HEMOPOIETIC STEM CELL MOBILIZATION IN MICE

MOBILISATIE VAN HEMOPOIETISCHE STAMCELLEN
IN DE MUIS

PROEFSCHRIFT

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Voor Beatrix,
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ABBREVIATIONS

BMC	bone marrow cells
BPA	burst promoting activity
CFU-s	colony-forming unit spleen
CoF	cobra venom factor
CSF	colony-stimulating factor
CSF-1	synonym of M-CSF
E-BFU	erythroid burst-forming unit
E-CFU	erythroid colony-forming unit
EO-CFU	eosinophil colony-forming unit
EPO	erythropoietin
G-CSF	granulocyte colony-stimulating factor
GM-CFU	granulocyte/macrophage colony-forming unit
GM-CSF	granulocyte/mcrophage colony-stimulting factor
HGF	hemopoietic growth factor
HIM	hemopoietic inductive microenvironment
HSC	hemopoietic stem cell
IgD	immunoglobulin D
IgM	immunoglobulin M
IL-3	interleukin 3
i.p.	intraperitoneal
i.v.	intravenous
KDO	2-keto-3-deoxyoctonate
LAP	lipid A associated protein
LPS	lipopolysaccharide
M-CSF	macrophage colony-stimulating factor
Meg-CFU	megakaryocyte colony-forming unit
Meg-CSF	megakaryocyte colony-stimulating factor
Mix-CFU	colonies containing cells of three or more lineages
PGE2	prostaglandin E2
PGF	prostaglandin F
PHZ	phenylhydrazine
PMAA	polymethacrylic acid
PMAA-STYR	polymethacrylic acid-styrene
SAF	stem cell activating factor
SHSF	splenic hemopoiesis stimulating factor

I. GENERAL INTRODUCTION

I.1 Hemopoiesis

Mature hemopoietic cells with the exception of lymphocytes have a finite lifespan and lack proliferative capacity; this necessitates the continuous production of these cells. As the demand for specific cells may vary, regulation of the type and number of cells produced is required. In the adult mouse, in which the experiments described in this thesis were performed, the bone marrow is the major site of hemopoiesis, but hemopoiesis is also observed in the spleen. Mature hemopoietic cells are generated by proliferation and differentiation of functionally less specialized immature cells. The most primitive cells are the pluripotent hemopoietic stem cells (HSC), operationally defined as cells capable of self-renewal, extensive proliferative ability and possessing the ability to generate mature cells of all lineages. HSC give rise to committed progenitor cells, i.e. cells which are restricted in their development to a specific cell lineage.

Proliferation and differentiation of committed progenitor cells eventually lead to the formation of mature cells of each lineage. As differentiation proceeds, the cells lose their proliferative capacity and the last stages of the development of mature cells proceed without proliferation. Mature and maturing cells are released into the blood stream to exert their function in various tissues or in the circulation.

In addition to mature cells, small numbers of progenitor cells and stem cells can be found in the blood stream. The generation of T lymphocytes proceeds somewhat differently from other hemopoietic cells. HSC in the bone marrow give rise to so-called pre-T cells which seed in the thymus where they proliferate and differentiate into mature immunocompetent T lymphocytes.

I.2 Hemopoietic organs

In the adult mouse the bone marrow and the spleen allow the prolonged proliferation and differentiation of hemopoietic stem cells. Both organs have an extravascular compartment, which is the breeding site of hemopoietic cells and an extensive vascular compartment which is largely comprised of relatively wide venous vessels, the so-called sinuses. Newly formed blood cells are delivered into the sinusoidal blood. Stem cells may cross the sinuses of spleen and bone marrow in two directions. This follows from the fact that stem cells are present in the peripheral blood, while i.v. injected stem cells can settle in the extravascular compartment of the bone marrow and spleen of lethally irradiated mice and man.

I.2.1 Bone marrow

The bone marrow has a complex vascular system within the hemopoietic tissue. According to De Bruyn et al. (1970) the arterial blood supply of the sinuses comes from the nutrient artery which penetrates the diaphysis of the long bones. Subsequently, it runs longitudinally in the center of the bone marrow and it sends off arterioles in a centrifugal direction. These vessels enter the shaft as capillaries and then turn back to form sinuses in the bone marrow. The sinuses are radially arranged in the bone marrow and empty into wider collecting sinuses, which open into a venous vessel which runs alongside the central artery and leaves the bone marrow through the same foramen where the nutrient artery entered. A second source of blood supply originates from a periostal capillary network. Capillaries from this network enter the Haversian canals which run obliquely to the long axis of the bone to connect with the sinuses. The wall of the sinuses consists of a continuous layer of endothelial cells resting on an incomplete basement membrane, the abluminal side of the basement membrane being incompletely covered by a layer of adventitial cells. Hemopoietic cells fill the tissue spaces between the sinuses, the so-called hemopoietic cords. They are supported by a framework of reticular cells, reticular fibres and ground substance. The transmural passage of blood cells involves migration through the body of the endothelial cells (Weiss, 1970, De Bruyn et al., 1971; Campbell, 1972; Weiss and Chen, 1975). Under normal conditions few immature cells leave the hemopoietic cords. The mechanism of the highly selective transmural passage is not completely understood.

