THE NATURE AND FUNCTION OF FIBROBLASTOID RETICULAR CELLS IN THE HEMOPOIETIC STROMA

DE AARD EN FUNCTIE VAN FIBROBLASTAIRE RETICULAIRE CELLEN IN HET HEMOPOIETISCHE STROMA

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE GENEESKUNDE
AAN DE ERASMUS UNIVERSITEIT ROTTERDAM
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. M.W. VAN HOF
EN VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN.
DE OPENBARE VERDEDIGING ZAL PLAATSVINDEN OP
WOENSDAG 6 NOVEMBER 1985 TE 15.45 UUR

DOOR

ALDERT HENRICK PIERSMA

Geboren te Soest

1985

Offsetdrukkerij Kanters B.V.,
Alblasserdam

Promotiecommissie:

Prof. Dr. O. Vos, promotor
Prof. Dr. J. Abels
Prof. Dr. D.W. van Bekkum
Prof. Dr. R. Benner
Dr. R.E. Ploemacher, co-promotor

Dit proefschrift werd bewerkt binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam.

Het onderzoek werd mede mogelijk gemaakt door financiële steun van de Nederlandse Stichting voor Medisch Wetenschappelijk Onderzoek (FUNGO).

aan mijn ouders

aan Marion

aan onze kinderen

CONTENTS

APPENDIX PAPERS Page

VOORWOORD/PREFACE

Gaarne wil ik op deze plaats al degenen bedanken die op welke wijze dan ook aan het tot stand komen van dit proefschrift hebben meegewerkt. Enkele van hen wil ik met name noemen.

Prof. Vos, ik ben U erkentelijk voor de mogelijkheden en de ruimte die U mij bood voor mijn werk. Ik deed nooit tevergeefs een beroep op U en dat waardeer ik in hoge mate.

Prof. Abels, Prof. van Bekkum en Prof. Benner dank ik voor de nauwgezette wijze waarop zij het manuscript van dit proefschrift hebben beoordeeld.

Rob Ploemacher, jouw gedegen kennis van zaken betekende een belangrijke steun in de rug. Ik ben je zeer dankbaar voor je betrokkenheid en voor de waardevolle suggesties waarmee je het onderzoek stimuleerde.

Kelvin Brockbank, I greatfully acknowledge your positive contributions to the present studies, particularly the stimulating discussions we had on the subject.

Cecile Ottenheim, jouw bijdrage in het laatste jaar van het onderzoek leverde een grote hoeveelheid resultaten op. Ik ben je bijzonder dankbaar voor de grote inzet waarmee je met name vele weefselcoupes maakte en kleurde.

Naast de twee laatstgenoemde dank ik ook mijn verdere kamergenoten Wim Molendijk, Karin Brakel-van Peer, Hans de Jong, Peter Nikkels, Jane Voerman en René Brons voor vele hand- en spandiensten, maar vooral voor de prettige sfeer waardoor ik met plezier terugdenk aan deze periode.

In het bijzonder dank ik Cary Meijerink-Clerkx, die het vele typewerk aan artikelen en proefschrift verrichtte. Telkens opnieuw verwonderde je mij door de snelheid en nauwgezetheid waarmee je werkte. Je was een onmisbare schakel bij het gereedkomen van dit proefschrift.

Els van Vliet en Marleen Melis dank ik voor hun inleiding in en hulp bij hybridoma- en ELISA- werk; Bep Smit dank ik dat ze mij chromosoompreparaten leerde maken en Pim Visser dank ik voor fraaie electronenmicroscopie en tekeningen.

De muizen en ratten konden rekenen op de goede zorgen van Joop Bos, Joop Brandenburg en Marry en Yvonne Steinvoort.

Tar van Os stond garant voor fotografisch werk van hoge kwaliteit met een opmerkelijke korte levertijd. Ook de audiovisuele dienst leverde een bijdrage in de fotografie. Rein Smid verzorgde vele bestellingen en Piet Hartwijk loste verschillende technische problemen op.

Mevrouw Godijn, Jopie Bolman, Lia de Wever en Marry Steinvoort zorgden dat schoon glaswerk steeds voorhanden was en droegen verder bij aan een goede sfeer door het zetten van thee en koffie.

Een aparte plaats neem jij in, Marion. Mijn waardering voor jouw zorg en toewijding is moeilijk in woorden te vatten. Je maakte een thuis voor de kinderen en mijzelf, en was een voortdurende stimulans voor mijn werk. Ik dank je voor je hulp bij het corrigeren van manuscripten, waarmee je verder bijdroeg aan een vlotte afronding van de promotie. Marten en Dorine dank ik voor de welkome afleiding die ze mij steeds boden, door te zijn wie ze zijn.

Mijn ouders ben ik dankbaar voor de wijze waarop ze mij onderwezen en lieten onderwijzen, waarmee ze me op de goede weg leidden. Behalve hen dank ik verdere familieleden en vrienden voor vele blijken van belangstelling.

ABBREVIATIONS

BFU-E	burst-forming unit erythrocytes
BPA	burst-promoting activity
CFU-c	colony-forming unit in culture
CFU-E	colony-forming unit erythrocytes
CFU-F	colony-forming unit fibroblasts
CFU-GM	colony-forming unit granulocytes/macrophages
CFU-MEG	colony-forming unit megakaryocytes
CFU-s	colony-forming unit in the spleen
Con A	concanavalin A
CSA	colony-stimulating activity
CSF	colony-stimulating factor
EPO	erythropoietin
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
HCGF	hemopoietic cell growth factor
HS-P	hemopoietic stroma for proliferation of CFU-s
IL-1	interleukin-1
IL-2	interleukin-2
IL-3	interleukin-3
M-CSF	macrophage colony-stimulating factor
SAF	stem cell-activating factor
TPO	thrombopoietin
TSF	thrombopoiesis-stimulating factor

GENERAL INTRODUCTION

1. HEMOPOIESIS

In the blood stream several types of mature blood cells can be recognized. These include erythrocytes, granulocytes, lymphocytes, monocytes and platelets. Their limited lifespan necessitates a continuous production of these cells throughout life. In mice, proliferation and differentiation of hemopoietic stem and progenitor cells occurs mainly in the bone marrow, but to some extent also in the spleen. Ever since the last phase of embryogenesis (Chapter 2) these organs contain undifferentiated pluripotent hemopoietic stem cells, each of which can give rise to all types of differentiated progeny. An important basic feature of these stem cells is their ability for self-renewal, i.e. they can give rise to other stem cells which are similarly undifferentiated. This concept guarantees the continuous availability of hemopoietic stem cells for the proliferation and differentiation into mature progeny. Selfrenewal, proliferation and differentiation in the hemopoietic system are regulated by humoral (Chapter 3) and stromal (Chapter 4) influences. Furthermore, these mechanisms account for hemopoietic cell requirements in stress situations (Chapter 5).

The knowledge about regulatory mechanisms in hemopoiesis has improved greatly since basic clonal assay systems became available during the sixties. In 1961, Till and McCulloch described the development of clonally derived nodules on spleens of lethally irradiated mice that had received a bone marrow cell graft. The number of nodules was proportional to the size of the graft (Siminovitch et al., 1963). These 'spleen colony-forming unit' (CFU-s)-derived nodules could contain erythrocytic, granulocytic, megakaryocytic and lymphocytic cells (Curry and Trentin, 1967; Fowler et al., 1967; Wu et al., 1967), as well as CFU-s, and were therefore considered hemopoietic stem cell-derived. Furthermore, CFU-s are capable of rescueing lethally irradiated mice (Metcalf and Moore, 1971). Recently, the validity of the CFU-s assay has been disputed with respect to the time after cell injection at which the spleen nodules should be counted. A proportion of these colonies have been shown to be transient (Magli et al., 1982), and nodules visible at day 12 seem to be derived from younger stem cells than day 8 colonies (Mulder et al., 1984). Furthermore, the cell cycle status seems to influence the lodging of stem cells in the spleen (Monette and DiMello, 1979). Nevertheless, the CFU-s assay is still the most widely used assay for measurement of hemopoietic stem cell numbers.

Another important development has been the establishment of *in vitro* clonal assays for hemopoietic cells. In 1966, Bradley and Metcalf as well as Pluznik and Sachs described the development in semisolid culture medium of granulocytic and monocytic cell clones from murine bone marrow cells. At present, also for the erythrocytic (Axelrad et al., 1974), megakaryocytic (Metcalf et al., 1975b; McLeod et al., 1976) and lymphocytic (Metcalf et al., 1975a; Rozenszajn et al., 1975) lineages clonal assays have been developed, each depending on the presence in the culture medium of specific regulator molecules. Furthermore, colonies containing two or more hemopoietic differentiation lineages can now be grown *in vitro*, which are derived from multipotent progenitor cells (Fauser and Messner, 1978; Hara and Ogawa, 1978; Metcalf et al., 1979; McLeod et al., 1980). These assays have greatly facilitated the study of regulation of hemopoiesis.

2. ONTOGENY OF HEMOPOIESIS

During embryogenesis hemopoietic stem cells (as measured by the spleen colony assay) can be found in several organs. The sites of earliest detection are the hemopoietic islands in the yolk sac, where CFU-s are present between days 8 and 12 of murine development (Moore and Metcalf, 1970). Between days 10 and 14 the peripheral blood contains detectable CFU-s numbers (Niewisch et al., 1970; Moore and Metcalf, 1970). At day 12 of development the embryonic liver becomes recognizable and readily contains CFU-s which decline in number onto day 15, peak again around birth and subsequently disappear from the liver (Silini et al., 1967; Niewisch et al., 1970). In the spleen CFU-s can be found from day 17 of development, the highest numbers being found at day 5 after birth. Eventually CFU-s numbers decline to the adult level of 2-3 CFU-s per 10^5 nucleated cells (Niewisch et al., 1970). From the 17th day of gestation onward the bone marrow becomes detectably populated with hemopoietic stem cells, and yields between 20 and 30 CFU-s per 10^5 nucleated cells during adult life (Metcalf and Moore, 1971). The peak in CFU-s numbers observed at day 11-12 of gestation in yolk sac, blood, and liver, coincides with fetal hemoglobin production. Conversely, the second liver CFU-s peak as well as splenic and bone marrow hemopoiesis are associated with adult hemoglobin synthesis (Niewisch et al., 1970).

The primary origin of hemopoietic stem cells has been a matter of dispute. Two theories have been put forward (Niewisch et al., 1970). Firstly, the unicentric theory proposes that the yolk sac stem cell is the common precursor of all other stem cells. The yolk sac stem cell-derived cells would be able to migrate into the embryo and to grow in the fetal liver, and finally build up adult hemopoiesis. This view is supported by the sequential presence of CFU-s in yolk sac, blood and fetal liver, respectively. Furthermore, yolk sac stem cells have been shown to be capable of repopulating the entire hemopoietic system of a lethally irradiated recipient (Moore and Owen, 1967; Tyan, 1968). Tissue culture experiments have suggested that cell migration from yolk sac to liver may account for the switch from fetal to adult hemoglobin production (Kovach et al., 1967; Barker, 1968).

Alternatively, the multicentric theory assumes that the stem cells of yolk sac, liver and spleen evolve independently from endogenous precursors (Niewisch et al., 1970). In this case, the yolk sac stem cell would vanish after differentiation into embryonic erythroblasts. Evidence for this theory was presented by Dieterlen-Lievre (1975) who grafted quail embryos on chick yolk sacs at a stage where circulation had not yet been established. In these 'yolk sac chimaeras' the hemopoietic organs were colonized by quail hemopoietic cells and not yolk sac cells. Further investigations on embryonic quail-chick chimaeras in which the relative contribution of both species to the chimaeras was varied, indicated that each hemopoietic organ rudiment is colonized by precursor cells derived from adjacent territories, being the mesoderm plate between the 10th and 17th somite (Martin et al., 1980). Actually, the intraembryonic ventral blood island mesoderm which is the direct extension of the extraembryonic yolk sac mesoderm, appears to contribute to an embryonic erythrocyte population that progessively declines during development (Turpen et al., 1981). On the contrary, the dorsal anterior mesoderm gives rise to the hemopoietic lineages found in closely related organs as the embryonic thymus, pro- and mesonephros, spleen and heart (Martin et al., 1980; Turpen et al., 1981). This concept of different origins of fetal and adult hemopoiesis mirrors the presence of fetal and adult hemoglobin respectively. In spite of these considerations, definitive proof for either theory has not yet been established (Turpen et al.,

1981). One could envisage that very early in development a unique determinative event gives rise to stem cells that eventually become distributed throughout dorsal, ventral and extra-embryonic mesoderm. In these sites they may subsequently give rise to progeny of fetal or adult type hemopoiesis at different stages of development, depending on the nature of their local microenvironment.

3. HUMORAL REGULATION OF HEMOPOIESIS

Self-renewal, proliferation and differentiation in the hemopoietic system are regulated by a variety of hormone-like factors. Many of these regulators influence hemopoiesis in a rather nonspecific way, such as prostaglandins and corticosteroids. Most of the factors to be reviewed here exert their activity in certain differentiation stages of a specific hemopoietic lineage.

3.1. Erythropoiesis

Erythropoietin (EPO) was the first specific hemopoietic regulator described (Reissman, 1950; Stohlman et al., 1954). This 60,000-70,000 dalton sialic acid-containing glycoprotein (Goldwasser and Kung, 1968), which is produced mainly in the kidney (Mirand and Prentice, 1957), induces erythroid differentiation. This effect can be easily detected in mice, when EPO is administered after suppression of erythropoiesis by hypertransfusion-induced polycythemia (Fried et al., 1957; Stohlman et al., 1968), starvation (Fried et al., 1957), or hypoxia (Cotes and Bangham, 1961). EPO induces and regulates hemoglobin synthesis in progenitor cells that are already committed to the erythroid lineage (Stohlman et al., 1968; Metcalf and Moore, 1971). EPO stimulates erythroid colony formation *in vitro* (Stephenson et al., 1971; Axelrad et al., 1974; Gregory, 1976). The sensitivity of erythroid progenitor cells to EPO increases during differentiation (Iscove, 1977; Wagemaker et al., 1977). Thus, the youngest *in vitro* clonable erythroid progenitor cell which forms multi-unit colonies *in vitro*, the erythroid burst-forming unit (BFU-E) is not responsive to EPO, whereas the erythroid colony forming unit (CFU-E) which is situated further down the differentiation pathway, does respond to EPO (Wagemaker et al., 1977).

Colony formation *in vitro* by BFU-E is dependent on a second erythroid regulator which has been termed burst feeder activity (Wagemaker et al., 1977), burst forming activity (Wagemaker, 1980) or burst-promoting activity (BPA). The activity was first detected in cultures stimulated by supernatant from pokeweed mitogen-stimulated murine spleen cell cultures (Johnson and Metcalf, 1977) and human leukocyte-conditioned medium (Aye, 1977), where colony formation by BFU-E appeared not correlated with EPO levels. BPA is also produced by T cells (Nathan et al., 1978), macrophages and monocytes (Murphy and Urabe, 1979; Zuckerman, 1980; Kurland et al., 1980) as well as adherent bone marrow (stromal) cells (Wagemaker et al., 1977; Gordon et al., 1980; Werts et al., 1980). BPA induces BFU-E to proliferate *in vitro* (Axelrad et al., 1978; Iscove, 1978; Linch and Nathan, 1984) resulting in progeny that acquires responsiveness to EPO in a few divisions (Wagemaker, 1980). Furthermore it stimulates hemoglobin synthesis (Porter and Ogawa, 1982). The responsiveness of erythroid cells to BPA declines with maturation (Wagemaker, 1980) which is the opposite of the sensitivity to EPO mentioned earlier. BPA has been partially purified (Iscove et al., 1982; Porter and Ogawa, 1982) from WEHI-3 cell line-conditioned medium and human bone marrow-conditioned medium. Only the latter preparation exclusively stimulated the erythroid differentiation pathway. The specificity of BPA, and its interchangeability with other colony-stimulating activities is still a matter of controversy (Iscove et al., 1982; Metcalf, 1984). An *in vivo* role for BPA was suggested by the finding that latex particle-induced changes in marrow BFU-E numbers were preceded by corresponding changes in BPA (Ploemacher et al., 1979).

3.2. Myelopoiesis

Granulocyte-macrophage colony formation *in vitro* is dependent on the presence of colony-stimulating factor (CSF). The existence of this factor was demonstrated by Pluznik and Sachs (1965) and Bradley and Metcalf (1966). CSF is found in serum (Robinson et al., 1967; Foster et al., 1968) and urine (Metcalf and Moore, 1971), whereas all tissues contain and synthesize detectable amounts of CSF (Nicola et al., 1979; Burgess and Metcalf, 1980). In mice, macrophages, mitogen-stimulated lymphocytes, fibroblasts (Pluznik and Sachs, 1965) and endothelial cells have been shown to produce CSF (Burgess and Metcalf, 1980). Biochemical studies (Metcalf and Moore, 1971) have indicated that CSF is a glycoprotein. The molecular weights of CSF preparations from different murine tissues appeared to be either 45,000 or 23,000 daltons. After removing sialic acid residues all molecular weights were found around 23,000 daltons (Nicola et al., 1979). At present a number of CSF preparations have been (partially) purified and were found to have a different spectrum of effects (reviewed by Metcalf, 1984). GM-CSF (Burgess et al., 1977) stimulates granulocyte-macrophage colony formation, but also the early stages of erythroid, eosinophil and megakaryocyte colony formation. M-CSF (Stanley and Heard, 1977) solely influences monocyte-macrophage colony formation. Since M-CSF is a prominent product of fetal tissue (Johnson and Metcalf, 1978) this may be the fetal form of CSF (Burgess and Metcalf, 1980). G-CSF (Metcalf and Nicola, 1983) essentially has the same effect profile as GM-CSF, but is weaker and transient for all precursors except a subset of granulocyte precursors (Metcalf, 1984). CSF is required for the *in vitro* survival and proliferation of granulocyte-macrophage progenitors (CFU-GM) (Burgess and Metcalf, 1980). CSF stimulates cycling and proliferation of CFU-GM in a dose-dependent manner (Burgess and Metcalf, 1980). Although the *in vitro* effects of CSF have thus been described in more detail the *in vivo* effects are still unclear (Burgess and Metcalf, 1980).

3.3. Thrombopoiesis

The first evidence for specific regulators of thrombopoiesis arose from experiments in which thrombocytopenic human serum induced a several fold increase in blood platelet counts two to three days after administration to normal mice (Kelemen et al., 1958). This finding has now been extended to many observations on megakaryocyte-stimulating activities in sera and urine from patients with thrombocytopenia and aplastic anemia (Enomoto et al., 1980; Hoffman et al., 1981). The active substance, named thrombopoietin (TPO) (Kelemen et al., 1958) was heat-labile and non-dialyzible. Later on evidence was presented that a factor with similar characteristics, present in spleen-conditioned medium, could stimulate *in vitro* megakaryocyte colony formation (Metcalf et al., 1975b). Other sources for this factor, which is also being referred to as megakaryocyte CSF (Lieschke, 1982) or thrombopoiesis-stimulating factor (TSF; Enomoto et al., 1980) are media conditioned by WEHI-3 cells (Williams et al., 1978), L-cells (Nakeff and Daniels-McQueen, 1976), a liver cell line (Penington, 1979), and certain EPO-preparations (McLeod et al., 1976). Yet, TPO and EPO can be separated since TPO is not sensitive to neuraminidase-treatment whereas neuraminidase abolishes EPO-activity *in vivo* (Goldwasser et al., 1974; Enomoto et al., 1980). Furthermore, EPO has been reported to inhibit megakaryocyte colony formation *in vitro* under certain conditions (Messner et al., 1982). The *in vitro* formation of megakaryocyte colonies has been shown to be dependent on two factors (Williams et al., 1978, 1982; Levin, 1983). TPO, a 35,000 dalton molecule, promotes megakaryocyte

maturation, whereas another substance described as the 100,000 dalton 'megakaryocyte potentiator' (Williams et al., 1982) seems necessary for triggering the CFU-MEG into proliferation (Levin, 1983). This concept of two regulators acting in different differentiation stages is analogous to the situation described for erythropoiesis.

3.4. Lymphopoiesis

Lymphopoiesis occurs in the thymus for T cells and in the bone marrow for B cells. In birds the Bursa of Fabricius supports B cell differentiation. Removal of thymus and/or bursa has profound depleting effects on lymphopoiesis and immunocompetence (St.Pierre and Ackerman, 1965; Miller and Osaba, 1967). These functions can be partially restored by reimplantation, infusion of tissue extracts and implantation of diffusion chambers containing tissue of these organs (St.Pierre and Ackerman, 1965; Metcalf and Moore, 1971). The last finding is suggestive of the existence of specific humoral factors for T and B cell differentiation. In vitro clonal growth of B and T lymphocytes (Metcalf et al., 1975a; Rozenszajn et al., 1975) is possible in the presence of mitogens such as phytohaemagglutinin (Rozenszajn et al., 1975), lipopolysaccharide, purified protein derivative or dextran sulphate (Rozenszajn et al., 1977). However, medium from mitogen-stimulated lymphocyte cultures was also able to sustain colony formation, thus providing evidence for a lymphocyte-derived growth factor (Sredni et al., 1978; Watson et al., 1979). At present, two factors are known for their T lymphoid colony-stimulating activity. Interleukin-1 (IL-1), a 10,000-30,000 dalton molecule which is produced by antigen- or mitogen-stimulated monocyte-macrophages, stimulates production by T helper cells of Interleukin-2 (IL-2), a 90,000-100,000 dalton molecule. In addition, IL-1 induces IL-2 receptor expression in antigen- or mitogen-stimulated T helper and T killer cells, which thus become sensitive to the proliferative stimulus of IL-2 (Rozenszajn et al., 1981; Smith, 1984). B lymphocyte production has also been shown to depend on growth and differentiation factors, but these studies are relatively preliminary as compared to those on T lymphocyte regulators (Smith, 1984).

3.5. Stem cell proliferation

The selfrenewal of hemopoietic stem cells is regulated by at least one humoral factor. In 1974, Cerny described an activity in medium conditioned by activated T cells that stimulated stem cell proliferation in vivo and in vitro. Furthermore, in vitro multiplication of stem cells could be induced by embryonic fibroblast-conditioned medium (Löwenberg and Dicke, 1977) human leukocyte-conditioned medium (Wagemaker and Peters, 1978), post-endotoxin serum and murine spleen-conditioned medium (van Bekkum et al., 1979). Initial experiments indicated that this stem cell activating factor (SAF) was separable from CSF on the basis of a smaller molecular weight (<10,000; Löwenberg and Dicke, 1977).

A second factor which also stimulates hemopoietic stem cell proliferation is called Interleukin-3 (IL-3). This factor was initially defined as the factor in Con A-spleen-conditioned medium that induces the 20α-hydroxysteroid-dehydrogenase in cultures of immature splenic T lymphocytes (Ihle et al., 1981). IL-3 is essentially separable from CSF, promotes differentiation of 'early stem cells' and supports the in vitro production of CFU-s (Ihle et al., 1983).

In a third independent line of research medium conditioned by the myelomonocytic cell line WEHI-3 was shown to contain an activity that promoted the growth of very young committed progenitor cells (Greenberger et al., 1979;

Dexter et al., 1980). This hemopoietic cell growth factor (HCGF) was shown to be distinct from CSF (Dexter et al., 1980). Recently convincing evidence has been obtained indicating that SAF, IL-3 and HCGF are identical or at least very closely related moieties (Dorssers et al., 1983; Garland and Dexter, 1984). They support stem cell proliferation and early progenitor proliferation, but essentially lack the CSF effect on later progenitors.

4. STROMAL REGULATION OF HEMOPOIESIS

In the hemopoietic organs blood-forming cells are packed within a stromal framework built up by a number of cell types and an intercellular matrix. Several observations have indicated a specific role for the hemopoietic stroma in the regulation of hemopoiesis. Stromal cells, including endothelial cells, macrophages, reticular fibroblasts, and adipocytes, have a variety of regulatory functions. Apart from mechanical support, they control migration of hemopoietic cells and play a role in regulation of hemopoietic differentiation. These functions are mediated by humoral factors as well as cellular interactions. This chapter briefly reviews structural and functional aspects of the hemopoietic stroma in bone marrow and spleen.

4.1. Evidence for the existence of a functional hemopoietic stroma

The most obvious evidence for a specific hemopoietic stroma is the fact that hemopoiesis is restricted to certain organs. The restriction is already evident during ontogeny, where yolk sac, liver, spleen and bone marrow are consecutively populated with hemopoietic cells. In adult mice the bone marrow is the most important site of hemopoiesis, but also the spleen contributes to hemopoiesis. In irradiated mice, hemopoietic cells home preferentially in bone marrow and spleen after intravenous infusion (Till and McCulloch, 1961). Apparently, in hemopoietic tissue, a stroma exists which possesses the proper unique set of requirements to sustain hemopoiesis. This is further stressed by findings which have been interpreted to suggest that within the stroma 'niches' exist that maintain hemopoietic cells or stimulate them into a certain differentiation pathway (Trentin, 1970, 1971). When lethally irradiated mice are injected intravenously with bone marrow cells, in a few days hemopoietic colonies develop in the stroma of spleen and bone marrow. These colonies are at first restricted to one hemopoietic lineage, depending on the type of niche they have filled. Upon expansion the colonies become multilineage in character, new lineages being formed at the periphery of the colonies, where progenitor cells are pushed into other niches with different stimulating potential (Trentin, 1970, 1971). On the whole, within the spleen erythroid colonies outnumber granuloid colonies (3:1), whereas in the bone marrow the reverse is true (1:2) (Curry et al., 1967; Wolf and Trentin, 1968).

When the marrow cavity is mechanically evacuated hemopoiesis is not resumed before establishment of a regenerated stroma (Tavassoli et al., 1974; Patt and Maloney, 1975). High dose local irradiation causes a late and permanent aplasia as a result of stromal injury (Knospe et al., 1968). In some cases, aplastic anemia patients do not benefit from transfused identical twin-derived bone marrow cells, possibly due to stromal deficiency (Appelbaum et al., 1980). All these studies strongly suggest stromal requirements for hemopoiesis.

Studies in which stromal tissue is transplanted to ectopic sites provide further information about the specific function of the hemopoietic stroma. Ectopically transplanted marrow or spleen pieces firstly reestablished their stroma before hemopoietic cells were trapped and hemopoiesis became detectable (Tavassoli and Crosby, 1968). After transplantation of whole spleens eventually the same erythroid:granuloid ratio is found as in the normal spleen (Wolf and Trentin, 1968; Ploemacher et al., 1982). When cellulose acetate membranes, coated with adherent cells derived from the hemopoietic stroma, were implanted in the peritoneal cavity they supported hemopoietic cell growth to a larger extent than when such membranes were coated with other adherent cells

(Knospe et al., 1978). Furthermore, implantation under the kidney capsule of fibroblastoid stromal cells of spleen or bone marrow origin lead to the formation of hemopoietic foci with stroma characteristic of the respective donor organs (Friedenstein et al., 1974a). These studies suggest that the implanted material contained specific properties of stromal elements.

Two mutant mouse strains, Sl/Sl^d and W/W^v are useful experimental models for the study of the hemopoietic stroma. Both strains are anemic, the Sl/Sl^d has a stromal defect but normal hemopoietic cells, whereas the W/W^v strain has defective hemopoietic stem cells and a normal stroma. Both systems are cross-correcting both in vivo and in vitro (Bernstein et al., 1968; Tavassoli et al., 1973; Dexter and Moore, 1977), again suggesting an important role for the hemopoietic stroma in the regulation of hemopoiesis.

4.2. Structure of the hemopoietic stroma

The hemopoietic tissue of the bone marrow is surrounded by a reticular cell layer covering the internal surface of the bone. Besides reticular cells, this layer also contains osteoblasts, macrophages, lymphocytes, as well as hemopoietic stem cells (Weiss and Sakai, 1984). The bone-lining reticular cells are connected with the meshwork of reticular cells present throughout the marrow parenchyma. These cells are closely associated with the marrow sinuses, where exchange between bone marrow and blood occurs. The blood supply of the bone marrow is delivered by the nutrient artery, that enters the marrow through the nutrient canal in the cortical bone (Lichtman, 1981). It branches into descending and ascending arteries, from which radial arteries again enter the bone canalicular system. These bone capillaries, which are further supplied with arterial blood from periosteal arterioles and muscular arteries, enter the marrow sinuses. The sinuses eventually join in the central sinus which leaves the marrow and leads into the venous circulation. Hemopoiesis takes place in the spaces between the highly branching sinusoidal network. Sinuses are composed of a complete luminal layer of endothelial cells and an incomplete abluminal layer of adventitial reticular cells, separated by an interrupted basement membrane. The endothelial cells are broad and flat, often overlapping or interdigitating, and interconnected with cellular junctions (Tavassoli, 1979). These cells have very thin areas, where both plasma membranes lie in close approximation. These areas provide sites of low resistance where holes in the endothelium may arise through which hemopoietic cells may penetrate into the blood stream. In addition these fenestrae as well as endocytotic activity in these cells provide mechanisms whereby materials from the blood stream can enter the hemopoietic tissue (Lichtman, 1981). The adventitial reticular cells form a discontinuous cell layer at the abluminal side of the sinuses. They are in close contact with the endothelium on the one hand, and with hemopoietic cords on the other. These cords are supported mechanically by reticular fiber-producing reticular cells. Cell traffic between hemopoietic tissue and blood passes by the adventitial reticular cells. These cells have been shown to be capable of major volume changes in response to hemopoietic stress, and may thus regulate hemopoietic cell egress into the blood (Weiss, 1970). Reticular cells possess organelles associated with protein synthesis (Weiss and Sakai, 1984). It is possible that not only fibrous matrix is produced but also mediators of hemopoietic differentiation. The intimate association of the reticular cell network with hemopoietic cells favours interactions of this kind. Erythroblastic islets and megakaryocytes lie just outside sinuses. Granulocytic progenitors lie in the midst of hemopoietic cords. Lymphocytes, monocytes and likely stem cells are clustered around arterial vessels. Macrophages occur throughout the marrow (Weiss, 1976) and also con-

tribute to the hemopoietic stroma. They are found as central cells in erythroblastic islands, and take part in the adventitial cell layer as well as the reticular network that supports the hemopoietic cords (Weiss, 1976). Fat cells, probably arising from adventitial reticular cells that accumulate fat, are situated outside sinuses. Murine marrow contains very few of these adipocytes, in comparison to for instance rat or human marrow (Lichtman, 1981). The number and size of adipocytes control hemopoietic volume, a mechanism nicely suited for a rigid bone-lined organ (Weiss and Sakai, 1984). The arterial vessels in the marrow are accompanied by myelinated and non-myelinated nerve fibers which regulate the tonus of arteries. The Schwann cells of myelinated fibers comprise another reticular cell type in the bone marrow (Lichtman, 1981). The structure of the bone marrow in addition to its specific functional characteristics make this organ the primary site of hemopoiesis.

The spleen possesses a capsule of connective tissue with fibroblastic cells and extracellular matrix, analogous to the bone-lining reticular cells in the bone marrow. From the capsule trabeculae of similar composition penetrate the inner structure of the spleen, and provide further mechanical support. The capsule contains smooth muscle which can cause rapid efflux of blood cells (Weiss and Sakai, 1984). The splenic blood supply is delivered by the splenic artery which enters the spleen at the hilus and branches off in trabecular arteries. These vessels further ramify into central arteries around which the white pulp, the lymphoid compartment of the spleen, is situated. The central arteries branch into terminal arterioles and capillaries that end in the red pulp, the non-lymphoid hemopoietic compartment, or in the marginal zone between red and white pulp. There the capillaries either end freely in the splenic parenchyma (the open circulation) or extend into the network of splenic sinuses (the closed circulation) (Chen and Weiss, 1973). In the red pulp efferent blood is collected in sinuses that come together in trabecular veins and finally in the splenic vein that leaves the spleen at the hilus. As in the bone marrow endothelium-associated reticular cells build up the parenchyma of the splenic hemopoietic tissue in the red pulp. They produce a supporting extracellular matrix, and adhere to one another by means of junctional complexes. Reticular cells are contractile, they contain microfilaments, and are innervated by adrenergic nerves that are supported by axon-bearing reticular cells (Weiss and Sakai, 1984). The contractility may be important in squeezing stored blood out of the spleen. The splenic red pulp further contains macrophages that, in addition to reticulum and endothelium, are involved in regulation of the considerable cellular traffic in the spleen. Hemopoietic cells are distributed in a characteristic pattern. Erythroblastic islets are found throughout the red pulp. Granuloid precursors are situated in proximity to trabecules and capsule. Megakaryocytes are mainly associated with the splenic capsule. These patterns imply the existence of different hemopoietic niches presented by different stromal cell populations in different sites within the hemopoietic stroma.

The structure of bone marrow and splenic stroma are strikingly similar. Yet there are major differences (Weiss, 1976). The bone marrow has a closed circulation whereas the splenic red pulp contains both open and closed circulation. Thus, the splenic parenchyma may be considered as part of the blood stream, such in contrast to the marrow stroma. The splenic cords contain more mature blood cells whereas the marrow stroma is mainly filled with immature hemopoietic cells. The endothelium is more cuboidal with a more rigorous basement membrane and also reticular fibers are more abundant in spleen than in bone marrow. Cell traffic throughout the splenic sinus occurs primarily from outside in and the pathway is between cells, whereas the sinuses of the marrow support two-way traffic and transmural cell passage occurs through

their cytoplasma rather than between cells. These differences in stromal structure underlie differences in hemopoietic regulation, and might explain why in the bone marrow erythropoiesis is less prominent than granulopoiesis in a ratio of 1:2 (Wolf and Trentin, 1968), whereas the spleen favors erythropoiesis over granulopoiesis in a ratio 3:1 (Curry et al., 1967).

4.3. Cell types in the hemopoietic stroma

Endothelial cells form the inner lining of the sinuses in the hemopoietic stroma. These cells express the factor VIII-related antigen on their surface, have substantial amounts of alkaline phosphatase, and synthesize collagen types I and IV in addition to fibronectin and laminin. These characteristics discriminate these cells from other stromal cell types. The primary function of endothelium is the formation of a border between blood and hemopoietic tissue. Accordingly, interchange between both compartments is regulated by these cells. Hemopoietic cell egress from the marrow parenchyma is thought to occur through fenestrae in the endothelial cells (Lichtman, 1981). These fenestrae can be associated with microfilaments (De Bruyn et al., 1971) suggesting an active role for the endothelium in cell traffic. Endothelial cells have active endocytosis, through which the hemopoietic stroma can also be exposed to externally derived materials (De Bruyn et al., 1975). Umbilical cord vein endothelium has been shown to produce GM-CSF (Knudtzon and Mortensen, 1975); if this is also true for stromal endothelium, these cells may contribute to hemopoietic cell differentiation. The notion that microcirculation is essential for hemopoiesis stems from experiments in which the marrow was locally irradiated with a high radiation dose (Knospe et al., 1966, 1968). This treatment resulted in an immediate aplasia due to hemopoietic cell death, followed by complete repair through influx of hemopoietic cells within a few weeks. However, a secondary aplasia followed which was caused by a destroyed microcirculation. Thus an adequate sinusoidal endothelium is required for the continuation of hemopoiesis.

Macrophages are the only stromal cells that develop from hemopoietic cells. They are derived from monocytes that may have circulated in the blood before differentiation into mature stromal macrophages. Specific hemopoietic cell markers distinguish macrophages from other stromal cells. These include Mac-1 (Springer et al., 1979) and the Ia antigen which is correlated with the antigen-presenting function of macrophages to lymphocytes (Calderon et al., 1975). Furthermore, these cells are characterized by abundant acid phosphatase activity. Their phagocytic capacity makes these cells important in clearing debris from the hemopoietic tissue. They contribute to the hemopoietic reticulum by production of collagen type I (Bentley, 1984). Macrophages are often centrally located in erythroblastic islets (Bessis, 1958), and enclose erythroblasts with long extended membranous processes (Ploemacher et al., 1977). The central macrophage is phagocytic, its heterolysosomes may contain erythroblast nuclei. Erythroblastic islets are located close to the adventitial layer of the sinusoids. Macrophages can extend into the sinusoidal lumen, thus regulating cellular traffic into and out of vessels (Weiss and Sakai, 1984). Macrophages secrete regulatory molecules for hemopoiesis, such as CSF which is stimulatory, and prostaglandin E_2 which is inhibitory for myelopoiesis (Kurland and Moore, 1977).

