randomly dispersed throughout the hepatic parenchyma. At this stage in development the erythroblasts were arranged around a central hepatocyte, not a macrophage. Both hepatocytes and macrophages can function as a

central reticular cell of a blood island (Emura et al 1985). This arrangement is thought to be essential for differentiation of both the erythroid cells and the hepatocytes. Blood cell precursors arranged around a central macrophage were not evident until the 14th day of gestation, however throughout development the number of clusters with a central hepatocyte far outnumbered those with a central macrophage. The outstanding morphological characteristic of a central macrophage was the presence of large numbers of inclusions of breakdown products of ingested red cells, extruded nuclei of red cells, lysosomes and phagosomes. The central cells have long cytoplasmic processes which extend in all directions to embrace the maturing cells in its vicinity. Some immature blood cell precursors were observed to be completely enveloped in the cytoplasmic processes. Intimate associations of blood cell precursors with the cytoplasmic processes were evident in all the erythroblastic islands. Desmosome-like attachments (Fukuda et al 1974) and gap junctions between the central cells and the adjacent blood cell precursors were numerous, thus there is potential for the transfer of substances between the cells of the islands.

The number of cells which constituted a cluster was high in the 14 and 15 day fetal livers, on average 10-15 cells. This could be due to the high numbers of haematopoietic cells present in fetal liver relative to the numbers of macrophages and hepatocytes present. The number of cells in each cluster was observed to decrease as fetal development proceeded. This is most probably due to the increase in numbers of hepatocytes and macrophages combined with the decrease in number of haematopoietic cells. Gradients of differentiation were observed in the clusters. The most immature cells occupied positions close to the central cell, some cells were entirely surrounded by the cytoplasmic processes of the central cells. More mature cells occupied peripheral positions however they usually remained

in close association with at least one cytoplasmic process of the central cell. These observations suggest that the early precursors are more dependent on the central cell and as they mature they migrate, possibly along the cytoplasmic processes of the central cell (Shaklai 1990), to occupy more peripheral positions within the clusters. Granuloid, lymphoid and monocytic cells occupied peripheral positions within the mixed clusters when haematopoietic activity was high. Only when the number of erythropoietic cells had decreased were they seen to occupy more central positions. It may be that myelomonocytic and lymphoid cells are less dependent on the central cell than erythroblasts or that the large numbers of erythroid cells restricts cells of other lineages to peripheral positions.

### 3.5.2 Mixed and Lineage-Restricted Clusters

The cell clusters in fetal and newborn liver were not restricted to haematopoietic cells of a single lineage (Jones 1970; Grossi 1985).Granulocytes and increasing numbers of monocytes observed in the 14 day fetal liver, formed constituent parts of cells clusters. The myelomonocytic cells were also closely associated with the cytoplasmic extensions of the central cells. Cytoplasmic and desmosome-like communications between the processes of the central cell and the myelomonocytic cells were abundant. When lymphoid cells first appeared in the newborn liver, they too were observed as an integral part of the cell clusters. Only when the number of erythroid cells was declining did the cells of other lineages occupy more central positions.

In the 10 day neonatal liver the cell clusters exhibited marked morphological differences from those observed earlier. Only single lineage clusters were observed surrounded by hepatocytes and the number of cells in each cluster was reduced. Cells were not observed arranged around a

central macrophage. The cell clusters themselves were restricted to isolated foci within the parenchyma, however the intimate associations between the hepatocytes and the adjacent blood cell precursors remained abundant. The presence of single-lineage colonies in neonatal liver have been described previously (Grossi et al 1985; Rossant et al 1986). The authors suggest that progenitor cells committed to specific lineages are present in the neonatal liver and that these precursors give rise to discrete foci of single-lineage cells. An alternative explanation would be that multipotential precursors are restricted to specific lineages by local environmental differences. However this seems unlikely in that within haematopoietic foci of the 10 day neonate, clusters of all three lineages were observed, erythroid clusters were often seen in the vicinity of granuloid clusters, not restricted to a local erythroid environment as the latter hypothesis would suggest.

### 3.5.3 Cellular Interactions in Vitro

Intricate cellular interactions have also been observed in vitro. Granulopoiesis takes place in close proximity to macrophages, and hepatocytes in FL cultures. Granulopoietic islands have been observed using time lapse video, where the central macrophage was observed to phagocytose dead granulocytes (Allen 1992). Interactions are also observed with blanket cells.

As previously mentioned, addition of anaemic mouse serum permits erythropoiesis in these cultures. In vitro erythroblastic islands have been reported in cultures initiated this way (Allen & Dexter 1982). The production of red cells in the long term cultures appears to mimic almost exactly their production in vivo. All red cell maturation is observed to take place on the surface of the adherent layer in association with macrophages

(Allen 1992), to form in vitro erythroblastic islands. The erythroblasts within the islands have been observed to undergo mitosis in synchrony, this was not reported in the granulopoietic islands. In addition erythroblast-macrophage interactions were not transient like the granulocyte-macrophage interactions observed (Allen & Testa 1991). Thus it is feasible that direct and continuous association with macrophages is essential for proliferation and differentiation of erythroblasts.

Cytoplasmic extensions of macrophages have been observed to be associated with the haematopoietic cells of the island. Like erythroblastic islands observed in vivo, numerous membrane contacts have been reported between macrophages and early erythroblasts, while more mature cells are not so intimately associated with the extensions (Allen & Dexter 1982). Macrophages have been observed to phagocytose extruded nuclei and rhopheocytosis has also been observed (Breton-Gorius et al 1991). Vesicular activity at closely apposed membranes and gap junctions has been observed (Rosendaal 1991; Allen 1992), gap junctions have also been reported between endothelial cells and macrophages (Hasthorpe et al 1991).

The extensive observation of erythroblastic and granulocytic islands both in vivo and in vitro has led to a general acceptance that true haematopoiesis takes place in a similar manner. The focal nature of the islands, taken together with the cellular interactions described, observations of rhopheocytosis, desmosomes and gap junctions in addition to recently demonstrated receptors for cell binding strongly suggest the central macrophage has an important role to play in haematopoietic regulation, in addition to those currently recognised- providing a substratum for cell differentiation, removal of extruded nuclei and cell debris, aiding passage of mature erythrocytes into the venous sinuses (Shaklai 1989).

### 3.5.4 Function of the Haematopoietic Islands

Three physiological functions have been ascribed to erythroblastic islands:- a metabolic function which introduces ferritin and other molecules into the erythroblasts (Bessis & Breton-Gorius 1962). Secondly the central macrophage is necessary for phagocytosis of expelled nuclei and thirdly phagocytosis of old red blood cells (Bessis 1973). Thus the central macrophage plays the roles of both a "nurse cell" and the grave of the red cell.

The latter two functions are universally accepted. In the fetal liver on the 15th day of gestation and in subsequent stages, macrophages were observed both in the process of engulfing extruded nuclei and also to contain ingested nuclei and all sorts of cell debris within their cytoplasm. A phagocytic function of the central hepatocytes has not been clearly demonstrated, however they play a very important metabolic role in the differentiation of blood cell precursors. The metabolic role of the erythroblastic islands is much more difficult to prove and has remained a very controversial aspect for many years.

The term rhopheocytosis was introduced to describe the presence of coated vesicles containing ferritin in erythroblasts. However electron microscopy is static and to this day the direction of transfer of the ferritin cannot be accurately determined. Bessis proposed that ferritin constituted an iron-reserve which was passed from macrophage to erythroblast for the synthesis of haemoglobin (Bessis 1973). As the erythroblasts mature and become advanced in haemoglobin formation they no longer require this supply of ferritin. The observations of 15 day fetal liver stained with Pearls Prussian Blue are in accordance with this theory. The cells in closest proximity to the central cell were more intensely stained than the more

mature cells on the periphery. The fact that numerous ferritin molecules are present in the cytoplasm of the proerythroblast at the time of initiation of haemoglobin (Hb) synthesis suggest that ferritin does not represent an excess of iron not required for (Hb) synthesis (Breton-Gorius 1991). However other groups dispute this.

Zamboni (1965) reported that as rhopheocytic vesicles were so rare they represented aggregates of excess iron not required for (Hb) synthesis. This excess is then removed by the central cell. Electron microscopic observations of ferritin in immature erythroblasts have indicated that the iron content of the cells was greater than was necessary for Hb synthesis (Mohandas 1991). Thus the less intently stained erythroid cells observed on the periphery of cell clusters in the 15 day fetal liver may contain sufficient ferritin for Hb synthesis and the excess may have been transported from these cells to the central cell. Zamboni (1965) only observed iron exchange between central cells and erythroblasts advanced in Hb formation. Intracellular ferritin is observed in 0.01% of circulating erythrocytes of normal individuals but in 3% of circulating erythrocytes in splenectomised individuals (Pollycove 1991). Thus the spleen may complete the removal of reticulocyte ferritin. This would account for the presence of some intensely stained erythrocytes in the hepatic sinusoids of the 8 day neonate.

It is known that circulating Hb is maintained by reclamation of iron from the Hb of phagocytosed erythroblasts and nuclei by the central macrophages. This is then promptly re-released for utilisation by immature cells (Pollycove 1991). It is possible therefore that ferritin is transported in both directions. Intercellular components initially required for normal development and intermediate metabolism of immature blood cell precursors, may be provided by the central cell. At later stages in development these products may become unnecessary and their removal

may be essential for further maturation to progress. The central cells would also be responsible for the removal of these waste products. Thus the ubiquitous presence of the central cell in the haematopoietic islands may be essential for normal intermediary metabolism and function of its adjacent blood cell precursors.

Macrophages are now known to produce multiple molecules such as Epo, G-CSF, M-CSF, GM-CSF and IL-1 (Rich 1986; Nathan 1987). Zucali et al (1977) demonstrated that production of Epo and colony stimulating factor by murine fetal liver macrophages was maximal in day 14 and day 15 FL cultures. The erythropoietic stimulating activity was capable of stimulating both in vivo and in vitro erythropoiesis (Zucali et al 1980). Vogt, Pentz & Rich (1989), demonstrated that murine macrophages were positive for the Epo gene, therefore it is plausible that the central macrophage could be the source of growth factor for its adjacent erythroid cells. Macrophages can respond to varying oxygen concentrations by increasing or decreasing production of Epo and CSF accordingly (Rich 1986). In hypoxic conditions an increase in size and enhancement of phagocytic activity is observed (Yoffey & Yaffey 1980).

Medlock & Haar (1983), also proposed the fetal hepatocyte as a likely candidate for synthesis and secretion of substances important to the haematopoietic functioning of the liver. This would explain the close associations observed in this investigation, of hepatocytes and haematopoietic cells both in vivo and in vitro. Megakaryocytes have been reported to respond to Epo and possess receptors for Epo (Krantz 1991). The close associations observed between hepatocytes and megakaryocytes in vivo in all stages of liver examined may thus be Epo mediated.

In addition to the array of stimulatory molecules produced by macrophages, inhibitory molecules, TNF-α, IL-1α, TGF-β and Interferons α and γ, are also secreted (Nathan 1987). Through their abundance, distribution, motility and versatility, macrophages can influence an array of diverse activities from induction of haematopoiesis to cell death, in addition to their roles in the immune and inflammatory responses.

The focal nature of the cell clusters both in vivo and in vitro and the close range cellular interactions involved, suggest that ECM molecules and components of the cell surface are also involved in haematopoietic regulation. How the central cells recognise the cells with which they become intimately associated has not been elucidated. In addition macrophages must have the capacity to distinguish between an expelled nucleus and mature erythrocytes, otherwise a high degree of red cell destruction would result.

It is thought that very early precursor cells, the proerythroblasts may be associated with erythroblastic islands (Breton-Gorius et al 1991), however the mechanism involved in the erythroblast macrophage binding as yet remains unclear. It is known that macrophages and erythroid progenitors express receptors for fibronectin and thrombospondin, in addition to the respective macrophage ligands, these molecules could be potentially involved in the formation of an erythroblastic island. Crocker et al (1990, 1991) reported that interaction of fetal liver cells with fetal liver macrophages occurs through a divalent-cation dependent adhesion receptor, the molecular nature of which is unknown to date. Such a receptor is also involved in erythroid-macrophage interactions in the adult bone marrow however, a second receptor is also involved in the adult. This receptor is divalent-cation independent and is known to be a plasma

membrane glycoprotein expressed specifically by bone marrow macrophages.

The ECM of the adherent layer of LTBMC consists of collagens types I, III, and IV, laminin, fibronectins and proteoglycans (Zuckerman & Wicha 1983). The collagens are produced by all stromal cell types, fibronectin is produced by endothelial cells, macrophages and fibroblasts while laminin is produced by endothelial cells only. The importance of these molecules for the integrity of the adherent layer is evident, perturbation of collagen synthesis impairs the outgrowth of the adherent layer. When an intact stromal layer is treated with antibody against fibronectin, the adherent layer is completely destroyed (Zuckerman et al 1985).

Heparan sulphate is the most abundant glucosaminoglycan (GAG) chain of the proteoglycans which constitute the adherent layer in murine LTBMC, while chondroitin sulphate predominates in human LTBMC (Gallagher, Spooncer & Dexter 1983). Gordon, Riley & Clark (1988) demonstrated that heparan sulphate was necessary for binding blast-colony forming cells, (Bl-CFC), to stromal layers in vitro. This binding was stage specific and reinforced the idea that lineage and stage specific microenvironments exist in the bone marrow and also that segregation of different haematopoietic progenitors facilitates their regulation and interaction with appropriate growth factors (Gordon et al 1987; Roberts et al 1988; Dexter et al 1990). Zuckerman & Wicha (1983), reported that haematopoiesis in long term cultures was preceded by extensive deposition of ECM by stromal cells by almost 1 week.

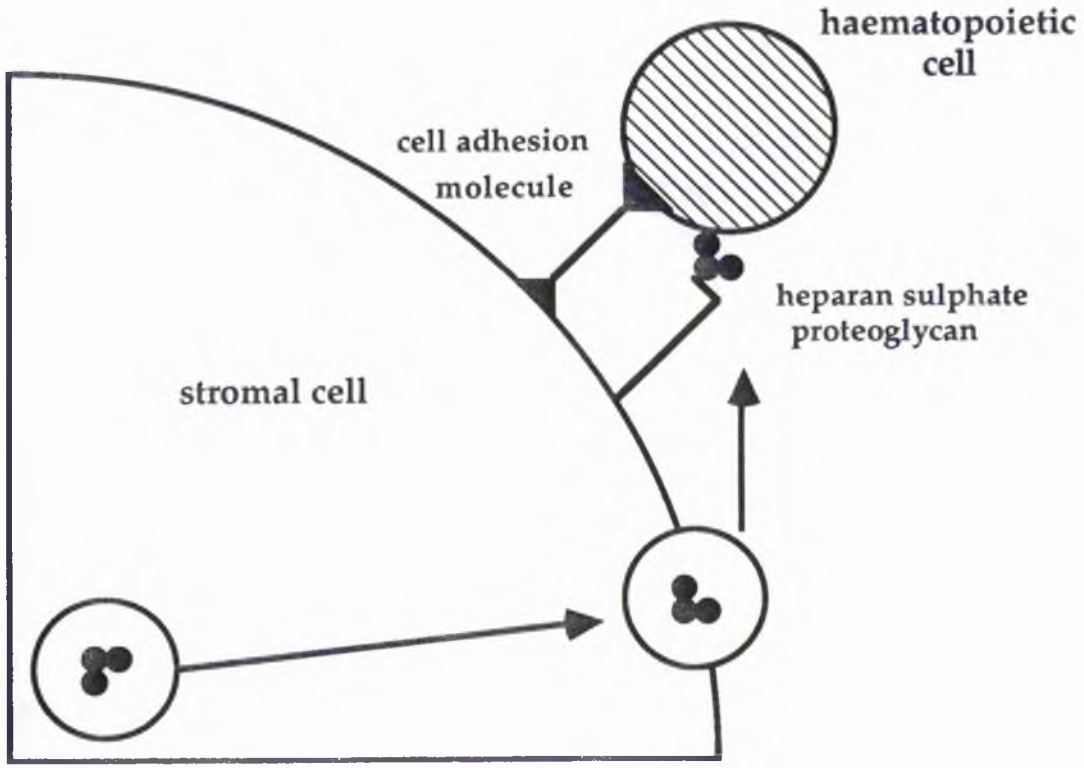
Fibronectin is synthesised by a large number of cells including endothelial cells, macrophages and hepatocytes. One of the main functions attributed to fibronectin is the property to bind cells together, it also has binding

capacities to collagens, GAGs and proteoglycans, thus it is regarded as an ECM promoter of cell attachment. It has to strike a balance between promoting anchorage of cells and permitting their migration. Early erythroid cells posses receptors for fibronectin unlike circulating red blood cells (Vuillet-Gaugler et al 1990). Thus one can speculate that attachment to the ECM can regulate cell migration within the marrow and loss of expression of adhesion receptors during differentiation will favour migration of mature cells (Coulombel et al 1992).

It is likely that bone marrow haematopoiesis is regulated through co-operation of locally synthesised growth factors, adhesion molecules and ECM proteins and the crucial stem cell stromal cell interactions, in short the haematopoietic inductive microenvironment. Most growth factors are locally synthesised by medullary stromal cells and can be secreted in a soluble form, which binds to heparan sulphate proteoglycans for presentation to haematopoietic cells, this mechanism was proposed by Dexter et al (1990) and is illustrated in Fig. (3.3-1).

In contrast it has been recently shown that human haematopoietic progenitors did not require direct contact with the bone marrow stroma for long term in vitro haematopoiesis (Verfaille 1992). However repeated addition of cytokines was necessary to maintain haematopoiesis in these cultures. Thus the main role of the stromal cells may be to promote the differentiation of haematopoietic progenitor cells. In addition Zipori & Lee (1988), suggested that stem cell self-renewal is maintained by novel stromal cell factors distinct from known colony stimulating factors. The biological importance of actual haematopoietic cell associations with stromal cells and ECM proteins, and the functional mechanism for proliferation and differentiation are unknown.

## Fig. 3.3-1 Haematopoietic Regulation in the Bone Marrow Microenvironment




Adult bone marrow
(proposed by Dexter et al 1990).

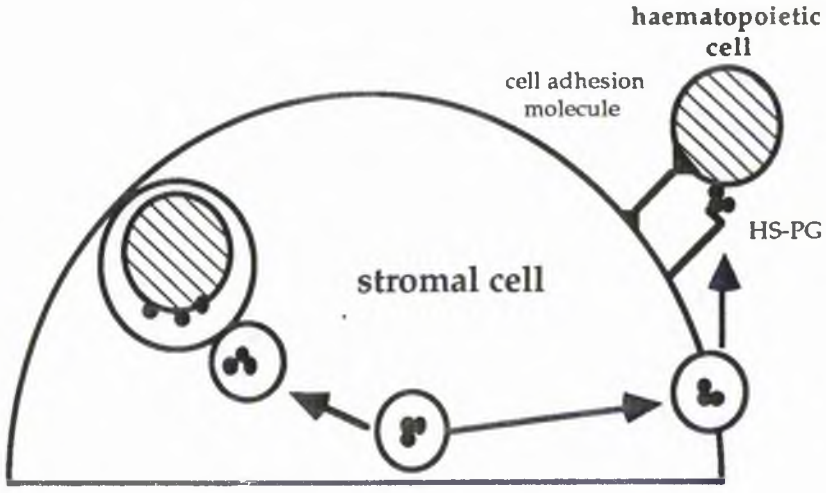
Regulatory factors are synthesised by the stromal cell and secreted in a soluble form. The regulatory factors bind to heparan sulphate proteoglycan for presentation to the haematopoietic cell, which retained in close proximity by cell adhesion molecules

While the ECM and adherent stromal layer of LTBMC has been the subject of intense investigation in recent years, in LTFLC however, these properties have not been so well documented. It is known that macrophages and hepatocytes can both produce fibronectin and regulatory factors for haematopoiesis. However information is lacking on the other ECM components and cell adhesion molecules of fetal cultures. Gordon et al (1988), demonstrated that FL-GAGs were inefficient in binding GM-CSF, in comparison to BM-GAGs. In another series of experiments Gordon and her associates (1990) found that binding of very early progenitors to marrow stromal layers did not appear to involve known cell adhesion molecules. Thus in the early stages of haematopoiesis, in particular fetal haematopoiesis, GAGs may not be essential for presentation of growth factors and cell adhesion molecules may not be required to maintain the integrity of the clusters. The morphological observations of fetal liver haematopoiesis undertaken both in vivo and in vitro, in the present investigation reveal the importance of the cellular interrelationships between macrophages and blood cell precursors as well as between hepatocytes and blood cell precursors. The form and arrangement of the cytoplasmic extensions, the presence of gap junctions, rhopheocytic vesicles and desmosome-like attachments are consistent with extensive interchange of material between the central cells and the blood cells precursors which surround them.

Whereas in adult bone marrow, under steady state conditions, regulatory factors appear to be fixed in relation to their target cells by specific molecules, as Dexter has envisaged (1990). In stimulated bone marrow, the more intimate relationships between differentiating erythroblasts and stromal cells (Yoffey & Yaffey 1980), may provide circumscribed microenvironments in which regulatory factors can be localised.

Similarly in fetal liver, the localisation of regulatory factors within circumscribed micoenvironments may be important for the regulation of haematopoiesis in addition to the mechanism proposed by Dexter (fig. 3.3-2).

Fig. 3.3-2 Haematopoietic Regulation in Fetal Liver




Haematopoietic regulation in fetal liver may involve circumscribed microenvironments of haematopoietic cells and stromal cells in which growth factors can be localised, in addition to the mechanism proposed by Dexter.

# Chapter 4

## Haematopoietic Colony Forming Cells

The earliest precursors of blood cells cannot be adequately characterised using morphological criteria. It is therefore necessary to exploit operational assays in order to obtain information about these cells upon which the maintenance of haematopoiesis is dependent (Wright & Lord 1977). The proportion of stem cells which are in the cell cycle is regulated by locally produced factors (Devalia & Linch 1991). Stimulators and inhibitors have been demonstrated which act specifically at the stem cell level (Dexter 1989). The stimulators and inhibitors regulate the proportion of cells in cycle, in response to the body's demands. In normal murine bone marrow the proportion of CFU-S synthesising DNA is very low, probably reflecting quiescence in the stem cell pool. In contrast, the haematopoietic stem cells are actively cycling in murine fetal liver (Thomas, Cork & Riches 1981).

This investigation was devised to evaluate the kinetic properties of high proliferative potential colony forming cells (HPP-CFC) and of granulocyte-macrophage colony forming cells (GM-CFC) derived from murine fetal and neonatal liver and adult bone marrow. The standard "suicide assay" technique as detailed in Chapter 2 was used to determine the proportion of cells in DNA synthesis.

Single cell suspensions of murine fetal and neonatal liver and bone marrow were incubated with cytosine arabinoside, Ara-C, which is cytotoxic to DNA synthesising cells. The difference in the number of colonies produced by control samples and Ara-C treated samples gives a measure of

the proportion of cells in the S-phase. Colonies produced which are in excess of 2mm diameter are considered to be derived from the HPP-CFC population. These HPP colonies contain more than 50, 000 cells. A dramatic decrease in the number of these large colonies produced by Ara-C treated samples is observed (Fig. 4.1-1).

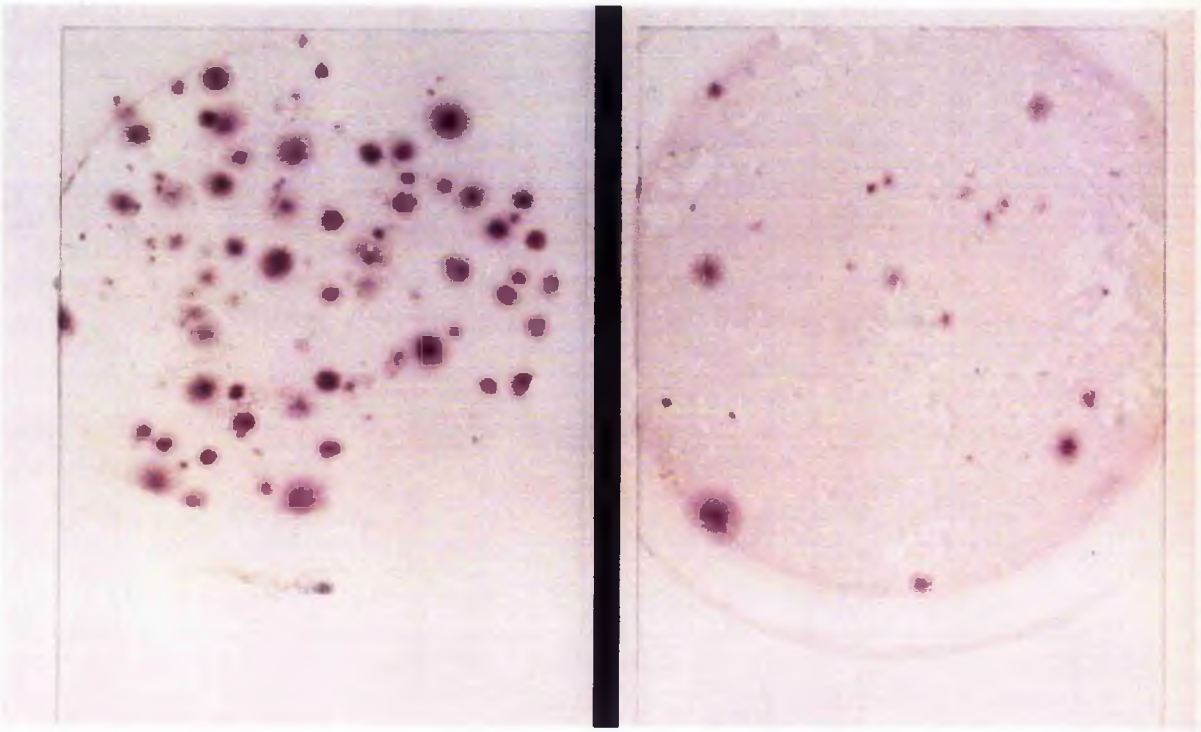


**Fig. 4.1-1** Reduction of HPP-CFC Colony Formation by Ara-C

The sample of fetal liver on the right has been treated with Ara-C, the sample on the left has not.