I.2.2 Spleen

The splenic parenchyma is surrounded by a fibrous capsule which sends off trabeculae that penetrate into the organ. Branches of the splenic artery enter the hilus and run in the trabeculae, where they divide. Subsequently they enter the parenchyma and become surrounded by a sheath of lymphoid cells. This periarteriolar lymphoid tissue is part of the white pulp of the spleen which further consists of the follicles and the marginal zone. The other major compartment is the red pulp which is involved in hemopoietic and phagocytic activity (Seifert and Marks, 1985). Splenic arteries branch to form penicillar arteries, straight vessels which radiate in different directions. These arteries become capillaries which partly end in the sinusoidal cords (open circulation) and partly into the venous sinuses (closed circulation) which drain into veins. The wall of splenic sinuses also consists of endothelial cells, a basement membrane and adventitial cells. In the open part of the circulation the cells are thought to pass the sinus wall toward the sinuses through slits between the endothelial cells (Chen and Weiss, 1973).

I.3 Hemopoietic stem cells

When lethally irradiated mice are injected with a sufficient number of syngeneic bone marrow cells, nodules consisting of differentiating erythroid, granuloid or megakaryocytic cells will form on the spleen about 5-14 days after injection (Till and McCulloch, 1961). Studies done on 10-14 day colonies using chromosome markers have shown that these colonies are of single cell origin (Becker et al., 1963; Fowler et al., 1967; Wu et al., 1967; Chen and Schooley, 1968). Colonies older than 10 days often contain cells of various lineages (Curry and Trentin, 1967). The cells forming spleen colonies have been called colony-forming unit spleen or CFU-s. Self-replication of colony-forming cells could be deduced from the presence of colony-forming cells within spleen colonies (Siminovitch et al., 1963). Chromosome marker studies indicated that lymphoid cells (Wu et al., 1968; Lala and Johnson, 1978) and mast cells (Kitamura et al., 1981) belong to the progeny of spleen colony-forming cells. CFU-s can rescue lethally irradiated mice (Metcalf and Moore, 1971). The above mentioned observations were taken as evidence that spleen colony-forming cells (CFU-s) are representative of pluripotent hemopoietic stem cells. Some doubt has arisen whether all colonies less than 10 days old are formed by stem cells since a portion of them seems to be transient and to contain no early progenitor cells (Magli et al., 1982; Mulder et al., 1985; Priestly and Wolf, 1985). There is evidence that human pluripotent stem cells also form splenic colonies after bone marrow transplantation

(Antin et al., 1985).

I.4 Progenitor cells

The study of committed progenitor cells became possible with the establishment of in vitro clonal assays. Pluznik and Sachs (1965) as well as Bradley and Metcalf (1966) independently described the growth of colonies containing granulocytes and macrophages from murine bone marrow cells immobilized in a culture medium containing agar. Clonal assays for the erythrocytic (McLeod et al., 1974), megakaryocytic (Metcalf et al., 1975a), lymphocytic (Metcalf et al., 1975b; Rozenszajn et al., 1975), eosinophilic (Metcalf et al., 1974) and mast cell (Nakahata et al., 1982c) lineages were subsequently developed. Later colonies containing cells from three lineages, apparently derived from multipotential cells (Mix-CFU) could be grown (reviewed by Johnson, 1984). Similar colonies have been grown from human blood and bone marrow cells (Fauser and Messner, 1979). More recently colonies containing almost exclusively blast cells, which show no signs of differentiation, have been cultured. These cells have extensive capacity for self-renewal and the ability to generate mixed-erythroid colonies (Nakahata and Ogawa, 1982a; Keller et al., 1984). Both Mix-CFU and blast cell colonies contain CFU-s (Metcalf et al., 1978; Humphries et al., 1979; Nakahata and Ogawa, 1982), the latter more than the first. The cells forming these colonies are thought to be a subpopulation of CFU-s (Johnson, 1984).

I.5 Regulation of hemopoiesis

I.5.1 Hemopoietic growth factors

Proliferation and differentiation of cells forming colonies in vitro depend on the presence of hemopoietic growth factors (HGF) in the culture medium. The use of colony assays has led to the development of schemes describing the relationships between progenitors and their HGFs. Such a scheme according to Stanley and Jubinski (1984) is shown in figure 1. Certain growth factors, such as erythropoietin, are considered to be lineage-specific since they are restricted to acting on one particular cell lineage. Other growth factors, like IL-3 are multilineage-specific causing multipotential cells to proliferate and differentiate along more than one lineage.