The reticular cell is the predominant stromal cell type in the hemopoietic tissue. It includes the adventitial reticular cells of the sinuses and other vessels, the reticular cells that form the bone-lining cell layer in the marrow and the splenic capsule and trabeculae, and reticular cells forming the adventitia of nerve sheats. The reticulum which they provide consisting of

highly-branching reticular cells plus extracellular matrix, is the major internal mechanical backbone of the hemopoietic stroma. In recent years there has been an extensive discussion about the nature and terminology of reticular cells (Weiss and Chen, 1975; Castro-Malaspina et al., 1980; Lichtman, 1981; Xu et al., 1983). This discussion arises from the notion that these cells do not seem to have specific characteristics that are not shared by any other cell type. Thus, reticular cells are merely described morphologically, and by the lack of characteristics that are specific for other stromal cell types (Tavassoli and Friedenstein, 1983). Reticular cells derive their name from their ability to produce reticular fibers that can be visualized using silver staining. These fibers contain collagen type III. Upon in vitro explantation bone marrow and spleen cells can form an adherent cell layer (to be discussed in more detail elsewhere) consisting largely of fibroblastic cells. They are probably derived from the reticular cell populations in the hemopoietic stroma. Detailed characterization of these fibroblastic cells has shown that they are neither macrophages nor endothelium. They produce collagen types I and III (Castro-Malaspina et al., 1980; Bentley, 1984), they synthesize fibronectin, whereas at least a subpopulation contains high levels of alkaline phosphatase (Westen and Bainton, 1979). They do not carry the factor VIII-related antigen, nor do they produce laminin or collagen type IV, being specific characteristics for endothelial cells. Furthermore, studies of chromosome composition of marrow cultures in human myeloproliferative diseases (Maniatis et al., 1969; Van Slyck et al., 1970; de la Chapelle et al., 1973; Jacobson et al., 1978) and transplantation studies in rodents (Friedenstein et al., 1978) indicate that these cells are not derived from the hemopoietic stem cells. They further do not carry the Mac-1 antigen for the granulocyte-macrophage lineage, are not phagocytic and contain hardly detectable acid phosphatase. These features discriminate reticular cells from macrophages. Reticular cells have a slow turn over under normal circumstances (Haas et al., 1969; Weiss and Sakai, 1984), and are relatively radioresistant (Friedenstein et al., 1974b).

Apart from providing the framework of the hemopoietic tissue these cells are involved in the regulation of cell traffic between the blood stream and the hemopoietic tissue. However, little is known about regulatory functions of this cell type with respect to hemopoiesis, although reticular cells are the most widely distributed stromal cells. Fibroblastoid reticular cells may carry specific properties which are related to the presence of hemopoiesis. Evidence for this notion was provided by Friedenstein et al. (1974a) who implanted cultured fibroblastoid cells derived from bone marrow and spleen under the kidney capsule. The cell pellets gave rise to stromal tissue representative of the organ of origin. Bone marrow fibroblasts would give rise to bone and stroma formation. This focus attracted host-derived hemopoietic cells and hemopoiesis became established. Splenic fibroblasts developed into a reticular stroma, that attracted host hemopoietic cells that gave rise predominantly to lymphopoiesis. It is possible that reticular cells provide specific sites or 'niches' within the stroma where pluripotent hemopoietic stem cells reside (Schofield, 1978), fostered via specific cellular interactions by the surrounding microenvironmental cells.

Cultured fibroblastic stromal cells have been shown to produce CSA (Wilson et al., 1974; Brockbank and van Peer, 1983) and support in vitro hemopoiesis (Zipori et al., 1981a, b, c). The latter authors have studied the interaction between stromal cell layers and myeloid progenitor cells. These authors observed inhibition of granulocyte-macrophage colony formation when hemopoietic and stromal cells were in direct contact. An agar layer placed between both cell populations or addition of monosaccharides to the cultures relieved the

inhibition. These data led to the conclusion that cell-bound glycoproteins or glycolipids were involved in the inhibition of colony formation. Furthermore, Zipori and Sasson (1981c) found compact or tight colonies which contained relatively many myeloid precursor cells, suggesting that self-renewal of progenitor cells was being stimulated in their cultures. Blackburn and Patt (1977) described an increased maintenance of hemopoietic stem cells over a 24 hr period when incubated in medium conditioned by bone marrow-derived fibroblastoid cells. The relation between this effect and the possible presence of IL-3 is as yet unknown.

One difficulty with the interpretation of *in vitro* studies employing primary fibroblastoid adherent stromal cell cultures is the presence of macrophages and endothelial cells in the adherent cell layers. The regulatory properties of these cell types may have contributed to observations such as those reviewed above. This notion has led to studies employing stromal cell lines for the description of interactions between hemopoietic cells and fibroblastoid stromal cells *in vitro*. By the use of stromal cell lines contamination with other cell types can be precluded. However, apart from the consideration that not all cellular functions *in vitro* may be readily extrapolated to the *in vivo* situation, such studies start from the questionable assumption that these putatively transformed lines still possess their 'normal' regulatory functions. Several fibroblastic cell lines with regulatory capacities for *in vitro* hemopoiesis have been described (Cronkite et al., 1982; Kodama et al., 1982; Lanotte et al., 1982a,b; Hines, 1983). Such lines usually produce colony-stimulating factors. The production rate thereof may be related to cell cycle status. Lanotte et al. (1982b) found highest CSF production during the period of growth arrest. Garnett et al. (1982) described a prostaglandin-like inhibitor of granulocyte/macrophage colony formation, produced by a preadipocyte cell line. Proliferation of hemopoietic stem cells was stimulated by a preadipocyte line (Kodama et al., 1982) and this effect was mediated by cellular contacts between the cell line and hemopoietic cells. Thus stromal cell lines may be useful tools in investigations of interactions between hemopoietic and stromal cells.

Adipocytes in the hemopoietic stroma are often located parasinal, which makes it likely that these cells originate from adventitial reticular cells by adipogenesis (Weiss and Chen, 1975; Lichtman, 1981). They control the hemopoietic volume, aromatize testosterone to estrogen, and appear to induce neutrophilic granulopoiesis (Lichtman, 1981). Furthermore, adipocytes seem to inactivate CSF (Mendelow et al., 1980). *In vitro* adipogenesis in fibroblastic stromal cells requires the presence of hydrocortisone in culture. However, unlike preadipocytes from other organs marrow fat cells are not induced by insulin (Greenberger, 1978; 1979). Adipose conversion is a common characteristic of many fibroblastoid stromal cell lines (Cronkite et al., 1982; Lanotte et al., 1982a; Kodama et al., 1982; Hines, 1983). Lanotte et al. (1982b) found a decrease in CSA-production during fat accumulation. The role of fat cells in *in vitro* hemopoietic systems has been a matter of dispute (Touw and Löwenberg, 1983).

4.4. Functional assays for the hemopoietic stroma

The most straightforward way to measure the capacity of the hemopoietic stroma to sustain hemopoiesis is the measurement of hemopoietic stem cell numbers during and after recovery from potentially damaging treatments. The rate of recovery of CFU-s numbers as well as the final plateau CFU-s numbers per organ can be taken as measures for stromal function provided that the stem cell population is not damaged. This assay has been applied extensively

to study radiation effects on hemopoietic stroma (Knospe et al., 1968; Vos, 1972; Kedo et al., 1976; Ploemacher et al., 1983). It gives a direct measure of stromal function *in situ* without the necessity of stromal cell proliferation. In this respect this assay differs from most if not all presently available assays for stromal function.

When intact hemopoietic organs, spleen or femur, are implanted subcutaneously, the hemopoietic cells disappear from the grafted organ and stromal cells die to a large extent. This is followed by clearance of cellular debris, proliferation of residual mesenchymal tissue, formation of sinusoids and finally reconstruction of hemopoiesis (Tavassoli and Crosby, 1968; Maniatis et al., 1971; Amsel and Dell, 1971). The newly established stroma is likely to be donor-derived, whereas the hemopoietic cells are host-derived (Amsel and Dell, 1971; Friedenstein et al., 1968; Fried et al., 1973b). The extent to which hemopoietic progenitor cells grow in these grafts is determined by the functional capacity of the stroma. Thus, the number of hemopoietic progenitors (CFU-s or CFU-c) in these grafts present six weeks after subcutaneous implantation is taken as a measure for stromal function (Chamberlin et al., 1974). Fried et al. (1976) have referred to this assay as measuring the functional capacity of the hemopoietic stroma to sustain proliferation of CFU-s (HS-P), suggesting that merely the proliferation of CFU-s is quantitated. However, also lodging and survival of CFU-s contribute to the actually measured CFU-s numbers, so that one should rather speak of the hemopoietic stroma for 'accumulation' of CFU-s. Besides these considerations it should be noted that this assay is critically dependent on the proliferation of surviving stromal cells for the establishment of a new functional stroma, and therefore can be considered as an assay for the proliferative capacity of stromal cells. This implant regeneration assay has been applied in several experimental situations. Radiation damage to the stroma has been studied extensively using this technique (Fried et al., 1973b, 1976; Chamberlin et al., 1974; Kedo et al., 1976; Ploemacher and van Soest, 1979) and furthermore the stroma of mice with inherited anemias has been analyzed with this assay (Fried et al., 1973a; Ploemacher and Brons, 1984).

Alternatively, stromal function can be assayed by allowing bone marrow or spleen cells to form an *in vitro* adherent cell layer which can support the proliferation of hemopoietic stem and progenitor cells (Dexter et al., 1976; Keller et al., 1983). Within two or three weeks after inoculation an adherent cell layer is formed which is composed of endothelial cells, macrophages, reticular cells and adipocytes (Allen and Dexter, 1976; Dexter et al., 1977). These stromal cell layers can be grown in such a way that they are devoid of hemopoietic cells (Greenberger et al., 1983). When normal bone marrow cells are added as a second inoculum to these adherent cell cultures, the supporting capacity of the adherent layer for proliferation of hemopoietic cells can be measured by quantifying hemopoietic (progenitor) cell production at weekly intervals. Hemopoiesis in such cultures can last for several months and can only be effective when stromal cell layers from hemopoietic tissues are present: in the absence of adherent cells, or in the presence of embryonic fibroblasts no longterm maintenance of hemopoiesis is found (Dexter, 1982; Greenberger et al., 1983). Longterm bone marrow cultures are dependent on the proliferation of stromal cells for the establishment of an adherent cell layer. Therefore, similar to the implant regeneration assay discussed earlier, the proliferative capacity of the stromal cells is a parameter that influences the results of this assay when employed to measure *in vivo* inflicted damage. Proliferation of stromal cells is not necessarily involved when established adherent cell layers are treated in order to study *in vitro* damage. The composition of the adherent layers with respect to the relative contribution of

several cell types may be different from the in vivo situation, depending on the in vitro proliferative capacities of these cell types. Thus, effects measured in longterm bone marrow cultures may not be applicable to the in vivo situation a priori. Nevertheless, this system allows detailed analysis of hemopoietic-stromal cell interactions in vitro (Allen, 1981). Dexter and Moore (1977) demonstrated duplication of the Sl/Sl^d defect. Furthermore, the effects of in vivo and in vitro induced damage by e.g. irradiation (Reincke et al., 1982; Greenberger et al., 1982) or cytostatic agents (Hays et al., 1982; Anderson et al., 1982), and the effects of virus particles in these cultures have been studied extensively (Greenberger et al., 1983).

During the early seventies culture techniques have been described in which fibroblastoid colonies were grown from hemopoietic and non-hemopoietic organs (Friedenstein et al., 1970, 1974b; Luria et al., 1971; Metcalf, 1972; Wilson et al., 1974). These colonies developed in foetal calf serum-containing medium without specific growth stimulators, and were either grown in liquid medium or semisolid agar or methylcellulose-containing medium. The colonies were composed of cells with a fibroblastoid morphology and are usually referred to as derived from fibroblastic colony-forming units (CFU-F). In most studies the CFU-F is defined as a cell which has the ability to form a colony containing at least fifty fibroblastoid cells within ten days of culture. Extensive characterization of these CFU-F colonies has indicated major differences between laboratories, especially with regard to the presence of macrophages and endothelial cells (Castro-Malaspina et al., 1980; Xu et al., 1983; Kaneko et al., 1982), which may in part be due to species differences. The application of semisolid medium seems to diminish the number of adherent macrophage colonies. Within a certain range of plating concentration there exists a linear relationship between the number of cells plated and the number of CFU-F colonies developing (Metcalf, 1972; Friedenstein, 1976; Brockbank et al., 1983b). Therefore the CFU-F assay allows quantitative determination of fibroblastoid progenitors. The numbers of CFU-F per million nucleated bone marrow cells vary between 1.5 (Friedenstein et al., 1970) and 50 (Wilson et al., 1974). The fibroblastoid cells within a CFU-F colony are most likely derived from reticular stromal cells. From the CFU-F numbers mentioned above it is clear that only a small fraction of reticular cells do form an in vitro CFU-F colony. It should be noted that reticular stromal cells are a cell population that turns over slowly in situ (Haas et al., 1969) which on the one hand may be an explanation for the relatively low CFU-F numbers measured, and on the other hand stresses that the assay is selective for a subpopulation of stromal cells which is able to proliferate in vitro. Furthermore, the proliferative capacity of stromal cells which is measured in the CFU-F assay, may or may not be correlated to their functional properties with respect to the regulation of hemopoiesis. The CFU-F assay has been used to study the effects on the hemopoietic stroma of radiation (Friedenstein et al., 1976; Wilson et al., 1974), cytostatics (Wilson et al., 1974; Haworth et al., 1982; McManus and Weiss, 1984), bleeding and immunization (Friedenstein et al., 1974b), bacterial endotoxin (Brockbank et al., 1983a), as well as aging (Mets and Verdonk, 1981; Brockbank et al., 1983b). In addition the assay has been applied to pathological material, especially with respect to hematologic malignancies (Greenberg et al., 1978; Castro-Malaspina et al., 1982; Haworth et al., 1982; Nagao et al., 1983a,b).

An assay which detects radiation damage to splenic hemopoietic stroma is the accumulation of CFU-s six days after injection of sublethal doses of bacterial endotoxin. The increase of splenic CFU-s numbers can approximate a hundredfold (Vos et al., 1972). It has been suggested that in addition to immigration this increase is established by local proliferation of CFU-s (McCulloch et al., 1970), which is mediated by a humoral factor that is indu-

ced by lipid A, a component of endotoxin (Staber and Metcalf, 1980). This humoral factor has been shown to initiate cycling of CFU-s (Molendijk and Ploemacher, 1984). Upon irradiation splenic CFU-s accumulation after endotoxin treatment is diminished. This effect is probably due to stromal damage rather than stem cell damage (Ploemacher et al., 1983), firstly because additionally injected bone marrow cells did not improve the response, secondly because hemopoietic spleen colonies are smaller in irradiated animals even after injection of a supplement of normal bone marrow cells, and thirdly because parabiosis between an irradiated and a normal animal does not result in recovery of the CFU-s accumulation response in the irradiated partner. It is uncertain whether the response is dependent on proliferation of stromal cells. Increases in CFU-F numbers after endotoxin treatment have been demonstrated (Brockbank et al., 1983a), but their functional relationship to CFU-s accumulation is not clear, although one could envisage that increasing hemopoietic cell numbers in the spleen may require expansion of the stromal cell pool. This CFU-s accumulation assay cannot be employed for measurement of bone marrow stromal function, since the CFU-s accumulation is far lower in this organ (Vos et al., 1972) which is due in part to the rigidity of the organ.

5. PERTURBATION OF HEMOPOIESIS

When the hemopoietic system or the mature blood cell compartment are damaged by exogenous agents compensatory reactions are required in order to reestablish the steady state situation. Such reactions can provide model systems by which regulatory mechanisms in the hemopoietic system can be unraveled. This chapter reviews some effects on hemopoiesis of three agents that have been used in the present investigations as tools to study regulation of hemopoiesis.

5.1. Radiation

The effects of irradiation on hemopoietic cells versus stromal cells are quite different. This difference is mainly caused by the fact that stromal cells are essentially non-proliferating *in situ* (Haas et al., 1969) whereas most hemopoietic cells are continuously proliferating. This renders the latter population more prone to mitotic death due to radiation-induced genetic damage. The gamma radiation survival curve for bone marrow CFU-s has a D_o of 1.15 Gy (Till and McCulloch, 1961). Essentially similar data have been obtained for CFU-s from other organs (Metcalf and Moore, 1971). The D_o for *in vitro* clonable stromal cells amounts between 2 and 3 Gy (Metcalf 1972; Wilson et al., 1974; Friedenstein et al., 1976, 1981). It is possible that the radiation sensitivity of stromal cells as measured by *in vitro* clonogenic assays is higher than the *in situ* sensitivity because in the *in vitro* assays normally quiescent stromal cells are triggered into proliferation, which reveals damage that may not become apparent *in situ* (Tavassoli, 1982). Fried et al. (1976) and Ploemacher and Van Soest (1979) using the implant regeneration assay, showed that persistent stromal damage in bone marrow and spleen was evident, with radiation doses of 5 Gy or higher. Lower doses allowed complete reconstitution of stromal function as measured by CFU-s and CFU-F numbers per organ (Fried et al., 1976; Friedenstein et al., 1976). With 10 Gy or higher doses the recovery of CFU-s and CFU-F as well as implant regeneration are incomplete (Maloney and Patt, 1972, Werts et al., 1977; Chamberlin et al., 1974; Fried et al., 1973b) in a dose dependent fashion. Knospe et al. (1966) studied the histologic effects of supralethal local irradiation on rat femurs. One week after 10 Gy irradiation hemopoietic elements had disappeared from the irradiated areas. At two weeks a slight recovery of hemopoiesis followed, in the immediate vicinity of sinusoidal structures. The recovery was transient, and after two to three months no sinusoids and hemopoietic elements were left. At this stage reticular cell proliferation was seen which became more abundant with time. At six months regeneration of sinusoids with associated islands of hemopoiesis was observed. This process went on until a well-defined sinusoidal network was rebuilt, however with more irregular distribution than in control stroma. The course of regeneration was similar with higher doses up to 20 Gy, the damage becoming more severe, and the recovery less prominent. The regeneration described here resembles that of the spleen after necrosis (Wolf, 1982), where proliferation of fibroblastic reticular cells precedes formation of new vascular channels and filling with hemopoietic and lymphopoietic cells. Radiation effects on the stromal CFU-F population size in the bone marrow are characterized by an initial dip in CFU-F numbers, followed by a (partial) recovery after some weeks (Friedenstein et al., 1976; Werts et al., 1977). This recovery likely coincides with the heretofore mentioned reticular cell proliferation (Knospe et al., 1966; Wolf 1982). From the data discussed in this paragraph it is evident that hemopoiesis is critically dependent on the pre-

sence of a well-functioning sinusoidal system, which in turn is supported by the presence of a reticular stroma.

5.2. Phenylhydrazine

Phenylhydrazine induces hemolytic anemia followed by a marked growth of the spleen (Jandl et al., 1965). The splenomegaly can be attributed partly to trapping of red cell debris and its sequestration by the mononuclear phagocyte system, but it is further accompanied by increases in both CFU-s and BFU-E numbers (Rencricca et al. 1970; Hodgson et al., 1972; Hara and Ogawa, 1976; Ploemacher et al. 1977). Concomitantly in the bone marrow CFU-s and BFU-E numbers have been reported to decline (Rencricca et al., 1970; Hara and Ogawa, 1976) whereas the blood contained elevated CFU-s and BFU-E, but not CFU-E numbers (Ploemacher et al., 1977; Hara and Ogawa, 1976). These findings have been explained as to indicate that stem cell and early erythroid progenitor cell traffic from the bone marrow via the blood into the spleen occurs after phenylhydrazine treatment in order to intensify erythropoiesis to compensate for the hemolysed cells. Furthermore, CFU-s numbers in the liver increased, most likely by influx of bone marrow-derived CFU-s via the blood (Ploemacher et al., 1977). Both in liver and bone marrow compensatory erythropoiesis occurred intra-sinusoidal following influx of monocytic cells that developed into central macrophages of erythroid islets (Ploemacher et al., 1977). These effects were followed by increases in CFU-c in the spleen (Hara and Ogawa, 1976), increased reticulocyte counts in the blood (Rencricca et al., 1970) and ultimately reestablishment of the steady state situation within 2 weeks. Phenylhydrazine-induced experimental hemolytic anemia provides a useful tool in the study of hemopoietic cell traffic between organs via the blood stream.

5.3 Bacterial endotoxin

Bacterial endotoxins have marked stimulatory effects on hemopoiesis. After injection of bacterial lipopolysaccharides the numbers of CFU-s in the blood increased within minutes, probably by mobilization from the hemopoietic organs (Vos et al., 1972). After returning to control levels a second rise lasting several days followed which is partially accomplished by CFU-s proliferation (Vos et al., 1972). Concomitantly, splenic and hepatic CFU-s numbers increased markedly and femoral CFU-s numbers decreased (Vos, 1978; Vos et al., 1980). Hemopoietic progenitors of all lineages increased in number in the spleen (Staber and Metcalf, 1980). Furthermore, increased serum levels of a number of hemopoietic cell growth-stimulating factors have been measured after lipopolysaccharide administration, (some of) which may be mediating the effects of the endotoxin (Staber and Metcalf, 1980). It has been shown that post-endotoxin serum contains a factor that initiates cycling of CFU-s (Molendijk and Ploemacher, 1984). The effects of endotoxin on stromal clonogenic cells partly mimic those mentioned for the second rise of CFU-s. In the spleen CFU-F numbers increased along with increasing total cellularity, whereas their numbers were depressed in the bone marrow (Brockbank et al., 1983a). Treatment with 0.3 - 0.5 mg of lipopolysaccharide caused extensive stromal damage to the bone marrow (Ploemacher, unpublished observations). Histologically this was evident by disappearance of most hemopoietic cells and the well-organized sinusoidal microcirculation, while a randomized distribution of erythrocytes throughout the stromal parenchyma was observed. Hence, it seems likely that also after endotoxin treatment the bone marrow hemopoietic stroma has to be rebuilt to a large extent.

INTRODUCTION TO THE APPENDIX PAPERS

The experimental work of this thesis, described in the appendix papers, is aimed at characterization of the nature and function of fibroblastic reticular cells in the hemopoietic stroma. A number of experimental models was employed to elucidate different aspects of these cells.

In the first appendix paper characteristics are described of fibroblastoid colony-forming cells (CFU-F) from murine bone marrow and their *in vitro* progeny. The data indicate that these fibroblastoid cells differ from macrophages and endothelial cells, and can be discriminated from fibroblastoid cells from other organs.

The second appendix paper describes three monoclonal antibodies that specifically recognize a determinant on non-phagocytic and phagocytic reticular cells in the hemopoietic stroma of fetal and adult mice. Reticular stromal cells with this specific determinant(s) are restricted to sites of adult-type hemopoiesis.

In the third appendix paper the regulatory capacity of a spleen-derived fibroblastoid cell line on *in vitro* myelopoiesis is described. This cell line is shown to produce two soluble regulators and one cell-associated activity by which *in vitro* myelopoiesis is modulated.

The fourth appendix paper describes the transplantability of CFU-F after lethal total body irradiation and bone marrow transplantation. Evidence is presented indicating that CFU-F can efficiently lodge in the recipients bone marrow.

The fifth appendix paper represents a study of the long-term recovery of CFU-F after lethal total body irradiation and bone marrow transplantation. This recovery is slow and incomplete, independent of graft size and presence of adherent stromal cells in the graft. If present in the graft, donor stromal cells do contribute to the recipients stroma, but they do not influence the total CFU-F recovery. Subsequent treatment with a sublethal dose of endotoxin allows full recovery of the CFU-F population.

The sixth appendix paper describes the kinetics of hemopoietic and stromal cells in phenylhydrazine-treated mice. In addition, evidence is presented that under these conditions CFU-F migrate into spleen and bone marrow via the blood stream.

In the seventh appendix paper a strong correlation is described between CFU-F numbers and the regenerative capacity of ectopically transplanted stroma in femurs of mice treated with several doses of gamma irradiation.

GENERAL DISCUSSION

The investigations described in this thesis were performed in order to expand knowledge concerning the nature of fibroblastoid cells in the hemopoietic stroma of mice and furthermore to study functional properties of this stromal cell population with respect to regulation of hemopoiesis.

The nature of fibroblastic reticular stromal cells in the non-lymphoid hemopoietic stroma has been studied in detail (appendix papers 1 and 2). These cells lack specific characteristics of macrophages and endothelial cells and can be discriminated from fibroblastoid cells in other organs according to their expression of the T200 and Thy-1 antigens. They produce extracellular matrix components characteristic for fibroblasts. In addition they express at least one specific determinant for the hemopoietic stroma which is recognized by the ER-HR1, 2 and 3 monoclonal antibodies. The notion that the hemopoietic stroma contains this stromal cell class with a tissue-specific phenotype suggests that these cells may have a specific function in contributing to a residence for hemopoietic stem cells and/or may regulate certain hemopoietic pathways (Ploemacher et al., 1985). The determinant recognized by ER-HR1, 2 and 3 may be functionally related to an essential hemopoietic event, since it is not present in all sites where hemopoietic stem cells reside, but is strictly related to sites where adult type hemopoiesis occurs.

The intimate association of hemopoietic cells with stromal cells allows local regulatory mechanisms to occur between these cells. Such regulation has been studied *in vitro* employing a fibroblastoid stromal cell line (appendix paper 3). This line exerted humoral stimulatory activities as well as short-r range inhibition on *in vitro* myelopoiesis. Zipori (1981a) employed a similar system with primary adherent bone marrow cell cultures as the stromal component. He proposed a model in which the extent of proliferation and differentiation of hemopoietic progenitor cells is determined by their actual position within gradients of stimulatory and inhibitory soluble regulators present in the stroma. Such gradients would arise via local regulator production by stromal cells and diffusion from these cells. Indeed, our double layer experiments confirmed the existence of a gradient of stromal cell-derived CSA, whereas in close proximity to the stromal cells inhibition of proliferation occurred.

In this context the spatial organization of hemopoietic tissue is of importance. It has been shown that hemopoietic cell types have specific radial distribution patterns (Lord et al., 1975) as have clonable stromal cells, although for the latter cells the actual data are controversial (Xu and Hendry, 1981; Brockbank et al., 1982). Thus each progenitor cell type may be spatially related to certain stromal cell types that contribute to regulation of its differentiation pathway. In other words, specific microenvironments may exist within the stroma, each with their own inductive and/or conductive properties (Trentin, 1970; Tavassoli, 1975; Ploemacher, 1978). The spatial distribution of CFU-F did not correlate with that of ER-HR1, 2 and 3 positive cells. This notion suggests that CFU-F is a specific minor subpopulation of the stromal reticular cell pool, which is essentially non-proliferating *in situ* (Haas et al., 1969). Clearly data obtained with CFU-F should not simply be extrapolated to the entire stroma.

The studies presented here further show that clonable fibroblastoid stromal cells are not completely fixed within the stroma. They reached the recipients hemopoietic stroma after intravenous bone marrow transplantation (appendix papers 4 and 5). Furthermore, migration into bone marrow and spleen was observed after induction of hemolytic anemia (appendix paper 6), suggest-

ing that circulating CFU-F may contribute to expansion of stromal support in stress hemopoiesis. These observations are in agreement with some studies which indicate migration of stromal cells (Werts et al., 1977; Keating et al., 1982; Feiner et al., 1983), but disagree with other observations. For instance local irradiation induced a persistent aplasia in the marrow (Knospe et al., 1966, 1968). After regeneration of subcutaneously implanted hemopoietic stroma the stromal cells were shown to be donor-derived whereas the hemopoietic cells immigrated from the hosts hemopoietic tissue (Fried et al., 1973b). Similarly, Sl/Sl^d mice in parabiosis with normal littermates did not show any improvement in growth of injected stem cells following irradiation, although normal proliferation occurred in the normal littermates (McCulloch et al., 1965). Obviously under these conditions, the relevant stromal component, which need not to be the CFU-F population, had not been transplanted. One could envisage that influx of stromal cells into hemopoietic tissue is only possible when open sites are available, as is suggested by the complete recovery of the CFU-F population in the femoral stroma of lethally irradiated mice after endotoxin-induced reduction of CFU-F numbers (appendix paper 5). This might explain why in some experimental situations migration was proven whereas in others it was not demonstrable. In the latter cases the stromal cells that might be replaced by CFU-F were possibly still viable leaving no gaps for immigrant CFU-F.

Nevertheless, it remains unclear whether the observed immigration of CFU-F is necessary for stromal recovery. The present studies indicate hat stromal cell-depleted grafts were not inferior to normal bone marrow grafts with respect to the growth of the CFU-F population after bone marrow transplantation (appendix paper 5). Again it should be realized that CFU-F are a subpopulation of stromal cells selected on the basis of an artificial feature, viz. in vitro proliferative capacity. This capacity is not necessarily related to stromal function in terms of regulatory properties towards hemopoiesis. On the other hand, the recovery of hemopoiesis after lethal total body irradiation and bone marrow transplantation is subnormal, and the hemopoietic responses to bleeding (Gong et al., 1969) and endotoxin (Ploemacher et al., 1983) are defective, as is true for the CFU-F numbers. Furthermore, CFU-F numbers were found to be highly correlated with regenerative capacity of transplanted hemopoietic tissue (appendix paper 7), confirming that in this case CFU-F numbers can be taken as a measure of proliferative capacity of the hemopoietic stroma as a whole. Thus, clonable fibroblastoid reticular cells are likely involved in the regeneration of the hemopoietic stroma after irradiation.

In some situations a decrease in CFU-F numbers can be indicative of stromal damage (e.g., irradiation, appendix paper 7). DeGowin and Gibson (1978) described suppression of stromal colony numbers in mice bearing an extramedullary tumor. Such decreases have also been found in human leukemia (Nagao et al., 1983a), but this is not a general phenomenon (Castro-Malaspina and Moore, 1982). In aplastic anemia patients no changes in clonable stromal cells were found (Gordon and Gordon-Smith, 1981). Increases in stromal colony numbers have been described in myelofibrosis (Castro-Malaspina and Moore, 1982). Changes in CFU-F may be mediated by humoral activities induced by the disease (DeGowin et al., 1981; Castro-Malaspina and Moore, 1982; Nagao et al., 1983b). These studies are interesting with respect to stromal regulation and moreover in view of the possibility that changes in CFU-F numbers could be taken as a diagnostic parameter in hemopoietic disorders.

Assays for stromal function which are not dependent on cellular proliferation are urgently required in order to be able to study the specific role of the hemopoietic stroma with respect to regulation of hemopoiesis. One possible approach is the identification of subpopulations of stromal cells by spe-

cific antibodies such as those described in this thesis. Such probes may assist in the functional description of specific cellular interactions between hemopoietic cells and stromal cells which mediate the regulation of proliferation and differentiation of hemopoietic stem and progenitor cells.

SUMMARY

In general, mature hemopoietic cells have a limited lifespan, and therefore continuous production of these cells throughout life is necessary. All blood cells are derived from pluripotent hemopoietic stem cells. The proliferation and differentiation of hemopoietic stem cells in adult mice occurs mainly in the bone marrow and to some extent also in the spleen. During embryonic life hemopoietic stem cells are found in yolk sac, liver, spleen and bone marrow consecutively.

Obviously the hemopoietic organs contain a stromal framework which allows hemopoiesis to occur, and which may exert regulatory influences upon the proliferation and differentiation of hemopoietic stem cells and progenitor cells. The stroma of non-lymphoid hemopoietic tissues contains three major cell types. Endothelial cells form the inner lining of the extensive sinusoidal network. Fibroblastic reticular cells are found as the outer lining of the sinusoids, and these cells penetrate the hemopoietic tissue with long cellular extensions to form yet another intense network, in which also macrophages take part.

This thesis describes investigations to elucidate the nature of fibroblastic reticular cells in the hemopoietic stroma as well as functional properties of these cells with respect to regulation of hemopoiesis. Appendix papers 1 and 2 represent studies in which the nature of *in vitro* cultured fibroblastoid stromal cells and their *in vivo* counterparts was investigated. These cells are shown to be different from macrophages and endothelial cells. Their fibroblastoid nature was demonstrated by the presence of specific extracellular matrix components. Furthermore, they differ from fibroblastoid cells from other organs on the basis of the presence of surface markers that are found on certain hemopoietic cell classes. Finally, monoclonal antibodies were produced against fibroblastoid stromal cells, which appeared to specifically detect at least one determinant on stromal cells within hemopoietic organs including embryonic organs containing transient hemopoiesis, with the exception of some scattered staining in subcutaneous connective tissue. Together these studies support the notion that hemopoietic organ fibroblastoid reticular cells comprise a unique cell population.

Functional properties of this cell population with respect to regulation of hemopoiesis were studied *in vitro* using a fibroblastoid stromal cell line which was derived from the spleen. As shown in appendix paper 3 this cell line modulated *in vitro* myelopoiesis (i) by elaboration of colony stimulating activity for granulocyte-macrophage differentiation; (ii) by elaboration of a prostaglandin-related soluble inhibitor of this process and (iii) by means of a short-range inhibition exerted by the fibroblastoid cells on the proliferation of granulocyte/macrophage progenitor cells in their immediate vicinity. The fibroblastoid cell line-dependent formation of myeloid colonies that contained relatively many immature cells pointed out that proliferation as well as differentiation of progenitor cells were regulated by the cell line.

After lethal total body irradiation bone marrow transplantation is necessary to reconstitute the hemopoietic system. In appendix paper 4 evidence is presented that fibroblastoid colony-forming stromal cells (CFU-F) present in the graft reach the recipients hemopoietic stroma and eventually comprise about half of the total CFU-F population. These cells may contribute to the recovery of the irradiated stroma. The irradiated stroma partially recovered but remained damaged over one and a half year after treatment as measured by the number of CFU-F in the femoral bone marrow, which is a measure for proliferative capacity of the stroma. Appendix paper 5 shows that

donor-derived stromal cells are not absolutely required for the partial recovery of CFU-F numbers, since the effect of stromal cell-depleted grafts was similar to that of normal bone marrow grafts. When irradiated and subsequently reconstituted mice were treated with bacterial endotoxin, a complete recovery of femoral CFU-F numbers followed. This finding was explained by suggesting that the irradiated stroma still contained viable stromal cells which had lost their in vitro colony-forming capacity due to radiation damage. These cells would not need to be replaced by CFU-F. Disruption of the stroma with endotoxin would then require repopulation by stromal cells that apparently had the ability to form in vitro colonies.

The transplantability of stromal cells via the intravenous route suggests that such cells may be able to migrate between hemopoietic organs in stress situations where expanded stromal support is needed. This possibility was examined, as shown in appendix paper 6, in mice suffering from a hemolytic anemia induced by treatment with phenylhydrazine. Under these circumstances extensive mobilization of hemopoietic cells occurred, which was shown to be accompanied by immigration of CFU-F into spleen and bone marrow. The organ of origin of these migrating stromal cells was not revealed in these studies. The influx of stromal cells into hemopoietic organs during hemopoietic stress may represent a mechanism to expand stromal support for hemopoiesis.

The regeneration of hemopoietic tissue after irradiation is initiated by reticular stromal cell proliferation, as was shown by histological studies. An extension of these studies is presented in appendix paper 7, where the regenerative capacity of ectopically transplanted femurs is compared to CFU-F numbers in femurs six weeks after receiving various doses of gamma-radiation followed by bone marrow transplantation. These assays, which are both dependent on the proliferation of stromal cells, correlated well, which suggests that CFU-F may be involved in the regeneration of the hemopoietic stroma. The size of the bone marrow graft injected after irradiation did not influence the results of both stromal assays, indicating that donor-stromal cells were not absolutely required for stromal recovery.

These investigations lead to a number of conclusions. Fibroblastoid reticular cells in the hemopoietic stroma comprize a unique cell population, which is proposed to regulate hemopoiesis through elaboration of humoral as well as close range activities. These stromal cells can migrate into irradiated stroma via the blood stream and comigrate with hemopoietic cells into hemopoietic organs in a hemopoietic stress situation. The regenerative capacity of the hemopoietic stroma following irradiation is correlated with the number of fibroblastoid colony-forming cells present in the stroma. It is stressed that assays measuring stromal proliferative capacity do not necessarily measure hemopoietic support capacity of the stroma. These studies indicate that fibroblastoid reticular cells in the hemopoietic stroma influence the proliferation and differentiation of hemopoietic cells.