# Results

## 4.1 HPP-CFC

A considerable proportion of HPP-CFC derived from fetal liver were found to be cycling in contrast to cells derived from adult bone marrow, of which only a small proportion were in S-phase (Table 4.1-1, Fig. 4.1-2).

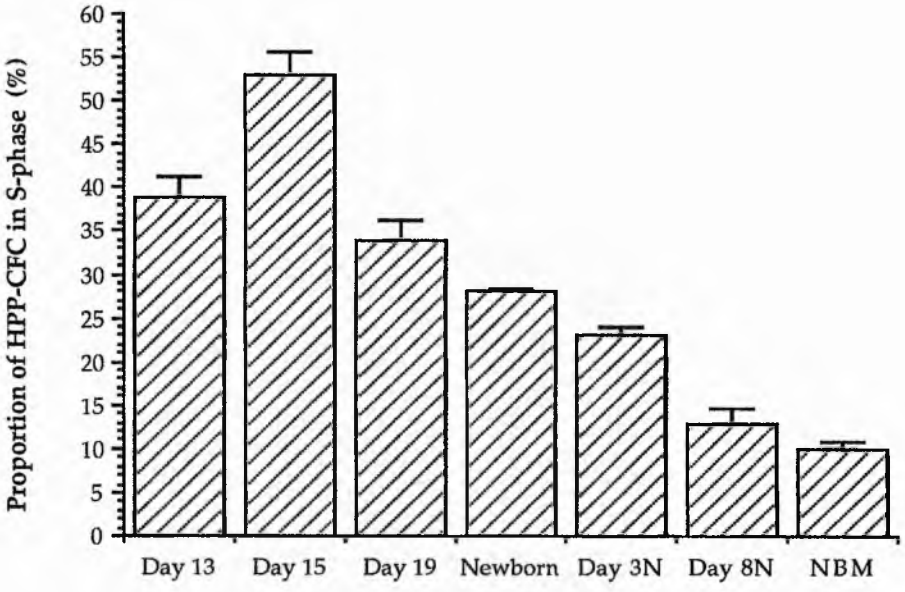
39% of HPP-CFC derived from 13 day fetal liver were engaged in DNA synthesis. The proportion of cycling cells increased to a maximum of 53% in the 15 day fetus, this value then declined to 34% in 19 day fetal liver. As development proceeded, a corresponding decline in the proportion of cycling cells was observed, from 23% in the newborn liver down to a minimum of 13% in 8 day neonatal liver. This value corresponds to the 10% of HPP-CFC engaged in DNA synthesis detected in normal adult bone marrow.

Anova showed a significant difference in the mean scores ($F = 53.66$, $d=6$, $p < 0.0001$). Tukey's post-hoc testing of comparisons revealed that the proportion of cells synthesising DNA in the 15 day fetal liver was significantly different from all other values obtained. The values obtained for 13 day fetal liver, 19 day fetal liver and newborn liver were not significantly different. The proportion of cells synthesising DNA in the newborn liver was significantly different from the values obtained for adult bone marrow. The proportion of cells engaged in DNA synthesis in the 3 day neonate, the 8 day neonate and adult bone marrow were not significantly different.

**Table 4.1-1** Proportion of HPP-CFC in S-phase in Fetal and Neonatal Liver and Adult Bone marrow

| Tissue Sample | % HPP-CFC in S-phase (mean ± S.E.) | No. of Experiments |
|---|---|---|
| 13 Day Fetal Liver | 39 ± 2.4 | 7 |
| 15 Day Fetal Liver | 53 ± 2.6 | 10 |
| 19 Day Fetal Liver | 34 ± 2.4 | 6 |
| Newborn Liver | 28 ± 0.4 | 4 |
| 3 Day Neonatal Liver | 23 ± 1.0 | 4 |
| 8 Day Neonatal Liver | 13 ± 1.7 | 3 |
| Adult Bone Marrow | 10 ± 0.7 | 8 |

**Fig. 4.1-2** Proportion of cycling HPP-CFC in Fetal and Neonatal Liver and in Adult Bone Marrow

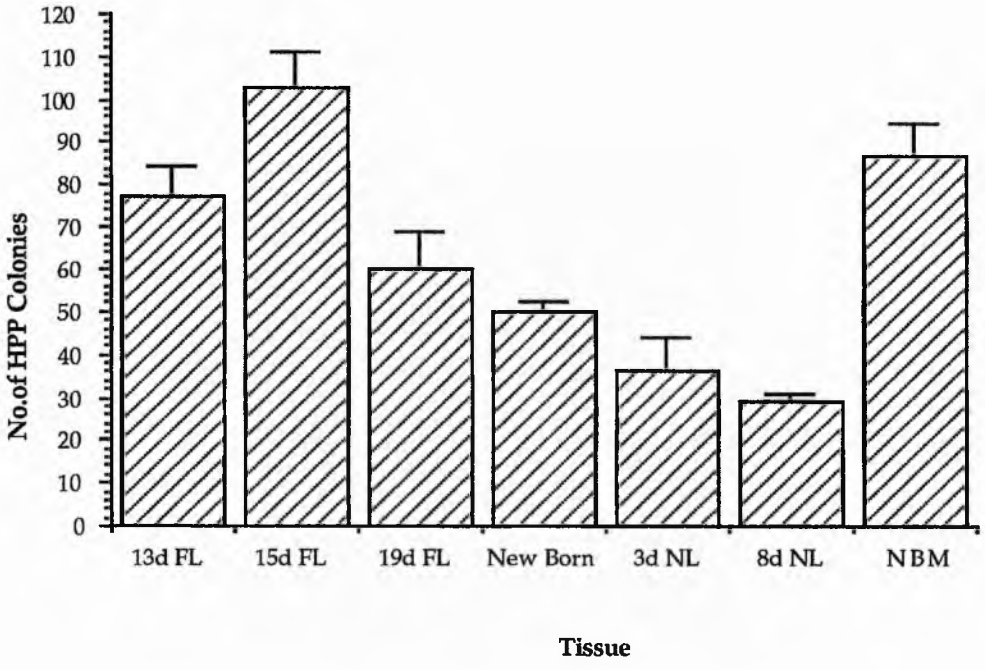


Colony production was maximal, (103 colonies/$10^5$ cells), in fetal liver on the 15th day of gestation (Table 4.1-2, Fig. 4.1-3). This corresponds to the maximum proportion of HPP-CFC synthesising DNA. Colony production in the liver samples declined with developmental age, only 50 colonies/$10^5$ cells were produced by newborn liver. As neonatal development proceeded colony production further declined from 36 colonies in the 3 day neonate to a minimum of 29 colonies /$10^5$ cells in the liver of the 8 day neonate, corresponding to the decline in the proportion of cells synthesising DNA. In adult bone marrow however a high number of colonies were produced (87 /$10^5$ cells) despite the low number of DNA synthesising cells in this tissue.

**Table 4.1-2** Number of HPP-CFC Colonies Produced / $10^5$ cells

| Tissue Sample | No. of Colonies Mean ± S.E. | No. of Experiments |
|---|---|---|
| 13 Day Fetal Liver | 77 ± 7.2 | 7 |
| 15 Day Fetal Liver | 103 ± 8.3 | 10 |
| 19 Day Fetal Liver | 60 ± 8.0 | 6 |
| Newborn Liver | 50 ± 2.4 | 4 |
| 3 Day Neonatal Liver | 36 ± 7.1 | 4 |
| 8 Day Neonatal Liver | 29 ± 1.6 | 3 |
| Adult Bone Marrow | 87 ± 7.4 | 8 |

**Fig.4.1-3** Number of HPP-CFC Colonies Formed / $10^5$ Cells




## 4.2 GM-CFC

The proportion of GM-CFC synthesising DNA was evaluated in murine fetal liver on the 15th day of gestation, on the day of birth and in normal adult bone marrow (Table 4.2-1, Fig. 4.2-1).

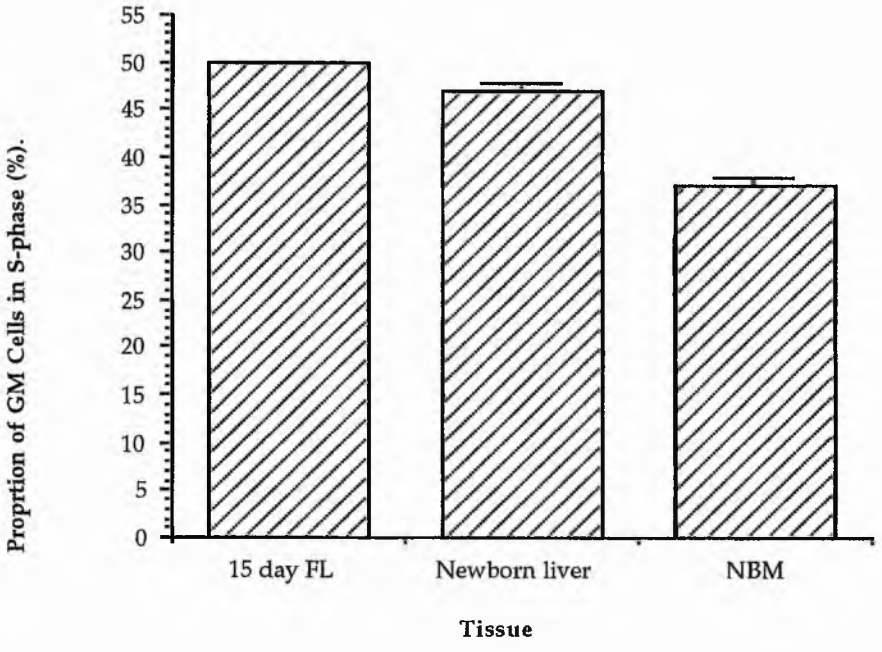
The proportion of GM-CFC in S-phase was high in tissue derived from all three samples. The highest proportion of cycling cells was detected in liver of the 15 day fetus (50%), this value declined to 47% in the newborn liver. Adult bone marrow was found to have the lowest proportion of DNA synthesising cells (37%). Although the differences in the proportion of GM-CFC synthesising DNA in the three tissue samples were not dramatic, Tukey's post-hoc testing of analysis of variance showed that the values obtained for both liver samples were significantly different to those of adult

bone marrow (F=92.39, df=2, p<0.0001). There was no significant difference in the values obtained for 15 day fetal liver and newborn liver.

**Table 4.2-1** Proportion of GM-CFC Engaged in DNA Synthesis in Fetal and Newborn Liver and in Adult Bone Marrow

| Tissue Sample | % GM-CFC in S-phase Mean ± S.E. | No. of Experiments |
|---|---|---|
| 15 Day Fetal Liver | 50 ± 0.0 | 3 |
| Newborn Liver | 47 ± 0.8 | 4 |
| Adult Bone Marrow | 37 ± 0.7 | 3 |

**Fig. 4.2-1** Proportion of cycling GM-CFC in Fetal and Newborn Liver and in Adult Bone Marrow

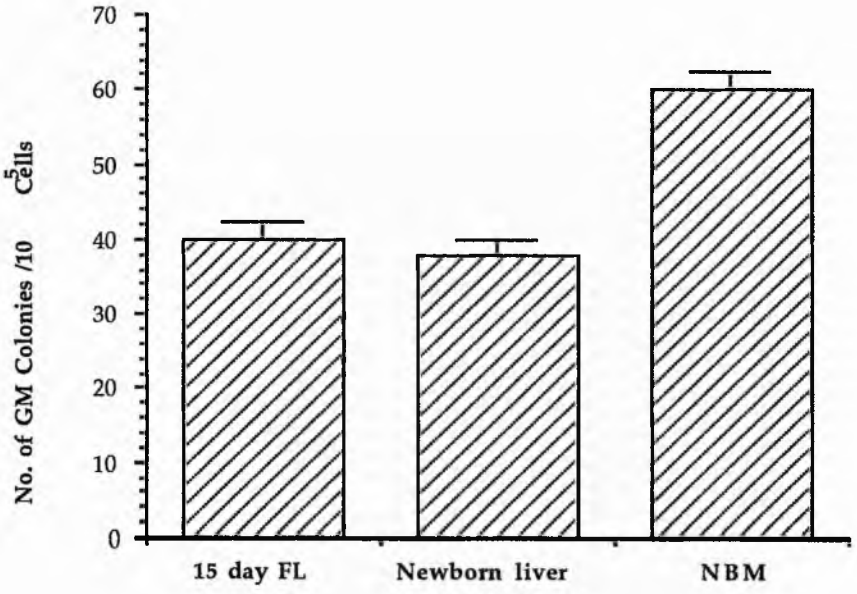


In contrast to the decline in the proportion of cycling GM-CFC in fetal and newborn liver, there was no significant difference in GM-CFC colony production in the two samples, $p > 0.3$, (Table 4.2-2, Fig. 4.2-2). Fetal liver produced an average of 40 colonies $/10^5$ cells, while newborn liver produced 38 colonies $/10^5$ cells. Adult bone marrow produced a significantly higher number of colonies (60 / $10^5$ cells), than either of the two liver samples ($p \ll 0.001$).

**Table 4.2-2** Number of GM-CFC Colonies Produced $/10^5$ Cells

| Tissue Sample | No. of Colonies Mean ± S.E. | No. of Experiments |
|---|---|---|
| 15 Day Fetal Liver | 40 ± 2.3 | 3 |
| Newborn Liver | 38 ± 2.1 | 4 |
| Adult Bone Marrow | 60 ± 2.6 | 3 |

**Fig. 4.2-2** Number of GM-CFC Colonies Produced $/10^5$ cells



# Discussion

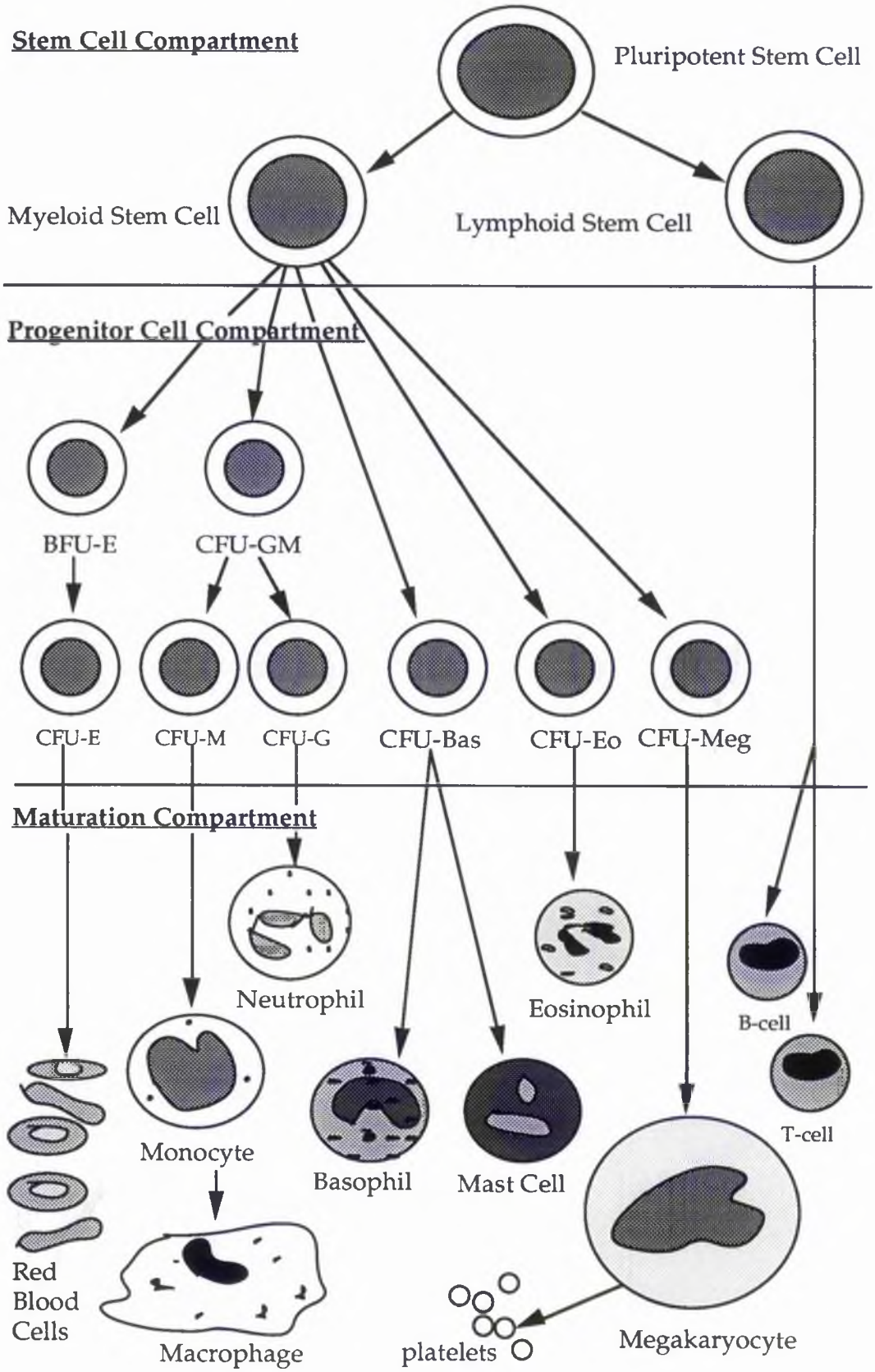
## 4.3 HPP-CFC

The in vivo CFU-S assay has permitted the investigation of murine haematopoietic stem cell behaviour and regulation. This assay is however restricted by its in vivo nature and species limitation. More recently, the use of combinations of haematopoietic colony stimulating factors (CSFs), has led to the cloning of a developmentally early haematopoietic precursor cell population, which is characterised by its high proliferative potential. These HPP-CFC are considered to be a component of the stem cell compartment (Fig. 4.3-1).

To date three subpopulations of HPP-CFC have been described (McNeice et al 1990). HPP-$CFC_1$, which is responsive to IL-1 and CSF1, is considered to be the most primitive of the HPP-CFC populations. HPP-$CFC_1$ in turn differentiate to produce HPP-$CFC_2$, responsive to IL-3 and CSF1. It is proposed that HPP-$CFC_2$ differentiate to produce a third subpopulation which is responsive to hemolymphopoietic growth factor (HLGF) and CSF1. This HPP-$CFC_3$ population is a more mature lineage restricted population, which differentiates to produce GM-CFC (McNeice et al 1988, 1990).

The kinetic properties of the HPP-$CFC_2$ population, responsive to IL-3 and CSF1, were evaluated in this investigation. Conditioned medium containing a combination of WEHI 3B, as a crude source of IL-3, and L929, as a crude source of CSF1/M-CSF, was used to stimulate the production of colony forming cells with a high proliferative potential. Ara-C was used to eliminate cells which were engaged in DNA synthesis.

**Fig. 4.3-1** The Organisation of the Haematopoietic System



The difference in colony numbers produced in the absence and in the presence of Ara-C gives an indication of the proportion of HPP-CFC which were synthesising DNA.

The proportion of HPP-CFC which were synthesising DNA was found to be high in the 13 day fetal liver (39%), this increased to a maximum of 53% in the liver of the 15 day fetus, then declined progressively as development proceeded. At birth the number of HPP-CFC in S-phase had declined to 28% and the numbers of HPP-CFC synthesising DNA further declined in the neonatal period to reach a minimum of 13% in the 8 day neonatal liver. In adult bone marrow the majority of stem cells are quiescent this was confirmed by the finding that only 10% of the HPP-CFC were synthesising DNA.

HPP colony production was greatest in the 15 day fetal liver (103 colonies/$10^5$ cells), corresponding to the highest proportion of cells in S-phase. Colony production declined with fetal development to a minimum of 29/$10^5$ cells in the liver of the 8 day neonate. This observed decline in colony production mirrors the decline in the proportion of cells synthesising DNA. In adult bone marrow the number of HPP colonies produced, (87/$10^5$ cells), was greater than any of the liver samples, with the exception of the 15 day fetus. Although a large number of HPP-CFC colonies were produced by bone marrow cells, the HPP-CFC derived from bone marrow are quiescent. In contrast only rapidly cycling 15 day fetal liver HPP-CFC produced colonies on this scale. The high number of colonies in the bone marrow compared to the liver indicates that the bone marrow contains a larger population of HPP-CFC than the liver. The majority of these cells in the bone marrow are quiescent only 10% were in S-phase. In the fetal liver however the majority of cells were actively

cycling, correlating with previous reports of in vivo CFU-S studies (Riches, Cork & Thomas 1981).

The high proportion of HPP-CFC which were synthesising DNA in the 15 day fetal liver coincided with the morphological evidence of extensive hepatic haematopoiesis at this stage in development. Morphological investigations have revealed a gradual decline in hepatic haematopoiesis as development proceeds. This corresponds to the decline in the number of HPP-CFC engaged in DNA synthesis as development progresses.

In the 8 day neonatal liver only 13% of HPP-CFC were in S-phase, at this stage the liver has ceased to be the main site of haematopoiesis. The proportion of cycling HPP-CFC present in the 8 day neonate approximates to the 10% of HPP-CFC synthesising DNA in the adult bone marrow. Thus it is reasonable to assume that like the stem cells of normal adult bone marrow, the considerably smaller number of stem cells in the 8 day neonate are quiescent.

The high proportion of HPP-CFC synthesising DNA in the 15 day fetal liver coincides with the availability of a stem cell specific stimulator of DNA synthesis. The decrease in the proportion of HPP-CFC synthesising DNA in the 8 day neonatal liver coincides with the disappearance of this putative regulator (Dawood et al 1990). The stimulator from 15 day fetal liver is produced by plastic adherent cells and by fractionated cells in the 1.064 and 1.076 $g/cm^3$ density bands and it has the ability to switch quiescent normal bone marrow cells into cycle in vitro. The stimulatory activity declines from the 15th day of gestation, it can be detected in 1 week old neonatal liver, however after this time its production ceases and it cannot be detected in liver 3 weeks after birth.

In normal adult bone marrow the stem cells are quiescent, only 10-15% of CFU-S are in S-phase (Thomas et al 1981). The reduced proportion of cycling cells is maintained by an inhibitor of DNA synthesis, which can reduce the proportion of CFU-S in regenerating bone marrow or fetal haematopoietic tissue (Lord et al 1976; Riches et al 1981; Tejero et al 1984). Maintenance of an increased proportion of cycling CFU-S in regenerating tissue, physiologically stressed tissue or fetal tissue is brought about by a stimulator of DNA synthesis (Wright & Lord 1977). Both the inhibitor and the stimulator are produced by macrophages (Lord 1988).

There is evidence that the control of proliferation occurs locally as the kinetic properties of haematopoietic stem cells can differ at different sites within the same animal (Wright & Lord 1977). The stimulator and the inhibitor are not thought to interact directly, instead the stimulator acts on inhibitor-producing cells leading to a reduction in the production of inhibitor. Like wise the inhibitor interacts with stimulator-producing cells to decrease stimulator production (Tejero et al 1984). Stem cell numbers are considered to play an important role in this regulatory process. In regenerating bone marrow, when the number of CFU-S has recovered to their normal value, a negative feedback signal leads to the inhibition of stimulator production. Similarly when CFU-S numbers are low, inhibitor production is decreased and stimulator production is increased (Toksoz et al 1980). However, this mechanism of regulation cannot operate in fetal liver.

Stimulator production in fetal liver has been shown to be maximal on the 15th day of gestation, when the numbers of CFU-S are highest (Dawood et al 1990). This correlates with the high number of HPP-CFC observed in the 15 day fetal liver. Thus in the fetal liver microenvironment a high number

of stem cells does not lead to reduced stimulator production. The number of CFU-S in fetal liver has been reported to decrease from the 15th day of gestation onwards. A similar result was obtained with HPP-CFC numbers, which were maximal on the 15th day of gestation and had declined considerably by the end of the first neonatal week. In the bone marrow microenvironment such a reduction in stem cell number would increase stimulator production and increase the output of the stem cell compartment. However in the studies of Dawood et al (1990), stimulator had decreased by the end of the first week of the neonatal period. This decrease in stimulator production correlates with their reported depletion of CFU-S numbers, in a similar manner to the reduction of HPP-CFC numbers in this investigation.