The continued presence of lineage-specific HGFs is

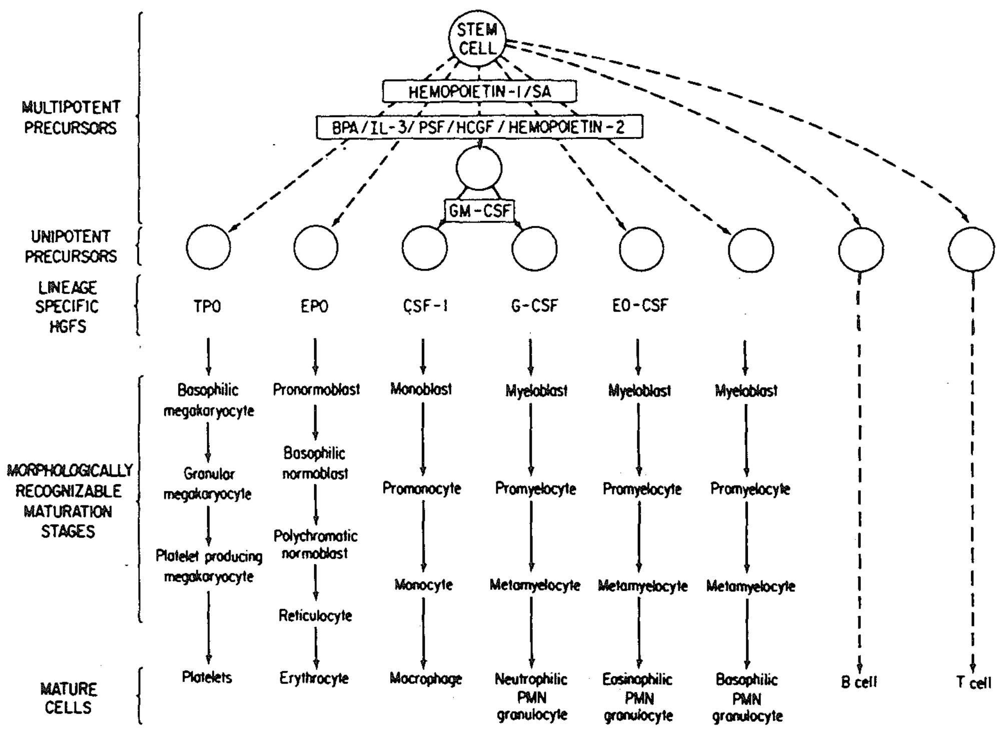

Figure 1. Schematic representation of haemopoiesis indicating the stages regulated by the known HGFs. SA, synergistic activity; BPA, erythroid burst-promoting activity; IL-3, interleukin 3; PSF, P-cell-stimulating factor; HCGF, haemopoietic cell growth factor; CSF, colony-stimulating factor; GM, neutrophilic granulocyte–macrophage; TPO, thrombopoietin; EPO, erythropoietin; G, neutrophilic granulocyte; EO, eosinophilic granulocyte.

required for both the proliferation and differentiation of cells within the lineage. Erythropoietin (EPO) was the first lineage specific humoral regulator of hemopoiesis described (Reissman, 1950; Stohlman et al., 1954). EPO induces the formation of small colonies of erythrocytes by the erythroid colony forming unit (E-CFU). The youngest clonable erythroid progenitor cell, the erythroid burst-forming unit (E-BFU), requires a second regulator, variously termed burst feeder activity (Wagemaker et al., 1977), or burst promoting activity (BPA) (Iscove, 1977). BPA stimulates proliferation and differentiation of E-BFU which give rise to progeny which acquires EPO responsiveness and at the same time loses BPA responsiveness (Axelrad et al., 1978; Iscove, 1978; Wagemaker, 1980; Linch and Nathan, 1984). Proliferation of E-BFU independent of EPO has also been shown in vivo (Wagemaker and Visser, 1980). Moreover, changes in bone marrow E-BFU numbers after injection of latex particles were found to be preceeded by corresponding changes in BPA levels (Ploemacher et al., 1979). These data may indicate a role in vivo for BPA.

The formation of colonies consisting of granulocytes and/or macrophages is dependent on the presence of colony-stimulating factor (CSF). Its existence was first shown by Pluznik and Sachs (1965) and Bradley and Metcalf (1966). In mice, GM-colony stimulating factors (GM-CSF) with apparent similar biochemical properties are synthesized in numerous tissues of the body (Nicola et al., 1979). In addition, GM-CSF can be found in the urine and serum (Metcalf, 1984). In vitro GM-CSF is detectable in the supernatant of fibroblasts (Pluznik and Sachs, 1965), mitogen-stimulated lymphocytes, macrophages and endothelial cells (Burgess and Metcalf, 1980). GM-CSF has been shown to stimulate the formation of colonies containing granulocytes and macrophages from single cells (Burgess et al., 1977). In addition, at high concentrations it can stimulate eosinophil colony formation and at relatively low concentrations it can initiate but not sustain cell division in multipotential, erythroid and megakaryocytic progenitors (Metcalf, 1984). M-CSF or CSF-1 (Stanley and Heard, 1977) stimulates the growth of colonies consisting of macrophages only. G-CSF has essentially the same effects as GM-CSF except that it does stimulate a subpopulation of granulocytic precursors and has but a weak and transient effect on all progenitor cells stimulated by GM-CSF (Metcalf and Nicola, 1983). CSF-1, GM-CSF and G-CSF have been purified to homogeneity (Burgess et al., 1977;

Stanley and Heard, 1977; Nicola et al., 1983). The role of CSF in vivo it is not yet clear (Burgess and Metcalf, 1980).

The stimulation of thrombopoiesis in man after injection of serum from a thrombocytopenic patient provided evidence for a humoral stimulator of thrombopoiesis (Keleman et al., 1958). This observation was later confirmed by others (Evatt et al., 1974; Enomoto et al., 1980; Shreiner et al., 1980). The active principle of this serum has been called thrombopoietin (Keleman et al., 1958).

The formation of megakaryocytic colonies in vitro is promoted by urine, serum and plasma from thrombocytopenic patients (Enomoto et al., 1980; Hoffman et al., 1981; Kawakita et al., 1982; Messner et al., 1982; Kimura et al., 1984). The active substance has been called Meg-CSF. In addition Meg-CSF can be obtained from a variety of tissue sources and cell lines (Metcalf et al., 1975a; Nakeff and Daniels-McQueen, 1976; Williams et al., 1978, 1982; Schrader et al., 1980).

Megakaryocyte colony formation seems to be dependent not only on Meg-CSF which promotes the maturation of Meg-CFU, but also on a factor named megakaryocytic potentiator which triggers Meg-CFU into proliferation (Williams et al., 1978, 1982).