SAMENVATTING

Gedifferentieerde hemopoietische cellen hebben in het algemeen een beperkte levensduur, waardoor een voortdurende produktie van deze cellen gedurende het hele leven nodig is. Alle bloedcellen zijn ontstaan uit pluripotente hemopoietische stamcellen. De proliferatie en differentiatie van hemopoietische stamcellen vindt in volwassen muizen hoofdzakelijk plaats in het beenmerg, en tot op zekere hoogte ook in de milt. Gedurende de embryonale ontwikkeling komen hemopoietische stamcellen achtereenvolgens voor in dooierzak, lever, milt en beenmerg.

Klaarblijkelijk bevatten de hemopoietische organen een stromaal netwerk dat hemopoiese mogelijk maakt, en dat regulerende invloeden zou kunnen uitoefenen op de proliferatie en differentiate van hemopoietische stamcellen. Het stroma van niet-lymfoide hemopoietische weefsels bevat drie belangrijke celtypen. Endotheliale cellen vormen de binnenste bekleding van het uitgebreide sinusoidale netwerk. Fibroblastaire reticulaire cellen vormen de buitenbekleding van sinusoiden, en deze cellen dringen met lange celuitlopers door in het hemopoietische weefsel waarbij opnieuw een intensief netwerk gevormd wordt, waarin ook macrofagen een rol spelen.

Dit proefschrift beschrijft een onderzoek naar de aard van fibroblastaire reticulaire cellen in het hemopoietische stroma, zowel als functionele eigenschappen van deze cellen met betrekking tot regulatie van de hemopoiese. Artikelen 1 en 2 vertegenwoordigen studies waarin de aard van *in vitro* gekweekte fibroblastaire stromale cellen en hun *in vivo* tegenhangers werd onderzocht. Deze cellen blijken te verschillen van macrofagen en endotheliale cellen. Hun fibroblastair karakter werd aangetoond door de aanwezigheid van specifieke extracellulaire matrix componenten. Verder verschillen ze van fibroblastaire cellen uit andere organen wat betreft de aanwezigheid van oppervlakte determinanten die op bepaalde hemopoietische celtypen gevonden worden. Tenslotte werden tegen fibroblastaire stromale cellen monoclonale antilichamen bereid die tenminste één determinant herkenden welke specifiek was voor stromale cellen in hemopoietische organen, inclusief embryonale organen waarin tijdelijk hemopoiese voorkomt. Een uitzondering hierop vormen enige cellen in het onderhuidse bindweefsel. Tesamen ondersteunen deze studies de notie dat fibroblastaire reticulaire cellen in hemopoietische organen een unieke celpopulatie zijn.

Funktionele eigenschappen van deze celpopulatie met betrekking tot regulatie van de hemopoiese werden bestudeerd *in vitro* door gebruik te maken van een fibroblastaire stromale cellijn verkregen uit de milt. Zoals wordt aangetoond in artikel 3 moduleerde deze cellijn de *in vitro* myelopoiese door het uitscheiden van 'colony-stimulating activity' voor granulocyt-macrofaag differentiatie, door uitscheiding van een prostaglandine-achtige oplosbare remmer van dit proces, en door middel van een remming op korte afstand door de fibroblastaire cellen van de proliferatie van granulocyt-macrofaag voorlopercellen in hun direkte omgeving. De vorming, geinduceerd door de fibroblastaire cellijn, van myeloiede kolonies die relatief veel onvolledig gedifferentieerde cellen bevatten, gaf aan dat zowel proliferatie als differentiatie door de cellijn gereguleerd worden.

Na letale totale lichaamsbestraling is beenmergtransplantatie noodzakelijk voor herstel van het hemopoietische systeem. In artikel 4 wordt aangetoond dat fibroblastaire kolonie-vormende cellen (CFU-F) die aanwezig zijn in het transplantaat, het hemopoietische stroma van de ontvanger bereiken, en uiteindelijk ongeveer de helft van de totale CFU-F populatie kunnen uitmaken. Deze cellen dragen mogelijk bij aan het herstel van het bestraalde stroma. Het

bestraalde stroma herstelde gedeeltelijk maar bleef beschadigd gedurende meer dan anderhalf jaar na behandeling zoals gemeten aan het aantal CFU-F in het beenmerg van de femur, hetgeen een maat is voor de proliferatieve capaciteit van het stroma. Artikel 5 laat zien dat stromale cellen van de donor niet noodzakelijk zijn voor het gedeeltelijke herstel van de CFU-F aantallen, daar het effect van transplantaten waaruit stromale cellen gedepleteerd waren vergelijkbaar was met dat van normale transplantaten. Wanneer bestraalde en vervolgens gereconstitueerde muizen behandeld werden met bacteriële endotoxine volgde een volledig herstel van de CFU-F populatie in de femur. Deze bevinding werd verklaard door aan te nemen dat het bestraalde stroma nog levende stromale cellen bevatte die hun capaciteit om in vitro kolonies te vormen hadden verloren door stralingsschade. Deze cellen zouden niet vervangen behoeven te worden door CFU-F. Verstoring van het stroma met endotoxine vereiste echter repopulatie door stromale cellen, die blijkbaar wel in staat waren om in vitro kolonies te vormen.

De mogelijkheid om stromale cellen te transplanteren via intraveneuze injectie suggereert dat deze cellen wellicht in staat zijn te migreren tussen hemopoietische organen onder stress omstandigheden waar verhoogde stromale ondersteuning nodig is. Deze mogelijkheid werd onderzocht, zoals beschreven in artikel 6, in muizen met een hemolytische anemie die geinduceerd was door behandeling met phenylhydrazine. Onder deze omstandigheden vond uitgebreide mobilisatie van hemopoietische cellen plaats die gepaard ging met immigratie van CFU-F in milt en beenmerg. De oorsprong van deze migrerende stromale cellen werd niet duidelijk uit deze studies. De influx van stromale cellen in hemopoietische organen tijdens hemopoietische stress kan een mechanisme vertegenwoordigen om de stromale ondersteuning van de hemopoiese te versterken.

De regeneratie van hemopoietisch weefsel na bestraling wordt ingezet door proliferatie van reticulaire stromale cellen, zoals is aangetoond met histologische studies. Een uitbreiding van deze studies wordt beschreven in artikel 7, waarin de regeneratieve capaciteit van ectopisch getransplanteerde femurs wordt vergeleken met CFU-F aantallen in femurs zes weken na behandeling met verschillende doses gammastraling gevolgd door beenmergtransplantatie. Deze assays, die beide afhankelijk zijn van de proliferatie van stromale cellen, kwamen goed overeen, hetgeen suggereert dat CFU-F betrokken zouden kunnen zijn bij de regeneratie van het hemopoietische stroma. De grootte van het beenmergtransplantaat, toegediend na bestraling, beinvloedde de resultaten van geen van beide stromale assays, wat erop wijst dat stromale cellen van de donor niet absoluut noodzakelijk waren voor stromaal herstel.

Deze studies leiden tot een aantal conclusies. Fibroblastaire reticulaire cellen in het hemopoietische stroma vormen een unieke celpopulatie, die de hemopoiese zou kunnen reguleren door produktie van zowel humorale activiteiten als door invloed op korte afstand. Deze stromale cellen kunnen via de bloedsomloop bestraald stroma bereiken en migreren samen met hemopoietische cellen naar hemopoietische organen in een hemopoietische stress situatie. De regeneratieve capaciteit van het hemopoietische stroma is gecorreleerd met het aantal fibroblastaire kolonievormende cellen in het stroma. Het feit wordt benadrukt dat assays die stromale proliferatieve capaciteit meten niet noodzakelijkerwijze de capaciteit van het stroma meten om de hemopoiese te ondersteunen. Dit onderzoek laat zien dat fibroblastaire reticulaire cellen in het hemopoietische stroma invloed uitoefenen op de proliferatie en differentiatie van hemopoietische cellen.

REFERENCES

Allen, T.D., Dexter, T.M. (1976). Cellular interrelationships during in vitro granulopoiesis. Differentiation 6:191.

Allen, T.D. (1981). Hemopoietic microenvironments in vitro: ultrastructural aspects. In: CIBA Foundation Symposium 84:38.

Amsel, S., Dell, E.S. (1971). Bone marrow repopulation of subcutaneously grafted mouse femurs. Proc. Soc. exp. Biol. Med. 138:550.

Anderson, R.W., Matthews, K.I., Crouse, D.A., Sharp, J.G. (1982). In vitro evaluation of hematopoiesis in mice treated with busulfan or nitrogen mustard. Biomedicine 36:149.

Appelbaum, F.R., Fefer, A., Cheever, M.A., Sanders, J.E., Singer, J.W. et al. (1980). Treatment of aplastic anemia by bone marrow transplantation in identical twins. Blood 55:1033.

Axelrad, A.A., McLeod, D.L., Shreeve, M.M., Heath, D.S. (1974). Properties of cells that produce erythrocytic colonies in vitro. In: Hemopoiesis in culture (ed. W.A. Robinson), U.S. Government Printing Office, Washington, 226.

Axelrad, A.A., McLeod, D.L., Suzuki, S., Shreeve, M.M. (1978). Regulation of the population size of erythropoietic progenitor cells. In: Differentiation of normal and neoplastic hematopoietic cells (eds. B. Clarkson, P.A. Marks, J.E. Till), Cold Spring Harbor, New York, 155.

Aye, M.T. (1977). Erythroid colony formation in cultures of human marrow: effect of leukocyte conditioned medium. J. Cell. Physiol. 91:69.

Barker, J.E. (1968). Development of the mouse hematopoietic system. I. Types of hemoglobin produced in embryonic yolk sac and liver. Devel. Biol. 18:14.

Bentley, S.A. (1984). Studies of bone marrow stromal cells in vitro. In: Myelofibrosis and the biology of connective tissue. Alan Liss Inc., New York, 179.

Bernstein, S., Russell, E., Kiegley, G. (1968). Two hereditary mouse anemias (Sl/Sl^d and W/W^v) deficient in response to erythropoietin. Ann. N.Y. Acad. Sci. 149:475.

Bessis, M. (1958). L'ilot érythroblastique, unité functionelle de la moelle osseuse. Rev. Hematol. 13:8.

Blackburn, M.J., Patt, H.M. (1977). Increased survival of hemopoietic pluripotent stem cells in vitro induced by a bone marrow fibroblast factor. Br. J. Haematol. 37:337.

Bradley, T.R., Metcalf, D. (1966). The growth of mouse bone marrow cells in vitro. Aust. J. Exp. Biol. Med. Sci. 44:287.

Brockbank, K.G.M., Piersma, A.H., van Peer, C.M.J. (1982). The regional distribution of fibroblastoid progenitors in murine femoral marrow revisited. Biomedicine 36:369.

Brockbank, K.G.M., Ploemacher, R.E., van Peer, C.M.J. (1983a). Splenic accumulation of stromal progenitor cells in response to bacterial lipopolysaccharides. Exp. Hematol. 11:366.

Brockbank, K.G.M., Ploemacher, R.E., van Peer, C.M.J. (1983b). An in vitro analysis of murine hemopoietic fibroblastoid progenitors and fibroblastoid cell function during aging. Mech. Aging Devel. 22:11.

Brockbank, K.G.M., van Peer, C.M.J. (1983). Colony-stimulating activity production by hemopoietic organ fibroblastoid cells in vitro. Acta Haematol. 69:369.

Burgess, A.W., Camakaris, J., Metcalf, D. (1977). Purification and properties of colony stimulating factor from mouse lung conditioned medium. J. Biol. Chem. 252:1998.

Burgess, A.W., Metcalf, D. (1980). The nature and action of GM-CSFs. Review. Blood 56:947.

Calderon, J., Kuly, J.M., Lefko, J.L., Unanue, E.R. (1976). The modulation of lymphocyte functions by molecules secreted by macrophages. I. Description and partial biochemical analysis. J. Exp. Med. 142:151.

Castro-Malaspina, H., Gay, R.E., Resnick, G., Kapoor, N., Meyers, P. et al. (1980). Characterization of human bone marrow fibroblastoid colony-forming units and their progeny. Blood 56:289.

Castro-Malaspina, H., Gay, R.E., Jhanwar, S.C., Hamilton, J.A., Chiarieri, D.R., Meyers, P.A., Gay, S., Moore, M.A.S. (1982). Characterization of bone marrow fibroblast colony-forming cells (CFU-F) and their progeny in patients with myeloproliferative disorders. Blood 59:1046.

Castro-Malaspina, H., Moore, M.A.S. (1982). Pathophysiological mechanisms operating in the development of myelofibrosis: role of megakaryocytes. Nouv. Rev. Fr. Hematol. 24:221.

Cerny, J. (1974). Stimulation of bone marrow haemopoietic stem cells by a factor from activated T cells. Nature 249:63.

Chamberlin, W., Barone, J., Kedo, A., Fried, W. (1974). Lack of recovery of murine hemopoietic stromal cells after irradiation-induced damage. Blood 44:385.

Chen, L.T., Weiss, L. (1973). The role of the sinus wall in the passage of erythrocytes through the spleen. Blood 41:529.

Cotes, P.M., Bangham, D.R. (1961). Bio-assay of erythropoietin in mice made polycythaemic by exposure to air at a reduced pressure. Nature 191:1065.

Cronkite, E.P., Harigaya, K., Garnett, H., Miller, M.E., Honikel, L., Shadduck, R.K. (1982). Production of CSF by a murine bone marrow cell line derived from Dexter adherent layer and other properties of this cell line. (Baum, S.J., Ledney, G.D., Thierfelder, S., eds.) Experimental Hematology Today, 11.

Curry, J.L., Trentin, J.J. (1967). Hemopoietic spleen colony studies I. Growth and differentiation. Devel. Biol. 15:395.

Curry, J.L., Trentin, J.J., Wolf, N.S. (1967). Hemopoietic spleen colony studies: II. Erythropoiesis. J. Exp. Med. 125:703.

De Bruyn, P.P.H., Michelson, S., Thomas, T.B. (1971). The migration of blood cells of the bone marrow through the sinusoidal wall. J. Morphol. 113:417.

De Bruyn, P.P.H., Michelson, S., Becker, R.P. (1975). Endocytosis, transfer tubules and lysosomal activity in myeloid sinusoidal endothelium. J. Ultrastruct. Res. 53:133.

DeGowin, R.L., Gibson, D.P. (1978). Suppressor effect of an extramedullary tumor on bone marrow erythropoiesis and stroma. Exp. Hematol. 6:568.

DeGowin, R.L., Gibson, D.P., Knapp, S.A., Wathen, L.M. (1981). Tumor-induced suppression of marrow stromal colonies. Exp. Hematol. 9:811.

De la Chapelle, A., Vuopio, P., Borgstrom, G.H. (1973). The origin of marrow fibroblasts. Blood 41:783.

Dexter, T.M., Allen, T.D., Lajtha, L.G. (1976). Conditions controlling the proliferation of hemopoietic stem cells in vitro. J. Cell. Physiol. 91:335.

Dexter, T.M., Moore, M.A.S. (1977). In vitro duplication and 'cure' of hemopoietic defects in genetically anaemic mice. Nature 269:412.

Dexter, T.M., Wright, E.G., Krisza, F., Lajtha, L.G. (1977). Regulation of hemopoietic stem cell proliferation in long-term bone marrow cultures. Biomedicine 27:344.

Dexter, T.M., Garland, J., Scott, D., Scolnick, E., Metcalf, D. (1980). Growth of factor-dependent hemopoietic precursor cell lines. J. Exp. Med. 152:1036.

Dexter, T.M. (1982). Stromal cell associated hemopoiesis. Review. J. Cell Physiol. suppl. 87.

Dieterlen-Lievre, F. (1975). On the origin of hemopoietic stem cells in the avian embryo: an experimental approach. J. Embryol. exp. Morphol. 33:607.
Dorssers, L., Burger, H., Wagemaker, G. (1983). Identity of murine stem cell activating factor (SAF) and interleukin-3 (IL3) and common specificity for pluripotent hemopoietic stem cells. Exp. Hematol. 11:suppl., 357.
Enomoto, K., Kawakita, M., Kishimoto, S., Katayama, N., Miyake, T. (1980). Thrombopoiesis and megakaryocyte colony stimulating factor in the urine of patients with aplastic anemia. Br. J. Haematol. 45:551.
Fauser, A.A., Messner, H.A. (1978). Granuloerythropoietic colonies in human bone marrow peripheral blood and cord blood. Blood 52:1243.
Feiner, R.H., Strober, S., Greenberg, P.L. (1983). Murine granulopoiesis after fractionated total lymphoid irradiation and allogeneic bone marrow transplantation. Exp. Hematol. 11:410.
Foster, R., Metcalf, D., Robinson, W.A., Bradley, T.R. (1968). Bone marrow colony stimulating activity in human sera. Results of two independent surveys in Buffalo and Melbourne. Br. J. Haematol. 15:147.
Fowler, J.H., Wu, A.M., Till, J.E., McCulloch, E.A., Siminovitch, L. (1967). The cellular composition of hemopoietic spleen colonies. J. Cell. Physiol. 69:65.
Fried, W., Plzak, L.F., Jacobson, L.O., Goldwasser, E. (1957). Studies on erythropoiesis. III. Factors controlling erythropoietin production. Proc. Soc. exp. Biol. Med. 94:237.
Fried, W., Chamberlin, W., Knospe, W.H., Husseini, S., Trobaugh, F.E. (1973a). Studies on the defective hemopoietic microenvironment of Sl/Sl^{d} mice. Br. J. Haematol. 24:643.
Fried, W., Husseini, S., Knospe, W.H., Trobaugh, F.E. (1973b). Studies on the source of hemopoietic tissue in the marrow of subcutaneously implanted femurs. Exp. Hematol. 1:29.
Fried, W., Chamberlin, W., Kedo, A., Barone, J. (1976). Effect of radiation on hemopoietic stroma. Exp. Hematol. 4:310.
Friedenstein, A.J., Petrakova, K.V., Kuralesova, A.I., Frovola, G.P. (1968). Heterotopic transplants of bone marrow. Analysis of precursor cells for osteogenic and hemopoietic tissues. Transplantation 5:230.
Friedenstein, A.J., Chailakhyan, R.K., Lalykina, K.S. (1970). The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinet. 3:395.
Friedenstein, A.J., Chailakhyan, R.K., Latsinik, N.V., Panaysuk, A.F., Keilissborok, I.V. (1974a). Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. Transplantation 17:331.
Friedenstein, A.J., Deriglasova, U.F., Kulagina, N.N., Panasyuk, A.F., Rudakova, S.F., Luria, E.A., Rudakov, I.A. (1974b). Precursors of fibroblasts in different populations of hemopoietic cells as detected by the *in vitro* colony assay method. Exp. Hematol. 2:83.
Friedenstein, A.J. (1976) Precursor cells of mechanocytes. Int. Rev. Cytol. 44:327.
Friedenstein, A.J., Gorskaja, U.F., Kulagina, N.N. (1976). Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp. Hematol. 4:267.
Friedenstein, A.J., Ivanov-Smolenskii, A.A., Chailakhyan, R.K., Gorskaja, U.F., Kuralesova, A.I., Latsinik, N.W., Gerasimov, U.W. (1978). Origin of bone marrow stromal mechanocytes in radiochimeras and heterotopic transplants. Exp. Hematol. 6:440.
Friedenstein, A.J., Latzinik, N.V., Gorskaja, U.F., Sidorovich, S.Y. (1981). Radiosensitivity and postirradiation changes of bone marrow clonogenic stromal mechanocytes. Int. J. Rad. Biol. 39:537.

Garland, J.M., Dexter, T.M. (1984). Promotion of hemopoietic progenitor cells by haemopoietic cell growth factor (HCGF) and relationship to 'interleukin 3'. Exp. Hematol. 12:76.

Garnett, H.M., Cronkite, E.P., Harigaya, K. (1982). Regulation of the CSA produced by a murine marrow-derived cell line (H-1). Proc. Natl. Acad. Sci. 79:1548.

Goldwasser, E., Kung, C.H-K. (1968). Progress in the purification of erythropoietin. Ann. N.Y. Acad. Sci. 149:49.

Goldwasser, E., Kung, C.H-K., Eliason, J. (1974). On the mechanism of erythropoietin induced differentiation. J. Biol. Chem. 249:4202.

Gong, J.K., MacVittie, T.J., Vertalino, J.E. (1969). A method for determining residual injury in the hematopoietic system of the X-irradiated rat. Radiat. Res. 37:467.

Gordon, M.Y., King, J.A., Gordon-Smith, E.C. (1980). Bone marrow fibroblasts, fat cells and CSA. Br. J. Haematol. 46:151.

Gordon, M.Y., Gordon-Smith, E.C. (1981). Bone marrow CFU-F in aplastic anemia. Colony growth and stimulation of GM-CFC. Br. J. Haematol. 49:465.

Greenberg, B.R., Wilson, F.D., Woo, L., Jenks, H.M. (1978). Cytogenetics of fibroblast colonies in Ph1-positive chronic myeloid leukemia. Blood 51:1039.

Greenberger, J.S. (1978). Sensitivity of corticosteroid-dependent insulin-resistant lipogenesis in marrow preadipocytes of obese-diabetic db/db mice. Nature 275:725.

Greenberger, J.S. (1979). Corticosteroid-dependent differentiation of human marrow preadipocytes in vitro. In Vitro 15:823.

Greenberger, J.S., Gans, P.J., Davisson, P.B., Moloney, W.C. (1979). In vitro induction of continuous acute promyelocyte leukemia cell lines by Friend or Abelson murine leukemia viruses. Blood 53:987.

Greenberger, J.S., Ecker, R.J., Otten, J.A., Tennant, R.W. (1982). In vitro quantitation of lethal and physiological effects of total body irradiation on stromal and hemopoietic stem cells in continuous bone marrow cultures from Rf mice. Int. J. Rad. Oncol. Phys. 8:1155.

Greenberger, J.S., Eckner, R.J., Shakeeny, M.A., Cohen, G.I. (1983). Role of stromal and hemopoietic stem cells in Friend spleen focus-forming virus effects in continuous bone marrow culture. Leuk. Res. 7:621.

Gregory, C.J. (1976). Erythropoietin sensitivity as a differentiation marker in the hemopoietic system: studies of three erythropoietic colony responses in culture. J. Cell. Physiol. 89:289.

Haas, R.J., Bohne, F., Fliedner, T.M. (1969). On the development of slowly turning over cell types in neonatal rat bone marrow (using ^{3}H-TdR incorporation). Blood 34:791.

Hara, H., Ogawa, M. (1976). Erythroid precursors in mice with phenylhydrazine-induced anemia. Am. J. Hematol. 1:453.

Hara, H., Ogawa, M. (1978). Murine hemopoietic colonies in culture containing normoblasts, macrophages and megakaryocytes. Am. J. Hematol. 4:23.

Haworth, C., Morris-Jones, P.H., Testa, N.G. (1982). Longterm bone marrow damage in children treated for ALL: Evidence from in vitro colony assays (GM-CFC and CFU-F). Br. J. Cancer 46:918.

Hays, E.F., Hale, L., Villarreal, B., Fitchen, J.H. (1982). Stromal and hemopoietic stem cell abnormalities in longterm cultures of marrow from busulfan-treated mice. Exp. Hematol. 10:383.

Hines, D.L. (1983). Lipid accumulation and production of CSA by the 261AD cell line derived from mouse bone marrow. Blood 61:397.

Hodgson, G.S., Bradley, T.R., Telfer, P.A. (1972). Hemopoietic stem cells in experimental hemolytic anemia. Cell Tissue Kinet. 5:283.

Hoffman, R., Mazur, E., Bruno, E., Floyd, V. (1981). Assay of an activity in the serum of patients with disorders of thrombopoiesis that stimulates formation of megakaryocyte colonies. New Engl. J. Med. 305:533.

Ihle, J.N., Pepersack, L., Rebar, L. (1981). Regulation of T cell differentiation: in vitro induction of 20-α-hydroxysteroid dehydrogenase in splenic lymphocytes is mediated by a unique lymphokine. J. Immunol. 126:2184.

Ihle, J.N., Keller, J., Oroszlan, S., Henderson, L.E., Copeland, T.D. et al. (1983). Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, P-cell-stimulating factor activity, colony stimulating factor activity and histamine-producing cell-stimulating factor activity. J. Immunol. 131:282.

Iscove, N.N. (1977). The role of erythropoietin in regulation of population size and cell cycling of early and late erythroid precursors in mouse bone marrow. Cell Tissue Kinet. 10:323.

Iscove, N.N. (1978). Erythropoietin-independent stimulation of early erythropoiesis in adult marrow cultures by conditioned media from lectin-stimulated mouse spleen cells. In: Hematopoietic Cell Differentiation (Golde, D.W., Cline, M.J., Metcalf, D., Fox, C.F., eds). Academic Press, New York, 37.

Iscove, N.N., Roitsch, C.A., Williams, N., Guilbert, J. (1982). Molecules stimulating early red cell, granulocyte, macrophage, and megakaryocyte precursors in culture: similarity in size, hydrophobicity and charge. J. Cell. Physiol. suppl. 1:65.

Jacobson, R.J., Salo, A., Fialkow, P.J. (1978). Agnogenic myeloid metaplasia: a clonal proliferation of hematopoietic stem cells with secondary myelofibrosis. Blood 51:189.

Jandl, J.H., Files, N.M., Bell Barnett, S., MacDonald, R.A. (1965). Proliferative response of the spleen and liver to hemolysis. J. Exp. Med. 122:299.

Johnson, G.R., Metcalf, D. (1977). Pure and mixed erythroid colony formation in vitro stimulated by spleen conditioned medium with no detectable erythropoietin. Proc. Natl. Acad. Sci. 74:3879.

Johnson, G.R., Metcalf, D. (1978). Sources and nature of GM-CSF in fetal mice. Exp. Hematol. 6:327.

Kaneko, S., Motomura, S., Ibayashi, H. (1982). Differentiation of human bone marrow CFU-F and their roles in hemopoiesis in vitro. Br. J. Haematol. 51:217.

Keating, A., Singer, J.W., Killen, P.D., Striker, G.E., Salo, A.C., Sanders, J., Thomes, E.D. et al. (1982). Donor origin of the in vitro hemopoietic microenvironment after bone marrow transplantation in man. Nature 298:280.

Kedo, A., Barone, J., Fried, W. (1976). Regeneration of CFU-S in the marrow of mice exposed to 300 rads after having recovered from 950 rads. Cell Tissue Kinet. 9:541.

Kelemen, E., Cserhati, I., Tanos, B. (1958). Demonstration and some properties of human thrombopoietin in thrombocythaemic sera. Acta Haematol. 20:350.

Keller, G.M., Johnson, G.R., Phillips, R.A. (1983). Hemopoiesis in spleen and bone marrow cultures. J. Cell. Physiol. 116:7.

Knospe, W.H., Blom, J., Crosby, W.H. (1966). Regeneration of locally irradiated marrow. I. Dose dependent longterm changes in rat, with particular emphasis upon vascular and stromal reaction. Blood 28:398.

Knospe, W.H., Blom, J., Crosby, W.H. (1968). Regeneration of locally irradiated marrow. II. Induction of regeneration in permanently aplastic medullary cavities. Blood 31:400.

Knospe, W.H., Husseini, S., Trobaugh, F.E. (1978). Hematopoiesis on cellulose ester membrane (CEM). II. Enrichment of the hematopoietic microenvironment by the addition of selected cellular elements. Exp. Hematol. 6:601.

Knudtzon, S., Mortensen, B.T. (1975). Growth stimulation of human bone marrow cells in agar culture by vascular cells. Blood 46:937.

Kodama, H., Amagai, Y., Koyama, H., Kasai, S. (1982). Hormonal responsiveness of a preadipose cell line derived from newborn mouse calvaria. J. Cell. Physiol. 112:83.

Kovach, J.S., Marks, P.A., Russell, E.S., Epler, H. (1967). Erythroid cell development in fetal mice: ultrastructural characteristics and hemoglobin synthesis. J. Molec. Biol. 25:131.

Kurland, J., Moore, M.A.S. (1977). Modulation of hemopoiesis by prostaglandins. Exp. Hematol. 5:357.

Kurland, J., Meyers, P.A., Moore, M.A.S. (1980). Synthesis and release of erythroid colony and burst-potentiating activities by purified populations of murine peritoneal macrophages. J. Exp. Med. 151:839.

Lanotte, M., Scott, D., Dexter, T.M., Allen, T.D. (1982a). Clonal preadipocyte cell lines with different phenotypes derived from murine marrow stroma: factors influencing growth and adipogenesis in vitro. J. Cell. Physiol. 111:177.

Lanotte, M., Metcalf, D., Dexter, T.M. (1982b). Production of monocyte-macrophage CSF by preadipocyte cell lines derived from murine marrow stroma. J. Cell. Physiol. 112:123.

Levin, J. (1983). Murine megakaryocytopoiesis in vitro: an analysis of culture systems used for the study of megakaryocyte colony-forming cells and of the characteristics of megakaryocyte colonies. Blood 61:617.

Lichtman, M.A. (1981). The ultrastructure of the hemopoietic microenvironment of the marrow, a review. Exp. Hematol. 9:391.

Lieschke, G.J. (1982). Kinetics of the formation of pure and heterogeneous megakaryocyte colonies by megakaryocyte progenitor cells in vitro. Exp. Hematol. 10:78.

Linch, D.C., Nathan, D.G. (1984). T-cell and monocyte-derived burst-promoting activity directly act on erythroid progenitor cells. Nature 312:775.

Lord, B.I., Testa, N.G., Hendry, J.H. (1975). The relative spatial distribution of CFU-s and CFU-C in the normal mouse femur. Blood 46:65.

Löwenberg, B., Dicke, K.A. (1977). Induction of proliferation of haemopoietic stem cells in culture. Exp. Hematol. 5:319.

Luria, E.A., Panasyuk, A.F., Friedenstein, A.Y. (1971). Fibroblast colony formation from monolayer cultures of blood cells. Transfusion 11:345.

Magli, M.C., Iscove, N.N., Odartchenko, N. (1982). Transient nature of early hemopoietic spleen colonies. Nature 295:527.

Maloney, M.A., Patt, H.M. (1972). Persistent marrow hypocellularity after local X-irradiation of the rabbit femur with 1000 rad. Radiat. Res. 50:284.

Maniatis, A.K., Amsel, S., Mitas, W.J., Coleman, N. (1969). Chromosome pattern of bone marrow fibroblasts in patients with chronic granulocytic leukemia. Nature 222:1278.

Maniatis, A.K., Tavassoli, M., Crosby, W.H. (1971). Origin of osteogenic precursor cells in extramedullary marrow implants. Blood 38:569.

Martin, C., Beaupain, D., Dieterlen-Lievre, F. (1980). A study of the development of the hemopoietic system using quail-chick chimeras obtained by blastoderm recombination. Devel. Biol. 75:303.

McCulloch, E.A., Siminovitch, L., Till, J.E., Russell, E.S., Bernstein, S.E. (1965). The cellular basis of the genetically determined hemopoietic defect in anemic mice of genotype Sl/Sl^{d}. Blood 26:399.

McCulloch, E.A., Thompson, M.M., Siminovitch, L., Till, J.E. (1970). Effects of bacterial endotoxin on hemopoietic colony-forming cells in the spleen of normal mice of genotype Sl/Sl^{d}. Cell Tissue Kinet. 3:47.

McLeod, D.L., Shreeve, M.M., Axelrad, A.A. (1976). Induction of megakaryocyte colonies with platelet formation in vitro. Nature 261:492.

McLeod, D.L., Shreeve, M.M., Axelrad, A.A. (1980). Chromosome marker evidence for the bipotentiality of BFU-E. Blood 56:318.

McManus, P.M., Weiss, L. (1984). Busulphan-induced chronic bone marrow failure: changes in cortical bone, marrow stromal cells and adherent cell colonies. Blood 64:1036.
Mendelow, B., Crobnicki, D., delaHunt, M., Katz, J., Metz, J. (1980). Characteristics of bone marrow stromal cells in suspension and monolayer cultures. Br. J. Haematol. 46:15.
Messner, H.A., Jamal, N., Izaguirre, C. (1982). The growth of large megakaryocyte colonies from human bone marrow. J. Cell. Physiol. suppl. 1:45.
Metcalf, D., Moore, M.A.S. (1971). Hemopoietic cells. North-Holland Publishing Company, Amsterdam, London.
Metcalf, D. (1972). Formation in agar of fibroblast-like colonies by cells from the mouse pleural cavity and other sources. J. Cell. Physiol. 80:409.
Metcalf, D., Nossal, G.J.V., Warner, L.N., Miller, J.F.A.P., Mandel, T.E., Layton, J.E., Gutman, G.A. (1975a). Growth of B lymphocyte colonies *in vitro*. J. Exp. Med. 142:1534.
Metcalf, D., MacDonald, H.R., Odartchenko, N. (1975b). Growth of mouse megakaryocyte colonies *in vitro*. Proc. Natl. Acad. Sci. 72:1744.
Metcalf, D., Johnson, G.R., Mandel, T.E. (1979). Colony formation in agar by multipotential hemopoietic cells. J. Cell. Physiol. 98:401.
Metcalf, D., Nicola, N.A. (1983). Proliferative effects of purified granulocyte colony-stimulating factor (G-CSF) on normal mouse hemopoietic cells. J. Cell. Physiol. 116:198.
Metcalf, D. (1984). The hemopoietic colony-stimulating factors. Elsevier, Amsterdam, New York, Oxford.
Mets, T., Verdonk, G. (1981). *In vitro* aging of human bone marrow derived stromal cells. Mech. Aging Dev. 16:81.
Miller, J.F.A.P., Osoba, D. (1967). Current concepts of the immunological function of the thymus. Immunol. Rev. 47:437.
Mirand, E.A., Prentice, T.C. (1957). Presence of plasma erythropoietin in hypoxic rats with or without kidney(s) and/or spleen. Proc. Soc. Exp. Biol. N.Y. 96:49.
Molendijk, W.J., Ploemacher, R.E. (1984). Effect of serum from mice treated with lipopolysaccharide on cycling of CFU-s *in vitro*. Exp. Hematol. 12:794.
Monette, F.C., DiMello, J.B. (1979). The relationships between stem cell seeding efficiency and position in cell cycle. Cell Tissue Kinet. 12:161.
Moore, M.A.S., Owen, J.J.T. (1967). Chromosome marker studies in the irradiated chick embryo. Nature 215, 1081.
Moore, M.A.S., Metcalf, D. (1970). Ontogeny of the haemopoietic system: Yolk sac origin of *in vivo* and *in vitro* colony forming cells in the developing mouse embryo. Br. J. Haematol. 18:279.
Mulder, A.H., Visser, J.W.M., van den Engh, G.J., Bauman, J.G.J. (1984). Prothymocytes can develop from day 12 CFU-s but not from day 8 CFU-s. Exp. Hematol. 12:414.
Murphy, M.J. Jr., Urabe, A. (1979). Modulatory effects of macrophages on erythropoiesis. In: *In vitro* aspects of erythropoiesis (Murphy, M.J. Jr., ed.). Springer Verlag, New York, 189.
Nagao, T., Yamauchi, K., Komatsuda, M. (1983a). Serial *in vitro* bone marrow fibroblast cultures in human leukemia. Blood 61:589.
Nagao, T., Yamauchi, K., Komatsuda, M., Noguchi, K., Shimizu, M., Yonekura, S., Nosaki, H. (1983b). Inhibition of human bone marrow fibroblast colony formation by leukemic cells. Blood 62:1261.
Nakeff, A., Daniels-McQueen, S. (1976). *In vitro* colony assay for a new class of megakaryocyte precursor: colony-forming unit megakaryocyte (CFU-M). Proc. Soc. exp. Biol. Med. 151:587.

Nathan, D.G., Chess, L., Hillman, D.G., Clarke, B., Breard, J., Merler, E., Housman, D.E. (1978). Human erythroid burst-forming unit: T cell requirement for proliferation in vitro. J. Exp. Med. 147:324.

Nicola, N.A., Burgess, A.W., Metcalf, D. (1979). Similar molecular properties of granulocyte-macrophage colony-stimulating factors produced by different mouse organs in vitro and in vivo. J. Biol. Chem. 254:5290.

Niewisch, H., Hajdik, I., Sultanian, I., Vogel, H., Matioli, G. (1970). Hemopoietic stem cell distribution in tissues of fetal and newborn mice. J. Cell. Physiol. 76:107.