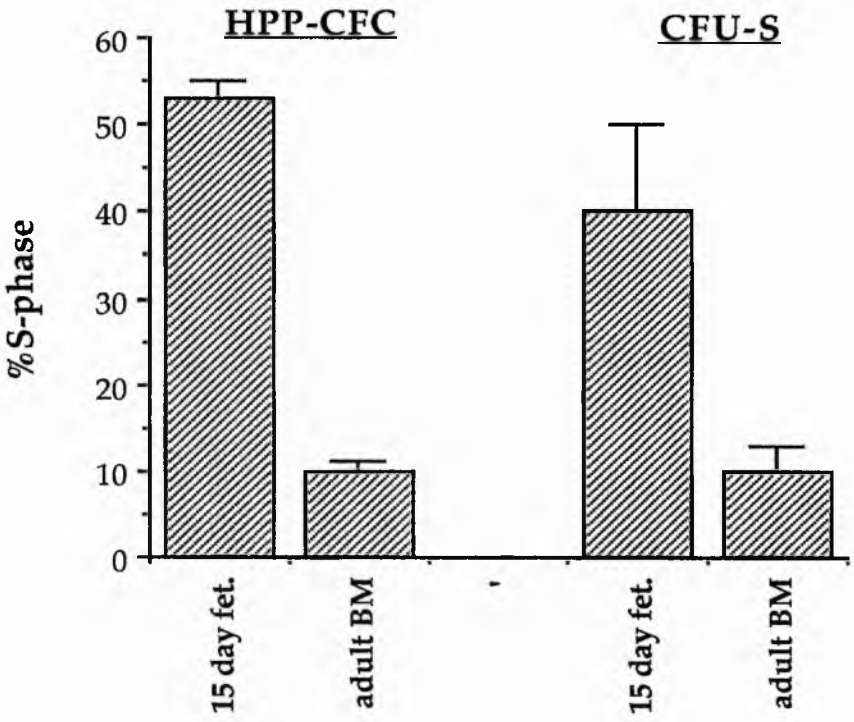
The number of HPP-CFC produced by adult bone marrow was considerably higher than those produced by fetal and neonatal liver, with the exception of the 15 day fetal liver. Only a small proportion of bone marrow cells were in S-phase (10%), it is possible that the high number of HPP-CFC in adult bone marrow leads to inhibition of the production of a stimulator of HPP-CFC proliferation. Thus in adult bone marrow an inverse relationship appears to exist between the number of stem cells and stimulator production, unlike that observed in fetal liver where a high number of stem cells existed concomitantly with a high degree of stimulatory activity

The kinetic properties of the in vitro HPP-CFC population are similar to the in vivo CFU-S population, in both fetal liver and in adult bone marrow (Fig. 4.3-2). In the 15 day fetal liver 30-40% of CFU-S are synthesising DNA, in the adult bone marrow however only 10-15% of CFU-S are in S-phase (Thomas et al 1981). The difference in the proliferative status of CFU-S derived from fetal liver and those derived from bone marrow is very closely correlated with the difference in the proliferative status of HPP-CFC

derived from 15 day fetal liver and HPP-CFC derived from adult bone marrow. The kinetic properties of CFU-S and HPP-CFC are also closely correlated in both cycling 15 day fetal liver and in quiescent adult bone marrow.

The numbers of HPP-CFC present declined during the latter part of gestation, after reaching a peak in the liver of the 15 day fetus. This reduction parallels the decline in the number of CFU-S from the 15th day of gestation which has been reported previously. About $4 \times 10^4$ HPP-CFC colonies were derived from the liver of the 15 day fetus (103 colonies $/10^5$ cells) and about $7.9 \times 10^3$ from neonatal liver on day 8 (29 colonies $/10^5$ cells). The size of the HPP-CFC population is comparable with that of CFU-S population, which forms colonies within 10 days. On the 15th day of gestation the liver cells would be expected to form 4-10 CFU-S colonies $/10^5$ cells (Thomas, Smith & Sumpster 1976), assuming an F factor of 15-20, this would represent a population of $2.4 \times 10^4$ - $8 \times 10^4$ CFU-S.

**Fig. 4.3-2** Kinetic properties of HPP-CFC and CFU-S Derived from 15 Day Fetal Liver and Adult Bone Marrow




There is also evidence for HPP-CFC self-renewal and sensitivity to the stem cell specific CFU-S stimulator and inhibitor (Robinson & Riches 1991). However the magnitude of the HPP-CFC stimulation and inhibition was less than that of CFU-S. This finding may suggest that the HPP-CFC are a more primitive population than CFU-S. Within the CFU-S population there is a hierarchical sensitivity to the regulators. The primitive day 12 CFU-S is more sensitive to inhibitor than stimulator while the more mature day 7 CFU-S is more sensitive to stimulator. Such a hierarchical arrangement may exist in fetal liver. The primitive HPP-$CFC_1$ may be more sensitive to inhibitor while the more mature HPP-$CFC_2$ population, assayed in this investigation may be more sensitive to stimulator.

HPP-CFC were originally operationally distinguished from other haematopoietic progenitor cells by their resistance to 5 FU treatment, their requirements for multiple haematopoietic growth factors acting in synergy and their ability to form colonies >1mm in vitro, containing in excess of 50, 000 cells. It was suggested that they were a slowly cycling population, similar to marrow repopulating cells, but very different from CFU-S which are markedly depleted by treatment with 5 FU (Bradley & Hodgson 1979).

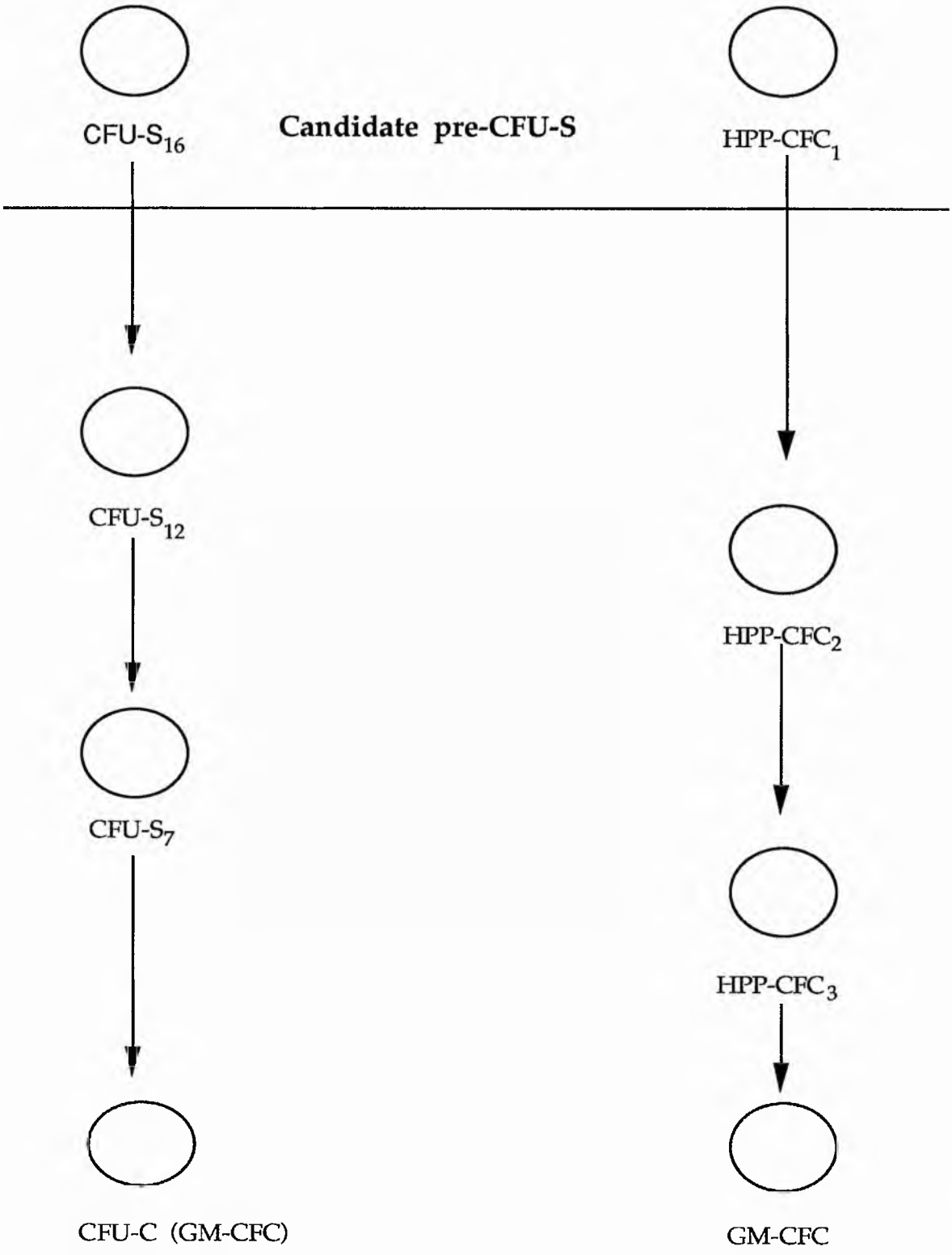
However more recently subpopulations of HPP-CFC have been defined (McNeice et al 1988; Falk & Vogel 1988), and it has been revealed that only the most primitive subpopulation, HPP-$CFC_1$, is resistant to treatment with 5 FU. The other HPP-CFC populations with more restricted growth factor requirements are severely depleted following 5 FU treatment, like CFU-S (McNeice et al 1988). Rhodamine-123 (Rh) uptake has also been used as a parameter to determine the hierarchical order of haematopoietic stem cells. Primitive transplantable stem cells with long term haematopoietic reconstituting ability, in vivo, are Rh-dull. Rh-bright cells have a rapid but limited haematopoietic reconstituting capacity. Investigations have revealed that HPP-$CFC_1$ are Rh-dull, while HPP-$CFC_2$ and more restricted populations are Rh-bright (Bertoncello et al 1992). The very primitive day 16 CFU-S, a candidate pre-CFU-S stem cell subset, like the HPP-$CFC_1$ population are also Rh-dull (McNeice et al 1990).

When developing colonies derived from HPP-$CFC_1$ are harvested they have been found to contain myeloid, erythroid and megakaryocytic progenitors in addition to transplantable haematopoietic stem cells. HPP-$CFC_2$ colonies contain mainly macrophage precursors and have a reduced ability to sustain long-term survival of lethally irradiated mice, compared to HPP-$CFC_1$ (Bertoncello 1992). The exact interrelationship of HPP-$CFC_3$,

with the other subpopulations is unclear. This subpopulation is present in murine bone marrow at a higher incidence than the other populations suggesting it is a more mature population. It has been proposed that they are located between the HPP-$CFC_2$ population and the GM-CFC progenitors (McNeice et al 1990)

It is likely that the HPP-CFC compartment is a continuum in which gradual changes in growth factor requirements occur as the HPP-CFC mature and differentiate. Within this continuum, the most primitive cells have the greatest long-term repopulating ability and are most sensitive to proliferation and differentiation stimuli (Wright & Lord 1992). The HPP-CFC are among the most primitive stem cells identified in vitro to date, having many characteristics similar to CFU-S. The HPP-$CFC_1$ subpopulation is regarded as a very primitive cell population, Bradley & Hodgson (1979), reported them to be highly quiescent. Recent studies have confirmed the quiescence of this subpopulation and proposed HPP-$CFC_1$ as candidate pre-CFU-S cells (McNeice et al 1990). The more mature HPP-$CFC_2$ population investigated in the present study display kinetic properties similar to the CFU-S in both fetal liver and in adult bone marrow, suggesting it is a developmentally early haematopoietic precursor cell population (Fig. 4.3-3).

**Fig. 4.3-3** Proposed Hierarchical Organisation of HPP-CFC, CFU-S and GM-CFC



## 4.4 GM-CFC

GM-CFC are a more mature precursor cell population than the HPP-CFC population. GM-CFC will form colonies in vitro providing a suitable source of colony stimulating activity is available, IL-3, GM-CSF, CSF1 or G-CSF. A combination of growth factors is not required for colony production. GM-CFC will produce colonies of granulocytes, macrophages or granulocytes and macrophages, the proportions of which can be altered by using sub-optimal concentrations of growth factors (Heyworth & Spooncer 1993).

A high proportion of GM-CFC, (50%), were engaged in DNA synthesis in the 15 day fetal liver, this proportion then decreased to 47% in the newborn liver, although small this reduction was statistically significant. Thus in comparison to the dramatic decline in proportion of cycling HPP-CFC, the proportion of GM-CFC actively synthesising DNA remains relatively high. The proportion of GM-CFC synthesising DNA in fetal and newborn liver was higher than that of adult bone marrow (37%). The difference in the proportion of cycling GM-CFC derived from fetal liver and adult bone marrow was not as dramatic as the difference in kinetic properties of HPP-CFC derived from fetal liver and adult bone marrow. These results suggest that GM-CFC, in fetal and newborn liver and in adult bone marrow are actively cycling. In contrast to the HPP-CFC derived from adult bone marrow which were quiescent. These results correlate with previous reports of the kinetic properties of GM-CFC (Cork et al 1982).

There was virtually no difference in the number of GM-CFC colonies produced by fetal and newborn liver, 38 and 40 colonies respectively. However adult bone marrow produced considerably more colonies than either fetal or newborn liver. As with the HPP-CFC data this suggests that

adult bone marrow contains more GM progenitors than either fetal or newborn liver, however a lower proportion of these cells are in cycle. Fetal liver and newborn liver both contain fewer GM progenitors, a higher proportion of which are in cycle.

The proportion of GM-CFC synthesising DNA remained high in both fetal and newborn liver. This coincides with the myelopoietic phase of hepatic haematopoiesis observed morphologically (Chapter 3).

The values obtained for the proportion of proliferating GM-CFC in murine fetal liver closely correlate with results obtained in human fetal liver. Cork et al (1982), demonstrated that in human fetal liver at > 14 weeks gestation, more than 30% of GM-CFC were synthesising DNA. In human fetal liver of 11-14 weeks gestation, only 5% of GM-CFC were in S-phase. In human fetal bone marrow however, the proportion of GM-CFC in S-phase remained high throughout (30%). This variation of GM-CFC proliferation in the two organs suggests that regulation of GM-CFC proliferation is under local control. Furthermore incubation of normal bone marrow with 11-14 week fetal liver conditioned medium, reduced the proportion of GM-CFC in S-phase to < 10%. While medium conditioned with fetal liver >14 weeks gestation did not alter the proportion of cycling GM-CFC in normal bone marrow, it promoted the proportion of GM-CFC in S-phase in a non-cycling GM-CFC population. Incubation of non-cycling GM-CFC with fetal liver of 11-14 weeks gestation did not promote proliferation.

Unlike the HPP-CFC population, GM-CFC are not sensitive to the CFU-S inhibitor. However Cork et al (1982) reported that supernatants from fetal liver of 11-18 weeks gestation produced a stimulator of CFU-S which increased the proportion of CFU-S in S-phase from < 18% to > 33%. Thus while human fetal liver of 11-14 week gestation produces an inhibitor of

GM-CFC, it also produces a stimulator of CFU-S. This demonstrates the specificity of these regulators of GM-CFC and CFU-S. Furthermore, the GM-CFC stimulator and inhibitor are produced by non-adherent cells (Cork et al 1982) while the CFU-S regulators are produced by adherent cells (Wright & Lord 1977; Dawood et al 1990).

The GM-CFC population does not display kinetic properties similar to the HPP-CFC population. The proportion of HPP-CFC synthesising DNA in fetal and neonatal liver decreased as development advanced and only 10% of HPP-CFC were in S-phase in adult bone marrow. In contrast, the proportion of GM-CFC synthesising DNA in the liver decreased only slightly from the 15th day of gestation to birth and also remained high in adult bone marrow. The GM-CFC population is a more mature haematopoietic progenitor cell population than either HPP-CFC or CFU-S populations. GM-CFC are considered to be a differentiation product of HPP-$CFC_3$ (M$^c$Neice et al 1990). The incidence of GM-CFC does not change significantly in liver on the 15 day of gestation and in newborn liver. The incidence of GM-CFC in the adult bone marrow was significantly higher than in either fetal or newborn liver. This observation, together with the less specific growth factor requirements for GM-CFC than HPP-CFC, provides further evidence that GM-CFC are a more mature lineage restricted population.

The observations of Toksoz et al (1980) on CFU-S and CFU-C are similar to those reported here for HPP-CFC and GM-CFC. CFU-C are considered to be the descendants of CFU-S (Meldrum, Thomas & Riches 1977). The proportion of GM-CFC in regenerating bone marrow were observed to retain a high cycling rate throughout, while CFU-S were actively proliferating initially then subsequently declined to a steady state minimal proliferation rate. This observation suggests that regulators exist which are

specific for CFU-S and HPP-CFC but have no effect on the more mature GM-CFC.

## 4.5 Regulation of Haematopoietic Colony Forming Cells

Control of the proliferative status of HPP-CFC and GM-CFC appears to be regulated by a balance between haematopoietic growth factors and a number of inhibitory molecules. As previously mentioned regulation is considered to be local. The inhibitor and stimulator of CFU-S described by Wright & Lord (1977) are produced by macrophages. The CFU-S inhibitor has recently been demonstrated to be macrophage inflammatory protein (MIP-1$\alpha$), which is produced in normal bone marrow but not in regenerating bone marrow (Graham et al 1990). The stimulator detected in fetal liver cells was produced by adherent cells (Cork et al 1982; Dawood et al 1990). While the GM-CFC inhibitor and stimulator reported in human fetal liver was produced by non-adherent cells (Cork et al 1982).

In addition to growth factors, a number of other stem cell specific regulators have been defined. TGF-$\beta$ is known to inhibit all subpopulations of HPP-CFC (Keller et al 1990). $PGE_2$ has been shown to inhibit GM-CFC proliferation, the same inhibition can also be obtained using Lactoferrin (Metcalf 1987). The leukaemic associated inhibitor (LIA), is produced by a range of normal cells and myeloid leukaemic cells. LIA inhibits GM-CFC proliferation but not leukaemic cell proliferation (Devalia & Linch 1991). LIA has many similar properties to the GM-CFC inhibitor detected in early human fetal liver, the possibility that these inhibitors represent the same factor has not been dismissed (Cork et al 1982). Haemoregulatory peptide has also been shown to inhibit proliferation of GM-CFC, in addition this peptide also has an inhibitory effect on CFU-S proliferation (Axelrad 1990).

AcSDKP has been shown to prevent the entry of murine CFU-S into DNA synthesis, thus it can protect cells from damage by sublethal irradiation or cytotoxic agents. AcSDKP has no effect on cells already engaged in DNA synthesis (Lenfant et al 1989).

Positive and negative regulators of haematopoiesis, acting in concert, thus appear to provide a mechanism for rapid, reversible and specific proliferative responses to changes in haematopoietic demands. In addition it has been shown that regulation particularly of the stem cell compartment is elicited, in part by essential interactions with the supporting stroma of the haematopoietic tissue. The exact role of the stroma and the effects of the factors produced by stromal cells on HPP-CFC and GM-CFC remains to be determined.

# Chapter 5

## Effects of Fetal Liver Extract on Normal and Irradiated Bone Marrow and Leukaemic Cells

The observations of Meng and his associates, (in Thomas 1993), revealed that the rate of complete remission in patients with acute leukaemia was higher (65%) in a group of patients who received infusions of fetal liver cells than in the patients who did not receive this treatment (36%). Furthermore increasing the number of infusions of fetal liver cells during periods of short duration was found to be more beneficial to the patients in that the period of severe anaemia was shorter and blood product consumption was smaller (Thomas 1993). These observations suggest infusion of fetal liver cells has beneficial effects on patients with acute leukaemia and aplastic anaemia.

Haematopoietic reconstitution, using fetal liver infusions without evidence of engraftment have also been reported. Wu et al (1985) reported recovery of patients with aplastic anaemia who were given infusions of fetal liver cells. Engraftment of the fetal liver cells was not observed and it was concluded that these cells are efficacious in promoting the recovery of haematopoiesis in functional deficiencies of the bone marrow. Aplastic anaemia has also been successfully treated by infusions of fetal liver conditioned medium (Kochupillai et al 1985). Engraftment of the fetal liver cells did not take place, thus the fetal liver infusion stimulates haematopoietic recovery of the bone marrow. Erythropoiesis was the first

and most dominant response observed followed by myelopoiesis, unlike bone marrow transplantation where myelopoiesis is dominant. Thus following fetal liver cell infusion, the bone marrow recovery exhibits a fetal pattern of growth.

This investigation was conducted to determine the effects of murine fetal liver extract (FLE) on normal and irradiated bone marrow and on leukaemic cells. In order to establish whether fetal liver extract could influence the formation of HPP-CFC by bone marrow rendered hypoplastic by irradiation, adult CBA mice were given various doses of $\gamma$ irradiation and sacrificed on the fourth day post-irradiation. A 10% concentration of FLE (1ml FLE + 9ml supplemented Dulbeccos medium), was added to bone marrow cell suspensions and the HPP-CFC content was determined using the standard HPP assay, FLE was not added to control groups.

Normal bone marrow was incubated with 10% FLE and serial half dilutions of FLE to a minimum concentration of 0.31% to determine whether diluting FLE would alter its effects. HPP-CFC numbers were determined as before.

The effect of FLE on a cloned myelomonocytic leukaemic cell line was also investigated to ascertain the influence of FLE on colony formation by leukaemic cells. Leukaemic cells were incubated with concentrations of FLE and assayed for clonogenic cell production (Chapter 2). The difference in colony numbers between control and treated groups was attributed to the effect of FLE.

## Results

### 5.1 Effect of FLE on Normal and Irradiated Bone Marrow

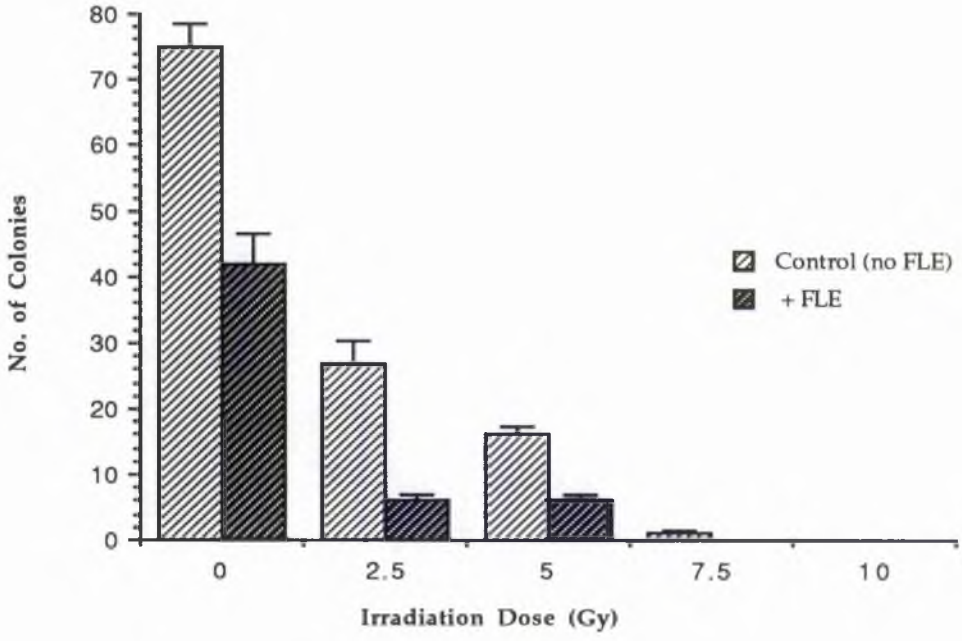
Addition of FLE to irradiated bone marrow reduced the numbers of HPP-CFC colonies formed (Table 5.1-1 & Fig. 5.1-1). The number of HPP colonies formed by normal bone marrow, which had not been irradiated was significantly reduced from 75 to 42 in the presence of FLE ($p < 0.0001$). A 78% reduction in HPP colony formation in the presence of FLE was observed in bone marrow which had received 2.5 Gy $\gamma$ irradiation. Colony numbers were reduced from 27 in the control samples to 6 in the FLE treated samples ($p < 0.0001$). A 63% reduction in colony number was also observed in bone marrow given a 5 Gy dose of irradiation, from 16 colonies in the control samples to 6 colonies in the presence of FLE ($p = 0.001$). The effect of FLE in bone marrow samples given 7.5 Gy and 10 Gy doses of irradiation could not be evaluated as the high irradiation doses perturbed colony production in both the control and the FLE treated samples. Colony production in both control and FLE treated bone marrow was observed to decrease as the dose of irradiation was increased. An increase in the number of HPP-CFC produced in the presence of FLE was not observed at any dose of irradiation.