In vivo stem cells represent a predominantly non-cycling population (Becker et al., 1965). In vitro, cycling of CFU-s in liquid cultures can be stimulated by the supernatant of lectin-activated T-cells (Cerny, 1974; Cerny et al., 1975). The active principle was named stem cell-activating factor (SAF) (Cerny, 1974). SAF-like activity was also found in the supernatant of lectin-activated T-cell hybridomas and spleen cells (Schrader and Clark-Lewis, 1982; Wagemaker, 1980), embryonic fibroblasts (Löwenberg and Dicke, 1977) and human leucocytes (Wagemaker and Peters, 1978).

It has recently been shown that Interleukin 3 (IL-3) purified to homogeneity stimulates proliferation of CFU-s in vitro (Garland and Crompton, 1983; Ihle et al., 1983). There is convincing evidence that IL-3 is identical to SAF derived from mitogen-stimulated lymphocytes (Dorssers et al., 1983; Clark-Lewis et al., 1985). In addition to its effect on stem cells, IL-3 stimulates colony formation by various progenitors including multipotential progenitors and blast cell colony forming cells (Greenberger et al., 1985; Suda et al., 1985). It is interesting that the same activities in WEHI-3 conditioned medium which apparently reside on one molecule, termed BPA (Iscove, 1978; Iscove et al., 1982). It therefore appears likely that IL-3 en BPA are identical. According to Suda et al. (1985), IL-3 does not trigger multipotential progenitors, which are probably a subpopulation of CFU-s, into cycle, nor did it influence their differentiation, but it appeared to be necessary for their continued proliferation. However, in contrast to this observation, Greenberger et al. (1983) did describe triggering of these cells into cycle by IL-3. On the basis of the properties reported for these factors it seems likely that IL-3 (Ihle et al., 1982), P cell stimulating factor (Schrader et al., 1981), hemopoietic growth factor (Bazill et al., 1983) and hemopoietin 2 (Bartelmez et al., 1985) are identical.

Recently another factor distinct from IL-3, called hemopoietin 1 was isolated, which stimulates the formation of mixed colonies (Bartelmez and Stanley, 1985; Jubinsky and Stanley, 1985). It has no detectable colony stimulating activity itself, but can synergize with other hemopoietic growth factors. It seems similar in properties to a factor described as synergistic activity by Kriegler et al. (1982)

Factors which modulate the proliferation of CFU-s in vitro have also been detected in the supernatant of explanted bone marrow cells (Wright and Lord, 1979). It is interesting that the supernatant of bone marrow cells containing cycling CFU-s stimulated proliferation of resting cells, whereas the supernatant of resting bone marrow cells inhibited proliferation of cycling CFU-s. There are not yet enough data to establish the role of the above mentioned factors with the exception of EPO and thrombopoietin, in the regulation of proliferation and differentiation of HSC and progenitor cells in vivo.

I.5.2 Microenvironment

In adult mice hemopoiesis is mainly restricted to bone marrow and spleen. In both sites hemopoietic cells are suspended within a stromal framework consisting of a number of cell types and an intercellular matrix of ground substance and fibers. Stromal cells include endothelial cells, macrophages, reticular fibroblasts and adipocytes. There is evidence that the hemopoietic microenvironment influences proliferation and differentiation of hemopoietic stem cells. Spleen colonies are committed to one line of differentiation for the first 8 to 10 days (Becker et al., 1963; Curry et al., 1967). Subsequently more lines of differentiation may develop. These findings have been taken as evidence that certain areas within the hemopoietic milieu, called niches, exist which govern the commitment of stem cells (Trentin 1970, 1971). Secondary lines of differentiation would develop after a colony encroaches upon another niche.

Erythroid colonies outnumber granuloid colonies 3:1 in the spleen, whereas in the bone marrow the reverse is true (1:2) (Curry et al., 1967; Wolf and Trentin, 1968). Pieces of marrow transplanted into the spleen support colonies with an E:G ratio similar to bone marrow in situ. Colonies growing across the junction of marrow and splenic stroma show abrupt transition of lineage, i.e. erythrocytic in spleen stroma and granulocytic in marrow stroma (Wolf and Trentin, 1968). The same authors observed that after secondary transplantation of stem cells which had seeded in the bone marrow, the E:G ratios were still partly dependent on the organ where the stem cells seeded after the first transplantation. Spleen colonies in a subcutaneously transplanted spleen show the same E:G ratio as they do in a spleen in situ (Wolf and Trentin, 1968; Ploemacher et al., 1982).

The above mentioned observations prompted Trentin and coworkers (Trentin, 1970, 1971; La Pushin and Trentin, 1977) to postulate that commitment occurs in response to a cell-to-cell interaction between hemopoietic stem cells and stromal cells. This is the essence of the concept of the hemopoietic inductive microenvironment (HIM). Alternatively, commitment could be the result of locally produced short-range diffusible HGFs.

An effect of the local milieu, i.e. microenvironment, on the proliferation of CFU-s is suggested by the fact that proliferation of CFU-s between different sites of the body may differ (Croizat et al., 1970b). This has been observed after partial body irradiation and after the induction of hemolytic anamia (Rencricca et al., 1970; Gidali and Lajtha, 1972). Another piece of evidence is obtained from experiments with the Sl/Sl^{d} mutant mouse which has normal stem cells, but whose capacity for proliferation is diminished by a defective microenvironment (McCulloch et al., 1965, 1970; Harrison and Russel, 1972).

The observed heterogeneity of CFU-s numbers in individual spleen colonies (Siminovitch et al., 1963) has led to the stochastic model of stem cell proliferation and differentiation (Till et al., 1964). The heterogeneity is thought to reflect the operation of a random process governing stem cell self-renewal and differentiation. This theory states that the self-renewal of stem cells is governed by a stochastic process with a specific probability of stem cell renewal. This theory has lately been supported by the observed self-renewal and differentiation of primitive "stem cells" in vitro (Ogawa et al., 1983; Lim et al., 1984; Suda et al., 1984). The heterogeneity observed in the expression of differentiation programs of these cells suggests a random process.