Patt, H.M., Maloney, M.A. (1975). Bone marrow regeneration after local injury: a review. Exp. Hematol. 3:135.

Penington, D.G. (1979). Megakaryocyte colony culture using a liver cell conditioned medium. Blood Cells 5:13.

Ploemacher, R.E., van Soest, P.L., Vos, O. (1977): Kinetics of erythropoiesis in the liver induced in adult mice by phenylhydrazine. Scand. J. Hematol. 19:424.

Ploemacher, R.E. (1978). The erythroid hemopoietic microenvironments. Thesis, Rotterdam.

Ploemacher, R.E., van Soest, P.L. (1979). Haemopoietic stroma. I. Effects of radiation on proliferative and differential support capacity for haemopoiesis. IRCS Medical Science 7:234.

Ploemacher, R.E., van Soest, P.L., Wagemaker, G., van 't Hull, E. (1979). Particle-induced erythropoietin-independent effects on erythroid precursor cells in murine bone marrow. Cell Tissue Kinet. 12:539.

Ploemacher, R.E., Brons, N.H.C., van Soest, P.L. (1982). Relative stability of inductive properties versus adaptable support capacity for hemopoietic colony formation in the spleen. Exp. Hematol. 10:187.

Ploemacher, R.E., Brockbank, K.G.M., Brons, N.H.C., de Ruiter, H. (1983). Latent sustained injury of murine hematopoietic organ stroma induced by ionizing radiation. Hematologica 68:454.

Ploemacher, R.E., Brons, N.H.C. (1984). Characteristics of the CFU-s population in mice carrying the Sl^J allele. Cell Tissue Kinet. 17:1.

Ploemacher, R.E., Piersma, A.H., Brockbank, K.G.M. (1985). The nature and function of granulopoietic microenvironments. Blood Cells 10:in press.

Pluznik, D.H., Sachs, L. (1965). The cloning of normal mast cells in tissue culture. J. Cell. Comp. Physiol. 66:319.

Pluznik, D.H., Sachs, L. (1966). The induction of colonies of normal mast cells by a substance from conditioned medium. Exp. Cell Res. 43:553.

Porter, P.N., Ogawa, M. (1982). Characteristics of human erythroid BPA derived from bone marrow conditioned media. Blood 59:1207.

Reincke, U., Hsieh, P., Mauch, P., Kellmann, S., Chen, L.B. (1982). Cell types associated with fibronectin in long-term bone marrow cultures. J. Histochem. Cytochem. 30:235.

Reissman, K.R. (1950). Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia. Blood 5:372.

Rencricca, N.J., Rizzoli, V., Howard, D., Duffy, P., Stohlman, F. Jr. (1970). Stem cell migration and proliferation during severe anemia. Blood 36:764.

Robinson, W., Metcalf, D., Bradley, T.R. (1967). Stimulation by normal and leukemic mouse sera of colony formation in vitro by mouse bone marrow cells. J. Cell. Physiol. 69:83.

Rozenszajn, L.A., Shoham, D., Kalechman, Y. (1975). Clonal proliferation of PHA-stimulated human lymphocytes in soft agar culture. Immunology 29:1041.

Rozenszajn, L.A., Michlin, H., Kalechman, Y., Sredni, B. (1977). Colony growth in vitro of mitogen stimulated mouse B lymphocytes. Immunology 32:319.

Rozenszajn, L.A., Goldman, I., Kalechman, Y., Michlin, H., Sredni, B., Zeevi, A., Shoham, D. (1981). T lymphocyte colony growth in vitro: factors modulating clonal expansion. Immunol. Rev. 54:157.

Schofield, R. (1978). The relationship between the spleen colony forming cell and the hemopoietic stem cell: a hypothesis. Blood Cells 4:7.

Silini, G., Pozzi, L.V., Pons, S. (1967). Studies on the hemopoietic stem cells of mouse foetal liver. J. Embryol. Exp. Morphol. 17:303.

Siminovitch, L., McCulloch, E.A., Till, J.E. (1963). The distribution of colony-forming cells among spleen colonies. J. Cell. Comp. Physiol. 62:327.

Smith, K.A. (1984). Lymphokine regulation of T cell and B cell function. In: Fundamental immunology (Paul, W.E., ed.). Raven Press, New York, p. 559.

Springer, T., Galfré, G., Secher, D.S., Milstein, C. (1979). Mac-1: a macrophage differentiation antigen identified by monoclonal antibodies. Eur. J. Immunol. 9:301.

Sredni, B., Gopaz, J., Rozenszajn, L.A. (1978). Induction of B lymphocyte colony growth in vitro by thymus-derived stimulating factor. Eur. J. Immunol. 8:681.

Staber, F.G., Metcalf, D. (1980). Cellular and molecular basis of the increased splenic hemopoiesis in mice treated with bacterial cell wall components. Proc. Natl. Acad. Sci. 77:4322.

Stanley, E.R., Heard, P.M. (1977). Factors regulating macrophage production and growth. Purification and some properties of the CSF from medium conditioned by mouse L cells. J. Biol. Chem. 252:4305.

Stephenson, J.R., Axelrad, A.A., McLeod, D.L., Shreeve, M.M. (1971). Induction of colonies of hemoglobin-synthesizing cells by erythropoietin in vitro. Proc. Natl. Acad. Sci. 68:1542.

Stohlman, F. Jr., Roth, C.E., Rose, J.C. (1954). Evidence for a humoral regulation of erythropoiesis. Studies on a patient with polycythemia secondary to regional hypoxia. Blood 9:721.

Stohlman, F. Jr., Ebbe, S., Morse, B., Howard, D., Donovan, J. (1968). Regulation of erythropoiesis: XX kinetics of red cell production. Ann. N.Y. Acad. Sci. 149:156.

St.Pierre, R.L., Ackerman, G.A. (1965). Bursa of Fabricius in chicken: possible humoral factor. Science 147:1307.

Tavassoli, M., Crosby, W.H. (1968). Transplantation of marrow to extramedullary sites. Science 161:54.

Tavassoli, M., Ratzan, R.J., Maniatis, A., Crosby, W.H. (1973). Regeneration of hemopoietic stroma in anemic mice of Sl/Sl^{d} and W/W^{v} genotype. J. Reticuloendothel. Soc. 13:518.

Tavassoli, M., Maniatis, A., Crosby, W.H. (1974). Induction of sustained hemopoiesis in fatty marrow. Blood 43:33.

Tavassoli, M. (1975). Studies on hemopoietic microenvironments. Exp. Hematol. 3:213.

Tavassoli, M. (1979). The blood marrow barrier. Review. Br. J. Haematol. 41: 297.

Tavassoli, M. (1982). Radiosensitivity of stromal cells responsible for in vitro maintenance of hemopoietic stem cells in continuous long-term bone marrow cultures. Exp. Hematol. 10:435.

Tavassoli, M., Friedenstein, A. (1983). Hemopoietic stromal microenvironment. Am. J. Hematol. 15:195.

Till, J.E., McCulloch, E.A. (1961). A direct measurement of the radiation sensitivity of normal mouse bone marrow cultures. Radiat. Res. 14:213.

Touw, I.P., Löwenberg, B. (1983). No stimulative effect of adipocytes on hematopoiesis in longterm human bone marrow cultures. Blood 61:770.

Trentin, J.J. (1970). Influence of hematopoietic organ stroma (hematopoietic inductive microenvironment) on stem cell differentiation. In: Regulation of hematopoiesis, I. (Gordon, A.S., ed.). Appleton-Century-Crofts, Educational Division, Meredith Corporation, New York, 161.

Trentin, J.J. (1971). Determination of bone marrow stem cell differentiation by stromal HIM. Am. J. Pathol. 65:621.

Turpen, J.B., Knudson, C.M., Hoefen, P.S. (1981). The early ontogeny of hematopoietic cells studied by grafting cytogenetically labeled tissue anlagen: localization of a prospective stem cell compartment. Devel. Biol. 85:99.

Tyan, M.L. (1968). Studies on the ontogeny of the mouse immune system. I. Cell bound immunity. J. Immunol. 100:535.

Van Bekkum, D.W., van den Engh, G.J., Wagemaker, G., Bol, S.J.L., Visser, J.W.M. (1979). Structural identity of the pluripotential hemopoietic stem cell. Blood Cells 5:143.

Van Slyck, E.J., Weiss, L., Dully, M. (1970). Chromosomal evidence for the secondary role of fibroblastic proliferation in acute myelofibrosis. Blood 36:729.

Vos, O. (1972). Multiplication of CFUs in mice after X irradiation and bone marrow transplantation. Cell Tissue Kinet. 5:341.

Vos, O., Buurman, W.A., Ploemacher, R.E. (1972). Mobilization of CFUs into the peripheral blood of the mouse: effects of endotoxin and other compounds. Cell Tissue Kinet. 5:467.

Vos, O. (1978). Effect of bacterial lipopolysaccharide on proliferation of CFUs. Exp. Hematol. 6:688.

Vos, O., Luiten, F., Ploemacher, R.E. (1980). Lodging of CFUs under various circumstances in bone marrow, spleen and liver. Exp. Hematol. 8:860.

Wagemaker, G., Ober-Kieftenburg, V.E., Brouwer, A., Peters-Slough, M.F. (1977). Some characteristics of in vitro erythroid colony and burst-forming units. In: Experimental Hematology Today (Baum, S.J., Ledney, G.D., eds.) Springer Verlag, New York, 103.

Wagemaker, G., Peters, M.F. (1978). Effects of human leukocyte conditioned medium on mouse haemopoietic progenitor cells. Cell Tissue Kinet. 11:45.

Wagemaker, G. (1980). Early erythropoietin-independent stage of in vitro erythropoiesis: relevance to stem cell differentiation. In: Experimental Hematology Today (Baum, S.J., Ledney, G.D., van Bekkum, D.W., eds.) Karger, Basel, p. 47.

Watson, J., Gillis, S., Marbrook, J., Mochizuki, D., Smith, K.A. (1979). Biochemical and biological characterization of lymphocyte regulating molecules. I. Purification of a class of murine lymphokines. J. Exp. Med. 150:849.

Weiss, L. (1970) Transmural cellular passage in the vascular sinuses of rat bone marrow. Blood 36:189.

Weiss, L., Chen, L.T. (1975). The organization of hematopoietic cords and vascular sinuses in bone marrow. Blood Cells 1:617.

Weiss, L., Sakai, H. (1984). The hematopoietic stroma. Am. J. Anat. 170:447.

Werts, E.D., Johnson, M.J., DeGowin, R.L. (1977). Post irradiation hemopoietic repopulation and stromal cell viability. Radiat. Res. 71:214.

Werts, E.D., Gibson, D.P., Knapp, S.A., DeGowin, R.L. (1980). Stromal cell migration procedes hemopoietic repopulation of the bone marrow after irradiation. Radiat. Res. 81:20.

Westen, H., Bainton, D.F. (1979). Association of alkaline phosphatase positive reticulum cells in bone marrow with granulocytic precursors. J. Exp. Med. 150:919.

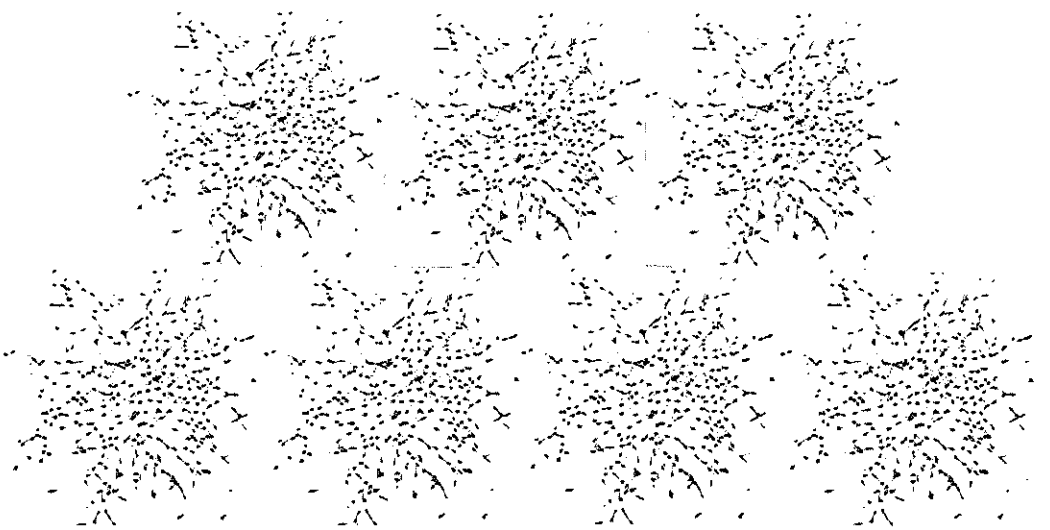
Williams, N., Jackson, H., Sheridan, A.P.C., Murphy, M.J. Jr., Elste, A., Moore, M.A.S. (1978). Regulation of megakaryopoiesis in longterm murine bone marrow cultures. Blood 51:245.

Williams, N., Eger, R.R., Jackson, H.M., Nelson, D.J. (1982). Two-factor requirement for murine megakaryocyte colony formation. J. Cell. Physiol. 110:101.
Wilson, F.D., O'Grady, L., McNeill, C., Munn, C.L. (1974). The formation of bone marrow derived fibroblastic plaques in vitro: preliminary results contrasting these populations to CFU-c. Exp. Hematol. 2:343.
Wolf, N.S., Trentin, J.J. (1968). Hemopoietic colony studies. V. Effect of hemopoietic organ stroma on differentiation of pluripotent stem cells. J. Exp. Med. 127:205.
Wolf, N.S. (1982). Dissecting the hemopoietic microenvironment. IV. Regeneration of splenic microstructure prerequisites and chronology of reconstruction. Exp. Hematol. 10:98.
Wu, A.M., Till, J.E., Siminovitch, L., McCulloch, E.A. (1967). A cytological study of the capacity for differentiation of normal hemopoietic colony-forming cells. J. Cell. Physiol. 69:177.
Xu, C.X., Hendry, J.H. (1981) The radial distribution of fibroblastic colony forming units in mouse femoral marrow. Biomedicine 35:119.
XU, C.X., Hendry, J.H., Testa, N.G., Allen, T.D. (1983). Stromal colonies from mouse marrow: characterization of cell types, optimization of plating efficiency and its effects on radiosensitivity, J. Cell Sci. 61:453.
Zipori, D. (1981a). Cell interactions in the bone marrow microenvironment: role of endogenous CSA. J. Cell. Biochem. 17:347.
Zipori, D., Sasson, T., Frenkel, A. (1981b). Myelopoiesis in presence of stromal cells from mouse bone marrow. I. Monosaccharides regulate colony formation. Exp. Hematol. 9:656.
Zipori, D., Sasson, T. (1981c). Myelopoiesis in presence of stromal cells from mouse bone marrow. II. Mechanism of glucose-dependent colony formation. Exp. Hematol. 9:663.
Zuckerman, K.S. (1980). Stimulation of human BFU-e by products of human monocytes and lymphocytes. Exp. Hematol. 8:924.

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren te Soest op 9 april 1957. De lagere school opleiding volgde hij grotendeels aan de Timotheusschool (hoofd: H. Piersma) te Alphen aan den Rijn. Het diploma Atheneum-ß werd behaald in juni 1975 aan het 'Christelijk Lyceum' (rector: Drs. A. Luttik) te Alphen aan den Rijn. In datzelfde jaar werd een begin gemaakt met de studie biologie met als tweede hoofdvak scheikunde (B4) aan de Rijksuniversiteit Utrecht, waarna het kandidaatsexamen werd afgelegd in juni 1978. Het doctoraaldiploma werd behaald in juni 1981 met als hoofdrichtingen experimentele tumorimmunologie (Prof.Dr. W. den Otter en Drs. H. van Loveren) en experimentele embryologie (Prof.Dr. N.H. Verdonk en Dr. C.A.M. van Dongen), en als bijvak experimentele farmacologie (Prof.Dr. J. van Noordwijk en Dr. L.P. Jager). Van augustus 1981 tot april 1985 bewerkte hij het in dit proefschrift beschreven onderzoek. Sinds 1 mei 1985 is hij als wetenschappelijk medewerker verbonden aan het Hubrecht Laboratorium (direkteur: Dr. S.W. de Laat) te Utrecht, alwaar hij onderzoek verricht aan de ontwikkeling van *in vitro* methoden voor de idenficatie van teratogene stoffen en hun werkingsmechanismen.

APPENDIX PAPERS



APPENDIX PAPER I

CHARACTERIZATION OF FIBROBLASTIC STROMAL CELLS FROM MURINE BONE MARROW

A.H. Piersma, K.G.M. Brockbank, R.E. Ploemacher, E. van Vliet,
C.M.J. Brakel-van Peer and W.J. Visser

Department of Cell Biology & Genetics, Erasmus University, Rotterdam.

In: Experimental Hematology 13, 237-243, 1985.

SUMMARY

Several properties of fibroblastic colony-forming units (CFU-F) from murine bone marrow and their in vitro progeny were evaluated. CFU-F had a high buoyant density relative to total bone marrow cells, they were non-cycling in situ, and adhered to nylon wool. The fibroblastic cells stained positively for fibronectin, lipid, alkaline phosphatase and nonspecific esterase, whilst phagocytosis assays were negative and ultrastructural analysis failed to reveal desmosomes. These properties contrasted bone marrow-derived fibroblastic cells to both endothelial cells and macrophages. Fibroblastic cells derived from several hemopoietic organs and skin were screened for antigenic determinants present on hemopoietic cells using monoclonal antibodies. Mac-1 and B220 were absent from all fibroblastic cells studied, whereas the Forsmann and Pgp-1 antigens were always present. Thy-1 was not detected on bone marrow-derived fibroblasts, but was present on fibroblastic cells derived from other sources. T200 was found on all hemopoietic organ-derived fibroblastic cells, but not on those derived from blood and skin. Thus, analysis of antigenic determinants allowed distinction between fibroblastic cells from different organs.

INTRODUCTION

Hemopoietic organ fibroblasts constitute a functional component of the hemopoietic stroma. These cells can transfer the hemopoietic stroma upon transplantation (1). Mesenchymal fibroblastoid cells are involved in the regeneration of hemopoietic organs following ectopic transplantation or injury induced by medullary marrow ablation (2-4). Furthermore, fibroblasts are present in adherent cell layers that have the ability to maintain in vitro hemopoiesis (5). In vitro colony formation assays have facilitated the detection of fibroblastic colony-forming cells (CFU-F) (6,7). Using these assays perturbations of CFU-F numbers under various circumstances have been studied (8-11). However, thorough characterization of CFU-F and their progeny is required in order to assess the nature of these fibroblastoid cells more precisely. The present study documents further characteristics of murine femoral CFU-F and of CFU-F-derived fibroblastoid cells in vitro. In particular, fibroblastic cells from several hemopoietic organs and skin were screened for antigenic determinants present on hemopoietic cells. We report that the spectrum of hemopoietic cell markers on fibroblastic cells depends on the organ from which they were derived.

MATERIALS AND METHODS

Mice and cell preparations. Male F1(CBA/Rij x C57BL/Rij) mice between 10 and 15 weeks old were used. Bone marrow cell (BMC) suspensions were prepared by flushing femur shafts with balanced salt solution (BSS) + 5% fetal calf serum (FCS). BMC were dispersed by repeated flushing through a 23 gauge syringe needle, washed once and resuspended in α-medium (α-modification of Dulbecco's modification of Eagles medium) + 5% FCS. Spleen cell suspensions were prepared by mincing spleens with scissors and pressing them through a nylon mesh sieve. Cell suspensions were washed once and resuspended in α-medium + 5% FCS.

CFU-F assay. Fibroblastic colony-forming units (CFU-F) were assayed by culturing 5×10^5 BMC or 5×10^6 spleen cells per ml of α-medium supplemented with 20% FCS and 0.8% methylcellulose in triplicate cultures for ten days in a humidified incubator containing 5% CO_2 in air at 37°C. Then the dishes were washed with cold phosphate-buffered saline (PBS), fixed with methanol, and stained with 10% Giemsa. Colonies containing at least 50 fibroblastic cells were counted with an inverted microscope.

Hemopoietic progenitor assays. Granulocyte/macrophage colony-forming units (CFU-GM) were assayed by culturing 10^5 BMC per ml under similar conditions for 7 days in α-medium + 10% FCS, 1% BSA and 10% Concanavalin A-stimulated murine spleen-conditioned medium. When determining erythroid burst-forming units (BFU-E), this medium was further supplemented with 0.5 units/ml erythropoietin and 10^{-4}M 2-mercaptoethanol.

Density separation. Buoyant density separation of BMC was performed as follows: Centrifugation tubes containing 3ml of a Ficoll 400 solution in PBS (pH 7.4) were subsequently overlayered with 2ml of a BMC suspension containing 2×10^7 nucleated cells per ml α-medium + 5% FCS. After 600g centrifugation for 20 minutes, fractions were collected.

S-phase analysis. For analysis of the proportion of CFU-F that was synthesizing DNA, hydroxyurea (HU), 900 mg/kg (12) in BSS or BSS alone was injected intravenously (i.v.) into mice, which were killed one hour later for determination of CFU-F numbers in bone marrow and spleen, as described above. The data are expressed as mean ± standard error of six organs determined individually.

Nylon wool filtration. Nylon wool columns were prepared by filling 6ml syringes with 0.6g nylon wool from Leuko-pak leukocyte filters (Fenwal). Columns were incubated at 37°C with α-medium + 20% FCS for 30 minutes before use. BMC suspensions in α-medium + 20% FCS (2×10^8 BMC per column at maximum) were then layered on the column and allowed to mix in with the nylon wool. After a 45 minutes incubation at 37°C nonadherent cells were eluted with 45 ml of α-medium + 20% FCS at a rate of 1ml per minute.

Phagocytosis assays. Phagocytosis assays were performed by incubating fibroblastic cells, grown for ten days on cover slips, for 30 min in 20% of a 1:60 dilution of Indian ink (Pelikan negro) or in 10% Latex (Sigma) at 37°C. Coverslips were then rinsed thoroughly with medium, and the cells were fixed with methanol for 10 min and stained with Giemsa stain. Control coverslips carried subconfluent peritioneal macrophages derived from the same mouse strain by rinsing cells from the peritoneal cavity with 6ml of BSS containing

5% FCS. Macrophages had been allowed to adhere overnight in α medium plus 20% FCS at 37°C.

Enzyme stains. Acid phosphatase (13), nonspecific esterase and alkaline phosphatase (14) stains were performed as originally described.

Fibronectin stain. Fibronectin was revealed by incubating unfixed CFU-F colonies with a conjugate of gelatin and fluorescein isothiocyanate (FITC) for 30 min at 37°C according to the method described by Hsieh et al. (15). After washing with PBS the cells were counterstained with 1% (wt/vol) toluidine blue in methanol (pH 5) for 5 min, then after a triple wash in double distilled water (DW) the slides were covered with 40% glycerol in DW (pH 7.4), and viewed with a fluorescence microscope.

Electron microscopy. For electron microscopy CFU-F colonies grown in plastic petri dishes were fixed for 1 hr in 3% glutaraldehyde in 0.1M cacodylate buffer at pH 7.3 and 4°C, washed in 0.1M cacodylate buffer and post-fixed in 1% OsO_4 for 1 hr. After a further wash in 0.1M cacodylate buffer the cells were incubated with 1% tannic acid in 0.05M cacodylate buffer at pH 7.0 and room temperature to improve tissue contrast (16). All steps in the incubation procedure were performed at pH 7.3 and 4°C. Dehydration via an ethanol series was followed by Epon embedding. To release the polymerised Epon (with cells) from the plastic, the dishes were alternately cooled in liquid nitrogen and heated in boiling water. Colonies were selected with an inverted microscope and cylinders, containing the selected cells, were drilled from the Epon and mounted in Epon blocks. Ultrathin sections were cut on a Reichert OM U_2 microtome and stained with a saturated solution of uranyl-acetate in water and lead citrate. The sections were studied with a Philips EM 400 electron microscope.

Cell preparation for staining with monoclonal antibodies. Target cells for staining with monoclonal antibodies were prepared as follows. Femoral bone marrow, spleen, or thymus single cell suspensions were cultured in 25cm^2 tissue culture flasks at 10^7 nucleated cells per 3ml α-medium + 20% FCS per flask for two weeks. Skin fibroblasts were grown from newborn mouse skin explants in α-medium + 20% FCS for two weeks. Blood was taken from the anterior eye chamber of ether anaesthetized mice. Red blood cells were sedimented at unit gravity for thirty minutes after mixing 1:1 with 0.2% methylcellulose in α-medium. After a wash in α-medium + 20% FCS the supernate cells were cultured in α-medium + 20% FCS + 0.8% methylcellulose. After ten days of culture the fibroblastic colonies that developed (11,25) were passaged with the aid of a rubber policeman and subcultured in 25cm^2 flasks. All subcultures were incubated for two weeks in a humidified incubator containing 10% CO_2 in air at 37°C. Cells were then scraped with a rubber policeman and placed on sixty-well Terasaki trays at 10^5 nucleated cells in 20 µl of α-medium + 20% FCS per well. After adherence overnight in an incubator, as above, the cells were fixed in 0.08% glutaraldehyde, washed three times with 0.05% Tween-20 in PBS and stored at 4°C in PBS containing 1% gelatin until used for monoclonal antibody staining. These procedures resulted in adherent cell populations with fibroblastic morphology. The absence of adherent macrophages in our fibroblast cultures is supported by the lack of Mac-1 antigen on the cells (Table 1).

Monoclonal antibody staining. Monoclonal rat-anti-mouse antibodies used were anti-Mac-1, clone M1/70 (17), anti Thy-1, clone 59AD22 (18), anti B220, clone RA3-3A1 (19), anti T-200, clone 30G12 (18), anti Forsmann, clone M1/22.25 (20), anti Pgp-1, clone 142/5.1 (21). Binding of antibodies was detected by staining with rabbit-anti-rat immunoglobulin conjugated to horse radish peroxidase (DAKO) and diaminobenzidine tetrahydrochloride (22) and viewing under a fluorescence microscope. Staining was compared with cells that had undergone the same procedure with the exception that no antibody was applied.

RESULTS

Characteristics of CFU-F. The density distribution of CFU-F in murine bone marrow was studied by employing multiple buoyant density separations. As shown in Figure 1, BMC had a peak density of 1.09-1.10 g/ml, whereas CFU-F peaked between 1.09 and 1.11 g/ml. In the 1.10-1.11 g/ml fraction, a thirty-fold enrichment in CFU-F numbers was obtained. After incubation of BMC on nylon wool no CFU-F could be detected in the eluate of the nylon wool columns (Fig. 2), whereas hemopoietic progenitors (BFU-E and CFU-G/M) were not reduced in relative numbers. After administration of hydroxyurea CFU-F numbers were not changed in bone marrow (48.3 ± 7.3 per 10^6 nucleated cells in experimental mice versus 54.8 ± 7.3 in control mice) and spleen (0.51 ± 0.08 per 10^6 nucleated cells in experimental mice versus 0.50 ± 0.10 in control mice), providing evidence that *in situ* CFU-F are essentially a non-cycling cell population.

Characteristics of fibroblastic cells in CFU-F colonies. May-Grünwald and Giemsa stained CFU-F colonies consisted of polygonal cells with large ovoid nuclei that contained five to ten nucleoli each (Figure 3). Phagocytic properties of CFU-F progeny were studied employing colloidal carbon particles and latex beads. The fibroblastic cells did not take up either particles or beads during 30 minutes of incubation, whereas control incubations using peritoneal macrophage cultures demonstrated extensive phagocytosis. Specific enzyme stains revealed that in CFU-F colonies acid phosphatase activity was very weak or absent, whereas nonspecific esterase was mildly positive. The alkaline phosphatase stain revealed variable positivity between fibroblastic cells within each colony. Cells in the center stained strongly positive, whereas towards the periphery cells stained weaker. These results contrasted with stains on peritoneal macrophages which stained strongly positive for acid phosphatase and negative for alkaline phosphatase. The presence of fibronectin was demonstrated by making use of the specific binding properties of this compound to gelatin (15).

Oil red O staining demonstrated the presence of many small fat droplets in the fibroblastic cells. Electron microscopy confirmed the presence of lipid-positive granules in CFU-F derived fibroblastic cells (Figure 4c). Ultrastructurally the nuclear profiles demonstrated a thin rim of condensed chromatin and at least one prominent nucleolus, while the cytoplasm contained rough and smooth endoplasmic reticulum, mitochondria, dense bodies, lipid droplets and the occasional multi-vesicular body (Figure 4a). Fibrous extracellular matrix was also observed (Figure 4b). These ultrastructural observations on murine CFU-F-derived fibroblastic cells are in agreement with those of Xu et al. (23). Pinocytotic or exocytotic structures resembling coated pits and vesicles were frequently seen (Figure 4b). Thorough screening of membrane profiles in sections containing confluent fibroblastic cells did not provide evidence for junctional complexes.

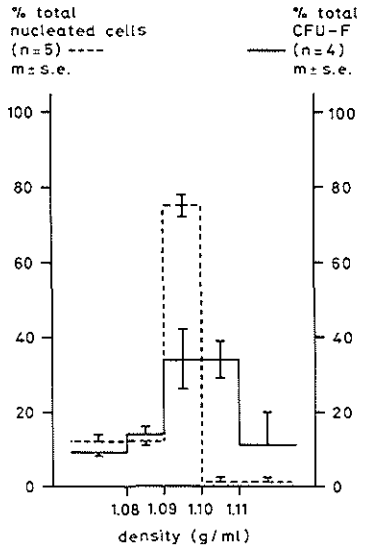


Figure 1. Density distribution of CFU-F as compared with total nucleated cells in murine femoral bone marrow, determined by multiple Ficoll density separation. n = number of experiments.

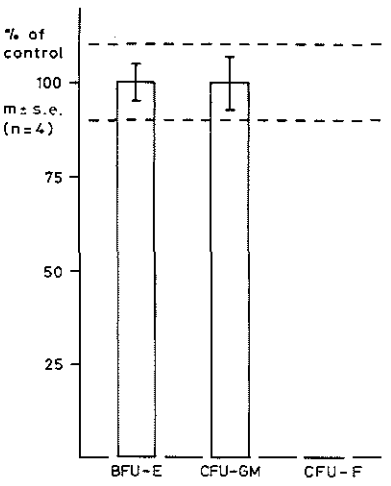


Figure 2. Effect of nylon wool adherence on hemopoietic and stromal progenitor cells. Data are expressed as progenitors present in the non-adherent fraction in per cent of normal bone marrow cells. n = number of experiments.

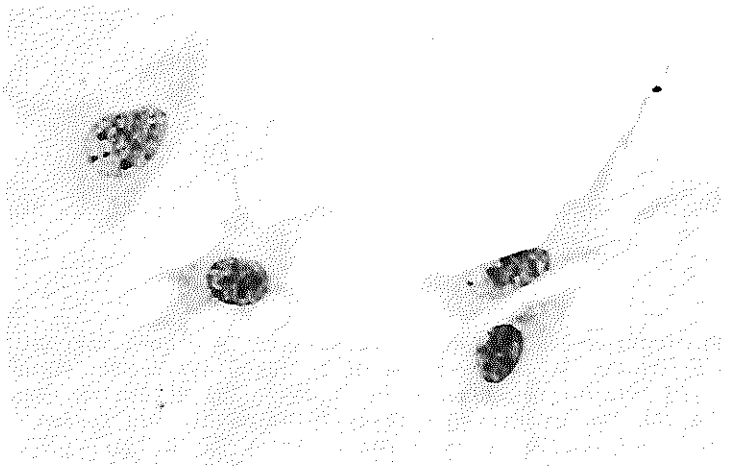


Figure 3. Light microscopic picture of fibroblastic cells in a bone marrow derived CFU-F colony, stained with standard Giemsa stain (x64).

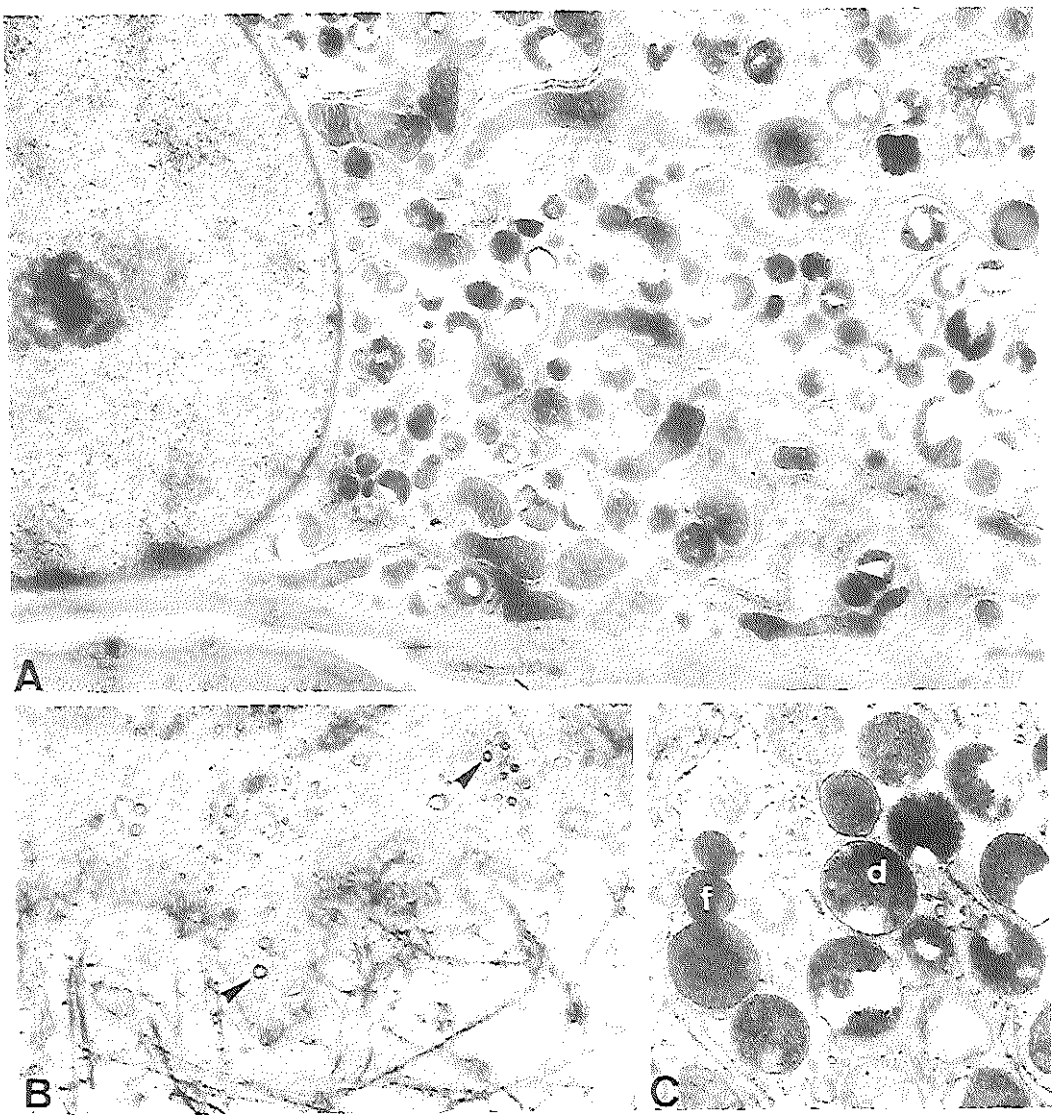


Figure 4. Transmission electron micrograph of fibroblasts in a bone marrow-derived CFU-F colony. (A) An Overview of a fibroblastic cell showing nuclear and cytoplasmic details (x 11,940). (B) A section through the edge of a cell demonstrating extracellular matrix and coated pits (examples indicated by arrows) and vesicles (x 18,080). (C) A group of three lipid droplets (f) and several dense bodies (d) (x 30,430).

Antigenic determinants on fibroblastic cells. Fibroblastic cells from hemopoietic organs and skin were screened for antigenic determinants present on hemopoietic cells using the immunoperoxidase technique (Table 1). Mac-1 and B220 were absent from all fibroblastic cells studied, whereas the Forsmann and Pgp-1 antigens were always present. Thy-1 was not detected on bone marrow fibroblastic cells but was present on the other fibroblastic cells. T200 was found on hemopoietic organ-derived CFU-F progeny, but not on blood and skin-derived fibroblastic cells.