**Table 5.1-1** Number of HPP-CFC Colonies Formed /$10^5$ Irradiated Bone Marrow Cells

| Dose of Irradiation | | Control BM | BM + FLE | Reduction (%) |
|---|---|---|---|---|
| 0.0 Gy | (n=10) | 75 ± 3.5 | 42 ± 4.7 | 44 ± 6.1 |
| 2.5 Gy | (n=10) | 27 ± 3.5 | 6 ± 0.8 | 78 ± 2.8 |
| 5.0 Gy | (n=10) | 16 ± 1.2 | 6 ± 1.1 | 63 ± 3.7 |
| 7.5 Gy | (n=10) | 1 ± 0.5 | 0 | 0 |
| 10.0 Gy | (n=10) | 0 | 0 | 0 |

Data expressed as mean ± SE n = number of experiments

**Fig. 5.1-1** Number of HPP-CFC Colonies Formed/$10^5$ Irradiated Bone Marrow Cells



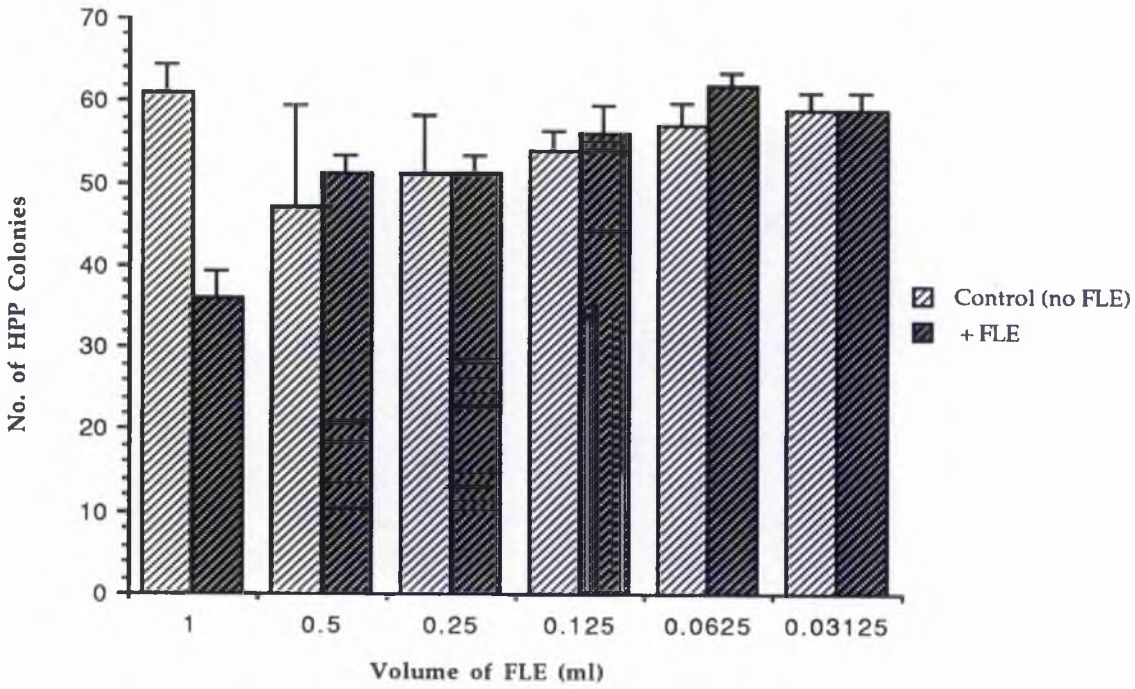

The inhibitory effect of FLE was observed to be dependent on the volume of FLE used. (Table 5.1-2 & Fig. 5.1-2). Inhibition of HPP-CFC colony growth was optimal when 1ml of FLE was used. A 41% reduction in colony numbers was observed from 61 colonies in the untreated samples to 36 colonies in the FLE treated samples ($p = 0.019$). At all other concentrations of FLE there was no significant difference in colony production in the control and FLE treated groups, $p \gg 0.275$. Decreasing the volume of FLE used, from 0.5ml down to 0.031ml, had no significant effect on the formation of HPP-CFC colonies $p \gg 0.296$. FLE did not promote the growth of HPP-CFC colonies at any concentration used.

**Table 5.1-2** Number of HPP-CFC Formed /$10^5$ Normal Bone Marrow Cells inthe Absence and Presence of FLE

| Volume of FLE (ml) | Control BM | BM + FLE | No. of Experiments |
|---|---|---|---|
| 1.0 | 61 ± 3.3 | 36 ± 3.3 | 4 |
| 0.5 | 47 ± 12.6 | 51 ± 2.2 | 4 |
| 0.25 | 51 ± 7.2 | 51 ± 2.4 | 4 |
| 0.125 | 54 ± 2.5 | 56 ± 3.4 | 4 |
| 0.062 | 57 ± 2.7 | 62 ± 1.6 | 4 |
| 0.031 | 59 ± 2.1 | 59 ± 2.1 | 4 |

Data expressed as mean ± S.E.

**Fig. 5.1-2** No. of HPP-CFC Formed by Normal Bone Marrow in the Absence and Presence of FLE



## 5.2 Effect of FLE on SA2 Leukaemic Cell Line

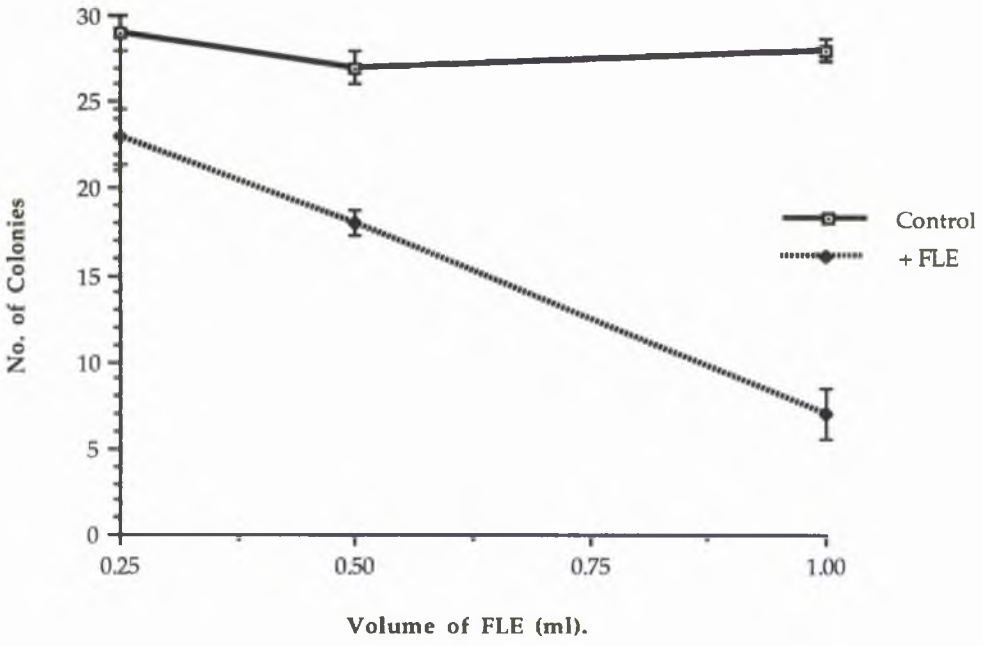
The presence of FLE significantly reduced the production of SA2 colonies, $p \ll 0.005$, (Table 5.2-1 & Fig. 5.2-1). Addition of 0.25ml of FLE to the SA2 cells resulted in a 21% reduction in the number of colonies produced. More effective inhibition of colony formation was demonstrated in SA2 cells which had been incubated with 0.5ml of FLE, when colony production was reduced by 33%. The most dramatic effect was observed when SA2 cells were incubated with 1ml of FLE. There was a 77% reduction in colony numbers from 29 colonies in the controls to 7 colonies in the SA2 treated samples.

**Table 5.2-1** Number of Colonies Formed by 200 SA2 Leukaemic Cells in the Presence and Absence of FLE

| Volume of FLE (ml) | Control SA2 | SA2 + FLE | Reduction (%) |
|---|---|---|---|
| 0.25 (n=6) | 29 ± 0.7 | 23 ± 1.2 | 21 ± 1.8 |
| 0.50 (n=6) | 27 ± 1.4 | 19 ± 1.4 | 33 ± 3.2 |
| 1.00 (n=6) | 29 ± 0.9 | 7 ± 1.1 | 77 ± 4.8 |

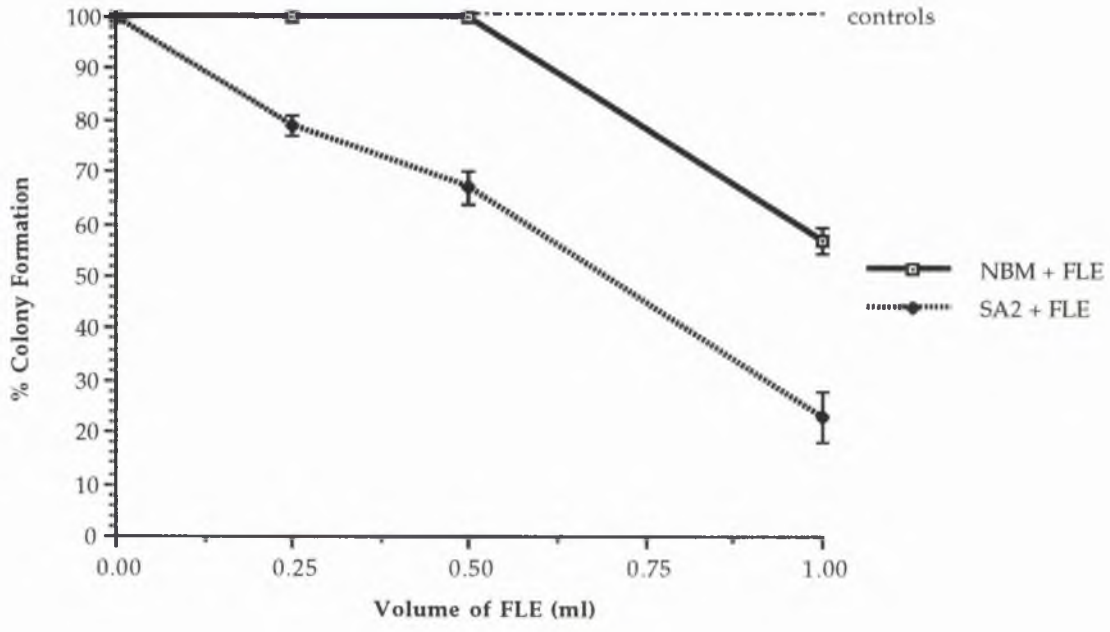
Data expressed as mean ± SE n = number of experiments

**Fig. 5.2-1** Effect of FLE on Colony Formation By SA2 Leukaemic Cells




Fetal liver extract showed a marked difference in suppression of SA2 leukaemic cells and HPP-CFC derived from normal bone marrow (Fig. 5.2-2). Colony formation of SA2 cells was significantly inhibited at all concentrations of FLE used. Growth of HPP-CFC was only suppressed by a 10 % concentration of FLE, however not to the same degree as the suppression of the SA2 cells with this concentration of FLE.

Fig. 5.2-2 Comparison of the Inhibitory Effects of FLE on Colony Formation of Normal Bone Marrow Cells and Leukaemic SA2 Cells




## Discussion

### 5.3 Haematopoietic Regulatory Factors in Fetal Liver

At volumes of less than 1ml, crude fetal liver extract had no effect on the formation of HPP-CFC by normal bone marrow. However 1ml of crude FLE reduced HPP formation by 41%. The effect of FLE on bone marrow rendered hypoplastic by irradiation was more dramatic. HPP formation by bone marrow given 2.5 and 5 Gy γ irradiation was considerably depleted following treatment with FLE. The inhibitory effect was most dramatic in

bone marrow given a 2.5 Gy dose, which was regenerating at the time of use. Bone marrow given a 5 Gy dose would not be fully regenerating at four days post-irradiation. However the number of colonies produced by FLE treated bone marrow given 5 Gy, was not different form the number produced by FLE treated bone marrow given 2.5 Gy. Colony production in 7.5 and 10 Gy samples was severely depleted in both the control and FLE treated groups. There was no evidence that infusions of crude fetal liver extract could improve aplastic anaemia.

While the number of HPP colonies was reduced in the FLE treated samples, the size of the colonies had increased (personal observations). Most of the HPP colonies were >5mm, which suggests that while FLE may reduce the primitive HPP-CFC formation, the number of more mature progenitor cells produced is increased by FLE treatment. Increased output of progenitor cells is vital for reconstitution of irradiated bone marrow. 1ml of crude murine FLE may induce decreased proliferation of HPP-CFC but increase differentiation of these primitive cells to produce essential marrow-repopulating progenitor cells.

Haematopoietic regulation in fetal liver has been the subject of intense investigation in recent years A stimulator of CFU-S has been detected in the 30-50K dalton fraction of murine FLE, while inhibitor is detected in the 50-100K dalton fraction. The concentration and effects of the stimulator decrease with gestation as the liver ceases to be the main site of haematopoiesis (Dawood et al 1990). In addition to the CFU-S stimulator and inhibitor, murine and human fetal liver have been shown to produce inhibitor and stimulator of GM-CFC production. Supernatants from fetal liver early in gestation produce inhibitor of GM-CFC and supernatants from late fetal liver produce stimulator of GM-CFC (Cork et al 1986).

EGM-CSF and burst promoting factor are released by adherent murine fetal liver stromal cells in vitro at around the 15th day of gestation (Thomas 1993). These factors promote differentiation of stem cells into various haematopoietic lineages in addition to stimulating proliferation of quiescent CFU-S (Zhao & Wu 1990). Murine fetal liver stromal cells have also been shown to produce interleukins 1-7, with the exception of IL-5, and stem cell factor at levels almost 20 times higher than bone marrow stromal cells (Gutierrez-Ramos, Olsson & Palacios 1992).

The presence of regulatory factors of CFU-S in murine and human fetal liver has been well documented, however regulation of the HPP-CFC population has not been so well documented. Normal quiescent bone marrow HPP-CFC have been shown to respond to the CFU-S stimulator, while actively cycling HPP-CFC in regenerating bone marrow respond to the CFU-S inhibitor (Robinson & Riches 1991). The CFU-S stimulator can be isolated from regenerating bone marrow cells which are actively cycling. In 15 day fetal liver, haematopoietic cells are also in cell cycle, however the crude fetal liver extract used in this investigation did not promote an increase in HPP-CFC production by normal bone marrow cells. It is possible that fetal liver contains additional haematopoietic regulators, the effects and composition of which have not yet been determined. In the crude form, the overall effect of fetal liver extract appears to inhibit proliferation of HPP-CFC, it may however promote differentiation of these cells.

Arginase is known to be produced in abundance by fetal liver (Wu, Pei & Cong 1989). Arginase has been shown to prevent the growth of normal mammalian cells or tumour cells in culture, by depleting essential amino acids such as arginine and its precursors in the culture medium. Wu et al (1989) demonstrated a significant content of arginase in human fetal liver

and reported CFU-GM growth was severely depressed following incubation with fetal liver supernatant. Fractionation of fetal liver revealed that arginase activity was high in the 60K dalton fraction which significantly reduced CFU-GM growth and completely suppressed growth of leukaemic HL-60 cells. The inhibitory activity observed in this fraction of human fetal liver is consistent with the inhibitory activity detected in the 50-100K dalton fraction of murine fetal liver (Dawood et al 1990). The 30-60K dalton fraction of human FLE did not significantly reduce the growth of HL-60 cells and arginase could not be detected in this fraction. CFU-S stimulator is detected in the corresponding fraction in murine FLE. However Wu and his associates reported 100% inhibition of the leukaemic cell line in the presence of a < 10K dalton fraction of fetal liver, in which arginase activity could not be detected. In contrast to the devastating effects of this fraction on leukaemic cells, the effects on CFU-GM growth were minor. Therefore it would appear that in addition to arginase another inhibitor of HL-60 growth is present in crude human fetal liver extract.

Murine FLE has been demonstrated to have suppressive effects on normal bone marrow cells, on irradiated bone marrow and on leukaemic SA2 cells. 1ml of FLE depresses colony production in both normal and irradiated bone marrow and severely depresses production of colonies by leukaemic cells. At volumes of 0.5ml or lower, FLE has no effect on the formation of HPP-CFC by normal bone marrow cells. In contrast, FLE was observed to have an inhibitory effect on SA2 leukaemic cells at all concentrations. The inhibitory effect was more pronounced as the concentration of FLE was increased, from a reduction in colony formation of 21% with 0.25ml FLE to 77% with 1ml FLE. The inhibitory effects of FLE were more pronounced on irradiated bone marrow than on normal bone marrow cells. Optimal effects of FLE were observed in bone marrow given 2.5 Gy and 5 Gy $\gamma$ irradiation.

Both SA2 cells and bone marrow given moderate doses of irradiation are in cycle, therefore it is reasonable to assume that the inhibitory effects of FLE are more pronounced on cells which are cycling

The human FLE used by Wu et al, like the murine FLE in the present investigation, were crude supernatants from centrifuged fetal liver cells. A 1ml FLE inhibited growth of HL-60, CFU-GM and in the present investigation SA2 leukaemic cells and HPP-CFC from normal and irradiated bone marrow. The FL supernatants in both cases were collected directly from centrifuged fetal liver cells without any fractionation or incubation at 37°C, and are therefore very crude and very different from fetal liver conditioned medium and fractionated fetal liver cells. There have been many reports of stimulatory effects of fetal liver conditioned medium and from specific fractions of both human and murine fetal liver.

Infusion of fetal liver has been demonstrated to improve remission of acute myeloid leukaemia and acute leukaemia patients recovering from chemotherapy and aplastic anaemia (Kochupillai et al 1985; Meng et al 1985). In both investigations there was no evidence of engraftment of the fetal liver cells. Izzi et al (1985), reported haematopoietic reconstitution of aplastic anaemia and acute leukaemia following fetal liver transplantation. The fetal liver cells did not engraft, however they permitted the patients to overcome a period of profound aplasia following the conditioning regime, by promoting haematopoiesis in the host. The fetal liver cell suspensions used by Izzi et al, which had been incubated at 37°C, did not present any anti-leukaemic effects. In contrast the crude murine fetal liver extract used in the present investigation had a dramatic suppressive effect on the growth of leukaemic cells.

The inhibitory effect of FLE on the leukaemic SA2 cells was more pronounced than its suppressive effect on HPP-CFC derived from adult bone marrow (Fig. 5.2-2). Growth of SA2 cells was depleted at all concentrations of FLE while only 10% FLE had a significant effect on the growth of normal bone marrow. This may suggest selective suppression of the leukaemic cell line rather than normal bone marrow by murine FLE. Similar effects of crude human FLE have been reported by Pei and Wu (1990). A low molecular weight fraction of FLE suppressed the growth of both human and murine leukaemic cell lines, however CFU-GM growth was only mildly suppressed. The suppressive effect of the human FLE was also observed to increase as the concentration of FLE was increased.

Pei & Wu also demonstrated the suppressive effect of human FLE on the growth of leukaemic cells in long term culture. The number of CFU-GM was reduced initially but increased after the 5th day in culture. Providing further evidence that the low molecular weight fraction of fetal liver plays an important role in the selective degeneration of leukaemic cells. Touraine, Royo and Gitton (1990), have also reported anti-leukaemic effects of fetal liver cells. Leukaemic mice injected with fetal liver showed a dramatic improvement, with almost entire ablation of the leukaemia. The fetal liver cells mounted a considerable reaction to the leukaemic cells without causing GvH reaction, this finding has very important clinical significance.

The inhibitory effect of FLE on leukaemic cells has important clinical applications in the treatment of leukaemic patients. At present allogeneic bone marrow transplantation is the most effective method of treating leukaemia, however in many cases the lack of HLA-identical donors, pose obvious problems for successful transplantation. Autologous bone marrow

transplantation has been considered as a viable alternative, using large doses of irradiation and cytotoxic drugs to purge the leukaemic cells from the bone marrow before re-infusion into the patient. However it is not always possible to remove all the leukaemic cells and the potential for relapse exists. Multiple infusions of fetal liver suspensions in leukaemic patient following large doses of chemotherapy have resulted in a high incidence of complete remission (Wu et al 1989).

The natural tumour suppressor in human fetal liver detected by Wu & Pei (1989) may also exist in murine fetal liver. Investigations could be performed on a < 10K dalton fraction of murine fetal liver in order to establish whether it selectively suppressed leukaemic cells. The suppressive effects of FLE on leukaemic cells described by Wu & Pei and in the present investigation could potentially be used to purge leukaemic cells for autologous bone marrow transplantation in the treatment of leukaemia. Both human and murine FLE inhibit the growth of leukaemic cells lines and have less detrimental effects on the growth of normal bone marrow cells. The disappearance of leukaemic cells in long term culture could prove to be a viable means of detecting ablation of the leukaemic cells from the bone marrow and thereby purifying the stem cells prior to re-infusion into the patients. Optimal culture conditions for stem cell proliferation would have to be established and the number of stem cells to be infused into the patient must be determined.

## 5.4 Fetal Liver Transplantation

Transplants of haematopoietic stem cells derived from fetal liver can reconstitute bone marrow function in patients with SCID, immunodeficiencies, some inborn errors of metabolism and following

cancer chemotherapy. Fetal liver transplants are unequivocally associated with less GvHD than corresponding bone marrow transplants and thus have been considered as a feasible alternative to bone marrow transplantation when an HLA-matched donor is not available. The high proportion of cycling cells in fetal liver and the absence of mature T-Lymphocytes confer potential advantages for fetal liver transplants over conventional bone marrow transplants.

Reconstitution of T-cell function in SCID patients and bare lymphocyte syndrome has been reported (Touraine et al 1987). Transplants of fetal liver and thymus from the same donor have been demonstrated to enhance immune reconstitution in SCID patients by providing a syngeneic environment for the differentiation of transplanted stem cells into lymphocytes. Fetal liver transplants have been used to treat various inborn errors of metabolism with only limited success, benefits for the patients were only partial and transient. Inborn errors of metabolism have also been significantly improved by these dual organ-derived cell transplants (Touraine et al 1985). However multiple transplants have to be performed in order to establish engraftment of the donor cells.

Fetal liver transplants have also been performed on patients with acute leukaemia and aplastic anaemia (Gale 1985). The incidence of engraftment is higher in leukaemic patients. Patients with aplastic anaemia have an intact immune system and thus graft rejection is not really surprising. Leukaemic patients have a functionally depressed immune system due to the disease and the pretransplant conditioning regimen. Improved engraftment of fetal liver cells may therefore be possible by intensifying the pre-and post-transplant immune suppression. Fetal liver transplantation has been demonstrated to induce haematopoietic and immune reconstitution in humans (Gale 1985; Touraine et al 1987; Roncarolo et al

1991), sheep and horses (Royo et al 1987), dogs (Prummer et al 1985; Cain & Champlin 1989) and mice (Thomas 1971, 1976). However failure of engraftment is an all too often outcome of fetal liver transplantation and considerably higher numbers of fetal liver cells have to be infused in comparison to the number of cells used for bone marrow transplants.

More recently in utero transplantation of fetal liver cells have been successfully performed, this technique takes advantage of the immunological tolerance of the fetal recipients. Harrison et al (1989) reported successful engraftment of fetal liver cells in rhesus monkeys with no evidence of GvHD. Successful in utero transplantation has also been reported in humans. Touraine (1991), performed transplantation of fetal liver cells from 7-10 week old fetuses, in patients with immunodeficiencies, thalassemia major and bare lymphocyte syndrome. So far results are very encouraging, donor cell engraftment has been demonstrated in transplants for all of the forementioned conditions. Thus engraftment difficulties which have hampered the advance of fetal liver transplants especially in bare lymphocyte syndrome may not be encountered with in utero transplants. In addition to the increased probability of graft take with in utero transplantation of fetal liver cells other advantages include, improved isolation of the fetus in the uterus rather than maintenance in a sterile bubble in a hospital and also the fetus itself provides the optimal environment for development of the transplanted fetal cells (Touraine et al 1992).

## 5.5 Prospects

Murine embryonic stem (ES) cells are a blastocyst derived cell line which can differentiate to produce all the cells of an entire fetus, following aggregation with developmentally compromised tetraploid embryos. The

ES-derived embryos die before birth, however the embryonic liver is a source of haematopoietic progenitors which can reconstitute lethally irradiated adult mice. The ES cells can be maintained in vitro and thus could provide a continuous source of stem cells (Forrester et al 1991). The c-myb proto-oncogene has recently been demonstrated to play an important role in maintaining the proliferative state of haematopoietic progenitor cells. Alteration of the c-myb gene in ES cells causes severe anaemia in the resultant mutant mice. This anaemia could not be detected on the 13th day of gestation, however severe affects were apparent by the 15th day of gestation. It can therefore be assumed that the c-myb gene affects definitive haematopoiesis in the fetal liver and not primitive haematopoiesis of the yolk sac (Mucenski et al 1991).

These findings in ES cells suggest that fetal liver haematopoietic progenitors are susceptible targets for gene transfer and these progenitors become resident in the bone marrow of the adult. Introduction of foreign genes into haematopoietic stem cells may lead to a better understanding of gene expression and the pathogenesis of genetic disorders (Clapp et al 1991). There is also potential for correcting genetic defects in vivo. Insertion of functioning genes into deficient cells may prove to be a favourable alternative to allogeneic cell transplants for some inborn errors of metabolism. One of the main obstacles to overcome at the present time is the maintenance of corrected gene expression in the hosts.

It is reasonable to assume that in the next few years conventional bone marrow and fetal liver transplantation may be replaced by transplantation of pure stem cells which can be manipulated to enhance their stimulatory and inhibitory effects. The stem cells could be grown and maintained in culture eliminating the search for compatible donors and maintenance of extensive registers. Research on the murine ES cell line may eventually

lead to the establishment of a human cell line with similar properties to fetal liver cells (Thomas 1993). These cells could be maintained in culture and used for transplantation, genetic transfer and synthesis of specific regulatory factors to cure or alleviate haematopoietic and genetic disorders.

# Chapter 6

## Adhesion of HPP-CFC to Stromal Layers in Vitro

The extravascular stroma of the bone marrow and the fetal liver, serve as haematopoietic microenvironments in the two organs, and are required for specific lodgement, proliferation and differentiation of haematopoietic stem cells in vivo. The importance of the stroma is demonstrated in long term cultures in vitro, in which the development of an adherent stromal layer is essential for haematopoietic stem cell proliferation and differentiation. The haematopoietic microenvironment of the stromal layer consists of cells and the extracellular matrix.

The ability of adherent stromal layers derived from fetal liver and normal bone marrow, to retain HPP-CFC seeded onto the layers and provide a suitable microenvironment for the proliferation and differentiation of the stem cells was investigated. Stromal layers derived from fetal liver on the 15th day of gestation and from normal adult bone marrow were established and allowed to reach confluence. The layers were irradiated with a known dose of $\gamma$ irradiation and re-seeded with a known concentration of haematopoietic cells derived from fetal liver or adult bone marrow 6-10 days later. After 2 hours the supernatant was removed from the layers and assayed for the presence of HPP-CFC. The difference in colony number produced from haematopoietic samples which had not been incubated with a stromal layer and samples which had been incubated with a stromal layer for two hours gives a measure on the number of stem cells which had adhered to the stromal layer (Chapter 2, pp 53-55).

