THE ERYTHROID HEMOPOIETIC MICROENVIRONMENTS

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CHAPTER I

General Introduction

Introduction

Hemopoietic differentiation

The cells present in the blood can be divided into three classes – red cells, white cells and platelets. Within the white cell class a subdivision can be made into granulocytes (neutrophils, eosinophils and basophils), lymphocytes and monocytes. In the normal adult the production of each mature cell type is mainly achieved by proliferation and differentiation of hemopoietic progenitor cells in the bone marrow and, in case of mice, also in the spleen. When morphological and functional differentiation of these progenitor cells has occurred the maturing cells are usually kept at the site of formation in the hemopoietic tissues for a certain period before they are released to the blood stream. In addition to these mature cells also immature cells may circulate in the blood in small numbers.

It has been demonstrated with chromosome marker techniques that pluripotent murine stem cells can give rise to all identifiable blood cells (Becker, McCulloch and Till, 1963), including lymphocytes (Ford et al., 1966; Micklem et al., 1966; Wu et al., 1968a; Abramson et al., 1977). Also tissue macrophages and plasma cells originate from pluripotent hemopoietic stem cells. The stem cells have the capacity for extensive proliferation resulting in self-replication (Caffrey Tyler and Everett, 1966; Metcalf and Moore, 1971), although this seems not to be infinite (Siminovitch et al., 1964; Metcalf and Moore, 1971; Botnick et al., 1976).

The stem cell can undergo a step of determination, which is called "commitment"; this limits the cell to a certain pathway of differentiation. The committed cells are called progenitor cells which do not manifest any morphological feature specific for one differentiation line but have become responsive to a specific humoral regulator. They have lost the possibility to return to a state of non-commitment and to enter into the non-proliferating G_0 phase of the cell cycle. The regulation of the commitment and proliferation of stem cells appears to be a complex interaction of inhibitory and stimulatory factors which makes it possible to respond rapidly to the variable body demands. In general regulatory factors are considered to belong to two categories, i.e. (1) humoral factors and (2) microenvironmental factors. The present opinion is, that the commitment of stem cells and the regulation of stem cell and early progenitor cell numbers possibly is a function of microenvironmental factors, whereas the size of the differentiated cell clone produced by each committed cell is possibly regulated by humoral factors (Metcalf and Moore, 1971; Tavassoli, 1975; Lord et al., 1976).

Note on the microenvironmental regulation of hemopoiesis

The microenvironmental factors are the subject of the largest part of the introduction to the experimental work described in the present thesis. They include the influence of stromal structure on hemopoiesis; this involves regulation of tissue pH and oxygenation and regulation of the production of short range factors. Probably the microenvironmental influence on hemopoiesis can be affected by long range neural and hormonal actions; a complex interaction between developing hemopietic cells and the microenvironmental stroma is assumed to exist locally.

Note on the humoral regulation of hemopoiesis

The humoral factors, that are involved in the regulation of hemopoiesis, show a great diversity in structure and source of production in the body. Moreover, a clear distinction between long range humoral factors and short range microenvironmental factors cannot precisely be made. This indicates, that microenvironmental and humoral regulation of hemopoiesis overlap and that certain aspects of both regulatory influences can not always be studied separately. Among the humoral factors involved in hemopoietic regulation certain steroids have been demonstrated to affect hemopoiesis without an intermediate action of a specific hemopoietic hormone (Levere et al., 1967; Byron, 1970; 1971; Paulo et al., 1974; Moriyama and Fisher, 1975; Udupa and Reissman, 1975). It is suggested that the ability of some steroids to increase the proportion of cells in the hemopoietic hormone-sensitive phase of the cell cycle is probably the mechanism responsible for the hemopoietic effects of these agents (Dunn and Napier, 1976).

Although the existence of specific humoral regulators has been established and some of these substances are able to make a committed precursor cell to mature *in vitro* – in the absence of stromal cells from hemopoietic tissues – it is neither well documented on which precursor cell stage the humoral factors act nor by which mechanism they act. However, the action of two humoral regulators has been thoroughly investigated, i.e. erythropoietin and colony stimulating factor. These factors, which are glycoproteins, probably have no action on stem cells but only on committed cells.

Erythropoietin is supposed to induce erythroid differentiation in a morphologically not defined cell compartment, which is the intermediate between the stem cell compartment and the morphologically recognizable erythroid compartment (Stohlman et al., 1968; Stohlman, 1970; Lajtha et al., 1971; Wagemaker, 1976). Probably it also stimulates the hemoglobin synthesis and the DNA synthesis rate in morphologically recognizable erythroblasts (Stohlman, 1970; Hegemann and Dörmer, 1976). Erythropoietin *in vivo* is defined as a substance, which is able to induce a burst of hemoglobin synthesizing cells in a polycythemic mouse that has no recognizable, immature erythroid cells. The erythropoietin is mainly produced in the kidney in response to a hypoxic stimulus (Mirand and Prentice, 1957).

However, other sites of production have been suggested, either defined, e.g. the Kupffer cells of the liver (Peschle et al., 1976; Naughton et al., 1977), the submandibular glands (Zangheri et al., 1973; Zangheri, 1977), the carotid body (Tramezzani et al., 1971), or not defined (Mirand and Murphy, 1970).

The colony stimulating factor is an *in vitro* defined substance, which stimulates the growth of cell clones from granulocyte and macrophage precursor cells in semisolid media (Pluznik and Sachs, 1966; Bradley and Metcalf, 1966). Several studies suggest, that the factor does not act simply as a trigger for cellular division but must be present in the cultures for the whole duration of incubation (Robinson et al., 1967; Metcalf and Foster, 1967a; Rosendaal, 1977). There are several indications that this factor has also an *in vivo* action on granulopoiesis (Metcalf and Stanley, 1971; Morley et al., 1971; Vogler et al., 1972). Because of the availability of a highly sensitive *in vitro* assay system for colony stimulating activity, much is known of its distribution in body fluids and organs in normal and abnormal states. The stimulating activity is detectable in serum of normal mice (Metcalf and Foster, 1967b) and in human serum (Foster et al., 1968) or urine (Metcalf and Stanley, 1971). *In vitro* the stimulating activity is excreted by many cell types, e.g. neonatal mouse-kidney cells (Bradley and Metcalf, 1966), fibroblasts (Pluznik and Sachs, 1965), cells in pregnant uterus, lung, spleen and bone marrow (Chan and Metcalf, 1972; Sheridan and Metcalf, 1973) and many other tissues (Metcalf and Moore, 1973). There exists strong experimental evidence that serum colony stimulating factor is not derived from a single organ but is a secretory product of a cell type, e.g. reticuloendothelial cells, which many organs have in common (Metcalf and Moore, 1971). Kurland et al., (1977) established that mononuclear phagocytes elaborate colony stimulating factors. It has been indicated, that the various colony stimulating factors described in the literature have one entity in common, which is responsible for the biological activity (Van den Engh, 1976).

In addition to the humoral factors that have a stimulatory effect on erythropoiesis and granulopoiesis, also specific inhibitors have been described for either erythropoiesis (Krzymowski and Krzymowska, 1962; Lewis et al., 1969b; Lindemann, 1971; Lindemann and Laerum, 1976; Lord et al., 1977) or for granulopoiesis (Rytöma and Kiviniemi, 1968, MacVittie and McCarthy, 1974; Vogler and Winton, 1975; Lφvhaug and Bφyum, 1977; Broxmeyer et al., 1977; Kurland et al., 1977).

Humoral regulators for thrombopoiesis (Abilgaard and Simone, 1967; Evatt and Levin, 1969; McDonald, 1976) and lymphopoiesis (cf. Van Bekkum, 1975) probably also exist. The thrombopoietic factor is called thrombopoietin and has been detected amongst others in normal serum and urine (Odell et al., 1961; Linman and Pierre, 1962) and in serum from patients with acute blood loss (Toi, 1956). The lymphopoietic factor appears to be a thymic hormone that probably stimulates the appearance of differential markers (T cell specific surface antigens) on precursor T-lymphocytes (Goldstein et al., 1971; Bach et al., 1971; Komura and Boyse, 1973). The specificity of the latter two hormones is not fully established and effects of erythropoietin on thrombopoiesis have also been reported (Choi and Simone, 1973;

Krizsa and Cserháti, 1976) although these data have been contradicted (Evatt et al., 1976).

Finally it must be noticed that apart from the specific humoral regulators of hemopoietic cells there are many non-specific factors such as nutritional factors (Kondi et al., 1963; Pearson, 1967; Adams, 1970; Mant and Faragher, 1972; Bell et al., 1976), including vitamins (cf. Herbert, 1970), which have profound effects on hemopoietic cells. Specific and non-specific factors form multiple control mechanisms of hemopoiesis, which regulate progenitor cell activation into a sequence of maturing cells and influence the rate of differentiation at later stages of the cell maturation pathway.

Embryonic Aspects of the Hemopoiesis

Chronological sequence of organs involved in hemopoiesis

During the embryonic period of a mammal, hemopoiesis is first observed in blood islands generally confined to yolk-sac mesoderm (Attfield, 1951; Borghese, 1959). In human embryos isolated foci of erythropoiesis are sometimes observed throughout the extraembryonic mesoblastic tissue (Bloom and Bartelmez, 1940). In most vertebrates yolk-sac hemopoiesis embraces erythropoiesis, granulopoiesis and megakaryocyte production. Lymphopoiesis does not occur in the yolk-sac. In the mouse yolk-sac hemopoietic activity is restricted to the erythropoiesis at 7-12 days gestational age.

Following the decline of yolk-sac hemopoiesis the liver becomes the major embryonic hemopoietic organ in most mammals (Rifkind et al., 1968; 1969; Niewisch et al., 1970). In the mouse liver erythropoiesis begins between 10-11 days gestation with megakaryocytes and granulocytes appearing shortly thereafter. Liver hemopoietic activity subsides rapidly after birth, but the murine liver may contain erythrocytic foci up to more than 1 week after birth (Borghese, 1959).

At the time that hemopoiesis is extensive in the liver, the splenic hemopoietic activity develops. In the mouse erythropoiesis is detectable in the spleen at 15 days gestation but by 17 days granulopoiesis predominates. Lymphocytes first appear in this organ at or shortly after birth.

Shortly before birth, by the 17th day of gestation in mice, the bone marrow becomes populated by granulopoietic cells but erythropoiesis is not observed until after birth. In the postnatal life of most mammals the marrow becomes the exclusive site of hemopoietic activity, with an exception of lymphopoiesis, which is also present in lymphoid organs including the spleen.

However, in some species, e.g. the mouse, hemopoiesis is also observed in the spleen of healthy adults. Since the mouse is the subject of investigation in this thesis and most knowledge on the microenvironmental aspects of hemopoiesis has been obtained in small vertebrates, the more detailed information in the following will be confined to the mouse, unless otherwise indicated.

Stem cell migration

During embryogenesis all sites of hemopoiesis, including lymphoid tissues, are dependent on colonisation by circulating stem cells originating *de novo* in the yolk-sac blood islands (Moore and Metcalf, 1970; Niewisch et al., 1970; Moore and Owen, 1965). At later stages following the decline in yolk-sac hemopoiesis, the expanding stem cell population in foetal liver contributes to the circulating stem cell pool and leads to colonization of spleen and bone marrow; probably also lymphoid tissue is colonized by circulating stem cells. Postnatally the expanding marrow function replaces the liver as a source of circulating stem cells and becomes the only long term renewal site for stem cells and the main site of hemopoiesis (Moore et al., 1970; Niewisch et al., 1970). There is evidence that interchange of stem cells and their descendants between the several hemopoietic compartments (bone marrow, spleen, thymus and lymph nodes) may be limited in the steady state (Ford et al., 1966; Carsten and Bond, 1968; Patt and Maloney, 1970; Micklem et al., 1975a, 1975b) and that there may be a considerable degree of mobility after (partial) body irradiation (Barnes et al., 1959; Goodman and Hodgson, 1962; Wu et al., 1968; Edwards et al., 1970).

Evidence for the existence of hemopoietic microenvironments

From the previous paragraph it may be concluded that hemopoietic organs must have a vascular trapping mechanism (Trentin, 1970) to establish an equilibrium between circulating cells and the stem cell compartment. Such a mechanism will probably consist of a series of complex cell regognition and interaction processes. The diversity of hemopoietic differentiation seen throughout development appears to be an organotypic characteristic determined by the environment presented to the immigrant stem cells. These considerations make it likely that hemopoiesis will be only present in certain restricted organs meeting specialized criteria for lodging, sustaining and directing the proliferation and differentiation of immigrant stem cells and for allowing the delivery of mature cells. The stromal cells of hemopoietic organs must play a highly specialized role in hemopoiesis by creating special local conditions ("microenvironments"), which favour the development of the different blood cell types. Evidence for the existence of microenvironments can be drawn from experiments by Knospe et al. (1966) and Jenkins et al. (1970), who observed a secundary and permanent late aplasia of areas of bone marrow and spleen, which received very high doses of local irradiation. Also the delayed return of hemopoietic stem cells and hemopoiesis to transplanted pieces of hemopoietic tissue until the late stage of regeneration of the characteristic organ stroma is in agreement with the role of the stroma in hemopoiesis (Knospe et al., 1968; 1972; Tavassoli and Crosby, 1968; Maloney and Patt, 1972; Wolf, 1974; Morley and Blake, 1974; Wolf and Lagunoff, 1974).

Clinical evidence has come from Fernbach and Trentin (1962) who reported the

failure of transfused bone marrow, obtained from an identical twin, to take and grow in the other twin with drug-induced pancytopenia.

Much knowledge concerning the existence and functional properties of a hemopoietic microenvironment has been derived from the studies on "Steel" mice. These mice suffer from a genetically determined (Bennett, 1956; Russell and Bernstein, 1966) macrocytic anemia and show abnormal megakaryocyte kinetics (Ebbe et al., 1973), whereas the granulopoiesis is slightly affected. Foetal liver and marrow show marked reduction in hemopoiesis. Stem cells of "Steel" mice with genotype SL/SL^d injected into heavily irradiated wild type mice produce normal numbers of spleen colonies and lead to hemopoietic recovery in the irradiated recipient. Conversely, when wild type stem cells are transplanted into irradiated SL/SL^d mice a very low spleen colony number is observed in the spleen and to a lesser extent in the bone marrow (McCulloch et al., 1965a; 1965b; 1965c, Sutherland et al., 1970). McCulloch et al., (1965a) showed that the impairment of colony growth in the latter chimeras is not due to a defect in the humoral regulation of erythropoiesis, since SL/SL^d mice in parabiosis with wild type mice did not show any improvement in growth of injected stem cells following irradiation, despite normal proliferation in the wild type parabiotic partner. However, implantation of spleen stroma from non-anemic littermates restored hemopoiesis at the implantation site (Bernstein, 1970; Wolf, 1974). The "Steel" defect apparently is an environmental factor that interferes with proliferation of hemopoietic cells in the spleen and to a lesser degree in the marrow (Altus et al., 1971; Knospe et al., 1976; McCarthy et al., 1977). Moreover, this environmental factor probably is cellular in nature, is more radioresistant than stem cells and supports the growth of stem cells. These statements have been derived from studies on transplanted femurs and spleens of both SL/SL^d and wild type origin into both SL/SL^d and wild type hosts (Fried et al., 1973a; 1973b). It appeared that eight weeks after transplantation the wild type grafts incorporated more radioactive iron, as a measure of hemoglobin synthesis in erythroid cells, than did the SL/SL^d ones. Freezing of the tissues prior to implantation abolished their ability to support hemopoiesis. X-irradiation of femur and spleen donors with 950 R reduced the growth of stem cells in the grafts, whereas grafts from donors exposed to 2500 R supported no stem cell growth at all.

Hemopoiesis does not only occur in special organs but there is also clear evidence for complete or regional specialisation within these organs. In the murine lymph nodes only lymphopoiesis occurs. In the spleen granulopoiesis is concentrated under the spleen capsule and along the subcapsular ends of the trabeculae whereas erythropoiesis is more diffusely scattered throughout the red pulp. The lymphoid follicles of the spleen generate clones of antigen-reactive and antibody-forming lymphocytes. This morphological evidence of segregation strongly suggests that different microenvironments exist within the spleen, each of which favours the proliferation of a distinct cell type.

In the context of the present discussion the term "microenvironment" denotes a specialized region of a hemopoietic organ, which differs from adjacent areas in the

organ or from other organs and which creates the local conditions within which stem cells can multiply and give rise to differentiating descendants. This microenvironment may embrace anything from a few cells to probably millions of cells in an entire organ. However, examination of microscopic sections of hemopoietic organs has shown that the organ can usually be divided into replicate compartments of microscopic dimension, which contain relatively small numbers of cells. Tavassoli (1975) has pointed to the fact that, whereas the term hemopoietic microenvironment has become synonymous with the hemopoietic milieu of an entire organ, it should more correctly be restricted to the designation of specific microareas or "niches" within the organ that induce hemopoietic differentiation. Each microenvironment induces stem cells to differentiate into one of the five lines of hemopoiesis; erythroid, granuoid, monocytic, megakaryocytic or lymphoid. Trentin (1970) introduced the term Hemopoietic Inductive Microenvironment, which was coined to describe the stromal influence on the commitment of stem cells. The commitment-inducing stimulus may be defined as an effect upon the multipotent stem cells by the surrounding microenvironment, which determines the direction of differentiation to be taken by its progeny (Wolf, 1974). This inductive function of the microenvironment is clear in lethally irradiated mice, which are injected intravenously with bone marrow cells. After a few days hemopoietic colonies develop in the bone marrow and spleen, which are clonal in nature (Becker et al., 1963, Chen and Schooley, 1968; Wu et al., 1967) and are formed by stem cells that have pluripotentiality for the all lines of hemopoiesis. For the first 8 to 10 days almost each colony is committed to a single line of differentiation, e.g. in the spleen: erythroid, neutrophilic or eosinipholic granuloid or megakaryocitic (Becker et al., 1963, Curry et al., 1967; Nowell et al., 1970; Trentin, 1970; 1971). These colonies show a tendency to "grow outward", i.e. immature elements are more frequently found on the periphery, forming a "shell" around more differentiated cells of the series (Curry and Trentin, 1967). As the colonies expand they develop second lines of differentiation probably because they encroach upon another type of microenvironment (Trentin, 1970; 1971). Each type of colony can be shown to contain stem cells, which can produce all colony types when retransplated (Lewis and Trobaugh, 1964). However, it has never been demonstrated that every single colony contains a detectable stem cell number.

There is morphological evidence that colonies of specific hemopoietic lines have a preference for certain locations in the spleen. Granulocytic colonies are usually found along the trabeculae of the spleen or in subcapsular sheets and less frequently within an atrophic lymphoid follicle and are predominantly neutrophilic. Erythroid colonies may appear anywhere within the red pulp and megakaryocyte colonies are usually found just beneath the splenic capsule (Curry and Trentin, 1967).

Another argument for the fact the definite areas in hemopoietic organs are reserved for only one line of hemopoiesis is the replacement of erythroid colonies by nests of undifferentiated cells in hypertransfused mice, which have been irradiated and reconstituted (Schooley, 1964; Liron and Feldman, 1965; Bleiberg et al., 1967; Curry et al., 1967). These undifferentiated cells appear to be erythropoietin-sensitive but do

not show erythroid maturation due to the absence of a sufficient level of erythropoietin in the hypertransfused animals. The suppressed presumptive erythroid colonies remain small and immature but do not become granuloid colonies.

The bone marrow and spleen hemopoietic environment differ from each other. In the bone marrow granuloid colonies predominate (erythroid: granuloid ratio = 0.5) (Wolf and Trentin, 1968). In the spleen the erythroid colonies predominate (erythroid: granuloid colony ratio = 3) (Curry et al., 1967). When whole spleens are transplanted subcutaneously they support hemopoiesis and give rise to spleen colonies with the same erythroid: granuloid ratio as does spleen *in situ* (Wolf and Trentin, 1968). These authors demonstrated also that pieces of marrow stroma, which were transplanted into the spleen, support hemopoiesis with an erythroid: granuloid ratio similar to that of bone marrow *in situ*. The specific properties of spleen and marrow were demonstrated in these experiments by the fact, that single colonies growing across the junction of marrow and spleen stroma showed abrupt transitions of hemopoietic types, with erythropoiesis in the spleen stroma and granulopoiesis in the marrow stroma. Moreover, Rauchwerger et al. (1973) observed that rat marrow cells injected into irradiated mice give both erythroid and granuloid spleen colonies with a ratio typical for murine spleen. However, when rat marrow cells were injected into irradiated rats almost only erythroid spleen colonies were observed, that grew much more slowly than mouse spleen colonies. The microenvironmental influence on hemopoiesis was also clearly demonstrated by the experiments of Meck et al. (1973). These authors induced hemopoiesis by injection of marrow cells into the omentum of irradiated mice and found largely granulopoietic colonies even when the experimental hosts were injected with large doses of erythropoietin (Meck and Laissue, 1977). In contrast, ten days i.p. transplants of spleen fragments from irradiated mice were predominantly erythropoietic (Brecher et al., 1972).

Seki (1974) demonstrated that inteperitoneal cellulose acetate membranes become coated with macrophages and fibroblasts, and support hemopoietic colony formation following irradiation and intraperitoneal injection of bone marrow cells. The granuloid colonies outnumber erythroid colonies (erythroid: granuloid colony ratio = 1: 20).

These observations suggest, that the lodging, proliferation and differentiation of pluripotent stem cells into specific lines of hemopoiesis are determined by the microenvironemntal circumstances in the hemopoietic tissues. The several types of microenvironments occur in a distinctive pattern and frequency inherent to the hemopoietic organ. It has been demonstrated by Wolf (1974) that the three microenvironmental functions mentioned above can be examined separately and, in some cases, be shown to operate independently of each other. It was shown that the lodgment of stem cells in genetically anemic SL/SL^d mice as well as in wild type mice was similar. However, the erythroid commitment appeared somewhat abnormal in the SL/SL^d spleen and the proliferation of stem cells was strongly reduced in SL/SL^d mice (Fried et al., 1973a; Wolf, 1974; McCarthy et al., 1977).

The nature of the determining effect is presumed to require close proximity or

contact of the cells, which produce the inducing factors, and the cells to be induced. The regulation of stem cell distribution in hemopoietic organs is probably the most basal property of the microenvironment since selective binding permits the stem cell to closely interact with inducing factors. The commitment and the proliferative stimulus on stem cells are suggested to be local in nature (Gidali and Lajtha, 1972; Lord et al., 1976) and are likely to occur at a cellular level, i.e. they are controlled by interaction of undefined stromal cells and hemopoietic cells through a process of cell-cell interaction. This interaction may operate via a direct cell-to-cell contact or may be mediated by signals acting at a very short range. The latter possibility has been promoted by studies of Matioli and colleagues who compared stem cell renewal in irradiated and nonirradiated parts of 4000 R partly irradiated murine spleens (Matioli et al., 1973; Matioli and Vasudevan, 1974; Matioli, 1976; Matioli and Rife, 1976). Following complex reasoning the authors concluded that stem cells must be very close to, or in contact with, other specialized cells ("sources" of a stimulus) in hemopoietic tissues, so that the sources regulated the renewal of stem cells by microdiffusion. The sources seem to vary widely in respect to radiobiological and functional properties, i.e. some sources decay rapidly at a dose of 4000 R, whereas other sources decay at a much slower rate even at a dose of 8000 R (Rife et al., 1975; Matioli and Rife, 1976).

Wagemaker (1977b) stated, that the dependency of the *in vitro* growth of erythroid Burst Forming Units (BFU-E, which is a very primitive cell type) on cell concentration suggests that an active principle associated with bone marrow cells is required for burst formation. This activity, which has been called burst feeder activity (BFA) was not abolished by irradiation up to 20 Gy. The cellular nature of BFA was demonstrated by means of cell separation employing continuous density gradient centrifugation and by freezing and thawing procedures. These data show that BFU-E lack the capacity to respond directly to erythropoietin *in vitro* and may imply that the early differentiation of erythroid progenitor cells is controlled by a local, cellular mechanism. Possibly this distinct cell population is responsible for giving the commitment stimulus to stem cells inducing them to erythropoietin-responsiveness. In addition to the indicative local role of stromal cells on stem cell proliferation, there exists evidence for promoting effects of a thymus hormone (Zipori and Trainin, 1975), of thymus cells (Lord and Schofield, 1973; Manyko et al., 1973) and of factors secreted from activated T-lymphocytes (Cerny, 1974), on stem cell proliferation in bone marrow from normal or thymectomized mice.

Microenvironments can be shut down or reactivated to meet changing demands for blood cell production. For instance, in chronic hemolytic diseases where the life span of red cells is shortened, the human marrow compensates by increased erythropoiesis. Areas of normally atrophic marrow become hemopoietic (Dacie, 1962; Hashimoto, 1962). Also murine marrow fragments, autotransplanted in a subcutaneous site during phenylhydrazine-induced acute hemolysis, regenerate and give rise to a larger hemopoietic nodule than when autotransplanted during normal steady-state hemopoiesis (Tavassoli et al., 1972). Moreover, the compensatory

extramedullary blood production in human liver, lymph nodes, spleen (Jordan, 1942) and paravertebral tissue in some pathological states demonstrates, that hemopoiesis is not confined to specific hemopoietic organs. However, it is not known, whether the ectopic organs, which are temporarily hemopoietic, also possess microenvironments with inductive properties, or simply function as places where progenitor cells can mature.

In contrast, microenvironments can be shut down, as is the case in patients and rabbits with prolonged, severe starvation and patients suffering from anorexia nervosa, where hypoplasia of hemopoietic cells and characteristic gelatinous transformation of marrow have been described (Pearson, 1967; Finkel and Mitus, 1968; Adams, 1970; Tavassoli et al., 1976).

In summary, the overall production of blood cells in scattered microenvironments appears to be regulated by a series of humoral and microenvironmental control systems involving specific feedback control. In addition, the hemopoietic tissue stroma in which hemopoiesis occurs, is probably able to adapt its volume in relation to changing hemopoietic demands. The study of Tavassoli et al. (1972) is suggestive for the fact that the hemopoietic tissue volume is controlled by a direct influence of hemopoietic cells on the tissue stroma.

On this place it has to be noted that a considerable number of experimental hemotologists nowadays are rather critical about the existence of a hemopoietic microenvironment. According to these scientists the size of each progenitor cell compartment is regulated by feedback systems and the commitment is an intrinsic property of the stem cell. One stem cell would develop a scala of surface determinants for all hemopoietic hormones, and the differential pathway, which will be taken by this stem cell, is determined by a competitive action of several hormones (Van Zant and Goldwasser, 1977). However, if erythropoietin was to increase the commitment of pluripotential or multi-committed stem cells toward erythropoiesis, then this would be at the expense of the alternative pathway toward granulopoiesis. The concept of such a "stem cell competition", whether the stem cells are multi-committed or not, is not supported by the experiments of Iscove (1977a) who monitored the response of early committed erythroid and granulocyte/macrophage precursors to manouvres as bleeding and hypertransfusion by measuring their numbers as well as their proliferative activity. The incidence of relatively mature erythroid precursors (CFU-E) showed significant changes after bleeding and hypertransfusion, whereas the responses of more primitive erythroid progenitors (BFU-E) together with the granulocyte/macrophage progenitors (CFU-C) showed contrasting patterns under similar conditions. Moreover, no competitive effect of the addition of erythropoietin or colony stimulating activity *in vitro* on the BFU-E and CFU-C colony incidence was observed. Finally, as judged by colony appearance *in situ*, almost all of the colonies, which formed in the presence of both stimulators, appeared to be either exclusively erythroid or exclusively granulocyte/macrophage.

In conclusion, there exist good reasons to assume that the microenvironment triggers the stem cell to an early committed, i.e. hormone-sensitive, state and that

the stem cell has not an intrinsically determined commitment. However, the available data on this problem do not allow a final judgment concerning the existence of a hemopoietic microenvironment.

Considerations on the microscopical anatomy of hemopoietic tissues

At any attempt to understand the nature of the hemopoietic microenvironments in the bone marrow and the spleen one should consider the structural arrangement of the cells constituting these areas. The microenvironments of the bone marrow and the spleen appear to be so constructed as to allow the entry into and exit from the circulation of stem cells. Moreover, mature cells may enter the blood whereas undifferentiated precursor cells in general do not. The nature of the mechanisms determining the release and trapping of cells in the microenvironment is far from resolved. However, evidence exists that cell-surface structure, stickiness and active and passive motility of hemopoietic cells are involved in these processes. Besides their role in the production of hemopoietic cells, both spleen and bone marrow have an important function in the clearance of damaged cells, particulate matter and antigens from the blood.

Bone marrow

The vascular organisation of the bone marrow is complex and is still not clearly understood. In this organ a venous sinusoidal system is present that communicates with afferent arterioles and empties into veins. In the bone marrow radially directed arterioles branches off from the central longitudinal artery and run toward the periphery of the marrow cavity. The arterioles lie near the center of the hemopoietic cords. These hemopoietic cords consist of large branched reticular cells which form a spongy network whose interstices are filled with hematopoietic cells and free macrophages, embedded in ground substance. An arteriole, its surrounding cord and its draining vascular sinus may be considered to form a "unit" or "lobule" of marrow (Weiss and Chen, 1975). There appears to be a direct continuity between arterial termination and vascular sinusoid (Branemark, 1959) which means that the bone marrow has a closed circulation.

The vascular sinusoids of the marrow consist of a trilaminar wall of lining or littoral cells (endothelial cells), basement membrane and a layer of adventitial cells enclosing a central lumen. The adventitial cell layer is incomplete and the constituting cells, which are reticular cells, may undergo swelling and extend from this layer by which a degree of compartmentalising of the perisinusoidal spaces, i.e. the hematopoietic cords (Weiss, 1965) is produced. The walls of the capillaries and sinusoids are not continuous but have lacunae which may allow relatively free entry and exit of cells (Hudson and Yoffey, 1966). In the course of massive reticulocyte release in anemia or granulocyte release following endotoxin treatment increasing

numbers of apertures develop in these walls (Weiss, 1965). After phlebotomy a marked reduction in the area covered by adventitial cells has been recorded coinciding with the early reticulocyte response to anemia (Tavassoli, 1977).

Within the hemopoietic cords there exists a great deal of order. Megakaryocytes lie against the outside wall of vascular sinuses without the interposition of either adventitial cell or basement membrane and release platelets into the circulation (Wright, 1910). Platelets may be delivered in strips through the apertures in the endothelial lining whereas the strips separate into individual platelets in the lumen of the sinus (Weiss, 1965). Erythroblasts also lie against the outside surface of the sinus wall. They are found in clusters called erythroblastic islets (Bessis, 1958; 1973). These clusters consist of a central macrophage surrounded by one or more tiers of erythroblasts (Le Charpentier and Prenant, 1975). The macrophage extends cytoplasmic processes, which envelope each of the erythroblasts in its islet. Granulocytes develop away from the sinus wall near the middle of the hemotopoietic cords. When they mature they appear to move toward the venous sinuses. In early histological studies of the marrow several authors have distinguished a generative hemopoietic zone in the subendosteal region from a maturational zone adjacent to the sinusoids (Wislocki, 1921; Wienbeck, 1938; Lennert, 1952). The earliest recognizable granulopoietic cells were found predominantly in the subendosteal region, whereas metamyelocytes and mature granulocytes were found mainly in the central region (Wienbeck, 1932; Lennert, 1952). These observations are in good agreement with those of Shackney et al. (1975; 1976), who demonstrated a preferential accumulation of slowly proliferating immediate precursors of mature granulocytes in the central region of the bone marrow, and the studies of Lord et al. (1975) in which the early granuloid committed cells (CFU-C) were found predominantly halfway the radius from the marrow axis to the bone surface. Monocytes, lymphocytes and stem cells (Lord et al., 1975; Shackney et al., 1975; Weiss and Chen, 1975) likely are situated in the marginal parts of the marrow, which are lying close to the bone matrix. These cells are believed to surround the radially directed arterioles forming a periarterial cellular sheath (Weiss, 1970). Fat cells occur adventitial to vascular sinuses and appear to be reticular cells which accumulate fat (Weiss, 1977).

Spleen

The spleen consists of a capsule and trabeculae enclosing the splenic parenchyma. This parenchyma may be divided into two major zones:
(1) the white pulp, which containts mainly lymphoid cells and
(2) the red pulp, in which mainly granulocytes, erythrocytes and thrombocytes and their precursors are found. The white pulp will not be further discussed at this place because this is beyond the scope of this thesis. For detailed information the reader is referred to Van Ewijk (1977). The red pulp contains arterial vessels, sinuses and hemopoietic cords. The arterial vessels branch radially from the central arterioles, which in turn are ramifications of trabecular arteries. Through the latter arteries the

blood enters the splenic pulp from the splenic artery. In contrast to the situation in the bone marrow, it is believed that the spleen possesses both a closed and an open circulation. The closed circulation is similar to that of the bone marrow, where arterioles communicate directly with the lumen of venous sinuses, which in turn empty into veins (Simon and Pictet, 1964). Within the open circulation arterioles open into extravascular spaces, the red pulp cords, whence blood filters back into the vascular bed through gaps in the sinus wall (Weiss, 1963; Thomas, 1967). Sinuses and cords have an architecture, which is similar to that of bone marrow. Also here, the adventitial reticular cells both provide a cellular cover to the cordal surface of the basement membrane and branch into hemopoietic cords contributing to the reticular meshwork of the cords (Weiss, 1965). Free macrophages are present in the cords and are mostly surrounded by erythroblasts. The hemopoietic cells are found in characteristic patterns. Erythroblasts occur in scattered aggregates throughout the red pulp. Immature granulocytes predominantly occur directly under the splenic capsule, along the splenic trabeculae and sometimes in atrophic lymphoid follicles. Megakaryocytes are mainly situated under the splenic capsule and in a minor part throughout the red pulp. It is not fully documented if there exists a similar degree of organization of hemopoietic cells in the splenic cords as was described for the bone marrow cords.

Whereas the integrity of the bone marrow is ensured by the bony shaft, the supporting framework of the spleen consists of a skeleton of fibroblasts, smooth muscle cells, and collagen and elastin fibres, intimately associated with reticular cells.

Concept of the hemopoietic microenvironment in general

Combining these morphological considerations with histochemical and biochemical studies, McCuskey et al. (1972a) proposed a new concept of the hemopoietic organ stroma. According to these authors, the hemopoietic microenvironment is morphologically composed of:
(1) a microvascular compartment,
(2) a connective tissue compartment and
(3) neural elements associated with both the blood vessels and the stroma.
The microvascular compartment is made up of arterioles, capillaries, sinusoids and venules and is responsible for regulating all that enters and leaves the compartment as well as tissue pH and oxygenation. Dilatation or constriction of the vessels, which may or may not be mediated by neural control, may create local conditions, which influence the metabolic behaviour of the connective tissue compartment. The latter compartment is composed of cells and the extracellular products, that arise from these cells, i.e. fibers and ground substances.

Because of the paucity of available information, one can only speculate on the nature of the stromal elements that affect hemopoietic cells in their proliferation

and differentiation. These stromal elements could be a constituent of the microvasculature, e.g. adventitial cells and endothelial cells, or could belong to the connective tissue compartment or both. Therefore, in the following paragraphs of this chapter the stromal components will be separately discussed in relation to their possible role in hemopoietic regulation.

The microvascular compartment

Experimental evidence exists, that in hemopoietic tissues the presence of a sinusoidal microcirculation is essential for hemopoiesis. Tavassoli and Crosby (1968) observed survival of marrow tissue fragments when these were autotransplanted to various extramedullary sites. Hemopoietic cells disappear from these implants within 48 hours. Soon capillaries penetrate the implant from the surrounding tissue and establish an intense circulation throughout the implant. Surviving reticular cells, however, proliferate and reconstruct the marrow's sinusoidal microcirculation (Tavassoli and Crosby, 1968; Tavassoli and Weiss, 1971). The surviving stromal cell population, which consists probably out of reticular cells, appears to be distinct from, and does not differentiate into, hemopoietic cells (Fried et al., 1973b). Hemopoietic repopulation of the marrow implants occurs only after its sinusoidal microcirculation has been re-established. Evidence for the origin of the hemopoietic cells in these implants is not conclusive. Several investigators studied the hemopoietic cells repopulating an implanted marrow and concluded that they were either cells immigrating from other hemopoietic sites (Friedenstein et al., 1968), or derived from both the host animal and cells in the implanted marrow plug or femur (Amsel and Dell, 1971; Fried et 1973b; Brecher et al., 1976).

The experiments of Knospe et al. (1966; 1968; 1972) and Maniatis et al. (1971) confirmed the former statement that the establishment of a sinusoidal microcirculation is a prerequisite for hemopoiesis. These investigators demonstrated that high doses of local irradiation (4000 rads to rat tibia) cause (1) an immediate localized aplasia, which is the result of destruction of hemopoietic cells and followed by (2) complete repair at two weeks. However, after two months a permanent aplasia followed as a result of a destroyed microcirculation. When two weeks after local irradiation bits of marrow from both irradiated and unirradiated tibiae were autotransplanted side by side to subcutaneous tissue, the marrow from the unirradiated tibia survived and reconstructed the sinusoidal microcirculation, whereas the marrow from the irradiated tibia did not survive. Thus, two weeks after high doses of local radiation, although the marrow is repopulated with normal hempoietic cells, the supporting sinusoidal structures have an insufficient repair potential. In this context the observation is interesting that human endothelial cells failed to replicate *in vitro* after 1000 rad irradiation (DeGowin et al., 1976), since these cells are an important component of the microvasculature. Unlike hemopoietic stem cells, sinusoidal cells are fixed tissue cells; they cannot migrate from other parts of the body to repair the damaged areas. A comparable damage of the hemopoietic organ stroma as is due to

950 or 1000 rads X-irradiation (Maloney and Patt, 1972; Fried et al., 1973a; Chamberlin et al., 1974; Kedo et al., 1976) may occur after cyclosphosphamide treatment and persists even after apparent hemopoietic recovery occurs (Tavassoli, 1975; Fried et al., 1976). In these studies the hemopoietic organ stroma appeared to be more resistent to X-irradiation or cyclophosphamide treatment than the hemopoietic stem cell, but, in contrast to stem cells, the organ stroma recovers more slowly or not at all from the induced damage. Werts et al. (1977), in discussing their experiments on local irradiation of mouse femurs with doses up to 10,000 rad, doubted whether sinusoidal destruction was directly causing the permanent aplasia noted by Knospe et al. (1966) and the hypocellularity noted by Maloney and Patt (1972) under comparable circumstances as in their own experiments. Werts and coworkers suggested, that initially the damage to stromal cells (i.e. adherent cells) created a microenvironment that was not supportive for a normal level of hemopoiesis. This suggestion was based upon their observations, that the survival and replication of bone marrow adherent cells *in vitro*, similar to the fibroblastic plaque cells initially described by Friedenstein et al. (1974a) and Wilson et al. (1974), was severely depressed after 5,000 - 10,000 rad irradiation and was comparable with a decrease in nucleated cell counts, ^{59}Fe uptake and erythroblasts counts in the irradiated femurs at the same time. Although it may be tempting to consider these fibroblasts as representatives of the microenvironmental influence on hemopoiesis (see Friedenstein et al. 1974b and Werst et al., 1977) these cells may be *in vitro* representatives of reticular cells, which constitute an important part of the sinusoidal lining in the femoral marrow.

The notion of the importance of the unique microcirculation in bone marrow and spleen for hemopoiesis has great value in the knowledge of the pathogenesis of marrow aplasia which follows the use of chemotherapeutic agents for acute leukemiae. Tavassoli (1974) suggested that the expansion of the leukemic cell population not only displaces the normal hemopoietic cells, but also destroys the microcirculation of the marrow. Thus, even when chemotherapy has eliminated the leukemic cell population, the marrow may be not capable of supporting hemopoiesis and a period of aplasia results. In acute granulocytic leukemia, at least, injury to the microcirculation was established by Chen et al. (1972).

There are several indications that the microvasculature of the hemopoietic organs is not a fixed structure but that dynamic changes in the architecture and types of vessels occur (Brånemark, 1959; Van Dyke and Harris, 1969; McClugage et al., 1971; Weiss and Chen, 1975). Using a chamber, that was implanted in a rabbit tibia, the group of McCuskey (McCuskey et al., 1971b, 1971c; McClugage and McCuskey, 1970) was able to examine the bone marrow in situ with direct microscopy, whereas the optical images could also be televised and cinerecorded. Ten weeks after the chamber implantation, regeneration of bone marrow, that had entered the gaps in the chamber from the surrounding pre-existing marrow, was completed. At this time the chamber contained: (1) osteoid and cancellous bone, (2) hypocellular marrow spaces, and (3) large sinusoids and venules located within the marrow spaces. However, after bleeding of the host or erythropoietin administration the large

venules and venous sinusoids were replaced by dilated or polygonal networks of sinusoids with a rapid blood flow, supplied by small arterioles or capillaries that run from surrounding spicules of bone into the marrow spaces. An increased cellularity (hemopoietic and fat cells) was associated with this change in vascularity. The authors suggested that the increase in arterioles and capillaries was caused by increased circulating levels of erythropoietin and that the developing red cell precursors must have adequate oxygenation to respond maximally to erythropoietin.

In agreement with the above observations are the studies of Michelsen (1967; 1968; 1969) who reported that marrow with active hemopoiesis always had a higher rate of blood flow. He suggested that differences in pressure and resistance during different periods of activity of bone marrow may be due to changes in the vascular architecture, since vascular resistance decreased with increased hemopoietic demand. This could be a result of vascular neoformation or dilatation of existing vessels, or both.

The connective tissue compartment

The supporting framework of the spleen consists of reticular and collagenic fibers of the trabeculae and the capsule. The framework of the bone marrow is formed by the bony shaft and the bone trabeculae, between which the hemopoietic tissue is located. The hemopoietic tissue between the supporting framework consists mainly of sinusoids that intertwine the hemopoietic cords. These cords show a vast, delicate network of starlike reticular cells having long, irregular protoplasmic processes running in all directions and uniting one cell with another. Some reticular fibers occur between these cells. Occasionally small arteries or veins are seen in the cords. The connective tissue associated with the small arteries and veins, as well as the wall of the vessels themselves, provide a basic, tough weak, framework for the hemopoietic tissue. The meshes in the reticular network are filled with hemopoietic cells and further with macrophages, fibroblasts and mesenchymal cells, which are, together with reticular cells, embedded in ground substance. There is some reason to believe that these different stromal cell types do not occur at random in hemopoietic organs. According to Trentin (1977) macrophages predominate in the splenic sites at which erythrocytic colonies occur after X-irradiation and reconstitution, whereas another stromal cell type occurs mainly in granulocytic colonies. To avoid any confusion on the terminology of these connective tissue cell types, some characteristics of these cells – as have been proposed by Weiss and Chen (1975) – will be given in the following.

(1) Reticular cells are branched cells, fibroblastic in character, which are associated with reticular fibers. They show minor phagocytosis and are contractile. When reticular cells are localized at the outside surface of the vascular endothelium they are called adventitial cells. The adventitial cells are capable of becoming fatty. Reticular fibers are a combination of collagen and mucopolysaccharides, produced by the reticular cells.