DISCUSSION

CFU-F from murine bone marrow were characterized by a wide buoyant density distribution relative to total BMC. In a high density fraction CFU-F were enriched. These results are in agreement with what has been described for human bone marrow-derived CFU-F by Castro-Malaspina et al. (24) who reported a wide density distribution of CFU-F and also a dense fraction in which no CFU-GM were found although it contained 10% CFU-F. A similar wide density distribution was reported for another mouse strain (25), but in this case CFU-F tended to a more buoyant region than total BMC. CFU-F were extremely adherent to nylon wool, which is in agreement with data on plastic surface adherence of murine (1) and human (24) CFU-F. The ability of hydroxyurea to kill CFU-F has been demonstrated in mice treated with bacterial lipopolysaccharides or the BSS employed as a carrier for the lipopolysaccharides (11). In this study S-phase analysis of bone marrow and spleen cells using the hydroxyurea method indicated that CFU-F are not cycling in situ in normal animals. Haas et al. (26) and Castro-Malaspina et al. (24) found similar results for rat bone marrow fibroblastic reticular cells and human bone marrow CFU-F, respectively.

The in vitro progeny of CFU-F had a fibroblastoid morphology, produced fibronectin, contained oil Red O positive lipid granules, and lacked the ability to phagocytose latex and carbon particles. These findings agree with reports on murine (23,27) and human (24) CFU-F progeny. Fibronectin is involved in a variety of cell attachment phenomena (28) and a recent study (29) suggests that attachment of early hematopoietic cells to bone marrow connective tissue may be mediated by fibronectin. Stains for lysosomal enzymes, which were partially performed in previous murine (30) and human (24) studies provided further evidence that CFU-F progeny are not macrophages. It is interesting to note that Westen and Bainton (31) have described a fibroblastoid reticulum cell in association with cells of the granulocytic series that is alkaline phosphatase positive and most concentrated in the endosteal marrow. CFU-F are also more frequent in endosteal marrow (32) and in vitro CFU-F colonies are alkaline phosphatase-positive and produce granulocyte/macrophage colony stimulating factor (33).

Bone marrow CFU-F-derived fibroblastic cells were further characterized by the presence of hemopoietic cell determinants that were visualized using monoclonal antibodies (Table 1). Markers that are specific for mature blood cells (Mac-1 for macrophages and granulocytes, Thy-1 for T cells, B220 for B cells) were not found on bone marrow fibroblastic cells whereas antigens, known to be present also or exclusively on immature hemopoietic cells (T-200, Forsmann, Pgp-1) (34) were uniformly present on these fibroblastic cells. However, the possibility that these preparations were contaminated with some immature hemopoietic cells adhering to their cell surface has not been completely ruled out.

TABLE 1

ANTIGENIC PROPERTIES OF FIBROBLASTIC CELLS DERIVED FROM SEVERAL ORGANS[a]

Antibody	Reference	Antigen	Bone marrow	Spleen	Thymus	Blood	Skin	Specificity hemopoietic cells (34)
M1/70	16	Mac-1	-	-	-	-	-	macrophages, granulocytes
59AD22	17	Thy-1	-	+	+	+	+	T cells
RA3-3A1	18	B220	-	-	-	-	-	B cells
30G12	17	T200	+	+	+	-	-	all except erythrocytes, CFU-S
M1/22.25	17	Forsmann	+	+	+	+	+	erythrocytes and their progenitors
142/5.1	19	Pgp-1	+	+	+	+	+	CFU-S, GM-progenitors, mature granulocytes and macrophages

[a] All determinations were done at least three times in triplicate.

Staining for the T200 and Thy-1 markers made it possible to discriminate between fibroblastic cells derived from the various hemopoietic organs and skin. Friedenstein et al. (1) have shown that fibroblasts cultured in vitro, when retransplanted under the kidney capsule would give rise to stromal tissue, representative of the organ from which they were originally derived; which indicates that the fibroblasts had organ-specific properties. The marker studies presented here demonstrate antigenic differences between fibroblastic stromal cells from different organs, which may be related to differences in functional requirements for each organ.

ACKNOWLEDGMENTS

We thank Prof.Dr. O. Vos for critically reviewing this manuscript and Mrs. Cary Meijerink-Clerkx for typing the manuscript.

This investigation was supported by The Netherlands Foundation for Pure Research (ZWO).

REFERENCES

1. Friedenstein AJ, Chailakhyan RK, Latsinik NB, Panasyuk AF, Keiliss-Borok ZV (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17, 331-340.
2. Tavassoli M, Crosby WH (1968) Transplantation of marrow to extramedullary sites. Science 161, 54-56.
3. Amsel S, Dell ES (1971) Bone marrow repopulation of subcutaneously grafted mouse femurs. Proc Soc Exp Biol Med 138, 550-552.
4. Patt HM, Maloney MA (1970) Reconstitution of bone marrow in a depleted medullary cavity. In: Hemopoietic cellular proliferation, ed. Stohlman FR JR; Grune & Stratton, NY, 56-66.
5. Dexter TM, Allen TD, Lajtha LE (1976) Conditions controlling the proliferation of hemopoietic stem cells in vitro. J Cell Physiol 91, 335-344.
6. Friedenstein AJ, Chailakhyan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea pig bone marrow and spleen cells. Cell Tissue Kinet 3, 395-403.
7. Wilson FD, O'Grady L, McNeill C, Munn SL (1974) The formation of bone marrow derived fibroblastic plaques in vitro: preliminary results contrasting these populations to CFU-C. Exp Hematol 2, 343-354.
8. Friedenstein AJ, Gorskaya UF, Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp Hematol 4, 267-274.
9. DeGowin RL, Gibson DM (1978) Suppressive effect of an extramedullary tumor on bone marrow erythropoiesis and stroma. Exp Hematol 6, 568-575.
10. Piersma AH, Ploemacher RE, Brockbank KGM (1983) Transplantation of bone marrow fibroblastoid stromal cells in mice via the intravenous route. Br J Haematol 54, 285-290.
11. Brockbank KGM, Ploemacher RE, van Peer CMJ (1983) Splenic accumulation of stromal progenitor cells in response to bacterial lipopolysaccharide. Exp Hematol 11, 358-363.
12. Benestad HB, Warhuus K, Reikvam A (1977) Hydroxyurea (HU) in experimental hematology. I. Characterization of in vitro and in vivo effects on progenitor and transit cells. Exp Hematol 5, 408-414.

13. Lojda A, van der Ploeg M, van Duyn P (1967) Phosphates of the naphtol AS series in the quantitative determination of alkaline and acid phosphatase activities 'in situ' studied in polyacrylamide model systems and by cytospectrophotometry. Histochemie 11, 13-32.
14. van Duyn P, Pascoe E, van der Ploeg M (1967) Theoretical and experimental aspects of enzyme determination in a cytochemical model system of polyacrylamide films containing alkaline phosphatase. J Histochem Cytochem 15, 631-645.
15. Hsieh P, Segal R, Chen LB (1980) Studies of fibronectin matrices in living cells with fluoresceinated gelatin. J Cell Biol 87, 14-22.
16. Simionescu N, Simionescu M (1976) Galloylglucoses of low molecular weight as mordant in electron microscopy. I. Procedure and evidence for mordanting effect. J Cell Biol 70, 608-621.
17. Springer T, Galfrē G, Secher DS, Milstein C (1979) Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur J Immunol 9, 301-306.
18. Ledbetter JA, Herzenberg LA (1979) Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. Immunol Rev 47, 63-90.
19. Coffman RL, Weissman IL (1981) B220: A B cell-specific member of the T200 glycoprotein family. Nature 289, 681-683.
20. Springer T, Galfrē G, Secher DS, Milstein C (1978) Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of novel leukocyte differentiation antigen. Eur J Immunol 8, 539-551.
21. Trowbridge IS, Lesley J, Schulte R, Hyman R, Trosser J (1982) Biochemical characterization and cellular distribution of a polymorphic, murine cell-surface glycoprotein expressed on lymphoid tissues. Immunogenetics 15, 299-312.
22. van Ewijk W, van Soest PL, van den Engh GJ (1981) Fluorescence analysis and anatomic distribution of mouse T lymphocyte subsets defined by monoclonal antibodies to the antigens Thy-1, Lyt-1, Lyt-2 and T-200. J Immunol 127, 2594-2604.
23. Xu CX, Hendry JH, Testa NG, Allen TD (1983) Stromal colonies from mouse marrow: characterization of cell types, optimization of plating efficiency and its effects on radiosensitivity. J Cell Sci 61, 453-466.
24. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MAS (1980) Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood 56, 289-301.
25. Zipori D, Bol S (1979) The role of fibroblastoid cells and macrophages from mouse bone marrow in the in vitro growth promotion of hemopoietic tumor cells. Exp Hematol 7, 206-208.
26. Haas RJ, Bohne E, Fliedner TM (1969) On the development of slowly turning over cell types in neonatal rat bone marrow. Blood 34, 791-805.
27. Werts ED, DeGowin RL, Knapp SK, Gibons DP (1980) Characterisation of marrow stromal (fibroblastoid) cells and their association with erythropoiesis. Exp Hematol 8, 423-433.
28. Yamada KM, Olden K (1978) Fibronectin-adhesive glycoproteins of cell surface and blood. Nature 275, 179-184.
29. Bentley SA, Tralka TS (1983) Fibronectin-mediated attachment of hematopoietic cells to stromal elements in continuous bone marrow culture. Exp Hematol 11, 129-138.
30. Friedenstein AJ, Latsinik NW, Grosheva AG, Gorskaya UF (1982) Marrow microenvironment transfer by heterotopic transplantation of freshly isolated and cultured cells in porous sponges. Exp Hematol 10, 217-227.

31. Westen H, Bainton DF (1979) Association of alkaline phosphatase-positive reticulum cells in bone marrow with granulocytic precursors. J Exp Med 150, 919-937.
32. Brockbank KGM, Piersma AH, van Peer CMJ (1982) The regional distribution of fibroblastoid progenitors in murine femoral marrow revisited. Biomedicine 36, 369-371.
33. Brockbank KGM, van Peer CMJ (1983) Colony-stimulating activity production by hemopoietic organ fibroblastic cells in vitro. Acta Haematol 69, 369-375.
34. van den Engh G, Bauman J, Mulder D, Visser J (1983) Measurement of antigen expression of hemopoietic stem cells and progenitor cells by fluorescence activated cell sorting. In: Hemopoietic stem cells. eds. Killman, Cronkite, Mullerberat, Munksgaard, Copenhagen, p. 135-147.

APPENDIX PAPER II

MONOCLONAL ANTIBODIES IDENTIFY SPECIFIC DETERMINANTS ON RETICULAR CELLS IN MURINE EMBRYONIC AND ADULT HEMOPOIETIC STROMA

A.H. Piersma, R.E. Ploemacher, K.G.M. Brockbank and C.P.E. Ottenheim

Department of Cell Biology & Genetics, Erasmus University, Rotterdam.

Submitted for publication

SUMMARY

Monoclonal antibodies were raised against fibroblastoid stromal cells derived from murine bone marrow. The fibroblastoid nature of these cells was demonstrated by the presence of collagen types I and III and fibronectin, and the absence of collagen type IV and laminin. Three antibodies to these fibroblastoid cells exclusively recognized hemopoietic stromal cells in splenic red pulp and bone marrow of adult mice, with the exception of the occasional cell in subcutaneous connective tissue. *In vivo*, a part of these reticular stromal cells phagocytize colloidal carbon particles. In addition, stromal cells were successfully stained in embryonic tissues which contain transient hemopoiesis, including the lateral plate mesoderm and the hepatic anlage, but not the extra-embryonic hemopoietic tissue of the yolk sac. Thus, monoclonal antibody staining correlated with the presence of adult type hemopoiesis but not fetal type hemopoiesis. These observations support the idea that the stroma of hemopoietic organs carries unique determinants which relate specifically to the presence of adult type hemopoiesis.

INTRODUCTION

Proliferation and differentiation of pluripotent hemopoietic stem cells in adult mice is normally confined to bone marrow and spleen. It is unknown what specific properties make these organs the residence of hemopoietic stem cells. Friedenstein et al. (1974) transplanted fibroblastoid stromal cells from spleen and bone marrow under the kidney capsule and observed the formation of hemopoietically functional stromal foci characteristic of the corresponding hemopoietic organ. This led them to the suggestion that fibroblastoid stromal cells in hemopoietic organs may carry determinants which specifically characterize the hemopoietic stroma. Here we describe three monoclonal antibodies raised against *in vitro* cultured primary fibroblastoid cells derived from murine bone marrow hemopoietic stroma.

MATERIALS AND METHODS

Murine male F1 (CBA/Rij x C57BL/Rij) bone marrow cells were cultured in α-medium (α modification of Dulbecco's minimal essential medium) supplemented with 20 per cent foetal calf serum at 10^7 nucleated cells in 3 ml of medium

per 25 cm^2 tissue culture flask. Medium was changed weekly. Within four weeks confluence was reached. Cells were then scraped and suspended in phosphate-buffered saline (PBS) and the contents of ten flasks was used for each immunization. Louvain rats were immunized by intraperitoneal injections with cell suspensions at days 0, 30, 60, 61, 62 and spleens were taken and suspended at day 64. Immune spleen cells were fused to P3-X63-Ag8.653 myeloma cells and hybridoma clones were grown in HAT medium, as described before (Van Vliet et al., 1980).

Clones were selected for their production of antibodies against cultured fibroblastoid bone marrow cells using an adapted version of the micro ELISA described by Van Soest et al. (1984). Briefly, confluent adherent bone marrow cell cultures, selected on morphological grounds for low macrophage contamination, were scraped and cells were allowed to adhere overnight on Terasaki trays and were subsequently fixed with 0.08% glutaraldehyde in PBS. First step antibodies were contained in hybridoma-supernates, second and third step reagents were rabbit-anti-rat-Ig-horse radish peroxidase (DAKO, Denmark) and diaminobenzidine, respectively.

Specific antibodies used to identify extracellular matrix components included rabbit-anti-human fibronectin (BRL, no. 6071SA), rabbit-anti-mouse laminin (BRL, no. 6265SA), and guinea pig-anti-human collagen type I, guinea pig-anti-human collagen type III, and rabbit-anti-human collagen type IV. The antibodies to collagens were produced by Dr. J.-M. Foidart (Liege, Belgium) and obtained through Dr. C.A.M. van Dongen (Utrecht, The Netherlands). Binding of these antibodies was tested in the micro ELISA system using protein A-β-galactosidase and 4-methyl-umbelliferyl-galactoside as second and third step reagents. In this assay murine bone marrow fibroblasts were compared to murine peritoneal macrophages and calf vascular endothelial cells.

Frozen sections (5 µm) of murine tissues were stained with rat anti-mouse monoclonal antibodies using a three-step ELISA method employing rabbit-anti-rat-Ig-horse-radish peroxidase and diaminobenzidine as indicated above. Mac-1 staining involved the M1/70 antibody (Springer et al., 1978) in the first step. Factor VIII related antigen was detected with a two-step ELISA, using rabbit-anti-human factor VIII antibodies conjugated to horse radish peroxidase (DAKO, Denmark) in the first step, and diaminobenzidine in the second. These anti-factor VIII antibodies cross-reacted with murine endothelium in frozen tissue sections. Staining was intensified by a further incubation in 0.5% $CuSO_4$ in physiological salt solution. Then preparations were fixed in 25% glutaraldehyde, dehydrated and embedded in Entellan.

Acid and alkaline phosphatase were detected as previously described by Van Duyn et al. (1967).

The immunoglobulin class of the monoclonal antibodies was determined by Ouchterlony double immunodiffusion in agar between tenfold concentrated hybridoma supernates and Ig class specific antibodies (Miles Laboratories, GB).

RESULTS

Cultured fibroblastoid bone marrow cells were used as the immunogen for producing monoclonal antibodies. This cell type has been characterized in detail in an earlier study (Piersma et al., 1985). The cultures were checked for contamination with other adherent stromal cells. Endothelial cells were not present as indicated by the absence of anti-factor-VIII related antigen. Macrophages, identified by staining with anti-Mac-1 antibodies, amounted maximally ten per cent. This cell type could be easily discriminated from the majority of fibroblastoid cells by their smaller and more rounded morphology.

TABLE 1

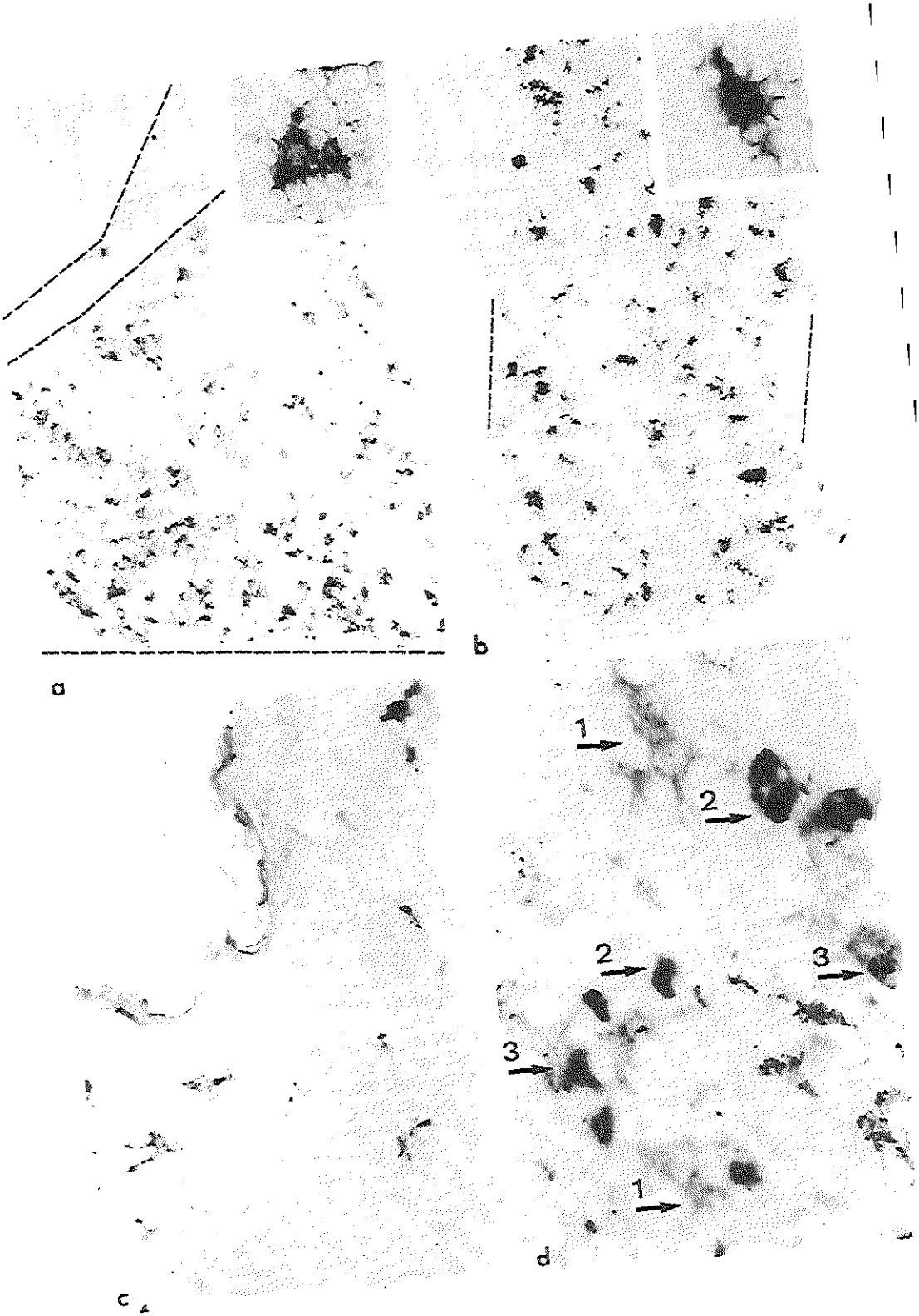
EXTRACELLULAR MATRIX COMPONENTS IN CULTURED ADHERENT CELLS

Antigen	Bone marrow fibroblasts	Peritoneal macrophages	Vascular endothelium
Collagen I	+	+	+
Collagen III	+	-	-
Collagen IV	-	-	+
Fibronectin	+	-	+
Laminin	-	-	+

The fibroblastoid nature of the latter cells was further proven by staining extracellular matrix components with specific antibodies (Table 1). The presence of collagen types I and III and fibronectin together with the absence of collagen type IV and laminin are typical fibroblastoid features, which also contrast them to macrophages and endothelial cells. In frozen sections three monoclonal antibodies to cultured fibroblastoid bone marrow cells, named ER-HR1, 2 and 3 specifically stained reticular cells branching extensively between non-staining hemopoietic cells in murine splenic red pulp (Fig. 1a) and bone marrow (Fig. 1b), in addition to an occasional cell in subcutaneous connective tissue (Fig. 1c).

When spleen and bone marrow frozen sections were stained with anti-factor-VIII related antigen, anti-Mac-1 antigen or for acid or alkaline phosphatase activity, the staining patterns were clearly different from those obtained using ER-HR1, 2 and 3 (not shown). These discrepancies indicate that these monoclonals did not solely recognize either the endothelium (factor VIII and alkaline phosphatase-positive) or macrophages (Mac-1 and acid phosphatase-positive) or the alkaline phosphatase-positive reticular cells described by Westen and Bainton (1979). Furthermore, when tissues derived from mice that had been injected with 0.2 ml indian ink one hour before termination were stained, our monoclonals detected both non-phagocytic and phagocytic reticular cells (Fig. 1d). The three antibodies revealed the same staining pattern and all belonged to the IgG2c subclass as determined in agar double immunodiffusion tests using subclass-specific anti-Ig-antibodies. No staining activity was found in the splenic white pulp and marginal zones, thymus, peripheral and mesenteric lymph nodes, Peyer's patches, duodenum, pancreas, muscle, liver, lung, kidney, testis and brain.

The distribution of the determinant during embryonic development was of particular interest (Table 2). The yolk sac of 9 day old embryos, which has been shown to contain blood islands with hemopoietic stem cells (Metcalf and Moore, 1971), failed to reveal a positive reaction after monoclonal antibody staining. In the embryonic liver around day 14 of development a few positive patches were observed. The number of these patches increased gradually until birth (Fig. 1e), but they were absent in the adult liver. In the spleen staining with the monoclonals was observed already shortly after establishment of the organ around day 17 of embryonic development (Fig. 1f), and became confined to the red pulp at the onset of splenic lymphopoiesis after birth. In the bone marrow staining was first observed around birth, and increased unto the adult stage. Positive staining was also observed in mesoderm-derived sub-


a
b
c
d
1
2
2
3
3
1

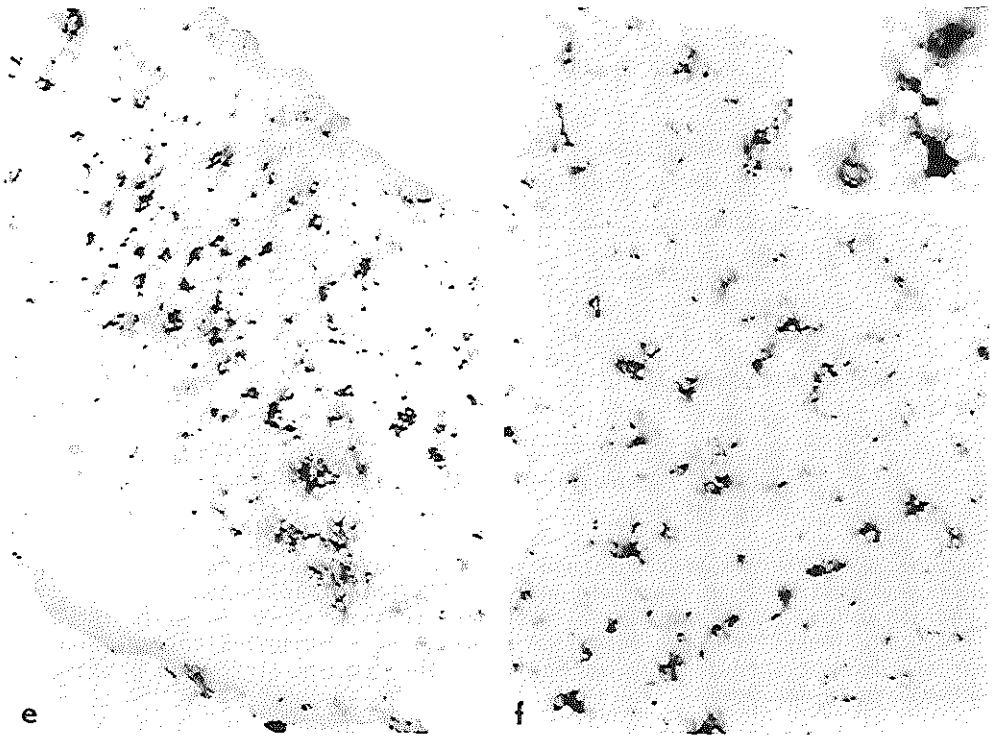


Figure 1. Frozen sections of murine tissues stained with an enzyme-linked immunosorbent assay using the monoclonal antibody ER-HR1:
(a) adult spleen, 40x objective; inset 100x; linings of white pulp, marginal zone and splenic capsule are indicated with dotted lines.
(b) adult bone marrow plug 40x; inset 100x; dotted lines indicate endosteal regions.
(c) adult skin, 63x.
(d) bone marrow plug of an adult mouse injected with 0.1 ml indian ink iv one hour before death, 100x; numbers indicate (1) cells staining with ER-HR1 only, (2) cells containing phagocytized carbon particles only, (3) cells staining with ER-HR1 that also contain phagocytized carbon particles; this picture was printed relatively light in order to allow discrimination between ER-HR1 staining and carbon particles.
(e) newborn spleen 40x.
(f) newborn liver, 40x; inset 100x.

TABLE 2

EXTENT OF MONOCLONAL ANTIBODY STAINING IN HEMOPOIETIC ORGANS AT VARIOUS STAGES OF DEVELOPMENT

(foetal) age	d. 9	d. 14	d. 17	birth	adult
yolk sac	-[a]	-	-	0	0
liver	0	+	+	++	-
splenic red pulp	0	0	+	++	+++
bone marrow	0	0	-	+	++

a. - = no staining; + = positive staining, relative intensity expressed by number of plusses; 0 = not relevant.

cutaneous connective tissue in the region situated roughly caudal from the neck and rostral from the liver anlage. This positivity became prominent shortly before birth and was still present to some extent in adult tissue (Fig. 1c). Apart from the described positivity in hemopoietically active organs and the subcutaneous connective tissue, no positivity was encountered elsewhere in embryos.

DISCUSSION

Monoclonal antibodies were produced against fibroblastoid cells derived from murine hemopoietic stroma. Their fibroblastoid nature was shown by determination of extracellular matrix components produced by these cells. The presence of collagen types I and III is generally agreed to be indicative of fibroblastoid cells (Bentley and Foidart, 1980; Castro-Malaspina et al., 1980). Our results discriminate fibroblasts from endothelium and macrophages, the alternative adherent stromal cell types (Allen and Dexter, 1976). Three monoclonal antibodies recognized at least one specific determinant on a population of non-phagocytic and phagocytic reticular cells in the hemopoietic stroma. These findings support the suggestion by Friedenstein et al. (1974) that stromal cells may carry determinants that are specifically related to the presence of hemopoiesis.

The function of ER-HR1, 2 and 3 positive cells within the hemopoietic stroma is as yet unknown. Interestingly, the staining pattern of ER-HR1, 2 and 3 in embryonic tissues coincides neither with the presence of hemopoiesis in the yolk sac blood islands (Metcalf and Moore, 1971) nor with the early peak of liver hemopoiesis before day 14 of embryonic development (Niewisch et al., 1970). These early hemopoietic events, however, are likely to contribute only to transient embryonic hemopoiesis and coincide with foetal hemoglobin production (Niewisch et al., 1970). On the other hand, the second burst of liver hemopoiesis, peaking around birth, as well as splenic hemopoiesis, both of which contribute to definitive adult hemopoiesis and coincide with adult hemoglobin production (Niewisch et al., 1970), are in good correlation with the staining pattern of ER-HR1, 2 and 3. It is remarkable that our monoclonal antibodies stain lateral plate mesoderm-derived connective tissue. This region has been implied to give rise to the hemopoietic stem cells that serve definitive hemopoiesis (Dieterlen-Lievre, 1975; Martin et al., 1980; Turpen

et al., 1981), as well as lymphopoiesis (Lassila et al., 1979; Kubai and Auerbach, 1983). The mesoderm anlage which reaches from the extra-embryonic yolk sac mesoderm to the intra-embryonic lateral plate mesoderm seems to comprise a continuum throughout which hemopoietic stem cells reside, and their probability of contributing to definitive hemopoiesis increases with their proximity to the lateral plate mesoderm. Thus, it has been suggested (Turpen et al., 1981) that extra-embryonic stem cells serve transient hemopoiesis, whereas intra-embryonic stem cells residing in places with ready access to the heart, aorta, cardinal veins, liver, spleen and thymus mainly give rise to definitive hemopoiesis. Although extra-embryonic stem cells may serve transient embryonic hemopoiesis, they have the capacity to repopulate the entire hemopoietic system (Moore and Owen, 1967; Tyan, 1968). Since the determinant of the hemopoietic stroma which is recognized by ER-HR1, 2 and 3 is specifically present in sites of definitive adult-type but not fetal hemopoiesis, these monoclonal antibodies provide a unique potential for further study of the hemopoietic stroma and its interactions with hemopoietic cells.

ACKNOWLEDGMENTS

We thank Dr. J.-M. Foidart and Dr. C.A.M. van Dongen for generously providing us with the specific antibodies. We thank Prof.Dr. O. Vos for critically reviewing this manuscript and Mrs. C.J.M. Meijerink-Clerkx for excellent secretarial assistance.

REFERENCES

Allen TD, Dexter TM. Cellular interrelationships during *in vitro* granulopoiesis. Differentiation 6, 191-194 (1976).

Bentley SA, Foidart JM. Some properties of marrow-derived adherent cells in tissue culture. Blood 56, 1006-1012 (1980).

Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Charieri D, Mckenzie S, Broxmeyer HE, Moore MAS. Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood 56, 289-301 (1980).

Dieterlen-Lievre FJ. On the origin of hemopoietic stem cells in the avian embryo: an experimental approach. J Embryol Exp Morphol 33, 607-619 (1975).

Friedenstein AJ, Chialakhyan RK, Latsinik NV, Panasyuk AF. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. Transplantation 17, 331-340 (1974).

Kubai L, Auerbach R. A new source of embryonic lymphocytes in the mouse. Nature 301, 154-156 (1983).

Lassila O, Eskola J, Toivanen P. Prebursal stem cells in the intra-embryonic mesenchyme of the chick embryo at 7 days of incubation. J Immunol 123, 2091-2094 (1979).

Martin C, Beaupain D, Dieterlen-Lievre F. A study of the development of the hemopoietic system using quail-chick chimaeras obtained by blastoderm recombination. Devel Biol 75, 303-314 (1980).

Metcalf D, Moore MAS. Haemopoietic cells. North Holland Publishing Company, Amsterdam, London, 1971.

Moore MAS, Owen JJT. Chromosome marker studies in the irradiated chick embryo. Nature 215, 1081-1082 (1967).

Niewisch H, Hajdik I, Sultanian I, Vogel H, Matioli G. Hemopoietic stem cell distribution in tissues of fetal and newborn mice. J Cell Physiol 76, 107-116 (1970).

Piersma AH, Brockbank KGM, Ploemacher RE, van Vliet E, Brakel-van Peer CMJ, Visser WJ. Characterization of fibroblastoid stromal cells from murine bone marrow. Exp Hematol 13, 237-243 (1985).

Springer T, Galfrē G, Secher DS, Milstein C. Monoclonal xenogeneic antibodies to murine cell surface antigens: identification of a novel leukocyte differentiation antigen. Eur J Immunol 8, 539-551 (1978).

Tyan ML. Studies on the ontogeny of the mouse immune system. I. Cell-bound immunity. J Immunol 100, 535-542 (1968).

Van Duyn P, Pascoe E, van der Ploeg MJ. Theoretical and experimental aspects of enzyme determination in a cytochemical model system of polyacrylamide films containing alkaline phosphatase. J Histochem Cytochem 15, 631-645 (1967).

Van Soest PL, de Josselin de Jong J, Lansdorp PM, Van Ewijk W. An automatic fluorescence micro-ELISA system for quantitative screening of hybridoma supernatants using a protein-A-β-galactosidase conjugate. Histochem J 16, 21, (1984).

Van Vliet PW, Van Ewijk W, Rozing J, Van Soest PL. in Peeters H (editor) Proc Colloquium Protides Biological Fluids, Pergamon Press, Oxford 1980, pp. 553-557: Monoclonal antibodies to cell surface antigens on cells of the mouse lymphoid system. Histophysiological applications (1980).

APPENDIX PAPER III

REGULATION OF IN VITRO MYELOPOIESIS BY A HEMOPOIETIC STROMAL FIBROBLASTIC CELL LINE

A.H. Piersma, K.G.M. Brockbank and R.E. Ploemacher

Department of Cell Biology & Genetics, Erasmus University, Rotterdam.

In: Experimental Hematology 12, 617-623, 1984

SUMMARY

An adherent cell line (AP63) derived from murine spleen was characterized as fibroblastic, and several of its properties distinguished it from other adherent cells (i.e., macrophages and endothelial cells). The ability of the AP63 cells to regulate in vitro myelopoiesis was investigated. Medium conditioned by the cell line (CM) induced granulocyte/macrophage (GM) colonies demonstrating the production of colony-stimulating activity by AP63 cells. A relatively large proportion of these colonies had a 'tight' morphology and contained many early myeloid cells and cells capable of secondary cluster and colony formation. CM also contained a prostaglandin-like inhibitor of colony formation. Furthermore AP63 cells inhibited GM colony formation by bone marrow cells in their immediate vicinity, whereas colony formation was stimulated at greater distances. These observations may reflect in vivo regulatory properties of hemopoietic stromal fibroblasts with respect to proliferation and differentiation of GM progenitor cells.

INTRODUCTION

Fibroblasts of the hemopoietic stroma are considered to play a role in the regulation of hemopoiesis. Friedenstein et al. (1) found that upon transplantation under the kidney capsule bone marrow fibroblasts could form a stroma in which hemopoiesis became established. Blackburn and Patt (2) were able to show that a 'marrow fibroblast-derived factor' increased the 24hr survival of hematopoietic stem cells. Wilson et al. (3) suggested that stromal fibroblasts could produce colony-stimulating activity (CSA). Further evidence for this notion was established recently in our laboratory (4).

A major drawback to the interpretation of recent work on stromal cells with respect to the role of fibroblasts is the mixed nature of the cell populations employed (3,5,6). In the present study, a murine splenic fibroblastic cell line is characterized and its capacity to regulate the formation of granulocyte/macrophage (GM) colonies is investigated. We report on the elaboration of stimulating and inhibitory activities that mediate the effects of this pure fibroblastic stromal cell population on myeloid colony formation.

MATERIALS AND METHODS

AP63 cell line. The AP63 cell line was derived from a splenic adherent cell culture of male F1 mouse (CBA/Rij x C57BL/Rij) origin. Cells were fed weekly with the α-modification of Dulbecco's modification of Eagles medium (α-medium) + 20% foetal calf serum (FCS). After a three months period in which no cell proliferation was noted, one culture grew to confluence. Cells were detached by treatment with 0.25% trypsin in phosphate buffered saline for 5 min at 37°C and passaged into twice the original volume and surface area in α medium + 20% FCS. This treatment was repeated weekly. The doubling time of the cultures averaged seven days and was stable during the course of the present study. Cells used for experiments had undergone seven to thirteen passages. Conditioned media (CM) were obtained from confluent AP63 cultures of at least two weeks' age, that had been refed with α-medium + 20% FCS seven days previously.

Phagocytosis assays. Phagocytosis assays were performed by incubating subconfluent cells cultured on cover slips for 30 min in 20% of a 1:60 dilution of Indian ink in α-medium (Pelikan 17 Negro) or in 1% (wt/vol) 0.8μm Latex beads (Sigma) at 37°C. Coverslips were then rinsed thoroughly with medium and cells were fixed with methanol for 10 min and stained with May Grünwald and Giemsa solution. Control coverslips carried subconfluent peritoneal macrophages derived from the same mouse strain by washing cells from the peritoneal cavity with 6ml balanced salt solution (BSS) containing 5% FCS. These cells were allowed to adhere overnight in α-medium + 20% FCS at 37°C.