Stromal layers derived from Day 17 fetal liver and Day 19 fetal liver were established to determine whether these layers could support haematopoietic cells seeded onto them in a similar manner to the Day 15 fetal liver layers. Cell suspensions of 15 day fetal liver or adult bone marrow were incubated with the adherent layers for two hours. As before the adhesion of HPP-CFC was determined by a reduction in colony numbers in the experimental groups when the supernatants were assayed.

## Results

### 6.1 Adhesion of HPP-CFC to Bone Marrow and 15 Day Fetal Liver Stroma

500, 1, 000 or 5, 000 HPP-CFC derived from fetal liver, normal adult bone marrow or regenerating bone marrow were incubated with stromal layers derived from 15 day fetal liver or adult bone marrow. The difference in colony number produced by experimental and control groups is attributed to adhesion of the HPP-CFC to the stromal layer. The proportions of HPP-CFC which adhered to the irradiated stromal layers are illustrated in Table 6.1-1 and Fig. 6.1-1.

**Table 6.1-1** Proportion of HPP-CFC which Adhere to Stromal Layers (%)

| No. of HPP seeded | BM->BM stroma | BM->FL stroma | FL->BM stroma | FL->FL stroma | RBM->BM stroma |
|---|---|---|---|---|---|
| 500 | 74 ± 1.3 | 72 ± 1.0 | 100 ± 0.0 | 73 ± 3.5 | 72 ± 1.1 |
| 1000 | 43 ± 1.3 | 53 ± 0.9 | 89 ± 0.9 | 55 ± 1.9 | 47 ± 2.7 |
| 5000 | 21 ± 2.9 | 31 ± 2.5 | 72 ± 0.8 | 27 ± 1.6 | 26 ± 3.0 |

When a concentration of 6.25 x $10^5$ cells derived from adult bone marrow, containing approximately 500 HPP-CFC, was incubated with an irradiated bone marrow stromal layer, 74% of the HPP-CFC adhered to the bone marrow layer. Seeding 1.25 x $10^6$ bone marrow cells, containing approximately 1, 000 HPP-CFC, onto a bone marrow layer resulted in adhesion of 43% of the HPP-CFC to the layer. The proportion of HPP-CFC which adhered to bone marrow stromal layers was further reduced to 21% when 6.25 x $10^6$ bone marrow cells (~ 5, 000 HPP) were incubated with an irradiated bone marrow layer. Adhesion of bone marrow cells to a fetal liver derived stromal layer was not significantly different from adhesion to a bone marrow stromal layer. 72% of 5, 000 HPP, 53% of 1, 000 HPP and 31% of 5, 000 HPP seeded, adhered to the fetal liver stromal layer.

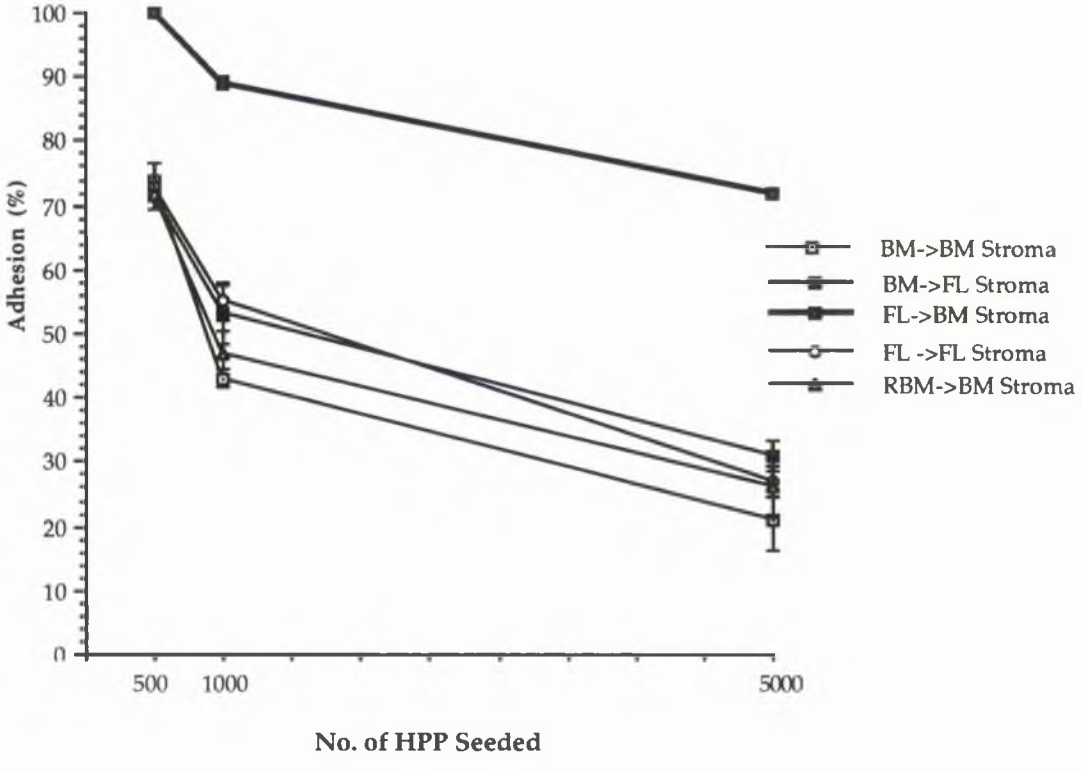
Incubating 5 x $10^5$ cycling fetal liver cells, containing approximately 500 HPP-CFC with a stromal layer derived from fetal liver resulted in adhesion of 73% of the HPP-CFC to the fetal liver layer. The proportion of HPP-CFC which adhered to the fetal liver layer was significantly reduced to 55% when 1 x $10^6$ fetal liver cells (~1, 000 HPP) were seeded onto the layer. Seeding 5 x$10^6$ (~ 5, 000) fetal liver cells onto the layer further reduced the proportion of cells which adhered to the layer to 27%. Very similar results were obtained when regenerating bone marrow cells were seeded onto a stromal layer derived from adult bone marrow. 72%, 47%, and 26% of the 500, 1, 000 and 5, 000 HPP respectively, adhered to the bone marrow stromal layer.

When 500 fetal liver derived HPP-CFC were seeded onto bone marrow layers, no HPP colonies could be detected in the supernatants, it was thus assumed that all the HPP-CFC present had adhered to the stromal layer. The proportion of adherent HPP-CFC was reduced to 89% when 1, 000 HPP-

CFC were seeded onto the bone marrow layers, this was further reduced to 72% when 5, 000 HPP-CFC were used. Anova and Freidmans non-parametric testing showed that the proportion of FL derived HPP-CFC which adhered to bone marrow stromal layers was significantly different from all other combinations ($p < 0.0001$), regardless of the concentration of HPP. It would thus appear that cycling fetal liver cells preferentially adhere to stromal layers derived from adult bone marrow.

The proportions of adherent cells were found to decrease as the concentration of HPP-CFC was increased in every combination tested. These proportions were significantly different at each concentration, regardless of combination ($p < 0.0001$).

**Fig. 6.1-1** Proportion of HPP-CFC which Adhere to Irradiated Stromal Layers



While the proportion of adherent cells decreased as the concentration of HPP-CFC was increased, the actual number of HPP-CFC which bound to the stromal layers increased as the concentration of stem cells was increased (Table 6.1-2, Fig. 6.1-2).

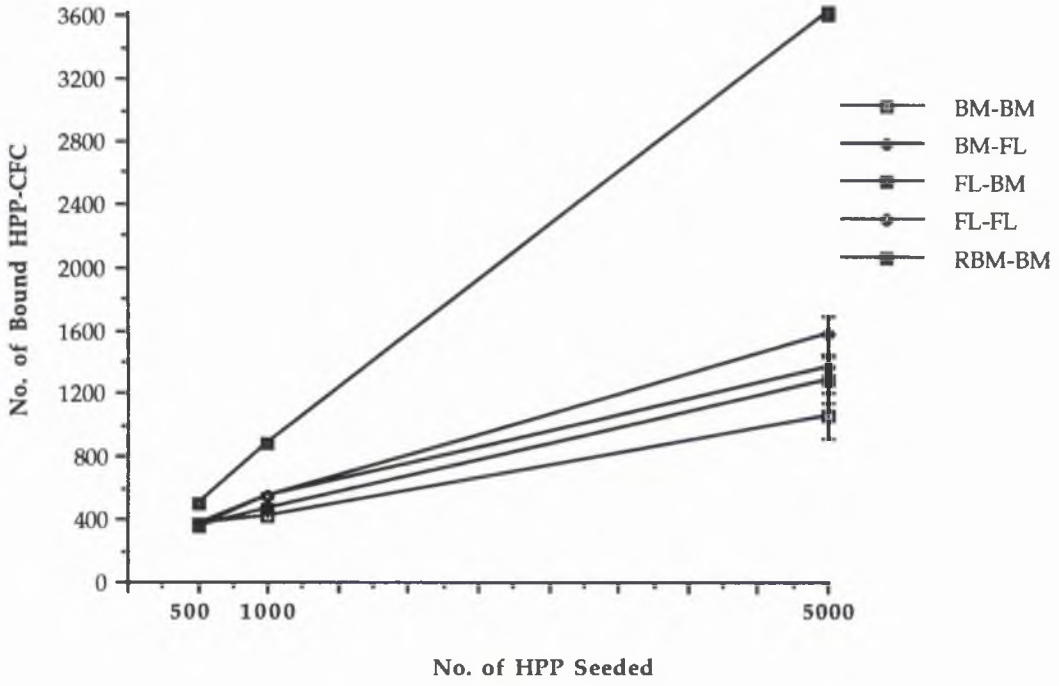
**Table 6.1-2** Number of HPP-CFC which Adhere to Irradiated Stromal Layers

| No. of HPP seeded | BM->BM stroma | BM->FL stroma | FL->BM stroma | FL->FL stroma | RBM->BM stroma |
|---|---|---|---|---|---|
| 500 | 368 ± 6.4 | 361 ± 4.8 | 500 | 363 ± 17.3 | 360 ± 5.7 |
| 1000 | 426 ± 13.3 | 543 ± 8.7 | 890 ± 8.9 | 548 ± 18.5 | 474 ± 27.5 |
| 5000 | 1060 ± 143 | 1570 ± 124 | 3618 ± 39 | 1360± 80 | 1290 ± 151 |

The results demonstrate that bone marrow stromal layers can accommodate at least 3, 600 HPP-CFC derived from fetal liver but when 1, 000 fetal liver HPP-CFC were seeded onto bone marrow stroma only 890 HPP-CFC adhered to the layer. Similar trends were observed in all other combinations, however the number of HPP-CFC which adhered to the stromal layers were markedly reduced. Fetal liver stroma could retain at least 1570 BM HPP and 1360 FL HPP, however only retained a proportion of the initial concentration of stem cells when 500 and 1, 000 HPP were seeded onto the stroma. Similarly bone marrow stroma could accommodate at least 1050 HPP derived from bone marrow and 1300 HPP derived from regenerating bone marrow but adhesion of such numbers of HPP-CFC was

only observed when an initial inocula of 5, 000 HPP-CFC were seeded onto the bone marrow layers.

**Fig. 6.1-2** Number of HPP-CFC which Adhere to Irradiated Stromal Layers




## 6.2 Ability of Late Gestational Fetal Liver Stromal Layers to Support Haematopoiesis

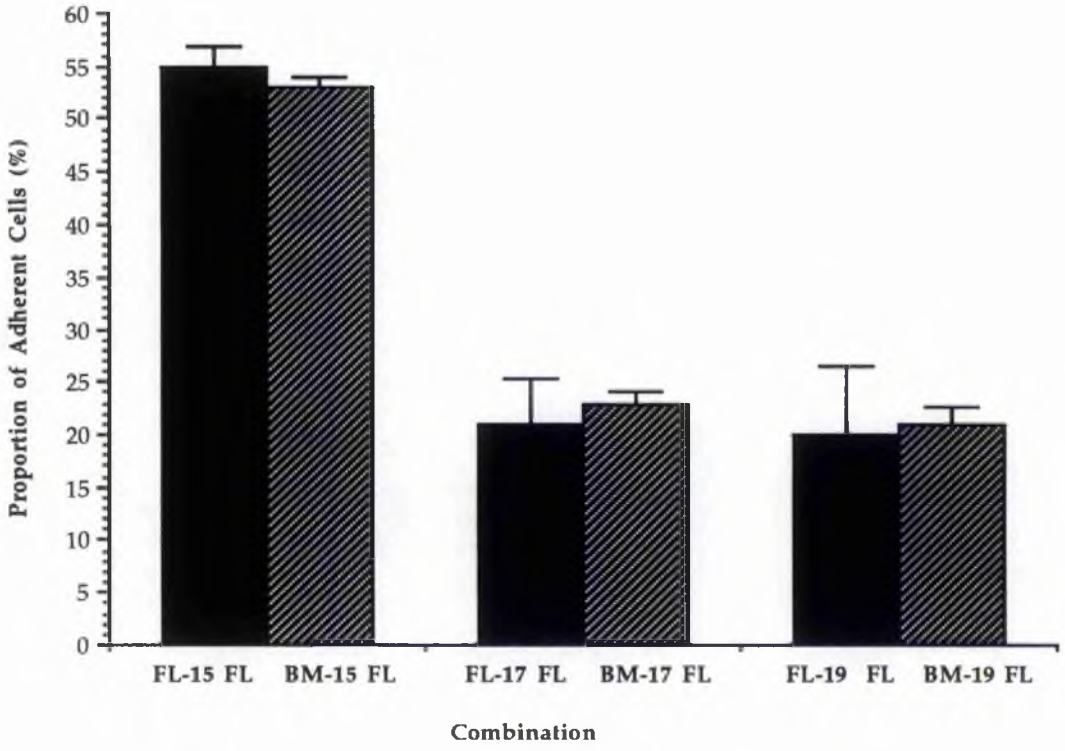
Haematopoietic cell suspensions of fetal liver or bone marrow, containing approximately 1, 000 HPP-CFC, were observed to adhere to stromal layers derived from Day 17 and Day 19 fetal liver (Table 6.1-2 and Fig. 6.1-2). 21% of HPP-CFC derived from 15 Day fetal liver adhered to stromal layers

derived from Day 17 fetal liver and 20% adhered to stromal layers derived from Day 19 fetal liver. 23% of HPP-CFC derived from adult bone marrow adhered to Day 17 fetal liver stroma and 21% adhered to Day 19 fetal liver stroma. Anova showed there was no significant difference in the adhesive properties of stroma derived from Day 17 and Day 19 fetal liver, nor in the ability of the layers to retain haematopoietic cells derived from fetal liver or bone marrow ($p = 0.99$). The proportion of HPP-CFC, derived from fetal liver or bone marrow, which adhered to the Day 17 and Day 19 fetal liver stroma was significantly reduced in comparison to the proportion of fetal liver or bone marrow HPP-CFC, which adhered to stromal layers derived from Day 15 fetal liver ($p < 0.0001$). This suggests that 15 day fetal liver has a greater supportive capacity for haematopoietic stem cells than stromal layers derived from fetal liver later in gestation.

**Table 6.2-1** Proportion of HPP-CFC which Adhere to Day 17 and Day 19 Fetal liver Derived Stromal Layers

| Combination | Proportion of Adherent HPP-CFC (%) Mean ± SE | No. of Experiments |
|---|---|---|
| FL-> Dy 15 FL stroma | 55 ± 1.9 | 5 |
| FL-> Dy17 FL stroma | 21 ± 4.2 | 8 |
| FL-> Dy19 FL stroma | 20 ± 6.4 | 8 |
| BM-> Dy15 FL stroma | 53 ± 0.9 | 5 |
| BM-> Dy17 FL stroma | 23 ± 1.1 | 4 |
| BM-> Dy19 FL stroma | 21 ± 1.6 | 4 |

Fig. 6.2-1 Adhesion of Fetal Liver and Bone Marrow HPP-CFC to Stromal Layers Derived from Day 15, 17 and 19 Fetal Liver




## Discussion

### 6.3 Adhesion of Haematopoietic Cells to Bone Marrow and Fetal Liver Microenvironments in vitro

Long-term bone marrow cultures demonstrate the crucial role of the stromal cells in supporting proliferation and differentiation of haematopoietic precursor cells in vitro. Stromal cell lines derived from bone marrow (BM) and fetal liver (FL) have been established by several groups to investigate the cellular and molecular interactions with

haematopoietic cells and to determine any morphological or functional differences in the stroma derived from both organs (Dexter et al 1977; Shaklai 1989; Vogt et al 1991; Allen 1992; Hata et al 1993). The morphological characteristics of stromal layers derived from adult bone marrow and fetal liver at 15, 17 and 19 days of gestation are detailed in Chapter 3. This Chapter discusses the adhesive properties of BM and FL stroma.

HPP-CFC derived from 15 day FL or adult BM were observed readily to adhere to stromal layers derived from either 15 day FL or adult BM. There was no significant difference in the adhesion of HPP-CFC derived from normal bone marrow to adherent layers derived from bone marrow or fetal liver, regardless of the concentration of stem cells incubated with the layer. Likewise, there was no significant difference in the adhesion of HPP-CFC derived from regenerating bone marrow to bone marrow stroma or in the adhesion of HPP-CFC derived from fetal liver to fetal liver stroma, for all three concentrations of stem cells used. However the loss of HPP-CFC derived from fetal liver which were incubated with stromal layers derived from adult bone marrow was significantly greater than all the other combinations investigated, suggesting preferential adhesion of fetal liver cells to bone marrow stroma.

The proportion of HPP which adhered to the stromal layers was dependent on the number of HPP seeded onto the layers. When only 500 HPP were seeded onto the stromal layers a high proportion of HPP adhered. This proportion was moderately reduced when 1000 HPP were seeded onto the layers and was further reduced when 5000 HPP were seeded onto the layers. This trend was consistently observed in all the different combinations of haematopoietic progenitor cells seeded and adherent stromal layers. The proportion of FL derived HPP which adhered to BM stromal layers

remained high throughout, although there was a reduction in this proportion as the number of FL cells was increased. When 5000 FL derived HPP were seeded onto bone marrow layers the proportion which adhered was as high as seeding 500 FL or BM derived HPP onto FL derived stroma, or seeding 500 BM or regenerating BM HPP onto BM stroma.

While the proportion of HPP-CFC which adhered to the stromal layers decreased as the concentration of stem cells was increased, the actual number of HPP-CFC which bound to the stromal layers increased linearly with concentration in all five combinations. This indicates that a number of HPP-CFC did not adhere to the stromal layers even when the layers had sufficient binding sites to accommodate all the HPP-CFC. Both bone marrow and fetal liver stromal layers could accommodate at least 1000 HPP-CFC, however when 1000 HPP-CFC of either bone marrow or fetal liver origin were seeded onto the layers, only a proportion of the initial concentration were retained by both layers. The decrease in proportion of adherent HPP-CFC as their numbers were increased could suggest that stromal layers have a specific number of niches in which haematopoietic stem cells can be accommodated. Using higher concentrations of haematopoietic cells could lead to saturation of the stromal layer, with all the binding sites occupied by haematopoietic progenitors. However the efficiency of FL derived HPP binding to BM stroma was not markedly reduced even at high concentrations ($5x10^6$) of HPP seeded per flask. Gordon et al (1987) used concentrations of up to $10^7$ cells, in 35mm petri dishes, and reported that in practice the number of binding sites in the stroma is not a limiting factor. It would therefore appear that while bone marrow and fetal liver stromal layers have niches for support of considerable numbers of haematopoietic stem cells, extensive

haematopoietic cell binding was only observed in the preferential adhesion of fetal liver stem cells to bone marrow stromal layers.

The kinetic properties of HPP derived from FL and those derived from BM are very different. A high proportion of HPP-CFC in fetal liver are actively cycling, while in the bone marrow HPP are quiescent. The difference in kinetic properties could account for the increased adhesion of FL stem cells to BM stromal layers. The proportion of HPP-CFC synthesising DNA is also high in regenerating BM (~ 40 %), however there was no significant difference in the adhesion of regenerating BM or normal BM HPP to bone marrow stromal layers. The selective adhesion to BM stromal layers thus appears to be a phenomenon unique to FL derived HPP-CFC.

Stromal layers derived from adult bone marrow and fetal liver can effectively support haematopoiesis in vitro. Haematopoietic stem cells seeded onto the layers must first adhere to the stromal layer prior to any proliferation or differentiation (Bentley 1982). This investigation has revealed that haematopoietic stem cells will effectively bind to stromal layers derived from both adult bone marrow and 15 day fetal liver. However adhesion of HPP-CFC to stromal layers derived from fetal liver on the 17th and 19th days of gestation was significantly reduced compared to adhesion of progenitor cells onto 15 day fetal liver stroma. Seeding BM or FL cell suspensions (both containing ~ 1, 000 HPP-CFC) onto 15 day FL stroma resulted in adhesion of 53% and 55% of HPP respectively. When the same initial inocula were seeded onto stroma derived from 17 day FL stroma, only 23% of BM and 21% of FL HPP-CFC adhered to the layer. Day 19 FL stroma was effective in retaining only 21% of BM and 20% of FL HPP-CFC from an initial concentration of 1, 000 HPP.

These results suggest that while fetal liver has a great supportive capacity for haematopoietic cells in vitro, this capacity declines as gestation proceeds. As haematopoietic activity in the bone marrow is initiated, there is essentially no need for the fetal liver to remain a haematopoietic site and thus the capacity of the liver to support haematopoiesis declines. This finding is consistent with the morphological changes observed in fetal liver in late gestation and neonatal liver (Chapter 3) and also with the decline in both the number and cycling status of HPP-CFC in late fetal and neonatal liver (Chapter 4).

Within the haematopoietic microenvironment, dynamic cellular interactions are constantly occurring, involving stem cells, progenitor cells and more mature cells physiologically interacting with stromal cells, glycoproteins and ECM. These interactions serve to regulate normal haematopoiesis by allowing the communication of regulatory information, migration and subsequent homing of stem cells within specific organs for presentation of specific growth factors. Membrane interactions between progenitor cells and stromal cells are complex and known to involve cellular components in addition to polysaccharides and fibrous proteins of the ECM (Roberts et al 1987, 1988: Torok-Storb 1992).

Bone marrow stromal cells produce specific ECM components, fibronectin, laminin, hemonectin, collagens and proteoglycans. The observations that individual cellular elements have only a limited capacity to support haematopoiesis compared to the entire stromal layer, suggests that the ECM plays a role in facilitating cell lodgement and homing to the marrow (Roberts et al 1988). Haematopoiesis can be modulated via ECM production, which is thought to be under the control of growth factors. TGF-β

stimulates collagen and proteoglycan synthesis and α-interferon increases glycosaminoglycan synthesis by stromal cells (Axelrad 1990).

The importance of attachment of haematopoietic progenitor cells to the stromal layer has been emphasised (Bentley 1982; Heard et al 1982). Attachment is thought to be highly selective and essential for the subsequent proliferation and differentiation of the haematopoietic cells. CFU-E and BFU-E show significant attachment to fibronectin while CFU-GM interact with hemonectin a different ECM component (Vuillet-Gaugler et al 1990). Growth factors may accumulate in the ECM of stromal cells as GM-CSF is bound to glycosaminoglycans produced by marrow stromal cells (Gordon et al 1987). Blast progenitors adhere to stromal layers grown in the presence of methylprednisolone (MP) but not to stromal layers grown in its absence, while more mature progenitors do not show any tendency to adhere to either type of stromal layer (Gordon et al 1985). These observations suggests that progenitor cells at different stages of maturation may occupy different microenvironments in the marrow and that different types of microenvironment, like haematopoietic cells, can be classified by their cell and growth factor binding capacities. The structure of the marrow may determine the distribution of haematopoietic lineages and alterations in the stromal cells may influence the differentiation patterns of progenitor cells.

Other groups have reported differences in the ability of haematopoietic progenitor cells to bind to stroma. Hardy & Minguell (1993) found the bipotential FDCP cell line bound very well to bone marrow stroma while multi-potential FDCP-mix and B6 Sut cell lines did not bind so well. The difference in the binding potential of the progenitor cells was attributed to differences in their cell surface membrane structures. Morphological examination of the haematopoietic stromal cell interactions revealed

numerous microvilli and "ruffle-like structures" on the surface of the haematopoietic cells which appeared to interact with the stromal cells. Microvilli were also observed on the stromal cells and appeared to interact with the projections from the haematopoietic cells. Thus it would appear that intimate associations between haematopoietic cells and stromal cells observed in vivo also occur in long term cultures. Interactions have also been described between progenitor cells microvilli and ECM components of fibroblasts.

Studies by Hata et al (1993), have demonstrated the ability of 15 day fetal liver hepatocytes to support BM and FL cells. Separation of the haematopoietic progenitor cells from the stroma by Millicell filters had no adverse effects on the maintenance of haematopoiesis, suggesting that the fetal hepatocytes release soluble growth factors into the culture. GM-CSF and M-CSF were detected and assumed to be released by the hepatocytes, however IL-1, IL-3 and IL-6, which are well documented haematopoietic regulators were not detected. It is possible therefore that binding of haematopoietic cells to fetal liver stroma is not essential for their subsequent proliferation and differentiation. Thus fetal liver stromal cells may be able to exert regulatory factors on progenitor cells in their close proximity without the need for cell adhesion molecules.