(2) Fibroblasts closely resemble reticular cells and tend to produce more collagen and less mucopolysaccharides than reticular cells. Especially the young fibroblasts are contractile cells.

(3) Macrophages are primarily defined on the basis of their content of a great number of lysosomes and their active endocytosis. They can form foreign body giant cells or osteoclasts. Macrophages secrete collagenase and a spectrum of regulatory molecules. The macrophage precursor is the monocyte, which originates from the bone marrow, is able to circulate in the body and becomes a macrophage when it starts phagocytosis. In contrast to fibroblastic cells as fibroblasts and reticular cells, the macrophages belong to the mononuclear phagocyte system (Van Furth et al., 1972).

Vascular sinuses appear very early in developing bone marrow. Chen and Weiss (1975) observed in human fetuses that soon after the vascular sinuses can be observed, macrophages and reticular cells appear in the perisinal connective tissue lying between the venous sinuses and calcified cartilage. "Presumed stem cells" then appear and this connective tissue becomes the hemopoietic marrow.

Reticular cells

It has been postulated by Weiss (1977) from morphological observations that reticular cells are able to trap and induce the differentiation of hemopoietic stem cells and sort the differentiating hemopoietic cells into characteristic locations in their spongy network. This postulation was based upon the observed morphological association of "apparent stem cells" and reticular cells in human fetuses (Chen and Weiss, 1975). Moreover, Weiss (1977) observed with transmission and scanning electron microscopy frequent association between reticular cells and developing eosinophilic granulocytes in rats with an eosinophilic granulocytosis induced by injection of larvae of *Trichinella spirales*. Further it has been suggested that in lymphoid organs there are cells, which closely resemble reticular cells and determine the homing and detainment of lymphocytes at their cell surface (Veerman and Van Ewijk, 1975; Friesz, 1976; Van Ewijk, 1977). This reticular cell type is the "interdigitating cell", which occurs in the thymus-dependent compartments of several lymphoid organs (Kaiserling and Lennert, 1974; Kaiserling et al., 1974; Hoefsmit, 1974; Veerman, 1974; Hoefsmit and Gerver, 1975; Mueller-Hermelink et al., 1976; Friesz, 1976) and the "dendritic cell", occurring in the peripheral part of lymphoid germinal centers (Nossal and Ada, 1971; Abe and Ito, 1973; Van Rooyen, 1973; Hoefsmit, 1974; Van Ewijk, 1977). According to Friess (1976) the interdigitating cell may trigger blast transformation and mitosis in the surrounding lymphocytes by means of the secretion of asialoglycoproteins. Veerman and Van Ewijk (1975) tentatively suggested that the dendritic cell contributes to the B lymphocyte microenvironment involved in antigendependent B cell proliferation.

Although the postulation of Weiss and the other suggestions may be speculative, it is probable that reticular cells have important functions in hemopoietic organs.

Caffrey et al. (1966) showed that reticular cells did not participate in the production of hemopoietic stem cells and were not stimulated to proliferate after sublethal irradiation. These authors could not observe any reduction in intensity or incidence of the label in reticular cells one year after all reticular cells had been labeled by repeated tritiated thymidine treatment. These cells fulfil the criteria of "fixed" cells showing minimum proliferation and considerable radioresistance and may therefore represent a stable population within a microenvironment which is engaged in intimate interaction with hemopoietic stem cells regulating differentiation and proliferation.

As indicated before, the reticular cells form a reticular meshwork of the hemopoietic cords and they presumably produce reticular fibers with which they are associated. As reticular cells are fibroblastic cells, they have well developed cytoplasmic filaments, as have the endothelial cells of the vascular sinuses, and therefore are likely contractile cells (Chen and Weiss, 1972; 1973; Weiss and Chen, 1975). By their contraction they may change the contour and dimensions of the hemopoietic cords, perhaps squeezing hemopoietic cells toward the sinus wall aiding their passage across the sinus wall into the circulation. This latter function could be preferably exerted by the reticular cells that form an adventitial cover for the sinuses. Tavassoli (1977) supposed, that these adventitial cells may be passively involved in hemopoietic cell delivery to the sinuses as he observed a marked reduction in the sinus wall area covered by adventitial cells coinciding with the early reticulocyte response to phlebotomy. From the study of Chamberlain et al. (1975) and Leblond et al. (1975) it appeared that such a reduction of the adventitial cell cover may be due to an early direct effect of erythropoietin. The progressive incompleteness of the adventitial layer of the sinus wall would then permit the reticulocytes to gain acces to more enthelial surface and the pores. The lifting of the adventitial cell processes from the abluminal surface of the endothelial layer was supposed to be the result of contraction of microfilaments in the reticular cells. Although it is indicated that erythropoietin possibly is capable to exert a direct effect on the splenic microvasculature, the effect could also be an indirect one, that is, mediated by the release of a vasoactive metabolite from erythropoietin sensitive hemopoietic cells such as suggested by McCuskey et al. (1973). The release of the granulocytes from the marrow in response to the leukopenia induced by endotoxin occurs also concomitantly with a marked reduction of the adventitial cover of the sinuses (Weiss, 1970).

The reticular cells may vary in volume, being gelatinous or fatty, thereby controlling the volume for hemopoiesis (Weiss and Chen, 1975; Tavassoli, 1977). When the increase in volume is large-scale, the marrow turns white (gelatinous) or yellow (fatty). It has to be noticed, however, that the gelatinous transformation of bone marrow is mainly represented by large extracellular deposits of a gelatinous substance, probably mucopolysaccharides (Tavassoli et al., 1976). It has been suggested that the development of fat spaces originating from the fusion of reticular cells was a prerequisite to formation of hemopoietic cells. Tavassoli et al. (1977) advanced the hypothesis that the composition of lipid in the adipose cells of the human bone

marrow (Tavassoli, 1976) may determine the ability and the extent of the marrow contained in different bones to respond to the expansion of blood forming elements under physiologic circumstances. These authors found more myristate and palmitate in the red marrow than in the yellow marrow, which is rich in the mono-unsaturated analogues of these fatty acids. It appeared that lipid in fat cells of red marrow is more easily resorbed than in yellow marrow during starvation (Cohen and Garner, 1965) and that a phenylhydrazine-induced hemolysis leads to resorption of lipid contained in fat cells of red marrow whereas the yellow marrow is relatively stable (Tavassoli et al., 1972; 1976). Moreover, the latter authors pointed at the possible direct role of the marrow lipid structure in the regulation of hemopoietic cell proliferation as these substances are suggested to be bioregulators of cell growth and proliferation (Holley et al., 1974; Inbar and Schinitzky, 1974; Leder and Leder, 1975).

Macrophages

In addition to reticular cells, the hemopoietic cords contain also a number of other connective tissue cell types. An important connective tissue cell type is the macrophage. Its precursor originates in the bone marrow as the promonocyte, which differentiates into a monocyte (Van Furth and Cohn, 1968). The monocytes can be released to the blood. They may undergo maturation into macrophages, among which are the Kupffer cells of hepatic sinusoids (Volkman and Gowans, 1963; Kinsky et al., 1969; Warr and Sljivić, 1974), although in unstimulated animals the resident macrophages probably are self-sustaining (Volkman, 1976). Macrophages are increasingly being recognized as multifunctional cells. They are secretory as well as phagocytic. They have a capacity to elaborate a spectrum of regulatory molecules, which have mutually antagonistic actions on hemopoiesis (Calderon and Unanue, 1975; Moore, 1976) and other eukaryote cell systems in general (Keller et al., 1976). For instance, the immune response needs cooperation between lymphocytes and physically intact "accessory cells" (macrophages), which produce high molecular weight factors promoting proliferation of immunocompetent cells (Calderon et al., 1975; Ellner et al., 1975; Lipsky et al., 1976). Another example of the regulatory properties of macrophages derives from the study of Kurland et al. (1977). These authors indicated that mononuclear phagocytes may both have a positive (by means of colony stimulating factor elaboration) and a negative (by means of Prostaglandin E elaboration) feedback control of committed granulocyte/macrophage progenitor cell proliferation.

Erythroblasts are aggregated around central macrophages in bone marrow (Bessis and Breton-Gorius (1962), spleen (Orlic et al., 1965) and yolk sac (Sorenson, 1961) and presumably in fetal liver (Naito and Wisse, 1977); a concomitant development of erythropoiesis and phagocytic properties of fetal liver macrophages has earlier been noticed (Zamboni, 1965), although also an intimate contact between liver parenchymal cells and developing hemopoietic cells was seen (Fukuda, 1974). Frequently more than one tier of cells surrounds the macrophage with the most

differentiated cells at the outside (Le Charpentier and Prenant, 1975). The association between these two cell types is very close, with erythroblasts almost completely surrounded by macrophage cytoplasmic processes (Bessis and Breton-Gorius, 1962). Erythrophagocytosis occurs frequently and is probably related to the macrophage property to recognize effete, damaged or abnormal erythroid cells (Cavallin-Stahl et al., 1974). In this sense macrophages may have a surveillance function with respect to the hemopoietic system.

The central macrophages probably have an important but as yet undefined role in supporting or sustaining hemopoiesis. It has been suggested that this cell has trophic functions, transferring ferritin to the developing erythroblasts, but it seems probable that still earlier erythroid precursors have a relationship with these central macrophages. In the polycythemic mouse spleen, in which no erythroblasts occur, proerythroblast islands were seen around a central macrophage within 12-24 hours after erythropoietin treatment (Orlic et al., 1965). Thus, since the proerythroblastic islands developed so rapidly, presumably the erythropoietin-sensitive precursor cells are also organized in clusters around a central macrophage. After sublethal irradiation lodgment of "lymphoid cells" on bone marrow central macrophages in depleted islets was observed by Ben-Ishay and Yoffey (1974) in an electron microscopic study. These authors described a subsequent differentiation of these "lymphoid cells' toward the erythroid compartment and speculated about the possible inductive properties of central macrophages as had been done before by Curry and Trentin, (1967). However, an inductive function of central macrophages is rendered less likely by the observations of Knospe et al. (1968) and Maniatis et al. (1971) who described a normal hemopoietic regeneration in rat femors following 4000-6000 r local irradiation preceding a secundary aplasia two months later. Though macrophages are generally a slowly proliferating and relatively radioresistant population one should not expect that they would be intact following such high doses of irradiation. When it is assumed that macrophages do not survive high doses of irradiation, then obviously another stromal cell type with inductive properties would have led to the observed hemopoietic regeneration. The latter authors concluded, that the secundary aplasia two months after irradiation was presumably due to damage to a population of stromal cells with a very slow turnover, which were not replaced by circulating cells (which may be macrophages or their precursors), since only local injection of bone marrow could prevent the secundary aplasia and loss of sinusoidal structure. However, this presumption ignores an alternative consideration, which is based upon other observations. That is, it remains to be seen whether the secundary aplasia after exposures in the 1000 rad range is due to the loss of specific inductive or regulatory functions of fixed stromal components of the microenvironment, as has been suggested by Knospe et al. (1968), or that it merely reflects sinusoidal damage. As the evolving hypocellularity after irradiation is associated with diminished blood flow (Van Dyke and Harris, 1969; McClugage et al., 1971; Patt and Maloney, 1975), an environment could be created, that is possibly inappropriate to establish hemopoiesis at all. The effect of local bone marrow injections at the damaged sites could

also be explained as a deposition of undamaged reticuloendothelial cells, which are capable to restore the microcirculation. Thus, the local irradiation experiments of Knospe et al. (1968) evidently do not exclude an inductive function of macrophages. Moreover, as to the radiosensitivity of macrophages, graded doses of X-irradiation up to 10.000 r had no effect on the uptake and little effect on the catabolism of some proteins by mouse peritoneal macrophages in vitro (Schmidtke and Dixon, 1972), which indicates that macrophages may survive high doses of irradiation. Additionally support for the notion that macrophages are relatively radioresistant was delivered by studies on mitogen-induced activation of thymus-derived lymphocytes (Rosenstreich et al., 1976). It was shown, that this lymphocyte activation depends upon the presence of macrophages ("accessory cells") and that these accessory cells remain functionally active even at irradiation doses of 2500 r or higher.

Although it is at present unknown whether macrophages have inductive properties, there is considerable evidence to indicate that macrophages may be particularly important in facilitating interaction between cells of identical or different populations. Macrophages are required for an immune response *in vitro* to sheep red cells, a response requiring interaction of thymus-derived antigen-sensitive cells and bone marrow-derived antibody-forming precursor cells (Shortman et al., 1970). It has been suggested by Metcalf and Moore (1971) that the macrophages in this system release an aggregation factor, since Dutton (1969) observed that the macrophage requirement in such a system could be replaced by media conditioned by macrophages. In this context the role of macrophages in general hemopoietic homeostasis may be considered to operate by facilitating close interaction between stem cells and/or progenitor cells with the inducing cell component of the hemopoietic microenvironment. Alternatively they may facilitate close interaction or aggregation between cells at the same stage of differentiation. Such an interaction would permit a specific control of population size by either stimulatory or inhibitory signals (Metcalf and Moore, 1971).

The macrophage capacity to recognize cell membrane abnormalities, which may be due to damage, ageing or mutation and thus to specifically phagocytize or detain certain cell types could also serve as a mechanism which regulates cell traffic to and from the hemopoietic microenvironment, i.e. the macrophage population could serve as a trap or sieve for circulating cells and possibly also for stem cells.

In vitro adherent stromal cells

In an attempt to separate and characterize the fixed, non-circulating cells of the organ stroma, Friedenstein et al. (1970; 1974a; 1976), Luria et al. (1972), Wilson et al. (1974) and Wilson and O'Grady (1976) observed that the adherent stromal cells from bone marrow, spleen, thymus and lymph nodes form *in vitro* fibroblastic plaques. After transplantation of such *in vitro* cloned autochthonous fibroblasts under the kidney capsule, Friedenstein et al. (1974b) observed development of bone and bone marrow or a typical splenic or lymphoid organ structure at the

injection site, depending upon the source characteristics of injected fibroblasts. This indicates that the fibroblasts are stromal cell precursors and are responsible for the transfer of hemopoietic microenvironment. An alternative explanation, which was not considered by these authors, is that these fibroblasts are creating the basal circumstances (e.g. microcirculation) in which the cells, that have inductive and regulatory properties, can lodge. However, it is not clear if fibroblasts function *in vivo* as stromal precursors after local injury of the hemopoietic organ stroma by irradiation or mechanical depletion. Possibly mesenchymal cells, apparently derived from nearby enlarging Haversion canals in depleted bone marrow, are the source of the regenerating organ stroma (Röhlich, 1941; Patt and Maloney, 1975). The evidence for an early influx of cells from the Haversion system was provided by autoradiographic analysis after tritiated thymidine labeling (Patt and Maloney, 1970). Using a similar marrow depletion technique, Meyer-Hamme et al. (1971) found no evidence of stromal cell migration from neighbouring marrow to the sites where the marrow was removed. Contradictory to the observations of Maloney and Patt (1968) and Patt and Maloney (1975), Brecher et al. (1976) concluded that (1) hemopoietic stem cells or (2) putative mesenchymal common precursors of fibroblasts, osteoblasts and hemopoietic progenitor cells do not reside in the Haversion system and play no role in the repopulation of mechanically depleted femurs. Taken together, in the *in vivo* situation only contradictory evidence exists concerning the role of fibroblasts of mesenchymal fibroblast precursors in the regeneration of locally damaged hemopoietic organ stroma.

The fibroblasts may be considered to represent a non-migrating cell type (Werts, Johnson and McGowin, 1977), but their precursors can be migratory. Hentel and Hirschhorn (1971) have shown that hemopoietic cells can give rise to fibroblasts by using a cytogenetic analysis of fibroblasts arising in cultures of bone marrow bearing the Ph-1, 1-3 translocation of C group trisomy markers. The incidence of these cytogenetic markers in the fibroblasts varied between the extremes of 0 and 100%. However, hemopoietic cells do not contribute as precursors to the regeneration of the organ stroma of marrow implants (Maniatis et al., 1971), although they may interact with stromal cells to stimulate the regeneration of the supporting stroma in autotransplanted marrow fragments in subcutaneous sites (Tavassoli et al., 1972).

Although fibroblasts may be considered by some authors to be stromal cell precursors *in vivo*, there is still no indication that these cells are able to promote stem cell proliferation, commitment and differentiation *in vitro*. Cultivation of bone marrow cells on a previously grown bed of fibroblast-like cells of bone marrow origin had no significant effect on the preservation of the hemopoietic stem cells or the dynamics of the change in their number, when compared to cultures of bone marrow cells in the absence of such fibroblast-like cells (Lubennikova and Domaratskaya, 1976). However, stem cell maintanance for several months *in vitro* has been described by Dexter and Testa (1976) and Dexter et al. (1977). Also in these experiments, bone marrow cells were allowed to establish an adherent population. No

fibroblasts were noted but three attached populations became apparent: (1) phagocytic mononuclear cells, (2) attached "epithelioid" cells and (3) aggregation of epithelial cells swollen to enormous proportions by the presence of numerous lipid-containing vacuoles. Later observations led to the conclusion that also mesenchymal cells were present and that the "epithelioid" cells probably represented endothelial and reticular cells (Dexter, 1977). Inoculation of freshly isolated syngeneic bone marrow cells on this established (but not confluent) adherent layer resulted in continued proliferation of cells (granulocytes and macrophages, released into the suspension) and continued production of stem cells. It was demonstrated that the presence of fat-containing aggregations was necessary for continued granulopoiesis and that cultures in full granulocyte production showed a characteristic clumping of granulocytes around these aggergates (Allen and Dexter, 1976). Moreover, pinocytotic vesicles and gap junctions were observed between the adjacent membranes of undifferentiated granulocytes and the epithelial cells themselves. It was concluded that the composite layer of three cell types is necessary for extended stem cell proliferation and granulopoiesis and may supply a necessary *in vitro* microenvironment for their proliferation and differentiation. It should be noticed that *in vivo* stem cell proliferation and maintenance is thought to be regulated by microenvironmental conditions.

The influence of the adherent cell layer on the maintenance of stem cells *in vitro* was clearly demonstrated by Dexter and colleagues (1977a) and Dexter and Moore (1977) using a model in which the maintenance of stem cells from SL/SL^d or W/W^v mice on an adherent stromal cell population from the same mice was examined. In both mouse strains a genetic determined anemia is present. However, in SL/SL^d mice as was discussed before, microeinvironmental defects are considered to be the major determinant of the anemia, whereas in W/W^v mice the stem cell compartment is defective. The anemia of the latter mice can be cured by transplantation of stem cells from SL/SL^d mice. It appeared that stem cell maintenance *in vitro* was severely reduced except when SL/SL^d stem cells were grown on an adherent stromal cell layer from W/W^v mice and as such supported *in vivo* observations.

Examinations of the interactions between isolated stromal cells from hemopoietic tissues and hemopoietic cells *in vitro* probably will resolve in the nearby future many questions regarding the existence and regulatory mechanisms of the microenvironmental control of hemopoiesis *in vivo.*

Intercellular groundsubstance

Within the connective tissue compartment the cells are embedded in a matrix of fibers and groundsubstance. This groundsubstance contains fluid, which is bound by proteins, mucopolysaccharides (glycosaminoglycans) and complexes of these substances (Laurent, 1977). The groundsubstance permits the transit of hormones, electrolytes and metabolites to and from the cells and plays an important role in regulating the transport of high-molecular weight material (Laurent et al., 1963).

There is increasing evidence that the composition of mucopolysaccharides influ-

ences hemopoietic proliferation and differentiation. McCuskey et al. (1971b; 1972a; 1972b) demonstrated histochemically specific alterations in the microvascular and connective tissue compartments of the hemopoietic microenvironment of the murine spleen and bone marrow during erythropoietic regeneration, suppression, or stimulation. As a result of these studies these authors proposed that hypoxia of hemopoietic tissue initiates a change in the composition of mucopolysaccharides present in the organ. In the murine spleen this results in a large increase in sulphated acid mucopolysaccharides. Such an environment favours mitotic activity of undifferentiated stem cells of both the erythroid and granuloid line. According to the authors reasoning, in the anemic animal there is a concomitant release of erythropoietin from the hypoxic kidney, which initiates vasodilatation resulting in increased blood flow and improved oxygenation. This vasodilation was suggested to result from the release of a metabolite, which may be adenosine. With the resulting increase in pO_2 (and a possible change in pH toward a higher value) there is a change in the production of mucopolysaccharides toward neutral types. Such an environment is favorable for erythropoiesis and permits erythroid committed cells to differentiate fully and mature. In the polycythemic animal, blood flow and tissue pO_2 in hemopoietic organs are low since the erythropoietin release is suppressed. The concentration of acid mucopolysaccharides remains elevated resulting in an environment in which granuloid cells differentiate.

Further support for this hypothesis has been obtained from studies of SL/SL^d and W/W^v genetically anemic mice (McCuskey and Meineke, 1973) using *in vivo* microscopic and histochemical methods. The observations suggested that a cell coating of sulfated acid mucopolysaccharides is required for early erythroid differentiation but that a microenvironment which contains excessive concentrations of sulfated acid mucopolysaccharides is not conductive to support the later stages of erythroid development. Schrock et al. (1973) demonstrated in a biochemical analysis of the spleen that when erythropoiesis is suppressed by hypertransfusion there is a significant increase in the concentration of two acid mucopolysaccharides, hyaluronic acid and chondroitin sulphate. In a biochemical study of spleens of mice with Friend leukemia, which have an erythroblastosis, Seno et al. (1974) reported also shifts in the mucopolysaccharide pattern in comparison to normal spleen. Whereas the spleen of normal mice and rats contain mainly chondroitin sulphates A, B and E, heparan sulphate and hyaluronic acid, the mucopolysaccharides of the leukemic mouse spleens were different from those of normal mice in the following aspects:
(a) The content of glucosamine-containing mucopolysaccharides, hyaluronic acid and heparan sulphate, was higher and
(b) chondroitin sulphate B was absent or present in only a trace amount. Possibly, the microenvironment reacts upon the differentiation stop in erythropoiesis in the leukemic mouse by decreasing the chondroitin sulphate B amount. These low concentrations of a sulphated acid mucopolysaccharide compound favour erythroid differentiation but the virus-infected cells have an intrinsic defect, which does not permit them to differentiate.

In patients with prolonged, severe self-induced starvation and also in rabbits with an experimentally-induced starvation, hypoplasia of hemopoietic cells and gelatinous transformation of marrow were noted (Tavassoli et al., 1976). Histochemical and ultrastructural studies indicated that the gelatinous substance consisted of acid mucopolysacchrides and was extracellular in nature (Pearson, 1967; Finkel and Mitus, 1968; Mant and Faragher, 1972; Tavassoli et al., 1976). Intracellular deposits of mucopolysaccharides, as are seen in the several mucopolysaccharidoses in humans, are not known to effect hemopoiesis (McKusick, 1972).

Mucopolysaccharides are not only found in hemopoietic organs but also in a large number of other tissues (Mathews, 1975) and are associated with the cell surface in plants, invertebrates and vertebrates (Lippman, 1965; Kraemer, 1971; Kojima and Yamegata, 1971). Cell surfaces may carry specific receptors for mucopolysaccharides or for the uptake of mucopolysaccharides (Pessac and Defendi, 1972; Wasteson et al., 1973). Mucopolysaccharides are believed to play an important role in developmental processes (Kondo et al., 1971; Sugiyama, 1972; Kosher and Searls, 1973; Höglund, 1976), and in cellular proliferation and differentiation in general. The mechanism with which they regulate cell proliferation and differentiation is unknown, although many speculations have been made in this respect. Lippman (1965) proposed a role of acid mucopolysaccharides at the cell surface in blocking and triggering cells for prophase events. The sulphate: carboxyl ratios of the mucopolysaccharide molecule, its special orientation and the relative concentration of particular available counterions would determine the net regulatory effect. According to Lippman (1965) Ca^{++} ions have to be preferably considered as counterions that can be released from the mucopolysaccharide-calcium complexing at the cell surface, to induce cytoplasmic and nuclear events that initiate prophase. The stimulating effect of calcium on the mitotic activity of bone marrow cells was postulated by Hunt and Perris (1973). Saiga and Kinoshita (1976) proposed, that mucopolysaccharides can induce the structural loosening and relaxation of template restriction in chromatin by binding directly to chromatin. Finally, the presumptive inhibitory action, that sulphated acid mucopolysaccharides exert in relation to erythropoiesis, may be related to the hypothesized adenyl cyclase-linked mechanisms, which may be involved in the modulation of the action of erythropoietin on erythropoietic progenitors *in vitro* (Brown and Adamson, 1977). An interaction of mucopolysaccharides with surface receptors on erythroid progenitors, differing operationally from erythropoietin-dependent colony forming cells, could then modulate the adenyl cyclase activation of these cells, resulting in an alteration of erythropoiesis.

In addition to a tentative direct regulatory role of mucopolysaccharides in hemopoiesis, evidence exists, that these substances may promote aggregation of several cell types *in vitro* (Pessac and Defendi, 1972). These authors speculated that aggregation factors even may be similar to mucopolysaccharides. It has been well established *in vivo* (Meijer et al., 1956) that mucopolysaccharides are present in the intercellular material and that in fact many cells are covered with a mucopolysac-

charide coat (Ito, 1965; Rambourg and Leblond, 1967). The surface coats of some ascites and polyoma-transformed cells, of cells transformed by adenovirus 12 and by simian virus 40, and of sponteneously transformed cells (Gasic and Baydak, 1962; Defendi and Gasic, 1963; Martinez-Palomo et al., 1969) have been shown to be thicker than those of the control cells. It is assumed that this material consists partly of mucopolysaccharides. Moreover, chicken cells, after transformation by Rous sarcoma virus, release more mucopolysaccharides than previously (Erichsen et al., 1961; Temin, 1965). These observations agree with the fact, that when malignant cells are tested for aggregation patterns, they give larger aggregates than control cells (Pejsachowicz et al., 1964; Kuroda, 1968). Thus cell-to-cell binding by mucopolysaccharides may regulate *in vitro* density-dependent inhibition of growth or may promote *in vivo* aggregation between cells at the same stage of differentiation, which permits the cells to exchange regulatory signals critical to their proliferation and differentiation. As discussed before, macrophages are supposed to release aggregation factors (Metcalf and Moore, 1971) and may in this context be considered as cells, which regulate the interaction between cells at the same stage of differentiation or between stem cells and/or progenitor cells and the hemopoietic microenvironment.

The neural compartment

The results of the investigations of McCuskey and colleagues suggest that the development of a mature neurovascular complex in bone marrow is critical to the establishment of a microenvironment within which hemopoiesis can occur (Miller and McCuskey, 1973). The neural influence is probably mediated through vasomotor activity (Foa, 1943a, 1943b; Miller and McCuskey, 1973). Concomitant with the development of a neurovasculature complex in bone marrow of neonatal rabbits scattered foci of early erythroblasts were seen and the marrow contained a high concentration of sulphated acid mucopolysaccharides. These results suggested that the formation of a competent neurovasculature in the marrow influenced the microenvironment to make it favorable for the initiation of hemopoiesis. It was noticed before that a microenvironment containing a high concentration of sulphated acid mucopolysaccharides appears to be a prerequisite for mitotic activity and differentiation of hemopoietic stem cells into early differentiated cells. The above suggestion is in agreement with the studies of Calvo and Forteza-Vilã (1969; 1970) who reported that the presence of Schwann cells and the myelination of nerves in the early neonatal animal may have some relation to the beginning of hemopoietic responsiveness. Garcia and Van Dyke (1961) found that newborn rats do not respond hemopoietically to hormonal stimulation prior to the 15th day after birth and Lucarelli et al. (1968) showed that erythrocyte production is not affected by nephrectomy, starvation or hypoxia during the first two weeks of life, which is in contrast with the response in adult animals.

Although Calvo (1968) and Calvo and Forteza-Vila (1969; 1970) suggested a

direct relationship between the nerve fibers and the hemopoietic cellular elements in adult mammals, Miller and McCuskey (1973) could not find support for their findings.

Halvorsen (1966) reported changes in reticulocyte counts, red cell mass and – although not consistent – in erythropoietin levels in adult rabbits after electrical stimulation of the hypothalamus, procedures in which lesions were introduced in the hypothalamic region followed by short-term hypoxia and after administration of pituitary and target hormone. He concluded that the central nervous system probably participated in the regulation of erythropoiesis. The clinical study of Gilbert and Silverstein (1965) indicated that this could be also valid for men. Stimulation of the lumbar portion of the sympathetic trunk resulted in a reduction of reticulocytes in the marrow and an increase in the blood of rats (Webber et al., 1970). From the studies of Halvorsen (1966) and Mirand et al. (1964) several possibilities emerge concerning the mechanism by which the central nervous system exerts its influence. The central nervous system could affect erythropoiesis through the pituitary gland and probably mediated by corticosteroids, through the autonomic nervous system or through an direct influence on erythropoietin production. The latter possibility was rendered less likely by the experiments of Feldman et al. (1966), who were unable to demonstrate an erythropoietin-like substance in the sera of rats that received an electrical stimulation of the posterior hypothalamic region and the mildbrain. Linke et al. (1965) and Miller and McCuskey (1973) suggested that the reported neural influences on hemopoiesis could be the result of vasomotor activity and resulting alterations in the microcirculation of blood through sinusoids. Such alterations might lead to changes in the levels of oxygenation of the hemopoietic microenvironment and subsequent shifts in the concentration of sulphated acid and neutral mucopolysaccharides as have been suggested by observations on the microcirculation in the murine spleen during erythropoietic regeneration and suppression (McCuskey et al., 1971a, 1972a).

Concluding remarks

Hemopoietic stem cells contain all the genetic information required for their further differentiation. The regulation of proliferation and differentiation requires exogenous stimuli, which are provided by both humoral factors and environmental influences. The diversity of hemopoietic differentiation suggests that we are dealing with a chain of different interactions leading to the formation of microenvironmental induction mosaics. Within the hemopoietic microenvironment several functions may be recognized. The regulation of stem cell lodgment (1) and proliferation (2) and the commitment of stem cells to the several lines of hemopoietic differentiation (3) are considered to reside in the *Hemopoietic Inductive Microenvironment (HIM)* and are likely to occur at a cellular level, i.e. they are controlled by interaction of not-defined stromal cells and hemopoietic cells through a process of cell-cell interaction.

This interaction may operate either via a direct cell-to-cell contact or may be mediated by signals acting at a very short range.

The proliferation and morphological differentiation of committed intermediate stages in hemopoietic differentiation appear to be sustained by humoral factors such as erythropoietin, thrombopoietin and colony stimulating factors. Although it is well documented, that the hormone-sensitive progenitor cells are capable of proceeding down the maturation pathway *in vitro* in the presence of an adequate hormonal stimulation, but without the presence of stromal elements, this does not exclude a microenvironmental regulation on these stages *in vivo*. The earliest distinguishable erythroid progenitor cells, i.e. the BFU-E (burst-forming unit that responds to erythropoietin; Axelrad et al., 1974; Heath et al., 1976; Iscove, 1977a), forms erythrocyte colonies *in vitro* after 7-14 days. However, there are indications that *in vivo* stem cells can differentiate into medium sized erythroblasts in 3-4 days (Hasthorpe and Hodgson, 1977). Thus, there is an indication that the *in vivo* circumstances may significantly shorten the maturation time. It is not excluded that these *in vivo* circumstances are partly of a humoral character as Iscove (1977a; 1977b) suggested that early erythroid progenitors exist in a "low" stage of proliferative activity, which is subject to erythropoietin-independent control of a non-dialysable serum activity. Other indications, that the microenvironment is involved in the regulation of the intermediate stages of hemopoietic differentiation, were delivered by *in vivo* microscopic, histochemical and biochemical studies (McCuskey and colleagues). These studies revealed that microenvironmental control of erythropoiesis (and possibly granulopoiesis and thrombopoiesis) occurs through interaction of mucopolysaccharide composition (of cell coats and groundubstance) and vasomotor activity (mediated by vasodilating substances or neural activity). The regulating activity of the microenvironment may be directly induced by changes in pH, oxygenation and/or hormones such as erythropoietin (Feleppa, 1970; 1971; McCusky, 1968; Chamberlain et al., 1975). Moreover, the microenvironment is considered to control the later stages of hemopoietic process (maturation and delivery) at a structural level, i.e. these processes require the unique microstructure of the hemopoietic organs. The possibility exists that also a thymus humoral factor (Zipori and Trainin, 1975) or thymus-derived lymphoid cells within the hemopoietic microenvironment influence proliferation, differentiation or maturation of stem cells. In support of this possibility are the suggestions that thymocytes or cells with theta antigenic surface determinants are required in the promotion of murine stem cells into erythrocytes (Goodman and Grubbs, 1970; Lord and Schofield, 1973; Wiktor-Jedrzejczak et al., 1977) and that a suppressor T cell is involved in the regulation of hemopoiesis (Goodman et al., 1977).

The possible influence, which the microenvironment exerts in relation to the proliferation and differentiation of committed progenitor cells, to maturation, detainment and delivery may be considered to belong to the Hemopoietic Conductive Microenvironment (HCM). It is not clear if the HIM differs from the HCM. However, with regard to the location of the HIM and the HCM

the observations are interesting, that bone marrow cell populations conform to a well-defined spatial organization corresponding to the chronologic relationships between marrow cells (Wislocki, 1921; Wienbeck, 1938; Lennert, 1952; Carsten and Bond, 1968 Van Dijke, 1968; Lord and Hendry, 1972; Lord et al., 1975; Shackney et al., 1975; 1976) and a migration of differentiating cells from the subendosteal region to the central region. This maturation occurs concomitant with growth retardation and maturation of the migrating cells. The notion of such a spatial organized maturation favours the idea, that the functional capacity of the HIM is optimal in the subendosteal reagions of the marrow, whereas that of the HCM increases toward the central part. Thus, it is tempting to theorize, that HIM and HCM stromal elements are partly spatially separated, probably showing a certain overlap.

Comparison of the above observations on the spatial organization of bone marrow cells with studies on the circulation in the bone marrow appear to provide a tentative definition of the microcirculation characteristics of the HIM and HCM. In tissues in which kinetic gradients and their spatial representation have been demonstrated by radioautographic methods, the region of active cell proliferation commonly lies in close proximity to the microvascular blood supply (Shackney et al., 1975). Several investigators have demonstrated in the bone marrow the presence of a capillary network and arteriolar endings at the junction of the osteoid and hemopoietic regions (Stodtmeister et al., 1956; Branemark, 1959; de Bruyn et al., 1970; Weiss and Chen, 1975). These observations support the opinion that the HIM microvasculature mainly consists of capillaries and arterioles, whereas that of the HCM is mainly sinusoidal. Applicating this to the splenic microenvironment one should expect the zone of stem cell lodging, proliferation and commitment to be on the conjunction of the red pulp and the marginal parts of the white pulp, i.e. the marginal zone. It is here, that the capillaries and terminal arterioles, which are branching off from the central arteriole in the T lymphocyte dependent area enter the red pulp. From this area the committed precursor cells may migrate toward the central regions of the several red pulp compartments while they mature. The mature cells are able to leave the splenic red pulp by entering the large splenic sinusoids, which are connected with splenic venes. These venes enter the trabecular venes, which are continuous with the splenic vene outside the organ. It is not likely that the region of active cell proliferation is situated along the trabeculae, since arterioles only leave the trabeculae through the white pulp compartment. Supporting evidence for this proposed migration pattern of developing hemopoietic cells in the spleen could emerge from autoradiographic spleen studies of normal and lethally irradiated and reconstituted mice in which the proliferative capacity of hemopoietic cells and their localization is studied. Unfortunately, data on this subject are scarce. However, Suzuki et al. (1973) divided murine spleens into three fragments, i.e.

(1) a capsular fragment, which contains all the white fibrous mass of the spleen, including the trabeculae,

(2) an outer part, including the outer capsule, and

(3) an inner part.
After *in vitro* incubation with erythropoietin for 48 hours the incorporation of radioactive iron into heme was studied in each of the three fragments of spleens from polycythemic and anemic mice. In the spleens of polycythemic mice the highest heme synthesis was found in the capsular part whereas the spleens of anemic mice showed the highest heme synthesis in the outer fragment. These observations lead the authors to the notion that erythropoietin-responsive cells have affinity for the fibrous capsular structure, which may be the white pulp, when they are in the undifferentiated stage.

In conclusion, there are many indications for the existence of a HIM but at present conclusive evidence has not been delivered. It will be clear that microenvironments create optimal circumstances for the immature blood cells to develop. Beyond this evident function all presumable functions of the hemopoietic microenvironment (i.e. stem cell commitment and regulation of the proliferative activity of stem cells as function of the HIM; modulation of the hormonal influences on committed precursor cells as function of the HCM) have to be investigated more extensively.

CHAPTER II

Introduction and Discussion of the Papers

Purpose of the investigations

In hemopoietic tissues several differential pathways from hemopoietic stem cells to mature end cells occur. The study on the microenvironmental regulation of proliferation and differentiation of these cells is difficult since a complex organization of hemopoietic cells together with several types of stromal cells and vasculature – the latter two with presumptive inductive and conductive functions – is present. Although very recently it has been indicated that *in vitro* adherent cells from bone marrow under certain culture conditions are capable to support stem cell maintenance and even enrichment and to sustain granulopoiesis (Allen and Dexter, 1976; Dexter et al., 1977; Williams and Rabellino, 1977), it is still not known which stromal cell type(s) represent(s) the microenvironment. However, studies with *in vitro* cultures of hemopoietic stromal elements have the disadvantage that only certain cell types may survive and others may obtain an "*in vitro* character", e.g. fibroblastic or epithelioid, probably with a concomitant loss or alteration of specific properties.

It will be clear that for *in vivo* studies the separation of only one hemopoietic differential pathway from the confusing mixture of other cell types in hemopoietic tissues may form a satisfactory approach to elucidate the indicated problems. Thus, the first purpose of investigation was to induce a specific line of hemopoiesis in an ectopic place, where proliferation and differentiation could be studied. A requirement for this system was, that continuous inflow of late progenitor cells into this ectopic compartment was limited since otherwise quantification of the proliferative capacity would not be interpretable. A first approach, a *Salmonella typhosa* endotoxin-induced granulopoiesis in the liver, did not meet this requirement as to the fact that 3-7 days after injection a large influx of mature granulocytes was observed. A more favourable system was found by using the experience of several authors, which observed ectopic erythropoiesis during severe anemias in men or during prolonged hypoxia in combination with splenectomy in mice. Since in laboratory mice ectopic erythropoiesis had never been described after phlebotomy-induced anemia, we have chosen a model, in which ectopic erythropoiesis was evoked by phenylhydrazine(PHZ)-induced hemolytic anemia.

At this point we tried to get answers to the following questions:

(1) Does the presence of erythroblasts at these ectopic places represents a full term erythropoiesis initiated by stem cells or does it represents an accidental accumulation of circulating erythroblasts which only undergo a terminal maturation? (appendix paper I, II, III).

(2) At which locations in these ectopic hemopoietic organs does erythropoiesis

occur with respect to the microcirculation? (appendix paper I, II, III).

(3) Are there specific stromal elements associated with any cell type belonging to the erythropoietic maturation pathway? (appendix paper I, II, III).
If so, are those stromal elements similar to the stromal cell type(s) present in the hemopoietic tissues? (appendix paper I, III).
Furthermore, are the mentioned stromal elements normally present at the same sites in the tissues in which ectopic erythropoiesis has been induced, or do they migrate toward these places? (appendix paper I, III).

(4) Is the presence of these stromal cell types an absolute requirement for ectopic erythropoiesis?
Do the stromal elements possess inductive or conductive properties or both? As long as specific regulatory properties of these cells cannot be demonstrated with biochemical, histochemical or microscopical methods, the kinetics of the onset of ectopic erythropoiesis will have to be investigated with special reference to the presence of hemopoietic cell types that initiate, and contribute to, the observed erythropoiesis (appendix paper II).

During the course of the investigations increasing evidence emerged concerning a relation between macrophages and erythropoiesis. As macrophages are involved in the regulation of ground substance composition, the observations of McCuskey and colleagues (see chapter I, Intercellular ground substance) on the role of mucopolysaccharides in erythropoiesis *in vivo* became relevant to our studies. Two additional purposes of investigation were formulated, that is:

(5) Does a causal relationship exist between the local amount of sulphated acid mucopolysaccharides (AMPS) and the extent of erythroid maturation, i.e. can erythropoietic activity be altered by regulating the amount of AMPS?
In addition, which AMPS compounds are active in this respect and in which concentrations?
The resolution of these questions requires an *in vitro* assay, in which the amount of several AMPS compounds can easily be defined and in which erythroid and also granuloid proliferation and differentiation are present at optimal hormonal stimulation (appendix paper IV).

(6) When it is assumed that macrophages possess a regulatory role in erythropoiesis then influencing the metabolic activity of macrophages *in vivo* may possibly induce alterations in erythropoiesis without an intermediate action of the hormone erythropoietin. This would indicate that microenvironmental factors or, more in general, erythropoietin-independent mechanisms, are involved in the regulation of erythropoiesis. When it can be demonstrated that only late erythrocyte progenitors are affected in this experimental set up, then this would be an indication for a presumptive HCM function. In contrast, when also the earliest erythroid precursors are affected, then the data would point to a HIM action on stem cells, committing them toward erythropoiesis. It was attempted to influence the metabolism of macrophages *in vivo* aspecifically by the administration of polystyrene latex particles to the experimental animals (appendix paper V).

Discussion of the experimental work

In this chapter the frame of the discussion is formed by the purposes of the investigation as formulated in chapter II. For a more extensive discussion the reader is referred to the separate discussions in the appendix papers. Fig. 1 (Chapter III) can be used as a guide to the position of precursor cells, which are mentioned in this chapter, in erythroid differentiation.

Question 1

A high mitotic activity in ectopic erythroblasts was found. Furthermore, measurements of the nuclear diameter of hepatic erythroblasts belonging to one distinct island showed a significant smaller deviation in the diameter sizes than would be expected from a random distribution of erythroblasts of all maturational stages (appendix paper I). No phagocytized remnants of immature erythroblasts were found in the central macrophages. The only phagocytized material was recognized as being derived from erythrocytes, reticulocytes and normoblast nuclei, which are – also under normal conditions – expelled from this late erythroblast type. No morphological evidence was obtained for the possibility, that lysis of erythroblasts occurred outside macrophages. At last, no morphological aberrations in the ectopic erythroblasts were observed as compared with steady state erythropoiesis in bone marrow and spleen. These observations indicate that erythroblasts at least are undergoing a number of divisions in the liver and suggest a synchronization in development of erythroblasts belonging to one island; the possibility that the erythroblasts are just being phagocytized by the central macrophages in the erythroid islets was rendered less likely.