It is conceivable that the microenvironment supplies factors to promote HSC proliferation and the growth of differentiated progeny without affecting commitment.

II. HEMOPOIETIC STEM CELL MOBILIZATION

II.1 Stem cells in the circulation

In 1951 Brecher and Cronkite demonstrated that lethally irradiated rats survived when they were subsequently brought in parabiosis with non-irradiated rats. Nowadays this can be interpreted as the first evidence for the passage through the blood of primitive hemopoietic cells from the shielded area to the irradiated bone marrow. These parabiosis experiments also demonstrated the migratory capacity of these cells. Repopulation of the hemopoietic tissues by cells of donor origin in lethally irradiated mice which were injected with peripheral leukocytes provided more direct evidence for the circulation of hemopoietic stem cells (Goodman and Hodgson, 1962). The number of hemopoietic stem cells in the blood stream totals 20-60 per mouse (Barnes and Loutit, 1967). Micklem (1966) noted a diurnal variation in circulating CFU-s numbers in mice with maximum numbers in the morning and minima in the afternoon. Experiments with the autorepopulation assay in which stem cells migrate from a lead-shielded area of bone marrow to the irradiated spleen, showed a rapid initial release, which was followed by a long-lasting outflow at a lower rate. From these experiments a steady state release of approximately 1.6 CFU-s/hr was calculated (Hanks, 1964; Hellman and Grate, 1968; Maloney and Patt, 1978). An approximately 2-fold increase in concentration of CFU-s in the blood of splenectomized mice (Vos et al., 1972) has been taken as evidence for the notion that a substantial fraction of CFU-s released from the bone marrow lodge in the spleen. Dorie et al. (1979), using a male-female parabiosis model, estimated a half-life of 1.7 hr for blood stem cells. This estimation was based on the disappearance of donor stem cells from the blood after separation of the parabionts. Separated parabionts were also used in the autorepopulation assay. In these experiments it was found that the fraction of donor stem cells recruited from shielded bone marrow for spleen colonization was significantly higher than the total marrow donor fraction. They explained their data by the hypothesis that there is an equilibrium between the stem cells in the circulation and a subpopulation of marrow stem cells. They suggested that the stem cells in the blood and spleen, because it had the same fraction of donor stem cells as blood and the earlier mentioned subpopulation of marrow stem cells all belong to the same pool. Splenectomy did not change the disappearance rate of CFU-s. However, bone marrow and blood CFU-s numbers increased by approximately 20%. This apparent compensatory response obscures the role of the spleen in the turnover of circulating stem cells (Dorie et al., 1979). Although it has been shown that stem cells may migrate from shielded marrow to irradiated marrow (Maloney and Patt, 1972), there appears to be a very limited interchange of stem cells between different marrow sites under steady state conditions (Micklem et al., 1975b,c).

Gidali et al. (1974) compared some properties of circulating CFU-s with those of bone marrow CFU-s. After X-irradiation peripheral blood CFU-s showed a lower Do than bone marrow CFU-s. Blood CFU-s have a higher 2 hr seeding efficiency and a higher cycling rate than bone marrow CFU-s as measured by the ^{3}H-thymidine suicide technique in vitro. These data support those of Lajtha et al. (1969). The repopulation potential of peripheral blood CFU-s was found to be lower than that of bone marrow CFU-s (Micklem et al., 1975a), although this could not be supported by Vos et al. (1981). The low repopulation ability of blood CFU-s was shared by splenic CFU-s and stem cells recruited from shielded marrow (De Vries and Vos, 1966; Lahiri and van Putten, 1969). These data and those of Dorie et al. (1979) support the idea that stem cells in the peripheral blood belong to the same pool of stem cells as spleen stem cells and the recruitable marrow stem cells.

II.2 Mobilization of stem cells

In the preceding chapter the presence of small numbers of hemopoietic stem cells in the peripheral blood was mentioned. Circulating HSC are a potential source of cells to be used in bone marrow transplantation. However, their easy availability may be offset by their sparcity and low proliferative ability. This problem might be circumvented by artificially increasing blood HSC numbers. The size of the stem cell population in the blood is assumed to represent a balance between inflow and outflow of stem cells to and from the hemopoietic sites. Evidence discussed in the previous paragraph indicates that stem cells are continuously being released from the bone marrow into the blood stream and possibly seed in the spleen. An increase of the number of circulating stem cells thus requires increased release from the bone marrow or interference with their seeding. In mice another possibility would be to mobilize stem cells from the spleen, however, the number of stem cells in the spleen is small as compared with the bone marrow (Metcalf and Moore, 1971).

A transient increase of circulating stem cells has been demonstrated after intravenous (i.v.) or intraperitoneal (i.p.) administration of various (chemical or natural) compounds, which can be roughly divided into the following categories: bacteria, bacterial and yeast wall products, polyanions, proteolytic enzymes and lectins.