The regulatory interactions between stromal cells and haematopoietic cells seem to be determined by mutual recognition and adhesive processes. The maturation of haematopoietic cells is thought to involve an orderly regression of cytoadhesive properties of stem cells and their progeny Allen & Testa 1991). Progenitor cells may thus respond to proliferation and differentiation signals by changes in their cytoadhesive properties, such changes may alter their capacity to bind to the stroma, promote migration to the sinus wall and subsequent release into the circulation. It is also

possible that progenitor cells themselves may modulate the expression of receptors on their cell surface for stromal cells and ECM, thereby determining their own differentiation pathway. Progenitor cells can synthesise chondroitin sulphate proteoglycan which is unique to these cells and is thought to play a role in the binding of progenitor cells to the stroma. Homing protein is present on the surface of CFU-S and CFU-GM, it is not found on marrow or spleen stromal cells. Homing protein shows specificity for galactosyl and mannosyl residues, both of which are essential for cell binding. Differentiation of erythroid cells is normally associated with loss of this protein (Tavassoli & Minguell 1991).

Among the ECM components, fibronectin is thought to be the most effective for inducing adhesion and re-organisation of cytoskeletal structures. Haematopoietic cells are thought to recognise the locus of the fibronectin-cell attachment domain and readily adhere to the stromal cells. Integrins which are structurally related to fibronectin are thought to be involved in the recognition and adhesion of haematopoietic cells to stromal cells. The fibronectin-adhesion property is known to deteriorate with erythroid cell development and thus end-stage cells can be released into the circulation (Vuillet-Gaugler et al 1990).

Hemonectin is a cytoadhesive glycoprotein which shows lineage and organ specific attachment properties for cells of the granulocyte lineage, which may localise granulocytic cells in close proximity to specific growth factor producing stromal cells. Hemonectin expression is also lost as maturation proceeds and release of mature granulocytes into the circulation ensues (Dexter et al 1990).

Collagen is an essential matrix protein for the proliferation and organisation of the marrow stromal cells. Collagens are thought to

participate in the adhesive interactions by binding directly to the progenitor cells or by acting as anchorage receptors for proteoglycans (Dexter et al 1990).

Proteoglycans play a major role in haematopoiesis however the mechanism of action of stromal proteoglycan is not well understood. They are thought to be involved in adhesion of progenitor cells to the stroma and to bind growth factors for presentation to the progenitor cells . Hence proteoglycans can direct compartmentalisation and molecular orientation of haematopoietic growth factors and subsequently direct specific lines of haematopoiesis (Gordon et al 1987; Roberts et al 1988).

AcSDKP which is known to inhibit quiescent CFU-S from entry into the cell cycle, has been shown to enhance binding of haematopoietic cells to stroma (Aizawa et al 1992). This enhancement is thought to occur through AcSDKP activation of stromal cells to express binding molecules (Lenfant et al 1989).

It is possible that the decreased adhesive properties of stromal layers derived from day 17 and day 19 fetal liver are due to decreased expression of one or a combination of ECM components, growth factors or proteoglycans. Expression of these molecules in late fetal liver derived stroma has not been documented. The findings of Hata et al (1993), that haematopoietic cells do not have to bind to 15 day fetal liver layers for regulation via growth factors, suggests that cell adhesion molecules are not necessary in long term fetal liver cultures. The reduced binding affinity of day 17 and day 19 fetal liver stromal layers may therefore be due to a reduction in growth factor production by the stromal cells rather than decreased numbers of cell adhesion molecules. Stromal cells derived from fetal liver deserve further investigation to clarify the expression of cell adhesion molecules and regulatory molecules, or lack of these factors and to

document the changes which occur in expression of such factors in haematopoietically declining fetal liver.

The mechanisms of functional binding of haematopoietic cells to stroma remain unclear. Homing of haematopoietic cells to bind to specific stromal cells is thought to be an important phenomenon in haematopoiesis (Zanjani et al 1993). In the present investigation, HPP-CFC derived from FL were found to selectively adhere to BM derived stroma. Selective adhesion of progenitor cells to BM stroma has been previously reported. Gordon et al (1985), reported selective adhesion of blast-CFC (Bl-CFC) to bone marrow stromal layers, grown in the presence of MP, containing numerous fat cells. More mature progenitors did not selectively adhere to these layers. Stromal layers grown in the absence of MP, which do not contain fat cells, did not remove Bl-CFC or mature progenitors from the supernatant. Similarly Riley & Gordon (1987), reported that Bl-CFC adhered efficiently to adult bone marrow stromal layers but did not adhere so efficiently to stromal layers derived from fetal liver or fetal bone marrow.

Van Den Heuvel et al (1991), demonstrated that stroma derived from haematopoietically active organs supported GM-CFC proliferation in vitro better than stroma from organs in which haematopoiesis was declining or had just begun. Adult bone marrow displayed the highest ability to support GM-CFC regardless of whether the progenitor cells were of fetal liver, splenic or bone marrow origin. The predominantly erythroid nature of the fetal liver microenvironment and lymphoid environment of the spleen could account for this difference (Thomas 1971).

Binding of Bl-CFC to BM stroma does not require calcium or magnesium ions nor does it require known cell adhesion molecules, suggesting that the requirements for interaction between stromal cells and progenitor cells are

provided entirely by the stromal layer (Gordon et al 1990). An inverse relationship between sialic acid content and adhesiveness has been reported (Gordon et al 1990). A high sialic acid concentration is thought to mask cell adhesion molecules on the more mature haematopoietic cells and thereby reduce adhesion. However the binding properties of Bl-CFC are not affected by neuraminidase treatment, which reduces sialic acid concentration, indicating that early haematopoietic progenitors may not express cell adhesion molecules (Gordon et al 1990). This finding has important implications in haematopoietic development.

Lack of expression, or masking, of cell adhesion molecules on fetal liver progenitor cells would prevent these cells from being "trapped" in the fetal liver environment and could promote migration of these cells from the liver to the spleen and bone marrow. Cell adhesion molecules expressed by haematopoietic cells are thought to be tissue specific which could explain the selective adhesion of Bl-CFC to adult bone marrow stromal layers (Bearpark & Gordon 1989). Expression of cell adhesion molecules by fetal liver progenitor cells has not been documented. Lack of expression of adhesion molecules by HPP-CFC would prevent the stem cells from remaining in the liver and promote migration to the bone marrow. Alternatively fetal liver HPP-CFC may express cell adhesion molecules which are specific for adult bone marrow receptors which could explain their selective adherence to bone marrow stroma.

While cell adhesion molecules may not be required, heparan-sulphate proteoglycan is however necessary for the binding of Bl-CFC to stroma and reinforces the idea that lineage and stage specific environments exist in the bone marrow (Gordon et al 1988). Segregation of haematopoietic progenitor cells in the bone marrow stroma, through specific binding molecules would facilitate their regulation and interaction with specific growth

factors, to promote the development of a particular cell lineage. Other proteoglycans may be instrumental in the homing of haematopoietic stem cells, while another class of membrane associated proteoglycans is thought to be involved in the developmental regulation of erythroid cells (Tavassoli et al 1990). ECM components form only one group of molecules involved in the homing phenomenon, others include homing protein and cytoadhesive molecules.

## 6.4 Homing

Homing of haematopoietic progenitor cells is the phenomenon which permits intravenous transplantation of bone marrow cell suspensions. Evidence suggests that homing is the initial event in haematopoiesis and is responsible for stem cell migration from the yolk sac to the fetal liver and subsequently to the spleen and bone marrow (Moore & Metcalf 1970). It is generally accepted that homing involves intimate membrane interactions between the haematopoietic cells and the stromal cells in order for self-renewal, proliferation and differentiation to occur (Tavassoli & Hardy 1990). Progenitor cells which home to the bone marrow must interact with the luminal surface of the sinusoidal epithelium separating the vascular and extravascular compartments of the bone marrow. The cells migrate through fenestrations in the endothelial layer into the haematopoietic compartment where they recognise and bind to lineage specific stromal cells (Tavassoli & Hardy 1990).

Homing protein is considered to be responsible for the initial recognition of stroma by haematopoietic cells and enables the stem cells to identify and subsequently lodge within a specific niche (Hardy & Minguell 1993). This interaction however is of relatively low affinity and stabilisation is

provided through ECM interactions which are of higher affinity (Tavassoli et al 1990). Incubation of haematopoietic progenitors with IL-3 and GM-CSF increases the density of homing protein. In lethally irradiated mice the concentration of homing protein is increased, and as a result the seeding efficiency of haematopoietic progenitor cells increases concomitantly with an increase in the adherence of these cell to stromal layers (Tavassoli & Minguell 1991).

Homing of transplanted haematopoietic stem cells to the bone marrow is blocked in the presence of galactosyl and mannosyl BSA, by inhibiting the action of the homing protein. In contrast homing of stem cells to the spleen is not affected by these synthetic neoglycoproteins (Aizawa & Tavassoli 1988). This finding suggests that different mechanisms are involved in the recognition and homing of transplanted stem cells in the two sites. It is likely that the mechanism of recognition of the fetal liver by haematopoietic stem cells and migration to this organ are also different.

While it is generally accepted that during the course of ontogeny haematopoietic cells migrate from the fetal liver and home to the bone marrow, the course and mechanism of this migration has not been delineated. As maturation proceeds the haematopoietic activity of the liver decreases (Chapters 3 & 4), this could be due to loss of its capacity for haematopoietic support and maintenance, as a result the stem cells migrate to the bone marrow which can provide a suitable microenvironment for proliferation and differentiation of stem cells. Alternatively the fetal liver haematopoietic stem cells may be pre-programmed to home to the bone marrow and ontogenic maturation of the stem cells may change their homing capacity. Such an ontogenic maturation could be expression of cell adhesion molecules which are specific for bone marrow receptors or other cell surface molecules which condition the homing and adhesion of fetal

liver stem cells to the bone marrow (Houssaint & Hallet 1988). Thus the decrease in haematopoietic activity of the fetal liver may be a result of egression of progenitor cells to the bone marrow. These two possibilities are not necessarily exclusive.

The present investigation has revealed that stromal layers derived from fetal liver on the 15th day of gestation can adequately support haematopoietic stem cells derived from fetal liver and from adult bone marrow. BM and 15 day FL stromal layers were both capable of binding and supporting bone marrow derived stem cells. Similar results have been reported for the support of both fetal liver and bone marrow derived GM-CFC by human fetal liver and adult bone marrow stromal layers (Slaper-Cortenbach et al 1987).

The preferential adhesion of fetal liver HPP-CFC to bone marrow stroma could be ascribed to the natural migration of fetal liver stem cells to the bone marrow in vivo. This observation is consistent with reports of selective adhesion and homing of sheep FL stem cells to the bone marrow in vivo (Zanjani et al 1993). This group demonstrated that haematopoietic stem cells derived from fetal liver, preferentially homed to the bone marrow when injected, intraperitoneally, into fetuses between 60-80 days of gestation (term 145 days), even though the fetal liver was the main site of haematopoiesis at this stage. Prior to development of the bone marrow, haematopoietic stem cells injected into sheep fetuses homed to the liver or the spleen. Zanjani and colleagues propose the existence of a hierarchy of homing sites, in which the bone marrow has the highest affinity for the homing of transplanted haematopoietic stem cells. A similar hierarchy of affinity for haematopoietic cells may be responsible for the homing of stem cells from the yolk sac to the fetal liver.

Alternatively the ECM proteins and cell adhesion molecules of the bone marrow stromal cells may bind fetal liver derived stem cells more effectively than stem cells of bone marrow origin. The expression of homing protein or adhesion molecule receptors on fetal liver progenitors has, as yet, not been delineated. Fetal liver stem cells may express a higher concentration of homing protein than bone marrow stem cells and consequently selectively migrate and adhere to the bone marrow.

Conversely, fetal liver haematopoietic cells may not express homing protein or specific cell adhesion molecule receptors. Differentiation in the erythroid lineage is associated with the loss of homing protein (Tavassoli & Hardy 1990). Erythropoiesis is the predominant form of haematopoiesis in the fetal liver, thus the fetal liver progenitors which have been maintained in an erythroid microenvironment may not express significant concentrations of homing protein. Lack of homing protein or adhesion molecule receptors would permit fetal liver progenitor cells to effectively bind to any part of the bone marrow stroma, unlike the selective binding of bone marrow stem cells to specific stromal environments. Hence bone marrow stroma may have the capacity to support considerably higher numbers of fetal liver stem cells which don not require such specific binding sites, compared to bone marrow stem cells. Selective adhesion of bone marrow stem cells to specific stromal sites permits the stroma to dictate the proliferation and differentiation of the stem cells along a particular lineage by presentation of specific growth factors. It is interesting to note that migration of stem cells to the spleen is not mediated through homing protein (Tavassoli 1990). This molecular recognition mechanism may exist only in the bone marrow, while fetal liver and splenic microenvironments may provide, as yet undetermined alternative mechanisms for the homing of stem cells.

## 6.5 Clinical Implications

Interactions of haematopoietic cells with stromal cells may have important clinical implications. Abnormal interactions could contribute to the evolution or manifestation of haematological disorders, either through defective stroma or unusual interactions of haematopoietic cells. Aberrant interactions of haematopoietic cells with stromal cells in patients with CML and AML have been effectively exploited for in vitro purging of bone marrow cells in these patients (Clark et al 1992). Studies by Gordon et al (1987), have revealed altered adhesive interactions between primitive progenitor cells and stromal cells in CML. Bl-CFC derived from CML patients adhere to stromal layers grown in the presence and absence of methylprednisolone.

This finding suggest that CML cells are less discriminating than normal progenitor cells and consequently have an advantage to normal cells as they can adhere to and proliferate at additional sites within the bone marrow stroma. The CML progenitors do not adhere to MP layers as efficiently as normal Bl-CFC (Gordon et al 1987). This finding has serious implications in the early release of CML cells into the blood, these blood-borne metastases result in secondary tumour growth at other sites within the body. Eaves et al (1986) demonstrated that progenitor cells from patients with CML undergo continuous cycling in cell culture both in the presence and in the absence of a stromal layer. Normal haematopoietic cells cycle only after medium changes in stromal cultures. These observations suggest that CML progenitor cell cycling is not regulated by stromal cells, leading to early expansion of CML, this is likely to play a crucial role in leukaemogenesis.

The use of new techniques to determine the molecular basis of cytoadhesive interactions between stem cells and stromal cells and to define the nature of defective interactions with haematopoietic stem cells and stromal cell in a number of haematological conditions should lead to advances in bone marrow transplantation therapy (Clark et al 1992).

Effective haematopoiesis is the product of interplay between haematopoietic stem cells and the supporting stroma, which provides a favourable environment for the maintenance of regulated proliferation and differentiation. Fetal liver stem cells seeded onto bone marrow stroma proved to be the most favourable combination for adhesion of stem cells and subsequent maintenance of haematopoiesis in the present investigation. It is not very surprising to find bone marrow stroma has a high affinity for fetal liver cells, otherwise migration of haematopoietic stem cells to the bone marrow might not occur and the initiation of haematopoietic activity in the marrow could be perturbed as a result. There would be no advantage to having a favourable soil for growth and maintenance of seeds if the soil was not sown with seeds to support (Tavassoli 1989).

# Chapter 7

# General Discussion

## 7.1 Haematopoietic Activity in the Fetus and Early Neonate

Haematopoiesis is sustained by a pool of pluripotent stem cells which are capable of extensive self-renewal to maintain their numbers and also differentiation into progenitor cells which are committed to various haematopoietic lineages. Pluripotent progenitor cells have been identified which differentiate into mature lineage restricted cells, which in turn feed into the mature blood cell pool. The complex mechanism controlling the proliferation and differentiation of stem cells and progenitor cells in mammals has not been fully elucidated. In both humans and mice a variety of glycoprotein regulators have been purified and the corresponding genes have been cloned (Plumb & Pragnell 1988).

Ontogenic development of the haematopoietic system involves a series of co-ordinated changes in embryonic and early fetal life. The first generation of haematopoietic cells proliferate in the yolk sac and extra-embryonic mesenchyme. Yolk sac haematopoiesis is then replaced by the liver which continues to accommodate blood cell production till birth (Keleman et al 1979).

The morphological investigations of fetal and newborn liver conducted in the present investigation revealed an increase in the haematopoietic activity of the fetal liver to a maximum on the 15th day of gestation. This activity was then observed to decline as gestational age advanced to a

minimum observed in the liver of the 10 day neonate. These findings are consistent with the changes in site of haematopoietic production during development. The murine liver has assumed haematopoietic activity from the yolk sac by the 10th day of gestation (Hata et al 1993). In the present investigation the number of haematopoietic cells together with the number of hepatocytes and macrophages increase steadily from the 12th day of gestation. In the liver of the 14 and 15 day fetuses, the parenchyma was densely packed with cells of all haematopoietic lineages, resulting in considerable distortion to the contours of the central hepatocytes or macrophages which were surrounded by haematopoietic cells, to form the classic erythroblastic islands first described by Bessis.

Erythroid cells were the predominant lineage of haematopoietic cells in the liver samples examined, especially in fetal liver, suggesting the fetal liver stem cells are restricted in their differentiation capacity or that the microenvironment is primarily inducive to erythropoiesis (Thomas 1970). The cells were observed arranged around a central macrophage or hepatocyte and closely associated with the cytoplasmic extensions of the central cell. Granulocytes and monocytes were present in significant numbers from the 14th day of gestation onwards. They were commonly observed on the periphery of erythroid clusters associated with the processes of the central cell. Megakaryocytes were also observed at this stage in development, usually in close association with hepatocytes. They were never observed as integral components of the cell clusters.

The number of haematopoietic cells in fetal liver was observed to decline from the fifteenth day of gestation. Granulocytes and monocytes occupied more central positions in the cell clusters as the number of erythroid cells decreased. Lymphoid cells were observed closely associated with mixed lineage clusters in the liver of the newborn mouse. Haematopoietic clusters

were restricted to single -lineage cells in the liver of the 10 day neonate. The cell clusters were no longer dispersed throughout the hepatic parenchyma but were limited to isolated areas in close proximity to the sinusoids (Rossant 1986).

While the number of haematopoietic cells decreased from the 15th day of gestation, a considerable increase in both size and number of hepatocytes was observed. This increase was also observed in vitro in long term fetal liver cultures. Hepatocytes which had been closely associated with haematopoietic cells were morphologically distinct from those which had not (Emura et al 1984). In newborn and neonatal liver, hepatocytes had a more regular outline than their fetal liver counterparts whose contours were distorted by the high number of haematopoietic cells. Adjacent hepatocytes were closely associated through apposition of their cells membranes or via interdigitating cytoplasmic processes. Cannalicular structures were observed between hepatocytes in vitro which may represent the in vivo bile cannaliculi. The intimate relations of adjacent hepatocytes could be associated with the change in function of the hepatocytes from a stromal element of a haematopoietic microenvironment to a parenchymal component of the metabolic liver (Medlock & Haar, 1983). At birth the bone marrow becomes the main site of haematopoiesis in mammals, while the liver assumes its function as the main metabolic organ of the body. The adult liver however retains its ability to function as a site of haematopoietic production in extreme circumstances.

The increase in the haematopoietic activity of fetal liver to reach a peak on the 15th day of gestation followed by a subsequent decline in activity in late fetal and neonatal liver was mirrored in studies of the kinetic properties of blood cell precursors derived from fetal and neonatal liver (Chapter 4).

Investigation of the cycling status of HPP-CFC derived from fetal, newborn and neonatal liver revealed a peak in the proportion of cells synthesising DNA in the liver of the 15 day fetus (53%), corresponding with the observed peak in haematopoietic activity. The proportion of cells in cycle progressively declined with gestational age to 28% in the newborn liver and further declined to 13% in the liver of the 8 day neonate. This decline in the proportion of HPP-CFC engaged in DNA synthesis corresponds to the morphological observations of minimal haematopoietic activity in the 8 day neonate, when the bone marrow has assumed the role as the main site of haematopoietic production. A similar trend was observed in the number of HPP colonies produced by fetal and neonatal liver. Fetal liver produced a high number of HPP colonies, this was maximal on the 15th day of gestation. Colony production then declined to a minimum in the liver of the 8 day neonate.

In contrast to the kinetic properties of HPP-CFC, the cycling status of GM-CFC did not alter dramatically from that of the 15 day fetal liver (50%) to that of the newborn liver (43%). This high proportion of cycling GM progenitors in late fetal and newborn liver correlates with the in vivo myelopoietic phase of haematopoiesis observed in 18 day fetal, newborn and neonatal liver.

## 7.2 Cellular Interactions in Haematopoiesis

An array of cellular interactions control the production of blood cells in haematopoietic organs. The stromal cells of each organ provide an appropriate environment for the proliferation and differentiation of stem cells. Haematopoietic cell interactions have been elucidated in vivo in

animal models and more recently in vitro using long term culture systems (Cline & Golde, 1979).

The first recognition of cellular associations and interactions during haematopoiesis was probably Bessis' report of the erythroblastic island in 1958. In the present investigation erythroblastic islands were numerous in haematopoietically active fetal liver, their numbers progressively declined with the decrease in haematopoietic activity in newborn and neonatal liver. In addition to Bessis' classic erythroblastic island, composed of a central macrophage surrounded by a ring of erythroblasts, many haematopoietic cells were observed to form clusters around a central hepatocyte in a similar manner. Indeed the number of haematopoietic clusters with a central hepatocyte outnumbered those with a central macrophage. Thus both hepatocytes and macrophages can function as the central cell of a haematopoietic island. This arrangement is thought to be essential for the differentiation of both erythroid cells and hepatocytes (Emura et al, 1985).

The central cells of the haematopoietic islands extended long cytoplasmic processes which were closely apposed to the adjacent haematopoietic cells and occasionally completely surrounded the blood cell precursors. These intimate associations are considered to play a significant role in the development of haematopoietic cells. Desmosomes and gap junctions were evident in regions of membrane apposition. These observations of direct cell-cell communication in erythroblastic islands have been previously reported, (Fukuda 1974: Rosendaal 1991), and their presence indicates the potential for the transfer of substances between the central cell and the developing haematopoietic cells.

The gradients of differentiation observed in the clusters suggests that less mature cells are more dependent on the central cell. Shaklai (1989), suggests the more mature cells may migrate along the cytoplasmic processes of the central cell to occupy peripheral positions within the islands. The clusters in fetal liver were not restricted to cells of the erythroid lineage, granuloid, monocytic and lymphoid cells were usually observed in peripheral positions (Jones 1970: Grossi et al 1985). It may be that these cells are less dependent on the central cell than the erythroblasts alternatively the large number of erythroblasts present may prevent occupation of central positions by cells of other lineages.

In the liver of the 10 day neonate, the cell clusters were associated with hepatocytes only and, like haematopoietic clusters in the bone marrow, were composed of blood cell precursors of a single lineage. The number and distribution of the clusters were restricted. These findings have been reported by Grossi et al (1985) and Rossant et al (1986), who suggest that progenitor cells in the 10 day neonate are not multipotential but committed to specific lineages and thus give rise to single-lineage clusters.

Erythroblastic islands have also been reported in vitro in long term bone marrow cultures (Allen & Dexter 1982). Like their in vivo counterparts, cytoplasmic extensions of the central macrophages were closely associated with the haematopoietic cells. Vesicular activity and gap junctions in regions of membrane apposition has been reported (Rosendaal 1991: Allen 1992). In the present investigation haematopoietic clusters were observed in vitro composed of central macrophages surrounded by granulocytes, in both bone marrow and fetal liver cultures. Granulocytes were also observed in close association with hepatocytes in fetal liver cultures, providing

further evidence that both macrophages and hepatocytes can support haematopoiesis.

Hardy & Minguell (1993), observed microvilli and "ruffle-like" structures on the surface of haematopoietic stem cells which interacted with microvilli on the surface of stromal cells. Similar interactions were observed between progenitor cell microvilli and ECM components of fibroblasts. It would thus appear that the electron microscopic observations of intimate associations between haematopoietic cells and stromal cells in vivo, also occur in vitro in long term culture.

The role of erythroblastic islands has been debated for many years. The scavenger role of the central macrophage in removing extruded nuclei and old blood cells is generally accepted, however the provision of growth factors and nutrients to developing blood erythroblasts has not yet been conclusively proven and remains controversial. Other unsolved problems relating to erythroblastic islands include:- is the central cell essential for differentiation and maturation of erythroblasts? Erythropoiesis can take place in vitro in the absence of macrophages, however the haemoglobin content and membrane organisation of the resulting erythroblasts are not akin to those of erythroblasts in vivo (Mohandas 1991). This finding suggests that the macrophages have an important role to play in the regulation of gene expression.

What factors produced by the central cell act on haematopoietic cells? Bessis' rhopheocytic vesicles were numerous between the central cells and adjacent blood cell precursors in the present investigation. The cells in closest proximity to the central cell were observed to have a greater concentration of ferritin than peripheral cells, suggesting ferritin is more essential for the earlier precursor cells (Breton-Gorius 1991). However the

direction of transfer of the ferritin could not be discerned. It is possible that the ferritin could travel in both directions from the central cell to the early precursors, as these cells mature they may no longer require high concentrations of ferritin, and excess ferritin could be removed by the central cell (Pollycove 1991).

Macrophages can produce a variety of regulatory molecules including Epo, G-CSF, M-CSF, GM-CSF, IL-1, TNF-α, TGF-β, fibronectin (Rich 1986; Nathan 1987). Epo and CSF production by murine fetal liver macrophages is reported to be maximal in cultures of day 14 and day 15 fetal liver cells (Zucali 1977). This correlates with the in vivo peak of erythropoiesis in the liver of the 15 day fetus in the present study. Macrophages respond to varying concentrations of oxygen by increasing or decreasing the production of Epo and CSF accordingly (Rich 1986).