With respect to the presence of erythroblast precursors in ectopic sites, using the spleen colony assay of Till and McCulloch (1961), a considerable increase in hepatic CFU-S could be demonstrated, mainly occurring between day 2 and 4 of the PHZ treatment. These hepatic CFU-S are likely to have migrated from the bone marrow to the liver, as is discussed in appendix paper II. No typical "presumptive stem cells" as described by Van Bekkum et al. (1971) were observed on the intravascullar CM during the observation period. However, with morphological methods it is almost impossible to find stem cells, occurring in a very low frequency among the other hemopoietic cells. Whether hepatic stem cell renewal is disregarded or not, the observed influx of CFU-S would be large enough to give rise to the observed erythroblast number on day 7. The 3 day delay between the peak in the number of CFU-S on day 4 and the highest erythroblast number on day 7 suggest that the erythroblasts originate in about 3 days from the stem cell population. This suggestion is supported by the fact, that also a 3 day period was observed between the shoulder in the CFU-S number around day 7 and the second lower peak in the erythroblast number on day 10. This period seems to be rather short, but Hasthorpe and Hodgson (1977) have shown that stem cells are capable of giving rise to erythroblasts by day 3 in the X-irradiated mouse spleen. However, when stem cells are

the first precursors of the hepatic erythroblasts, then much larger and much fewer erythroid colonies had to be expected, than were observed. The observed colony size, measuring up to about 60 erythroblasts, may be explained by the fact that (a) not stem cells but late erythroid precursor cells, limited in their proliferative capacity, initiate hepatic erythropoiesis, or that
(b) certain erythroid precursor cell stages have a migratory capacity, and upon reseeding within the liver give rise to several erythroid colonies, which are the offspring of one precursor cell. The migratory capacity of some late erythroid precursor cells is made likely by *in vitro* and *in vivo* observations. *In vitro,* one BFU-E forms a burst of small clusters which are spatially separated from each other. *In vivo*, we observed that of all the morphologically recognizable erythroblasts almost only the proerythroblasts are partly not connected to CM, but stick to endothelial walls. Contributing to the notion, that immigration of late erythroid precursor cells can not explain the onset of hepatic erythropoiesis, are the studies of Hara and Ogawa (1976; 1977). Neither in normal nor in PHZ-induced anemic mice these authors were able to find any erythrocytic colony-forming units (CFU-E) in the blood and they suggested that CFU-E do not participate in the migration of erythropoietic precursors in mice. From day 2 until day 5 we observed some large mononuclear blasts in the blood, that were not identical with proerythroblasts but were more undifferentiated. It is not clear if these cells were monocytic or erythrocytic, but the latter possibility is not supported by the studies of Hara and Ogawa. No erythroblasts were observed in the peripheral blood; however, it may be argued that this observation does not exclude a transit of erythroblasts to ectopic sites since erythroblasts may be present in the blood for days in very low numbers and therefore remain undetectable with routine light microscopy.

In addition to the possibility, that pluripotent stem cells initiate the hepatic erythropoiesis under anemic stress, a second likely possibility is supplied by the earlier mentioned studies of Hara and Ogawa (1976; 1977), who observed high numbers of BFU-E circulating in the blood on day 2 after two PHZ injections into mice. These BFU-E closely resemble stem cells both in their density and velocity sedimentation characteristics and possibly also in their multipotentiality (Wagemaker, 1977b). In contrast to earlier data (Iscove and Sieber, 1975; Heath et al., 1976), BFU-E are probably not at all (Iscove & Guilbert, 1977, Wagemaker, 1977b) sensitive to erythropoietin.

Adler et al. (1976) reported that hepatic CFU-S in mice with a radio-strontium-induced marrow aplasia decreased after splenectomy and were obsent in mice splenectomized prior to radio-strontium injection. This is implicative for the fact that the liver cannot support proliferation of CFU-S.

Although the above mentioned data suggest that CFU-S or BFU-E are tentative precursors of hepatic erythroblasts during anemic stress, it is not likely that the temporarily ectopic erythropoiesis is completely selfsustaining with concomitant stem cell renewal.

Question 2

Under normal conditions erythroblasts are found extravascularly in intersinusoidal areas, which are the hemopoietic cords, in fetal liver, spleen and bone marrow. In contrast, in the preceding yolk sac stage erythropoiesis is intravascular. This microenvironment is relatively hypoxic in comparison with the arteriolar and capillary areas. Under anemic stress, both in bone marrow and liver, erythropoiesis is present in the terminal parts of sinusoids and, especially in the liver, also in the central veins. This indicates that erythroblasts preferably are located in a somewhat hypoxic environment associated with a sinusoidal system.

Question 3

Ectopic erythroblasts are mostly located around central macrophages (CM) and as such from small erythroid islets which consist of a few to about 60 erythroblasts. This arrangement is similar to that of erythroid islets in bone marrow and spleen under normal conditions. A part of the proerythroblasts can be also found sticking to the endothelial wall without any connection with a CM. No other cell types, e.g. reticular cells, are present in intravascular islets. The presence and number of CM in the hepatic central venes and the marrow sinusoids and central veins is well correlated with the number of erythroblasts at these respective sites.

Morphological observations suggest that these CM develop from immigrated monocytes, that obtain phagocytic activity around day 3 of the phenylhydrazine (PHZ) treatment. According to Van Furth et al. (1972) these monocytes are generated in the bone marrow and may migrate with the blood to distinct locations in the body, where they may undergo functional specialization into macrophages. Under normal conditions only very small numbers of macrophages are present in the central veins of liver and bone marrow and in the marrow sinusoids. In support of the notion that the observed intravascular CM – at least in the bone marrow and in the central veins of the liver – are not resident macrophages, were the experiments in which colloidal carbon (CC) was injected into mice, before hemolytic anemia was induced. The CC was applied in low doses, at which it appeared, in preleminary experiments, to be exclusively phagocytized by macrophages and not to be demonstrable in reticular and endothelial cells, in blood granulocytes or monocytes. When CM would belong to the resident macrophage population or be derived from it, then the CM would also show carbon loading. However, when CM had arisen from monocytes, then no carbon would be present in the CM. The intravascular CM in the bone marrow did not contain carbon particles, whereas a part of the extravascular CM also showed no carbon loading after day 2. These observations indicate that all intravascular CM and a part of the extravascular CM in the bone marrow had originated from cells, that have no phagocytizing ability at the moment of carbon injection. The possibility that only a part of the macrophages was able to ingest carbon was rejected, as in subsequent studies with control mice all recognizable bone marrow macrophages showed carbon uptake after carbon injection.

Additional experimental data on the carbon uptake of liver macrophages, not presented in the appendix papers, are in good agreement with the observations on

the origin of the intravascular CM in the bone marrow. It appeared, that when 50 μl of colloidal carbon was injected into mice, 45 minutes prior to the initiation of the PHZ treatment, no carbon loading was observed of the CM in the central veins (CM-V) on day 7. However, in the sinusoids a part of the CM (CM-S) showed carbon labeling, whereas a part did not. When colloidal carbon was injected on day 3 of the PHZ treatment, only a part of the CM-V showed carbon uptake and a part did not contain carbon particles. From these experiments it may be concluded that:

(1) CM-V originate from cells, that had no phagocytizing capacity at the moment of carbon injection and
(2) CM-S represent a mixed population of cells, including both resident macrophages (Kupffer cells) and exudate macrophages, which are probably derived from monocytes.

The latter notion is supported by the observation, that 2 types of nuclei could be distinguished in CM-S, one of which resembled that of Kupffer cells (appendix paper I).

In addition to the morphological observations and experimental data on the carbon uptake of CM both of which indicates a monocytic origin of a part of intravascular and extravascular CM, also another observation, using an intrinsic macrophage marker, justified such a conclusion. This was the observation on the presence of paracrystalline inclusions in bone marrow macrophages of (C57BL/Rij x CBA/Rij) F1 mice (appendix paper III). These inclusion bodies, which are lysosomal in nature and develop in the first postnatal weeks, very probably occur in about half the number of laboratory mouse strains. When they are present, they are always observed in bone marrow macrophages and with a decreasing degree of probability in spleen, lymph nodes, lungs and liver (unpublished data). In (C57BL/Rij x CBA/Rij) F1 mice these inclusions almost exclusively occur in the greatest part of the femoral macrophage population. However, during hemolytic anemia the observed intravascular marrow CM did not contain inclusion bodes and neither did a small part of the extravascular marrow CM. These data are indicative for a monocytic origin of the latter macrophages.

These considerations indicate that CM are not a selfsustaining unique macrophage population that differs from other macrophages. The CM, both intravascular and extravascular, very probably may arise from monocytes and exert their function within an appropriate sinusoidal or venous tissue area.

Question 4

From appendix paper I, II and III it appears that during the first 2 days of the PHZ treatment an increase in the number of intravascular monocytic cells occurred together with a minor increase in intravascular erythroblasts. On day 3, when the first monocytic cells developed their phagocytic activity, a clear increase in the intravascular erythroblast number was noticed. Moreover, the number of intravascular CM and erythroblasts correlated well during the whole observation period.

These observations suggest, that the presence of functional macrophages is required for the onset and maintenance of intravascular erythropoiesis.

In additional experiments, not presented in the appendix papers, silica particles (< 5 μm; kindly provided by Dr. Ing. M. Reisner, Steinkohlenbergbauverein, Essen, G.F.R.), 2 mg/mouse and colloidal carbon, 0.04 or 0.2 ml, were administered i.v. and carragheenan (Sigma, St. Louis), 10 mg/mouse, suspended in a B.S.S. (Mishell and Dutton, 1967), was administered i.p. at different moments before or during the PHZ treatment. These substances are phagocytized by macrophages; colloidal carbon may be considered to be a relatively inert material, but carragheenan and silica cause lysis of macrophages when ingested. After recirculation carragheenan and silica may cause further damage to macrophages. When these particulate substances were injected before the initiation of the hemolytic anemia, the number of hepatic and marrow erythroblasts was significantly reduced on day 7, compared to control anemic mice. When carragheenan or silica were injected after the third day of the PHZ treatment a considerably stronger decrease (> 90%) of hepatic erythropoiesis occurred; thus the erythroid abolition is probably due to the damaged CM. These experiments suggest, that hepatic erythropoiesis is the most vulnerable to the effects of silica, carragheenan and – in a lesser extent – colloidal carbon when many phagocytizing CM are present, which is the case after 3 days, when the immigrated monocytes develop their phagocytic activity and become exudate CM. The mode of action of these particulate substances on the CM-erythroblast relationship is not fully understood, but a tentative explanation is given in appendix paper V. It is assumed that intracellular damage of CM after ingestion of silica or carragheenan causes weakening of their function to detain erythroblasts, whereas also a disturbance or alteration of a presumptive, regulatory function in the control of the population size of erythroid precursor cells may lead to ineffective or strongly diminished erythropoiesis, as is probably the case after latex and carbon ingestion (appendix paper V).

The presented data strongly suggest that the presence of central macrophages is an absolute requirement for ectopic erythropoiesis *in vivo*.

The question concerning the possible inductive or conductive properties of CM has to be considered in relation to the experimental conditions. In appendix paper I, II and III the properties of the intravascular CM occurring during anemic stress were specially investigated. In the literature simple helper functions are attributed to the CM, i.e.

(a) detainment of the differentiating erythroblasts, and prevention of their release into the circulation,
(b) phagocytosis of expelled normoblast nuclei and disranged erythroid elements,
(c) transfer of ferritin particles from the CM cytoplasm to the surrounding erythroblasts and
(d) representing a centre for resumption of erythropoiesis subsequent to a period of depressed red blood cell formation (Ben-Ishay, 1974) or during compensatory erythropoiesis at ectopic sites.

In our opinion the CM may also have a regulatory function in relation to erythroid maturation (appendix paper IV).

The possibility, that intravascular CM also possess inductive properties, i.e. are able to commit a pluripotential stem cell toward erythropoietin-sensitivity, is supported by the notion that CFU-S or BFU-E are tentative precursors of hepatic erythroblasts during anemic stress (see answer to question 1). CFU-S (and BFU-E?) need a committive induction stimulus toward the erythroid pathway and both cell types have to be triggered to erythropoietin-sensitivity. In consideration of this, it cannot be excluded or proved, that under certain conditions the liver may develop a HIM that has the property to lodge stem cells (a function of the HIM) and to commit them toward erythropoietin-responsiveness (which is also a function of the HIM). The liver probably is deficient in CFU-S maintenance (see answer to question 1), which is another function of the HIM. Since in the liver temporarily two of the three HIM functions (see chapter I, Evidence for the existence of hemopoietic microenvironments) may be assumed to be present, this would imply that the several HIM functions can be separated, as has also been suggested by Wolf (1974).

Question 5

In appendix paper IV it is demonstrated, that addition of several sulphated AMPS compounds to *in vitro* cultures of bone marrow cells may interfere with the EP-responsiveness of erythroid precursor cells, i.e. CFU-E. Only chondroitin sulphate-A, -B and -C and heparitin sulphate were effective in inhibiting the erythrocytic colony growth, resulting in a lower colony incidence, in relatively high concentrations. It was remarkable, that chondroitin sulphate -A and -B in lower concentrations appeared to stimulate the erythrocytic colony growth above the expected control value with optimal erythropoietin stimulation. It also appeared, that granulocytic colony formation was not affected under these circumstances or was affected in a different manner. That is, high concentrations of chondroitin sulphate-C had a slight stimulatory action on granulocytic colony growth – in contrast to the effect on the colony formation of CFU-E – whereas a reverse effect was suggested when high concentrations of hyaluronic acid were present in the cultures. McCuskey and colleagues postulated that the presence of neutral mucopolysaccharides is essential for erythroid maturation. If this postulate were correct, one would expect that in our *in vitro* experiments glycogen would exert a stimulatory effect on the erythrocytic colony formation. According to Pearse (1968) glycogen is a homoglycan, which can be considered to be a neutral (muco) polysaccharide. The experimental data in appendix paper IV principally indicate a regulatory role of sulphated AMPS and not of glycogen in erythroid maturation.

The action of the active sulphated AMPS compounds was found to be limited to a narrow concentration range. Extrapolation of these data to the *in vivo* situation suggests that ground substance factors may interfere with the humoral systemic influence on erythropoiesis.

The observed inhibitory amount of some AMPS compounds *in vitro* probably

also may occur *in vivo*. Schrock et al. (1973) found in a biochemical analysis of total hyaluronic acid and chondroitin sulphate in polycythemic mice respective amounts of 130.5 and 131.0 μg per g lipid-free spleen dry weight, which represents about 30 per cent of the spleen net weight. This implicates that the total AMPS amount per spleen may be about 5 μg at a spleen volume of 0.1-0.2 ml and according to Noordegraaf (1977) may even amount up to 50 μg per spleen with a weight of 20-40 μg chondroitin sulphate -A and -C per ml culture medium and even with smaller amounts of chondroitin sulphate -B and heparitin sulphate. Although it is realized, that AMPS is partly present in the cellular fraction and partly in the intercellular ground substance, it is likely that high concentrations of AMPS may occur locally in the spleen and probably also in the bone marrow. The presented data are strong indications for the existence of an Erythroid Hemopoietic Conductive Microenvironment, in which the composition of ground substance factors may modulate the erythropoietin-responsiveness of erythrocyte precursors. It is postulated that CM may play a regulatory function in the HCM by regulating the local amount and/or composition of AMPS in the narrow spaces between CM and surrounding erythroblasts.

Question 6

In appendix paper V it has been demonstrated, that injection of polystyreen latex particles into mice affects bone marrow erythropoiesis *in vivo*. Both a stimulatory and inhibitory action of the particulate substance, depending upon the injected doses, have been observed. No alterations in the colony numbers have been observed when latex was added *in vitro* to the culture system, in which CFU-E and CFU-C colony formation was determined. The particulate substance did not affect the systemic erythropoietin level in a way by which the alterations in erythropoiesis could be explained.

The earliest erythroid cell class that was affected, has been found to be the BFU-E. No significant alterations have been detected in the CFU-C compartment after latex injection. Injection of latex particles into mice led to a pronounced effect on the Burst Feeder Activity (BFA). After infusion of a low dose of latex a 50% increase in BFA was observed on day 2 and another on day 7 with subnormal levels found in between. Both BFA peaks preceded two discrete peaks of the BFU-E incidence on day 4 and 9 (340% of control) and at the same time of the CFU-E incidence (164% of control). After a high dose latex injection a decrease in BFA was observed and the BFU-E recovery on day 8 again was associated with a earlier peak in BFA around day 5. These observations have been interpreted as indicating that BFA probably is involved in the controll of the population size of BFU-E and possibly also partly that of the CFU-E *in vivo* (see Fig. 1, Chapter III). The notion, that BFA-associated cells are a prerequisite for burst formation *in vitro* by induction of the apparent erythropoietin-sensitivity on "early" BFU-E, allows the speculation that this dinstinct marrow populations may have a similar function *in vivo*. From this speculation and the observation of the two-day time lag between peaks of

BFA and BFU-E arises the opinion, that the inductive action on non-committed cells leds within two days toward erythropoietin-responsiveness. The observation that BFA-associated-cells possible are required for only two days *in vitro* to allow burst formation (Wagemaker, 1977a) is in fair agreement with this suggestion. According to their relative small size (6.7 to 8.4 μm as determined on the basis of a buoyant density of 1.083 $g.cm^{-3}$) it is not probable that the effector cells, which are associated with BFA, are macrophages (Wagemaker, 1977b). However, the *in vivo* action of colloidal carbon and polystyreen latex particles strongly suggests that macrophages are involved in a control of BFU-E population size. It has been demonstrated that the vehicles of the latex do not exhibit significant effects on the number of erythroid progenitors *in vivo*. Furthermore, inhibition of phagocytosis by corticosteroid administration to mice prior to injection of a low dose of latex, prevented the expected increase of the CFU-E number on day 3 after latex injection. These data suggest that particles have to be phagocytized in order to affect the BFA-associated cells. In conclusion, the possibility cannot be excluded or proved at present, that macrophages and BFA-associated cells represent the same marrow population *in vivo*.

As to the fact, that central macrophages share the closest contacts with erythroblasts and may function as a centre for resumption of erythropoiesis it is postulated that central macrophages may be involved in the commitment of pluripotent stem cells toward erythropoietin-responsiveness and/or possess a regulatory influence in the control of the BFU-E population size.

CHAPTER III

General discussion

Discussion

A relationship between functional properties of the hemopoietic organ stroma and the presence and extent of hemopoietic activity has been suggested by observations of several kinds over the years. The data presented in this thesis add to the notion of the existence of an Erythroid Hemopoietic Inductive Microenvironment and point to an *in vivo* action of a Hemopoietic Conductive Microenvironment for erythropoiesis. In the concluding remarks of Chapter I it was hypothesized that the HIM and HCM stromal elements may be spatially partly separated, with probably a certain overlap. The evidence presented in this thesis supports the assumption, that central macrophages in erythroid islest may represent both the inductive and the conductive microenvironmental influences on erythropoiesis (Fig. 1). According to this assumption manipulation of macrophage functions may affect erythropoiesis in different ways.
Possibly, reconsideration of the factors, which lead to hemopoietic protection during irradiation and affect the phenomenon of genetic resistance, can contribute to a better understanding of the macrophage role in erythropoiesis.

Clinical relevance of hemopoietic microenvironmental studies in the therapy of hemopoietic disorders

In recent years it has been suggested that hemopoietic defects may not only be due to intrinsic hemopoietic cell defects, but may also be generated by abnormalities in hemopoietic microenvironments (Tavassoli, 1975). These abnormalities may be congenital e.g. macrocytic anemia in SL/SL^d mice, Di George thymic aplasia (Di George, 1965; Lischner and Di George, 1969) and Nezelof thymic dysplasia (Nezelof et al., 1964), of which the latter two are associated with an absence, respectively a severe impairment of cell-mediated immunity. Probably, also abnormal granulopoiesis in RFM mice (Jenkins et al., 1969a; 1969b) belongs to the congenital abnormalities. Alternatively, abnormalities in hemopoietic microenvironments are suggested to be induced by irradiation, treatment with cytostatics and also by the expansion of a leukemic cell population in a hemopoietic organ. Some of these abnormalities extrinsic to the hemopoietic cells appear to be due to defects in humoral regulation or function in the cell milieu, e.g. in the case of chloramphenicol-, thiamphenicol- and chlorpromazine-induced leukopenia (Ratzen et al., 1974; Yunis and Gross, 1975). Also the studies of Greenberg and Mara (1977) suggest, that a stromal defect may be related to an inadequate local production of colony stimulating activity (CSA). These authors observed in sequential studies of chemotherapy-induced

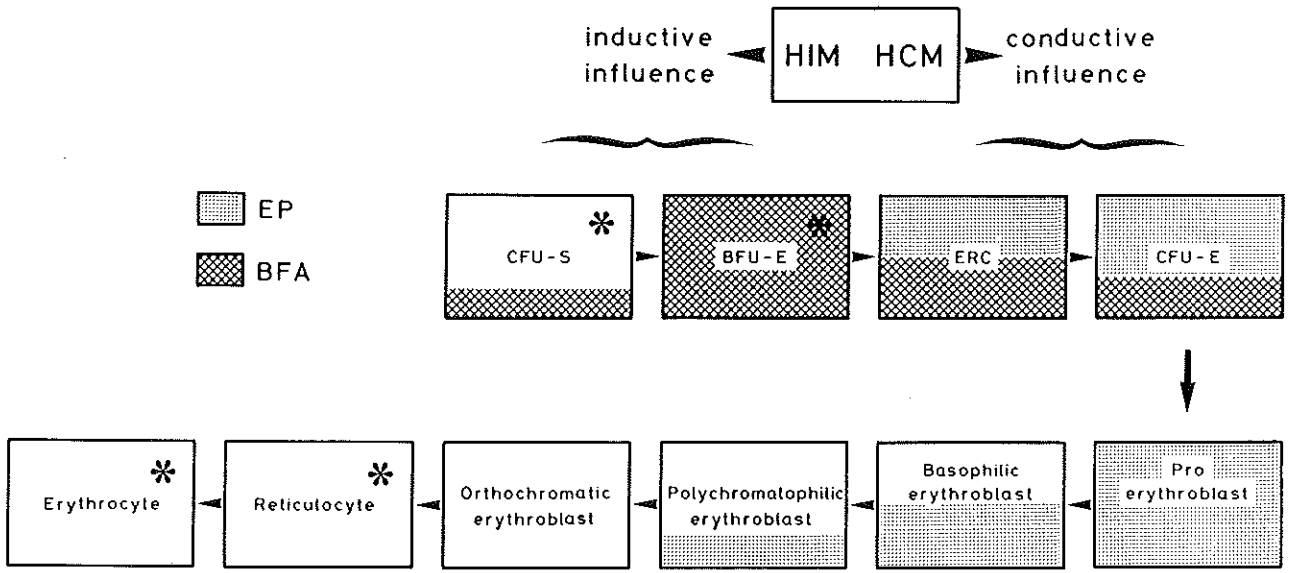
inductive influence
HIM HCM
conductive influence
EP
BFA
CFU-S
BFU-E
ERC
CFU-E
Erythrocyte
Reticulocyte
Orthochromatic erythroblast
Polychromatophilic erythroblast
Basophilic erythroblast
Pro erythroblast

Fig. 1

remissions in acute myeloid leukemia patients normal or increased marrow CSA provision, whereas intermittent low or diminishing marrow CSA provision was found in patients with short remissions. Other extrinsic defects were suggested to be derived from microcirculatory damage (Chen et al., 1972; Tavassoli, 1974) in acute leukemias or to be generated by stromal defects in the connective tissue compartment (McCuskey and Meineke, 1973; Fried et al., 1973a; Wilson and O'Grady, 1976).

The present evidence on damaging effects of irradiation and cytostatic treatments on the hemopoietic organ stroma of rodents may be of importance in clinical transplantation procedures. Although the succes of clinical bone marrow transplantation in practice is principally limited by the development of acute or delayed graft versus host desease, one should keep one's mind open to these damaging effects in case of ineffecient graft take or developing secundary hypoplasia after grafting. Thus, on consideration of the extrinsic factors that are involved in hemopoiesis, it is clear that a rational therapy for the multiple categories of hemopoietic defects will be consequently difficult to develop. Treatment of acute leukemias, thymic aplasia or dysplasia and certain forms of aplastic anemia (Jeannet et al., 1974; Sensenbrenner et al., 1977), neutropenia and pancytopenia possibly has to focus more on organ grafting procedures (in which pieces of thymic, splenic or bone marrow tissue are transplanted) more than on stem cell transplantation only, which has shown to be an effective treatment for hemopoietic intrinsic defects. On the other hand, when the extrinsic factors appear to be humoral defects, treatment with a purified hormone may conceivably overcome the hemopoietic defect in a number of cases. Hemopoietic cell cultures of patients with hematological disorders seem to be a helpful tool to discriminate between intrinsic and extrinsic hemopoietic cell defects. Moreover, sequential culture analysis will contribute to a better understanding of therapy-induced microenvironmental damage. Preferred discriminants in these sequential culture studies could be at present:

(1) the hormone provision of hemopoietic cells,

(2) the capacity of adherent stromal cells to support stem cell maintenance as can be

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Fig. 1 Schematic representation of a model in which the position of precursor cells in erythroid differentiation and of the assumed regulatory influence of the erythroid microenvironment in murine bone marrow is presented.

Cells on the upper line are not (easy) recognizable morphologically in contrast to the cells on the lower line. The position of BFU-E relative to CFU-S and ERC is hypothetical. The cell stages indicated by an asterisk are, under normal conditions, also present in the peripheral blood in detectable numbers.

The assumed erythropoietin-independent influence of the microenvironment (HIM and HCM), which possibly is exerted by central macrophages, on the development of CFU-S toward erythrocytes is indicated in the upper part of the figure. The estimated degrees of sensitivity of the different cell types to erythropoietin (EP) and burst feeder activity (BFA) are expressed as the areas respectively taken in by the simple and the complex lattice-work.

Explanation of abbreviations:

CFU-S, spleen colony forming unit ("stem cell"); BFU-E, erythroid burst forming unit; ERC, erythropoietin-responsive cell, which is *in vivo* defined as a cell type, which responds to EP injection into a polycythemic mouse by giving rise to proerythroblasts within 24 hours and may comprise some CFU-E; CFU-E; erythroid colony forming unit.

determined in current models (Dexter and Testa, 1976; Williams and Rabellino, 1977) derived from the system reported by Friedenstein et al. (1970) and

(3) the inductive capacity of 15 Gy γ-irradiation-resistant bone marrow cells, i.e. Burst Feeder Activity (Wagemaker, 1977), as it has been examined in appendix paper V.

Summary

Mature hemopoietic cells have a transport function or are engaged in the defense of the body against foreign microorganisms and substances. Because they are in general rather short-lived, a complicated cell renewal system is present during the entire life in order to ensure homeostasis. According to the current knowledge pluripotent hemopoietic stem cells are able to generate all types of blood cells and also macrophages. The proliferation and differentiation of stem cells and their progenitor cells is regulated by short range (microenvironmental) and long range (humoral) factors. The microenvironmental influence in hemopoiesis is considered to act on the stem cell level, determining

(1) the migration and lodgment of stem cells in their proper places,
(2) the stem cell proliferation and
(3) the commitment of a stem cell toward one specific cell line, inducing the development of the sensitivity for a specific hemopoietic hormone.

These microenvironmental influences are exerted by the so called Hemopoietic Inductive Microenvironments (HIM) of which the nature and morphology so far has not been revealed (Chapter I).

Indications exist, that alterations in one or more of the 3 compartment of hemopoietic organs, i.e. the microvascular compartment, the connective tissue compartment and the neural compartment, may affect hemopoiesis *in vivo*. Defects in these compartments may be congenital or induced by X-irradiation, cytostatic treatment or an expanding leukemic cell population. However, a regulatory role of stromal elements in progenitor cell proliferation and differentiation is considered to be of minor importance compared to the humoral influences on these progenitor cells. The regulation of progenitor cell maturation is described in this thesis to reside in a Hemopoietic Conductive Microenvironment (HCM) (Chapter I, Concluding remarks).

During fetal and postnatal life of a mammal erythroblasts occur in clusters around central macrophages (CM) in hemopoietic organs. The experimental work described in this thesis is related to the role of central macrophages in erythroid islands and the role of a presumptive erythroid HIM and HCM in erythropoiesis.

It has been demonstrated that CM also occur in ectopic erythropoietic islands during severe hemolytic anemia, and probably are generated from circulating monocytes. Moreover, the resident liver macrophages, i.e. Kupffer cells, have been indicated to temporarily act as CM under these circumstances. It appeared that CM probably are a prerequisite for erythropoiesis and it has not been excluded, that CM in ectopic locations as hepatic and bone marrow sinusoids and veins have the property to commit a stem cell toward erythopoietin-responsiveness (appendix papers I, II and III; Chapter II, questions 1-4).

In addition to the reasonable assumption, that CM may simply exert a detainment function, a surveyor function and a phagocytic function in relation to the surrounding erythroblasts, it has been postulated that

(a) CM probably are stromal elements of an erythroid HCM with a regulatory function in erythrocyte precursor maturation, and
(b) that CM possibly are stromal elements of an erythroid HIM, which induces stem cells toward erythropoietin-responsiveness.

Colloidal carbon and polystyrene latex particles administered to mice have been found to induce both a temporary, dose dependent, increase and decrease of erythropoiesis in the bone marrow. The modulating effect of these substances on erytropoiesis has been demonstrated to be an erythropoietin-independent phenomenon. The data indicated that latex particles may affect the control of the BFU-E (a very early erythrocyte precursor cell) population size *in vivo* by an erythropoietin-independent mechanism, which may be an inductive one. Very likely, this is also valid for colloidal carbon particles. The presented evidence supports the assumed activity of an erythroid HIM and represents the first indication that the activity of the erythroid HIM *in vivo* may be manipulated separately (appendix paper V; Chapter II, question 6).

In vitro experiments in which bone marrow cells were grown in the presence of variable amounts of a number of acid mucopolysaccharide compounds, have revealed that some of these compounds are able to increase or totally prevent erythroid colony formation, despite an optimal hormonal stimulation, whereas granulocytic colony formation is not or slightly opposite affected. It has been suggested that sulphated acid mucopolysaccharides in the ground substance, especially the chondroitin sulphates -A, -B and -C are regulating substances in the conductive influence that CM exert on erythropoiesis. It has been concluded, that the data reported in the appendix paper and in the literature justify the postulation of an erythroid HCM (appendix paper IV; Chapter II, question 5).

It has been noted, that one should be mindful of the fact, that the current therapies in human acute leukemia, aplastic anemia and combined immunologic deficiency disease possibly may damage the hemopoietic microenvironments and that therefore a systemic investigation on stromal hemopoietic regulation and factors, which influence this regulation, has to be promoted.

Samenvatting

Gedifferentiëerde hemopoietische cellen in zoogdieren hebben een transportfunctie of zijn betrokken bij de verdediging van het lichaam tegen lichaamsvreemde micro-organismen en stoffen. Omdat bloedcellen in het algemeen een korte levensduur bezitten, is een "cell renewal" systeem gedurende het gehele leven aanwezig om een homeostase te verzekeren.

Volgens de huidige kennis zijn pluripotente hemopoietische stamcellen in staat om zich te ontwikkelen tot alle typen bloedcellen en ook macrofagen. De proliferatie en differentiatie van stamcellen en hun nakomelingschap worden gereguleerd door factoren, die op korte afstand (micro-omgevingsniveau) en op lange afstand (humoraal niveau) werkzaam zijn. De micro-omgevingsinvloed op de hemopoiese wordt geacht werkzaam te zijn op stamcelniveau en bepaalt:

(1) de migratie en de hechting van stamcellen op de juiste plaatsen,
(2) de stamcelproliferatie en
(3) de inductieve stimulus, die een stamcel in de richting van een specifieke cellijn ontvangt, d.w.z. waarbij een stamcel gevoelig wordt voor de werking van een specifiek hemopoietisch hormoon.

Deze micro-omgevingsinvloeden worden door de zogenaamde Hemopoietische Inductieve Micro-omgeving (HIM) uitgeoefend. De aard en morfologie van de HIM zijn tot op heden niet ontraadseld (Hoofdstuk I).

Er bestaan aanwijzingen, dat verandering in één of meer der 3 compartimenten in hemopoietische organen, t.w.: het microvasculaire compartiment, het bindweefselcompartiment en het neurale compartiment, de hemopoiese *in vivo* kunnen beinvloeden. Defecten in deze compartimenten kunnen congenitaal aanwezig zijn of geinduceerd worden door X-bestraling, behandeling met cytostatica of door een uitgroeiende leukemische celpopulatie. Een regulerende rol van stromale elementen in de proliferatie en differentiatie van onrijpe bloedcellen wordt echter ondergeschikt geacht aan de humorale invloeden op deze cellen. De regulatie van de maturatie der voorlopercellen is in dit proefschrift voorgesteld als een functie van een Hemopoietische Conductieve Micro-omgeving (HCM).

Tijdens het foetale en postnatale leven van een zoogdier komen erythroblasten voor in groepjes rond centrale macrofagen (CM) in bloedvormende organen. Het experimentele werk, wat in dit proefschrift wordt beschreven, heeft betrekking op de rol van centrale macrofagen in erythroid eilandjes en de rol van een veronderstelde HCM en HIM op de erythropoiese.

Aangetoond kon worden dat CM ook in ectopische erythroide eilandjes voorkomen tijdens een ernstige hemolytische anemie en waarschijnlijk ontstaan uit circulerende monocyten. Bovendien wezen de waarnemingen op het feit, dat residente levermacrofagen (Kupffer cellen) tijdelijk een CM-functie kunnen uitoefenen onder deze omstandigheden. De CM vormen waarschijnlijk een voorwaarde voor het voorkomen van erythropoiese en het kon niet worden uitgesloten, dat CM op de ectopi-

sche plaatsen, zoals in de sinusoiden en centrale venen van de lever en het beenmerg, de eigenschappen bezitten om een stamcel te induceren tot erythropoietinegevoeligheid (appendix papers I, II en III, Hoofdstuk II, vragen 1-4).

In aanvulling op de veronderstelling, dat CM eenvoudigerwijs slechts een vasthoudfunctie, een opzichtersfunctie en een fagocytaire functie zouden kunnen bezitten in relatie tot de omringende erythroblasten, werd gepostuleerd dat

(a) CM wellicht stromale elementen zijn in een erythroide HCM, die een regulatoire functie uitoefenen op de maturatie van rode bloedcelvoorlopers en
(b) dat CM mogelijk stromale elementen in een erythroide HIM zijn, die stamcellen induceert tot erythropoietinegevoeligheid.

Colloidale kool- en polystyreen latexdeeltjes, toegediend aan muizen, veroorzaakten zowel een tijdelijke doses-afhankelijke toename als afname van de erythropoiese in het beenmerg. Aangetoond werd, dat het modulerende effekt van deze substanties op de erythropoiese een erythropoietine-onafhankelijk fenomeen is. De gegevens duiden erop, dat latexdeeltjes de controle van de populatiegrootte der BFU-E (een zeer vroege voorlopercel van erythrocyten) *in vivo* kunnen beinvloeden en dat hierbij een inductieve stimulus betrokken kan zijn. Zeer waarschijnlijk kan dit ook gelden voor colloidale kooldeeltjes. De aanwezige bewijsvoering ondersteunt de veronderstelde werkzaamheid van een erythroide HIM *in vivo* specifiek beinvloed kan worden (appendix paper V; Hoofdstuk II, vraag 6).

In vitro experimenten, waarin beenmergcellen werden gekweekt in de aanwezigheid van variabele hoeveelheden van diverse mucopolysacchariden, demonstreerden dat enkele van deze stoffen in staat zijn om de erythroide kolonievorming te stimuleren of totaal te onderdrukken ondanks een optimale hormonale stimulatie, terwijl de granulocytaire kolonievorming niet of enigszins tegengesteld wordt beinvloed.

Verondersteld wordt, dat gesulfateerde zure mucopolysacchariden in de grondsubstantie van bloedvormende organen, en wel speciaal de chondroitine sulfaten -A, -B en -C, regulatoire stoffen zijn binnen de conductieve invloed die CM uitoefenen op de erythropoiese. Er werd geconcludeerd, dat de gegevens, die vermeld worden in de appendix artikelen en in de literatuur, de postulering van een erythroide HCM rechtvaardigen (appendix paper IV; Hoofdstuk II, vraag 5).

De literatuurgegevens hebben geleid tot het inzicht, dat men erop bedacht moet zijn, dat de huidige therapie voor humane akute leukemieën, aplastische anemieën en ernstige gecombineerde immunologische deficiëntieziekten mogelijkerwijs de hemopoietische micro-omgeving kunnen beschadigen en dat daarom een systematisch onderzoek zou moeten worden bevorderd aan de stromale regulatie van de hemopoiese en de faktoren, die deze regulatie beinvloeden.

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Curriculum vitae

Na het behalen van het diploma HBS-B aan de Christelijke Scholengemeenschap „Groen van Prinsterer" te Vlaardingen in 1965 was de schrijver gedurende een jaar als laborant werkzaam bij het Radiobiologisch Instituut TNO te Rijswijk. Na in 1966 geslaagd te zijn voor het examen leerling-analist (zoölogische richting) te Utrecht werd in datzelfde jaar begonnen met de studie biologie aan de Rijksuniversiteit te Utrecht. Het kandidaatsexamen werd afgelegd in februari 1969. De doctoraalstudie omvatte de hoofdrichting Endocrinologie (uitgevoerd bij Dr. J.A.M. Mattheij en Dr. M. Terlou) en de bijvakken Pathologie (bij Prof. Dr. A. de Minjer) en Elektronenmikroskopie (bij Dr. F. Spies) benevens de werkzaamheden ter verkrijging van de aantekening Onderwijsbevoegdheid (bij Dr. H.J. Saaltink en Drs. W.J. Zweinenberg). Het doctoraalexamen werd in 1971 afgelegd.

Van 1968 tot 1974 was hij part-time leraar biologie, aanvankelijk aan de Christelijke Scholengemeenschap „Westland-Zuid" te Vlaardingen en vanaf 1971 aan de Christelijke Scholengemeenschap "Melanchthon" te Rotterdam.

Sinds oktober 1971 is de promovendus als wetenschappelijk medewerker werkzaam binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen deze vakgroep werd het onderzoek verricht dat in dit proefschrift is beschreven.

Abbreviations

AMPS	acid mucopolysaccharides (glycosaminoglycans)
BFA	burst feeder activity; enhances erythroid burst formation and is an absolute requirement for the early phase of burst formation *in vitro*; associated with relatively radioresistant bone marrow cells
BFU-E	burst forming unit; cell forming a macroscopically visible red colony *in vitro* consisting of erythroid clusters when cultured for 7-15 days in the presence of erythropoietin and a minimal number of BFA-associated cells
CFU-C	colony forming unit in culture; cell giving rise to a colony within 6 days, consisting of granulocytes and/or monocytes and macrophages in an *in vitro* assay
CFU-E	erythroid colony forming unit, giving rise to a small erythroid colony in EP-stimulated cultures within 3 days
CFU-S	colony forming unit in the spleen; cell giving rise to a colony in the spleen colony assay. Colonies have an erythroid, granuloid, megakaryocytic or mixed appearance and also occur in the bone marrow, although their incidence in the latter organ is not employed as a quantitive standard
CM	central macrophage, which may be found in erythroid islets
CM-V	central macrophage in the hepatic veins
CM-S	central macrophage in the hepatic sinusoids
E : G ratio	ratio of the incidence of erythroid and myeloid colonies
EP	erythropoietin
HCM	hemopoietic conductive microenvironment, which may be defined as the local influence of hemopoietic organ stroma, determing detainment and delivery of hemopoietic progenitor cells and mature cells and – in co-operation with humoral regulators – the proliferation and differentiation of committed progenitor cells
HIM	hemopoietic inductive microenvironment, which may be defined as the local influence of hemopoietic organ stroma, determining: stem cell lodging and mobilization, stem cell renewal and commitment of a stem cell toward a specific hemopoietic pathway
PHZ	phenylhydrazine chloride

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Morphological Investigation on Phenylhydrazine-Induced Erythropoiesis in the Adult Mouse Liver

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Summary. In adult mice suffering from a phenylhydrazine (PHZ)-induced hemolytic anemia, erythropoietic islands were observed in the liver. These islands were studied with the light and electron microscope. Within two days after the beginning of four daily injections of PHZ, erythroid elements appeared in the sinusoids and central veins. A maximum number of erythroblasts was found on day 7. Light and electron microscopic observations revealed that the erythropoietic islands consisted of centrally located macrophages (CM) with a Kupffer cell-like morphology, surrounded by erythroblasts, which were often of the same maturation stage. CM in central veins (CM-V) and in sinusoids (CM-S) were found to have a different morphology. The CM-V phagocytized less circulating red blood cells and were in contact with a smaller number of erythroblasts. Furthermore, the contact areas between erythroblasts and CM-S extended for a much longer distance than those between erythroblasts and CM-V. The progenitor cell for the CM-V is most likely a monocyte, since cells which were morphologically determined as monocytes were found to appear on the first day of the PHZ treatment and differentiated into macrophages within about 2 days. The origin of the CM-S population was less clear, but could be monocytic as well. These data are tentatively explained as a migration of a progenitor of a cellular component of the erythroid microenvironment into the liver after appropriate stimuli. In contrast to fetal liver erythropoiesis, erythroblasts in the adult liver occurred only incidentally extrasinusoidally. Furthermore, specialized membrane contacts between erythroblasts and CM or hepatocytes could not be observed in adult liver. Ferritin could not be detected on the erythroid cell membrane or located in coated vesicles. Also, no ferritin could be observed within or attached to the finger-

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like processes of CM. The observations suggest that the coated vesicles in erythroid elements are partly exocytotic vesicles and are not specific for ferritin transport. The morphological aspects of PHZ-induced extramedullary erythropoiesis is discussed in relation to the hemopoietic microenvironment.

Key words: Liver (Mouse) – Erythropoiesis – Microenvironment – Light and electron microscopy.

Introduction

Erythropoiesis has been the subject of numerous light and electron microscopic studies. Although extensive ultrastructural information has been published concerning the relationship between central macrophages (CM) and the surrounding erythroblasts in erythroid islets in the yolk sac (Sorenson, 1961), bone marrow (Bessis et al., 1956) and the spleen (Orlic et al., 1965), an interdependency is hypothetical. For example, a commonly mentioned feature, ropheocytosis (which is a pinocytotic process thought to be involved in ferritin uptake of maturing erythroid elements, the ferritin being delivered by the CM), can be observed without any demonstration of ferritin (Bessis et al., 1962). Recently, Sullivan et al. (1976) demonstrated an involvement of ropheocytotic vesicles in transferrin uptake without the involvement of macrophages. It is difficult to obtain morphological information about the kinetics and function of this relationship between macrophages and erythroblasts, since in all hemopoietic organs several lines of hemopoiesis are mixed and thus impossible to differentiate. Therefore, an experimental situation was attempted in which the erythroid differentiation line in vivo could be studied separately. This would also be helpful in elucidating the factors contributing to a specific hemopoietic inductive microenvironment (Wolf and Trentin, 1968). As Bozzini et al. (1970) mentioned that the adult liver was involved in the overall erythropoiesis of splenectomized mice exposed to hypoxia, we decided to study the liver as an extramedullary model of erythropoiesis.