II.2.1 Endotoxin

In 1943 Delauney described a marked leukopenia following injection of glycolipid from gram negative bacteria into guinea pigs. Leukopenia followed by leukocytosis was noted by Stetson (1951) after injection of meningococcal endotoxin in rabbits. The leukocytosis was due to the release of granulocytes from the bone marrow reserves. Later the effect of endotoxin on circulating polymorphonuclear cells has been documented in many species including humans (Athens et al., 1961; Mechanic et al., 1961). A biphasic increase of blood CFU-s in mice after i.v. injection of endotoxin was described by Vos et al. (1972). Peak numbers were found 1/2-3 hrs after injection with high doses (100-500 µg), which was earlier than the peak found after small (10 µg) doses. A second increase of blood CFU-s began on the second day and reached a maximum on day 3 when 30 µg was injected and on day 5 when 500 µg was injected. This second peak was found to coincide with a large increase of splenic CFU-s numbers. In splenectomized mice a similar rapid rise of blood CFU-s after endotoxin injection was seen, suggesting that they were mobilized from the bone marrow (Vos et al., 1972). Endotoxins derived from different bacteria had a similar effect on blood CFU-s although peak CFU-s numbers were different (Vos and Wilschut, 1979).

Mobilization of GM-CFU has been demonstrated after i.v. or i.p. endotoxin administration in mice, dogs and humans (Quesenberry et al., 1973a; Cline and Golde, 1977; McVittie and Walker, 1978). In mice a tenfold increase of splenic GM-CFU was noted 3 days after i.p. injection of 5 µg S.typhosa endotoxin. Detoxified endotoxin retains its mobilizing properties (Vos and Wilschut, 1979).

In mice, changes in CFU-s numbers in blood and spleen similar to those seen after endotoxin injection were demonstrated after administration of B.pertussis vaccine (Monette et al., 1972), Corynebacterium parvum (Eliopoulos et al., 1979) and the yeast wall product zymosan (Vos and Wilschut, 1979). The similarity between neutropenia produced by endotoxin and that caused by activated complement prompted studies of complement involvement in CFU-s mobilization. Injection of LPS or zymosan into mice caused a fall in serum C3 levels (Vos and Wilschut, 1979). When mice were depleted of complement by prior injection of cobra venom factor the rapid mobilization of CFU-s by these agents was inhibited (Wilschut et al., 1979). These data indicate that an intact complement system is required for CFU-s mobilization by LPS and zymosan.

Related to the above mentioned substances is the polyglycan "glucan" derived from Saccharomyces cerevisiae. Patchen and Lotzova (1980) demonstrated CFU-s mobilization upon injection of this agent.

II.2.2 Polyanions

The naturally occurring polyanion heparin as well as the synthetic polyanions dextran sulphate and polymethacrylic acid have been shown to induce lymphocytosis in various species including humans (Jansen et al., 1962; Ormai and de Clercq, 1969; Hagenbeek et al., 1976). Lymphocytosis occurs after i.v. as well as i.p. injection, with peak numbers found 3 hours after administration of the particular agent. Mobilization is not limited to lymphocytes, because granulocyte and monocyte numbers were increased as well although to a lesser extent (Van der Ham et al., 1977). The latter authors also demonstrated CFU-s mobilization after i.v. injection of dextran sulphate, polymethacrylic acid (PMAA) and the copolymer of polymethacrylic acid and styrene (PMAA-STYR). Peak CFU-s numbers occurred 2 hours after injection. GM-CFU mobilization by dextran sulphate in dogs followed essentially the same time course as CFU-s mobilization in mice (Ross et al., 1978). In these animals GM-CFU numbers increased sevenfold while mononuclear cells increased twofold suggesting a selective effect of dextran sulphate on this population of progenitor cells. The lymphocytosis by dextran sulphate is caused by a reduction of their rate of exit from the blood as demonstrated by the reduced homing following infusion of lymphocytes treated in vitro with dextran sulphate. PMAA on the other hand was found to increase the output of lymphocytes from the thoracic duct (Ormai and Palkovits, 1972). The negative charge of sulphated polysaccharides seems to be essential for their effect on the number of circulating lymphocytes (Bradfield and Born, 1974). Ross et al. (1978) have suggested that dextran sulphate attaches itself to the GM-CFU membrane to cause mobilization and/or reduce homing of these cells.

Although polyanions can activate the complement system both in vitro (Loos and Bitter-Suermann, 1976) and in vivo (Wilschut et al., 1979), decomplementation of mice could not or only slightly prevent CFU-s mobilization by dextran sulphate and PMAA-STYR (Wilschut et al., 1979), indicating that factors from the complement system do not mediate polyanion-induced CFU-s mobilization.

II.2.3 Proteolytic enzymes

Intravenous injection of the proteolytic enzymes proteinase and trypsin in mice causes mobilization of CFU-s as well as mature hemopoietic cells (Vos et al., 1972; Ploemacher et al., 1980). A rise in CFU-s numbers was seen within ten minutes after injection. No delayed CFU-s mobilization occurred. Prior decomplementation caused a moderate decrease of CFU-s mobilization while it sharply reduced the mobilization of mature cells (Ploemacher et al., 1980). Repeated trypsin injections decreased the number of CFU-s mobilized. This correlated with a decrease in bone marrow CFU-s numbers (Feher and Gidali, 1982). These data support the existence of an exhaustable pool of mobilizable stem cells in the bone marrow.

II.2.4 Lectins

Both concanavaline A and phytohemagglutinin (PHA) are able to mobilize CFU-s upon i.v. injection into mice independent of the complement system (Wilschut et al., 1979). Micklem (1966) also described an increase in CFU-s numbers in the blood after injection of PHA.