Specific stains. The presence of the Mac-1 antigenic determinant was tested with an enzyme-linked immunosorbent assay (ELISA) using the M1/70 antibody (7). Binding of antibodies was assayed employing rabbit-anti-rat-Ig conjugated with horse-radish peroxidase. The reaction was completed by adding orthophenylenediamine, which would oxidize to give a yellow reaction product. Staining for factor VIII-related antigen was performed using rabbit-anti-human factor VIII conjugated to horse radish peroxidase (DAKO) and diaminobenzidine-tetrahydrochloride resulting in a brown precipitate in positive reactions. The DAKO reagent has been shown to cross-react with mouse factor VIII (8). Negative controls incubated with diaminobenzidine-tetrahydrochloride did not reveal endogenous peroxidase activity. Acid phosphatase stain was performed according to the method described by Lojda et al. (9); nonspecific esterase and alkaline phosphatase stains were performed as described by van Duijn et al. (10). Fibronectin was detected by incubating a conjugate of gelatin and fluorescein isothiocyanate for 30 min at 37°C according to the method described by Hsieh et al. (11).

Electron microscopy. Subconfluent monolayers of fibroblasts in plastic petri dishes were fixed for 1 hr in 3% glutaraldehyde in 0.1M cacodylate buffer, washed in 0.1M cacodylate buffer and postfixed in 1% OsO_4 for 1 hr. After a further wash in 0.1M cacodylate buffer the cells were incubated with tannic acid (12). All steps in the incubation procedure were performed at pH 7.3 and 4°C. Dehydration via an ethanol series was followed by Epon embedding. To release the polymerised Epon (with cells) from the plastic, the dishes were alternately cooled in liquid nitrogen and heated in boiling water. Cell groups were selected with an inverted microscope and cylinders, containing the selected cells, were drilled from the Epon and mounted on dummy blocks. Ultrathin sections were cut on a Reichert OM U_2 microtome and stained with a saturated solution of uranyl-acetate in water and lead-citrate (13). The sec-

tions were studied with a Philips EM 400 electron microscope.

Bone marrow cells. Male F1(CBA/Rij x C57BL/Rij) mice between 10 and 15 weeks of age served as bone marrow donors. Mice were killed by cervical dislocation. Femurs were removed and flushed with BSS + 5% FCS. Single cell suspensions were prepared by aspirating the cells through a 23 gauge needle. Cells were washed once before use in experiments.

Granulocyte/macrophage-colony-forming unit (CFU-G/M) assay. CFU-GM colony assays were performed by culturing 5×10^4 or 10^5 BMC per ml of α-medium supplemented with 20% FCS, 1% bovine serum albumin (BSA) and 0.8% methylcellulose; 10% murine concanavalin-A (Con-A) spleen-conditioned medium or AP63CM was added as a source of CSA. After 7 days of incubation in a humidified incubator containing 10% CO_2 colonies containing at least 50 cells were counted. All cultures were performed in duplicate unless otherwise stated.

Double layers. A solution of 0.3% Difco bacto-agar in α-medium was prepared by heating . After having cooled to 42°C various volumes of this agar solution were layered over confluent AP63 cells, grown in 24 well tissue culture clusters (Costar) and left to gel for 30 min at room temperature. Then 2×10^4 bone marrow cells in 0.2 ml of α-medium + 20% FCS + 0.8% methylcellulose were placed over the agar layers and incubated as described for the CFU-GM assay.

Conditioned medium treatments. Components with mol.wt. <15.000 were depleted from CM using a Minicon B15 concentrator. Batches of CM were concentrated x100 and diluted to the original volume with α-medium. The CM was then filter-sterilised and used in CFU-GM assay cultures. Ether extraction of CM was performed by mixing equal volumes of analytical grade diethylether and CM and shaking every ten minutes for four hours. After overnight separation of the two liquid phases the medium was removed and residual ether was removed by vacuum suction.

Indomethacin incubation. Some batches of CM were produced in the presence of indomethacin (Sigma) which was dissolved in ethanol and diluted to 5µg indomethacin per ml of medium. Media for CFU-GM assay cultures contained a final volume of 0.04% (vol./vol.) ethanol. Control cultures were supplemented with 0.04% ethanol (vol./vol.).

RESULTS

Characterization of the AP63 cell line. A representative area of a May-Grünwald and Giemsa stained culture of AP63 cells is shown in Figure 1. Large, polygonal fibroblastic cells are visible, with clear nuclei that contain several nucleoli.

In ultrathin sections, observed by electron microscopy, the cells had a typical fibroblast morphology with nuclei containing large quantities of finely dispersed chromatin (Fig. 2). The most numerous and prominent cytoplasmic organelles were mitochondria, dense bodies, fat droplets and mildly dilated rough endoplasmic reticulum. The dense bodies frequently contained membrane rests. Oil red O staining confirmed the presence of lipid-positive granules in AP63 cells (not shown). Analysis of cell membrane profiles failed to demonstrate desmosomes. Similarly, Factor VIII-related antigen could not be detected on AP63 cells. These last two observations render it unlikely that

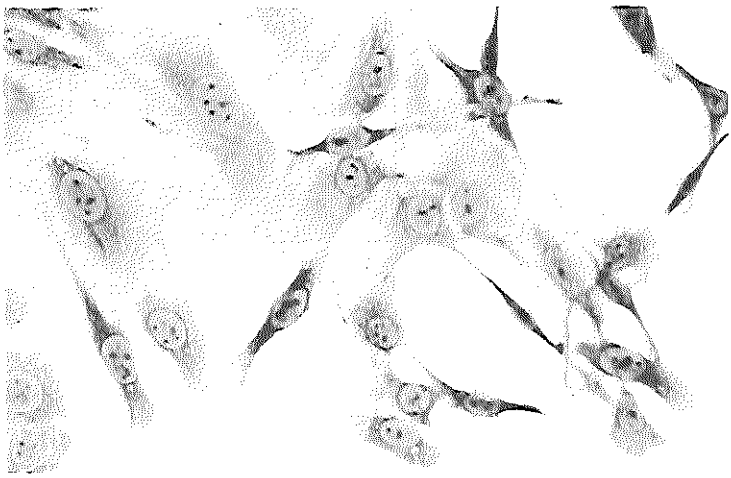


Figure 1. May-Grünwald and Giemsa stained subconfluent culture of AP63 cells. Magnification 400x.

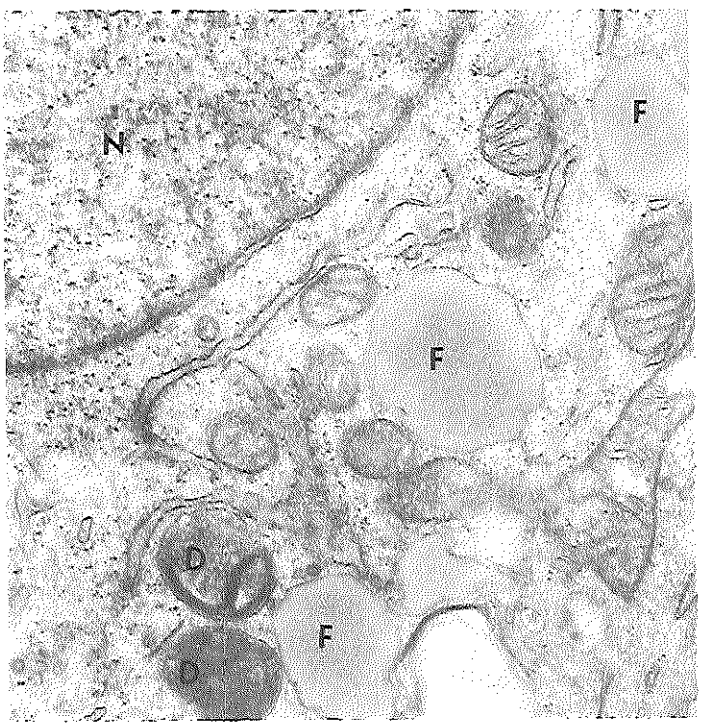


Figure 2. Electron micrograph of a section of an AP63 cell, the nucleus (N), some fat droplets (F) and dense bodies (D) are indicated. Magnification 31,600X.

TABLE 1

CHARACTERISTICS OF AP63 CELLS IN COMPARISON TO PERITONEAL MACROPHAGES

Characteristics[a]	AP63 cells	peritoneal macrophages
phagocytosis		
carbon	-	+
latex	-	+
acid phosphatase	-	+
alkaline phosphatase	+	-
nonspecific esterase	+	+
peroxidase	-	-
Mac-1 antigen	-	+
Factor VIII-related antigen	-	-
fibronectin	+	-

a. Each determination was performed at least three times.

AP63 cells are of endothelial origin.

In Table 1 a number of properties of AP63 cells are compiled and compared with macrophages. Phagocytosis assays were negative with AP63 cells, while peritoneal macrophage cultures had a massive uptake of both latex and carbon particles. In contrast with peritoneal macrophages, the AP63 cells were positive for alkaline phosphatase and fibronectin and negative for acid phosphatase and the Mac-1 macrophage antigenic determinant. Both AP63 cells and peritoneal macrophages had non-specific esterase activity but did not reveal peroxidase activity.

Regulation of in vitro myelopoiesis. CFU-GM colony formation in the presence of varying concentrations of crude AP63CM is shown in figure 3. At 4% CM the maximal colony number was reached, higher concentrations produced a dose-dependent inhibition of colony formation. In these cultures colonies were frequently observed, that consisted of a dense aggregate of round cells and that lacked the halo of single cells usually observed in CFU-GM colonies (Fig. 4). This type, which was designated a 'tight' colony, averaged up to 25% of the total number of colonies at all CM concentrations employed (Table 2). When components with mol.wt. of <15,000 daltons were depleted from the conditioned medium, it induced a far lower percentage of tight colonies (Table 2). Smears of tight colonies revealed that they consisted largely of immature cells, myelocytes and metamyelocytes of the granulocyte lineage. Representative cells are shown in Figure 5. Colonies were replated to determine whether they contained colony forming cells. Table 3 shows that upon replating of tight colonies extensive cluster and colony formation was found, and that a similar number of GM colonies with a dispersed morphology formed very few secondary clusters and colonies upon replating.

To obtain information about the nature of the substance that inhibited CFU-GM colony formation at higher AP63CM concentrations, AP63CM was treated in several ways, as shown in Table 4. After extraction of AP63CM with ether the inhibitory activity was still present. This result suggests that no major lipid moieties were associated with the inhibitory activity. This contrasts with the lipid-associated inhibitory activity in certain mouse sera found by

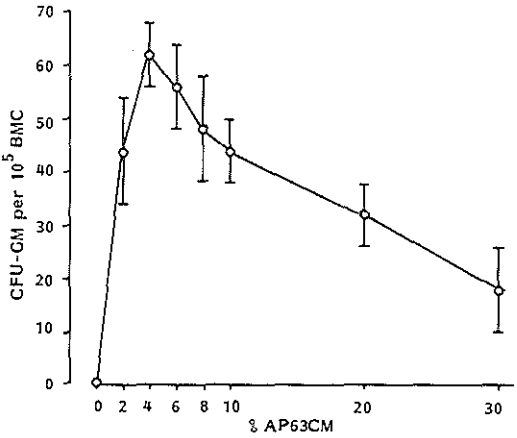


Figure 3. Dose-response curve of granulocyte/macrophage colony numbers versus percentage AP63 conditioned medium (vol./vol.) present in the cultures. Mean ± 1 SE of three experiments, each of which was carried out in duplicate.

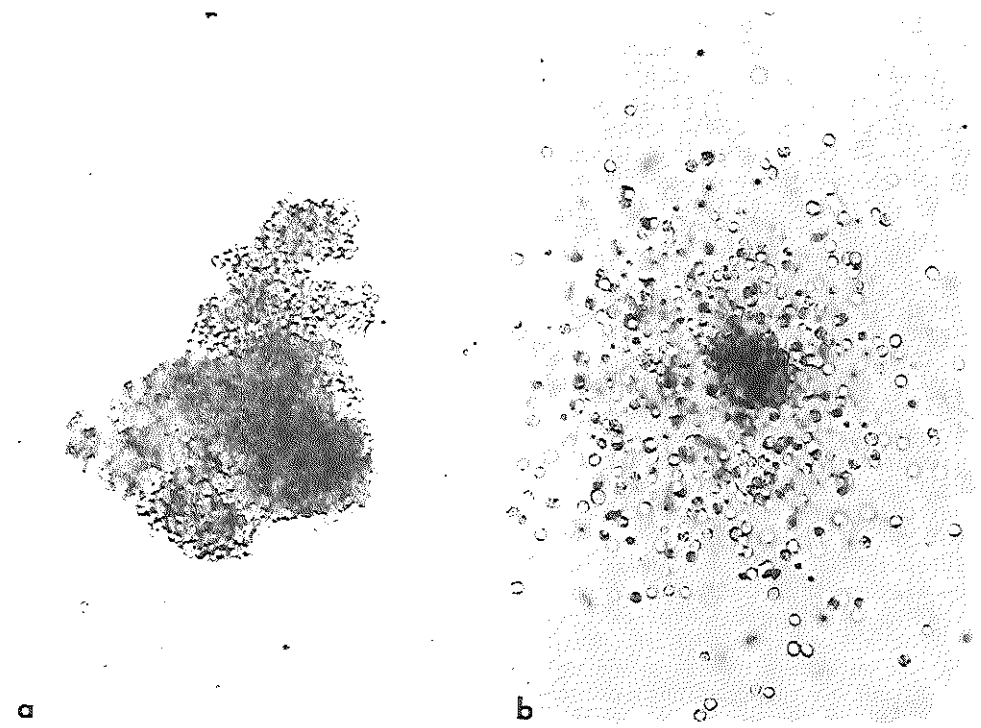


Figure 4. Morphology of tight (a) and dispersed (b) colonies raised in the presence of AP63 conditioned medium. Magnification 1000x.

TABLE 2

INCIDENCE OF 'TIGHT' COLONIES IN BONE MARROW CELL CULTURES STIMULATED WITH AP63 CONDITIONED MEDIUM

% conditioned medium	CFU-GM per 10^5 BMC	% tight colonies
4	77 ± 5	25.0 ± 2.5[a]
10	78 ± 7	23.9 ± 2.0
30	43 ± 8	24.5 ± 2.4
10[b]	71 ± 9	0.7 ± 0.9

a. Data are expressed as mean ± 1 SE of three experiments, each performed in quadruplicate.
b. Conditioned medium depleted of components with mol. wt. <15,000 daltons.

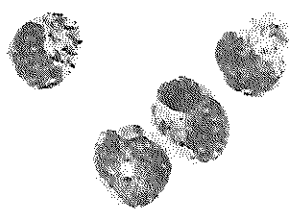


Figure 5. Main cell type present in tight colonies, raised in the presence of AP63 conditioned medium. Magnification 1000x.

Stanley et al. (14). However, when AP63CM was ultrafiltered with a <15,000 dalton retension size Minicon concentrator, the inhibitory activity was depleted, indicating that a CM component of <15,000 daltons was involved in the inhibitory effect. To establish whether the inhibitory activity was associated with prostaglandins, AP63CM was raised in the presence of indomethacin to prevent synthesis of prostaglandins. The CM produced in this fashion contained less inhibitory activity (Table 4). This observation suggests that a prostaglandin or another product of arachidonic acid metabolism may have caused the inhibition of colony formation.

GM colony formation was studied in the presence of AP63 cells. Colonies were formed only when the AP63 cells and the inoculated BMC were separated by an agar layer to prevent close cell contacts but not factor diffusion (Fig. 6). Bone marrow cells plated directly on AP63 cells failed to generate GM colonies. Also when prostaglandin synthesis by the adherent layer was inhibited by including indomethacin (5µg/ml) in the medium, both 24 hrs before and again at inoculation of the BMC layer, no colonies were formed. The smallest agar layer employed (1.25 mm) resulted in the formation of approximately 40 GM colonies per 2 x 10^4 BMC plated. An increase in the thickness of this intermediate agar layer caused a decrease in the number of GM colonies formed in the upper layer. Under these conditions approximately 20% of the colonies had a tight morphology independent of agar depth (17.5% ± 3.9% for 1.25 mm

TABLE 3

REPLATING OF TIGHT AND DISPERSED COLONIES RAISED IN THE PRESENCE OF AP63 CONDITIONED MEDIUM[a]

primary colony type	tight			dispersed	
Colony no.	secondary colonies[b]	secondary clusters[c]	colony no.	secondary colonies	secondary clusters
1	344	0	1	3	0
2	124	135	2	1	3
3	15	62	3	1	0
4	2	13	4	1	0
5	0	95	5	0	3
6	0	20	5	0	2
7	0	17	7	0	1
8	0	16	8	0	0
9	0	14	9	0	0
10	0	12	10	0	0
11	0	8	11	0	0
12	0	8	12	0	0
13	0	4	13	0	0
14	0	4	14	0	0
15	0	4	15	0	0
16	0	4	16	0	0
17	0	2	17	0	0
18	0	2	18	0	0
19	0	2	19	0	0
20	0	2	20	0	0

a. Colonies raised in the presence of conditioned medium were replated at day 7 of culture in medium containing 10% spleen conditioned medium as a CSA source. The table includes data from two experiments. The number of secondary colonies plus clusters obtained from tight colonies was significantly higher ($p < 0.005$) in comparison with those obtained from dispersed colonies plus clusters, however, the number of secondary colonies from replated tight colonies was not significantly different from those obtained from dispersed colonies.

b. Aggregates containing at least 50 cells at day 7 of secondary culture.

c. Aggregates containing 8-49 cells at day 7 of secondary culture.

agar, 23.2% ± 2.4% for 2.5 mm agar; M ± 1 SE, n=8). Colonies formed over deeper agar layers were all relatively small and were therefore not counted for the 'tight' and 'dispersed' designation.

DISCUSSION

A murine cell line (AP63) derived from splenic stroma has been characterized as fibroblastic. The line contrasted to macrophages in several aspects (Table 1) and it did not exhibit endothelium-associated cell junction structures or factor VIII-related antigen.

The AP63 cell line produced granulocyte/macrophage CSA in both soft gel

TABLE 4

TREATMENT AND PRECONDITIONING OF AP63 CONDITIONED MEDIUM TO REVEAL THE NATURE OF ITS INHIBITORY ACTIVITY[a]

Treatment	GM colonies per 10^5 bone marrow cells 5% CM	30% CM	inhibition
none	67 ± 2[b]	24 ± 4	+
ether extraction	68 ± 2	20 ± 3	+
ultrafiltration(<15KD depletion)	74 ± 3	77 ± 9	-
preconditioning of medium			
control	84 ± 4	32 ± 4	+
indomethacin	72 ± 4	60 ± 6	-

a. Presence of the inhibitory activity after treatment was determined by the differential capability of colony formation in 5% versus 30% conditioned medium (CM) present in bone marrow cell cultures.

b. Data represent the mean ± 1 SE of three experiments.

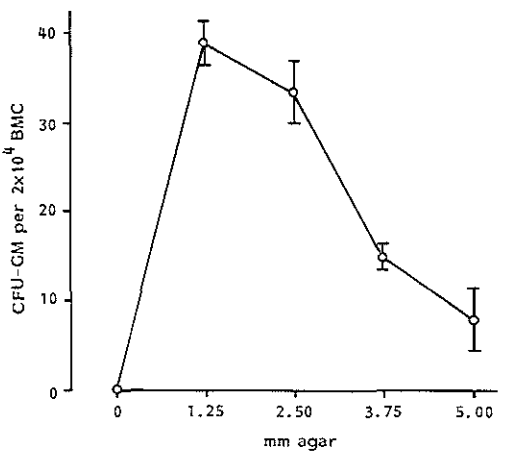


Figure 6. Granulocyte/macrophage colony formation by bone marrow cells placed over confluent AP63 cell layers that were covered with agar layers of variable depth. Mean ± 1 SE of two experiments, each of which was carried out in quadruplicate.

and liquid culture systems. In a previous report (4) primary cultures of hemopoietic organ fibroblasts were shown to produce CSA in a soft gel culture system. Most reports on CSA production in liquid longterm hemopoietic cell cultures over adherent bone marrow-derived stromal cell layers have been negative (15). Heard et al. (16) demonstrated CSA production in these long-term hemopoietic cultures when soft gel medium was placed over the adherent stromal cells but not in liquid medium conditioned by these cultures. Recently, Shadduck et al. (17) detected CSA in supernatants from longterm bone marrow cultures using a radioimmunoassay, but not employing a biological assay. In addition, Zipori (18) detected low levels of CSA in liquid cultures of adherent stromal cells in the absence of hemopoietic cells. These observations suggest that CSA may be difficult to detect in supernatants of longterm bone marrow cultures because of consumption by hemopoietic cells or the presence of inhibitory substances.

The inhibitory substance produced by AP63 cells has been identified tentatively as a prostaglandin-like molecule, since incubation of AP63 cells in medium containing indomethacin resulted in conditioned medium devoid of inhibitory activity. Prostaglandins have profound effects on hemopoiesis. The hemopoietic stem cell can be induced to proliferate (19), and erythropoiesis can be enhanced in vivo (20) as well as in vitro (21). Kurland and Moore (22) showed that in vitro myelopoiesis was inhibited by prostaglandins. Whereas these authors indicate the macrophage as a major producer of prostaglandins, our observations show that stromal fibroblasts may also produce significant amounts of these mediators.

AP63 cells also exerted a short range inhibition by which colony formation was completely abolished. This inhibitor was not related to prostaglandins, since incubation of the adherent cells with indomethacin could not overcome the inhibition. Separation of AP63 cells and BMC with an agar layer relieved this inhibition. The high colony numbers formed over the thinnest agar layer, point to a high CSA level in close proximity to the fibroblast layer. The decrease in colony number with increasing agar thickness implies the presence of a concentration gradient of CSA in these cultures. Zipori and Sasson (23) also reported short range inhibition of colony formation by marrow adherent cells. These authors proposed a model in which this inhibitor and CSA together regulate GM colony formation (24). The mixed stromal cell population however, did not allow them to identify the cell type(s) responsible for production of these factors. Using AP63 cells we show that fibroblasts can account for elaboration of both factors. In addition we report a PG-like soluble inhibitor of GM colony formation elaborated by the cell line, whereas the stromal cell layer (24) did not regulate GM-colony formation via prostaglandins. Garnett et al. (25) have reported a fibroblastoid stromal cell line that elaborated CSA and a prostaglandin-like inhibitor in addition to a second soluble non-prostaglandin inhibitor blocking the development of granulocytic colonies. However this cell line failed to exhibit short range inhibition of GM colony formation.

It is interesting to note that in double layer cultures Zipori and Sasson (23) found colonies, that contained many immature myeloid cells, and had a relatively high replating efficiency. These colonies may resemble the tight colonies described in our study which were also characterized by their contents of early myeloid cells and a high replating efficiency. Moreover, in our study tight colonies could be induced in the absence of the adherent cell layer, i.e. when cultures were stimulated with CM from the cell line. The distribution of tight colonies in our dose-response curve (Table 2) and double layer experiments was the same, which implies that the incidence of these colonies is independent of CM concentration, but may be related to the concentration ratio of

two or more regulators. Our finding that ultrafiltered CM induced a decreased incidence of tight colonies provides further evidence for this notion. The extent of proliferation versus differentiation in myeloid clones *in vivo* may be regulated by the ratio of CSA and inhibitor present in their hemopoietic microenvironment.

The data presented in this report show that the AP63 stromal fibroblastic cell line elaborates activities by which proliferation and differentiation of CFU-GM progenitor cells can be regulated. These observations may reflect *in vivo* properties of hemopoietic organ fibroblasts, by which myelopoiesis is regulated.

ACKNOWLEDGMENTS

We thank Prof.Dr. O. Vos for reviewing the manuscript critically, Mr. W.J. Visser and Mrs. C.M.J. Brakel-van Peer for expert technical assistance, and Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript. This investigation was supported by the Dutch Foundation for Pure Research (ZWO).

REFERENCES

1. Friedenstein, A.J., Chailakhyan, R.K., Latsinik, N.V., Panasyuk, A.K., Keilissborok, I.V. (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. Transplantation 17, 331-340.
2. Blackburn, M.J. & Patt, H.M. (1977) Increased survival of hemopoietic pluripotent stem cells *in vitro* induced by a bone marrow fibroblast factor. Br. J. Haematol. 37, 337-344.
3. Wilson, F.D., O'Grady, L., McNeill, C. & Muna, C.L. (1974) The formation of bone marrow derived fibroblastic plaques *in vitro*: preliminary results contrasting that population to CFU-C. Exp. Hematol. 2, 343-354.
4. Brockbank, K.G.M. & van Peer, C.M.J. (1983) Colony stimulating activity production by hemopoietic organ fibroblastoid cells *in vitro*. Acta Hematol. 69, 369-375.
5. DeGowin, R.L. & Gibson, D.P. (1981) PG-mediated enhancement of erythroid colonies by marrow stromal cells (MSC). Exp. Hematol. 9, 274-280.
6. Zipori, D. & Bol, S. (1979) The role of fibroblastoid cells and macrophages from mouse bone marrow in the *in vitro* growth promotion of haemopoietic tumor cells. Exp. Hematol. 7, 206-208.
7. Springer, T., Galfé, G., Secher, D.S. & Milstein, C. (1979) Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur. J. Immunol. 9, 301-306.
8. Zuckerman, K.S. & Wicha, M.S. (1983) Extracellular matrix production by the adherent cells of long-term murine bone marrow cultures. Blood 61, 540-547.
9. Lojda, Z., van der Ploeg, M. & van Duyn, P. (1967) Phosphates of the naphthol AS series in the quantitative determination of alkaline and acid phosphatase activities 'in situ' studied in polyacrylamide membrane model systems and by cytospectrophotometry. Histochemie 11, 13-32.
10. van Duyn, P., Pascoe, E. & van der Ploeg, M. (1967) Theoretical and experimental aspects of enzym determination in a cytochemical model system of polyacrylamide films containing alkaline phosphatase. J. Histochem. Cytochem. 15, 631-645.

11. Hsieh, P., Segal, R. & Chen, L.B. (1980) Studies on fibronectin matrices in living cells with fluoresceinated gelatin. J. Cell. Biol. 87, 14-22.
12. Simionescu, N. & Simionescu, M. (1976) Galloylglucoses of low molecular weight as mordant in electronmicroscopy. I. Procedure and evidence for mordanting effects. J. Cell. Biol. 70, 608-621.
13. Venable, J.H. & Coggeshall, R. (1965) A simplified lead citrate stain for use in electron microscopy. J. Cell Biol. 25, 407-417.
14. Stanley, E.R., Robinson, W.A. & Ada, G.L. (1968) Properties of the colony stimulating factor in leukaemic and normal mouse serum. Aust. J. Exp. Biol. Med. Sci. 46, 715-726.
15. Dexter, T.M., Spooncer, E., Toksoz, D. & Lajtha, L.G. (1980) The role of cells and their products in the regulation of in vitro stem cell proliferation and granulocyte development. J. Supramolec. Structure 13, 513-524.
16. Heard, J.M., Fichelson, S. & Varet, B. (1982) Role of CSA in murine long-term bone marrow cultures: evidence for its production and consumption by the adherent cells. Blood 59, 761-767.
17. Shadduck, R.K., Waheed, A., Greenberger, J.S. & Dexter, T.M. (1983) Production of colony stimulating factor in longterm bone marrow cultures. J. Cell. Physiol. 114, 88-92.
18. Zipori, D. (1982) Properties of bone marrow stromal cells and derived lines from SJL/J mice. Exp. Hematol. 10, 10 (abstract).
19. Feher, I. & Gidali, J. (1974) Prostaglandin E2 as stimulator of haemopoietic stem cell proliferation. Nature 247, 550-551.
20. Dukes, P.P., Shore, N.A. Hammond, D., Ortega, J.A. & Datta, M.C. (1973) Enhancement of erythropoiesis by prostaglandins. J. Lab. Clin. Med. 82, 704-712.
21. DeGowin, R.L., Gibson, D.P. & Knapp, S.A. (1981) PGE and the erythropoietic and stromal insufficiency induced by extramedullary tumor. J. Lab. Clin. Med. 98, 217-226.
22. Kurland, J. & Moore, M.A.S. (1977) Modulation of hemopoiesis by prostaglandins. Exp. Hematol. 5, 357-373.
23. Zipori, D. & Sasson, T. (1981) Myelopoiesis in presence of stromal cells from mouse bone marrow. II. Mechanisms of glucose dependent colony formation. Exp. Hematol. 9, 663-674.
24. Zipori, D. (1981) Cell interactions in the bone marrow microenvironment: role of endogeneous colony-stimulating activity. J. Supramolecular Structure and Cellular Biochemistry 17: 347-357-357.
25. Garnett, H.M., Cronkite, E.P. & Harigaya, K. (1982) Regulation of the colony-stimulating activity produced by a murine marrow-derived cell line (H-1). Proc. Natl. Acad. Sci. USA 79, 1545-1548.

APPENDIX PAPER IV

TRANSPLANTATION OF BONE MARROW FIBROBLASTOID STROMAL CELLS IN MICE VIA THE INTRAVENOUS ROUTE

A.H. Piersma, R.E. Ploemacher and K.G.M. Brockbank

Department of Cell Biology & Genetics, Erasmus University, Rotterdam.

In: British Journal of Haematology 54, 285-290, 1983

SUMMARY

Kinetics of fibroblastic colony-forming cells (CFU-F) were studied in mouse bone marrow after lethal total body irradiation and intravenous bone marrow transplantation. After an initial decrease, CFU-F numbers recovered, and plateaued five weeks post-treatment at ten per cent of normal values.

Using chromosome-marked donor bone marrow cells we found that one day after transplantation 72% of donor CFU-F had reached the recipients bone marrow, indicating a highly specific lodgment of CFU-F. Three months after transplantation donor CFU-F were still detectable, and comprised about half of the femoral CFU-F population.

It is concluded that CFU-F, a component of the haemopoietic microenvironment, are transplantable via the intravenous route.

INTRODUCTION

In vitro fibroblastic colony-forming cells (CFU-F) from bone marrow are regarded as being part of the hemopoietic stroma. Cultured bone marrow fibroblasts are able to transfer the hemopoietic microenvironment when transplanted beneath the renal capsule in guinea pigs (Friedenstein et al., 1974). In several *in vitro* systems, bone marrow adherent layers, consisting partly of fibroblasts, were shown to regulate *in vitro* hemopoiesis (Dexter et al., 1976; Reimann and Burger, 1979; Bentley and Foidart, 1980; Zipori et al., 1981). Furthermore, medium conditioned by bone marrow fibroblasts can increase *in vitro* CFU-s survival (Blackburn and Patt, 1977).

Hemopoietic stroma has been considered non-transplantable via the intravenous route in mice and man (Knospe et al., 1968; Friedenstein et al., 1978; Golde et al., 1980). Recently, Keating et al. (1982) showed that stromal cells of donor origin predominated in longterm marrow cultures from patients that had been given bone marrow transplantation. Here we report studies which show that after intravenous bone marrow transplantation in mice a large percentage of donor CFU-F lodges and remains in the host bone marrow.

MATERIALS AND METHODS

Mice. F1 (C57BL/Rij x CBA/Rij), CBA/N and CBA/H-T6T6 mice were obtained from the Department of Animal Breeding of the Erasmus University, Rotterdam, and the Medical Biological Laboratory TNO, Rijswijk (ZH), The Netherlands. In

all studies male mice at 18-20 weeks of age were used.

Bone marrow cells. Mice were killed by cervical dislocation. Femurs were taken and flushed with buffered salt solution (BSS) + 5% fetal calf serum (FCS) using a syringe with a 25 gauge needle. Cell clumps were dispersed by repeatedly flushing the cell suspension. Cells were then centrifuged and resuspended in α-medium + 20% FCS for culture purposes or in BSS + 5% FCS for transplantation purposes.

Irradiation. Mice received a lethal dose of 9 Gy total body γ irradiation (TBI) using a ^{137}Cs source, with a dose rate of 1.3 Gy/min. Radiation control mice died within two weeks after treatment.

Transplantation. F1 mice received an intravenous injection in a lateral tail vein of 4×10^7 nucleated bone marrow cells (BMC) from age-matched donors, suspended in 0.5 ml of BSS + 5% FCS. For subsequent cytogenetic studies 2.5×10^7 CBA/T6T6 chromosome-marked BMC were transplanted into syngeneic CBA/N recipients. In each of the above mouse strains the transplant size represented the contents of two femurs after removal of the epiphyses and metaphyses.

CFU-F assay. Fibroblastic colony-forming cells were quantitated by culturing BMC at a concentration of 5×10^5 or 10^6 nucleated cells in 1 ml of semisolid medium, consisting of α-medium, supplemented with 20% FCS and 0.8% methylcellulose in Costar Ø 35mm culture dishes in triplicate. For chromosome studies a glass coverslip was placed in each dish. Colonies containing fifty or more fibroblastic cells were counted on day 10 of culture, after washing the cells with phosphate buffered saline, fixation in methanol (10 min) and staining with 10% Giemsa.

Chromosome preparation. Cultured cells were synchronized at day 9 of culture. The semisolid medium was washed off under sterile conditions by dipping the coverslips into BSS + 5% FCS, and the cultures were refed with α + 20% FCS, supplemented with 10^{-7}M methotrexate (t = 0 hr). At 16 hr medium was changed for α + 20% FCS + 10^{-5}M thymidine. At 21 hr 0.1 µg Colcemid (Gibco no. 541) per ml medium was added to the cultures. At 22 hr medium was replaced with hypotonic medium (one part FCS + nine parts aquadest), and the cells were incubated for 30 min. All incubations were carried out at 37°C in a humidified 10% CO_2 in air incubator. Fixation was performed by slowly adding fixative (1 part glacial acetic acid + 3 parts methanol) to the hypotonic medium. Finally medium was changed to 100% fixative. After five changes in 100% fixative the liquid was removed and dishes were centrifuged for 10 min at 300 g. The coverslips were transferred to a heating table and left for 10 min at 80°C before being mounted on microscope slides and stained with 10% Giemsa. Each colony contained up to five interpretable mitoses, all of which were judged.

Indian Ink ingestion. Phagocytosis of carbon particles was studied by adding 20% (v/v) of a one in sixty dilution of India Ink (Pelikan 17 Negro) in α + 20% FCS to cultures after removal of the semisolid medium on day 9 of culture. Cultures were incubated for 30 min and then washed three times with PBS to remove noningested ink. Directly after this treatment chromosome preparation was initiated.

RESULTS

In the first week after irradiation femoral CFU-F numbers gradually declined to about 0.5% of normal values (Fig. 1). In radiation control mice this decrease was faster than in mice that received BMT. Control mice died between days 10 and 14. In transplanted mice CFU-F numbers recovered from the third week onwards to about 10% of normal values at five weeks. No further change in CFU-F numbers occurred with time.