This model raised questions as to how a suitable erythroid microenvironment can be re-established in an organ which had been involved in erythropoiesis for only two or three weeks in the fetal and postnatal life (Silini et al., 1967; Borghese, 1959).

The present communication deals with a description of the morphological events accompanying temporary erythropoiesis in the adult liver of mice made severely anemic by treatment with phenylhydrazine. PHZ is known to damage the cell membrane of mature red blood cells and results in precipitates of altered hemoglobin known as Heinz bodies in erythrocytes, the presence of which shortens their lifespan. Moreover, the reticulocyte population, being rapidly pushed out into the blood, does not have the usual time to mature and is composed of larger and more heterogeneous cells. These reticulocytes, which become the only circulating red blood cells for some days after the PHZ treatment and the erythrocytes into which they mature, are also more rapidly cleared from the circulation (Walter et al., 1972).

Materials and Methods

Experimental Animals

CBA/Rij male mice, 12–20 weeks old and weighing 22–28 g, were used. The mice were purchased from the Medical Biological Laboratory TNO, Rijswijk, The Netherlands.

Phenylhydrazine Treatment

Hemolytic anemia was induced by 4 daily intraperitoneal injections of 1 milligram phenylhydrazine chloride (PHZ; Merck, Darmstadt). The effect of the PHZ treatment on the erythropoiesis was evaluated by red blood cell and reticulocyte counts, hematocrite values, weighing of the spleen and differential bone marrow counts. The first day of the first injection was designated as day zero. At least 7 animals were studied every day for a period of 14 days.

Preparation for Microscopy

Fixation was carried out by perfusion through the portal vein under Nembutal (70 mg/kg body weight) anesthesia. The method of Wisse (1970) was used but with omission of the prewash. The fixation solution contained 1.5% undistilled glutaraldehyde (Fluka AG, Switzerland) in 0.075 M Millonig or cacodylate buffer, pH 7.2. After 3 min of perfusion, during which the liver became pale and solid, small blocks of liver tissue (1–4 mm^3) were prepared with a razor blade and transferred directly to a OsO_4 fixative solution buffered with 0.075 M Millonig or cacodylate to pH 7.2 for subsequent immersion fixation (1.5 h at 4° C). Dehydration in a graded ethanol series was performed rapidly and followed by embedding in Epon. Polymerization was carried out for 16 h at 37° C, followed by 8 h at 45° and another 48 h at 60°. The osmolality of the solutions was approximately 310 milliosmols as measured by an Advanced Osmometer.

Light Microscopy

1.5 µm sections from Epon embedded tissue were prepared for light microscopy with a Reichert OM U2 and OM U3 microtome. The sections were stained with an aqueous solution of toluidine blue adjusted to pH 8.6 with 0.1 M Borax buffer. Coverslips were mounted with Entellan instead of conventional mounting media in order to avoid bleaching of the stained sections.

Electron Microscopy

Sections for transmission electron microscopy were made with the same microtomes and contrasted with saturated uranyl acetate and lead hydroxide according to the method described by Venable (1965). A Philips EM 300 electron microscope was used for examination and photography.

For scanning electron microscopic studies, glutaraldehyde fixed livers from mice treated with PHZ 8 days previously were prepared according to Van Ewijk et al. (1975). The specimens were examined in a Cambridge MK IIa scanning electron microscope, operated at an accelerating voltage of 20 kV, with 200 µm diameter illuminating apertures. The specimens were studied in a tilted position of 45°.

Observations

A regimen of four daily intraperitoneal injections of 1 mg PHZ induced a severe hemolytic anemia in mice. Hematocryt values (Ht) were temporarily down to 28 (control Ht, 48) and subnormal to day 9. Affected red blood cells and their debris

were cleared from the circulation mainly by the liver, spleen and bone marrow. The anemia caused a marked increase of erythropoiesis in the spleen up to 20 times the control level on day 7. In the bone marrow 40 to 50% of the nucleated cells were erythroblasts from day 3 through day 7, which was nearly twice as much as in untreated mice.

In the liver erythroid cells were found to be present in central veins and sinusoids, close to the central veins. A part of the erythrophagocytic macrophages was surrounded by maturing erythroblasts, some erythroblasts contacted more than a single macrophage. When erythroid cells took an extra-sinusoidal location, they were regularly surrounded to a large extent by liver parenchymal cytoplasm.

The morphological alterations in liver Kupffer cells, central macrophages and erythroblasts during experimentally induced hemolytic anemia will be described in the present study. The quantitative effects will be published elsewhere.

Kupffer Cells

Twenty-four h after initiation of the PHZ treatment Kupffer cells showed an enlarged, pale nucleus and slightly basophilic cytoplasm. The area occupied by these cells was considerably enlarged to about 300% of the control area. This indicated an increased activity. Some cells contained phagocytized erythrocytes in variable degrees of degradation. The Kupffer cells showed more cytoplasmic extensions than were seen in control animals.

On day 2 the Kupffer cells contained many more erythrocytes and reticulocytes, but nucleated cells were never seen within their cytoplasm. The area occupied by their cytoplasm had increased to about 450% of the control value. These swollen cells developed increasing numbers of protrusions that whirled free into the sinusoidal lumen. Often damaged mature red blood cells were attached to their membranes. Various stages of degradation of phagocytized red blood cells could still be observed within the Kupffer cells. Several cells with the morphology of small lymphocytes were in close contact with macrophages.

Three days after initiation of the PHZ treatment many Kupffer cells were filled with phagocytized erythrocytes and reticulocytes. Occasionally, they seemed to fill up a total sinusoid. Their cytoplasmic surface had increased to about 700% of the control value. The amount of phagocytized material observed within Kupffer cells increased up to day 5 (Fig. 1). On this day a maximal increase of their cytoplasmic area was observed, which amounted to approximately ten times the original value. A decrease in the size of Kupffer cells was observed from day 5 to day 9, when it was still five times the control value.

Central Macrophages

On the first day of treatment, in the central veins and in the sinusoids, relatively small cells with a very indented nucleus similar to monocytes adhered to the endothelial wall. The karyoplasm was condensed at the periphery of the nucleus and contained one or more small nucleoli. The number of nuclear pores was small.

The cells were attached to endothelial cells through short microvilli-like processes. The outer surface of these cells was rather smooth but showed occasional microvilli (Fig. 2). This was in contrast to the Kupffer cells, which showed at the same time a more extensive ruffling of the outer membrane. No phagocytosis of erythrocytes was noted. In the cytoplasm a moderate amount of ribosomes and polyribosomes, partly bound to short profiles of rough endoplasmatic reticulum, was present. The rod-shaped mitochondria were spherical in transverse sections with a diameter of 0.26 µm and varied in length in longitudinal sections. Membrane-bounded, electron dense, round to oval bodies, with an average diameter of 0.18 µm, were located in the cytoplasm. Only occasionally, vacuoles with an electron lucent content were present. Some lysosomes contained membranous structures. A Golgi zone, which was usually very small, was present in the juxtanuclear cytoplasm, and regularly two centrioles were found in its vicinity. Directly beneath the cell surface an organelle free zone could be distinguished, that possessed a higher electron density than the rest of the cytoplasm. Microtubuli and filaments could be found in this area.

On day 2 the cytoplasm had increased in area. Some of the cells showed a less indented nuclear membrane. Their cell surface was still smoother than that of Kupffer cells at this time and possessed a moderate amount of pseudopod-like and microvilli-like processes (Fig. 3). The Golgi region was still small but seemed to be more active due to an increase in the number of small electron lucent vesicles.

On day 3 the number of cells had markedly increased compared to day 2. Mitotic figures were not observed. This increase preceded a similar rise in erythroblasts on day 4. The morphology of the cells had clearly changed. Most cells did not exceed the volume of a proerythroblast. In addition to an increase in the number of nuclear pores, they revealed numerous knotty and pseudopod-like extensions, while in some cells an ingested erythrocyte could be observed. The Golgi area in the cell center was enlarged and consisted of several active cysternal stacks. The nucleus was still polymorphic. It had a small rim of peripherally condensed chromatin and was located excentrically in the cell, close to the side where the cell was connected to the wall of the sinusoids or central veins. Great amounts of primary vesicles, apparently derived from the Golgi saccules, were found throughout the cytoplasm. Relatively short profiles of rough endoplasmic reticulum, which were irregularly dilated, occurred more frequently. The number of mitochondria per cell had increased. Pinocytosis was scarcely observed. The cells tended to form loose clusters in the central veins. This phagocytotic cell type, which could be observed to develop within 2 days out of a cell with the morphology similar to a monocyte, was surrounded by erythroblasts from day 3 on in the central veins. Therefore, we considered this cell type to be a CM. The relation to erythroblasts in erythroid islands will be described below.

Erythroblasts

One day after the first PHZ injection only very few erythroblasts could be observed in the sinusoids and none in the central venes. The first erythroblasts noted had a very immature appearance and differed light microscopically from large

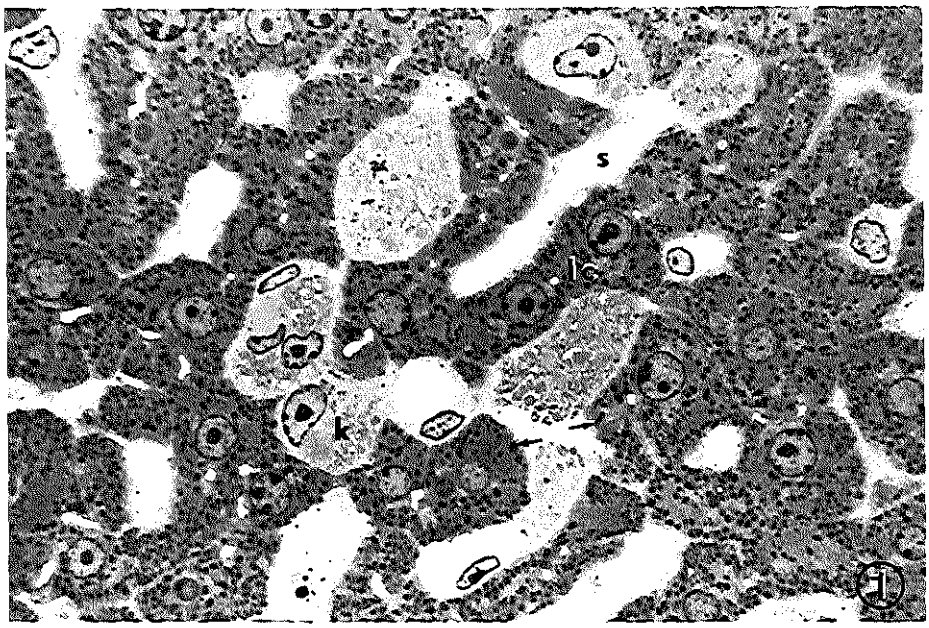


Fig. 1. Light microscopic photomicrograph of a liver area showing swollen Kupffer cells. *K* Kupffer cell; *lc* liver parenchymal cell; *arrows* mitochondria; *s* hepatic sinusoid. ×2310

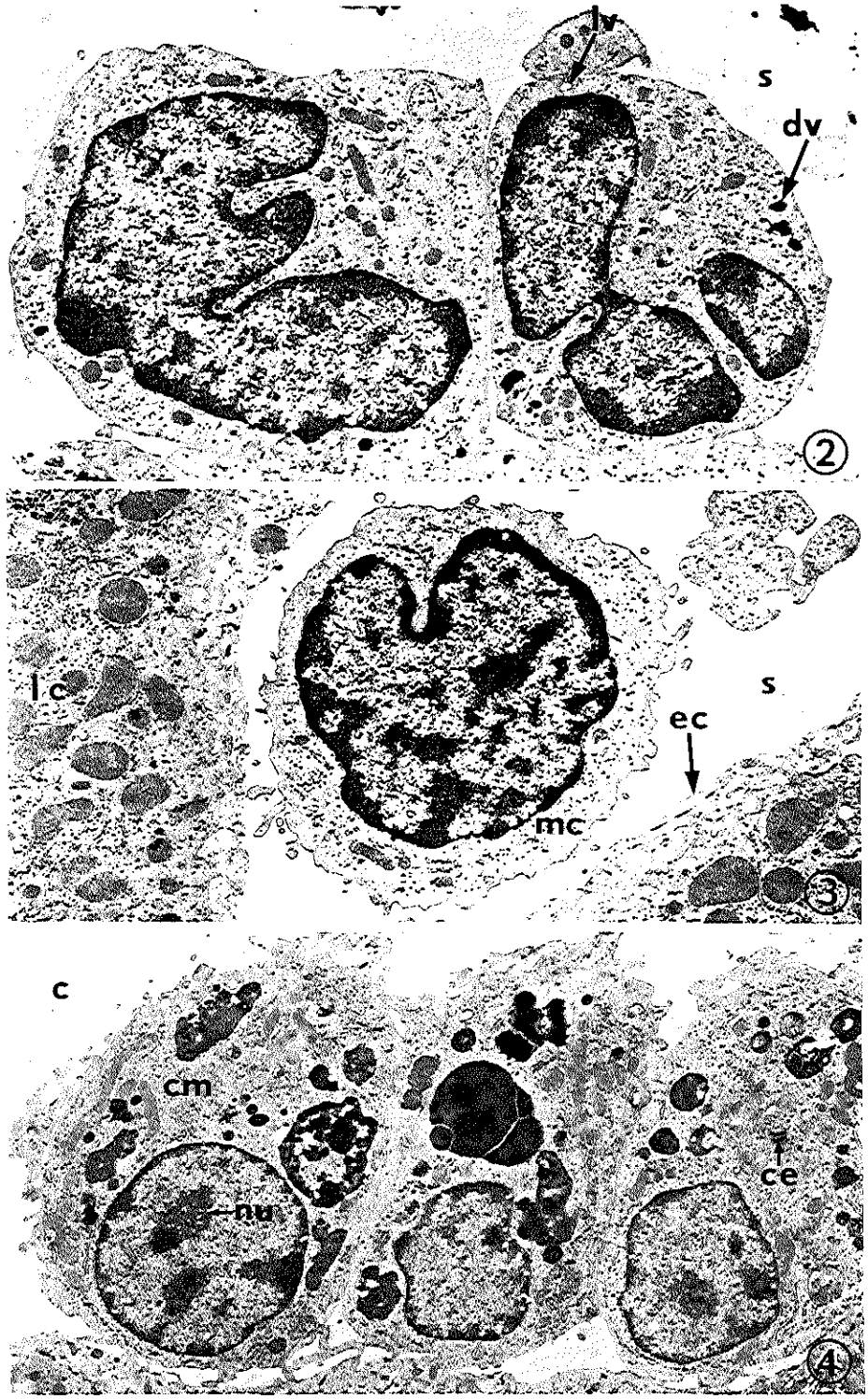
Fig. 2. Liver sinusoid. One day after the first injection of PHZ, cells that resemble monocytes frequently adhere to the endothelial lining of sinusoids and central veins. Note the irregularity of the nucleus and the smooth outer surface of the cells with occasional microvilli. The cell at the right shows dense vesicles (*dv*) and lucent vacuoles (*lv*). *s* Sinusoidal lumen. ×6390

Fig. 3. Hepatic sinusoid (*s*) lined by endothelial cells (*ec*). A characteristic mononuclear cell (*mc*), which can be seen in sinusoids and central veins on day 2. More ruffling of the outer membrane is apparent when compared to Figure 1. The processes lack cell organelles and are filled with a microfilamentous cytoplasm which is continuous with a rim of similar cytoplasm all along the periphery of the cell. The nucleus has become more spherical with an increase in the number of nuclear pores. *lc* Liver parenchymal cell. ×6140

Fig. 4. Central vein of the liver. Micrograph showing a cluster of several central macrophages (*cm*) on day 7. The erythroblasts around their periphery can be observed in subsequent serial sections. The nucleus is spherical with slight indentations, many nuclear pores and prominent nucleoli (*nu*). Short profiles of endoplasmatic reticulum may be distinguished mostly in the cell periphery. Illustrated are several stages in the breakdown of sequestered red blood cells, the remnants of which are mainly represented by membranous structures and aggregates of ferritin particles. Note the irregular outlines of the cells. *ce* Centriole; *c* lumen of central vene; *nu* nucleolus. ×4250

lymphocytes only in having more cytoplasm and being more basophilic. These cells contained a leptochromatic nucleus. Regularly, cells were observed that had a morphology transitional between cells resembling lymphoblasts and proerythroblasts (Fig. 5).

At day 2 the first large proerythroblasts could be found in the sinusoids but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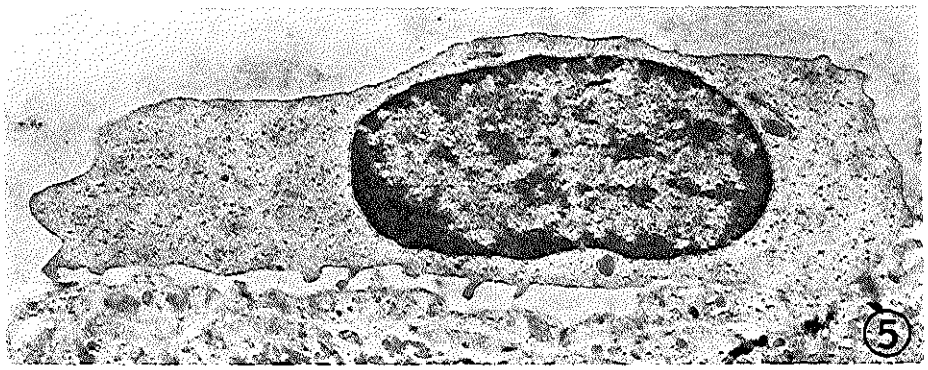


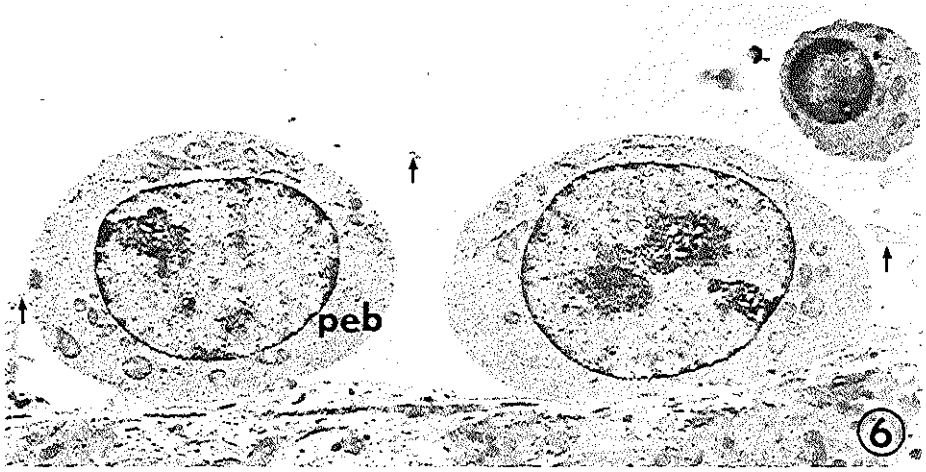
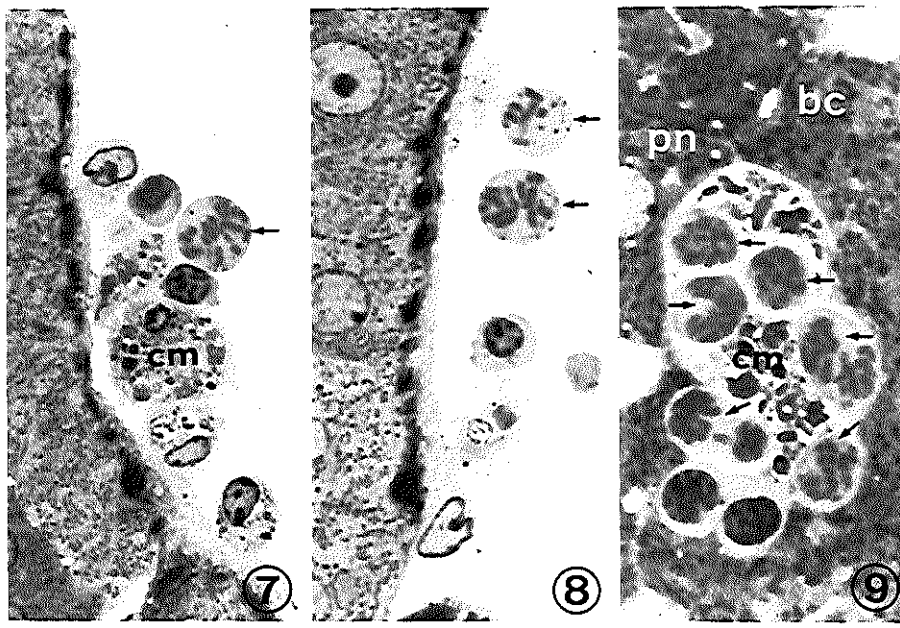


Fig. 5. A large transitional cell on day 5 in the central vein. ×6715

Fig. 6. Micrograph showing 2 proerythroblasts (*peb*) in contact with endothelium in a central vein. Processes of CM-V are swirling around this large cells (*arrows*). ×3842

not in the central veins. The first proerythroblasts were often seen attached as single cells or in clusters to the endothelial layer (Fig. 6). They were not always located close to CM. At this time more lymphocytes entered the liver and were sometimes found to be lying extrasinusoidally in the space of Disse. Occasionally, in the plane of section lymphocytes were surrounded entirely by cytoplasm of hepatocytes.

On the third day after the first PHZ injection a clear elevation of erythroblast number was observed. For the first time erythroblasts were seen in the central venes. The majority of these cells were proerythroblasts and basophilic erythroblasts. Using serial sections it was possible to demonstrate that most of the erythroid cells made contact with philopodia of CM without having contact with endothelial cells. Mitotic figures were frequently found (Figs. 7–9).



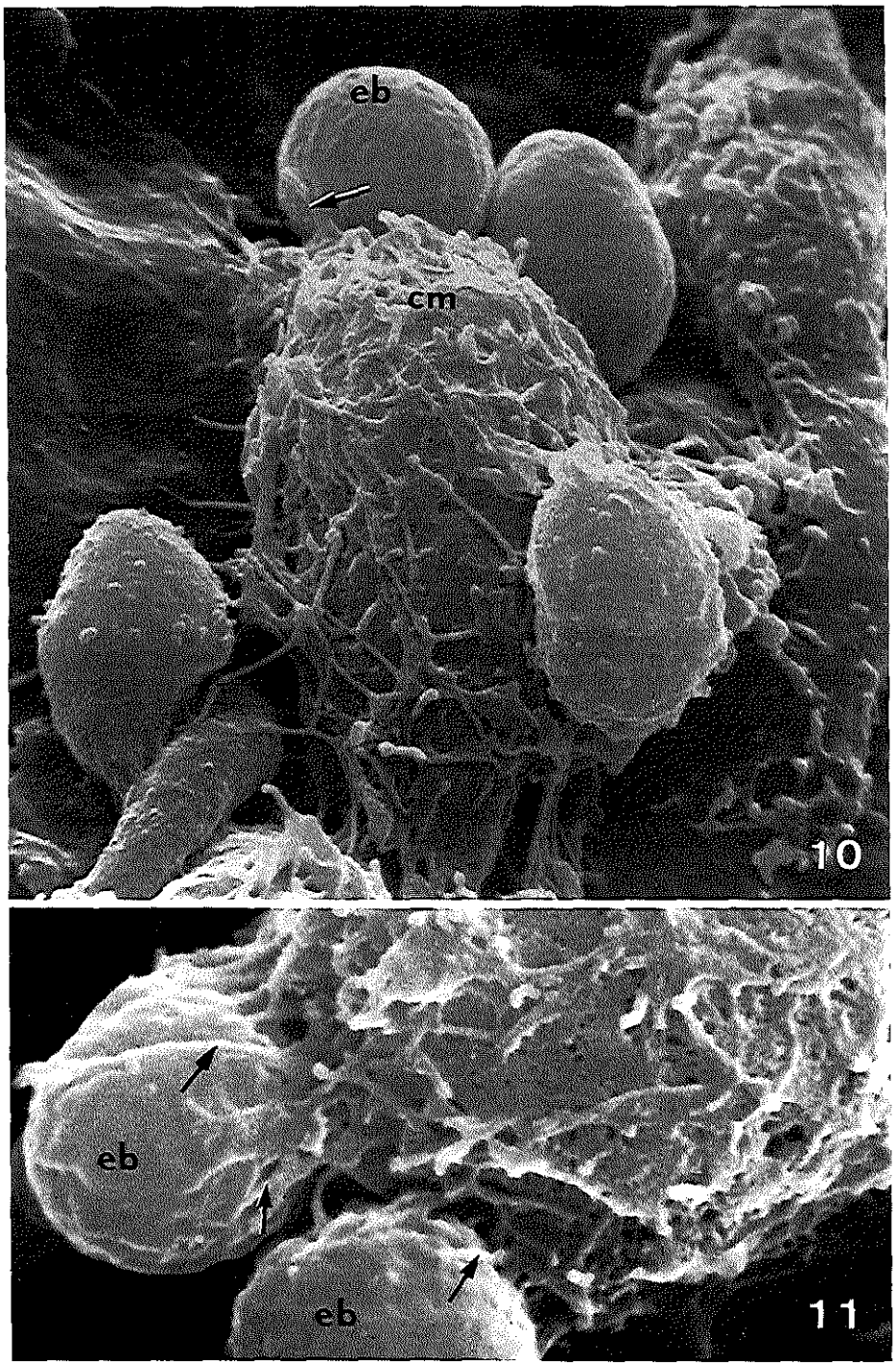
Figs. 7 and 8. Light microscopic photomicrographs of the same intravenously located erythroid islands on day 7, demonstrating the presence of several mitotic figures (*arrows*). Fig. 7, ×3150; Fig. 8, ×3654

Fig. 9. Same as in Figures 7 and 8, intrasinuoidally located erythroid islands on day 7. The CM-S shows more debris of phagocytized red blood cells than the CM-V in Figures 7 and 8. Note the irregular profiles of the hepatic cells close to the differentiating blood elements. *bc* bile canaliculus; *pn* parenchymal cell nucleus. ×3654

Erythroid Islands in the Central Venes

From day 4 through day 11 erythroblasts in the central veins were mostly found clustered at the periphery of one or more CM which had phagocytized considerable amounts of circulating red blood cells. However, in general the CM in the central veins (CM-V) had taken up less material than sinusoidal phagocytes at the same time (compare Figs. 7 and 9). The difference in the amount of sequestered material may be due to the location of the cells. CM-V came in contact with the blood which had already passed the sinusoidal phagocytes and was partly purified of damaged erythrocytes.

All stages of erythroid maturity, including extrusion of normoblast nuclei, were found. Microtubuli were frequently seen throughout the cytoplasm of the erythroblast. This indicates that the erythroblasts in the adult liver are more similar to such cells in adult bone marrow than to the cells in the fetal liver, since in the last type microtubuli are rarely present. Regarding the nuclear extrusion in normoblasts, very thin rims of cytoplasm with surface membranes were observed surrounding the expelled nuclei.



Figs. 10 and 11. Scanning electron micrographs of an erythroid island in the central vene on day 8 of the PHZ treatment. Thin projections of the CM-V (*arrow*) can be observed to cover parts of the surrounding erythroblasts. Note the elongated shape of the CM in the right field of the micrograph. *eb* Erythroblast. Fig. 10, ×5600; Fig. 11, ×12,600

The CM-V had an irregularly elongated shape (Figs. 4, 10). The ultrastructure (Fig. 4) revealed a moderately dense nucleoplasm with prominent nucleoli. which were often associated with the nuclear envelope. Condensed chromatin was found in a small rim at the nuclear periphery. Many nuclear pores were present. Toward day 7 the nuclei became more spherical with slight indentations. The cytoplasm possessed rod shaped or spherical mitochondria, of which the short axis had increased compared to the first three days (0.6 μm on day 5; 0.4 μm on day 7), and the long axis had a variable length. Short profiles of rough endoplasmic reticulum were always slightly dilated and covered by a moderate amount of ribosomes. Polyribosomes and some free ribosomes were dispersed throughout the cytoplasm, but especially in the cell periphery. Many small uniform vesicles, originating from extensive Golgi zones, and a much smaller number of dense granules could be observed. Membrane limited ingested material was present, which varied from recognizable whole erythrocytes and reticulocytes to smaller clumps of varying size, shape and density. The majority of the smaller clumps were typical of ferrogenous aggregates and micelles or admixtures of ferritin with material of varying electron density. Always two centrioles associated with the Golgi apparatus were observed. Engulfment of red blood cells into the cytoplasm of the CM was also observed. Fragmentation of cells outside the macrophages begins once the cells have been completely engulfed.

The CM-V covered parts of the surrounding erythroblasts with long finger-like cytoplasmic projections, which showed a strong ruffling of the outer membranes and often showed branching (Figs. 8, 10, 11). In the philopodia very few or no ribosomes or other cell organelles were seen. Outer membranes of both cell types scarcely apposed each other directly, and desmosomes or tight junctions were not observed (Fig. 12). Also, between the CM-V and the endothelium specialized cell membrane contacts were never demonstrated. The distance between the outer membranes of the CM-V and the surrounding erythroblasts was not smaller than 250 Å. Proerythroblasts were attached to CM-V as well as to the endothelium (Fig. 6); more mature erythroblasts only contacted the CM-V.

Ferritin particles were rarely observed on outer membranes or in the coated vesicles of erythroblasts, nor were they seen in or attached to the macrophage philopodia. Sometimes, membrane limited ferrogenous aggregates, which had the characteristics of hemosiderin granules, were present in erythroblasts. The small coated (in the literature often called "ropheocytotic") vesicles were found also in erythroid cell parts which were not adjacent to the CM-V. They frequently occurred in groups in the perinuclear region (Fig. 13) and close to mitochondria and the Golgi apparatus. In these regions the smooth surface of the vesicles was not as prominent as in the peripheral parts of the cytoplasm. The number of this kind of vesicles increased at the time that erythroblasts lost their mitochondria. In more mature erythroblasts extrusion of small membranous structures, similar to mitochondria, was occasionally noted. In all stages of erythroid maturation mitochondria showed dilation of the christae forming rounded structures (Fig. 16). These findings may indicate a possible origin of the coated vesicles out of aging mitochondria. In this respect at least a part of the coated vesicles would represent exocytotic vesicles, and the described process could be a process in which maturing erythroblasts loose their aged mitochondria.

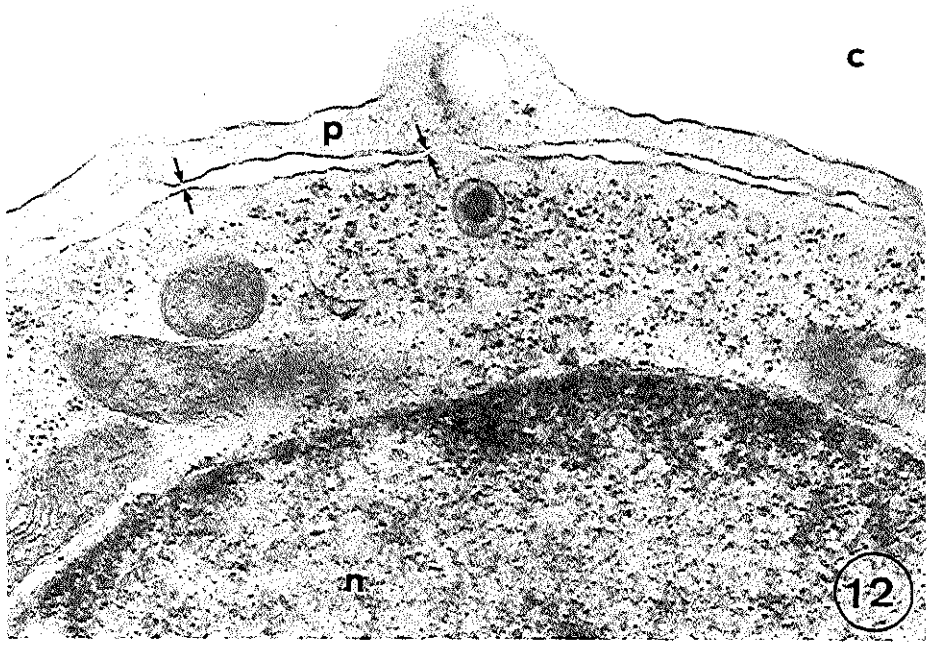


Fig. 12. Part of an erythroblast in contact with a CM-V process (*p*) on day 4. Note the irregular distance between the two cell types. The arrows point at the 250 Å space, which is the smallest distance found between the outer membranes. *n* Nucleus. ×44,220

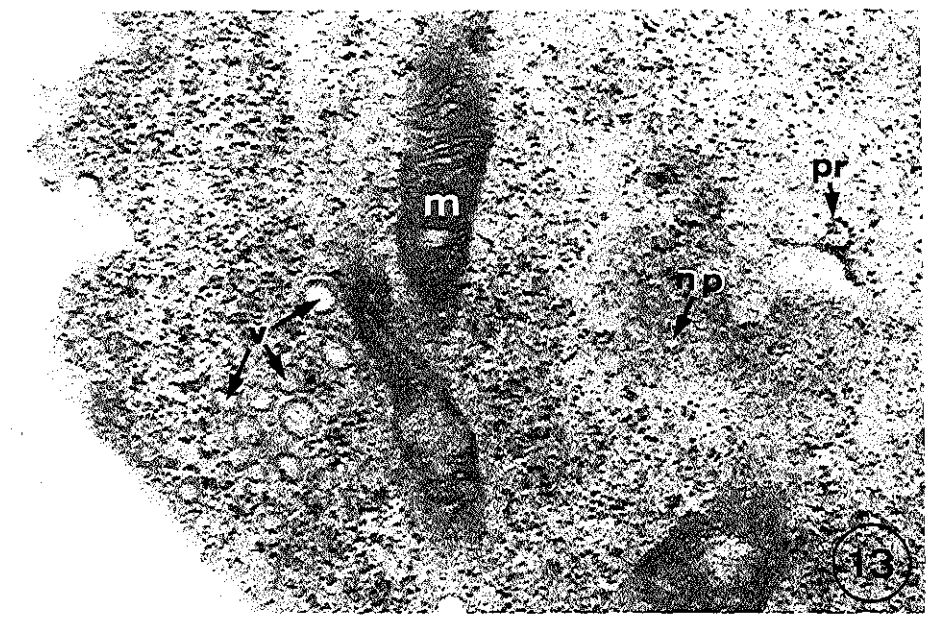


Fig. 13. Part of an erythroblast on a CM-V (not visible) on day 4. Demonstrated is the clustering of ropheocytotic vesicles (*v*). Both the outer membrane and the nuclear membrane are tangentially sectioned. *m* Mitochondrion; *np* nuclear pores; *pr* polyribosomes on the outer leaflet of the nucleus. ×40,000

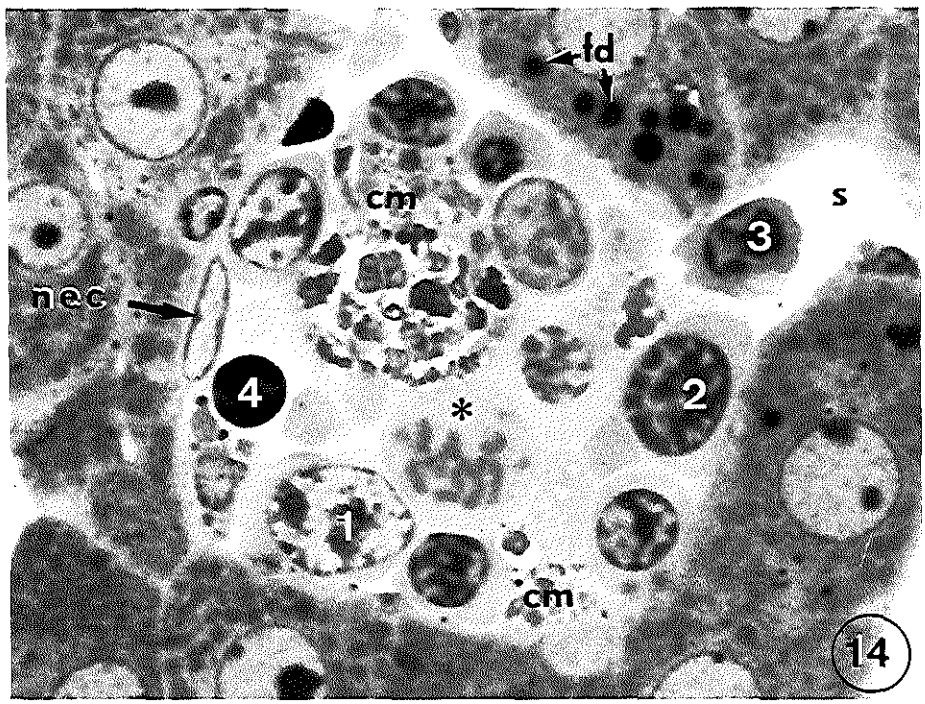


Fig. 14. Light microscopic photomicrograph of an erythroid island in a liver sinusoid on day 4 illustrating a great variation in erythroid developmental stages. *Asterisk* erythroid mitotic figure; *nec* endothelial cell nucleus; *fd* fat droplets in parenchymal cell; *1* proerythroblast; *2* basophilic erythroblast; *3* polychromatophilic erythroblast; *4* expelled normoblast nucleus. ×4356

The similar appearance of the cells surrounding an individual macrophage suggests that they were in approximately equivalent stages of development. Moreover, in some erythroid islands a high frequency of mitotic figures was observed (Figs. 7, 8).

Scanning electron microscopy on day 8 revealed, as the transmission electron microscopy also indicated, that a portion of the erythroblasts possessed short microvilli, whereas others only showed a slight ruffling of the outer membrane (Fig. 10). Light microscopically differential counts were made; on day 8 only 5% of the hemopoietic cells in the central veins were lymphocytes and segmented neutrophils. Thus, since 95% of the cells were erythroblasts, a reasonable impression of their morphology could be obtained on this day with the scanning electron microscope. The CM-V projections varied from knobs to plates that branched into small processes (Fig. 10). The entanglement of the projections on the macrophage surface suggested considerable motility of these digitating processes. With this technique it could be clearly demonstrated that the elongated CM-V often shared small contact areas with erythroblasts. Some macrophages were not surrounded by erythroid cells.

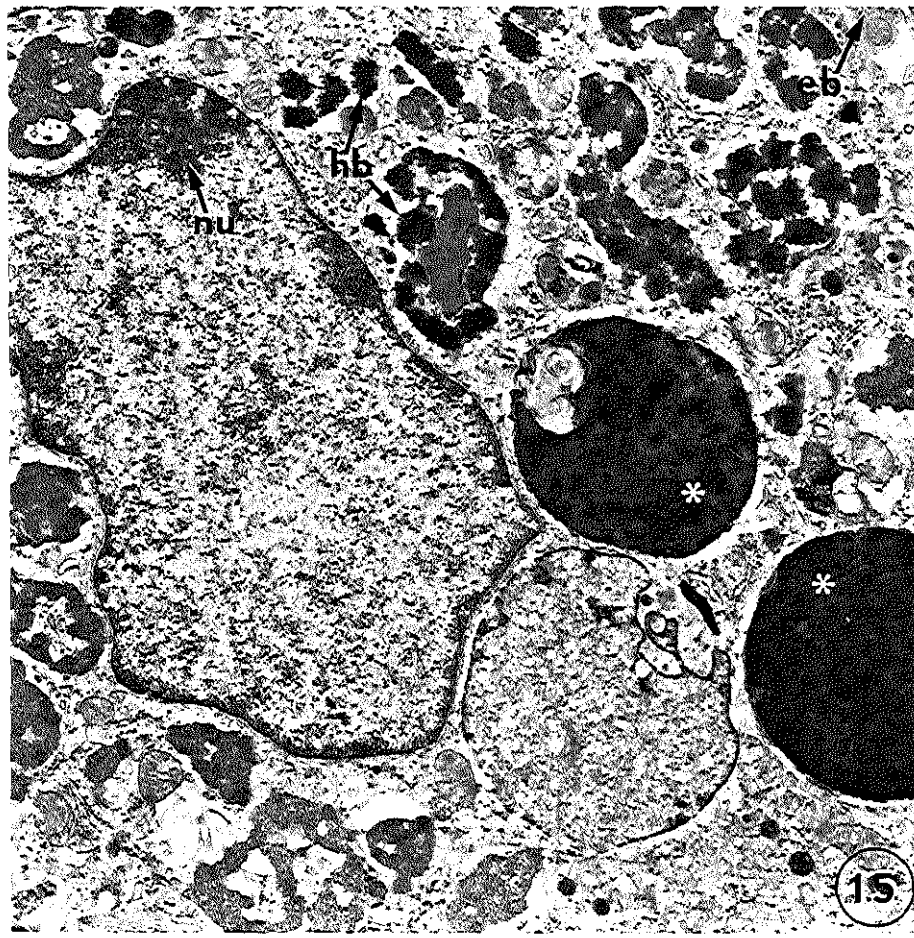


Fig. 15. Part of a CM-S on day 7. A part of a surrounding erythroblast (*eb*) can be seen in the upper right corner of the field. The CM-S contains the remainder of red blood cells in which the Heinz bodies (*hb*) can still be recognized after interiorization of the erythrocytes. *Asterisks* indicate two normoblast nuclei with a surrounding rim of cytoplasm. ×8150

Erythroid Islands in the Liver Sinusoids

In the sinusoids the number of erythroblasts forming islets was in general much greater than in the central veins (Figs. 9, 14) and sometimes amounted to about 100 cells per islet. As intravenously, from day 4 on erythropoiesis in the sinusoids showed all stages of maturation, including extrusion of normoblast nuclei. The number of erythroid elements surrounding a single CM often exceeded that which was found for a CM-V.