III. STRUCTURE AND BIOLOGICAL ACTIVITY OF ENDOTOXIN

III.1 Endotoxin

Endotoxin is a toxic substance produced by gram-negative bacteria. It is a lipopolysaccharide protein complex which is firmly bound to the bacterial cell wall. Generally endotoxin is released from organisms following lysis or other forms of desintegration. This is in contrast to exotoxins, which are synthesized and excreted by intact bacteria (Pfeiffer, 1892). Endotoxins are produced by a large variety of gram-negative bacteria predominantly enterobacteriaceae, pathogenic or non-pathogenic for animals or man, such as the genera Salmonella, Shigella, Escherichia, Klebsiella and others. Endotoxins have not been found in cell walls of gram-positive bacteria, mycobacteria or fungae (Morrison and Ryan, 1979). However, recently endotoxin-like material from the cell wall of the gram-positive organism Listeria monocytogenes has been purified (Wexler and Oppenheim, 1980). This "listerial-LPS" showed a striking similarity with LPS derived from gram-negative bacteria, both in its chemical composition and in its biological activities in vivo as well as in vitro.

The cell wall of gram-negative bacteria consists of a cytoplasmic inner membrane and a trilayer outer cell wall structure consisting of a peptidoglycan layer, linked by lipoproteins to a phospholipid bilayer (outer membrane) and an outermost lipopolysaccharide (LPS) layer (Braun, 1973). A schematic model of the enterobacterial cell wall is given in Figure 2.

The extraction of endotoxin from bacterial cell walls was initiated by Boivin et al. (1933) and by Morgan (1937). The Boivin procedure involving extraction with ice-cold trichloroacetic acid is still widely used. In 1952 Westphal et al. described the extraction of bacteria with hot (65-68^{o}C) phenol/water mixtures. This method is applicable to practically every endotoxin-producing organism. Later, Galanos and colleagues (1969) developed a variant of this method, especially for the extraction of rough mutant bacteria by using a mixture of phenol/chloroform/petroleum ether at room temperature. By means of all these procedures, endotoxin lipopolysaccharides complexed to protein or without it are obtained, which form colloidal suspensions in water. The protein-free lipopolysaccharide complexes are composed of 60-85% polysaccharide and about 15-40% lipid. In

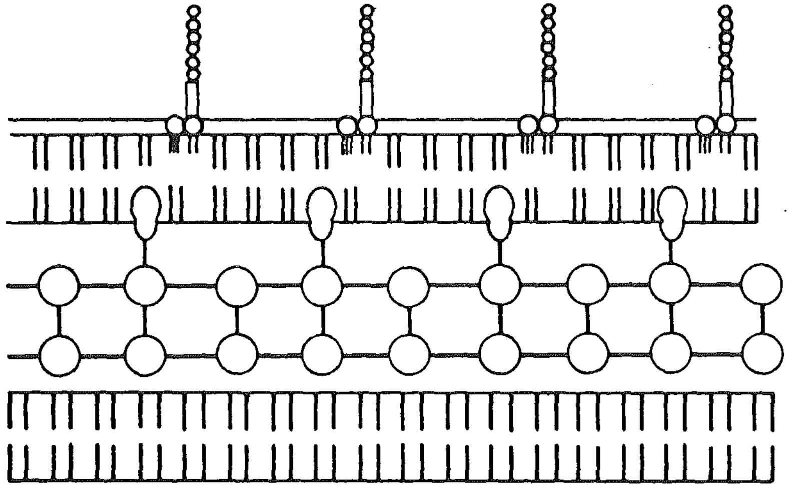

Figure 2 A model of the gram-negative cell envelope.

suspension lipopolysaccharides probably exist as high molecular weight aggregates especially in the presence of divalent cations (Mg^{++}, Ca^{++}).

III.2 Lipopolysaccharide (LPS)

Lipopolysaccharide molecules consist of three regions of contrasting chemical and biological properties (Westphal, 1975): the O specific polysaccharide, the core polysaccharide and the lipid region. A model of LPS as described by Westphal (1975) is shown in Figure 3. LPS carries the species-specific determinant, the O antigen, responsible for the specificity of antibacterial antibodies raised during immunization with bacteria. The O antigen consists of repeating oligosaccharide units; each unit consisting of three to six sugar moieties. The number of repeating units may vary between two and ten, even within the same bacterium (Westphal and Lüderitz, 1954; Jann et al., 1975). The composition of the oligosaccharide unit is extremely variable (reviewed by Lüderitz et al., 1971). The core region of Enterobacteriaceae such as Salmonella is a rather unique oligosaccharide built up from 10 or 11 monosaccharide units which comprise besides glucose, galactose and glucosamine, also a heptose and ketodeoxyoctonic acid (KDO). Little variation in the composition of the core region is seen in one genus (Galanos et al., 1977).

III.2.1 R mutants

The S ⟶ R mutation is a well-known spontaneous mutation by which the morphology of the colony formed by bacteria of the parent wild or S(mooth) strain changes from smooth into R(ough). It was found that on S ⟶ R mutation most or all sugars involved in the O specificity of the S lipopolysaccharide are lost (Lüderitz and Westphal, 1966) (Fig. 3). The R mutants appeared to have a block in the biosynthesis of the O-specific repeating units. The isolation of pure lipopolysaccharides from R mutants allowed the investigation of the structural prerequisites for endotoxic activity.