To determine whether donor CFU-F were involved in this recovery, chromosome marked CBA/T6T6 bone marrow cells were transplanted into 9 Gy irradiated syngeneic CBA/N mice. CFU-F colonies were developed from the chimaeric mice, and chromosome analyses were performed. In all instances all countable mitoses in any one colony were of one type, either T6T6 or N. These studies revealed (Table I) that one day after BMT the recipient bone marrow contained 63 ± 8% donor CFU-F, taking into account that the content of one femur averages $\bar{6}$ per

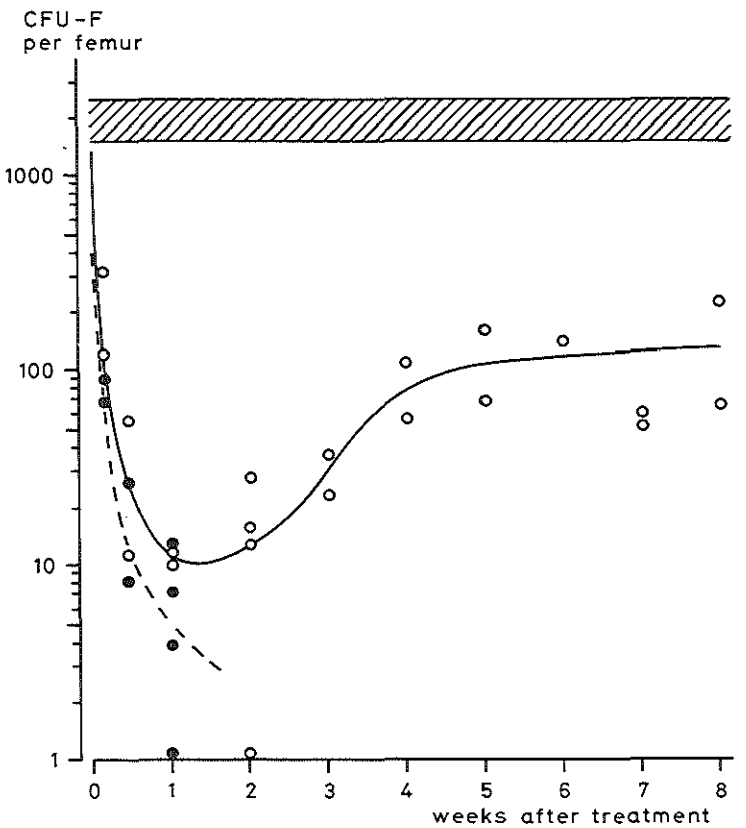


Figure 1. Kinetics of femoral CFU-F in (CBA/Rij x C57BL/Rij)F1 mice given either 9 Gy total body irradiation alone (●--●), or 9 Gy total body irradiation followed by reconstitution with 4 x 10^7 bone marrow cells intravenously (○—○). The hatched area represents the CFU-F level found in nonirradiated normal mice. Each point represents the mean of two mice assayed.

TABLE I

ORIGIN OF BONE MARROW CFU-F AFTER LETHAL IRRADIATION AND BONE MARROW TRANSPLANTATION

Days after treatment[a]	No. of host mice	CFU-F colonies total	CFU-F colonies donor[b]	CFU-F colonies host[b]	% donor CFU-F[c]
1	3	156	29	22	63 ± 8
59	3	36	8	9	47 ± 29
94	4	50	4	5	44 ± 28

a. CBA/N mice received 9 Gy total body irradiation and were reconstituted with 2.5×10^7 CBA/T6T6 bone marrow cells intravenously.
b. Number of interpretable colonies.
c. Mean ± 1 SE for individual mice.

cent of the total body bone marrow content. In order to obtain more information about the specificity of the seeding process of CFU-F in the medullary sites the one day seeding efficiency was estimated. Since 92 CFU-F could be retrieved from one femur one day after transplantation of 1340 CFU-F, it can be calculated that 72 per cent of the injected CFU-F had lodged in the hosts bone marrow.

In order to exclude the possibility that adherent haemopoietic cells (i.e., macrophages) formed metaphases among the CFU-F colonies, cultures derived from chimaeric mice were incubated with Indian ink before chromosome preparation. Within 70 colonies containing identifiable metaphases there were no metaphase cells containing phagocytosed ink particles. Thus, it is extremely unlikely that macrophage metaphases were scored in our chromosome analysis experiments. Macrophages containing phagocytosed ink particles were observed in some of the larger fibroblastic colonies.

DISCUSSION

We have demonstrated that donor CFU-F participate in the reestablishment of hemopoietic stroma after lethal TBI and BMT in mice. The CFU-F kinetics observed by us after 9 Gy TBI and BMT were similar to those reported by Friedenstein et al. (1976) after 1.5 Gy without BMT. After an initial decline due to radiation damage, CFU-F numbers recovered to and plateaued at ten per cent of normal levels. The finding that all metaphases in individual fibroblastoid colonies derived from chimaeric mice were of the same type provides evidence for their clonal origin. Chromosome staining of clonally derived fibroblastic colonies made it possible to designate the origin of each fibroblastic colony-forming cell. In addition it was established that hemopoietic cells did not contribute to the metaphases in the chromosome preparations. Nonadherent haemopoietic cells were washed off prior to chromosome preparation and adherent phagocytic cells were excluded by means of Indian ink ingestion. Although macrophages were found in some CFU-F colonies, they did not contain metaphase chromosomes. The apparent nonproliferative status of macrophages may be due to the culture conditions employed. Our chromosome marker studies suggest that 72% of donor CFU-F lodged in the hosts bone marrow, which implies a very high seeding efficiency. This observation is supported by the data of Werts et al.

(1980), who demonstrated that migration of CFU-F from unirradiated sanctuaries can occur in partially irradiated mice.

In a previous study on CFU-F transplantation via the i.v. route Friedenstein et al. (1978) could not show donor CFU-F in F1 (CBA x C57BL/6J) host mice that were reconstituted with CBA bone marrow cells using strain-specific antisera. In these experiments, relatively low doses of donor cells were transplanted, which would reduce the chance of donor CFU-F detection in the recipient bone marrow. Bentley et al. (1982), studying continuous bone marrow cultures from chimaeric mice, concluded that the in vitro haemopoietic microenvironment was of recipient origin. In this study, only 10^5 donor BMC were injected into the recipient mice. Our data show that such grafts would have contained approximately 5 CFU-F, which reduces the chance of detection after transplantation. Similarly, in two human studies donor CFU-F could not be found in male/female chimaeras (Wilson et al., 1978; Golde et al., 1980). However, in a recent study, Keating et al. (1982) showed that donor cells (macrophages, fibroblasts, endothelial cells) made up the majority of the adherent layer in longterm marrow cultures derived from leukemia patients that had been given BMT.

Our observations establish that in mice fibroblastic stromal cells are transplantable via the intravenous route. Whether or not these transplanted stromal cells contribute to the success of BMT awaits further investigation.

ACKNOWLEDGMENTS

The authors appreciate the advise of Prof.Dr. O. Vos in preparation of the manuscript. They also wish to thank Mrs. Cary Meijerink-Clerkx for typing the manuscript. This investigation was supported by the Netherlands Foundation for Pure Research (ZWO).

REFERENCES

Bentley, S.A., Foidart, J.-M. (1980) Blood 56, 1006-1012.
Bentley, S.A., Knutsen, T., Whang-Peng, J. (1982) Exp. Hematol. 10, 367-372.
Blackburn, M.J., Patt, H.M. (1977) Br. J. Haematol. 37, 337-344.
Dexter, T.M., Allen, T.D., Lajtha, L.G. (1976) J. Cell. Physiol. 91, 335-344.
Friedenstein, A.J., Chialakhyan, R.K., Latsinik, N.V., Panasyuk, A.F., Keiliss-borok, Z.V. (1974) Transplantation 17, 331-340.
Friedenstein, A.J., Gorskaya, U.F., Kulagina, N.N. (1976) Exp. Hematol. 4, 267-274.
Friedenstein, A.J., Ivanov-Smolenski, A.A., Chialakhyan, R.K., Gorskaya, U.F., Kuralesova, A.I., Latsinik, N.W., Gerasimov, U.W. (1978) Exp. Hematol. 6, 440-444.
Golde, D.W., Hocking, W.G., Quan, S.G., Sparkes, R.S., Cale, R.P. (1980) Br. J. Haematol. 44, 183-187.
Keating, A., Singer, J.W., Killen, P.D., Striker, G.E., Salo, A.C., Sanders, J., Thomas, E.D., Thorning, D., Fialkow, P.J. (1982) Nature 298, 280-283.
Knospe, W.A., Blom, J., Crosby, W.H. (1968) Blood 31, 400-405.
Reimann, J., Burger, H. (1979) Exp. Hematol. 7, 45-51.
Werts, E.D., Gibson, D.P., Knapp, S.A., DeGowin, R.L. (1980) Rad. Res. 81, 20-30
Wilson, F.D., Greenberg, B.R., Konrad, P.N., Klein, A.K., Walling, P.R. (1978) Transplantation 25, 87-88.
Zipori, D., Sasson, T., Frenkel, A. (1981) Exp. Hematol. 9, 656-663.

APPENDIX PAPER V

RECOVERY OF HEMOPOIETIC STROMAL PROGENITOR CELLS AFTER LETHAL TOTAL BODY IRRADIATION AND BONE MARROW TRANSPLANTATION IN MICE

A.H. Piersma, K.G.M. Brockbank, R.E. Ploemacher and C.P.E. Ottenheim

Department of Cell Biology & Genetics, Erasmus University, Rotterdam

In: Transplantation 40, in press, 1985.

SUMMARY

The recovery of fibroblastic colony-forming units (CFU-F) in murine bone marrow hemopoietic stroma was studied during eighteen months after 9 Gy lethal total body irradiation and reconstitution with syngeneic bone marrow cells. After an initial depletion CFU-F numbers increased from 10 per cent of normal values at three months to 40 per cent at 18 months after treatment, irrespective of graft size and presence of CFU-F in the graft. Fourteen months after treatment 35 per cent of all CFU-F present in the recipients bone marrow was donor-derived independent of graft size. When mice were treated with a high dose lipopolysaccharide-W three months after irradiation and bone marrow transplantation, CFU-F numbers decreased to hardly detectable levels within one day, and then recovered to normal numbers four weeks later, whereas untreated control mice still had low CFU-F numbers. These data suggest that after lethal total body irradiation the stroma still contained viable fibroblastic cells that had lost their in vitro colony-forming capacity as a result of radiation damage. In consequence there was no need for replacement of these fibroblastic cells by donor-derived or host-derived CFU-F. Only depletion of CFU-F from the bone marrow, as was induced with lipopolysaccharide, stimulated repopulation of the stroma with colony-forming fibroblastic cells.

INTRODUCTION

As has been demonstrated by Friedenstein et al. (1), hemopoietic organ-derived fibroblastic cells, after cloning in vitro, were able to transfer the hemopoietic stroma upon retransplantation under the kidney capsule. Thus, splenic fibroblasts gave rise to reticular stroma with host-derived lymphopoiesis, whereas bone marrow fibroblasts resulted in bone and marrow stroma formation with subsequent host-derived hemopoiesis. Since then fibroblastic colony-forming units (CFU-F) have been considered to represent an important cell population in the hemopoietic stroma.

Recently, a number of studies have shown that cells from the hemopoietic stroma were transplantable via the intravenous route. Upon total body irradiation (TBI) and bone marrow transplantation in man (2) and mice (3) as well as after total lymphoid irradiation and bone marrow transplantation (4), the hemopoietic stroma could be shown to contain donor-derived fibroblastoid cells.

The transplantability of stromal cells after TBI may be important for the subsequent restoration of radiation-induced stromal damage. In this study we

therefore measured the contribution of donor-derived CFU-F to the stroma of lethally irradiated and bone marrow reconstituted mice. It is postulated that donor-derived CFU-F were not required for rescueing lethally irradiated mice, because the stroma still contained functional fibroblastic cells that lost their in vitro colony-forming capacity as a result of radiation-induced damage.

MATERIALS AND METHODS

Mice. F1(C57BL/Rij x CBA/Rij), CBA/N and CBA/H-T6T6 mice were obtained from the Department of Animal Breeding of the Erasmus University, Rotterdam, and the Medical Biological Laboratory TNO, Rijswijk (zh), The Netherlands. In all studies male mice were 18-20 weeks of age at the first treatment.

Bone marrow cells. Mice were killed by cervical dislocation. Femurs were flushed with buffered saline solution (BSS) + 5% fetal calf serum (FCS) using a syringe with a 25 gauge needle. Cell clumps were dispersed by repeatedly flushing the cell suspension. Cells were then centrifuged and resuspended in α-medium + 20% FCS for culture purposes or in BSS + 5% FCS for transplantation purposes.

Nylon wool filtration. Nylon wool columns were prepared by filling 6 ml syringes with 0.6 g nylon wool from Leuko-pak leukocyte filters (Fenwal). Columns were incubated at 37°C with α-medium + 20% FCS for 30 minutes before use. BMC suspensions in α-medium + 20% FCS (2×10^8 BMC per column at maximum) were then layered on the column and allowed to mix in with the nylon wool. After a 45 minutes incubation at 37°C nonadherent cells were eluted with 45 ml of α-medium + 20% FCS at a rate of 1 ml per minute. The eluate never contained detectable CFU-F (5).

Irradiation. Mice received a lethal dose of 9 Gy total body gamma irradiation using a ^{137}Cs source, with a dose rate of 1.3 Gy/min. Radiation control mice died within 2 weeks after treatment.

Transplantation. F1 mice received an intravenous injection in a lateral tail vein of nucleated BMC from age-matched syngeneic donors, suspended in 0.5 ml of BSS + 5% FCS. For subsequent cytogenetic studies, CBA/T6T6 chromosome-marked BMC were transplanted into syngeneic CBA/N recipients.

Endotoxin-treatment. Mice were intravenously injected with 150 µg lipopolysaccharide-W (Salmonella Typhosa 0901; Difco) in 0.5 ml BSS three months after TBI and bone marrow transplantation.

CFU-F assay. Fibroblastic colony-forming cells were quantified by culturing BMC at a concentration of 5×10^5 or 10^6 nucleated cells in 1 ml of semi-solid medium, consisting of α-medium, supplemented with 20% FCS and 0.8% methylcellulose in Costar 35 mm culture dishes in triplicate. For chromosome studies, a glass coverslip was placed in each dish. Colonies containing fifty or more fibroblastic cells were counted on day 10 of culture, after washing the cells with phosphate-buffered saline, fixing in methanol and staining with 10% Giemsa. Femoral CFU-F numbers were calculated according to total cellularity per femur.

Chromosome preparation. Cultured cells were synchronized as follows. At day 9 of culture, the semisolid medium was washed off under sterile conditions by dipping the coverslips into BSS + 5% FCS and the cultures were refed with α-medium + 20% FCS, supplemented with 10^{-7} M methotrexate (t= 0 h). At 16 h the medium was changed for α-medium + 20% FCS + 10^{-5} M thymidine. At 21 h 0.1 µg Colcemid (Gibco no. 541) per ml of medium was added to the cultures. At 22 h the medium was replaced with hypotonic medium (one part FCS + nine parts aquadest) and the cells were incubated for 30 min. All incubations were carried out at 37°C with 10% CO_2 in air in a humidified incubator. Fixation was performed by slowly adding fixative (1 part glacial acetic acid + 3 parts methanol) to the hypotonic medium. Finally, the medium was changed to 100% fixative. After five changes in 100% fixative the liquid was removed and dishes were centrifuged for 10 min at 300 g. The coverslips were transferred to a heating table and left for 10 min at 80°C before being mounted on microscope slides and stained with 10% Giemsa. Each colony contained up to five interpretable mitoses, all of which were judged (3).

RESULTS

To determine whether grafted stromal cells were necessary for the recovery of the femoral CFU-F population in lethally irradiated and reconstituted mice, 9 Gy TBI mice were transplanted with adherent stromal cell-depleted bone marrow cells or normal bone marrow cells. As is shown in Figure 1, the recovery of the femoral CFU-F population was similar in both experimental groups as measured up to eight weeks after treatment. In a similar experiment (Figure 2) two bone marrow inoculum sizes -as well as a stromal cell-depleted bone marrow graft- were used. The recovery of CFU-F in the recipient femur took place slowly from 10 per cent at three months to approximately 40 per cent of normal values over a year after treatment. Again no significant difference was observed between the various experimental groups.

Subsequently we assessed the contribution of donor-derived CFU-F to the recipient femoral CFU-F population after this partial recovery. Therefore, CBA/N mice were lethally irradiated and reconstituted with CBA/T6T6 chromosome-marked bone marrow cells. Fourteen months later CFU-F assays were performed, and the origin of the colonies was determined after chromosome staining. From table 1 it appears that irrespective of graft size one third of the recipient femoral CFU-F population was donor-derived.

TABLE 1

ORIGIN OF BONE MARROW CFU-F 14 MONTHS AFTER LETHAL TOTAL BODY IRRADIATION AND BONE MARROW TRANSPLANTATION[a]

Graft size	No. of mice	CFU-F colonies total	donor	host	% donor CFU-F
10^6 BMC	3	220	14	40	35
2.5×10^7 BMC	3	197	18	47	38

a. CBA/N mice received 9 Gy total body irradiation and were reconstituted with CBA/T6T6 syngeneic bone marrow cells.

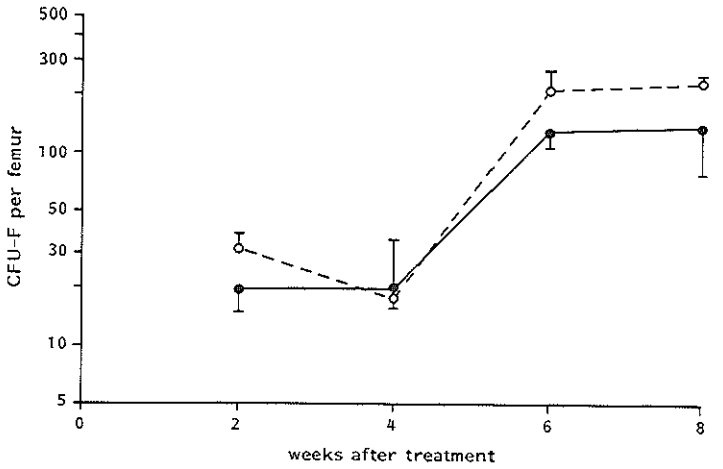


Figure 1. Femoral CFU-F numbers in mice after total body irradiation and reconstitution with 2.5×10^6 normal bone marrow cells (●) containing 65 ± 8 CFU-F per 10^6 nucleated cells or 2.5×10^6 nonadherent bone marrow cells (○) containing no detectable CFU-F. Mean ± standard error of three individually assayed mice per point.

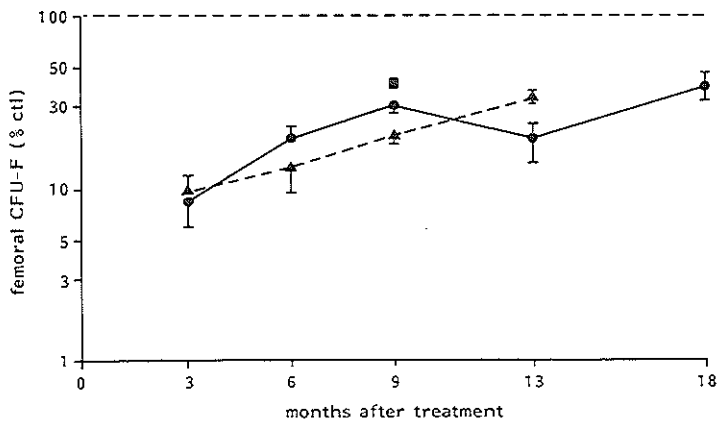


Figure 2. Femoral CFU-F numbers in per cent of untreated controls of mice after total body irradiation and reconstitution with 10^6 (●) or 2×10^7 (▲) normal bone marrow cells containing 71 ± 8 CFU-F per 10^6 nucleated cells, or 2×10^7 nonadherent (■) bone marrow cells containing no detectable CFU-F. Mean ± standard error of three individually assayed mice per point.

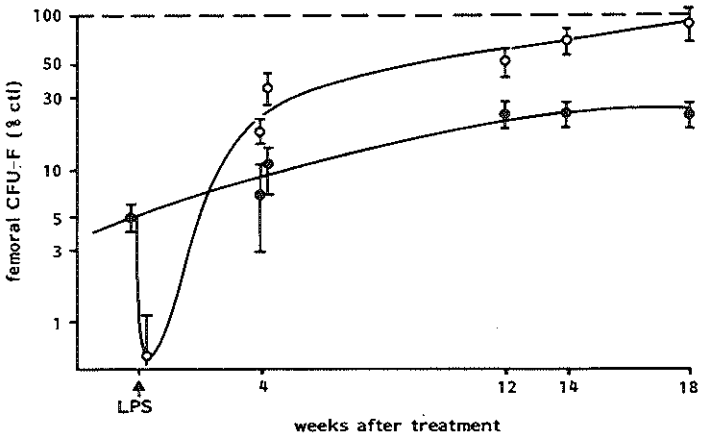


Figure 3. Femoral CFU-F numbers in per cent of untreated controls of 9 Gy total body irradiated mice reconstituted with 10^6 bone marrow cells, which were treated 3 months after irradiation with 150 µg LPS-W iv (○) or received no further treatment (●). Mean ± standard error of three individually assayed mice per point.

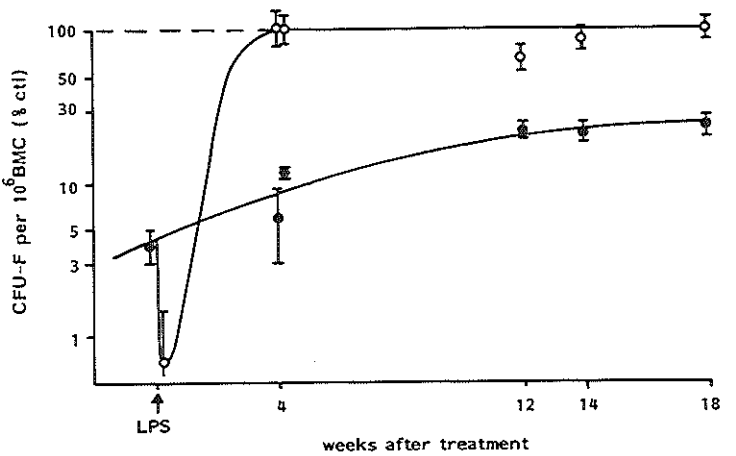


Figure 4. CFU-F numbers per 10^6 bone marrow cells in per cent of untreated controls of 9 Gy total body irradiated mice reconstituted with 10^6 bone marrow cells that were treated 3 months after irradiation with 150 µg LPS-W iv (○) or received no further treatment (●). Mean ± standard error of three individually assayed mice per point.

The residual stromal damage in the experiments presented sofar seems to contrast with the fact that hemopoiesis in irradiated mice returns almost to normal within a month. We hypothesized that the 'missing CFU-F' might be accounted for by stromal fibroblastic cells that retained their hemopoietic regulating properties but lost their in vitro colony-forming capacity as a result of radiation damage. If this would be the case, severe depletion of these fibroblastic cells might allow reconstitution of the CFU-F population. This possibility was tested by treating 9 Gy TBI mice, rescued with BMC, with 150 µg lipopolysaccharide-W three months later. In an earlier study (6) this compound was shown to reduce femoral CFU-F numbers in normal mice. As shown in Figure 3, we found a depletion of CFU-F numbers to 0.6% of control values one day after lipopolysaccharide treatment. By four weeks after lipopolysaccharide injection, the femoral CFU-F numbers had recovered to significantly higher levels than those in irradiated and bone marrow reconstituted control mice, and they continued to increase in the following weeks. Full recovery of CFU-F numbers was established 18 weeks after endotoxin treatment, whereas control mice had reached only 30% at that stage (figure 3). Calculated on the basis of cellularity CFU-F numbers had already reached the normal level after 4 weeks (figure 4) and they have remained at that level since, indicating that the recovery of CFU-F per femur after lipopolysaccharide treatment was limited by hypocellularity in the bone marrow, which lasted over 14 weeks.

DISCUSSION

This study documents the longterm recovery of fibroblastic stromal cells after lethal total body irradiation and syngeneic bone marrow transplantation in mice. In previous reports (3,7) we described an incomplete recovery of femoral CFU-F up to six months after treatment. The underlying study shows that CFU-F numbers were still below normal values 18 months after irradiation, independent of the amount of BMC grafted and also independent of the presence of adherent stromal cells in the graft (Figures 1 and 2). Obviously the hosts stromal cell compartment was able by itself to take care of the partial CFU-F repopulation. However, if present, grafted stromal cells did contribute to the recovery because in mice grafted with normal BMC donor-CFU-F made up 35-38% of the total CFU-F population (Table 1). This percentage was reached with both graft sizes employed, indicating that even a small number of grafted stromal cells could give rise to this recovery. Our data are comparable to those from Feiner et al. (4) who found that 30 weeks after total lymphoid irradiation and allogeneic bone marrow transplantation 25-35% of adherent stromal cells in irradiated sites were of donor origin. The study of Keating et al. (2) however demonstrated that in man after bone marrow transplantation the stroma became completely donor-derived. In this case pretreatment consisted of repeated cyclophosphamide injections in addition to irradiation, which may have had a more damaging influence upon the hemopoietic stroma of the host, allowing donor stromal cells to fully repopulate the hosts stroma.

These findings may be explained by assuming that in the irradiated stroma the fibroblastic cells remain viable after irradiation, whereas upon explantation for CFU-F assay their radiation damage makes proliferation for colony formation impossible (8). Thus, although deficient CFU-F numbers would be determined, the stroma would be functionally normal and would not need to be repopulated by CFU-F. When we tested this hypothesis after depleting the irradiated stroma with lipopolysaccharide, we indeed found a complete recovery of CFU-F in contrast to non-endotoxin treated mice (Figure 3). The discrepancy between stromal function in situ (9,10) and assayed stromal function upon ex-

plantation of a stromal component (8,11) after damage-inducing treatments is a general problem in microenvironmental research. Not only the CFU-F assay but also the assay that measures regenerative capacity of subcutaneously implanted hemopoietic organs as a parameter for stromal function (12) as well as in vitro longterm bone marrow culture techniques on adherent stromal cell layers (13) is dependent on proliferation of stromal cells. It may well be that their proliferative capacity does not correlate with stromal function in situ and this limits the applicability of these assays for measuring functional damage to the hemopoietic stroma. This notion is clearly illustrated by our findings employing the CFU-F assay.

The data suggest, that the incomplete recovery of femoral CFU-F numbers in lethally irradiated mice is not due to an inefficient repopulating capacity of donor-derived and host-derived stromal cells, but rather to the presence of viable fibroblastic stromal cells in the hemopoietic stroma that are unable to proliferate.

ACKNOWLEDGMENTS

The authors wish to thank Prof.Dr. O. Vos for critically reviewing the manuscript and Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript. This work was supported by the Netherlands Foundation for Medical Research (FUNGO-ZWO).

REFERENCES

1. Friedenstein, A.J., Chailakhyan, R.K., Latsinik, N.N., Panasyuk, A.F., Keilissborok, I.V. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 1974;17:311.
2. Keating, A., Singer, J.W., Killen, P.D., Striker, G.E., Salo, A.C., Sanders, J., Thomas, E.D. et al. Donor origin of the in vitro hemopoietic microenvironment after bone marrow transplantation in man. Nature 1982; 298:280.
3. Piersma, A.H., Ploemacher, R.E., Brockbank, K.G.M. Transplantation of bone marrow fibroblastoid stromal cells in mice via the intravenous route. Br. J. Haematol. 1983;54:285.
4. Feiner, R.H., Strober, S., Greenberg, P.L. Murine granulopoiesis after fractionated total lymphoid irradiated and allogeneic bone marrow transplantation. Exp. Hematol. 1983;11:410.
5. Piersma, A.H., Brockbank, K.G.M., Ploemacher, R.E., van Vliet, E., Brakel-van Peer, C.M.J., Visser, W.J. Characterization of fibroblastic stromal cells from murine bone marrow. Exp. Hematol. 1985;13:237.
6. Brockbank, K.G.M., Ploemacher, R.E., van Peer, C.M.J. Splenic accumulation of stromal progenitor cells in response to bacterial lipopolysaccharides. Exp. Hematol. 1983;11:358.
7. Ploemacher, R.E., Brockbank, K.G.M., de Ruiter, H. Latent sustained injury of murine hemopoietic organ stroma induced by ionizing irradiation. Haematologica 1983;68:454.
8. Piersma, A.H., Ploemacher, R.E., Brockbank, K.G.M. Radiation damage to femoral hemopoietic stroma measured by implant regeneration and quantitation of fibroblastic progenitors. Exp. Hematol. 1983;11:884.

9. Knospe, W.H., Blom, J., Crosby, W.H. Regeneration of locally irradiated bone marrow. I. Dose dependent, longterm changes in the rat, with particular emphasis upon vascular and stromal function. Blood 1966;28:398.
10. Sacks, E.L., Goris, M.L., Glatstein, E., Gilbert, E., Kaplan, H.S. Bone marrow regeneration following large field irradiation. Influence of volume, age, dose and time. Cancer 1978;42:1057.
11. Ploemacher, R.E., van Soest, P.L. Hemopoietic stroma. I. Effects of radiation on proliferative and differential support capacity for hemopoiesis. IRCS Medical Science 1979;7:234.
12. Chamberlin, W., Barone, J., Kedo, A., Fried, W. Lack of recovery of murine hemopoietic stromal cells after irradiation-induced damage. Blood 1974; 44:385.
13. Dexter, T.M., Allen, T.D., Lajtha, L.G. Conditions controlling the proliferation of hemopoietic stem cells in vitro. J. Cell. Physiol. 1976;91:335.

APPENDIX PAPER VI

MIGRATION OF FIBROBLASTOID STROMAL CELLS IN MURINE BLOOD

A.H. Piersma, R.E. Ploemacher, K.G.M. Brockbank, P.G.J. Nikkels and C.P.E. Ottenheim

Department of Cell Biology & Genetics, Erasmus University, Rotterdam.

In: Cell and Tissue Kinetics 18, in press, 1985.

SUMMARY

This paper describes the kinetics of fibroblastic colony-forming units (CFU-F) in murine blood after phenylhydrazine-induced hemolytic anemia and their subsequent migration into hemopoietic organs. Murine blood contained 5.3 ± 0.8 CFU-F per 10^6 nucleated cells. Absence of particle ingestion and factor VIII-related antigen in addition to the enzyme pattern in CFU-F-derived cells confirmed that these cells did not have a macrophage-like or endothelial nature. Phenylhydrazine treatment of mice resulted in a three fold increase in blood CFU-F numbers which was accompanied by increases in blood cellularity and granulocyte-macrophage progenitor numbers. When both partners of CBA/N and CBA/T6T6 mice in parabiosis had been treated with phenylhydrazine, spleens and femoral bone marrow of both mice were shown to contain partner-derived CFU-F. These data suggest that circulating CFU-F represent a stromal cell population which can migrate into hemopoietic organs.

INTRODUCTION

The presence of fibroblastoid cells in the blood stream has been demonstrated in guinea pigs (Maximow, 1928), humans (Paul, 1958), mice (Metcalf, 1972), rabbits (Friedenstein, 1976) and dogs (Klein et al., 1983). The possibility that these cells were released from blood vessel walls during blood sampling was excluded by Luria et al. (1971). Circulating fibroblastic cells may represent a stromal cell population that migrates between tissues and organs, however, conclusive evidence for this notion has sofar not been presented (Friedenstein, 1976).

In the present study we examined the kinetics of fibroblastic colony-forming units (CFU-F) in blood, spleen and bone marrow of mice after induction of hemolytic anemia with phenylhydrazine. Migratory properties of CFU-F were then studied by treating normal and chromosome-marked mice in parabiosis with phenylhydrazine and assessing the origin of spleen and bone marrow derived CFU-F afterwards. Thus we provided evidence for migration of CFU-F from one partner to the spleen and bone marrow of the other.

MATERIALS AND METHODS

Male (CBA/Rij x C57BL/Rij)F1 and CBA/N and CBA/T6T6 mice, 10-20 weeks old were used. The mice were ether anaesthetized and bled from the suborbital plexus. Blood was collected in heparinized tubes. Subsequently the mice were killed by cervical dislocation and spleens and femurs removed using sterile techniques.

Nucleated blood cells were counted with Bürker counting chambers. Blood cells were separated (Boyum, 1968) in 0.1% methylcellulose and 50% α-medium by unit gravity for 30 min at room temperature to sediment erythrocytes, and the supernatant cells were then collected. The spleens were pressed through a nylon mesh sieve and suspended in 6 ml of buffered salt solution (BSS) containing 5% foetal calf serum (FCS). Femoral marrow cells were obtained by flushing the marrow from femurs, after removal of the epiphyses, in BSS + 5% FCS. Single cell suspensions from spleen and bone marrow were prepared by repeated flushing of the cells through a 23 gauge syringe needle. The cells collected from blood, spleen and bone marrow were centrifuged and resuspended in α-medium containing 5% FCS. Nucleated cell counts were performed with a coulter particle counter, prior to dilution to the required cell concentration in α-medium + 5% FCS.

For CFU-F quantitation the cells were cultured in α-medium containing 0.8% methylcellulose and 20% FCS. One ml aliquots of culture medium containing $2\text{-}7 \times 10^6$ spleen cells, $2\text{-}7 \times 10^5$ bone marrow nucleated cells, or varying numbers of blood nucleated cells were plated in 35 mm Costar culture dishes and incubated at 37°C in an atmosphere consisting of 10% CO_2 in air. All determinations were performed in triplicate. On day ten of culture the dishes were rinsed with phosphate buffered saline, fixed in methanol and stained with 10% Giemsa. Fibroblastoid colonies containing at least fifty fibroblastoid cells were counted with an inverted microscope.

Particle ingestion and enzyme stains were performed as described (Piersma et al., 1985). Factor VIII-related antigen was determined with horse-radish-peroxidase conjugated to rabbit-anti-human factor VIII-related antigen (DAKO, Denmark), which crossreacted with endothelium in frozen sections of various F1 murine organs at a 1 : 10 dilution. Binding of the antibody conjugate was detected with diaminobenzidine.

For CFU-GM quantitation the cells were cultured in a tenfold lower concentration than in the CFU-F assay in α-medium supplemented with 10% FCS, 1% BSA and 10% murine Con-A-spleen conditioned medium in duplicate for seven days in an atmosphere consisting of 10% CO_2 in air at 37°C prior to counting colonies containing at least fifty cells using an inverted microscope.

Hemolytic anemia was induced by four daily intraperitoneal injections of one mg phenylhydrazine-hydrochloride (Merck, Darmstadt, Germany) in 0.5 ml BSS. Mice in parabiosis received two daily injections one week after parabiosis. Parabionts were established between CBA/N and CBA/T6T6 chromosome-marked mice according to the method of Benner et al. (1977) and chromosome preparations were performed as previously described (Piersma et al., 1983).

RESULTS

In twenty independent experiments murine blood cells were assayed for CFU-F (Table 1), and were found to contain 5.3 ± 0.8 (mean $\pm$ SE) CFU-F per 10^6 nucleated cells (range 1.1 to 13.6). The blood-borne CFU-F colonies consisted of cells with a typical fibroblastoid morphology i.e. large flattened polygonal cells containing a large nucleus with several nucleoli. Furthermore (Table

TABLE 1

CFU-F IN MURINE BLOOD[a]

Exp. no.	nucleated cells plated ($\times 10^{-6}$)	number of CFU-F colonies	CFU-F per 10^6 nucleated cells
1	2.1	6	2.9
2	4.5	51	11.3
3	1.6	19	11.7
4	0.8	11	13.6
5	4.5	11	2.5
6	2.3	6	3.5
7	3.4	4	1.2
8	4.4	13	3.0
9	1.4	3	2.1
10	1.6	8	5.2
11	3.8	17	4.5
12	9.9	67	6.8
13	5.1	45	8.8
14	2.8	18	6.5
15	5.3	29	5.5
16	4.7	21	4.4
17	7.3	25	3.4
18	5.7	6	1.1
19	4.4	16	3.6
20	4.0	17	4.3
mean $\pm$ SE			5.3 $\pm$ 0.8

a. Blood samples taken via the suborbital plexus were pooled from three mice per experiment, and erythrocytes were depleted by methylcellulose agglutination. Remaining nucleated blood cells were washed before plating in culture medium.