A part of the CM in the sinusoids (CM-S) was filled with mature red blood cells (Figs. 14, 15). The nuclei showing several prominent nucleoli, dispers

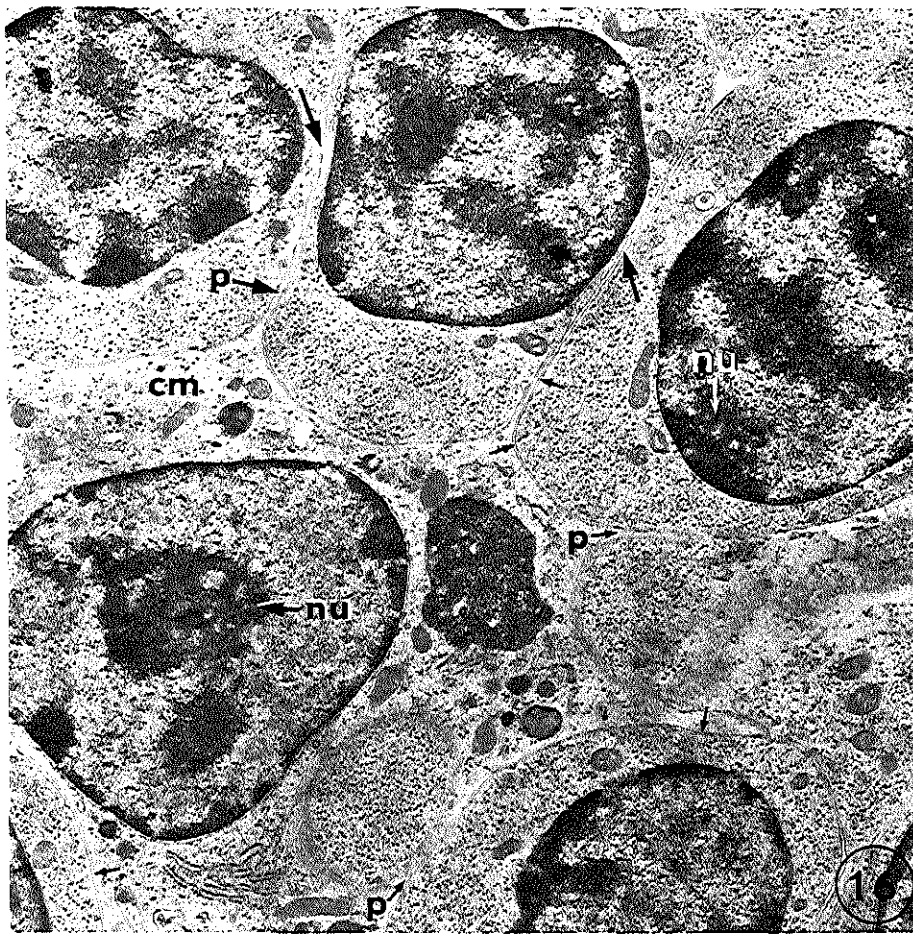


Fig. 16. Day 7. Typical arrangement of a stellate CM-S and surrounding early basophilic erythroblasts. Thin projections (*p*) of the "nurse cell" can be followed for some distance between the erythroblasts. Note the regular width between the CM and the erythroid elements (small arrows). This cleft is smaller than the one that is found between mutual erythroblasts (large arrows). ×8150

nucleoplasm and many nuclear pores, were often slightly indented by the great amount of phagocytized material.

Two types of nuclei could be distinguished in CM-S. One (Fig. 16) was similar to the CM-V nuclei illustrated in Figure 4 and had several prominent nucleoli in the center of the nucleus. A well defined small rim of condensed chromatin was interrupted by nuclear pores with clearly recognizable nuclear channels in the juxtanuclear cytoplasm. The second type of CM-S nucleus (Fig. 15) resembled that of Kupffer cells after day 1. The rim of condensed chromatin was smaller and less condensed whereas nuclear pores had less well defined nuclear channels compared to the first type of nucleus. Nucleoli were smaller in size and number and

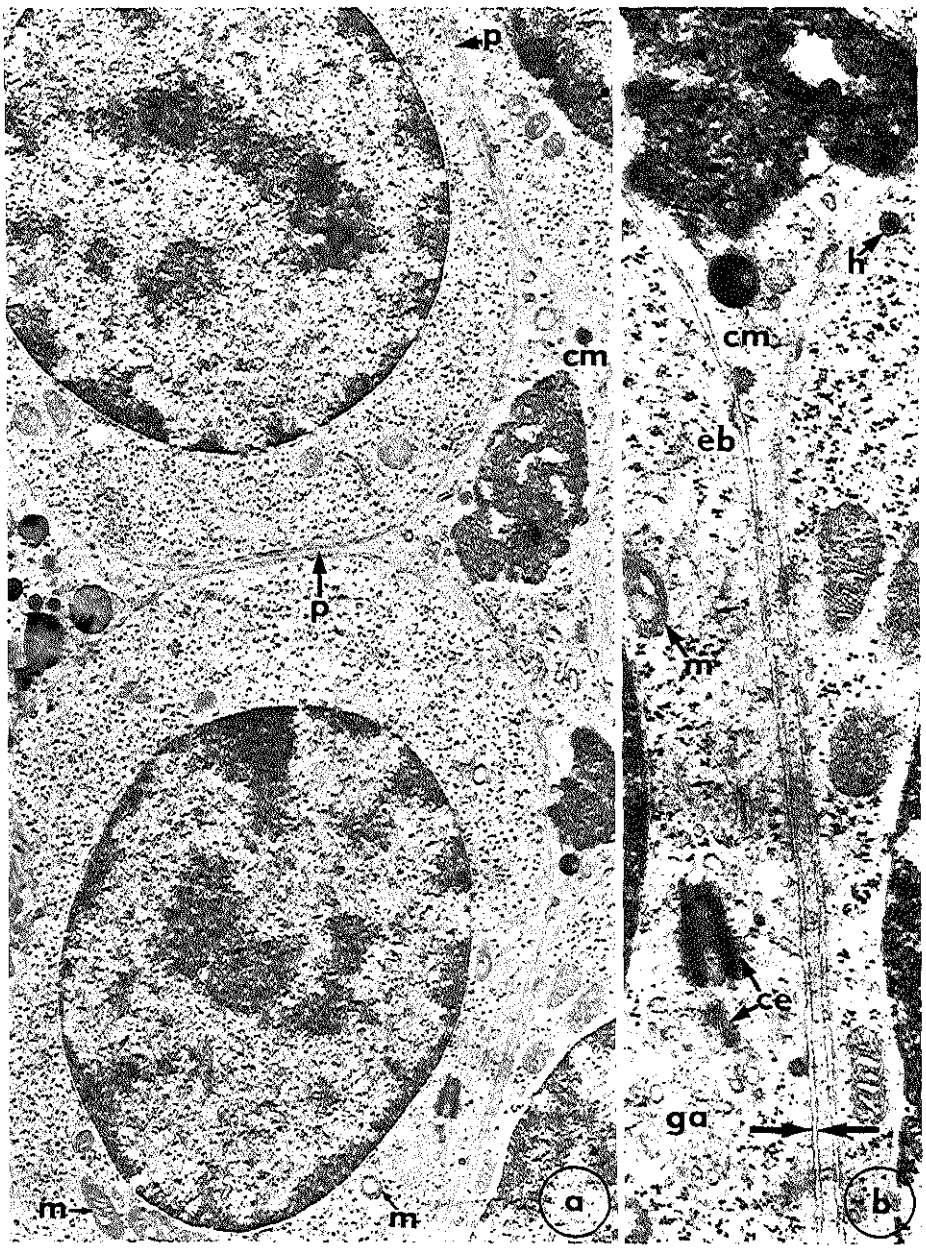


Fig. 17. a Day 7. Electron micrograph illustrating the regular thickness of CM-S projections (840 Å). Typical mitochondria (*m*) (not seen in any other type of cell in the liver) can be observed. × 11,570. **b** Detail from Figure 17a. Note the 250 Å cleft between the CM-S and erythroblast between large arrows. With this magnification microtubules can be recognized in the erythroid cytoplasm (small arrows). *ce* Centriole; *ga* Golgi apparatus; *h* hemosiderin granule. × 33,800

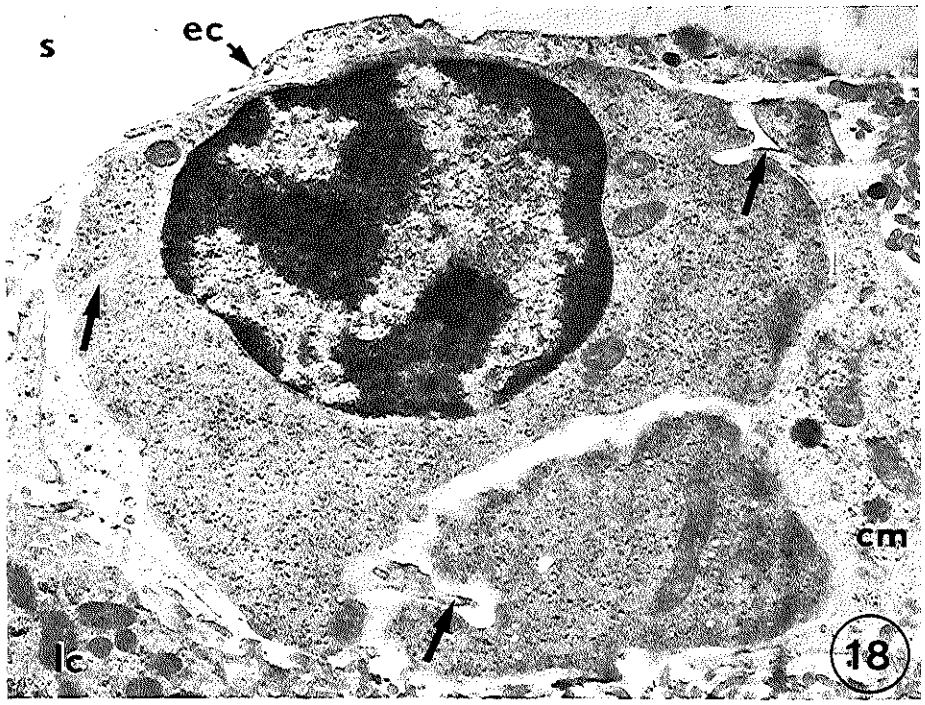


Fig. 18. Two polychromatophilic erythroblasts on day 7. In this extrasinusoidally located islet three short extensions indent the adjacent erythroid plasma membranes (*arrows*). *ec* Endothelial cell. ×11,570

were situated more peripherally in the nucleus. It is not clear whether these differences in the nuclear morphology of CM-S were due to differences in cellular activity or ontogeny. In general, the morphology of CM-S could not be distinguished from that of Kupffer cells. However, CM-S in older erythroid islands had occasionally phagocytized some expelled normoblast nuclei (Fig. 15). Only the cells which had differentiating erythroid elements at their periphery were considered to be CM-S.

The CM-S had a more stellate appearance than CM-V. This was probably due to a lack of space in the sinusoids so that proliferating erythroblasts deformed the CM-S. In the sinusoids a closer contact between erythroblasts and CM could be observed than in the central venes. CM-S often branched between the closely packed erythroblasts (Fig. 16). Their thin projections, resembling cytoplasmic sheets, had an average thickness of 840 Å. The cleft between the outer membranes of such a macrophage and the surrounding erythroblasts had a very regular width of 250 Å, while the distance between erythroblasts of the same island measured 370 Å (Figs. 16, 17). The regular distances (all standard errors of less than 8%) found between these cells were probably due to their glycoprotein coats, that could not be demonstrated with the present techniques. Rarely, a short fingerlike projection of a CM was seen indenting the outer membrane of an erythroblast.

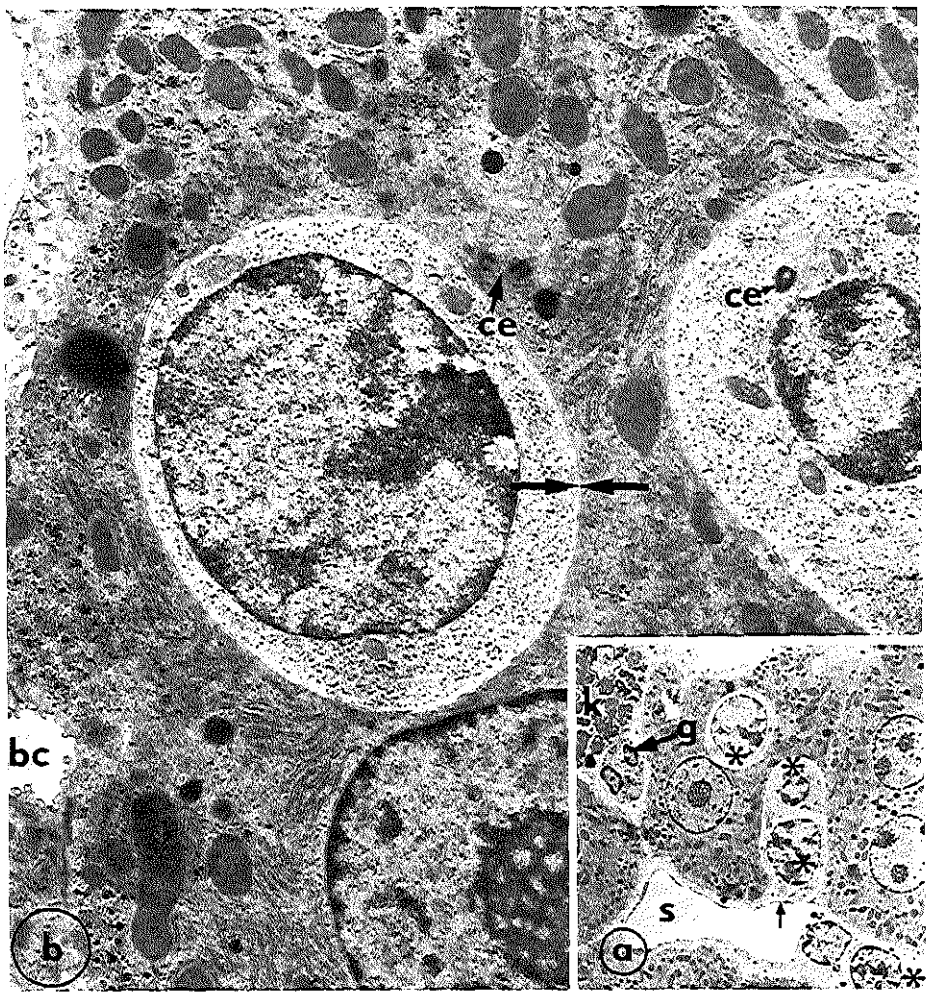


Fig. 19. a Micrograph showing a section through a cluster of erythroid cells in the hepatic parenchyma on day 7, separated from the sinusoid by an endothelial cell (*arrow*). *g* Neutrophil; *k* Kupffer cell; *asterisks* erythroblasts. ×1372. **b** Detail from Figure 19a. No littoral cell separates the erythroblast and the hepatocyte. Note the regular distance of 250 Å between the adjacent cells (between arrows). *bc* Bile canaliculus. ×8476

This type of cellular contact was only seen in extrasinusoidal islands (Fig. 18). In the thin macrophage projections neither ribosomes, other cell organelles nor ferritin could be observed. Cell contacts different from the types described above were not observed. The observations suggest that the amount of phagocytized material determines the extent with which the CM-S is able to surround the erythroblasts. According to this view a swollen cell filled with red blood cells has a restricted surface area to form cytoplasmic processes.

Often the erythroid elements in one island and even in islands in which more

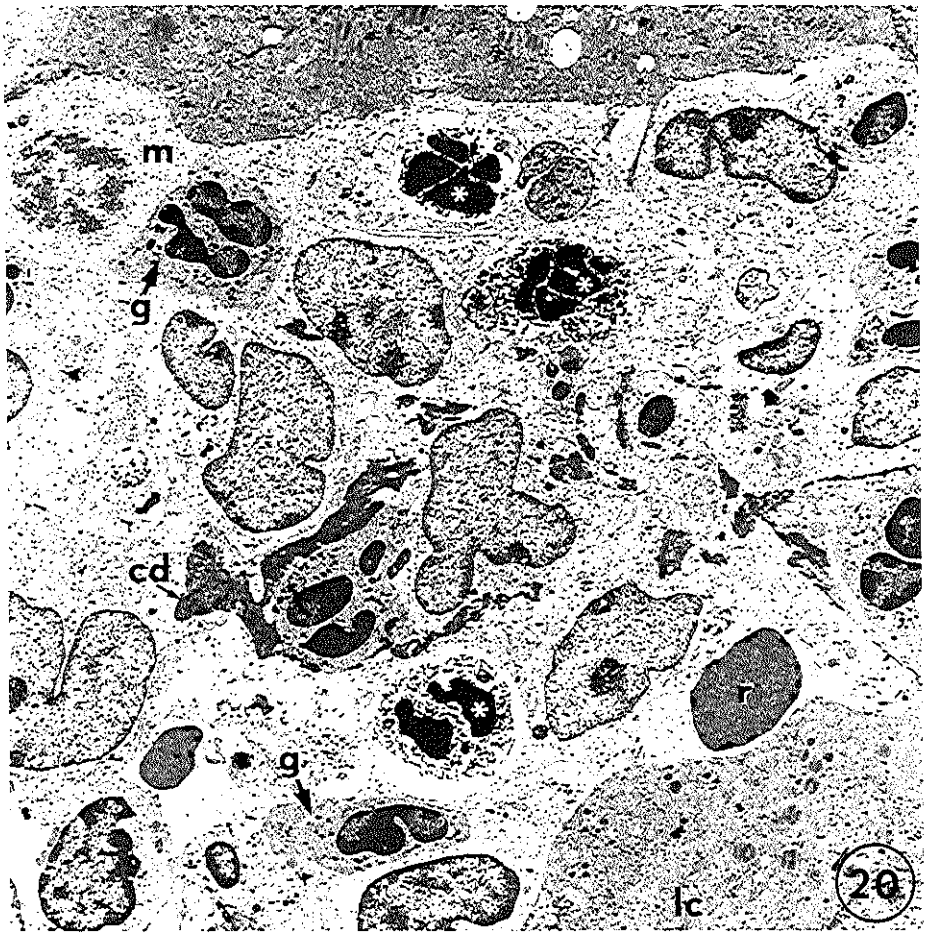


Fig. 20. Exudate between parenchymal cells on day 1. *Asterisks* indicate pyknotic neutrophils. *g* Segmented neutrophil; *m* mitotic figure in littoral cell; *cd* collagen deposits. × 3390

than one CM-S was present were in the same stage of development. Moreover, measurements on the nuclear diameter of erythroblasts belonging to one distinct island showed a significant smaller deviation than could be expected from a random distribution of erythroblasts of all maturational stages. This may suggest a synchronization in development of cells belonging to one islet. The frequency of mitotic figures in erythroblasts per island also suggested a synchronization of the cell cycle (Fig. 9). Erythroblasts in telophase were sometimes found to be connected by a cytoplasmic bridge with spindle remnants, of which the microtubuli sometimes could be followed to the newly formed nuclei. Fleming bodies were also present as a part of the cytoplasmic bridge.

As described for the intravenous erythropoiesis, no indications of a ferritin transport from the CM-S toward the surrounding erythroblasts could be de-

monstrated. The presence of intramitochondrial ferritin was in no case encountered. As to the coated vesicles similar observations as in intravenously located erythroblasts were found.

Especially after day 3 of the PHZ treatment some erythroid islands were observed to expand in such a way that the adjacent hepatic cell surfaces were thrown into irregular folds with littoral cells (Kupffer cells and endothelial cells; Kelly et al., 1962) located between (Fig. 9). Sometimes erythroblasts of a more immature type were found nearly completely surrounded by the cytoplasm of a hepatocyte without an intervening littoral cell (Fig. 19). In this case as well desmosomes or tight junctions as described by Fukuda (1974) in human fetal liver did not occur. The distance between the two cell types measured 250 Å.

Exudates

From day 1 until day 5 and sometimes after day 10 small exudates with invaded neutrophils intermingled with littoral cells, lymphoid cells and monocytes could be found (Fig. 20). The neutrophils were often pyknotic. The parenchymal cells in this area were peripherally vacuolated, and desmosomal contacts between adjacent hepatocytes around the bile canaliculi extended for a much longer distance than in non-affected liver areas. The observations suggest that in the first day after PHZ treatment not parenchymal cells, although locally vacuolated, but littoral cells are damaged due to the PHZ treatment, the debris being cleared by neutrophils. In some animals hepatocytes were observed to be destroyed after more than one week. A rise in the mitotic rate of liver parenchymal cells around day 14 indicated a recovery from this delayed destruction. The replication of littoral cells in the exudates, judged by the presence of mitotic figures in these cells (Fig. 20), showed that the liver phagocyte population is not fully dependent on immigrating cells.

Segmented neutrophils were not only found in exudates. Throughout the entire liver a peak in the number of these cells was observed on day 3. On day 2 an increasing number of lymphocytes entered the liver; the highest numbers were observed on day 4 and 5.

Discussion

Morphologic studies in the past two decades have confirmed the extravascular nature of erythropoiesis in the yolk sac (Sorenson, 1961), fetal liver (Ackerman et al., 1961; Chui et al., 1974; Fukuda, 1974; Grasso et al., 1962; Jones, 1959; Rifkind et al., 1969; Sorenson, 1960; Zamboni, 1965), bone marrow (Ben-Ishay et al., 1971A; Berman, 1967; Bessis et al., 1962; Pease, 1956; Sorenson, 1962; Tanaka et al., 1966) and the spleen (Djaldetti et al., 1972; Orlic et al., 1965; Pictet et al., 1969) of various mammals, including man. The results of the present study on the location of erythropoiesis in the liver indicate that, in contrast to the extravascular erythropoiesis described above, the presence and production of red blood cells induced in the adult liver occurs mainly intravascularly.

In none of the erythroid developmental stages were abnormalities seen as has been described in severe iron deficiency anemia (Hill et al., 1972), sideroblastic anemia (Sorenson, 1962), aplastic anemia (Frisch et al., 1974 A) and several hemoglobinopathies (Hollán et al., 1968; Frisch et al., 1974 B). Therefore, we have the impression that neither the PHZ-induced severe hemolytic anemia nor PHZ itself caused abnormalities in erythroblasts.

In both intrasinusoidal and intravenous erythroid islands the presence of phagocytizing macrophages could be demonstrated. In this respect, it may be of importance to note, that the first erythroblasts in the central veins were observed at a time when CM-V had developed their phagocytizing ability, namely on day 3. An identical coincidence was observed by Zamboni (1965) between the onset of human fetal liver erythropoiesis and Kupffer cell phagocytic activity.

Concerning the morphologic features of the erythroid islets in the adult liver, our studies demonstrated that they are more comparable with hemopoiesis elsewhere than with fetal liver erythropoiesis. Firstly, a number of studies point out the combined presence of CM and erythroid elements in the yolk sac (Sorenson, 1961), bone marrow (Bessis et al., 1956 A; Marmont et al., 1962; Sorenson, 1962) and the spleen (Orlic et al., 1965; Pictet et al., 1969). It is remarkable that in the fetal liver a cellular contact has never been described between developing erythroblasts and phagocytizing macrophages (Chui et al., 1974; Fukuda, 1974; Grasso et al., 1962; Zamboni, 1965). Moreover, microtubuli are present in hepatic erythroblasts in our study and are characteristic for adult erythropoiesis (Grasso, 1966) but were rarely seen in human fetal liver erythropoiesis (Fukuda, 1974). The manner in which erythroblasts are stereologically interrelated with CM has a similarity with the descriptions of Ben-Ishay et al. (1971 B) in rat bone marrow.

Several reports have been published in which a fingerlike process of the CM is described that indents the erythroblast membrane and may even extend deeper into the cytoplasm in human bone marrow (Bessis et al., 1956 B, 1957; Sorenson, 1961) and the guinea pig yolk sac (Sorenson, 1961). In our study this phenomenon was only occasionally observed in the space of Disse. In the adult liver some of the proerythroblasts did not make contact with a CM. Similar observations were made by Djaldetti et al. (1974) who observed that only erythroblasts of later maturational stages appear in islands between mesenchymal cells in embryonic spleens.

A generally described property of the CM is its ability to transfer ferritin, formed out of ingested erythrocytes, to erythroblasts. This phenomenon would occur on those places where membranes of both cell types are in close apposition. The ferritin uptake is thought to occur by means of "ropheocytosis", which is a pinocytotic process (Bessis et al., 1959; Policard et al., 1959; Sorenson, 1961). However, evidence in favor of such a transport mechanism could only clearly be seen in several hematological disorders (Bessis et al., 1962; Sorenson, 1962). In normal mouse bone marrow Berman (1967) could only occasionally demonstrate ropheocytotic vesicles in erythroblasts and at a considerable distance from a central reticulum cell. This observation is in accordance with the present study where only vesicles without ferritin could be found. These vesicles were also observed in cell sites not apposing the macrophage surface. Jones (1965) and other authors made the same observations. Therefore, the existence of coated vesicles in erythroblasts appears not to be connected with the presence of ferritin. Jandl

(1960) suggested the possibility that ropheocytotic vesicles containing ferritin could be indicative of a process with which erythroblasts are able to release an excess of accumulated ferritin due to non-specific uptake or an excessive or deranged transferrin incorporation. Sullivan et al. (1976) found evidence that ropheocytotic vesicles are involved in transferrin uptake from the serum. In this concept the CM is not necessary as a direct iron-donor as Lajtha (1955) already demonstrated.

Some authors have mentioned the occurrence of erythroid cell clusters around a single CM in which all elements display common morphologic features (Orlic et al., 1965). Moreover, sometimes several coronas of erythroblasts can be observed around a CM with the oldest elements being at the periphery (Bessis et al., 1962; Le Charpentier et al., 1975; Marmont et al., 1962; Marmont, 1960). Our observations support and extend these findings, although in the central veins never more than a single corona could be clearly observed. Orlic et al. (1965) interpreted this phenomenon for the erythropoietin-induced red blood cell formation in the mouse spleen as resulting from a wave of cell divisions out of identical progenitor cells that are triggered at the same time by erythropoietin. In our study a similar synchronization may be expected, since by injection of PHZ a heavy stimulation of erythropoiesis is created. The observations of Friederici (1958), who described a parallelism of the serum erythropoietin titer and the degree of anemia in PHZ-treated rabbits, confirm this view. However, in the initial period of the PHZ treatment, no great amounts of erythropoietin sensitive progenitors are thought to be available in the liver. A necessary migration of these cells or stem cells toward the liver will give rise to a more or less asynchronous lodgment and differentiation, resulting in maturation differences of separate erythroid islets in the liver. This difference favors the conclusions that only one progenitor cell gives rise to a single erythroid island by synchronized proliferation and maturation patterns. The several coronas around a macrophage, with the oldest elements being at the periphery, could reflect a cyclic regulation of proliferation inside a single island. Another explanation for the presence of several coronas is that they may be a reflection of the asynchronous lodgment and subsequent proliferation of more than one stem cell or erythroid progenitor cell in an island. Whether this phenomenon has anything to do with a macrophagic influence on erythroid kinetic patterns is not yet known.

In the present study using morphologic criteria it was not possible to detect stem cells with a morphology similar to that described by Van Bekkum et al. (1971) and Rubinstein et al. (1973). Light microscopically, a number of transitional stages were observed between cells that resembled lymphoblasts and proerythroblasts. However, we could not make a precise description of the differential changes toward proerythroblasts on the ultrastructural level. An indication that stem cells may be the progenitors for the erythropoiesis in the adult liver is given by the demonstration of high numbers of colony forming units on day 4 with the exogenous colonization technique of Till et al. (1961). These data will be published elsewhere.

The presence of erythroid mitotic figures and all erythroid stages that can be recognized morphologically, and which are partly synchronized per island, indicates a real erythropoiesis in the adult mouse liver after PHZ. Whether or not the

hemopoietic microenvironment in the liver is only temporarily able to sustain erythropoiesis cannot be concluded from our observations.

In the present study extrusions of vesicles with membranous inclusions that were similar to portions of mitochondria were seen in more mature erythroblasts. This phenomenon was also described by Jones (1959) in fetal human liver erythroblasts, as a process paralleling the extrusion of normoblast nuclei.

The deformed shape of some hepatocytes resulting from the presence of proliferating erythroid cells in the sinusoids has also been described in fetal liver by other authors (Ackerman et al., 1961; Grasso et al., 1962; Jones, 1959; Zamboni, 1965; Fukuda, 1974). The distances between mutual erythroblasts and hepatocytes were in accordance with the observations of Grasso et al. (1962) in fetal liver.

The ability of Kupffer cells to phagocytize large amounts of blood elements is not a common one. The greater part of erythroclasia under normal conditions takes place in the spleen. Only in pathologic states as in hemolytic anemias and after splenectomy is erythrophagocytosis extensive in Kupffer cells (Bessis et al., 1962) and bone marrow (Marmont, 1973; Marton, 1973), whereas the greatest part of clearance by means of mechanical trapping of Heinz body-containing red blood cells takes place in the sinus wall apertures of the mouse spleen (Klausner et al., 1975).

The regularly observed association of erythroblasts and CM indicates an interdependency of these cell types. Many different interpretations have been made for these observations. Two of them, namely 1) the macrophage property to detain the differentiating erythroblasts, preventing their release into the circulation and 2) the ability to phagocytize expelled normoblast nuclei and disarranged erythroid elements (Ben-Ishay, 1974; Weiss, 1965) are very suggestive, but the underlying processes are not clearly understood. Further, the macrophages may function to facilitate cell interaction between erythroblasts of the same stage of differentiation, permitting a specific control of local population size. Other considerations, in which the macrophages induce the differentiation of uncommitted stem cells and control the rate of entry and release of circulating stem cells and progenitor cells, are highly speculative. Ben-Ishay et al. (1974) using the electron microscope could demonstrate in bone marrow after sublethal irradiation, that lodgment of lymphoid cells on CM and subsequent differentiation of these lymphoid cells toward the erythroid compartment occurred. A practical interpretation for the postulated properties of a CM is that it requires a CM to recognize membrane (in-)compatibilities, whether these are due to damage, aging, somatic mutation or differentiation. A possible mechanism to confer specificity between cells was proposed by Mayhew (1974), who suggested the involvement of a polynucleotide base sequence. Moreover, some authors indicate an involvement of surface change properties or cell coat composition (McCuskey et al., 1973).

The general occurrence of CM in erythroid islands raises questions concerning the morphological features and origin of these cells in several organs. Morphologically, similar macrophages have been found in the several organs involved in erythropoiesis. The subplasmalemmal zone of higher electron density, that was found in CM and contained microfilaments and tubuli, has also been observed in mouse Kupffer cells by Singh (1974), rat Kupffer cells (Wisse, 1974), neutrophils

(Davies et al., 1973) and macrophages (Allison et al., 1971). It is thought that it provides the structural basis for the membrane movement necessary for endocytosis in these cells.

The appearance of monocytes, which seem to differentiate in about two days into phagocytizing CM in the adult liver after PHZ treatment, may represent the migration of a progenitor of a cellular component of the erythroid microenvironment. This phenomenon was most clearly demonstrated in the central veins. In the sinusoids these cells and Kupffer cells are very much alike after some days of PHZ treatment. Therefore, it is not clear whether Kupffer cells are involved in microenvironmental induction of erythropoiesis also. In the fetal liver Kupffer cells never function as a CM (Fukuda, 1974) nor do perisinusoidal mesenchymal cells. Observations of Van Furth et al. (1970), who described the possibility of monocytes to differentiate in vitro into macrophages within 48 h, and Parakkal et al. (1974), who studied changes in surface morphology during the in vitro differentiation of monocytes to macrophages, support the observed monocyte differentiation in the present study. Also in vivo, monocytes can differentiate into tissue macrophages according to the observations of Ebert et al. (1939). Kinsky et al. (1969) and Souhami et al. (1974) have shown that macrophage populations in the mouse liver can be augmented by precursor cells from the bone marrow. According to Van Furth et al. (1968; reviewed by Van Furth in 1970) the origin of monocytes is the bone marrow. From the preceding evidence we can propose a bone marrow origin of the CM in the adult liver during PHZ treatment. The concept of migration of a cellular component of the HIM has never been accepted as a rule. Wimer et al. (1969) found indications of migration after endosteal curettage in patients with myelofibrosis. In contrast, Knospe et al. (1966) demonstrated that the secondary aplasia, induced by heavy local irradiation of a long bone, could be cured only by transplantation of bone marrow into the affected area. Also, marrow fragments that were transplanted to extramedullary sites have been observed to survive by proliferation of the reticular cells of the surviving stroma after initial necrosis, but not by proliferation of invading stromal cells (Tavassoli et al., 1968). Knospe (1972) found indications that the presence of a sinusoidal system with an appropriate microenvironment determines the presence of hemopoiesis. In the adult liver the sinusoidal structure is not heavily damaged by PHZ, thus allowing erythropoiesis to occur if microenvironmental factors are present to sustain this. Possibly the invaded monocytes may function as progenitors of a cellular component of a microenvironment favorable for proliferation and differentiation of erythroblasts. We found no conclusive evidence for a function of liver CM that extends beyond a detainment of erythroblasts and erythrophagocytosis. However, an inductive function is not excluded.

As to the sinusoidal system in the liver it seems reasonable to suppose that Kupffer cells play a role in regulating the blood flow and as a consequence the local oxygenation. Rüttner et al. (1957) and Motta (1975) proved that Kupffer cells in the rat can control and even block the blood flow in parts of the liver by bulging into the lumen of the sinusoids. Our observations indicate that a somewhat hypoxic environment is favorable for proliferation and differentiation of erythroblasts that can morphologically be recognized, since these cells were found predominantly in the central veins and terminal parts of sinusoids.

In conclusion, several morphological phenomena of erythropoiesis were studied on PHZ-induced erythropoiesis in the liver. Microenvironmental factors involved in erythrocyte production in the liver were also investigated. However, although it is indicated that the macrophages may play an important role, no definite parameter for a suitable erythropoietic microenvironment can be given.

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Kinetics of Erythropoiesis in the Liver Induced in Adult Mice by Phenylhydrazine

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Phenylhydrazine treatment of normal mice elicited a rise in the numbers of CFU-S in blood, spleen and liver. High numbers of CFU-S were found in blood and liver 4 d after the first phenylhydrazine injection. CFU-S in the liver decreased slowly and were nearly absent after 2 weeks. Blood CFU-S returned to normal levels by day 6, whereas spleen CFU-S numbers remained high upto day 12 with a 20-fold increase being apparent between days 5 and 8. Bone marrow CFU-S numbers were relatively unaffected except for a dip between days 4 and 7 with a nadir at day 5 where numbers decreased to 50% of the control levels.

Approximately 40% of liver, spleen and blood CFU-S, present on the 4th d after initiation of phenylhydrazine treatment, were killed with a single dose of hydroxyurea whereas bone marrow CFU-S numbers were not significantly reduced by the drug.

Splenectomy performed before (21 d) or during phenylhydrazine treatment did not diminish the number of CFU-S found in the liver on day 4. A 3 d interval was observed between peak numbers of CFU-S and erythroblasts in the liver which suggests that hepatic CFU-S are able to undergo differentiation along the erythroid pathway. The presence of macrophages was correlated with that of erythroblasts in the hepatic central veins. These macrophages may be essential to the liver environment for induction of erythropoiesis.

Key words: anaemia – colony forming units – liver – murine erythropoietic tissue

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Repeated injections of phenylhydrazine (PHZ) induce severe anaemia in adult mice. The anaemia is associated with a wave of extra-medullary erythropoiesis in the liver which we have described morphologically in a previous study (Ploemacher & van Soest 1977a). Other observations of erythropoiesis in adult liver have been made in PHZ-treated salmon (Smith et al. 1971), in man where metastatic tumor displacement of bone marrow has occurred (Jordan 1942) and in splenectomized mice after hypoxic treatment (Bozzini et al. 1970).

An increase in the number of monocytes was observed in the liver where they underwent differentiation in the first 3 d into erythrophagocytizing macrophages, around which multiplication and differentiation of erythroblasts was observed (Ploemacher & van Soest 1977a). The presence of macrophages in erythroid islands has also been described in the yolk sac (Sorensen 1961), spleen (Orlic et al. 1965) and bone marrow (Le Charpentier & Prenant 1975). Moreover, macrophages con-

stitute the central part of intravascular erythroid islands in the bone marrow of PHZ-treated mice (Ploemacher & van Soest, 1977b).

We examined the relationship between the numbers of macrophages and the number of erythroblasts in the liver after PHZ treatment in order to determine the role of these cells with respect to the erythropoietic microenvironment.

A morphological investigation was done in order to identify the cells responsible for liver erythropoiesis (Ploemacher & van Soest 1977a). The presence of proerythroblasts in the liver during the first few days after initiation of PHZ-treatment was observed. However, this did not discount the possible stem cell or differentiated erythroid precursor cell origin of the liver erythroid cells. Hara & Ogawa (1976) reported a significant increase in the number of blood BFU-E in mice 2 d after initiation of a PHZ-treatment. We have investigated the changes in numbers and distribution of CFU-S in liver, spleen, bone marrow and blood of PHZ treated mice in order to gain further information about the origin of erythropoietic cells in the liver.

Materials and Methods

Mice

Male CBA/RIJ mice, 19 weeks old and with a wt. of 27-29 g were used for the spleen colony assay. For the histological studies male CBA/Rij mice, 12-20 weeks old and weighing 22-28 g were used. The mice were purchased from the Medical Biological Laboratory TNO, Rijswijk, The Netherlands.

PHZ-treatment

Haemolytic anaemia was induced by 4 daily intraperitoneal injections of 1 mg of phenylhydrazine chloride (PHZ) (Merck, Darmstadt). 4 injections of 0.5 or 0.1 mg daily or 3 daily injections of 1 mg were less toxic but caused a less pronounced erythropoiesis in the liver of the animals. The treatments induced a severe haemolytic anaemia that lasted for 8 d, whereas no mortality was observed. The day of the first injection was designated as day zero. On each day until day 14 at least 7 animals were sampled for microscopical studies.

Irradiation

The recipient mice received a lethal dose of 950 rad whole body irradiation generated in a Philips Müller MG 300 X-ray machine. Animals were irradiated in well-aerated circular Perspex cages. Physical constants of the irradiation were: 250 kV (constant potential); 11 mA; added filtration of 1.0 mm Cu; irradiation was corrected for field heterogeneity; focus object distance 53 cm; animals were irradiated at a dose rate of 30-35 rad/min. Maximal backscatter was achieved by placing the cage on a layer of 11 cm hardboard. During irradiation the dose was measured with a Baldwin Ionex dosemeter.

Total body perfusion

Total body perfusion under phenobarbital sodium (70 mg/kg body wt.) anaesthetization was carried out in one experiment. A balanced salt solution of Mishell & Dutton (1967) at room temp. was used to perfuse mice. The solution was brought into the left heart ventricle and permitted to flow out through the right atrium. Perfusion was carried out until the liver had become pale-yellow which occurred within 1 min.

Spleen colony assay

The number of CFU-S was determined by the spleen colony assay of Till & McCulloch (1961). Pooled cell suspensions of marrow, spleen and liver were made from 5 mice per point and diluted in balanced salt solution. Each blood sample used for the CFU assay was obtained from the suborbital plexus from mice under ether or, in the case of perfused mice, under phenobarbital anaesthesia. Blood samples were pooled and appropriate dilutions made so that injected vol. of 0.5 ml contained at the most 20 spleen colony forming units. The correct dilutions were estimated from previously performed pilot experiments. Cell suspensions were injected within 2 h after irradiation of the recipients. For liver suspensions the maximal amount which could be injected was restricted to a maximum of 1/20 of a liver per mouse; higher concentrations caused death from embolism. The spleens were removed 7 d after inj. and fixed in Telleyesnizky's solution. The colonies were counted under the low power objective of a stereo-microscope.

To test whether CFU-S in spleen, marrow, liver and blood were actively proliferating 4 d after initiation of PHZ-treatment, 4 PHZ treated donors received 900 mg/kg hydroxyurea (Rencricca et al. 1970) intraperitoneally 3 h before sacrifice.

Preparation for microscopy

Perfusion fixation was carried out through the portal vein under phenobarbital anaesthesia. The method of Wisse (1970) was used with slight modifications, as were described in a previous paper (Ploemacher & van Soest 1977a).

After fixation in respectively 1.5% glutaraldehyde (Fluka AG, Switzerland) and 1% OsO_4 in 0.075 M phosphate buffer (Millonig 1961, pH 7.2; 310 milliosmols) tissues were dehydrated in a graded ethanol series and embedded in Epon. 1.5 μm sections were prepared for light microsocpy with a Reichert OM U2 and OM U3 microtome. The sections were coloured with an acqueous solution of toluidine blue adjusted to pH 8.6 with 0.1 Borax Buffer. Coverslips were mounted with Entellan (Merck, Germany).

Determination of absolute and relative cell numbers in blood, spleen and bone marrow

The degree of anaemia was determined by counting the number of erythrocytes per ml in the peripheral blood and assessing the haematocrit value. Blood used for haematrocrits, erythrocyte counts and absolute and differential leucocyte counts was obtained from the suborbital plexus. The total number of nucleated cells in blood, spleen and bone marrow was determined with a Model B Coulter electronic particle counter. The percentage of different leucocyte types was determined in May-Grünwald-Giemsa stained blood smears and spleen and bone marrow sedimentation preparations, as described by Sayk (1960).

Determination of absolute numbers of cell types in the liver

The numbers of erythroblasts and central macrophages (CM) that were sectioned through the nucleus were counted in light microscopical preparations. The surface area of the liver sections was measured. By determining the vol. of the liver in each mouse on each day and the diameter of the nuclei of the various cell types, the total number of these cell types per liver could be calculated according to Weibel (1969).

Splenectomy

Mice were splenectomized under phenobarbital anaesthetization. Sham-splenectomized mice were treated exactly as splenectomized mice, except for the removal of the spleen.

Results

Spleen and liver weights

Liver wt. fell during the first 6 d and thereafter increased to that of controls by day 9. The wt. of the spleen increased up to day 7, coincident with an increased number of nucleated cells, due to excessive erythropoietic activity and erythroclasia. 14 d after the first PHZ injection the spleen wt. had almost returned to the pretreatment level (93 mg; control, 70 mg).

Changes in blood, spleen and bone marrow cell populations

From day 2 until day 5 some large mononuclear blasts could be observed in the blood. These cells had 1 or 2 distinct nucleoli in a leptochromatic nucleus and showed a more or less basophilic cytoplasm. They had a morphology which was similar to that of lymphoblasts or early proerythroblasts. A frequent appearance of neutrophil bands and an increase number of monocytes were characteristic for blood smears from days 3 and 4.

In the spleen as well as in the bone marrow an erythropoietic wave was observed with more than 40% of the total nucleated cell number in the bone marrow being erythroid on day 3 (control value, 27%) and 50% on day 8. On day 9 a sharp decline in the percentage of marrow erythroblasts occurred with normal levels being reached by day 12.

Cells in the liver

(a) Erythroblasts. In the central veins erythroblasts first appeared on day 3 reaching a peak number on day 5 (Figure 1). In the remainder of the hepatic tissue erythroblasts were apparent by day 1 reaching a peak level on day 7 followed by a lower plateau on days 8-10. Erythroblasts disappeared from the central veins on day 11 and from the rest of the liver on day 13. Determination of the diameters of liver erythroblasts and their nuclei revealed a largest diameter on day 7. This indicates that a high percentage of young erythroblasts was present.

A stimulation of the mitotic rate in erythroblasts was observed at days 4 and 7 (Figure 2). The first peak coincided with a peak mitotic activity in the spleen and bone marrow whereas the second peak lay in between two mitotic peaks at day 6 and a smaller one at day 8 in spleen and marrow (Figure 2). These observations suggest that hepatic erythropoiesis is subjected to similar regulatory mechanisms as those, which induce the periodic stimulation of mitoses in the spleen and the bone marrow.