Hepatocytes are considered to produce substances which are essential for the development of erythroblasts (Medlock & Haar 1983). Hata et al (1993), demonstrated the production of GM-CSF and M-CSF by murine fetal liver hepatocytes, providing further evidence for their role as a haematopoietic supporting cell. The focal nature of the haematopoietic cell clusters both in vivo and in vitro, together with the observations of cellular interactions, rhopheocytic vessicles, gap junctions and desmosomes, provides the ideal environment for the provision of growth and regulatory factors to the blood cell precursors by the central cells.

The mechanism by which the blood cell precursors recognise and remain in close association with the central cell is unclear. Recognition of stromal cells in the bone marrow by haematopoietic stem cells occurs by means of a homing protein which has galactosyl and mannosyl specificities (Hardy & Minguell 1993). Different recognition mechanisms are thought to be

involved in the hepatic and splenic microenvironments which as yet have not been investigated (Aizawa & Tavassoli 1988). Interaction of haematopoietic cells with macrophages in fetal liver is thought to occur through a divalent-cation dependent adhesion receptor, mediated by calcium. This receptor also operates in erythroblast-macrophage interactions in adult bone marrow, together with a divalent-cation independent receptor, which has been characterised as a glycoprotein produced exclusively by bone marrow macrophages (Crocker et al 1990, 1991).

ECM proteins consisting of collagens, laminin, fibronectin and proteoglycans are essential for the maintenance of haematopoiesis in long term bone marrow cultures (Zuckerman et al 1985). Fibronectin has an important role to play in the attachment of haematopoietic cells to stromal layers. Adhesion of CFU-E precursors is inhibited by antifibronectin antibodies (Coulombel et al 1992). Loss of fibronectin receptors results in the loss of adherence to the stromal layers. This phenomenon is especially important for reticulocytes which then migrate into the supernatant in long term cultures (Bernard 1991).

Heparan sulphate proteoglycan (HS-PG) is a CAM which is necessary for the binding of blast-CFC to stromal layers in vitro (Gordon et al 1988). Information on CAM's and ECM proteins of fetal liver cultures is however minimal. Macrophages and hepatocytes can both produce fibronectin and haematopoietic regulatory molecules. Thus blood cells precursors could attach to central cells and proliferate in response to specific growth factors presented to them. However Gordon et al (1990), reported that binding of very early progenitor cells to bone marrow stroma did not involve known CAM's. Thus binding of fetal liver haematopoietic cells to fetal liver stroma may not involve HS-PG, but instead as yet unknown CAM's.

Further investigation is required to elucidate the binding of fetal liver blood cell precursors to hepatic stromal cells.

The biological importance of attachment of haematopoietic cells to stromal cells and ECM proteins for proliferation and differentiation of stem cells is unknown. It is likely that at least some of the forementioned features will contribute to the elusive role played by the microenvironment in the overall process of haematopoiesis (Mel 1991).

## 7.3 Haematopoietic Microenvironments

Haematopoietic cell clusters form only a part of the haematopoietic inductive microenvironment, in which supportive stromal cells provide a favourable environment for the maintenance of haematopoiesis. The haematopoietic stroma is thought to be essential for the proliferation and differentiation of stem cells (Tavassoli & Takahashi 1982). The biological interactions of stromal cells with primitive haematopoietic progenitors can reconstitute all the haematopoietic lineages within an irradiated host (Greenberger 1991).

Fetal liver and bone marrow stroma both in vivo and in vitro provide suitable microenvironments for the proliferation and differentiation of haematopoietic stem cells. Maintenance of haematopoiesis in vitro was dependent upon the establishment of an intact stromal layer. Stromal layers of both bone marrow and fetal liver origin were found to support haematopoietic stem cells seeded onto them regardless of whether the stem cells were of bone marrow or fetal liver origin. Granuloid progenitors were observed in fetal liver in vivo, however there is very little granulopoiesis in this organ. In vitro the dramatic shift from erythropoiesis to

myelopoiesis suggests that all the necessary information for granulocyte production is present in the cells of fetal liver and that the culture conditions used promote their proliferation. It is not known how this change to granulopoiesis results, it could be through the selective survival or proliferation of cells producing the necessary factors for granulopoiesis. Alternatively it could occur through alteration of the regulatory pathways of the haematopoietic cells or the microenvironment to suppress erythropoiesis and promote granulopoiesis.

Stroma derived from day 17 and day 19 fetal liver could also support exogenous stem cells seeded onto them. However the haematopoietic activity in these cultures was sustained for a shorter period of time than in cultures of 15 day fetal liver stroma. This correlates with the in vivo observations of a morphological decline in fetal liver after the 15th day of gestation. The stromal environment of fetal liver on days 17 and 19 of gestation may be less suitable for haematopoiesis than that of day 15 and consequently the stem cells will migrate to the bone marrow and spleen where the stroma provides a more suitable microenvironment for haematopoiesis.

Haematopoietic stem cells seeded onto established stromal layers must first adhere to the layer prior to any proliferation or differentiation. Stromal layers derived from fetal liver on the 15th, 17th and 19th days of gestation and adult bone marrow could effectively bind a significant number of haematopoietic stem cells regardless of their origin. However stromal layers derived from day 17 and 19 fetal liver could not bind stem cells as effectively as day 15 fetal liver layers. The reduced haematopoietic activity in these layers could thus be a result of ineffective binding of haematopoietic stem cells to the layers. Thus the haematopoietic decline in fetal liver could be due, in part, to the less favourable stromal

microenvironment in late gestation, which has a reduced binding capacity for stem cells.

Haematopoietic stem cells which were derived from fetal liver were found to selectively adhere to stromal layers derived from adult bone marrow. This adhesion was significantly greater than all other combinations of stem cells and stromal layers investigated. The number of fetal liver HPP which adhered to the bone marrow stromal layers increased linearly as the number of HPP seeded was increased. Zanjani et al (1993), reported selective adhesion and homing of fetal liver stem cells to the bone marrow in vivo, at the time when fetal liver was the main site of haematopoiesis. Selective adhesion of Bl-CFC to bone marrow stroma has been reported by Gordon et al (1985). However mature progenitors did not display this selective adhesion.

The reasons for the selective adhesion of fetal liver stem cells to bone marrow stroma are unclear. Why should bone marrow stroma bind fetal liver cells more effectively than bone marrow stem cells? The difference in kinetic properties of cycling fetal liver HPP and quiescent bone marrow HPP cannot be a contributory factor, as RBM cells which are also in cycle did not display preferential adhesion to bone marrow stromal layers. Zanjani et al (1993) propose a hierarchy of homing sites in which the bone marrow has the highest affinity for homing of transplanted haematopoietic stem cells. Alternatively the fetal liver stem cells may be pre-programmed to migrate from the liver microenvironment to that of the bone marrow. Homing of stem cells to the bone marrow could be considered as part of the maturation process of fetal liver stem cells.

It is not known how one tissue loses its potential to support haematopoiesis while another tissue gains this potential. The decline in

haematopoietic activity of the liver could be a result of changes in the microenvironment making it less suitable for haematopoiesis, alternatively it could be a consequence of egression of stem cells to the bone marrow, or indeed a combination of both these factors (Houssaint & Hallet 1988). The applications of newer techniques to study the kinetics of haematopoietic cells during migration and gestational maturation should provide some insight into the change in site of blood cell production (Zanjani et al 1993).

The selective adhesion observed in the present investigation could be explained by the hypothesis that, under the conditions of this assay, a greater proportion of HPP-CFC derived from fetal liver are capable of adhering to stromal layers than HPP-CFC derived from adult bone marrow. Combining this factor with the possibility that BM stroma may contain a greater number of binding sites than fetal liver stroma provides an explanation why adhesion in the combination of fetal liver HPP and bone marrow stroma was significantly greater than all other stem cell-stromal combinations (Fig. 7.3). The actual functional mechanisms of haematopoietic stem cells binding to stroma are not known, however it is thought to be highly selective and essential for the development of haematopoietic cells. Membrane interactions of progenitor cells and stromal cells are very complex and are known to involve cellular components, ECM proteins and CAMs (Roberts et al 1988; Torok-Storb 1992).

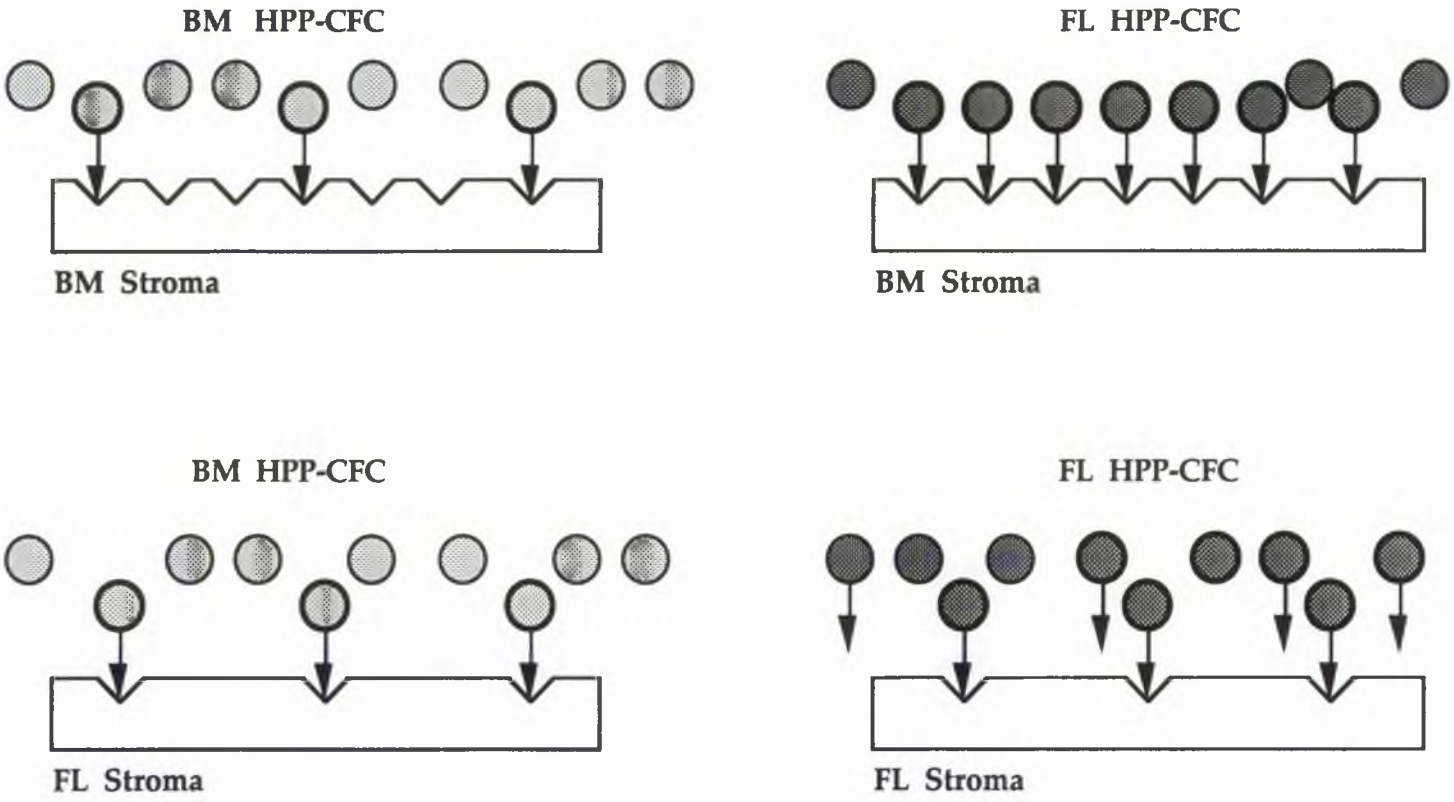



**Fig. 7.3 Selective Adhesion of Fetal Liver HPP.CFC to Bone Marrow Stroma**

Fetal liver may contain a greater number of HPP-CFC with the specific requirements for binding to stromal layers than bone marrow stem cells. In addition bone marrow stroma may have a greater number of binding sites available for HPP-CFC than fetal liver stroma. Hence greatest adhesion is observed when fetal liver HPP-CFC are seeded onto bone marrow stroma.

Progenitor cells attach to specific ECM proteins, CFU-E and BFU-E attach to fibronectin, CFU-GM however interact with haemonectin (Vuillet-Gaugler et al 1990). Bl-CFC adhere only in methylprednisolone grown stromal layers while mature progenitors do not display this preference. HS-PG is necessary for the binding of Bl-CFC to stroma (Gordon et al 1988), while another class of proteoglycan is thought to be involved in regulation of erythroid cells (Tavassoli et al 1990). Thus progenitor cells may occupy specific environmental niches within the bone marrow, each microenvironment could be classified by their cell binding and growth factor presentation capacities. In this manner the structure of the marrow could determine the distribution and differentiation of haematopoietic cells lineages (De Haan et al 1993). Alternatively progenitor cells may have the capacity to modulate the expression of stromal cell and ECM receptors on their cell surfaces, such as chondroitin sulphate and homing protein, and thereby determine their own differentiation pathway (Long 1992).

The morphological observations of fetal liver in vivo, in the present study, indicate that in contrast to the bone marrow microenvironment, lineage specific environmental niches do not exist in fetal liver. Granulocytes and monocytes were commonly observed as structural components of cells clusters together with erythroblasts. These mixed lineage clusters predominated in fetal liver, only in the liver of the 10 day neonate were single-lineage clusters observed, resembling those described in adult bone marrow.

The multiple types of haematopoietic cell interactions in the bone marrow microenvironment require the presence of specialised cell surface structures which mediate cell adhesion and transmit signals from the stromal cells to growth factors and ECM. These systems represent a

powerful mechanism for directing and localising cellular responses (Tavassoli et al 1990; Long 1992). Such a system has not been documented for haematopoietic regulation in the fetal liver microenvironment. It is not known whether fetal liver stem cells bind to fetal liver stromal cells in a manner similar to the binding of BM stem cells to BM stroma, or indeed whether functional binding is necessary in the hepatic microenvironment where the close associations of stem cells and stromal cells might suffice.

Expression of ECM molecules by fetal liver cells has received very little attention. Both fetal liver macrophages and hepatocytes can synthesise collagen, whether fetal liver macrophages have the capacity to produce other ECM proteins, like their bone marrow counterparts, is not known. Fetal liver GAGs have been shown to be inefficient in presenting GM-CSF to haematopoietic stem cells and thus may not be involved in growth factor presentation in the fetal liver microenvironment. Hata et al (1993) demonstrated that fetal liver macrophages and hepatocytes in vitro could release soluble growth factors which acted on haematopoietic cells in the vicinity.

Gordon's report (1990), that primitive Bl-CFC did not express known CAMs could also hold true for the primitive fetal liver stem cells. Lack of expression or masking of CAMs in the fetal liver environment would prevent the stem cells from being trapped and could promote their migration to the bone marrow. Alternatively during ontogenic maturation fetal liver cells may express CAMs which are specific for bone marrow receptors.

It is not known whether fetal liver stem cells express homing protein in the same concentration as bone marrow stem cells, if at all. Lack of expression of homing protein or specific CAMs by fetal liver stem cells

would permit these cells to bind effectively to any part of the bone marrow stroma and not be restricted to localised microenvironments like bone marrow stem cells. Hence bone marrow stroma may have the capacity to accommodate a greater number of fetal liver stem cells than those of bone marrow origin.

Fetal liver stem cells have properties which are similar to those of stem cells in patients with chronic myeloid leukaemia. CML progenitors are less discriminating than normal progenitors and thus can adhere to and proliferate in a large number of sites in the bone marrow which are not normally occupied by haematopoietic cells. Much remains to be learned regarding the role of ECM, CAMs and other as yet unidentified molecules in the binding of stem cells to stromal cells. Greater understanding of these interactions will increase our knowledge of normal stem cell biology and the behaviour of leukaemic cells.

## 7.4 Haematopoietic Regulation

Haematopoietic stem cells carry the entire genetic information which is required to amplify the stem cell subpopulation and to proliferate into differentiated progeny. The number of stem cells is maintained at a relatively constant level indicating that the mechanisms exist whereby stem cell numbers can be retained despite their differentiation into lineage restricted precursor cells (Clark et al 1992). Regulation of these processes is elicited in part by the intimate associations of the stem cells with stromal cells of that particular microenvironment. The microenvironment regulates stem cell and progenitor cell lodgement, proliferation, differentiation and release of mature blood cells into the circulation.

Intimate associations of stem cells and stromal cells are thought to be essential for the regulation of haematopoiesis. Stromal cells are known to produce growth factors essential for effective haematopoiesis (Verfaillie 1992). In addition ECM proteins and CAMs are thought to play important roles in binding of stem cells and presentation of growth factors. Loss of attachment to stromal cells will promote the migration of mature cells into the circulation (Coulombel 1992). Bone marrow haematopoiesis is considered to be regulated through co-operation of all these factors, though exactly how this co-operation is operative remains to be determined.

Most haematopoietic growth factors are locally synthesised by stromal cells, evidence suggests that growth factors can be cell associated as a complex linked to the stromal cell surface by proteoglycans. Alternatively the growth factors could be secreted as a soluble form which is then absorbed onto ECM components such as HS-PG (Roberts et al 1988). Both of these forms of growth factor presentation act as adhesive ligands binding the stem cells to the stromal cells. It is not known whether CAMs play a role in determining the lineage differentiation of the stem cells (Long 1992).

Stromal cells also play a role in returning primitive haematopoietic cells to a quiescent state. TGF-β and MIP-1α produced by stromal cells in culture are thought to be responsible for inhibition of primitive haematopoietic stem cell proliferation in concert with growth factors (Eaves et al 1991). Thus stromal cells can produce diverse and opposing local control functions to promote and inhibit stages of haematopoiesis. The actual mechanism by which the majority of stem cells are held in growth arrest is not known. The model of attachment without proliferation is one which could be most relevant to in vivo haematopoiesis (Greenberger 1991).

Haematopoietic regulation in the fetal liver microenvironment has not been so well documented. Fetal liver haematopoiesis was observed to involve intimate associations of stem cells with central macrophages and hepatocytes in the present investigation. Both fetal liver macrophages and hepatocytes can produce regulatory factors and fibronectin however information is lacking on the involvement of ECM proteins and CAMs in the fetal liver environment. The observations of gap junctions and desmosomes during EM studies of fetal liver are consistent with the local release of substances from the central macrophage or hepatocyte to the adjacent blood cell precursors. Rosendaal (1991) demonstrated dye coupling of stromal cells and haematopoietic cells and proposed that communication via gap junctions may play a role in maintaining precursor cells which would otherwise differentiate through exposure to growth factors. The findings of Hata et al (1993) that fetal liver stromal cells can release soluble growth factors into culture suggests that ECM proteins and CAMs may not play such a significant role in fetal liver haematopoiesis. The presence of mixed lineage clusters in fetal liver could be attributed to lack of expression of known CAMs by fetal liver stem cells. Fetal liver haematopoietic cells may express unidentified CAMs which permit progenitor cells of different lineages to associate with the same stromal cells.

Proliferation of haematopoietic stem cells appears to be controlled locally by factors which regulate the proportion of stem cells in cycle. The proportion of HPP-CFC synthesising DNA in adult bone marrow was only 10%, indicating they are quiescent. In contrast 53% of 15 day fetal liver HPP-CFC were actively cycling. A stem cell specific stimulator of DNA synthesis is present in fetal liver at this stage in gestation (Dawood et al 1990). Stimulator production declines from the 15th day of gestation, which

coincides with the decline and cessation of hepatic haematopoiesis in the neonate. Thus in the fetal liver microenvironment, a high proportion of cycling stem cells exists concomitantly with high concentrations of stimulator (Dawood et al 1990).

The stem cell regulatory mechanism is different in the bone marrow microenvironment. The quiescent stem cell population in the bone marrow is maintained by an inhibitor of DNA synthesis, which can reduce the proportion of cycling cells in regenerating tissue (Riches et al 1981). Stem cell cycling is increased by a stimulator of DNA synthesis in regenerating or stressed tissue (Wright & Lord 1977). Stem cell numbers play an important role in this regulatory process, a low number of stem cells results in decreased inhibitor production and increased stimulator production. Similarly high stem cells numbers decrease stimulator production and increase inhibitor production. An inverse relationship between stem cell number and stimulator production appears to exist in the bone marrow microenvironment, unlike that of fetal liver where a direct relationship is observed (Dawood et al 1990).

Positive and negative regulators of haematopoiesis acting in concert with the forementioned associations of stromal cells and stem cells provide mechanisms for rapid and specific proliferative responses to changes in haematopoietic demands. The elucidation of the requirements for optimal stem cell growth should result in improvements of culture systems to yield in vivo reconstitution of haematopoiesis.

## 7.5 Haematopoietic Stem Cells

The kinetic properties of the HPP-$CFC_2$ subpopulation, evaluated in this investigation were found to be very similar to those of the CFU-S population, both in fetal liver and adult bone marrow. The proportion of fetal liver derived HPP engaged in DNA synthesis (53%) correlates with the 30-40% of fetal liver derived CFU-S in S-phase (Thomas et al , 1981). In adult bone marrow only 10% of HPP were synthesising DNA, a similar reduction in the cycling status of bone marrow derived CFU-S has been reported (Thomas et al 1981). The reduction in HPP-CFC numbers throughout gestation mirrors the reduction in CFU-S numbers reported by Dawood et al (1990). In addition to the similarities in kinetic properties, HPP-CFC and candidate pre-CFU-S populations share many phenotypic characteristics including specific stem cell markers (Bertoncello et al 1992). The kinetic properties of HPP-CFC established in this investigation are also similar to those of early erythroid progenitors of fetal liver and bone marrow origin (Peschle et al 1981).

However bone marrow derived HPP-CFC have been shown to be less sensitive to the CFU-S regulators than the CFU-S population (Robinson & Riches 1991), and hence the HPP-CFC population may be a more primitive haematopoietic stem cell population than CFU-S. A hierarchy has been determined within the CFU-S population by their sensitivity to haematopoietic regulators, 5FU treatment and Rh-123 uptake. The very primitive day 16 CFU-S is more sensitive to inhibitor and are Rh dull. More mature CFU-S populations are more sensitive to stimulator and are Rh bright (Bertoncello et al 1992). A similar hierarchy has recently been determined in the HPP population. HPP-$CFC_1$ population, which is Rh dull and resistant to 5FU treatment has been proposed as a candidate pre-CFU-S

(McNeice et al 1990). The HPP-$CFC_2$ and more lineage restricted HPP-$CFC_3$ subpopulations are Rh bright and severely depleted by 5FU treatment (McNeice et al 1988; Bertoncello et al 1992). These similarities between the in vitro HPP-CFC and the in vivo CFU-S populations, confirm the proposal that HPP-CFC are a developmentally early haematopoietic precursor cell population with properties closely related to those of the true haematopoietic stem cell.

The GM-CFC population is known to be a more mature precursor cell population than the HPP-CFC. The results of the present study were in accordance with this. The number of GM-CFC engaged in DNA synthesis remained relatively high in fetal and neonatal liver, in sharp contrast to the reduction observed in the proportion of cycling HPP over the same period. GM-CFC derived from adult bone marrow were also found to be actively cycling, in accordance with previous reports (Cork et al 1982). Like HPP-CFC, the number of GM-CFC colonies produced by fetal and neonatal liver was lower than that of the bone marrow. It can be assumed therefore that fetal and neonatal liver contain fewer HPP and GM progenitors than adult bone marrow however, the proportion of cells in cycle is higher in fetal and neonatal liver than in the bone marrow. The kinetic properties of GM-CFC are similar to a more mature population of erythroid precursors (m-BFU-E) described by Peschle et al (1981). The proportion of cycling m-BFU-E is found to be high in both fetal liver and adult bone marrow.

Variations in concurrent GM-CFC proliferation in fetal liver and fetal bone marrow suggest stem cell regulation is controlled locally. Human fetal liver of 11-14 weeks gestation produces an inhibitor of GM-CFC proliferation, while liver > 14 weeks produces a GM-CFC stimulator (Cork et al 1982). GM-CFC, unlike HPP-CFC, are not sensitive to CFU-S regulators. Indeed fetal liver produces CFU-S stimulator and GM-CFC inhibitor concurrently

and these regulators are produced by adherent and non-adherent cells respectively (Wright & Lord 1977: Cork et al 1982). Similarly it is proposed that CFU-S regulators have no effect on the CFU-C population, direct descendants of CFU-S (Toksoz et al 1980). The CFU-C, like GM-CFC and m-BFU-E, retain a high cycling activity in adult bone marrow.

It is apparent that classes of haematopoietic progenitor cells exist, each with their own specific growth factor requirements and distinct kinetic properties. Marked differences in the kinetic properties of corresponding fetal and adult derived primitive progenitors were observed in the present investigation and have been previously reported (Peschle et al 1981: Thomas et al 1981). Ontogenic maturation of haematopoietic stem cells is associated with a decline in their proliferative activity. All fetal liver progenitor cells appear to be actively cycling, irrespective of their lineage and differentiation state, while in adult bone marrow differentiation of quiescent primitive stem cells is associated with an increase in the proliferative activity of the descendants.