TABLE 2

CHARACTERISTICS OF FIBROBLASTIC CELLS IN BLOOD-DERIVED CFU-F COLONIES

Carbon ingestion	-
Latex ingestion	-
Factor VIII-related antigen	-
Alkaline phosphatase	$\pm$
Acid phosphatase	$\pm$
Nonspecific esterase	-
Peroxidase	-

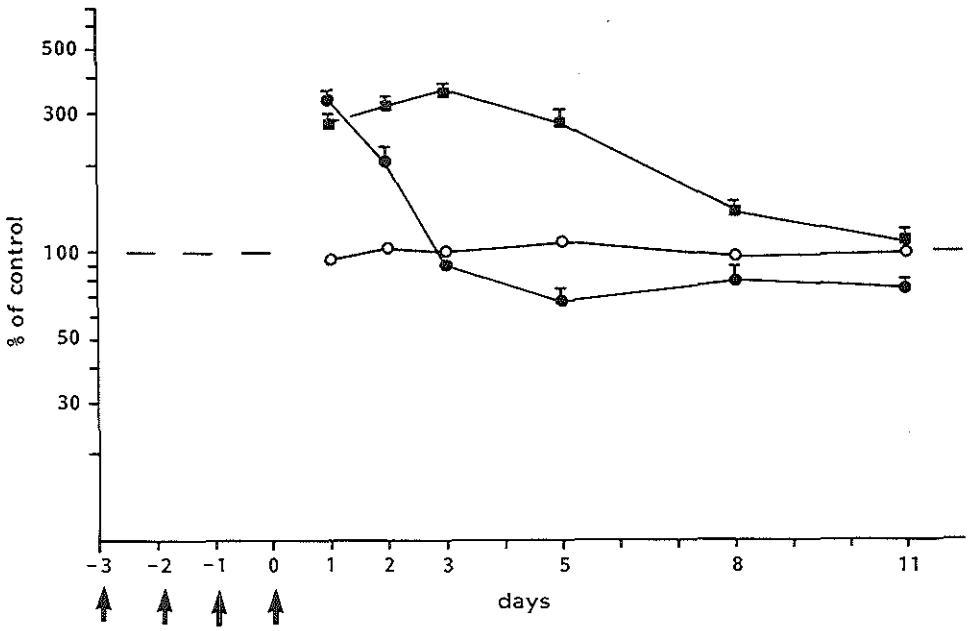


Figure 1. Nucleated cellularities of femoral bone marrow (○), spleen (■), and one ml blood (●) in mice treated with four daily injections (▲) of 1 mg PHZ per 0.5 ml BSS ip expressed as percentage of matched untreated controls ± standard error of three individual experiments. Each experiment involved three individual mice per point. Control cellularities averaged 5.8×10^6 per ml blood, 2.54×10^8 per spleen, and 2.72×10^7 per femur.

2) these cells were unable to ingest carbon and latex particles and could not be shown to carry the factor VIII-related antigen, which argues against a macrophage-like or endothelial nature of these CFU-F-derived cells. This point is further strengthened by their enzyme pattern which lacked detectable amounts of nonspecific esterase and peroxidase, whereas alkaline phosphatase was weakly positive in a minority of fibroblastic cells within a colony, and acid phosphatase was found to a limited extent through the colonies.

In Figure 1 the nucleated cell numbers of femur, spleen and blood are given for mice that had been treated with four daily injections of phenylhydrazine. Femoral cellularity was not changed, whereas splenic cellularity was increased threefold one day after treatment and decreased to normal levels between days 5 and 11. Blood cellularity was increased three to four fold at first and went back to subnormal levels by day 3 and remained as such throughout the study.

After phenylhydrazine treatment granulocyte/macrophage progenitor cell numbers (Figure 2) in the femoral bone marrow hardly changed. In the spleen they were elevated by five-fold, gradually returning to two-fold levels eleven days after treatment. In the blood these progenitors were increased by four fold at the first day after treatment and slowly returned to control values by the eleventh day.

Changes in CFU-F numbers following phenylhydrazine treatment are depicted in Figure 3. In the femur no significant changes were apparent. In the spleen

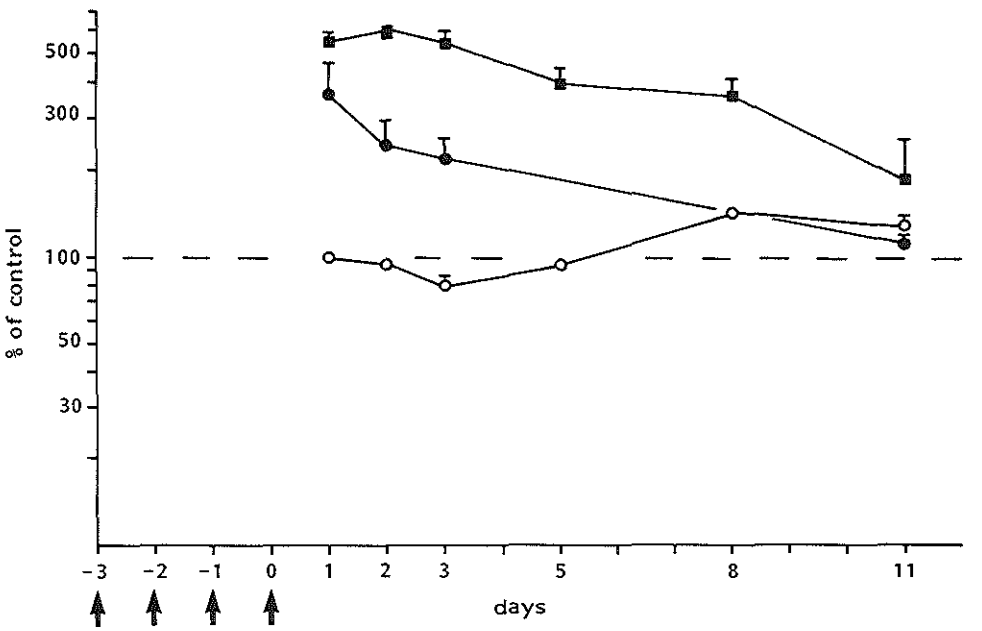


Figure 2. Granulocyte/macrophage colony-forming units in femoral bone marrow (○), spleen (■) and one ml blood (●) in mice treated with four daily injections (▲) of 1 mg PHZ per 0.5 ml BSS ip expressed as percentage of matched untreated controls ± standard error of three individual experiments. Each experiment involved three individual mice per point. Control CFU-GM numbers averaged 57 per ml blood, 9493 per spleen and 37177 per femur.

a threefold increase in CFU-F numbers at day one was followed by a decrease to normal levels at day three. The CFU-F in the blood were variably increased by up to three fold throughout the observation period.

In order to establish whether the increase in blood CFU-F numbers reflected a migrating population of stromal progenitor cells in which spleen and/or bone marrow served as target organs for CFU-F, we established parabiosis between CBA/N and syngeneic chromosome-marked CBA/T6T6 mice. After fusion of the blood streams of the partners, both were treated with phenylhydrazine, and one week later the spleens and femoral bone marrow were assayed for donor- and host-derived CFU-F. From Table 3 it can be seen that with one exception spleen as well as bone marrow of both partners in three parabionts contained significant amounts of partner-derived CFU-F, indicating that substantial migration from one animal to spleen and bone marrow of the other had occurred.

DISCUSSION

Under steady state conditions murine blood contained 5.3 ± 0.8 CFU-F per 10^6 nucleated blood cells (Table 1). This number is comparable to the data of Luria et al. (1971) for guinea pig blood. These authors showed that CFU-F incidence did not change with the number of punctures performed during blood

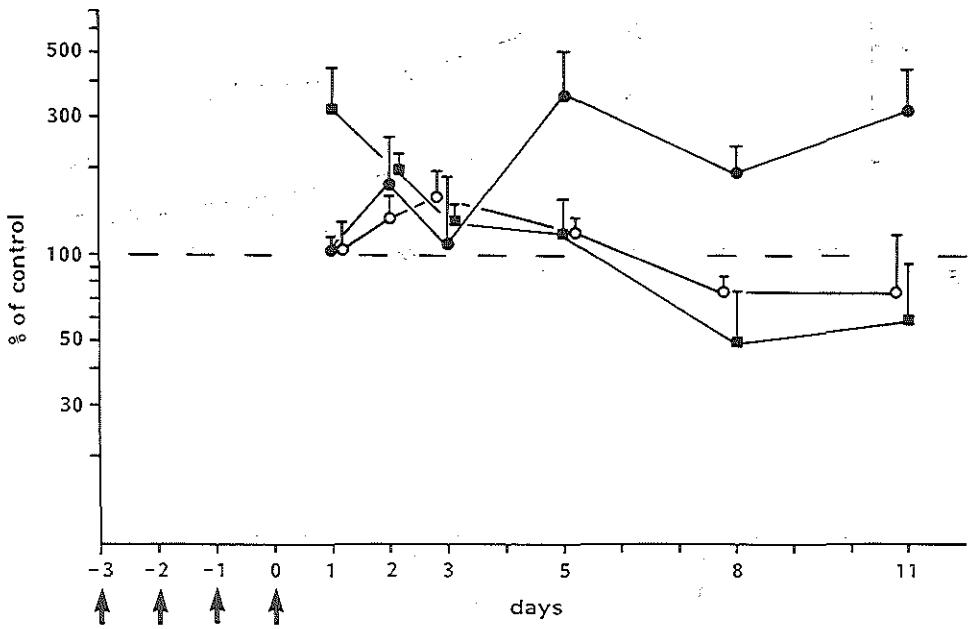


Figure 3. Fibroblastic colony-forming units in femoral bone marrow (○), spleen (■), and one ml blood (●) in mice treated with four daily injections (▲) of 1 mg PHZ per 0.5 ml BSS ip expressed as percentage of matched untreated controls ± standard error of three individual experiments. Each experiment involved three individual mice per point. Control CFU-F numbers averaged 18 per ml blood, 1262 per spleen, and 1272 per femur.

sampling, thus providing evidence that the fibroblastic colonies developed from circulating blood cells. The morphology of the CFU-F colonies and the characteristics listed in Table 2, as well as the absence of the macrophage determinant Mac-1 (Piersma et al., 1985), stress that these cells are not macrophage-like or endothelial in nature.

After induction of hemolytic anemia by phenylhydrazine treatment blood CFU-F numbers were increased by up to three fold, accompanied by an increased cellularity and increased granulocyte-macrophage progenitor numbers (Fig. 1-3). In the spleen similar effects were seen, whereas in the bone marrow these values had hardly changed. These observations confirm and extend studies by Rencricca et al. (1970), Hodgson et al. (1972), Hara and Ogawa (1976) and Ploemacher et al. (1977) who reported on hemopoietic stem and progenitor cell kinetics following phenylhydrazine-induced hemolytic anemia. Also after endotoxin treatment in mice, splenic CFU-F numbers were greatly increased (Brockbank et al., 1983). Friedenstein et al. (1974) reported an increase of CFU-F numbers in the bone marrow of mice after bleeding and in the lymph nodes of mice after immunization. In partially irradiated mice the irradiated part of the bone marrow was repopulated by fibroblastoid stromal cells much faster than the bone marrow of total body irradiated mice (Werts et al., 1980). Furthermore, upon intravenous bone marrow transplantation in man (Keating et

TABLE 3

ORIGIN OF CFU-F IN SPLEEN AND BONE MARROW OF PARABIOSED MICE[a]

Parabiont number	partner	organ	origin of CFU-F: donor	host	unknown	$\frac{\text{donor}}{\text{donor + host}} \times 100\%$
1	CBA/N	spleen	0	2	69	0
		bone marrow	3	10	45	23
	CBA/T6T6	spleen	4	11	160	27
		bone marrow	3	13	59	19
2	CBA/N	spleen	3	13	49	19
		bone marrow	9	23	56	28
	CBA/T6T6	spleen	7	17	40	29
		bone marrow	5	24	37	17
3	CBA/N	spleen	5	24	72	17
		bone marrow	13	52	74	20
	CBA/T6T6	spleen	3	11	37	21
		bone marrow	9	27	57	25

a. One week after parabiosis both partners received two daily injections of 1 mg phenylhydrazine in 0.5 ml BSS and were sacrificed for assay of CFU-F one week after this treatment.

al., 1982) and mice (Piersma et al., 1983) stromal cells were shown to lodge in the recipients bone marrow. The need for additional stromal support for hemopoiesis may require influx of stromal cells from other sites via the blood stream. The increase in blood CFU-F numbers after phenylhydrazine treatment supports this notion.

The study with phenylhydrazine-treated parabionts presented here provides conclusive evidence for the influx of blood-borne CFU-F into spleen as well as bone marrow (Table 3). We cannot exclude that CFU-F migration is a non-specific effect of phenylhydrazine-treatment. However, in view of the literature quoted above, migration of fibroblastoid stromal cells via the blood may represent a general phenomenon which serves the expansion of the hemopoietic stroma in stress-induced hemopoiesis.

ACKNOWLEDGMENTS

The authors wish to thank Prof.Dr. O. Vos for critically reviewing this manuscript, and Mrs. Cary Meijerink-Clerkx for typing the manuscript.

REFERENCES

Benner, R., van Oudenaren, A., de Ruiter, H. Antibody formation in mouse bone marrow. IX. Peripheral lymphoid organs are involved on the initiation of bone marrow antibody formation. Cell. Immunol. 34, 125, 1977.

Boyum, A. Separation of leukocytes from blood and bone marrow. Scand. J. Clin. Lab. Invest. 21, 1, 1968.

Brockbank, K.G.M., Ploemacher, R.E., van Peer, C.M.J. Splenic accumulation of stromal progenitor cells in response to bacterial lipopolysaccharides. Exp. Hematol. 11, 366, 1983.

Friedenstein, A.J., Deriglasova, U.F., Kulagina, N.N., Panasyuk, A.F., Rudakava, S.F., Luria, E.S., Radakov, I.A. Precursors of fibroblasts in different populations of hemopoietic cells as detected by the in vitro colony assay method. Exp. Hematol. 2, 83, 1974.

Friedenstein, A.J. Precursor cells of mechanocytes. Int. Rev. Cytol. 47, 327, 1976.

Hara, H., Ogawa, M. Erythropoietic precursor cells in mice with phenylhydrazine -induced anemia. Am. J. Hematol. 1, 453, 1976.

Hodgson, G.S., Bradley, T.R., Telfer, P.A. Hemopoietic stem cells in experimental hemolytic anemia. Cell Tissue Kinet. 5, 283, 1972.

Keating, A., Singer, J.W., Killen, P.D., Striker, G.E., Salo, A.C., Sanders, J., Thomas, E.D. et al. Donor origin of the in vitro hemopoietic microenvironment after bone marrow transplantation in man. Nature 289, 280, 1982.

Klein, A.K., Dyck, J.A., Stitsel, K.A., Shimizu, J., Fox, L.A., Taylor, N. Characterization of canine fetal lymphohematopoiesis: studies of CFUcm, CFUc and CFUf. Exp. Hematol. 11, 263, 1983.

Luria, E.A., Panasyuk, A.F., Friendenstein, A.J. Fibroblast colony formation from monolayer cultures of blood cells. Transfusion 11, 345, 1971.

Maximow, A.A. Cultures of blood leukocytes. From lymphocyte and monocyte to connective tissue. Arch. exp. Zellforsch. 5, 169, 1928.

Metcalf, D. Formation in agar of fibroblast-like colonies by cells from the mouse pleural cavity and other sources. J. Cell. Physiol. 80, 409, 1972.

Paul, J. Establishment of permanent cell strains from human adult peripheral blood. Nature 182, 808, 1958.

Piersma, A.H., Ploemacher, R.E., Brockbank, K.G.M. Transplantation of bone marrow fibroblastoid stromal cells in mice via the intravenous route. Br. J. Haematol. 54, 285, 1983.

Piersma, A.H., Brockbank, K.G.M., Ploemacher, R.E., Van Vliet, E., Brakel-van Peer, C.M.J., Visser, W.J. Characterization of fibroblastic stromal cells from murine bone marrow. Exp. Hematol. 13, 327, 1985.

Ploemacher, R.E., van Soest, P.L., Vos, O. Kinetics of erythropoiesis in the liver induced in adult mice by phenylhydrazine. Scand. J. Hematol. 19, 424, 1977.

Rencricca, N.J., Rizzoli, V., Howard, D., Puffy, P., Stohlman, F. Jr. Stem cell migration and proliferation during severe anemia. Blood 36, 764, 1970.

Werts, E.D., Gibson, D.P., Knapp, S.A., DeGowin, R.L. Stromal cell migration precedes hemopoietic repopulation of the bone marrow after irradiation. Radiat. Res. 81, 20,1980.

APPENDIX PAPER VII

RADIATION DAMAGE TO FEMORAL HEMOPOIETIC STROMA MEASURED BY IMPLANT REGENERATION AND QUANTITATION OF FIBROBLASTIC PROGENITORS

A.H. Piersma, R.E. Ploemacher and K.G.M. Brockbank

Department of Cell Biology & Genetics, Erasmus University, Rotterdam.

In: Experimental Hematology 11, 884-890, 1983.

SUMMARY

Radiation damage to femoral hemopoietic stroma in mice was measured six weeks after irradiation and reconstitution with syngeneic bone marrow by quantitation of fibroblastic progenitor cells (CFU-F), and by assaying hemopoietic progenitors and CFU-F numbers in regenerated subcutaneous femur implants. A dose-dependent effect of radiation on both preimplantation CFU-F numbers and implant regeneration was observed. Changes in bone marrow cell (BMC) graft size did not alter these stromal parameters. Therefore, transplanted CFU-F, which were present in the BMC graft, did not alter either the CFU-F content of femurs or their regenerative capacity. The strong correlation between CFU-F numbers and implant regenerative capacity after irradiation, leads us to suggest a function for CFU-F in femoral implant stromal regeneration.

INTRODUCTION

Radiation damage to hemopoietic stroma can be measured in different ways. One method is to assay stromal regenerative capacity by subcutaneous implantation of the irradiated hemopoietic organ into a syngeneic host. It is generally accepted that after subcutaneous implantation of femurs as well as femoral marrow plugs, the majority of hemopoietic cells in the implant disappear, then donor mesenchymal tissue proliferates, and hemopoiesis is reconstituted by host hemopoietic cells (1,2,3,4,5). The number of spleen colony-forming units (CFU-s, 6) present in the regenerated implants can be taken as a measure of stromal regeneration (7).

Radiation damage to hemopoietic stroma has also been determined by quantitation of fibroblastic colony-forming units (CFU-F) in the organ (8,9,10, 11), using *in vitro* colony formation assays. Hemopoietic organ fibroblasts have been shown to be able to transfer the hemopoietic microenvironment upon retransplantation under the kidney capsule (9). Furthermore, these cells have been implicated in the *in vitro* regulation of hemopoiesis (8,12,13).

In this study, a direct comparison is made between the CFU-F content and regenerative capacity of femurs derived from radiation-treated mice. We report a strong correlation between these two stromal assays, suggesting that CFU-F play a role in the regeneration of femoral implants.

MATERIALS AND METHODS

Male F1(CBA/Rij x C57BL/Rij) mice between 15 and 20 weeks of age were treated in groups of three animals. Total body γ-irradiation was performed using a ^{137}Cs source, with a dose rate of 1.3 Gy/min. Mice were intravenously reconstituted with fresh bone marrow cells (BMC) from syngeneic donors. Six weeks later, three femurs, one of each treated animal, were freed of muscle and connective tissue, and after removal of the epiphyses implanted subcutaneously in the ventral abdominal region in a normal syngeneic host and left for six weeks.

Bone marrow cells from the remaining femurs were pooled and used for quantitation of CFU-F. The assay was performed by culturing 5 x 10^5 BMC in one ml of medium, consisting of α-medium supplemented with 20% foetal calf serum (FCS) and 0.8% methylcellulose, in 35mm Costar tissue culture dishes for ten days in a 10% CO_2 in air humidified incubator at 37°C. Colonies containing fifty or more fibroblastic cells were counted after washing the cells with phosphate buffered saline, methanol fixation (10 min) and staining with 10% Giemsa.

Six weeks after implantation, femoral implants were removed and assayed for granulocyte-macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), CFU-s and CFU-F. Assays for CFU-GM were performed by culturing 10^5 BMC per ml of α-medium, supplemented with 1% BSA, 20% Con A-stimulated spleen-conditioned medium as a source of colony-stimulating activity, 20% FCS, and 0.8% methylcellulose in 35 mm Costar tissue culture dishes. After seven days of culture colonies containing at least fifty cells were counted. BFU-E assays were performed using the same culture medium as used for CFU-GM, however, the culture medium was further supplemented with one unit per ml of a step III preparation of sheep plasma erythropoietin (Connaught Labs., Ltd., Willowdale, Ontario, Canada) and 10^{-4}M β-mercaptoethanol. CFU-s assays were performed according to the method described by Till and McCulloch (6). Briefly, cells were injected iv into eight 9 Gy lethally irradiated recipient mice per experimental group. On day eight following transplantation the spleens of the recipients were removed and placed in Telleyesnizky's fixative. The number of macroscopic colonies was counted with the naked eye. CFU-F assays were performed as described above. All *in vitro* assays were done in triplicate.

RESULTS

Radiation effect on femoral CFU-F content. Femoral CFU-F numbers were assayed 6 weeks after total body irradiation (TBI) and transplantation with syngeneic BMC. Figure 1 shows that an inverse relationship was found between CFU-F number and radiation dose. No difference was observed when varying doses of BMC were grafted after 9 Gy irradiation.

Radiation effect upon ectopic femur regeneration. Implant regeneration was measured 6 weeks after TBI and transplantation with BMC by subcutaneous implantation of femurs into normal syngeneic recipient mice; six weeks after implantation the implants were assayed for their hemopoietic and stromal progenitor cell content. CFU-GM numbers decreased with increasing radiation doses (fig. 2). No influence of BMC graft size on implant CFU-GM content was found. Irradiation decreased the number of BFU-E and CFU-s in the implants and transplantation of the irradiated mice with varying doses of BMC did not alter the BFU-E and CFU-s content of the implants (table I). An inverse relationship was

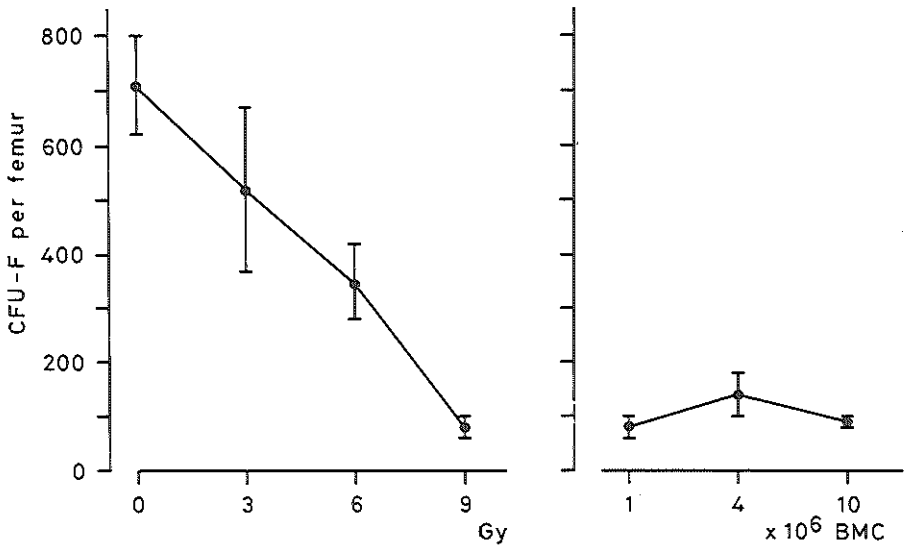


Figure 1. CFU-F content of femoral shafts six weeks after treatment with: (left) varying irradiation doses and reconstitution with 10^6 bone marrow cells (BMC); (right) 9 Gy total body irradiation and reconstitution with varying numbers of BMC. The data are expressed as the mean ± 1 standard error of three experiments in each of which three groups of three femurs were assayed separately.

also found between radiation dose and CFU-F numbers in the regenerated implants (fig. 3). For each radiation dose implants contained similar CFU-F numbers in comparison to the preimplantation femoral CFU-F numbers, assayed 6 weeks after irradiation (fig. 1,3). Variation of the BMC transplant size did not change the implant CFU-F content (fig. 3).

DISCUSSION

In this paper a comparison is made between CFU-F numbers and the regenerative capacity of implanted femoral shafts. Assays for stromal parameters were initiated six weeks after radiation treatment in order to allow femoral CFU-F numbers to stabilize. Earlier studies have shown that femoral CFU-F numbers undergo large changes during the initial weeks after treatment (10, 14). After an initial dip, CFU-F numbers returned to 10% of normal values 4 weeks after 9 Gy irradiation and bone marrow transplantation and remained at this level during the ensuing 6 months (14). The regenerative capacity of the stroma was measured by quantitation of CFU-GM content of implanted femurs. CFU-GM numbers appeared to be a reliable parameter in this assay, since they were found to correlate with both the BFU-E and CFU-s data obtained in the present study (table 1) and CFU-s and CFU-GM data from previous investigations (15,16).

The data presented here show that fibroblastic colony-forming cells in the bone marrow and the regenerative capacity of femoral hemopoietic stroma upon subcutaneous implantation have a similar sensitivity to radiation (fig.

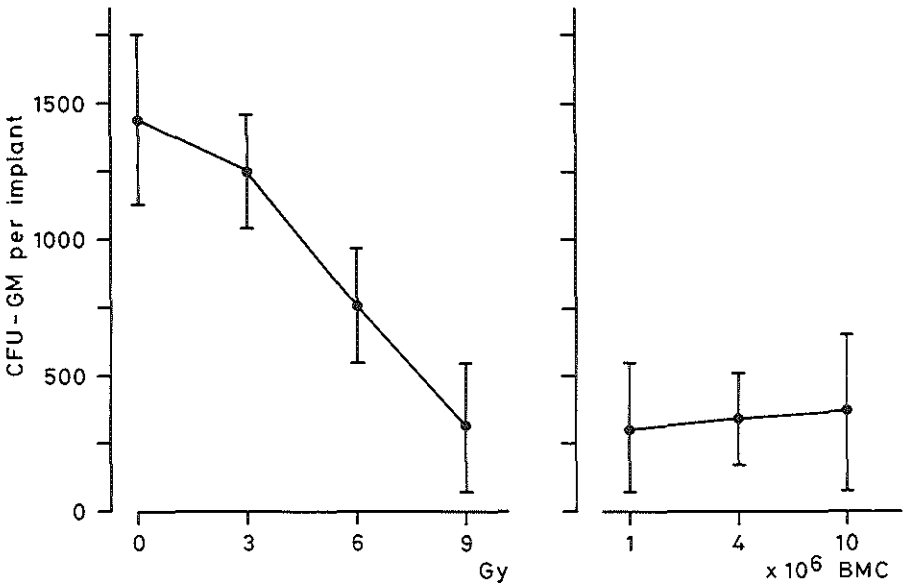


Figure 2. CFU-GM content of implanted femurs six weeks after implantation, that had been treated six weeks before implantation with: (left) varying irradiation doses and reconstitution with 10^6 bone marrow cells (BMC); (right) 9 Gy total body irradiation and reconstitution with varying numbers of BMC. The data are expressed as the mean ± 1 standard error of two experiments, in each of which three groups of three femoral implants were assayed separately.

1,2). The remarkable radiation sensitivity of the stroma, which is detectable with as little as 3 Gy, seems to contrast with earlier findings (17,18). However, in our study we used an assay system which is dependent on proliferation of stromal cells. Similarly, other studies (7,19-21) which have employed proliferative assay systems also indicate relatively high radiation sensitivities of hemopoietic stroma. The requirement for cell proliferation probably reveals a higher radiosensitivity because radiation damage becomes apparent only during mitosis.

CFU-F numbers in the implanted femurs as well as in irradiated femurs decreased with increasing radiation doses (fig. 3). Other studies in which a wider radiation dose range was employed showed that radiation doses below 3 Gy resulted in slightly increased CFU-s numbers in femoral implants (16), and also led to increased CFU-F numbers (140% of controls) in femurs 6 months after irradiation (11). These correlations suggest that CFU-F may be important in the regeneration of bone marrow stroma. Histological findings in extra-medullary marrow implants (1,22) and depleted marrow cavities (18), indicate that stromal reconstitution is initiated by proliferation of mesenchymal cells. This cell type may be functionally related to the CFU-F. Alternatively, it is also possible that both the reduction of CFU-F and of hemopoiesis in the implanted femurs are due to the loss of a third cell type, e.g. endothelium of small vessels (17), which may have lost its support capacity for both CFU-F and hemopoiesis.

Recently, we demonstrated the transplantation of murine CFU-F by the intravenous route and found that bone marrow-derived CFU-F have a high seeding

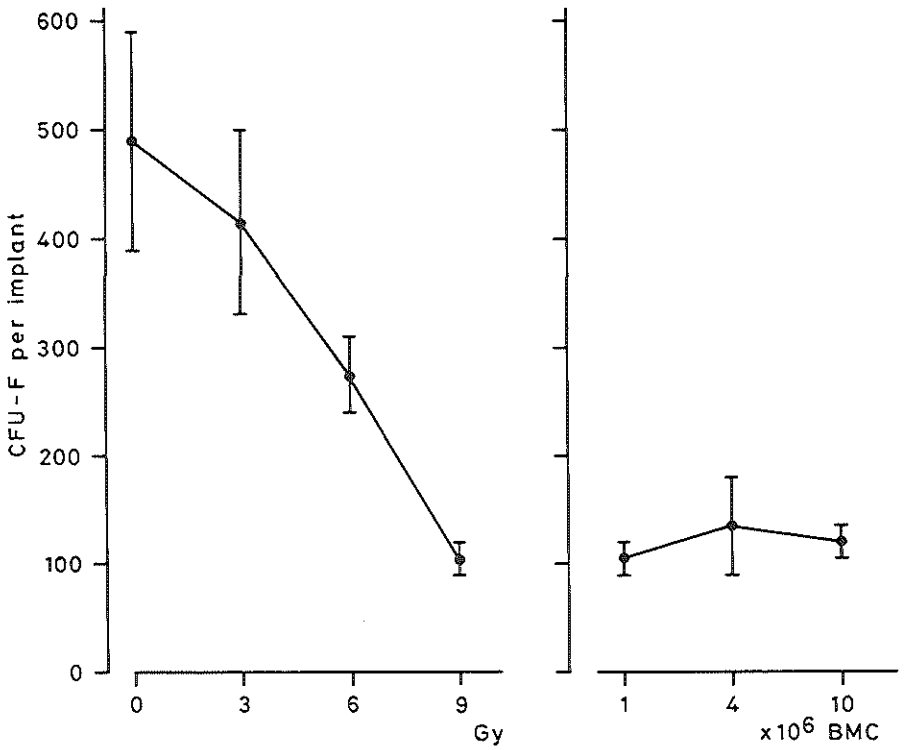


Figure 3. CFU-F content of implanted femurs six weeks after implantation, that had been treated six weeks before implantation with: (left) varying irradiation doses and reconstitution with 10^6 bone marrow cells (BMC); (right) 9 Gy total body irradiation and reconstitution with varying numbers of BMC. The data are expressed as the mean ± 1 standard error of three experiments, in each of which three groups of three femoral implants were assayed separately.

efficiency for bone marrow (14). One day after transplantation 72 percent of inoculated CFU-F had reached the hosts marrow and comprised approximately 50% of the CFU-F population from one day up to three months after treatment. However, in this study when we transplanted more than 10^6 BMC and thus more CFU-F after irradiation there was no increase in femoral CFU-F content six weeks later nor did it improve the regeneration of implanted femurs (fig. 1,2). This apparent anomaly may be explained by proposing that many CFU-F niches were still occupied by functional fibroblasts, which had lost their colony-forming capacity due to radiation-induced genetic damage. Thus, there would have been a limited number of niches available for remaining host CFU-F and transplanted donor CFU-F. The notion that the number of femoral CFU-F niches and their spatial distribution is rather constant is suggested by earlier studies on CFU-F during aging (23,24). The development of specific probes for the detection of CFU-F and their progeny *in situ* could facilitate the testing of this hypothesis.

TABLE 1

CFU-S AND BFU-E CONTENT OF FEMORAL IMPLANTS SIX WEEKS AFTER IMPLANTATION

Radiation dose (Gy)[a]	BMC dose	CFU-s per implant[b]	BFU-E per implant
0	10^6	1600±830[c]	180±2
9	10^6	82±11	44±35
9	10^7	57±2	36±3

a. Donors were treated six weeks before implantation with the indicated dose of irradiation and reconstituted with the indicated numbers of bone marrow cells.
b. The data are expressed as the mean ± 1 standard error of three experiments for CFU-s and two experiments for BFU-E. In each experiment three groups of three implanted femurs were assayed separately.
c. The 0 Gy group was significantly different ($p < 0.05$, Wilcoxon test) when compared with both of the 9 Gy treatment groups.

ACKNOWLEDGMENTS

The authors wish to thank Prof.Dr. O. Vos for critically reviewing this manuscript and Mrs. Cary Meijerink-Clerkx for typing the manuscript.

REFERENCES

1. Tavassoli M & Crosby WH (1968) Transplantation of marrow to extramedullary sites. Science 161, 54.
2. Amsel S & Dell ES (1971) Bone marrow repopulation of subcutaneously grafted mouse femurs. Proc. Soc. exp. Biol. (NY) 138, 555.
3. Maniatis A, Tavassoli M & Crosby WH (1971) Origin of osteogenic precursor cells in extramedullary marrow grafts. Blood 38, 569.
4. Friedenstein AJ, Petrakova KV, Kurolesova AI & Frolova GP (1968) Heterotopic transplants of bone marrow analysis of precursor cells for osteogenic and haematopoietic tissues. Transplantation 6, 230.
5. Fried W, Husseini S, Knospe WH & Trobaugh FE Jr (1973) Studies on the source of hematopoietic tissue in the marrow of subcutaneously implanted femurs. Exp. Hematol. 1, 29.
6. Till JE & McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat. Res. 14, 213.
7. Fried W, Chamberlin W, Kedo A & Barone J (1976) Effects of radiation on hematopoietic stroma. Exp. Hemat. 4, 310.
8. Wilson FD, O'Grady L, McNeill CH & Munn SJ (1974) The formation of bone marrow derived fibroblastic plaques in vitro: preliminary results contrasting these populations to CFU-C. Exp. Hematol. 2, 343

9. Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF & Keiliss-Borok IV (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. Transplantation 17, 331.
10. Friedenstein AJ, Gorskaja UF & Kulagina NN (1976) Fibroblast precursors in normal and irradiated mouse hemopoietic organs. Exp. Hematol. 4, 267.
11. Ploemacher RE, Brockbank KGM, Brons NHC & De Ruiter H (1983) Latent sustained injury of murine hemopoietic organ stroma induced by ionizing radiation. Hematologica 68, 454.
12. Blackburn MJ & Patt HM (1977) Increased survival of hemopoietic pluripotent stem cells in vitro induced by a bone marrow fibroblast factor. Br. J. Haematol. 37, 327.
13. Brockbank KGM, & van Peer CMJ (1983) Colony stimulating activity production by hemopoietic organ fibroblastoid cells in vitro. Acta Hematol. 69, 369.
14. Piersma AH, Ploemacher RE &Brockbank KGM (1983) Transplantation of bone marrow fibroblastoid stromal cells in mice via the intravenous route. Br. J. Haematol. 54, 285.
15. Chamberlin W, Barone J, Kedo A & Fried W (1974) Lack of recovery of murine hemopoietic stromal cells after irradiation-induced damage. Blood 44, 385.
16. Ploemacher RE & van Soest PL (1979) Hemopoietic stroma. I. Effects of radiation on proliferative and differential support capacity for haemopoiesis. IRCS Medical Science 7, 234.
17. Knospe WH, Blom J &Crosby WH (1966) Regeneration of locally irradiated bone marrow. I. Dose dependent, longterm changes in the rat, with particular emphasis upon vascular and stromal reaction. Blood 28, 298.
18. Patt HM & Maloney MA (1980) Reconstitution of bone marrow in a depleted medullary cavity. In: Hemopoietic Cellular Proliferation, ed.: Stohlman Fr, Jr. pp. 56-66. Grune and Stratton NY.
19. Werts ED, DeGowin RL, Knapp SK & Gibson DP (1980) Characterization of marrow stromal fibroblastoid cells and their association with erythropoiesis. Exp. Hematol. 8, 423.
20. Friedenstein AJ, Latsinik NV, Gorskaya UF &Sidorovich SY (1981) Radiosensitivity and postirradiation changes on bone marrow clonogenic stromal mechanocytes. Int. J. Radiat. Biol. 5, 537.
21. Tavassoli M (1982) Radiosensitivity of stromal cells responsible for in vitro maintenance of hemopoietic stem cells in continuous, longterm marrow culture. Exp. Hematol. 10, 435.
22. Steinberg MH, Robinson F, & Dreiling BJ (1973) Studies on the development of extramedullary marrow implants. Transplantation 15, 1.
23. Brockbank KGM, Ploemacher RE & van Peer CMJ (1983) An in vitro analysis of murine hemopoietic fibroblastoid progenitors and fibroblastoid cell function during aging. Mech. Aging Devel. 22, 11.
24. Brockbank KGM, Piersma AH & van Peer CMJ (1983) The regional distribution progenitors in murine femoral marrow revisited. Biomedecine 36, 369.