(b) Macrophages. In the erythroid islands erythroblasts are found around phagocytizing cells, which we called central macrophages (CM; Ploemacher & van Soest 1977a). The presence and number of CM were studied in central veins only. These

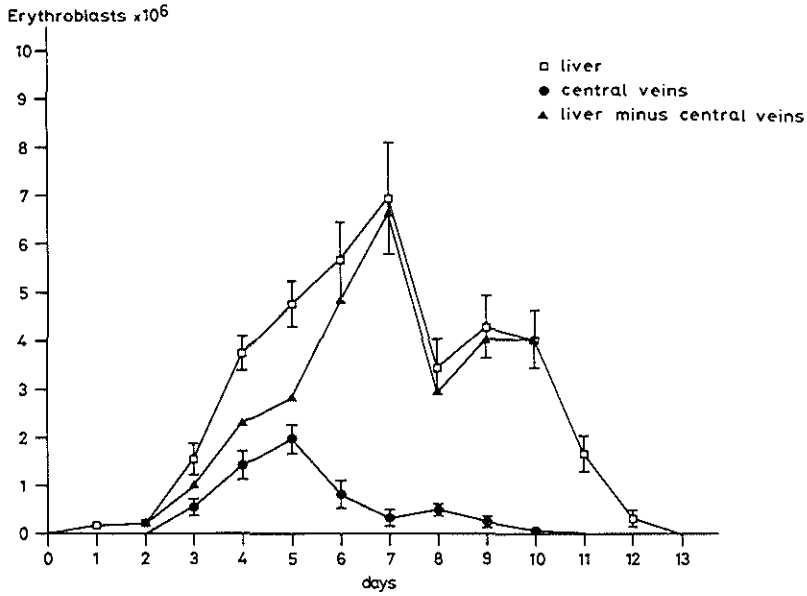

Figure 1. Absolute number of erythroblasts in the liver of PHZ-treated mice. The means of at least 7 animals (± SEM) are expressed in the graph. □ = total erythroblast number per liver; ○ = number of erythroblasts in the central veins; △ = number of intra- and extra-sinusoidal located erythroblasts, estimated by substracting the erythroblast number in the central veins from the total number per liver.

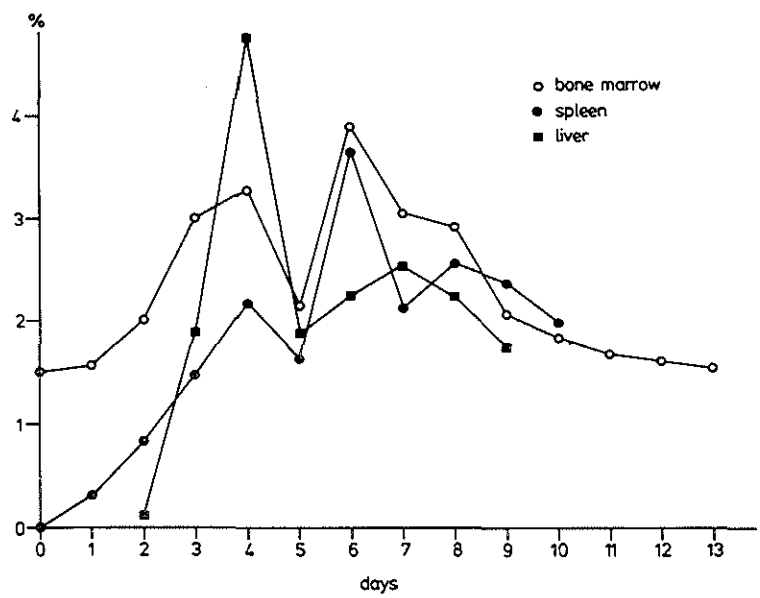

Figure 2. Mitotic indices of haemopoietic cells in liver, spleen and bone marrow expressed as the percentage of cells found in metaphase and anaphase. The means of 7 animals per experimental point are plotted. In each animal the percentage of mitoses was determined in 2000 cells, and in the liver in at least 500 cells.

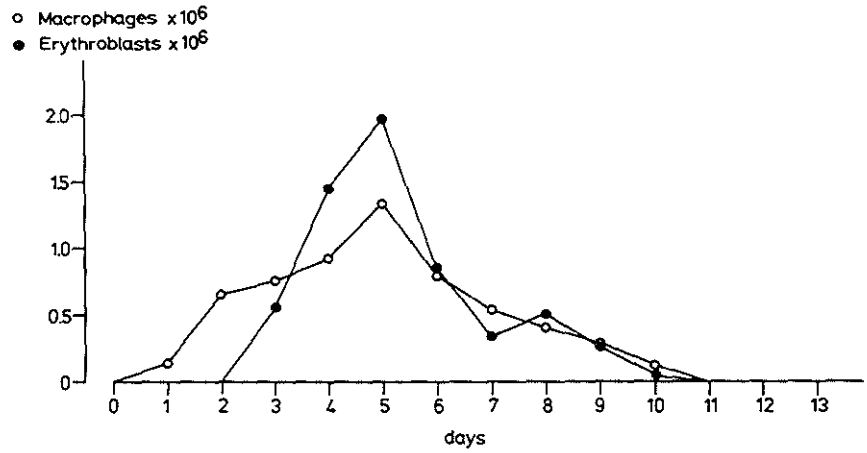

Figure 3. Correlation between the number of erythroblasts and macrophages (monocytes and macrophages) in the central veins of the liver after PHZ treatment. Each point represent the average cell number per liver determined in at least 7 animals.

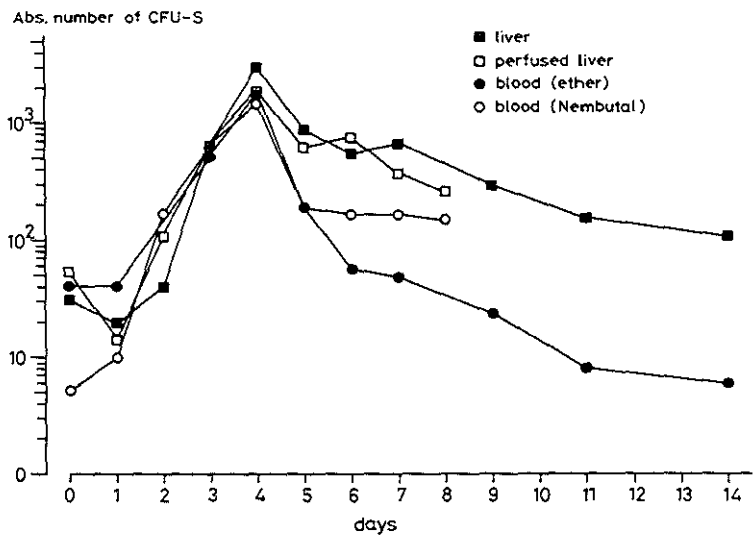

Figure 4. Stem cell content (CFU-S/liver, 1 ml blood) of the liver and blood of PHZ-treated mice. Standard errors were between 23 and 30%. In one experiment total body perfusion was carried out just before sacrifice. ■ = liver; □ = perfused liver; ● = blood from ether anaesthetized mice; ○ = retro-orbital blood from Nembutal anaesthetized mice just before total body perfusion. In each experiment the livers and blood of 5 donor mice were pooled.

cells are not easily distinguished from Kupffer cells in the sinusoids of the liver. All monocytes and macrophages that stuck to the endothelial wall, whether they were surrounded by erythroid elements or not, were counted. Figure 3 shows that the monocytic cells appear 2 d before erythroblasts can be found in the central veins. The absolute number of both macrophages and erythroblasts in the liver showed a rise up to day 5. Mitotic figures were never found in the macrophage population.

CFU-S in blood, spleen, bone marrow and liver

PHZ inj. induced a sharp increase in the number of blood and liver CFU-S (Figure 4) on day 3. In the blood the CFU-S level on day 2 was already higher than that at day 0. Their number in both blood and liver peaked on day 4 with values that were about 50 times as high as the untreated control values. Subsequently the number of blood CFU-S decreased rapidly.

In the liver the number of CFU-S parallelled that of the blood up to day 4. From day 6 the CFU-S number declined gradually during the subsequent days and was still slightly above controls at day 14. No pronounced differences were found between CFU-S numbers in perfused and non-perfused livers, which indicates that the high amount of stem cells found in the liver is not due to their presence in the blood in this organ.

In the spleen the number of CFU increased gradually until the 5th d and remained at that level up to day 8 (Figure 5). During this period a 20 fold increase in the number of colony forming units was observed compared to the number that was found in the spleen of control animals. After day 8 the CFU-S number declined. About normal numbers were found on day 14 (not indicated in Figure 5).

In the femoral bone marrow a slight and not significant elevation of CFU-S numbers was observed for the first 3 d after the initiation of the PHZ-treatment (day 3: 130% of the control value). On days 4-7 the femoral CFU-S numbers were below normal with lowest numbers found on day 5, when only 50% of the control CFU-S numbers was present (Figure 5). At day 8 the number was restored to normal.

4 d after initiation of PHZ-treatment (Table 1) 44.5% of the liver, 42% of the spleen and 39% of blood CFU-S were killed by a single dose of hydroxyurea, indicating, that a high proportion of CFU-S were in the DNA synthesis phase. Bone marrow CFU-S were not significantly affected by the drug.

Origin of CFU-S in the liver

To test whether the PHZ-induced stem cell rise in the liver was due to a migration of CFU-S from the spleen towards the liver, splenectomy was performed 3 weeks before or on day 0, 1, 2 and 3 of the PHZ-treatment. The numbers of CFU-S and erythroblasts were assayed on days 4 and 7 respectively. Table 2 shows that splenectomy either during or 3 weeks before the PHZ-treatment had no effect on the CFU-S

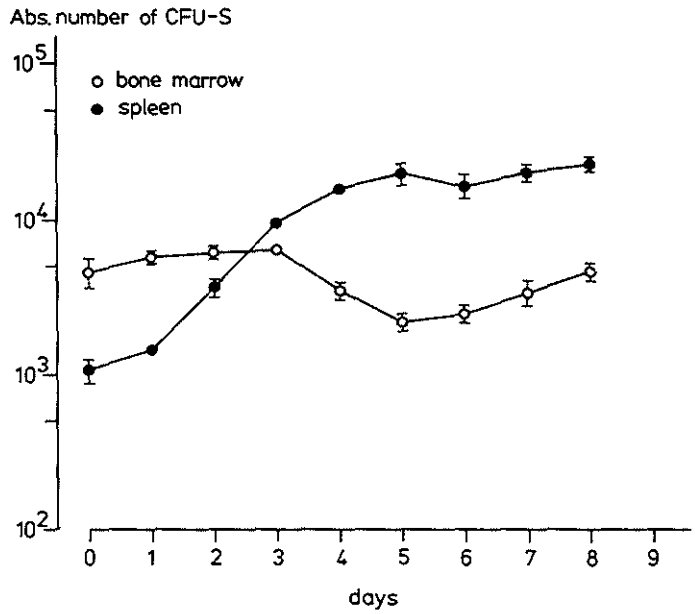

Figure 5. Stem cell content (CFU-S/femur, spleen ± SEM) of bone marrow and spleen of PHZ-treated mice. The means of two separate CFU assays are plotted; in each experiment the spleen and marrow of 5 donor mice were pooled. In one experiment total body perfusion was carried out just before sacrifice on each day.

number in the liver. However, the erythroblast number on day 7 was always suppressed when splenectomy or sham-splenectomy was performed on day 0, 1, 2 and 3 of the PHZ-treatment. This was thought to be due to starvation following the operation. Starvation did not influence significantly the CFU-S number on day 4

Table 1
Total CFU-S content of liver, spleen, bone marrow and blood: effect of hydroxyurea (OHU)
OHU was injected 3 h before sacrificing the mice on day 4
The organs and the blood of 5 donor mice were pooled

	PHZ	PHZ + OHU	% kill	P**
Liver	4245± 355*	2356± 285	44.49	0.005
Spleen	46770±3060	27225±1499	41.73	0.001
Femur	5230± 549	4700± 423	10.13	difference not significant
Blood (ml)	3060± 392	1866± 198	39.02	0.01
				Irradiation controls
Spleen colonies				0.00

* ± standard error of the mean.
** Statistical analysis compared results from individual colony counts, Wilcoxon test.

Table 2
Content of CFU-S and erythroblasts in the liver of PHZ-treated mice
Effect of splenectomy (Sx), sham-splenectomy (Sh.Sx) and starvation

		Day of operation											
		Sx					Sh.Sx					Starva-tion***	Control
		−21	0	1	2	3	−21	0	1	2	3		
CFU* x 10^3	Exp. 1	3.75	–	2.98	4.80	–	2.76	–	3.80	2.56	–	–	2.67
	2	3.69	–	3.01	3.57	–	3.10	–	3.35	3.19	–	2.50	3.28
	3	–	–	–	–	–	–	–	–	–	–	3.16	2.71
Mean CFU		3.72	–	3.00	4.19	–	2.93	–	3.58	2.88	–	2.83	2.89
Erythroblasts** x 10^6	4	10.30	–	3.00	3.23	3.90	8.50	–	–	4.45	5.15	–	9.12
	5	6.80	–	–	–	–	5.40	–	–	–	–	–	5.38
	6	–	–	2.10	2.45	–	–	–	2.85	3.56	–	3.21	5.42
	7	–	–	2.63	–	–	–	–	2.63	–	–	–	5.87
	8	–	1.66	–	–	–	–	2.21	–	–	–	2.76	4.98
Mean erythroblasts		8.55	1.66	2.58	2.84	3.90	6.95	2.21	2.74	4.01	5.15	2.99	6.15

* Each figure represents the stem cell number determined on day 4 from a pooled suspension of 3 donors.
** Each figure is the mean of determinations in 6 animals, determined on day 7.
*** Mice were starved from day 0-3.

but caused a suppression of the erythroblast number on day 7 (Table 2). Determination of the number of erythroblasts in liver, spleen and marrow on day 4 in starved and non-starved PHZ treated mice showed a depression of the erythroblast number in the spleen (45% depression), liver (51%) and bone marrow (12%) of starved animals as compared to non-starved mice.

Discussion

Although erythropoiesis is not observed in normal adult mouse liver we have demonstrated that this organ can sustain erythropoiesis under conditions in which there is a strong demand for red blood cell formation. On stimulation of erythropoiesis by PHZ administration the stem cell content of the liver is raised from approximately 40 to 2,500 CFU-S during 4 days of treatment. An increased number of CFU-S has also been found in regenerating liver of adult mice after treatment with carbon tetrachloride (Varon et al. 1975) and in the liver of repeated whole-body irradiated mice (Testa et al. 1977). The mechanism by which this increase in liver CFU-S occurs is unknown. Nevertheless, it may be considered that the liver CFU-S are dormant in vivo until stimulated to proliferate and thus give rise to an increase in number. An endogenous source of hepatic CFU-S after partial hepatectomy of adult mice was also suggested by Hays et al. (1975). However, it has been shown that a parallel increase in peripheral blood CFU-S also occurs which suggests that the CFU-S in the liver are being seeded into the liver from the blood. The perfusion of the liver did not significantly alter the number of CFU-S recovered from the liver, indicating that these CFU-S were not just due to the blood in this organ. It has been suggested by Hodgson et al. (1968) that blood stem cells, which are in high numbers in PHZ-treated splenectomized mice, are of marrow origin. Similarly it can be argued from our splenectomy experiments that the high numbers of liver CFU-S observed on day 4 were due to stem cell traffic from the marrow. The observations of Adler et al. (1976) that hepatic CFU-S in mice with a radio-strontium-induced marrow aplasia decreased after splenectomy and were absent in mice splenectomized prior to radio-strontium injection, imply that the liver cannot support proliferation of CFU-S.

Using a single dose of hydroxyurea, on day 4, approximately 40% of the stem cells in the blood, spleen and liver were killed whereas the numbers of bone marrow CFU-S were not significantly reduced ($p > 0.01$) from control levels (Table 1). Similarly Hodgson (1973) found that 5 d after initiation of PHZ-treatment in mice spleen CFU-S were sensitive to the lethal action of hydroxyurea while bone marrow CFU-S were not. This observed absence of cycling CFU-S in bone marrow may be due to preferential migration of cycling CFU-S from bone marrow to other haemopoietic sites, which are more conductive to stem cell cycling and erythropoiesis e.g. spleen and liver. The observation that subnormal levels of CFU-S were present in bone marrow from days 4 to 8 would be consistent with the notion of CFU-S migration from bone marrow. The increase of circulating and splenic CFU-S following

endotoxin treatment have also been suggested to be of marrow origin (Vos et al. 1972, Monette et al. 1972). Vos et al. (1972) have demonstrated that many stem cells are capable of lodging in the liver within 5 min after intravenous injection of a high number of marrow cells.

A 3 d period between the peak in the number of CFU-S and the number of erythroblasts in the liver suggests that the erythroblasts have originated, in the liver, from the stem cell population. Hasthorpe & Hodgson (in press) have shown that stem cells are capable of giving rise to medium sized erythroblasts by day 3 in X-irradiated mouse spleen. It is, however, also tenable that the hepatic erythroblasts were derived from immigrated blood BFU-E which are throught to be at an early stage in erythrocytic development and circulate in high numbers on day 2 after two PHZ injections into mice as was shown by Hara & Ogawa (1976). The possibility, that more mature erythrocytic cells (i.e. CFU-E and erythroblasts) represented the precursors of the hepatic erythroid islets is not plausible, since the latter authors could not demonstrate any blood CFU-E under the same conditions. Moreover, erythroblasts in the peripheral blood were not observed in the present study.

Our finding that both splenectomy and sham operation during PHZ-treatment elicitated an inhibition of the liver erythropoiesis can be explained as an effect of the operation. When the animals had been sham operated 3 weeks before the PHZ-treatment no suppression of erythroid cell production was observed. We demonstrated a similar inhibitory effect on the red cell production in the liver after 4 d of starvation, as was observed after splenectomy during PHZ-treatment. Starvation is known to suppress erythropoiesis in both spleen and marrow in mice (Fruhman & Gordon 1955, Naets & Wittek 1974). However, this depression is higher in the spleen and liver than in the marrow when erythroblast numbers are compared in starved PHZ-treated mice with non-starved PHZ-treated mice on day 4.

The data indicate that a suitable microenvironment for erythropoiesis can be temporarily sustained in the adult mouse liver. It is not clear if this microenvironment also has inductive and regulatory properties. The combined presence of central macrophages in erythroid islets of several haemopoietic tissues and the appearance of central macrophages in the liver and in marrow sinusoids (Ploemacher & van Soest, 1977b) concomitant with the onset of erythroblastic proliferation are indications for the fact that central macrophages are important cellular components of the erythroid microenvironment.

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III

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Morphological investigation on ectopic erythropoiesis in experimental hemolytic anemia

Morphologische Untersuchungen der ectopischen Erythropoese bei experimenteller hämolytischer Anämie

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Abstract

Mouse bone marrow – erythropoiesis – hematopoietic micro-environment

Normal erythropoiesis occurs extravascularly in the bone marrow and in the spleen of mice. The erythroblasts are located around central macrophages (CM). We have investigated at which locations in the mouse erythropoiesis may occur under anemic stress. Our study was focussed on the presence and origin of CM in ectopic erythroid islets in order to obtain insight in the microenvironmental factors determining the sites at which erythropoiesis can occur.

From day 2 until day 10 after initiation of a phenylhydrazine (PHZ)-induced hemolytic anemia intravascular erythroblasts of all differential stages were observed and were mostly located around CM. The CM appeared within 3 days at the intravascular sites.

The intravascular CM did not show the elongated paracrystalline inclusions that are typical for the CM in the marrow parenchyma. When colloidal carbon particles were i. v. injected prior to PHZ-treatment only extravascular CM showed carbon labelling, whereas intravascular CM did not contain carbon during the observation period of 10 days. The observations suggest that the intravascular CM do not belong to the resident macrophage population of the marrow and originate within 3 days from cells, probably monocytes, that are non-phagocytic at the moment of carbon injection. A monocytic origin is also suggested for the CM, which contributed to the observed increase in extravascular CM during the course of the anemia.

The present observations confirm earlier reports on the origin of intravascular CM in the liver of PHZ-treated mice under identical conditions.

The possible regulatory action of CM in erythroid maturation is discussed.

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Introduction

During the past decade considerable indirect evidence has been gathered concerning the role of the hemopoietic organ stroma in inducing and sustaining hemopoiesis [36]. According to the concept of McCuskey et al. [19] the hemopoietic microenvironment is morphologically composed of a microvascular compartment, a connective tissue compartment and neural elements associated with both the blood vessels and the stroma.

The nature of the cells within the hemopoietic connective tissue compartment is not defined up till now. Bessis and colleagues [8, 9, 27] described erythroblastic islands composed of developing erythroid cells surrounding ferritin-containing central macrophages in human marrow stroma. The centrally placed phagocytic cells extend irregular branching processes between a number of erythroblasts and reticulocytes in various stages of development. Such phagocytic cells have also been found in the yolk sac of guinea pigs [34], the spleen [23, 24] and bone marrow [4, 10, 35] of man and rodents. Moreover, central macrophages (CM) have been described in hepatic sinusoids and central veins of severely anemic mice [25, 26]. The presence of both CM and surrounding erythroblasts suggests an interrelationship of these cell types. It is believed that the CM have a function (1) in detaining the differentiating erythroblasts, preventing their release into the circulation, (2) in phagocytizing expelled normoblast nuclei and disranged erythroid elements [3, 39] and (3) as a centre for resumption of erythropoiesis subsequent to a period of depressed red blood cell formation [3]. However, detailed information concerning the origin of CM and their presence and role in ectopic erythropoiesis is still lacking.

In addition to macrophages, reticular cells in the bone marrow may also constitute an important element in the hemopoietic microenvironment. The reticular cells, i. e. adventitial cells and endothelial cells, are elongated, fixed cells and are associated with a fibrillar, extravascular connective tissue. The reticular cells of the bone marrow also show phagocytosis [14, 15, 18], although in a minor degree. According to Chen et al. [12] and Weiss [40] the close relationship of differentiating blood cells and reticular cells in human marrow might surmise that signals critical to hemopoiesis are exchanged across the contiguous membranes, inducing the differentiation of stem cells into a particular cell line.

Under normal conditions erythropoiesis is found extravascularly in the fetal liver, the spleen and the bone marrow. 24 hours after sublethal irradiation of rats, however, Ben-Ishay and Yoffey [7] observed some less mature erythroid cells entering the marrow sinusoids through the damaged endothelial lining. During the recovery phase of a phenylhydrazine (PHZ)-induced hemolytic anemia, Weiss [39] found entrance of normoblasts into the sinus space. Under similar conditions we found all stages of morphologically recognizable erythroblasts and erythroid mitoses in the central veins and sinusoids of the mouse liver [25] and also in the marrow sinusoids and veins. Indications have been found that the CM in the hepatic central veins differentiated out of immigrated monocytes in about two days. Although this could also be true for the CM in the liver sinusoids it was not excluded that the resident macrophages, i. e. Kupffer cells, may function as CM.

The present study is an attempt to investigate (1) whether the erythroblasts in the bone marrow sinusoids and veins are located around CM in erythroid islets and (2) whether these CM are a resident population of cells, which migrate out of the extravascular space after PHZ treatment or are differentiating out of monocytes. Finally, a possible presence of reticular cells in intravascular erythropoiesis in the bone marrow is studied.

Materials and methods

Experimental animals. CBA/Rij male mice, 20 weeks old weighing 28 g, and (C 57 BL/Rij × CBA/Rij) F 1 male mice, 19 to 20 weeks old weighing 28 to 30 g were used. The mice were purchased from the Medical Biological Laboratory TNO, Rijswijk and from the Laboratory Animals Centre of the Erasmus University, Rotterdam.

Phenylhydrazine treatment. To create a favourable condition for the induction of ectopic erythropoiesis, hemolytic anemia was induced by 4 daily intraperitoneal injections of 1 mg phenylhydrazine chloride (PHZ; Merck, Darmstadt) in 0.5 ml buffered salt solution (BSS) [22]. The day of the first injection was designated as day zero. Groups of 9 animals were studied each day for a period of 14 days. In addition, 9 control animals were studied. The effect of the PHZ treatment on the erythropoiesis was evaluated by red blood cell and reticulocyte counts, hematocrite values, weighing of the spleen and differential bone marrow counts.

Carbon injection. To discriminate between macrophages that were present before the PHZ treatment and macrophages that developed from a monocytic pool during the anemic period, carbon labelling of phagocytic cells was performed. Preliminary experiments showed that colloidal carbon doses of more than 0.05 ml per animal were unsuitable for this study since some blood monocytes also showed a minor phagocytosis of carbon particles.

1 hour before PHZ treatment a group of mice was injected with 0.05 ml diagnostic colloidal carbon from Searle Diagnostic (High Wycombe, England) dissolved in 0.45 ml BSS.

Groups of 3 mice were sacrified each day up to day 10.

Preparation for microscopy. Total body perfusion under Nembutal (70 mg/kg body weight) anaesthesia through the left heart ventricle, starting with a prewash of one ml of BSS (*p*H 7.2, 310 milliosmols) was carried out at room temperature. The fixation solution contained 1.5 % glutaraldehyde (Fluka AG, Switzerland) in 0.075 M MILLONIG [21] buffer, *p*H 7.2 and had an osmolality of 310 milliosmols as measured by an Advanced Osmometer. After 3 minutes of perfusion small tissue blocks (2 to 6 mm^3) were prepared with a razor blade from heart, kidneys, lungs, thymus, mesenterial and inguinal lymph nodes and Peyer's patches. Immediately after dissection of the left and right femora both epiphyseal ends were cut off and the bone marrow was gently pressed out by forcing the fixative solution with a 21 G needle through the femoral shaft. After an additional 2 hours fixation at 4° C the tissue blocks were postfixed (1.5 hours at 4° C) with 1 % OsO_4 in 0.075 MILLONIG buffered to *p*H 7.2 (310 milliosmols). Dehydration in a graded ethanol series was performed rapidly and followed by embedding in Epon. Polymerization was carried out for 16 hours at 37° C, followed by 8 hours at 45° and another 48 hours at 60°.

Light microscopy. 1.5 μm sections from Epon-embedded tissue were stained with an aqueous solution of toluidin blue.

Electron microscopy. Sections for transmission electron microscopy were contrasted with saturated uranyl acetate and lead citrate [29]. The sections were examined in a Philips EM 300 electron microscope, operated with an accelerating voltage of 60 kV and a 20 μm objective aperture.

Determination of the number of macrophages and erythroblasts in bone marrow. The numbers of macrophages and erythroblasts that were sectioned through the nucleus were counted in light microscopical preparations. The surface area of the sections was measured. The number of cells was expressed as number per area of 1 mm^2. No morphometric corrections were carried out on these numbers since significant differences in the mean nuclear diameter of macrophages on the various days after PHZ treatment could not be detected.

Results

The course of the anemia. The hematocrite fell from a normal value of 47 % on day 0 to 25 % on the fourth day after initiation of the PHZ treatment. From this day

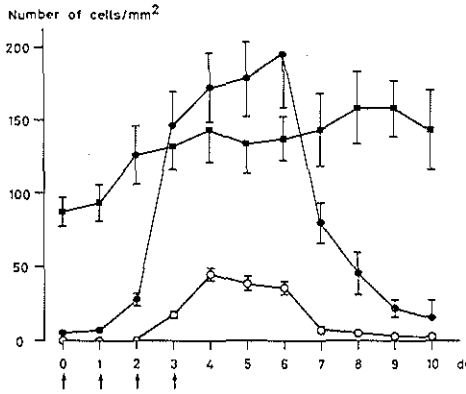


Fig. 1. Number of cells per area (1 mm²) measured in light microscopical bone marrow sections of CBA/Rij mice. Only cells showing a nucleus were counted. The standard errors of the means of at least 6 animals per day are indicated by bars. – ○: Intravascular macrophages. – ■: Extravascular macrophages. – ●: Intravascular erythroblasts.

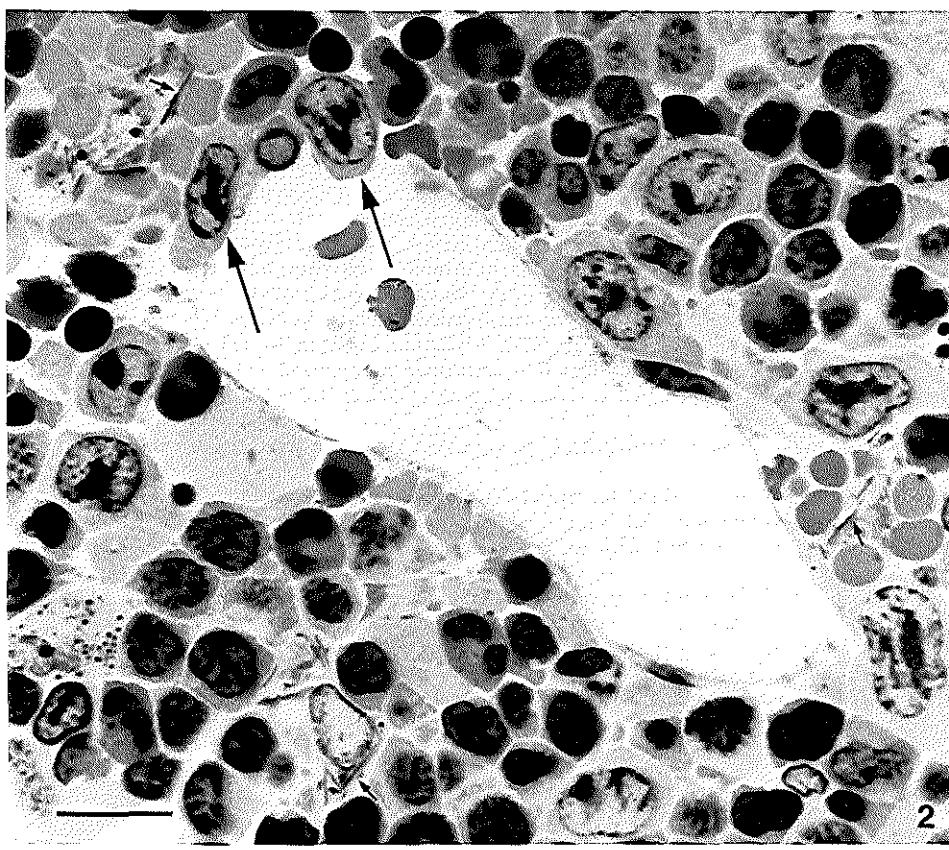


Fig. 2. Bone marrow of a F 1 mouse on day 4 of the PHZ treatment. Two proerythroblasts are probably crossing the thin sinus lining towards the lumen *(large arrows)*. Note the elongated inclusions in the extravascular macrophages *(small arrows)*. – Bar 10 μm.

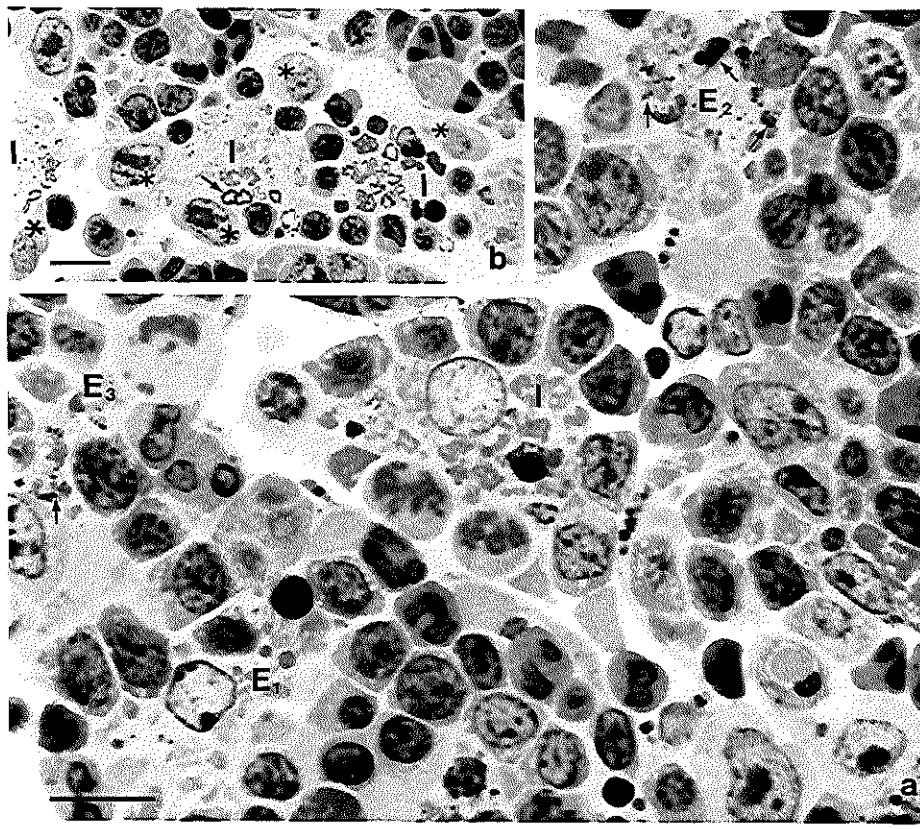


Fig. 3 a. Marrow from a F 1 mouse on day 6 that was injected with colloidal carbon prior to PHZ treatment. A large intrasinusoidal central macrophage (I) without carbon-labelling surrounded by erythroblasts can be observed. In the lower part of the islet two mitotic cells are present. The difference in nuclear morphology between intravascular (I) and extravascular central macrophages (E_I) is demonstrated. Two extravascular macrophages (E 2 and E 3) have ingested carbon *(arrows)* which cannot easily be recognized in the photographs. – Bar 10 μm. – **b.** Intravascular erythroid islet in marrow of a CBA/Rij mouse 6 days after initiation of the PHZ treatment. Ghosts of ingested erythrocytes *(arrows)* showing Heinz bodies can be noticed in the central macrophages (I). A number of immature red cells is present in the sinus *(asterisks)*. – Bar 10 μm.

onwards a recovery period was observed and on day 10 the hematocrite returned to normal values.

The spleen weight increased from a normal value of 70 mg to 450 mg on day 7. Pretreatment values were reached around day 16.

The PHZ treatment also elicited a rise in the number of erythroblasts in the bone marrow. Whereas in control mice erythroblasts constitute only 27 % of the nucleated cells in the marrow, they rose to 43 % on day 3 and 50 % on day 8. About a normal percentage was observed from day 12 onwards.

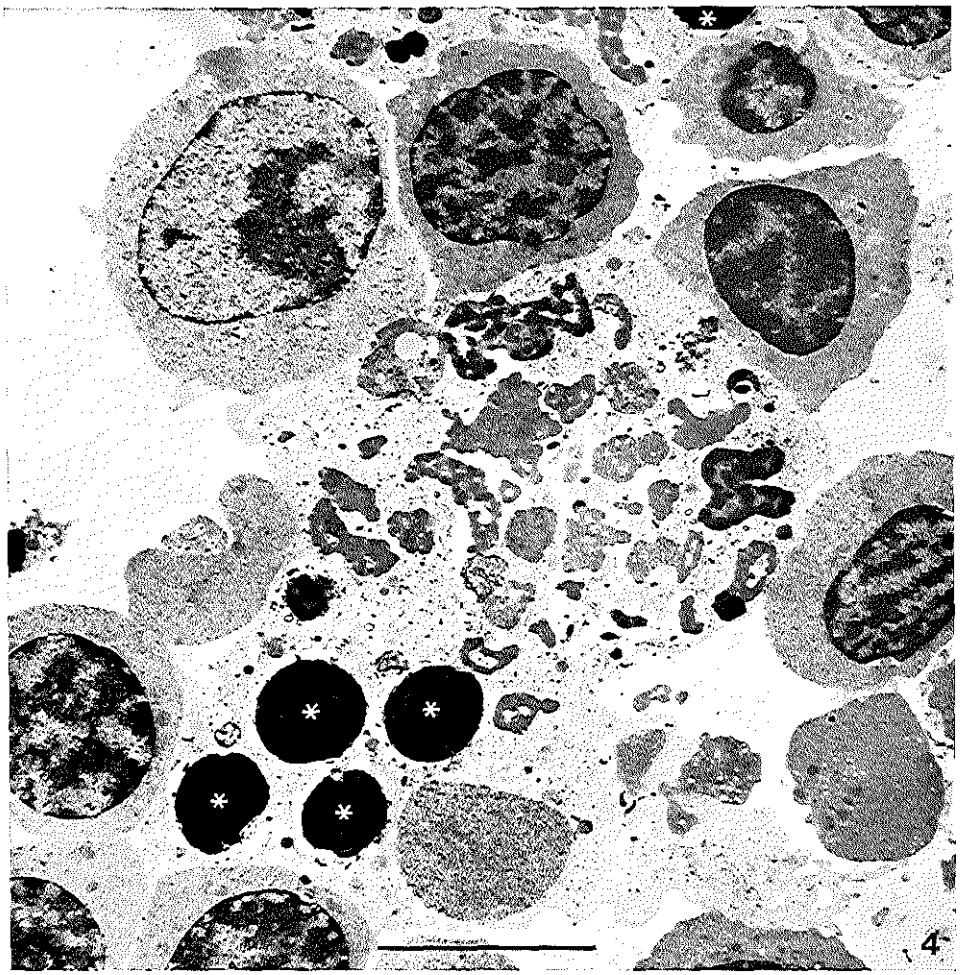


Fig. 4. Marrow from a CBA/Rij mouse on day 6. Erythroblasts can be noticed around intrasinal central macrophages that have ingested extruded normoblast nuclei *(asterisks)* and many erythrocytes. – Bar 5 μm.

Ectopic erythropoiesis. No erythropoiesis was observed in material obtained from heart, kidneys, lungs, thymus, Peyer's patches and inguinal lymph nodes. In mesenterial lymph nodes erythropoietic islets were sometimes observed after PHZ treatment.

Entrance of early erythroblasts into sinuses and veins of the bone marrow was observed from day 1 until day 6. As can be seen in Figure 1, a large increase in the number of intrasinal erythroblasts occurred on day 3 and some increase maintained up to day 6. After day 6 a rapid decrease was found and almost no intrasinal erythroblasts were observed on day 10.

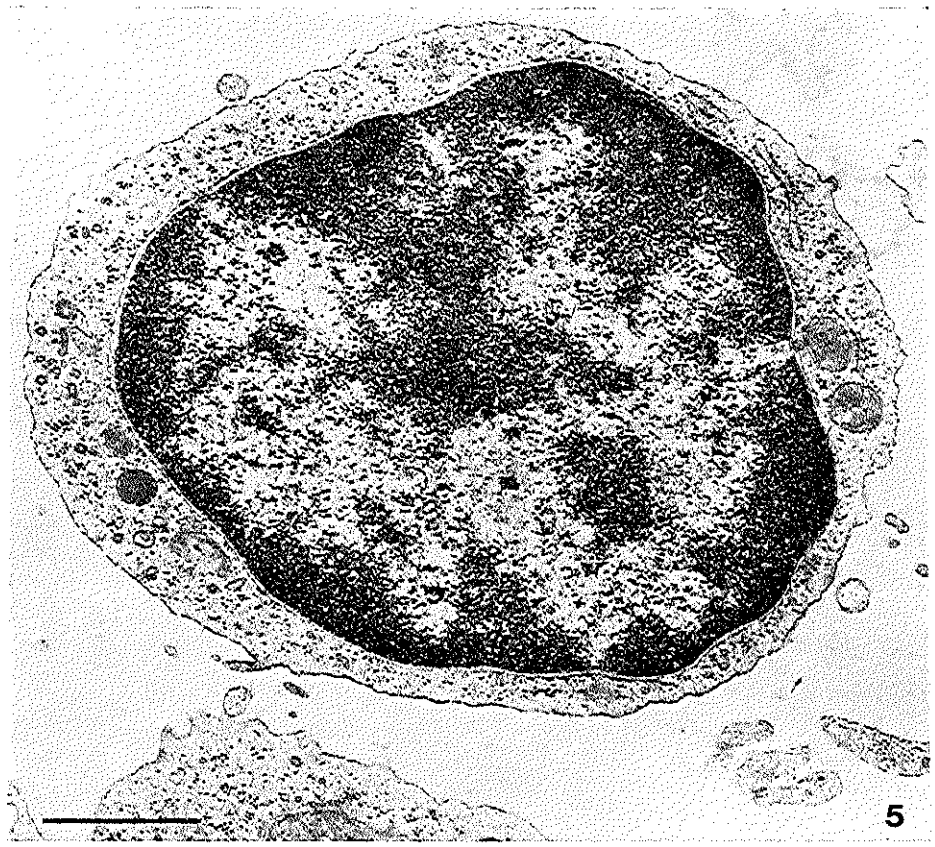


Fig. 5. Transitional cell in bone marrow sinus of a F 1 mouse on day 1. This cell probably represents a very early stage in the activation of a lymphocyte-like progenitor. Scattered monoribosomes and some polyribosomes are present. The cytoplasm contains short profiles of rough endoplasmatic reticulum and a few small mitochondria. – Bar 1 μm.

Some proerythroblasts were lying in apertures of the sinusoidal wall (Fig. 2), and sometimes showed uropods, in which all cell organelles were gathered. The uropods were always found in the extravascular part of the erythroblasts in passage through the wall. This observation is suggestive for the migration of early erythroblasts from the perivascular sites into the lumen of the sinuses and veins. Except for some proerythroblasts, the erythroblasts in sinuses and veins were in close contact with macrophages (Figs. 3 a, b, 4). These macrophages were rarely observed in marrow sinuses and never in central veins of control mice. Because such macrophages were frequently surrounded by erythroblasts and because they were centrally located in these erythroid islets, they were designated as CM. In the erythroblastic islets mitoses in erythroid cells were frequently seen (Fig. 3 a). The absence of nearly all polyribosomes was a characteristic feature of mitotic cells (Fig. 9 a). Rhopheocytosis was noted in all erythroid developmental stages and also during mitoses.