## 7.6 Regulatory Factors Produced by Fetal Liver

Fetal liver is known to produce a number of haematopoietic regulatory factors, CFU-S regulators (Dawood et al 1990), GM-CFC regulators (Cork et al 1982, 1986), EGM-CSF and BPF (Zhao & Wu 1990), Arginase (Wu et al 1990), Epo (Rich 1986) and all the interleukins except IL-5 (Guiterrez-Ramos et al 1992). These regulators have been isolated from fractionated fetal liver or detected in conditioned medium produced by fetal liver cells incubated at 37°C.

The CFU-S stimulator detected by Dawood et al (1990) was isolated from fractionated fetal liver cells and was found to be present at high concentrations on the 15th day of gestation. In the present investigation

however, a 10% concentration of crude 15 day fetal liver extract (FLE) was found to have an inhibitory effect on HPP formation by normal bone marrow cells, while lower concentrations had no effect. The inhibitory effect of FLE was more pronounced on regenerating bone marrow cells reducing colony formation by 78%. The overall effect of FLE on primitive HPP formation appears to be inhibitory. The size of the colonies produced in the presence of FLE had increased to 5mm on average. Thus while crude FLE may inhibit proliferation of primitive HPP it may promote the differentiation of stem cells.

The fetal liver extract was also observed to inhibit colony formation by SA2 leukaemic cells. Inhibition of SA2 formation was directly related to the concentration of FLE used. Greatest inhibition was observed when SA2 cells were incubated with 1ml FLE. Both SA2 leukaemic cells and RBM cells are actively cycling, while normal bone marrow cells are not. It would therefore appear that the inhibitory effect of crude FLE was more pronounced on actively cycling cells. Touraine et al (1990), reported complete leukaemic ablation following infusion of fetal liver cells into leukaemic mice.

Similar effects have been reported by Wu et al (1989) using human FLE. A 10% concentration of human FLE was found to inhibit CFU-GM growth and growth of the leukaemic HL-60 cell line. Fractionation of human fetal liver revealed the presence of arginase, a known inhibitor of normal and tumour cell growth, in the 60K dalton fraction. It was not detected in the 30-60 K dalton fraction, which had no suppressive effects on either normal or leukaemic cells. This is consistent with the detection of a CFU-S stimulator in this fraction (Dawood et al 1990). Leukaemic cell growth was completely inhibited in the presence of a <10 K dalton fraction of fetal liver however, while only a weak suppressive effect was observed on CFU-GM

growth. Arginase activity could not be detected in this fraction of human FLE. These findings suggest that fetal liver contains additional regulatory factors which as yet have not been identified. Izzi et al (1985) reported haematopoietic reconstitution of aplastic anaemia and acute leukaemia patients following fetal liver transplant, however the fetal liver cells did not have an anti-leukaemic effect. The fetal liver extract used by Izzi et al had been incubated at 37°C and cryopreserved, unlike the crude extract used in the present investigation.

It is thus apparent that haematopoietic stimulators and inhibitors are present in different molecular weight fractions of fetal liver, which can have different effects on a variety of cells. The results of Wu et al (1989) on the low molecular weight fraction indicate that an as yet unidentified suppressor exists which displays preferential suppression of leukaemic cells. Futhermore the distribution of this new inhibitor has been reported to be similar to arginase in that it is mainly detected outside the fetal liver cells or it could be stored in the ECM (Wu, Wong & Pei 1989). However the overall effect of crude murine FLE in the present investigation and human FLE (Wu et al 1989), was the inhibition of leukaemic and regenerating cells at all concentrations of FLE, while normal bone marrow cell proliferation was only affected by high concentrations of FLE.

The leukaemic suppressor in the low molecular weight fraction of human fetal liver may also exist in murine fetal liver. This deserves further investigation to determine its existence in murine FL and whether it effects are more pronounced in the fractionated form than in crude FLE. The anti-leukaemic effect of FLE has important clinical applications in the purging of leukaemic cells for autologous bone marrow transplantation.

## 7.7 Clinical Applications

Fetal liver and bone marrow are abundant sources of haematopoietic stem cells and progenitors, which in a suitable microenvironment can differentiate into various lines of blood cells. Stromal cell in both organs have important roles to play in the regulation and control of haematopoiesis. The mechanism which governs the switch in haematopoietic production sites from fetal liver to bone marrow remains elusive. Likewise why should the erythroid lineage predominate in fetal liver while granulopoiesis predominates in the bone marrow? Many questions remain unanswered regarding the complicated process of haematopoiesis in both fetal liver and bone marrow.

When considering the haematopoietic potential of the cell clusters observed in fetal liver and adult bone marrow, the molecular mechanisms underlying the cellular associations must also be considered, including recognition, adhesion, production and presentation of regulatory factors and release of mature progenitors. At least some, if not all, of these features make a significant contribution to the role played by the microenvironment in the overall process of haematopoiesis.

A number of haematological disorders can be attributed to abnormal interactions of stromal cells with haematopoietic cells, caused by progenitor cell defects or defective stroma. Clinical progress in this field is likely to advance through the application of molecular techniques to define the nature of abnormal stem cell-stromal interactions. Purging of bone marrow cells in leukaemic patients by suppressors such as FLE could be further enhanced by modification of the adhesive interactions between the leukaemic cells and the stromal cells.

Elucidation of optimal cell growth requirements would have enormous significance in transplantation therapy by allowing expansion of progenitors, endowed with properties of fetal liver cells, which could be transplanted to facilitate rapid engraftment and reduction of antigenic treatment. Primitive stem cells maintained in culture could be used for genetic transfer or could be manipulated to synthesise specific regulatory factors to alleviate genetic and haematological conditions when infused into the host.

# Chapter 8

# Conclusions and Further Work

## 8.1 Conclusions

The haematopoietic activity of murine fetal liver increases from the 10th day of gestation to reach a peak on the 15th day of gestation, when the number of actively cycling primitive stem cells is maximal. Hepatic haematopoiesis is predominantly erythroid in nature, however cells of all other lineages are also observed. There appear to be two waves of haematopoiesis in the fetal liver, the first is a wave of erythropoiesis, this is followed by a wave of myelopoiesis which never exceeds that of erythropoiesis.

As maturation proceeds, the haematopoietic activity of the liver declines together with a decline number and cycling activity of primitive stem cells. Megakaryocyte numbers are observed to decline concomitant with erythrocyte numbers while the number of hepatocytes increases. Hepatocytes which appeared late in gestation and had not been associated with haematopoietic cells were structurally different from those which had. The hepatocytes were changing to elements which function in the metabolic role of the liver, rather than supportive stromal cells in haematopoietically active liver. Stroma derived from fetal liver at the 17th and 19th days of gestation has a reduced capacity to support haematopoietic cells, compared to that of 15 day fetal liver. Thus in addition to the decline in haematopoietic cell number late in gestation,

the stromal elements of the fetal liver are also changing reducing their capacity to support haematopoiesis.

The bone marrow assumes the role as the main site of haematopoietic production at birth. There is some haematopoietic activity in neonatal liver, which closely resembles that of the bone marrow in that haematopoietic cell clusters are composed of single-lineage cells and these clusters are restricted to isolated foci. Primitive stem cells derived from neonatal liver are relatively quiescent like those of adult bone marrow. At this stage the liver functions as the main metabolic organ of the body with cessation of hepatic haematopoiesis as a result.

Intimate associations of haematopoietic cells with stromal cells are essential for haematopoiesis. Haematopoietic activity in vitro was dependent upon the presence of a confluent stromal layer to which haematopoietic cells must first adhere. Fetal liver and bone marrow stromal layers could adequately support blood cell production, regardless of the origin of the haematopoietic cells. Fetal liver haematopoietic cells selectively adhered to bone marrow stromal layers. This preferential adhesion could not be attributed to the difference in kinetic properties of fetal liver and bone marrow derived stem cells. Bone marrow stroma may have a greater number of binding sites for haematopoietic cells than fetal liver stroma and fetal liver stem cells may be more adherent than bone marrow stem cells. This preferential adhesion may promote the in vivo migration of haematopoietic cells from the fetal liver to the bone marrow.

Haematopoietic cells clusters were abundant in haematopoietically active fetal liver in vivo and in vitro. Gradients of differentiation could be discerned within the clusters, the more mature cells occupied peripheral positions and may therefore be less dependent on the central cell. Once mature, the cells leave the cluster environment and migrate towards the sinusoids for release

into the circulation. Macrophages and in particular hepatocytes function as central supporting cells of the haematopoietic clusters in the fetal liver microenvironment. Central macrophages are responsible for the removal of extruded nuclei and cell debris. Haematopoietic clusters in fetal liver were composed of mixed-lineage cells, thus lineage specific microenvironments do not exist in fetal liver, unlike neonatal liver and adult bone marrow.

The extensive areas of membrane apposition of central cells and blood cell precursors, where gap junctions and desmosomes were observed, provide ideal conditions for the transfer of regulatory factors between the cells. Rhopheocytic vesicles were numerous, indicating ferritin may be transferred between the central cells and adjacent blood cell precursors, however the direction of transfer could not be determined. The central cells may thus have the capacity to direct proliferation and differentiation of blood cell precursors with which it is associated, through provision of exogenous substances.

The kinetic properties of fetal liver stem cells differ from those of bone marrow stem cells. A high proportion of fetal liver HPP-CFC are engaged in DNA synthesis, while in the bone marrow microenvironment these stem cells are quiescent. Quiescence in the bone marrow population is maintained by proliferation inhibitors produced in response to high numbers of cycling stem cells, while in the fetal liver microenvironment a large cycling stem cell population is maintained by proliferation stimulators. The proportion of cycling stem cells in fetal liver declines as maturation proceeds. The kinetic properties of the in vitro HPP-CFC are very similar to those of the in vivo CFU-S, in both fetal liver and adult bone marrow, indicating the HPP-CFC population is a primitive stem cell population with very similar properties to the true haematopoietic stem cell. A more mature class of progenitor cells, GM-CFC, are actively cycling in both fetal and neonatal liver and also in adult bone marrow. These progenitor cells are regulated by specific factors which

have no effect on the HPP-CFC population. Thus while stem cells and progenitor cells are actively cycling in the fetal liver microenvironment, only progenitor cells are cycling in the adult bone marrow. However the actual number of stem cells and progenitor cells especially, is higher in adult bone marrow than fetal or neonatal liver.

Fetal liver is known to produce both haematopoietic stimulators and inhibitors. In a crude form, fetal liver extract had an inhibitory effect on regenerating bone marrow stem cells and leukaemic cells. The quiescent stem cell population of adult bone marrow was inhibited by 1ml of fetal liver extract, however at lower concentrations there was no effect. The overall inhibitory effect of fetal liver extract is more pronounced on actively cycling stem cells and leukaemic cells, this finding has important clinical applications in the treatment of leukaemia. Fetal liver extract did not promote the proliferation of stem cells, however colony size was increased in its presence. Thus while crude fetal liver extract inhibits proliferation of primitive cycling stem cells it may promote differentiation of these cells into more mature progeny.

## 8.2 Further Work

Fetal bone marrow stromal layers could be used to test their ability to bind HPP-CFC to establish whether the decrease in supportive capacity of day 17 and day 19 fetal liver stromal layers is accompanied by an increase in the supportive capacity of stromal layers derived from fetal bone marrow.

It may be possible to determine the number of binding sites available in stromal layers by increasing the number of HPP-CFC seeded onto the layers. When all the available sites for HPP-CFC are occupied, increasing the

concentration of HPP-CFC further should not effect the number of cells which bind to the layer, presumably a plateau will be observed.

Mature progenitor cells could be incubated with adherent stromal layers in order to determine whether they display preferential binding for bone marrow stromal layers.

Anti-fibronectin antibodies could be used to determine whether the binding of fetal liver blood cells precursors to fetal liver stromal layers requires fibronectin, which is necessary for adhesion of bone marrow stem cells to bone marrow stroma.

The < 10K dalton fraction of murine fetal liver extract could be investigated in order to establish whether it preferentially suppressed leukaemic cells rather than normal blood cells precursors. Also whether the effects of fetal liver extract on leukaemic cells are more pronounced in fractionated or crude forms.

The effects of murine fetal liver extract could be tested on more mature progenitors (GM-CFC) to establish whether it has different effects on primitive and more mature progenitor cells.

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# Appendix

## Publications

Blair A., Thomas D.B.: Morphological Relationships of Differentiating Blood Cell Precursors to Adjacent Macrophages and Hepatocytes. Journal of Anatomy **176**: 237, 1991.

Blair A., Suissa S., Thomas D.B: Morphological Relationships of Differentiating Blood Cell Precursors to Adjacent Macrophages and Hepatocytes in Murine Fetal Liver. Journal of Anatomy **179**: 249, 1991.

Blair A., Riches A.C., Robinson S.N., Thomas D.B: The Proliferative Status of Cells Derived from Murine Fetal Liver which Form Colonies In Vitro (high proliferative potential colony forming cells- HPP-CFC). Journal of Anatomy **182.1**: 129, 1993.

Blair A., Thomas D.B: The Adhesive Properties of Cells Derived from Murine Bone Marrow or from Murine Fetal Liver which form Colonies In Vitro (high proliferative potential colony forming cells - HPC). Proceedings of the 8th Symposium of Haemopoiesis: 26, 1993 .

Blair A., Brynmor Thomas D: A mechanism which may promote the transfer of haematopoiesis from the liver to the medullary cavities. Journal of Anatomy (in press)

**Morphological relationships of differentiating blood cell precursors to adjacent macrophages and hepatocytes in murine liver.** By ALLISON BLAIR (supervised by D. BRYNMOR THOMAS), University of St. Andrews.

Contrasting patterns of concurrent haematopoiesis in the liver and in the bone marrow of the human fetus have been attributed to differences in the environments available for the differentiation of blood cell precursors in the two situations (Thomas J. Anat **110**, 1971). These differences may reflect different relationships between blood cell precursors and stromal cells which have been demonstrated in vitro (Gordon et al, Exp. Hematol **18**, 1990). The present investigation has been devised to provide information about these relationships in the hope that it will contribute to an understanding of the interactions between the early precursors of blood cells and the cells in their immediate vicinity which appear to be of fundamental importance for the regulation of haematopoiesis (Dexter et al, Phil. Trans. Roy. Soc. Lond. **327B**, 1990).

Samples of liver were obtained from CBA mice on the fifteenth day of gestation, the day of birth and the eighth day of the neonatal period; fixed in glutaraldehyde; post-fixed in osmium tetroxide; embedded in araldite resin and prepared for examination using a Philips 301 electron microscope.

Extensive haematopoiesis was evident on the fifteenth day of gestation when distinctive clusters of cells were observed. These cells were arranged around central macrophages or around central hepatocytes. Cytoplasmic extensions from the macrophages and hepatocytes were intimately related to adjacent blood cell precursors, some of which were surrounded by these extensions. By

the end of the first week of the neonatal period, when the number of clusters had decreased, the cytoplasmic extensions were shorter and their arrangement was very much simpler. In the clusters of blood cell precursors differentiation gradients were observed, the most highly differentiated cells being furthest from the central cell. The form and arrangement of cytoplasmic extensions and the distribution of rhopheocytotic vesicles is consistent with the extensive interchange of materials between hepatocytes and blood cell precursors as well as between macrophages and blood cell precursors.

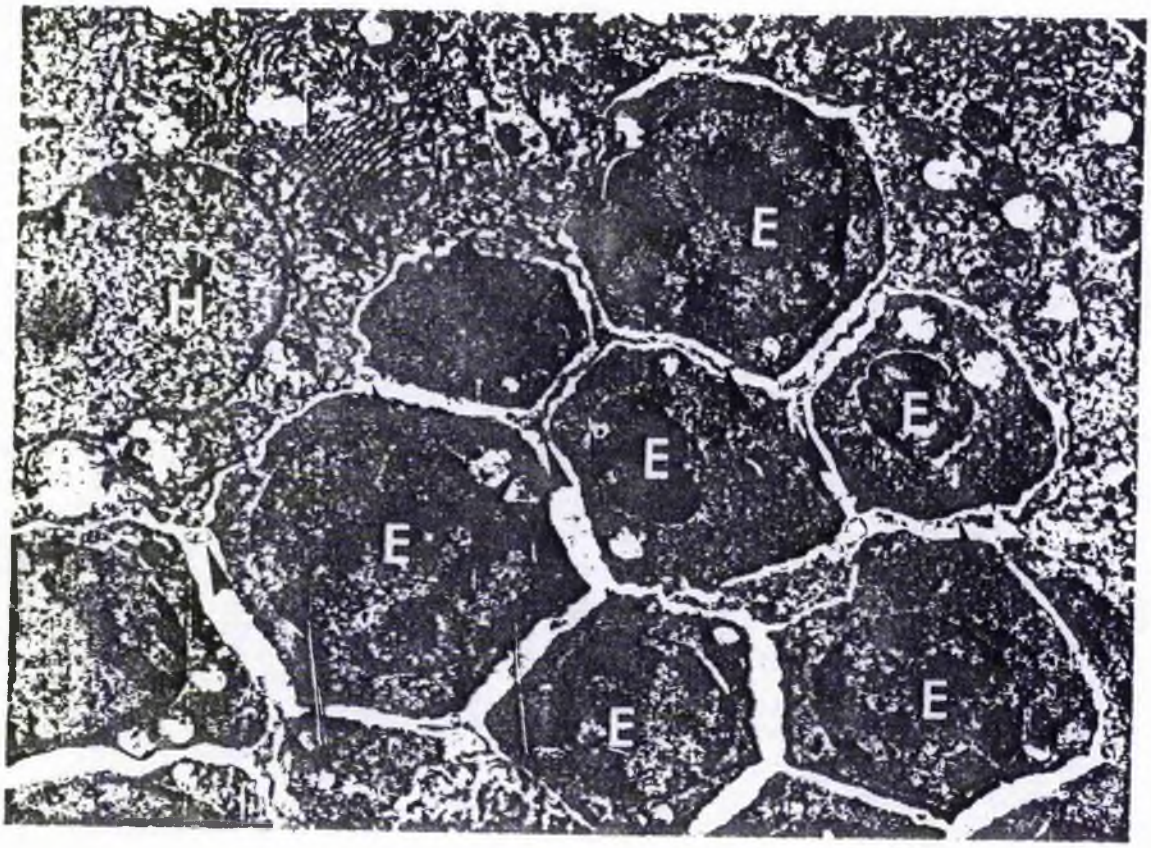
The cellular relationships which have been observed are compatible with the existence of environmental compartments in which single blood cell precursors or small groups of blood cell precursors can be subjected to the influence of regulatory factors in circumscribed microenvironments which are isolated from one another.

**An electron microscopical study of the relationships between differentiating blood cell precursors, macrophages and hepatocytes in murine fetal liver.** By ALLISON BLAIR, SAMANTHA SUISSA and D. BRYNMOR THOMAS, University of St. Andrews.

The interactions between blood cell precursors and the cells in their immediate vicinity, which appear to be of fundamental importance for the regulation of haematopoiesis (Dexter et al. Phil. Trans. Soc. Lond. **327B**, 1990), may be different in the liver from those in the bone marrow, reflecting different relationships in the two situations, which have been demonstrated in vitro by Gordon and her associates (Exp. Hematol. **18**, 1990). In the present investigation attention has been focused upon the relationships between blood cell precursors and stromal cells in murine fetal liver in vivo.

Samples of fetal liver were obtained from CBA mice on the fifteenth day of gestation, on the eighteenth day of gestation and on the day of birth; fixed in glutaraldehyde; postfixed in osmium tetroxide; embedded in araldite resin and prepared for examination using a Philips 301 electron microscope.

The cellular relationships which have been observed will be demonstrated. These relationships are consistent with extensive interchange of materials between hepatocytes (H) and blood cell precursors (erythroblasts E, fig. 1), as well as between macrophages and blood cell precursors, and are compatible with the existence of environmental compartments in which single blood cell precursors or small groups of blood cell precursors can be subjected to the influence of regulatory factors in circumscribed microenvironments which are isolated from one another by cytoplasmic extensions derived from stromal cells (arrows) (Blair and Thomas, J. Anat **237**, 1991).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**The Proliferative Status of Cells Derived from Murine Fetal Liver which form Colonies in vitro (high proliferative potential colony forming cells-HPP-CFC).** By ALLISON BLAIR, A.C. RICHES, S.N. ROBINSON and D. BRYNMOR THOMAS, School of Biological and Medical Sciences, University of St.Andrews.

The proliferative status of the cells derived from murine fetal liver which establish macroscopic colonies in the spleens of irradiated recipients (FL. CFU-S) is different from that of similar cells derived from murine bone marrow (BM.CFU-S). Whereas most of the FL.CFU-S are engaged in DNA synthesis, most of the BM.CFU-S are not. An in vitro assay has now been successfully used to study the kinetic properties of cells which are very similar to the kinetic properties of CFU-S (Robinson and Riches, Journal of Anatomy, 1991). In the present investigation this assay has been used to evaluate the kinetic properties of HPP.CFC derived from murine fetal liver during the last week of gestation and the first week of the neonatal period. The proportion of HPP.CFC in fetal liver which is synthesizing DNA falls from 53 ± 2% on day 15 of gestation to 13 ± 1% on
day 8 of the neonatal period, when it approximates to the proportion of HPP.CFC which is synthesizing DNA in the bone marrow of adult mice (10 ± 1%).

The high proportion of hepatic HPP.CFC synthesizing DNA during the latter pat of gestation coincides with the availability of a stem cell specific stimulator of DNA synthesis and the decrease in the proportion of HPP.CFC synthesizing DNA during the first week of the neonatal period coincides with the disappearance of this putative regulator (Dawood et al, Journal of Anatomy, 1990). The difference between the proliferative status of FL.CFU-S and that of BM.CFU-S is reflected in the difference between the proliferative status of HPP.CFC in murine fetal liver and that of HPP.CFC in the bone marrow of adult mice. The similarities between the kinetic properties of CFU-S and those of HPP.CFC derived from bone marrow which have previously been emphasised are reflected in similarities between the kinetic properties of CFU-S and those of HPP.CFC derived from murine fetal liver.

**The Adhesive Properties of Cells Derived from Murine Bone Marrow or from Murine Fetal Liver which form Colonies *in vitro* (high proliferative potential colony forming cells-HPC).** By ALLISON BLAIR and D.BRYNMOR THOMAS, School of Biological and Medical Sciences, University of St.Andrews.

The adhesive properties of blood cell precursors which form colonies in vitro (high proliferative potential colony forming cells-HPC) have been studied. The kinetic properties of these cells are similar to those of CFU-S to which they appear to be closely related (Blair et al, Journal of Anatomy 182: 129, 1993).

HPC and adherent stromal layers (ASL's) derived from murine fetal liver (FL) on the 15th day of gestation or from the bone marrow (BM) of young adult mice have been used for this purpose. HPC.FL and HPC.BM have each been incubated with FL.ASL's or BM.ASL's for 2 hours at 37°C. The reduction in the number of colonies established during the fourteen days following such incubation has been used to evaluate the adhesion of HPC to ASL's.

The loss of HPC, which can be attributed to adhesion, is similar when HPC.FL are incubated with FL.ASL's, HPC.BM are incubated with FL.ASL's or HPC.BM are incubated with BM.ASL's but the reduction in the number of colonies formed following the incubation of HPC.FL with BM.ASL's is very much greater suggesting preferential adhesion.

Such preferential adhesion of HPC.FL to BM.ASL's is consistent with the hierarchy of homing envisaged by Zanjani et al. (Blood 81: 399, 1993). The preferential adhesion which has been demonstrated would promote the in vivo transfer of haematopoiesis from the liver to the bone marrow.

**A mechanism which may promote the transfer of haematopoiesis from the liver to the medullary cavities.** By ALLISON BLAIR and D. BRYNMOR THOMAS. *School of Biological and Medical Sciences, University of St. Andrews*

The orderly transfer of haematopoiesis from extra-medullary sites to the medullary cavities during the course of ontogeny may be necessitated by a deterioration in the quality of the haematopoietic microenvironments available in the extra-medullary sites (Thomas *et al. Journal of Anatomy*, **155**, 1987); facilitated by preferential migration of blood cell precursors from the extra-medullary sites to the medullary cavities (Zanjani *et al. Blood*, **81**, 1993) and enhanced by the retention of blood cell precursors in the medullary cavities. The present analysis of the adhesion of early blood cell precursors to stromal cells maintained in vitro has been devised to contribute to an evaluation of the latter possibility.

Early blood cell precursors which form colonies in vitro (high proliferative potential colony forming cells = HPP.CFC) derived from murine fetal liver (FL) on the 15th day of gestation or from the bone marrow (BM) of young adult mice were incubated on adherent stromal layers (ASLs) derived from each of these two sources. Following the incubation of 5, 000 HPP.CFC on ASLs for 2 hours at 37°C:-

$27 \pm 1.6$ FL.HPP.CFC remained adherent to FL. ASLs

$31 \pm 2.5$ BM.HPP.CFC remained adherent to FL. ASLs

$21 \pm 2.9$ BM.HPP.CFC remained adherent to BM.ASLs

in contrast:-

$72 \pm 0.8$ FL.HPP.CFC remained adherent to BM.ASLs

similar results were obtained throughout the range from 500 to 5, 000 incubated HPP.CFC.

These results cannot be attributed to the difference in kinetic properties of HPP.CFC derived from fetal liver and from bone marrow. However they can be explained by supposing that the proportion of FL.HPP.CFC which are capable of adhering to ASLs is more than double the proportion of BM.HPP.CFC that are capable of doing so and that the number of binding sites available in unit area of BM.ASLs is more than double that for FL.ASLs. The remarkable affinity of FL.HPP.CFC for BM.ASLs is compatible with the notion that early precursors of blood cells are more likely to be retained and to accumulate in the medullary cavities than in fetal liver.