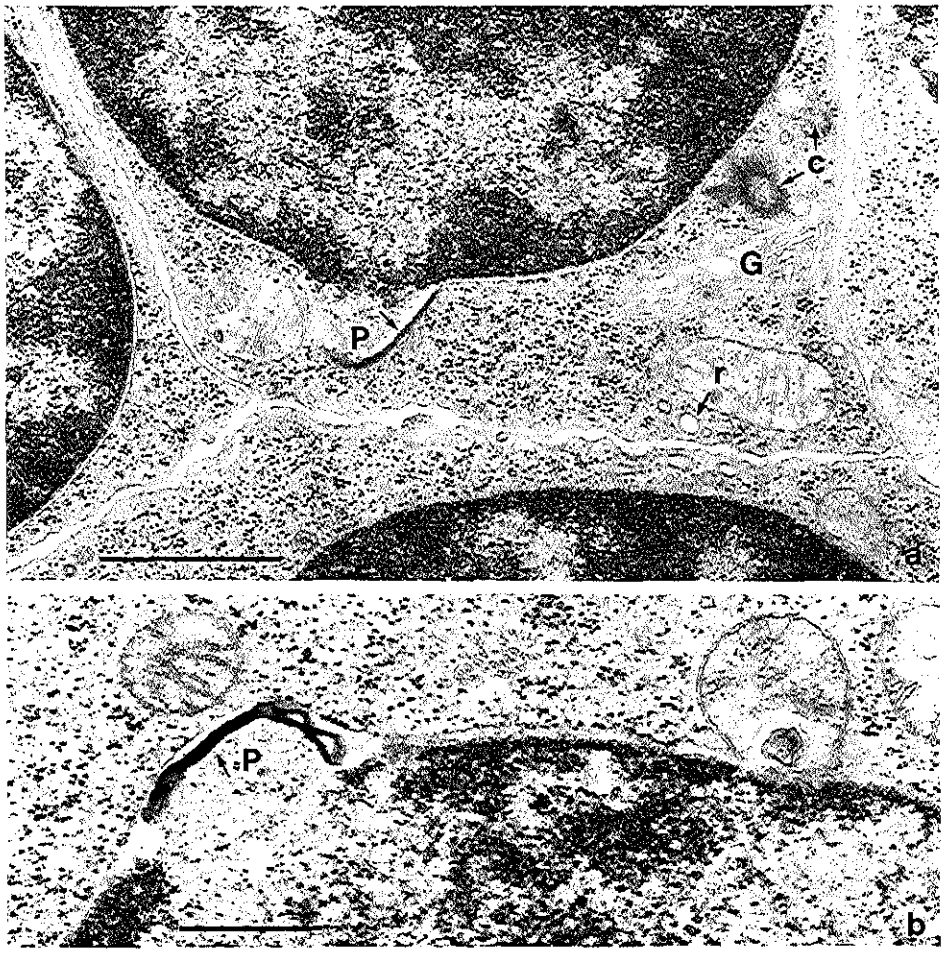


Fig. 6 a. Part of a cordal erythroblast in the marrow of a F 1 mouse on day 6. The field contains a nuclear process (P), which is partly marginated by an electron dense membranous structure just inside the outer nuclear membrane *(arrow)*. A continuous inner nuclear membrane can not be recognized in such a process. – r Ropheocytotic vesicle. – G Golgi area. – c Centriole. – Bar 1 μm. – **b.** Part of cordal erythroblast in the marrow of an untreated F 1 mouse. A membranous structure *(arrow)* is present in the delated perinuclear cisterna around a nuclear process. – Bar 0.5 μm.

Cells that had the morphology of stem cells (see VAN BEKKUM et al. [2] and RUBINSTEIN et al. [30]) were not observed around the CM. However, especially on day 1 transitional cells that had a morphology between cells that resembled lymphoid cells and proerythroblasts, were observed in the sinusoids (Fig. 5). The cytoplasm contained both mono- and polyribosomes, short profiles of rough endoplasmatic reticulum and a few small mitochondria. The size of transitional cells varied widely (10 to 16 μm diameter). These transitional cells resembled the cells described by BEN-ISHAY and

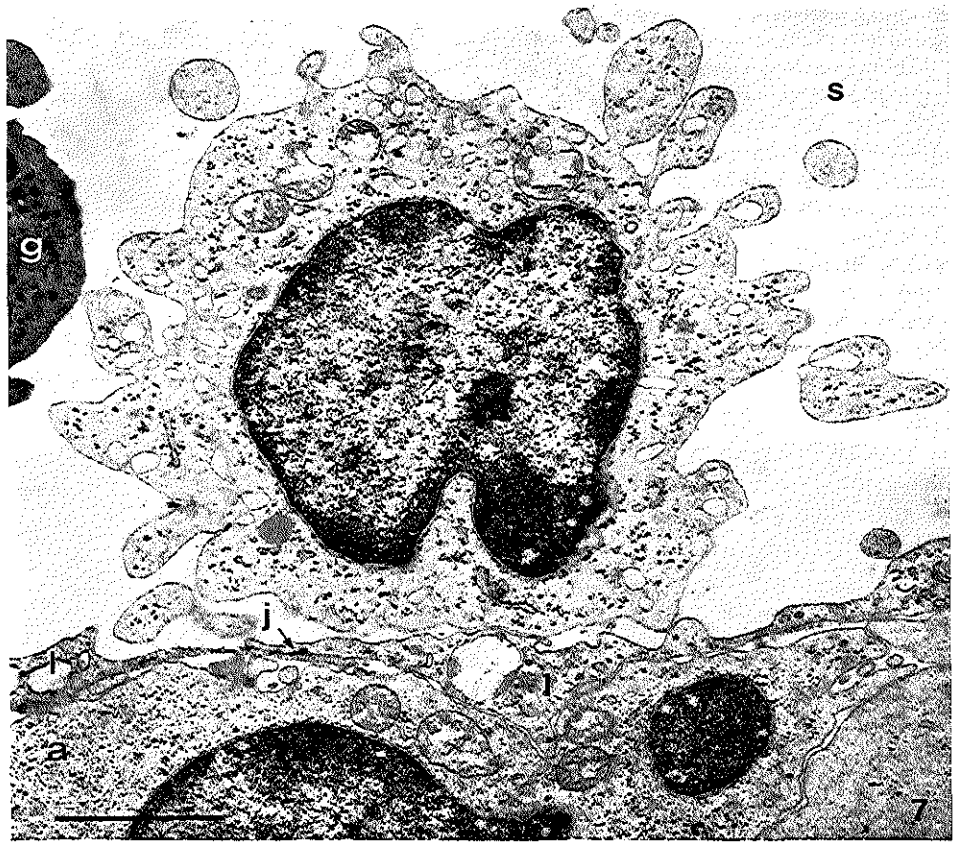


Fig. 7. Transitional cell between a monocyte and a macrophage in marrow sinus of F 1 mouse on day 2. Note the irregular nuclear outline, the moderate amount of marginal nuclear chromatin and the activity of the outer membrane. The cytoplasm is not yet containing remnants of phagocytized erythrocytes which are present in extravascular macrophages at this time. – s Sinal lumen. – L Lining cell. – a Adventitial cell. – j Junction between lining cells. – g Neutrophilic granulocyte. – Bar 2 μm.

colleagues [5, 6, 7]. Nuclear indentations, being characteristic features of transitional cells, according to these authors, were found in our study only occasionally.

Noteworthy, both in normal and anemic mice, peculiar nuclear protrusions were frequently observed in basophilic, polychromatophilic and orthochromatic erythroblasts (Figs. 6 a, b, 9 a, b, 10). These typical structures protruded more or less deeply into the cytoplasm and were lined by the inner and outer nuclear membrane. In the juxtanuclear part the protrusion was often filled with some condensed nuclear chromatin and the part that was distant from the nucleus was filled with amorphous material. One side was often limited by an electron-dense membranous structure. The observations on different erythroblasts suggested that the nuclear processes were tied off from the nuclei to become single membrane limited inclusions in which myelin figures,

probably representing the remnants of the inner nuclear membrane, and some amorphous and granular material were present (Fig. 10).

Central macrophages in erythroblastic islets in the bone marrow. CM occurring in marrow parenchyma and the lumen of sinuses and veins could be distinguished from each other light- and electronmicroscopically. Intravascularly the CM were present from day 3 until day 10 with a peak number on day 4 (Fig. 1). However, from day 1 on an increase in the number of monocytes and monocytic cells, attached to the endothelial lining, was noted. Cells that had a morphology transitional to monocytes and macrophages were frequently found after day 1 (Fig. 7). These cells showed an extensive ruffling of the cell membrane and possessed a larger Golgi area and more profiles of rough endoplasmatic reticulum when compared to monocytes. Furthermore, their nuclei were less polymorphic and contained less condensed chromatin than those of monocytes. Our observations suggested that disappearance of CM from the sinuses occurred by means of active migration towards the extravascular cords. The cells in this process passed the sinusoidal lining (Fig. 8).

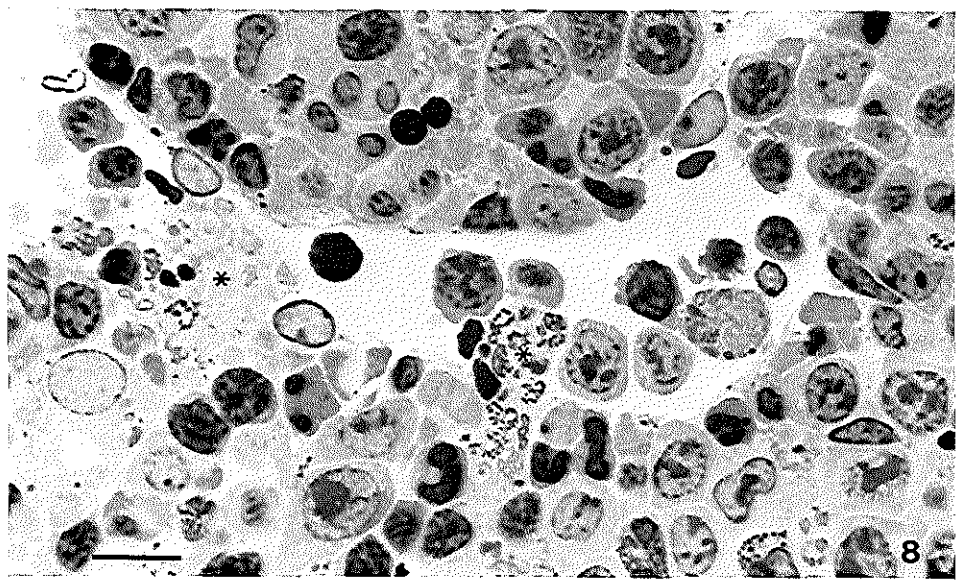
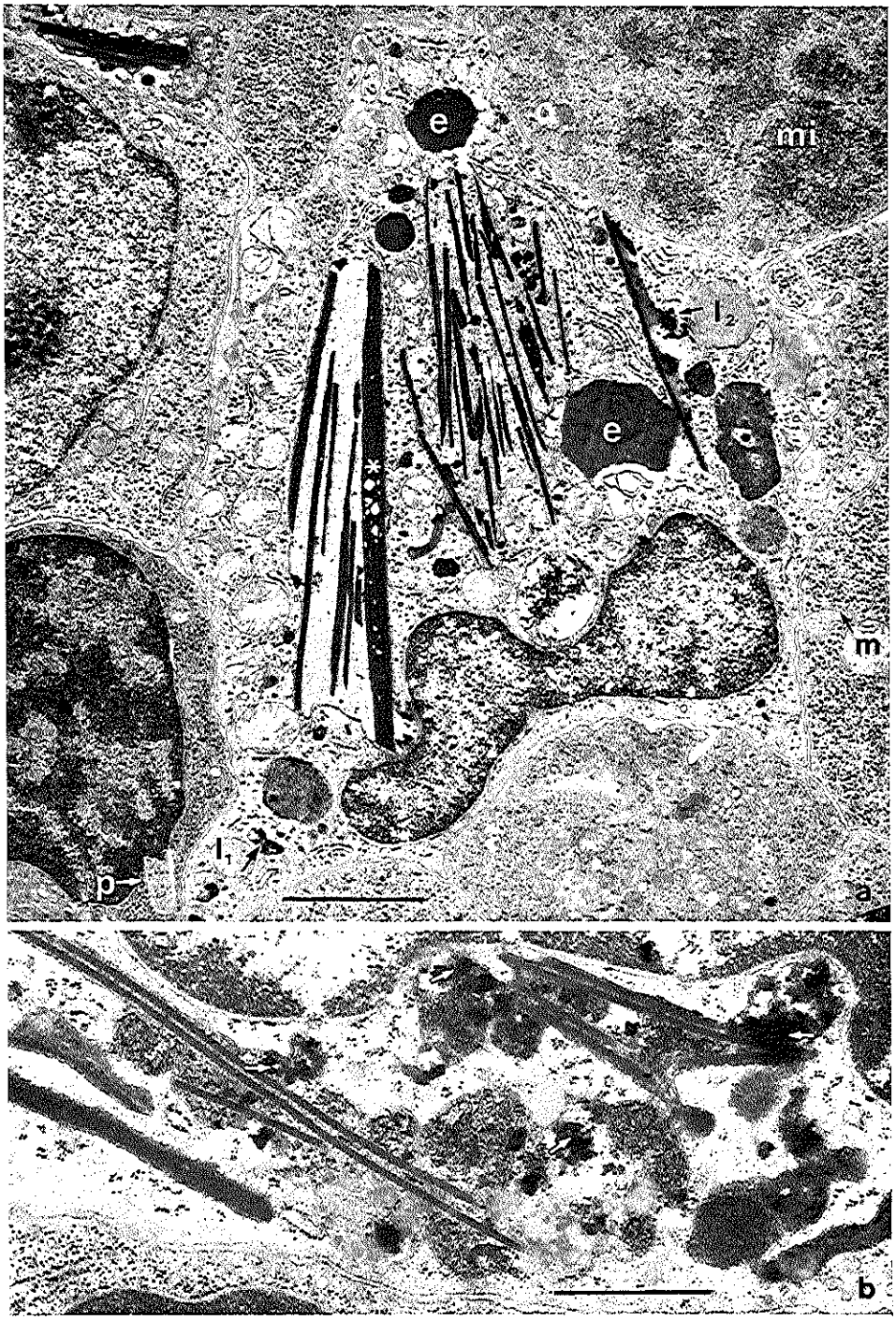


Fig. 8. CBA marrow on day 6 showing two macrophages *(asterisks)* that are located in lining apertures. One macrophage is partly surrounded by erythroblasts. The figure is suggestive for the migration of macrophages towards extravascular sites and would explain the disappearance of vascular macrophages after day 6 (see Fig. 1). – Bar 10 μm.

Fig. 9 a. Extravascular central macrophage in the marrow of an F 1 mouse on day 6. Colloidal carbon was injected 1 hour prior to initiation of the PHZ treatment. The carbon is present in separate lysosomes (l_1) and in the typical inclusions bodies (l_2). A macrophage process (m) indents an adjacent erythroblast membrane. A nuclear process (P) can be recognized in the lower left field. – Note the degradation of polyribosomes in an mitotic erythroid cell (mi) in the upper right. One rod-like inclusion has been cut breadthwise *(asterisk)*. – Note the membranes surrounding the typical inclusions. – e Ingested erythrocytes. – Bar 2 μm. – **b.** Part of an extravascular central macrophage. Carbon particles *(arrows)* and inclusion bodies are surrounded by the same membrane. – Bar 1 μm.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The number of extravascular CM per unit area had increased to about 150% on day 3 and 4. After day 5 a small second rise was observed towards 175% on day 8. Mitotic figures in CM were never observed. When compared to intravascular CM, the extravascular CM showed a more extensive contact with erythroblasts by means of long and slender sheet-like processes. Sometimes the adjacent erythroid plasma membranes were indented by short bud-like protrusions extending from the CM (Fig. 9 a).

Both in PHZ-treated and in non-treated F 1 mice the cytoplasm of the extravascular CM showed flat, elongated, crystal-like inclusions of electron dense material that were surrounded by single or multilaminated membrane systems and were associated with aggregates of hemosiderin. In contrast, these specific inclusion bodies were never seen in intravascular CM, suggesting that the intravascular CM did not originate from the extravascular CM (Fig. 9). In some inclusions a striation pattern could be observed in which the two directions of arrangement were separated by an angle of 60°. The spacing in these striation patterns measured 32 Å and 42 Å respectively. However, a structural interpretation was limited since the observations were based on electron microscopy of randomly oriented sections. The inclusions were embedded in an electron lucent or moderately dense substance. In unstained light microscopic slides the elongated inclusions appeared yellow, whereas in stained slides a metachromasia was present and they appeared light to dark green. The inclusions were not birefrigent. Pretreatment of mice with carbon resulted in the presence of carbon particles in the inclusion bodies (Fig. 9 a, b). Furthermore, fusions of these inclusion bodies with ferritin-containing electron-dense granules and with erythrocyte-containing vacuoles were observed in all PHZ-treated animals. The intravascularly located CM contained more ingested erythrocytes with Heinz bodies (compare Figs. 4 and 9 a). Both intravascular and extravascular CM evidently played a role in phagocytizing extruded normoblast nuclei (Figs. 3 a, 4, 10). The CM exhibited characteristics common to macrophages. Their nuclei were usually spherical and contained mainly marginally condensed chromatin. The intravascular CM nuclei contained less marginated nuclear chromatin when compared to extravascular CM (Fig. 3 a). In extravascular CM the nuclei were sometimes indented (Fig. 9 a) by ingested material or the paracrystalline inclusion bodies. Both in intravascular and extravascular CM, an extensive multifocal Golgi region was observed with cisternae, vacuoles and numerous electron-dense and lucent vesicles. The electron-dense vesicles were commonly found in extravascular CM. Ferritin micelles were distributed throughout the cytoplasm of the extravascular CM and in a lesser amount in the intravascular CM. In ferritin aggregates (hemosiderin form 1 [9]) regular patterns could be observed that were formed by planes of micellar rows with a spacing of 130 Å.

The space, measuring about 230 Å in width, between the extravascular CM and the surrounding erythroid cells was frequently filled with a flocculent material of moderate electron density (Fig. 10). Similar material was observed in the smooth endoplasmic tubules of the CM.

We investigated whether injection of the mice with colloidal carbon particles prior to PHZ treatment could discriminate between (1) an origin of CM from phagocytic cells, i. e. the resident macrophage population, and (2) an origin from cells, i. e. monocytes, that do not ingest colloidal carbon at the time of injection. As is illustrated in Figure 3 a intravascular CM were not containing carbon particles during the observation period of 10 days. After day 2 about 20% of the extravascular CM contained no carbon.

Adventitial cells. The trilaminar wall of bone marrow sinusoids consisted of lining endothelial cells and adventitial cells separated by an irregular discontinuous basement

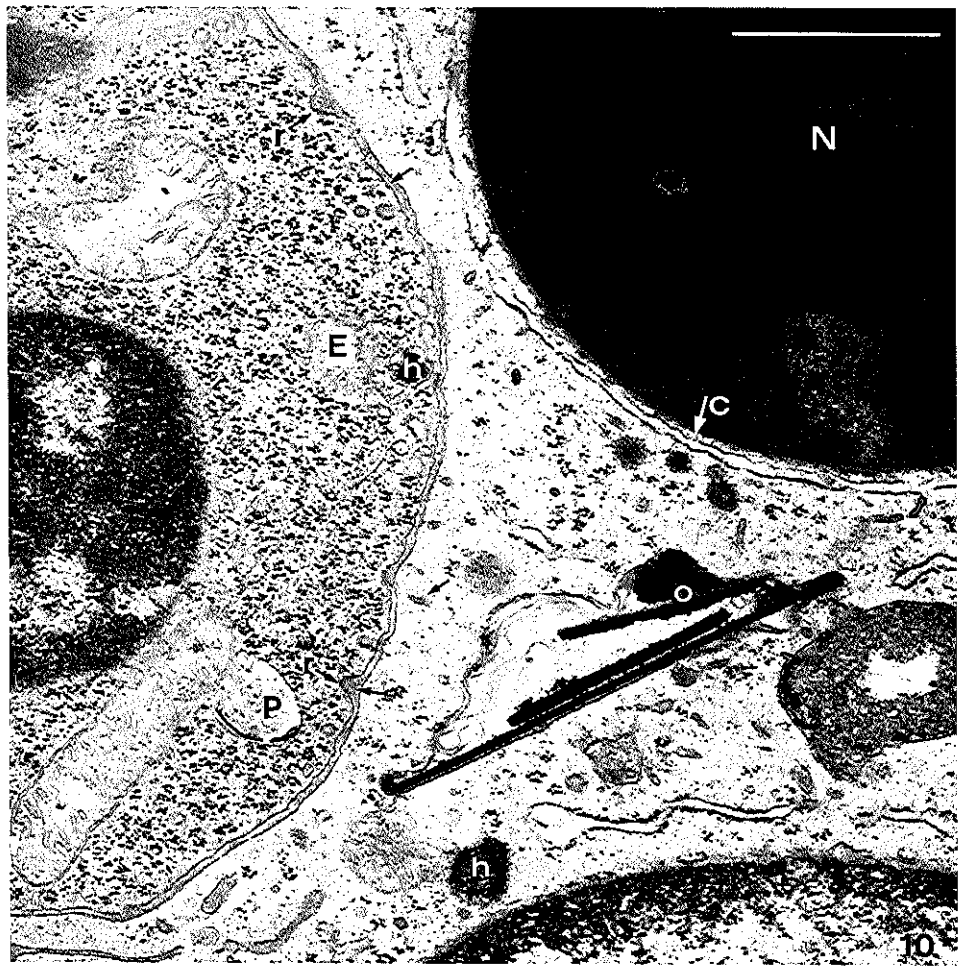

Fig. 10. Part of an extravascular central macrophage and erythroblast (E) in marrow of F 1 mouse on day 6. The erythroblast contains a nuclear process (P) which can only partly be seen in this section. A normoblast nucleus (N) with a surrounding rim of cytoplasm (c) is ingested by the macrophage. – Note the regular width of the space between the macrophage and the erythroblast. The space contains a flocculent material *(arrows)*, which is also present in the smooth tubules *(arrow)* within the macrophage cytoplasm and the pinocytotic holes in the erythroblast membrane (r). – h Hemosiderin. – Bar 1 μm.

membrane. The adventitial cells, which form the extravascular part of the sinusoidal wall, were not observed at intravascular sites after PHZ treatment. The adventitial cells are typical reticular cells with an extensive Golgi region, moderately long profiles of rough endoplasmatic reticulum, which are frequently dilated, and smooth endoplasmatic reticulum. Similar to the observations on extravascular CM, the smooth tubules contained flocculent material of moderate electron-density. This material was

also present extracellularly between erythroblasts and adventitial cells. The adventitial cells were often associated with collagen deposits especially at the endothelial side. After PHZ treatment some adventitial cells showed a very voluminous and electron-lucent cytoplasm (Fig. 11).

Discussion

In the present study it was demonstrated that intravascular erythroblasts of all morphologically recognizable developmental stages could be observed around central macrophages in the marrow of anemic mice. The erythroblasts were dividing and no indications of phagocytosis by the central macrophages could be detected. These observations indicate that the presence of intravascular erythroblasts under the described circumstances represents an intravascular erythroid maturation. It is not clear to which extent the extravascular hemopoiesis contributed to the overall red cell production in the animal after PHZ treatment. No erythropoiesis could be induced in the thymus, lymph nodes, lung, heart and kidneys under these circumstances. However, it has been described that erythropoiesis may occur in the mouse thymus [1, 32] and liver [25]. The observed increase in spleenweight may result from the combined action of various factors [11], among which are a heightened erythropoiesis and erythroclasia.

Our observations suggest that the early erythroblasts migrate out of perisinussoidal spaces of the marrow through the apertures of the sinusoidal wall into the sinuses. WEISS [39] postulated that the sinuses and intersinal cords form "a reciprocating system wherein portions may become vascular (sinal) and extravascular (cordal) depending upon the requirements for hemopoiesis. The reciprocation could be effected by the ready capacity of sinal walls and adventitial spurs to take form, change disposition and break down". We found no indications for such a reciprocation although in our study it is not excluded that the walls of the sinuses were altered by partial destruction through the action of PHZ, letting heretofore extravascular erythroblasts into the circulation. A breakdown of the cordal structure was rendered less likely by the observations, that in the marrow sinuses only a small amount of immature cells from non-erythroid differentiation lines were present.

The increase in the number of intravascular erythroblasts coincided with the appearance of intravascular CM as was also observed in the adult mouse liver [25]. Reticular cells were not observed intravascularly in the bone marrow. These observations are indicative for a strong relationship between CM and erythroblasts, in which the CM permits the erythroblasts to lodge around it and to mature without ingesting the immature red cells. It is not clear to which extent the observed transitional cells contribute to the formation of intravascular erythroblasts. The number of intravascular marrow erythroblasts determined on the various days after initiation of the PHZ treatment showed a greater correlation with the respective numbers in the sinusoids of the liver than with those in the central veins in that organ under identical conditions [26]. In the hepatic sinusoids a peak number was reached on day 7 and subsequently a rapid fall on day 8 was observed. It is probable that a sinusoidal system in which macrophages are present, represents a microenvironment, which is conducive for erythroid maturation during anemic stress. It should be noted that such an erythroid conductive microenvironment supports erythropoiesis and may or may not differ from the hemopoietic inductive microenvironment (HIM) of WOLF and TRENTIN [35] which induces differentiation of stem cells.

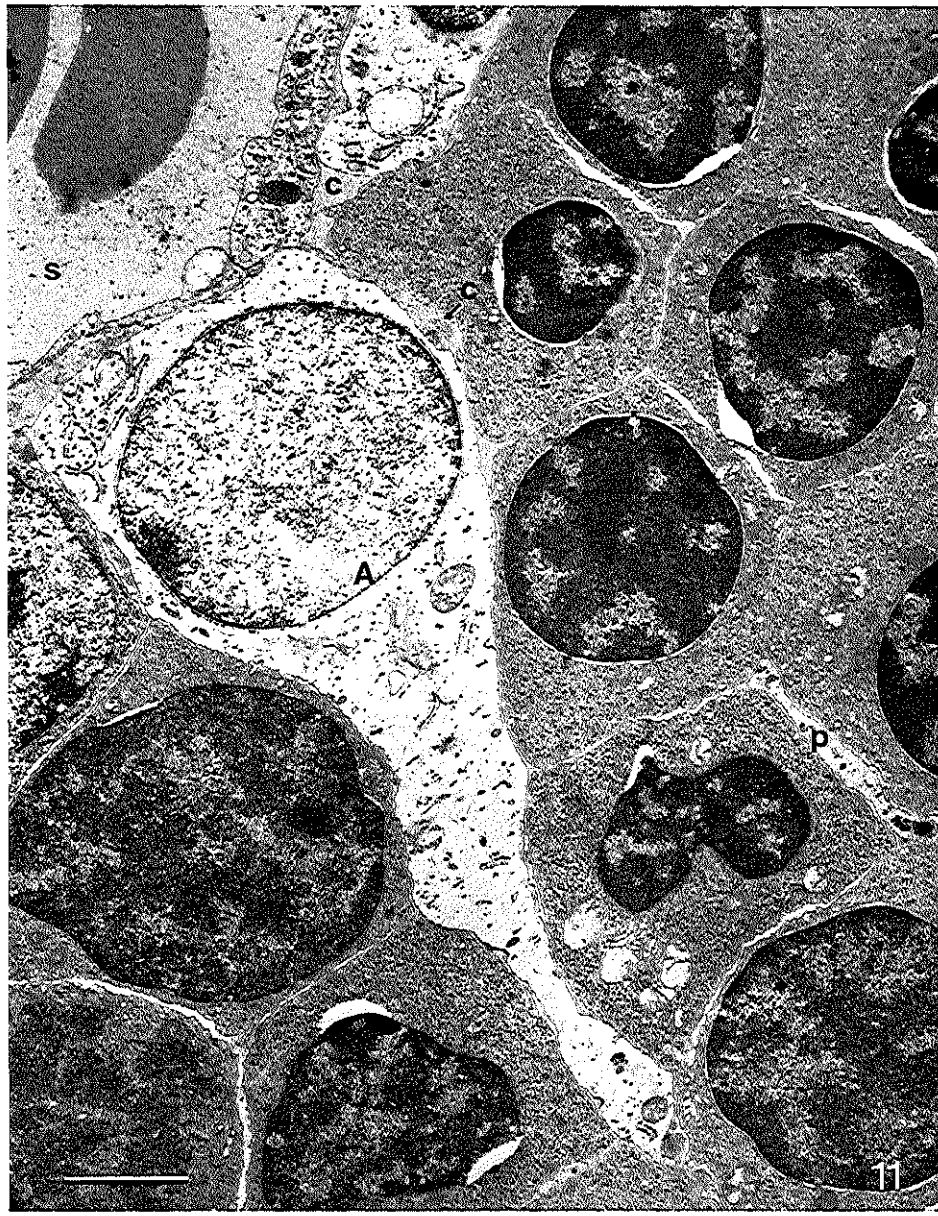

Fig. 11. Marrow of a F 1 mouse on day 6 that has received colloidal carbon prior to PHZ treatment. A rarified adventitial cell (A) is extending from the sinus lining into the parenchymal cord. No carbon has been ingested by this cell. – Note the very lucent cytoplasm when compared to the serum-containing sinal lumen (s). Erythroblasts surround the adventitial cell but are also contacting central macrophage processes (p). – c Collagen in ground substance. – Bar 2 μm.

We observed protrusions of the nuclear envelope in erythroblasts of both normal and anemic mice from the early basophilic stage on. This phenomenon suggested an exposition of nuclear material into the cytoplasm prior to the extrusion of the entire nucleus. Similar nuclear processes were earlier [39] observed in polychromatophils during PHZ-induced anemia in rats. However, it is not excluded that the nuclear protrusions are fixation artefacts.

The unique membrane-bound cytoplasmatic inclusions are common in marrow CM of (C 57 BL/Rij × CBA/Rij) F 1 mice at least after the first week of the postnatal life (PLOEMACHER and VAN SOEST, unpublished observations), and were also described in mouse bone marrow macrophages from C 57/BL-6 mice [10], NCS mice [13] and in rabbit spleen macrophages [33]. The fact that we observed colloidal carbon particles enclosed by the membranes that were surrounding these structures and the observation that they contain acid hydrolases [13], indicate that they are lysosomal in nature. A possible origin of these lysosomes from ingestion and partial digestion of effete erythrocytes by the macrophages was suggested by FEDORKO [13]. Such elongated inclusions are rarely found in CBA mice, even during the course of the hemolytic anemia. Moreover, in (C 57 BL/Rij × CBA/Rij) F 1 mice no obvious increase of the number of these lysosomes in CM was observed after PHZ treatment. The findings reported here may indicate that the occurrence of these lysosomes is not directly related to effete erythrocyte uptake by the CM. Moreover, a relation of similar inclusion bodies with granulocyte uptake in macrophages was proposed by ICHIKAWA [17]. The quantity of these inclusions in the macrophages was described to correlate with the age of the mice [42].

The intravascularly located CM could be distinguished from resident CM in the extravascular stroma in several ways. (a) The intravascular CM contained more phagocytized erythrocytes. This fact is probably due to their advantageous location in the circulation. (b) The contact area between CM and erythroblasts was larger in extravascular erythroid islets. This could be the result of a lack of space at these sites. (c) The observation, that intravascular CM did not show uptake of any carbon particles suggests that these CM originate from cells that had no phagocytizing ability at the moment of carbon injection. (d) The absence of the elongated inclusion bodies in intravascular CM indicates that the latter cells probably have not a resident macrophage origin. It should be noted that the presence of the inclusion bodies has been used in this study as a natural occurring macrophage label. Based on these morphological characteristics it is likely to assume that the origin of intravascular CM is monocytic. This is furthermore confirmed by the fact that the latter cells possess the ability to differentiate into macrophages in about 2 days [14]. Also in the liver under similar conditions morphological indications were found that CM in the central veins developed out of monocytes [25]. The first phagocytizing cells were observed intravascularly 3 days after initiation of the PHZ treatment both in this organ [25] as in the bone marrow. The number of extravascular CM increased after PHZ treatment and a part of these CM contained neither carbon nor elongated inclusion bodies after day 2. These observations suggest that the number of extravascular CM can be augmented from a monocytic cell pool in a period of elevated erythropoiesis. An increase in the number of erythroid islets was also observed in marrow suspensions of some PHZ-treated rats [18]. Thus, it seems possible that the progenitor, i. e. monocyte, of a cellular part of the erythroid conductive microenvironment is possibly able to migrate and is not bound to extravascular sites in murine spleen and bone marrow.

It is not known whether the intravascular CM exhibit similar functions as the resident extravascular CM in addition to the evident properties as phagocytosis and detaining

of developing erythroblasts. In fact, the question arises whether the resident CM is an unique macrophage type whose function is exerted only in erythroblastic islets. The concept of rhopheocytosis with which developing red cells would take up ferritin, delivered by the CM, probably is not valid for erythropoiesis in general [10, 25, 39], although ferritin may be present on the outer membrane and in the rhopheocytotic vesicles of erythroblasts. However, it is conceivable that substances other than ferritin might pass from the CM to the surrounding erythroblasts [6, 28]. We showed that the intercellular gap between the CM and the erythroblasts at certain sites contained a flocculent material, probably representing the remainder of mucopolysaccharides (MPS) [37, 43]. MPS is produced by CM and adventitial cells in the parenchymal stroma. It has been postulated that the amount of sulphated acid MPS in the hemopoietic microenvironment may affect the erythrocytic maturation both *in vivo* [19, 31] and *in vitro* [27]. Taken together, it seems likely that marrow CM in erythroblastic islets are involved in a regulatory action by affecting the local concentrations of acid MPS and probably also neutral MPS in the ground substance in which erythroblasts are embedded.

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IV

Studies of the Hemopoietic Microenvironments: Effects of Acid Mucopolysaccharides and Dextran Sulphate on Erythroid and Granuloid Differentiation *in vitro*

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The effects of acid mucopolysaccharides (AMPS) on *in vitro* erythrocytic and granulocytic colony formation of murine bone marrow cells have been studied. High concentrations of chondroitin sulphate A, B and C and heparitin sulphate partly or completely inhibited the response of CFU-E to erythropoietin stimulation whereas addition of heparin, hyalyronic acid and keratan sulphate II in concentrations up to 100 μg/ml did not elicite an inhibition of erythrocytic colony formation. The granulocytic colony formation was not significantly affected by AMPS-addition under these circumstances.

Low concentrations of chondroitin sulphate A and B evoked a stimulatory effect on the CFU-E number. The synthetic polyanion dextran sulphate did not affect the erythrocytic and granulocytic colony formation.

It is concluded that AMPS can affect the *in vitro* erythrocytic proliferation and differentiation in concentrations which do not affect the granulocytic maturation.

Since stromal cells, i.e. macrophages and reticular cells, in bone marrow *in vivo* have the ability to produce and remove AMPS in the extravascular matrix we postulate that stromal cells may be involved in the regulation of erythroid progenitor cell maturation.

Key words: Mouse – marrow – erythrocytic colony formation – granulocytic colony formation – polyanions

The microenvironments of a hemopoietic organ play a role in inducing (Curry & Trentin 1967) and probably also in sustaining hemopoiesis. For the most part only indirect evidence is available for the action of the hemopoietic organ stroma. Although considerable information has been gathered concerning the inductive function of the hemopoietic microenvironment (Tavassoli 1975), the function in sustaining hemopoiesis and regulation of the intermediate stages (proliferation and morphological differentiation) has not been adequately confirmed by experiment and is believed by many to be non-existent (Tavassoli 1975). Van Zant & Goldwasser (1976) postulated a promoting effect of splenic cells on hemoglobin synthesis by marrow cells *in vitro*. McCuskey et al. (1972a,b) McCuskey & Meineke (1973) and Schrock et al. (1973) have reported specific alterations of the amount of acid mucopolysaccharides (AMPS) in the murine spleen when erythropoiesis was regenerating, suppressed or stimulated. McCuskey et al. (1975) also reported shifts in AMPS in bone marrow. It was postulated that excessive concentrations of AMPS in the *in vivo* hemopoietic microenvironment are not conductive to the support of erythroid differentiation. Tavassoli et al. (1976) also suggested that the accumulated AMPS in the femoral marrow of severely starved humans and rabbits may provide an environ-

ment inconducive to erythropoiesis. However, a causal relationship between the presence of AMPS and the suppression of erythroid differentiation has never been demonstrated.

Using the spleen as an example, McCuskey et al. (1972a,b) classified the hemopoietic microenvironment into the following compartments (a) microvascular, (b) stromal or connective tissue and (c) neural; the classification was applied by them to bone marrow and ectopic sites. Since we are interested in the possible role that microenvironmental factors, especially those of the connective tissue compartment, play in the regulation of proliferation and differentiation of hemopoietic cells (Ploemacher & van Soest 1977a,b), we considered it worthwhile to study the effect of AMPS *in vitro* on the colony formation of murine bone marrow cells. The effect of a nontoxic (Ross et al. 1976) synthetic polyanion, i.e., dextran sulphate, was also included in our study.

Materials and Methods

Mice

(C57BL/Rij x CBA/Rij) F1 male mice, 20-22 weeks old and weighing 28-30 g were employed as donors for bone marrow suspensions. They were purchased from the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands.

Polyanions and other compounds

Dextran sulphate (mol.wt. 17,000) was a gift from Dr. A. Hegenbeek, Radiobiological Institute TNO, Rijswijk ZH), The Netherlands, The acid mucopolysaccharides chondroitin sulphate A(CS-A; mol.wt. 12,000), chondroitin sulphate B(CS-B; mol.wt. 27,000) chondroitin sulphate C(CS-C; mol.wt. 40,000), heparitin sulphate (HS; mol.wt. ~15,000), heparin (HEP; mol.wt. 11,000) keratan sulphate II (KS; mol.wt. ~10,000) and hyaluronic acid (HA; mol.wt. 230,000) were kindly provided by Dr. M.B. Mathews, Dept. of Pediatrics, University of Chicago, Illinois 60637, USA. Glycogen was obtained from GIBCO (Grand Island, N.Y., USA; Cat. 50750), D-glucose from Fluka AG (Germany) and D-galactosamin-hydrochlorid from Merck (Germany).

Plasma culture method for production of erythrocytic and granulocytic colonies in vitro

Pooled cell suspensions of femoral bone marrow in a buffered saline solution (BSS, cf. Mishell & Dutton, 1967) were made from three mice per experiment. Every polyanion was tested for its ability to affect the colony formation in three to eight separate experiments and the figures were made up by compiling the results from these experiments. The suspensions were diluted to give 10^5 cells/ml in an appropriate volume of plasma medium. The plasma culture system described by McLeod et al. (1974) was used. The plasma medium used was slightly modified in that NCTC-109 medium and Eagle's minimum essential medium were replaced by Dulbecco's medium (Flow Laboratories, U.K.). 0.25 u/ml Erythropoietin Step III (Connaught Medical Research Laboratories, Ontario, Canada) was included for the erythrocytic colony cultures. For culturing the granulocytic colonies 0.1 ml/ml mouse fibroblast conditioned medium (cf. Van den Engh 1974) was included instead of the kidney tubules conditioned medium. In each experiment the test materials were added at the beginning of the culture period to give a final concentration of 10^{-7} up to 100 μg/ml plasma medium. In addition, a series of cultures containing no additives were run.

In accordance with McLeod's sytem for culturing the erythrocytic colonies, microtiter plates

(Greiner, Nürtinger, Germany) were used with each well containing 0.1 ml of culture medium. For each experimental point the average colony number in 16 whole clots after 2½ days of culture was determined. The granulocytic colony number was determined by counting the average number of colonies in three Falcon 35 x 10 mm petri dishes containing 1 ml medium after 6 days of culture.

Preliminary experiments indicated that high amounts of CS-B, HS, HEP and DS added to the plasma cultures prevented clot formation. The polyanions exhibited this effect by their ability to bind to calcium ions, which are a prerequisite for fibrin formation. Under these circumstances the cells were permitted to settle down. The absence of clot formation did not influence the granulocytic colony counts, since these colonies were counted *in situ*. After culturing, however, it appeared impossible to remove the cells from erythropoietin-stimulated cultures for subsequent fixation benzidin and hematoxylin staining. Nucleated cell counts in fixed clots indicated a cell loss when more than 50% of the polyanion concentration, which just prevented clot formation, was added to the cultures. When extra calcium (up to 200 μg/ml $CaCl_2.2H_2O$) was added to these polyanion concentrations to permit adequate clotting, the inhibition of the erythrocytic colony formation was less. For this reason, these high polyanion concentrations were studied separately.

Incubation of cells with polyanions prior to culturing

In two series of experiments the cells were incubated for 2 h at 37°C with constant shaking at a concentration of 10^6 nucleated cells/ml in BSS containing 20% fetal calf serum. CS-A, CS-B, CS-C or DS, in final concentrations ranging from 10^{-4} up to 200 μg/ml, were added to the cell suspensions at the start of the incubation.

After incubation the cells were washed three times with BSS, and resuspended in the culture medium at a concentration of 10^5 nucleated cells per ml.

The average SEM in the percentage of erythrocytic colony numbers was 9.78%. The average SD in the percentage of granylocytic colony numbers was 11.44%. Colony numbers higher than 125% and lower than 78% of the control numbers were considered to differ significantly ($P < 0.05$) from the expected values when no additives were included.

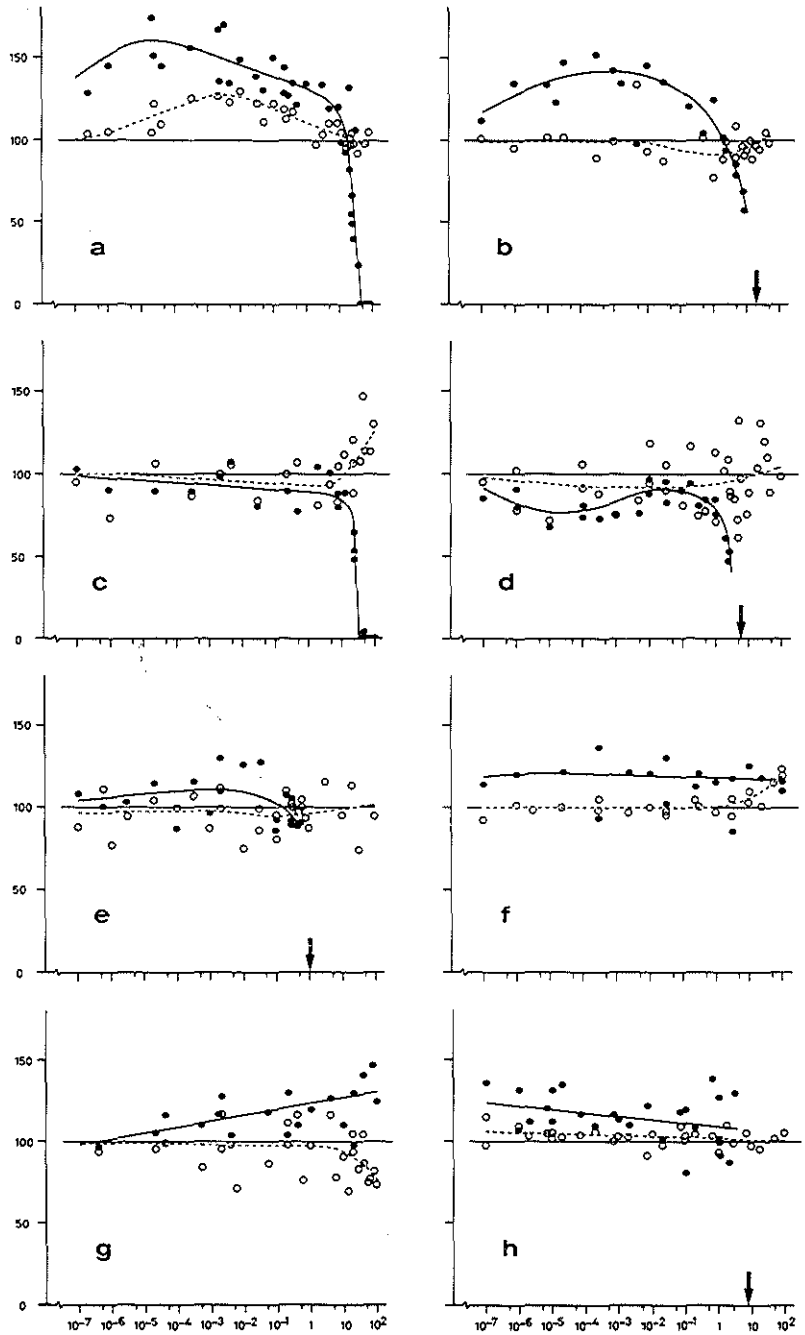
Results

Erythrocytic colony formation

Addition of 40 μg/ml or more CS-A or CS-C resulted in a complete inhibition of erythrocytic colony formation (Figures 1a,c). In the event of a decreased number or erythrocytic colonies the size of the colonies was smaller, indicating a suppressed proliferative activity of the erythropoietic cells. Concentrations of 5 to 10 μg/ml CS-B decreased the erythrocytic colony formation ($P < 0.05$). Addition of 20 μg/ml CS-B prevented clot formation (Figure 1b): thus concentrations of 10 μg/ml and higher were not studied.

Low amounts (10^{-7} up to 1 μg/ml) of CS-A and CS-B exhibited a stimulatory effect on the erythrocytic colony formation ($P < 0.05$). The enhancing effect was greatest at concentrations of 10^{-5} μg/ml for CS-A and 3×10^{-4} μg/ml for CS-B.

The addition of HS to the cultures in concentrations between 10^{-5} and 10^{-3} μg/ml slightly decreased ($P < 0.05$) the erythrocytic colony number (Figure 1d). A concentration of 3 μg/ml elicited a 60% inhibition of the CFU-E number ($P < 0.05$). HS prevented clot formation in amounts higher than 5 μg/ml, thus concentrations of 2.5 μg/ml and higher were not studied.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As opposed to the effects of CS-A, CS-B, CS-C and HS, no inhibitory effect was detected when HEP was added to the cultures in concentrations of 0.5 μg/ml or less (Figure 1e). Concentrations of more than 0.5 μg/ml were not tested due to the prevention of clot formation with HEP concentrations of 1 μg/ml and higher.

HA (Figure 1g) had a slightly enhancing effect on the CFU-E number ($P < 0.05$ for concentrations higher than 10 μg/ml); the effect increased with increasing concentrations. KS had a slight but not significant stimulatory effect on the erythrocytic colony number over the whole ranging of concentrations used (Figure 1f).

In Figure 1h the effects of the addition of a synthetic non-toxic polyanion, i.e. DS are presented. This polyanion had no effect on the sensitivity of CFU-E to erythropoietin. At a concentration of 8 μg/ml it prevented clot formation.

Granulocytic colony formation

As opposed to the effects of several AMPS compounds on the number of CFU-E, no inhibitory effects of AMPS on the CFU-C number were observed with polyanion concentrations between 10^{-7} μg/ml and the maximal concentrations. Even at concentrations where clot formation was prevented, no reduction of colony formation was observed although the colonies had a less aggregated appearance.

CS-C only slightly enhanced the colony number at concentrations of 25 μg/ml and higher, and CS-A exhibited a similar effect between concentrations of about 10^{-3} and 10^{-2} μg/ml ($P < 0.05$). In high concentrations HA slightly inhibited the colony formation. The other compounds had no effect.

Incubation with polyanions prior to culturing

We attempted to determine whether a short incubation of the bone marrow cells with polyanions prior to culturing would exert similar effects as culturing in the presence of polyanions. As is indicated in Table 1, incubation over a 2-h period at 37°C with concentrations from 10^{-4} up to 200 μg/ml of CS or DS did not significantly inhibit the erythrocytic or the granulocytic colony number ($P < 0.05$). The enhancement of the erythrocytic colony formation after incubation with high concentrations of CS-A and CS-B was probably due to the fact that triplicate washing after incubation caused the AMPS concentration to be diluted by a factor of between 10^4 and 5×10^4. In consequence, stimulatory amounts of CS-A and CS-B, were present in the culture medium.

Figure 1. Number of erythrocytic (•) and granulocytic (○) colonies after culture of bone marrow cells in the presence of several polyanions in a wide range of concentrations. The colony numbers are expressed as percentage of those of the control cultures without additives. Arrows in figures denote inability to passage further due to insufficient clotting and in consequence inability to count erythrocytic colony numbers. Plotted are the figures up to 50% of these concentrations. a. CS-A; b. CS-B; c. CS-C; d. HS; e. HEP; f. KS; g. HA; h. DS.

Table 1
Number of erythrocytic (E) and granulocytic (G) colonies after incubation with CS-A, CS-B, CS-C and DS before culturing

Polyanion Concentration[a]	CS-A		CS-B		CS-C		DS	
	E	G	E	G	E	G	E	G
10^{-4}	94.60[b]	101.16[b]	106.30	96.03	125.00	98.74	93.99	93.23
10^{-2}	106.91	95.24	89.08	93.48	111.23	96.68	114.07	92.95
10^{0}	142.70	94.19	119.95	110.25	103.61	91.52	104.95	107.81
10^{1}	147.65	110.28	137.30	90.14	112.50	97.02	104.21	105.15
2.10^{2}	131.33	114.02	142.62	90.29	109.45	89.11	106.36	114.22

[a] μg/ml medium
[b] The figures express the percentage of colonies relative to the number in control cultures containing no additives. The pooled results of two separate experiments are given.

Addition of calcium

Since calcium is believed to be involved in hormone-induced DNA synthesis and subsequent cell division (Lippman 1965, Hunt & Perris 1973), we considered the possibility that calcium binding by polyanions could lead to a decreased proliferation of CFU-E. From Table 2 it appears that addition of calcium to inhibiting amounts of CS-A, -B and -C did not affect the inhibitory action of these polyanions on the CFU-E number. The differences that were found when high calcium concentrations were added to CS-B concentrations of more than 10μg/ml could be correlated with the prevention of cell loss, due to a more efficient clotting. When 200 μg/ml of calcium was added to cultures that contained 50% or more of the HS, HEP and DS concentrations, which just prevented clot formation, also higher colony numbers were counted (not indicated in Table 2). This enhancing effect was positively correlated with the prevention of cell loss from the clots to which calcium was added. Furthermore, by adding extra calcium to the cultures neither the enhancing effects of CS-A and CS-B on the number of erythrocytic colonies nor the CFU-E number in cultures, to which no polyanions were added, appeared to be affected. In addition, the granulocytic colony formation was also not significantly altered by the addition of calcium (Table 2).

Addition of glycogen, D-glucose and D-galactosamin

In order to determine whether the stimulatory effect of low amounts of CS-A and -B was due to metabolic use of these polyanions by the proliferating erythroblasts, we studied the effect of glycogen, D-glucose and D-galactosamin on the number of erythrocytic and granulocytic colonies. G-galactosamin was concerned because it is a constituent of AMPS and CS-A, -B and -C in particular. Table 3 shows that addition of these compounds in concentrations between 10^{-7} and 100 μg/ml to the cultures

Table 2
Effect of addition of calcium on the number of erythrocytic (E) and granulocytic (G) colonies cultured in the presence of CS-A, -B and -C

Compound	µg/ml	0[a]		4.10^{-4}[a]		200[a]	
		E	G	E	G	E	G
CS-A	10^{-4}	138.17[b]	125.41[b]	134.70	122.60	148.01	129.60
	20	36.70	98.53	26.50	104.33	35.81	100.87
	40	0.00	105.08	0.00	92.29	0.00	104.77
CS-B	10^{-4}	151.92	88.78	145.49	112.81	138.23	133.17
	10	55.94	93.70	51.56	91.07	47.70	99.02
	15	33.15	88.10	30.28	102.06	46.30	100.44
CS-C	10^{-4}	88.00	86.64	106.42	91.23	93.99	105.20
	20	48.57	88.00	64.94	120.16	53.46	111.55
	40	0.00	107.56	0.00	113.47	0.00	113.02
		100.00	100.00	102.47	94.47	114.47[c]	108.63[c]

[a] µg $CaCl_2.2H_2O$/ml culture medium
[b] The figures express the percentage of colonies in relation to the number of colonies in control cultures containing no additives. Given is the average result of two separate experiments.
[c] Only 100 µg $CaCl_2.2H_2O$/ml culture medium was present.

did not evoke significant alterations in numbers of CFU-E or CFU-C, apart from 10 µg/ml D-galactosamin, which had a small enhancing effect on the CFU-C number ($P < 0.05$). These findings suggest that the stimulatory effects of CS-A and -B on the erythrocytic colony number are not produced by metabolic use of these AMPS compounds.

Table 3
Number of erythrocytic (E) and granulocytic (G) colonies after culturing cells in the presence of D-galactosamin (gal), D-glucose (gluc) or glycogen (glyc)

Concentration of compound	gal		gluc		glyc	
	E	G	E	G	E	G
10^{-7}[a]	101.93[b]	97.01[b]	89.03	103.70	105.34	108.31
10^{-4}	118.48	100.95	111.72	114.05	100.33	90.79
10^{-2}	103.97	125.15	91.44	109.33	94.97	94.10
10^{-1}	97.81	112.40	101.05	99.29	92.88	103.45
10^{0}	97.41	123.26	81.31	114.58	104.76	98.58
10^{1}	85.88	128.40	85.48	115.00	84.49	91.97
10^{2}	n.d.	n.d.	122.46	91.74	98.20	111.93

[a] µg/ml culture medium.
[b] Percentage of colony numbers in relation to control cultures containing no additives. The results of one experiment are given.

Discussion

The results of this study demonstrate that certain polyanions are able to affect the erythrocytic colony formation *in vitro*. An enhancing effect of the erythrocytic colony number was shown for CS-A and CS-B. Most striking was the observation that CS-A and CS-C could totally inhibit the sensitivity of CFU-E to erythropoietin. CS-A and CS-C also slightly enhanced the granulocytic colony formation. Our results are in agreement with the postulation of McCuskey & Meineke (1973) concerning the inhibiting effect of excessive concentrations of AMPS on the later stages of erythroid development *in vivo*.

Mucopolysaccharides occur as extracellular matrix molecules and are associated with the cell surface in plants, invertebrates and vertebrates (Lippman 1965, Kraemer 1971, Kojima & Yamagata 1971). AMPS are believed to play an essential role in developmental processes (Kosher & Searls 1973, Sugiyama 1972), and in cellular proliferation and differentiation in general. Lippman (1965) proposed a role of AMPS at the cell surface in blocking and triggering cells for prophase events. It is not clear in which way polyanions interfere with the erythropoietin-responsive cells but it may be by shielding of specific receptors for erythropoietin on the cell membrane, which results in a reduction of proliferation and differentiation (Shida & Shida 1976, McConnell 1971, Cuatrecasas 1971). This possibility is supported by a number of recent studies, which demonstrated the existence of receptors at cell surfaces that react with specific substances in a manner resulting in alteration of cell metabolism and proliferative capacity (Shida & Shida 1976, McConnell 1971, Cuatrecasas 1971, Morrison et al. 1965). Chang et al. (1974) suggested that a protein receptor on the external surface of responsive cells from rat bone marrow is required for the action of erythropoietin on these cells.

As to the nature of the stimulatory effects on the erythrocytic colony formation, exerted by low concentrations of CS-A and CS-B, the underlying mechanism is unknown. Since a metabolic effect is not indicated by our observations it may be speculated that shielding of receptor sites for an anti-erythropoietin serum activity enhances the effect of erythropoietin on cells sensitive to this factor. Krzymowski & Krzymowska (1962) have reported inhibitory effects of plasma of polycythemic animals, but the evidence for the existence of an erythropoiesis-inhibiting factor is not conclusive (Zivný & Nečas 1972). However, no information is available concerning the possible presence of such an inhibitor activity in the culture medium used.

The binding of calcium by polyanions might result in a lack of response of CFU-E to erythropoietin stimulation since calcium is believed to mediate hormone-induced DNA synthesis and subsequent cell division (Lippman 1965, Hunt & Perris 1973). According to Perris & Whitfield (1967) and Hunt & Perris (1973) calcium is able to stimulate mitoses in bone marrow cells. A role played by calcium has become less likely since our observations that addition of calcium did not affect the inhibitory and stimulatory effects of AMPS on the erythrocytic colony number or the colony growth in the absence of additives *in vitro*.

The effects of AMPS in the present study provide the first direct evidence in support of the hypothesis of McCuskey et al. (1972a,b, 1975) that the AMPS concentration in the bone marrow groundsubstance may affect the maturation of erythroid progenitor cells. Since reticular cells and macrophages have the property to excrete and ingest AMPS it is suggested that *in vivo* stromal cells are involved in the conductive action of some sulphated AMPS on erythropoiesis. The capacity of macrophages to elaborate a spectrum of regulatory molecules, which have mutually antagonistic actions on hemopoiesis, has been recognized earlier (Moore 1976). The central macrophages (Le Charpentier & Prenant 1975, Ploemacher & van Soest 1977a,b) have to be considered preferably as regulator cells since they maintain close connections with erythroblasts, which are located around them. These close contacts will minimize the necessary amounts of regulatory AMPS.

In summary, our observations suggest that, apart from the well-documented action of the humoral regulator erythropoietin, ground substance components, i.e. AMPS, may affect erythroid maturation.

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Particle-induced erythropoietin-independent effects on erythroid precursor cells in murine bone marrow

Running title: Regulation of Erythroid Progenitors

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Abstract

A possible regulatory action of phagocytic cells on erythropoiesis was investigated by infusion of inert polystyreen latex particles (LAT). LAT appeared to induce changes in the femoral content of erythroid progenitor cells. These changes were most pronounced in primitive erythroid progenitor cells (BFU-E) and appeared to be gradually damped in more differentiated populations (CFU-E and erythroblasts). LAT did not influence granulocyte/macrophage progenitor cells (CFU-C). The effects of LAT could not be attributed to changes in the systemic erythropoietin (EP) concentration. Administration of dexamethason could prevent the effect of low doses of LAT, suggesting that phagocytosis of the particles is essential to the observed effects.

Erythroid burst formation was previously found to be dependent on a bone marrow associated activity, termed BFA (burst feeder activity). BFA acts as an *in vitro* inducer of EP-responsiveness in BFU-E. In this study it was found that LAT-induced changes in femoral erythroid progenitor cell content were characteristically preceded by corresponding changes in BFA. It was concluded that BFA-associated cells probably play a role *in vivo* in the early differentiation of erythroid progenitor cells.

The present data are interpreted as direct *in vivo* evidence supporting a two-step regulatory model operating in erythropoiesis and provide evidence that phagocytic cells are a component of the Erythroid Hemopoietic Inductive Microenvironment.

Introduction

The presence of central macrophages (CM) in erythroid islands in murine yolk sac, fetal liver, spleen and bone marrow has been established in many morphological studies (Sorensen, 1961; Orlic, Gordon & Rhodin, 1965; Berman, 1967; Naito & Wisse, 1977). Also in experimentally induced hemolytic anemia the combined presence of CM and surrounding erythroblasts has been demonstrated in sinusoids and central venes of liver and bone marrow (Ploemacher & Van Soest, 1977a; 1977b; Ploemacher, Van Soest & Vos, 1977). CM have apparent simple functions as:
(1) selective detainment of erythropoietic cells until full maturation is achieved, and
(2) sequestration of expelled normoblast nuclei and disranged erythroid elements.

There is increasing evidence that macrophages have a capacity to elaborate a spectrum of regulatory molecules, which have mutually antagonistic actions on hemopoiesis (Calderon & Unanue, 1975; Ellner, Lipsky & Rosenthal, 1975; Moore,

1976; Kurland, Broxmeyer & Moore, 1977) and other eukaryote cell systems in general (Keller et al., 1976). It has been demonstrated by Miller et al. (1976), that repeated infusion of an emulsion of perfluorotributylamine induced extra-medullary hemopoiesis; it was suggested that "overloading" of phagocytic cells, and probably also non-phagocytic cells, induce the observed changes in hemopoiesis. However, a regulatory role of macrophages in erythropoiesis has not been reported up till now.

It is the purpose of this study to investigate whether a selective manipulation of macrophage functions causes specific alterations in the erythroid compartment. To this purpose inert polystyreen latex particles (LAT), which are predominantly phagocytized by macrophages, were intravenously administered to mice. The effects on hemopoiesis were measured by determining the femoral content of primitive (erythroid burst forming units, BFU-E) and more differentiated (erythroid colony forming units, CFU-E) erythroid progenitor cells, of primitive progenitor cells of the granulocyte/macrophage differentiation pathway (CFU-C), and of erythroid precursor cells (erythroblasts).

It was previously demonstrated that mouse BFU-E lack the capacity to respond directly to EP *in vitro*, but require the presence of a bone marrow-associated activity, which has been termed BFA (burst feeder activity). BFA was found to be associated with a non-dividing population of mouse bone marrow cells, characterized by a buoyant density of 1.0803 $g.cm^{-3}$ and a modal sedimentation rate of 4.7 $mm.h^{-1}$ (Wagemaker, in press). Circumstantial evidence *in vitro* indicates that the BFA-induced progeny of BFU-E becomes responsive to EP, leading to colonies consisting of up to 10^4 reticulocytes. This *in vitro* requirement of BFU-E would predict that *in vivo* BFU-E numbers are regulated by BFA rather than by EP. The relative *in vivo* unresponsiveness of BFU-E to changing levels of circulating EP has been demonstrated in a number of studies (Axelrad et al., 1974; Iscove, 1977; Iscove, in press; Wagemaker, 1977). It is demonstrated in this study that LAT-induced changes of femoral BFU-E content are preceded by corresponding changes in femoral BFA.

Materials and Methods

Mice

Male (C57BL/Rij x CBA/Rij) F1 mice, 17-20 weeks old and weighing 30-35 g were used. They were purchased from the Laboratory Animals Centre of the Erasmus University, Rotterdam, The Netherlands.

Particulate matter

Polystyrene latex particles (LAT; 0.234 μm diameter; SERVA, Heidelberg, Germany) were suspended in the balanced salt solution (BSS) described by Mishell and Dutton (1967). LAT did not appear to inhibit colony formation by CFU-C and CFU-E *in vitro* up to concentrations of 2 $mg.ml^{-1}$. Vehicles were prepared by centrifuging the relevant dilutions of the latex suspension at 27500 RCF in a MSE High Speed 25 Centrifuge and collecting supernates. Dexamethason sodium phosphate (Decadron phosphate, Merck, Sharp & Dohme, Haarlem, The Netherlands;

30 mg/kg body weight) was administered i.p. to decrease phagosome formation in macrophages (Wiener, Marmary & Curelaru, 1972; Klausner et al., 1975; Nicol & Bilbey, 1960; Raz & Goldman, 1976).

Polycythemic mice

Polycythemia was induced by 2 i.p. injections of 0.05 ml packed isologous erythrocytes on day 0 and day 1. The hematocrit was 69.5 ± 0.3 (mean ± SEM) at the day of sacrifice.

Erythropoietin

Erythropoietin (EP) step III, prepared from anemic sheep plasma (Connaught Medical Research Laboratories, Willowdale, Ontario, Canada), was used in CFU-E cultures and for *in vivo* administration. EP used in BFU-E cultures was human urinary erythropoietin, collected and concentrated by Centro de Estudios Farmacologicos y de Principios Naturales, Buenos Aires, Argentina, further processed and assayed by Hematology Research Laboratorium, Children's Hospital of Los Angeles, California, and authorized for distribution by the Division of Blood Diseases and Resources of the National Heart, Lung and Blood Institute, NIH, Bethesda, Md., USA., spec. act. 18.6 ± 1.2 units.mg^{-1}.

Assay for BFU-E and BFA

BFU-E were estimated *in vitro* as described by Wagemaker (1977). For collection and dilution of bone marrow cell suspensions the α-medium was replaced by BSS, resulting in a concentration of 20% BSS in the final culture medium. Briefly, 10^5 nucleated cells per ml were cultured in α-medium, supplemented with 10% (V/V) horse serum, 10% (V/V) fetal calf serum, 2% (W/V) bovine serum albumin, 3 x 10^5 M egg lecithin, 7 x 10^{-6} M human transferrin saturated with $FeCl_3$, 10^{-7} M $Na_2SeO_3.5H_2O$ and 10^{-4} M2-mercaptoethanol using 0.8% (W/V) methylcellulose as a semisolidifying agent. Optimal stimulation of erythroid burst formation was obtained with 1 unit EP ml^{-1}. The cultures were stimulated with irradiated (1500 rad) bone marrow cells at a concentration of 1.8 x 10^6. ml^{-1}, serving as a source of BFA. As previously reported BFA-associated bone marrow cells resist up to 2000 rad γ-irradiation, their effect approaching plateau levels at concentrations higher than 10^6.ml^{-1} (Wagemaker, in press). All cultures were performed in quadruplicate. The control incidence of BFU-E as determined following the described culture conditions averaged 33 BFU-E per 10^5 nucleated cells.

BFA was assayed by culturing 6 x 10^5.ml^{-1} irradiated (1500 rad) bone marrow cells in the presence of EP, using bone marrow cells from untreated control animals at a concentration of 10^5.ml^{-1} as target cells. It was demonstrated that the number of bursts induced is a linear function of the irradiated cell concentration between concentrations of 10^5 and 10^6.ml^{-1}, which indicates that the number of burst induced can be employed as a direct measure of the BFA-concentration of the cultures (Wagemaker, in press).

Assays for CFU-E and CFU-C

The improved plasma plot culture system according to the system introduced by McLeod, Shreeve & Axelrad (1974) was used. The collection and dilution medium was replaced by BSS. The plasma medium used was slightly modified in that NCTC-109 medium and Eagle's minimum essential medium was replaced by Dulbecco's medium (Flow Laboratories, U.K.). 0.25 U.ml^{-1} EP was included for the erythrocytic colony cultures. For culturing the granulocytic colonies 0.1 ml. ml^{-1} mouse fibroblast conditioned medium (cf. Van den Engh, 1974) was included instead of the kidney tubules conditioned medium. According to McLeods system for culturing erythrocytic colonies microtiter plates (Greiner, Nürtinger, Germany) were used with each well containing 0.1 ml of culture medium. For each experimental point the average colony number in

16 whole clots after 21/2 day of culture was determined by counting the average number of colonies after 6 days of culture in 3 Falcon 35 x 10 mm – petri dishes each containing 1 ml medium. The incidence of CFU-E in cultures of normal bone marrow was 400 per 10^5 nucleated cells; that of the CFU-C was 230 per 10^5 cells.

Determination of haematocrit and absolute and relative cell number

Blood used for haematocrit measurements was obtained from the suborbital plexus of etherized mice just before sacrifice. Absolute and relative cell numbers in marrow preparations were determined as described earlier (Ploemacher et al., 1977b).

Erythropoietin bioassay

Erythropoietin bioassay was performed as previously described (Wagemaker et al., 1972) using female ND2 mice 9-12 weeks of age. Briefly, polycythemia was induced by exposing the mice, in a closed cage of 65 x 45 x 25 cm, to an inflow consisting of 8% O_2 and 92% N_2 at a rate of 3 l/min for 9 days, 8 hr/day. On the fourth and fifth day after termination of this intermittent hypoxia, EP was injected intraperitoneally in five mice per experimental point in three divided doses at time intervals of 12 hr; 24 hr after the last injection, 1 μCi $^{59}FeCl_3$ in saline was intravenously injected; the mice were killed 24 hr later and peripheral blood ^{59}Fe uptakes determined. Responses obtained with mouse serum samples were calibrated against a standard preparation purified from plasma of phenylhydrazine-treated sheep, in turn calibrated against the 2nd Int. Ref. Preparation of Erythropoietin, kindly provided by the WHO International Laboratory for Biological Standards, Mill Hill, London.

Results

The dose-effect relationship for latex particles (LAT) was studied in the dose range between 10^{-2} and 3 x 10^2 mg dry weight/mouse, 3 days after LAT injection (Fig. 1). The response of the CFU-E number showed a sigmoid curve; 3.10^{-1} to 10^{-2} mg LAT appeared to be most effective in stimulating the CFU-E number detected, whereas 10 mg LAT exhibited the most pronounced inhibiting effect on these late erythrocyte precursor population. Although the erythroblast number was severely depressed following infusion of 30 mg LAT, no conclusions were drawn from this as to the fact that a 50 per cent mortality of the mice was observed within 3 days. The hematocrit and the committed granulocyte/macrophage precursors, i.e. CFU-C, were not significantly affected.

In fig. 2 the time course of the alterations in the erythroid progenitor cell compartment and CFU-C following infusion of 0.3 mg LAT is shown. From fig. 2a it appears that this low dose of LAT induced pronounced changes in BFA that preceded 2 discrete peaks in the femoral content of BFU-E (340% of control value). Subnormal levels of BFA between day 3 and 6 were reflected in a drop in the BFU-E incidence, but this did not fall to a subnormal level. LAT induced no detectable changes in the number of CFU-C detected. The time course of the CFU-E and erythroblast incidence both showed a peak increase on day 4, concomitantly with the changes in the BFU-E number detected, although the onset of the increase in erythroblast number showed a one day delay in relation to that of the CFU-E num-

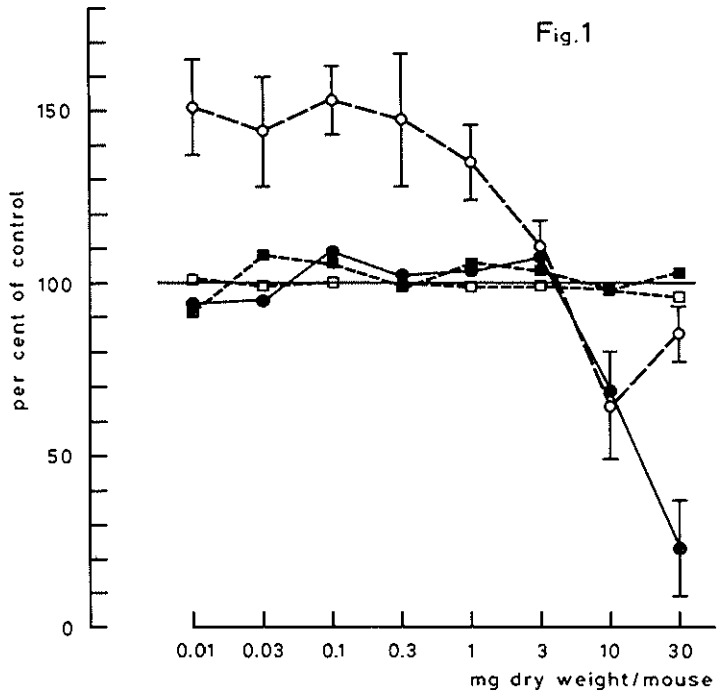

Fig. 1. Effect of i.v. injection of various doses of polystyreen latex particles (LAT) on the incidence of CFU-C (▪), CFU-E (○) and erythroblasts (●) per femur and the hematocrit (▫) as determined at 3 days after latex infusion. Each point represents the arithmetic mean of 3 separate experiments, in which three mice per dose were used. Control mice were injected with BSS. Vertical bars indicate SEM.

ber. A second increase in the CFU-E incidence occurred up to day 8, whereas erythroblast numbers remained at control values from day 5 on, as were the hematocrit values during the whole observation period. The effect of LAT on the erythroid compartment is shown to be damped with progressive maturation along the erythroid pathway (Fig. 3).

The time course of 10 mg LAT is shown in fig. 4. This LAT dose was very effective in suppressing erythropoiesis as measured by determination of the BFU-E and erythroblast numbers (Fig. 4b). BFA was subnormal during 4 days following LAT infusion. The recovery of BFA on day 5 and 6 again preceded the BFU-E recovery on day 8. In contrast to the CFU-E numbers detected, BFU-E numbers were only slightly below normal on the first days following LAT injection. However, from day 4 on BFU-E reflected the cyclic response of CFU-E. The CFU-C compartment size was not affected during the observation period (Fig. 4a).

The data presented in fig. 2 and 4 strongly suggest that changes in the number of BFU-E are a response to changes in BFA and that CFU-E may be also responsive to

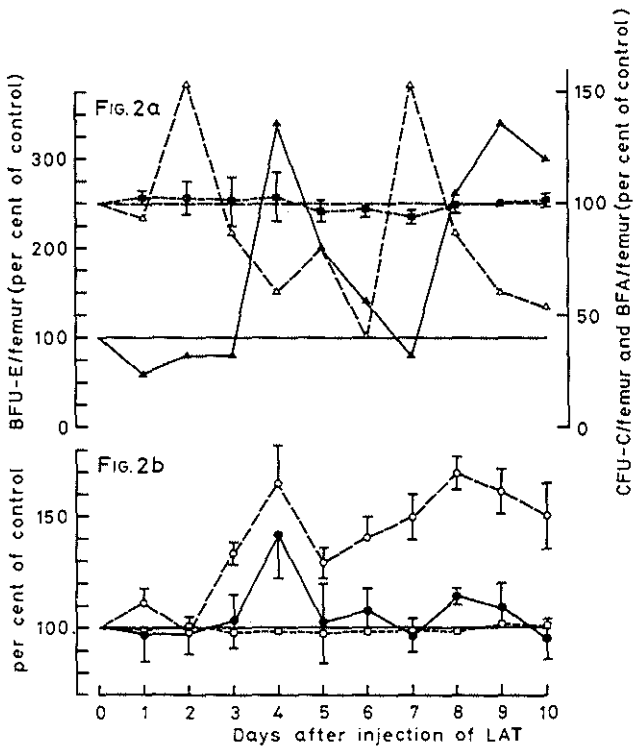

Fig. 2a. Changes in burst feeder activity (△) and the incidence of BFU-E (▲) and CFU-C (■) per femur following infusion of 0.3 mg dry weight LAT. Parameters are expressed as percentage of control values. Each point represents the arithmetic mean of two separate experiments. Vertical bars indicate SEM.

Fig. 2b. Effect of 0.3 mg LAT on the hematocrit (□) and the incidence of CFU-E (○) and erythroblasts (●) expressed as percentage of control values. Each point represents the arithmetic mean of three separate experiments. In each experiment the femoral marrow of at least three mice per day was pooled. Vertical bars indicate SEM.

BFA in a lesser extend. In a follow-up of this study it appeared, that the time course of the presented effects could be delayed by about one day. Although in such a case BFA was observed to be severely suppressed on day 1 after infusion of 10 mg LAT and BFU-E numbers were above normal at the same time, a similar BFA-responsiveness of BFU-E was suggested.

In order to investigate whether the observed effects of LAT were associated with endocytosis of the particles themselves by phagocytic cells, the vehicles of 0.3 and 10 mg LAT were administered to mice. As is shown in table 1, vehicle injection did not significantly affect the BFU-E and CFU-E incidence on day 3, indicating that LAT particles themselves induced the reported changes in erythropoiesis. Dexame-

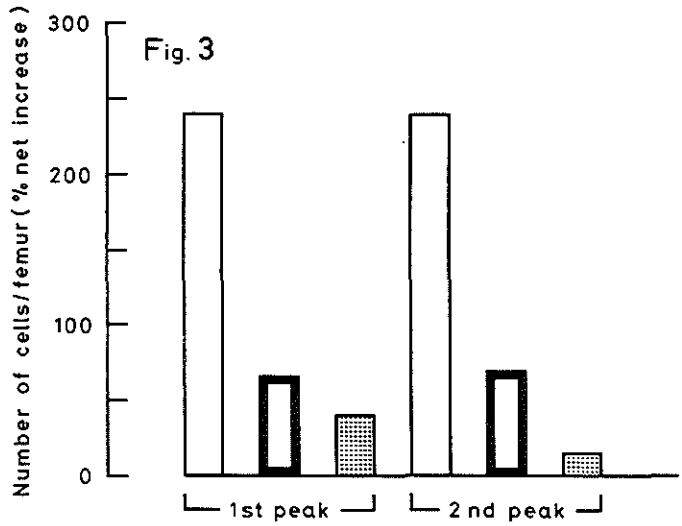

Fig. 3. Histogram showing the first and second peak increase of BFU-E (□), CFU-E (▫) and erythroblasts (▫) numbers detected following infusion of 10 mg LAT. Plotted are the 4th day (first peak) and 8th to 9th day (second peak) values, expressed as the percentage of the control changes in femoral erythroid progenitor cell incidence with increasing maturation.

thason phosphate pretreatment of mice totally prevented on day 3 the stimulatory effect of 0.3 mg LAT on the CFU-E number detected but not the inhibitory effect following injection of 10 mg LAT. Corticosteroid treatment alone sightly suppressed the femoral content of CFU-E. Probably, a single injection of dexamethason may be inadequate to prevent phagocytosis of a high dose of particles. Although the effects of corticosteroids have to be regarded with caution owing to their multiple effects *in vivo* these data are indicative for a particle-induced effect on erythroid progenitor cells.

As is apparent from table 2, the systemic EP level was not increased at day 3 after infusion of 0.3 mg of LAT. The EP values, that were found following injection of 10 mg of LAT, were not significantly lower than normal values. Also the EP level in LAT-injected anemic mice could not account for the somewhat lower femoral CFU-E number. These data clearly demonstrate that the effects on erythropoiesis exhibited by LAT were not mediated by changes in EP concentration. This conclusion was confirmed by the experiment shown in table 3, which demonstrates that the relative effect of LAT is independent of administration of a large dose of EP. Table 4 shows a similar experiment in polycythemic mice. It appears that LAT infusion resulted in a slight suppression of the CFU-E incidence in the absence of detectable levels of circulating EP, again confirming that the LAT-induced changes occur by an EP-independent mechanism. As was observed after EP administration to

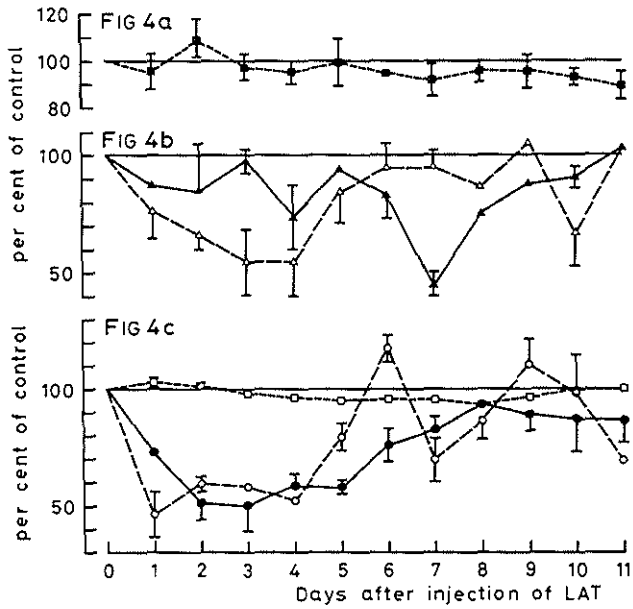

Fig. 4a,b. Femoral burst feeder activity (△) and incidence of BFU-E (▲) and CFU-C (■, Fig. 4a) per femur following 10 mg LAT infusion and expressed as percentage of control values. Each point represents the arithmetic mean of three separate experiments in each of which the marrow of 3 mice were pooled. Vertical bars indicate SEM.

Fig. 4c. Suppression of the femoral CFU-E (○) and erythroblast (●). incidence and the hematocrit values (□) after infusion of 10 mg LAT. The arithmetic mean of four separate experiments, each containing at least three mice per point, are expressed as percentage of control values. Vertical bars indicate SEM.

control mice (table 3) also here LAT caused a deficient response of the CFU-E compartment to EP in comparison to EP-injected polycythemic mice that had not received LAT. The relative increase of the femoral content of CFU-E after EP administration was comparable in normal and LAT-pretreated animals.

Discussion

The results presented in this paper demonstrate that injection of latex particles induces pronounced alterations in the erythroid compartment without affecting granulocyte/macrophage progenitor cells. Several arguments contribute to the notion that the observed effects are EP-independent phenomena. Firstly, no significant changes were observed in serum EP concentrations following infusion of LAT. Secondly, a high dose of LAT decreased the CFU-E number in polycythemic mice,

Table 1.
Effect of administration of the vehicles, corresponding with 0.3 and 10 mg latex (LAT), and the effect of dexamethason phosphate (Dexa) pretreatment on latex-induced changes in the femoral BFU-E, CFU-E and CFU-C incidence three days after infusion. Dexamethason phosphate was administered i.p. 1 hr before latex injection. The data of two separate experiments are shown.

Treatment	Colony-forming cells/femur		
	CFU-C	BFU-E	CFU-E
BSS (control)	35400 (32700)[a]	6150	145000 (111000)
Vehicle 0.3 mg LAT	36600 (33400)	6000	138800 (108000)
Vehicle 10.0 mg LAT	34500 (27900)	6750	125800 (92000)
BSS + 0.3 mg LAT	35300 (32200)	n.d.	240900 (149900)
BSS + 10.0 mg LAT	34900 (31600)	n.d.	110000 (64500)
Dexa + BSS	32500 (35600)	n.d.	122100 (83000)
Dexa + 0.3 mg LAT	34100 (31500)	n.d.	115600 (82600)
Dexa + 10.0 mg LAT	37300 (33700)	n.d.	92100 (60000)

[a]Data of second experiment are indicated in brackets.

which lack detectable levels of circulating erythropoietin. Furthermore, a high dose of EP could not abolish the decrease of CFU-E induced by a high dose of LAT. A major argument is provided by the effects of LAT on the femoral BFU-E numbers detected; BFU-E is known from *in vivo* and *in vitro* studies to be unresponsive to EP (Axelrad et al., 1974; Iscove, 1977; Wagemaker et al., 1977; Wagemaker, in press). Both at low and high doses of LAT BFU-E and CFU-E incidences were concomitantly influenced, indicating that the effects on BFU-E are not compensatory to an increased erythropoiesis or to an initial loss of more differentiated erythroid progenitor cells.

It was previously demonstrated that BFU-E requires the presence of both EP and

Table 2.
Erythropoietin (EP) levels, as determined by measuring the 24 hr peripheral blood ^{59}Fe uptake in posthypoxic polycythemic mice, and femoral CFU-E incidence in normal and anemic mice after LAT administration.

Pretreatment	Treatment	Days after injection	EP concentration $mU.ml^{-1}$	CFU-E/femur[a]
none	BSS	2	55 ± 11[b]	116100
none	0.3 mg LAT	3	59 ± 9	149800
none	10.0 mg LAT	2	50 ± 7	68500
cp[c]	BSS	2	147 ± 24	190700
cp	10.0 mg LAT	2	173[d] ± 13	168000

[a]determined on day 3 after injection
[b]standard error of the mean
[c]cardiac puncture one day before injection. Extirpation of 0.75 ml blood under ether anesthesia was followed immediately by i.v. injection of 0.3 ml Megimide (Nicholas Laboratories Ltd., England), 0.35 ml freshly prepared isologous serum and 0.05 ml BSS.
[d]difference with control anemic EP level not significant as tested with student "t" test.

BFA (Wagemaker, in press). It is thought that BFA is required in the initial stage of erythroid burst formation, whereas the action of EP becomes important in a later stage, in which hemoglobin synthesis is the predominant feature of erythroid maturation to the reticulocyte stage. Essentially on the basis of these data Iscove & Guilbert (in press) recently proposed a two-step regulation model for hemopoiesis, involving stem cell-type regulators (exemplified by BFA) versus pathway-specific regulators (exemplified by EP and CSF). In view of these ideas it is of particular interest that the present data provide *in vivo* evidence for a specific role of BFA-associated cells by demonstrating corresponding changes in femoral BFA and femoral BFU-E. At low doses of LAT the increase of BFA precedes the increase of BFU-E by 2 days. This kinetic feature, combined with current knowledge on the *in vitro* action of BFA, strongly suggests that the increase in erythroid progenitor cells occurs secondary to the increase of BFA. This conception is in agreement with the relative amplitudes (Fig. 3) of the LAT-induced increases of BFU-E (240% over control values), CFU-E (64%) and erythroblasts (41%), which may be explained from a decreasing responsiveness to BFA with the increasing dependence on EP, which accompanies progression in the erythroid differentiation pathway towards the reticulocyte. Although essentially similar, the picture at high doses of LAT is less clear. It was observed in preliminary studies that high doses of LAT, in contrast to low doses, exert opposite effects in bone marrow and spleen CFU-E populations. It is therefore not excluded that the results obtained with high doses of LAT are complicated by a redistribution of erythroid progenitor cells over the body. It is of relevance that high doses of LAT are apparently toxic, doses of 30 mg per mouse resulting in a significant mortality among the mice.

The apparent cyclic nature of the LAT-induced changes are at present not understood. It seems unlikely that reutilization of LAT causes the second wave of changes, because LAT does not cause lysis of cells, which have ingested this material. Morley et al. (1970), in analyzing the oscillatory nature of hemopoiesis, argued on the basis of a publication by Milsum (1966), that a simple type of negative feedback system, containing an absolute timedelay, will always show some oscillation following a

Table 3.
Effect of exogenous EP administration on femoral CFU-E incidence in mice preinjected with 10 mg LAT. The mean data of two separate experiments are shown. EP (5 U/mouse/day) was given i.v. at day 1 and 2 after latex infusion and mice were sacrificed at day 3.

Treatment	BSS[a]	EP
BSS	110.800 (100%)[b]	158.750 (100%)
LAT	66.000 (60%)	95.450 (60%)

[a]Control injection with buffered saline solution.
[b]In the two experiments the decreases due to LAT were 60% and 59% for BSS-treated animals and 56% and 64% for EP-treated animals.

Table 4.
Effect of EP administration on femoral CFU-E content of polycythemic mice that received 10 mg LAT at day 2 after the second i.p. injection with 0.5 ml packed erythrocytes. One U EP was given on day 3 and bone marrow was collected at day 5. The mean data of three separate experiments are shown.

Treatment	BSS	EP
BSS	29.000 ± 2930[a] (100%)	56.300 ± 14.900 (100%)
LAT	22.900 ± 5500 (77 ± 13%)	37.200 ± 6480 (66 ± 6%)

[a]Mean incidence of CFU-E per femur ± S.E.M.

brief perturbation, the period of the oscillation being approximately equal to twice the time-delay. In our data concerning the effects of low doses of LAT the "periodicity" of the changes is approximately twice the time-delay between changes in BFA and in BFU-E. Although more rigorous experimentation is required to unravel the nature of the apparent cyclic changes induced by LAT, the existence of a feedback system between BFA-associated cells and erythroid progenitor cells might be an atractive and logical hypothesis.

It is implicated in the present study that polystyreen latex particles have to be phagocytized in order to exhibit the observed effects on erythropoiesis. In a recent study (Ploemacher et al., manuscript in preparation) we found that infusion of colloidal carbon particles induced comparable EP-independent changes in erythroid progenitor cells in the bone marrow, whereas – in contrast to the effect of LAT – high doses elicited an increase in the number of stem cells (CFU-S) and granulocyte/macrophage precursors (CFU-C). The *in vivo* responsiveness of BFU-E and CFU-E to particle loading of macrophages might imply that:
(1) macrophages are involved in pre-erythropoietin-dependent regulation of erythroid progenitor cell proliferation, and
(2) that macrophages *in vivo* possibly may be equivalent to or belong to the distinct cell population associated with BFA *in vitro* and may represent a stromal marrow cell population belonging to the assumed Erythroid Hemopoietic Inductive Microenvironment (Trentin, 1971).

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