III.2.2 Lipid A

The lipid part of LPS, often called lipid A (Westphal and Lüderitz, 1954), is a long-chain fatty acid derivative of a phosphorylated hexosamine disaccharide backbone: 4-phosphoglycosaminyl-β-1.6-glucosamine-1-phosphate. The aminogroups are substituted with β-hydroxy-myristic acid. The 3 hydroxyl groups are esterified with lauric (C12), myristic

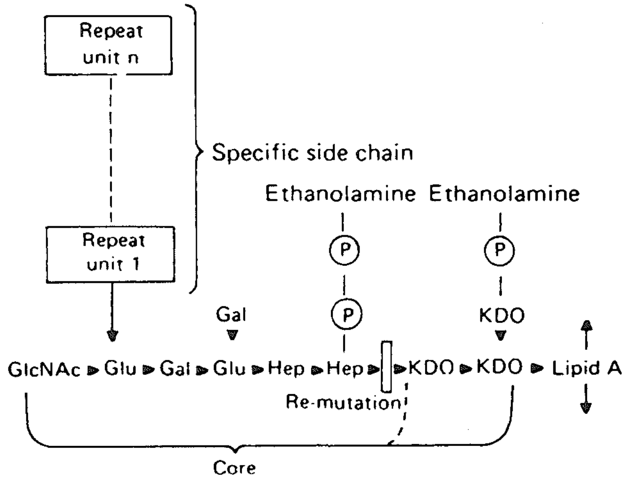

Figure 3.Structure of LPS

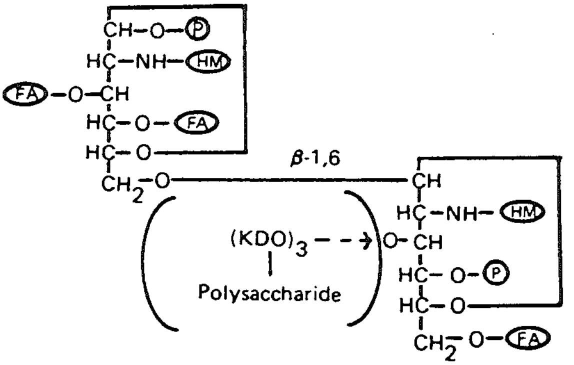

Fig 4 Structure of lipid A. FA:long chain fatty acid,HM:3hydroxymyristic acid,P:phosphate.

(C14), palmitic (C16) acid and an additional β-hydroxy-myristic acid (C14-OH) (reviewed by Lüderitz et al., 1973). One hydroxyl group of glucosamine is esterified by the double ester myristoyl-β-hydroxy-myristic acid (Rietschel et al., 1972). The structure of lipid A from a Salmonella lipopolysaccharide is shown in Figure 4. Lipid A of various strains was found to have the same disaccharide backbone (Hase and Rietschel, 1976). There is little variation in the fatty acid composition between different Salmonella species (Rietschel et al., 1972). These findings indicate that there is much less variation in the lipid part than in the polysaccharide part of LPS.

There are two sources of lipid A. Firstly, R mutants glycolipids containing only lipid A and KDO. Alternatively, lipid A can be isolated from any LPS by hydrolysis with mild acid (Westphal and Lüderitz, 1954). This treatment cleaves the ketosidic linkage of KDO to lipid A.

III.3 Endotoxin protein

As mentioned earlier endotoxin is a lipopolysaccharide-protein complex. The protein component attracted little attention because it was seen as an inert carrier of LPS. Wober and Alaupovic (1971) and Tsang et al. (1974) postulated that the protein is conjugated to LPS through lipid A by a phenol sensitive linkage. While investigating the immunologic properties of LPS, Skidmore et al. (1975) noted that certain LPS preparations had a mitogenic effect on spleen cells of mice from the LPS-low responder C3H/HeJ strain. The method of extraction used to prepare LPS critically affected the mitogenic activity. Butanol-extracted LPS had mitogenic activity while phenol-extracted LPS lacked this activity (Skidmore et al., 1975). Further extraction of mitogenic LPS preparations with phenol abolished the effect. An endotoxin protein with mitogenic properties from the phenol phase was independently described by Morrison et al. (1976) and by Sultzer and Goodman (1976). Further analysis of endotoxin protein from different strains of bacteria showed that it actually consisted of various proteins with molecular weights ranging from 8,000-80,000 (Morrison et al., 1980). Due to its protein nature and association with lipid A, endotoxin protein is often called lipid A-associated protein or LAP (Morrison et al., 1980). The presence or absence of LAP did not significantly alter the toxicity of LPS as measured by the local Schwartzmann reaction, but the LD50 of LAP-LPS for mice was one third of that of LPS.

Therefore LAP seems to be able to modulate the action of LPS, while LAP is relatively non-toxic when injected in mice at doses up to 2 mg (Morrison et al., 1976; Sultzer and Goodman, 1976; Goodman and Sultzer, 1979). I.v. injection of LAP caused neutropenia (Morrison et al., 1980). LAP induced an increase in serum CSF level and like LPS, it was found to stimulate splenic hemopoiesis (Staber et al., 1981). On the basis of weight the effects of LAP were less than of LPS (Staber et al., 1981).

III.4 Endotoxin structure and biological activity

Bacterial endotoxins were first defined by their ability to produce fever (pyrogenicity). Later it was recognized that endotoxins exert many different biological activities (reviewed by Morrison and Ulevitch, 1978). Information regarding the nature of the toxic component of endotoxin has been obtained in two ways: (1) by chemical modification of endotoxin and (2) by using R mutants. Tal and Goebel (1950) dissociated endotoxin in two ways: treatment with dilute acid which led to a toxic lipoprotein and a non-toxic polysaccharide and treatment with alcoholic alcali gave rise to non-toxic protein and a toxic lipopolysaccharide. It has been demonstrated in a number of ways that lipid A is the component of LPS responsible for its toxic activity. LPS can be detoxified by alkaline hydrolysis while the antigenic activity of the polysaccharide remains intact (Neter et al., 1956). Later it was shown that this treatment removes the ester-linked fatty acids from lipid A (Neter et al., 1956; Rietschel et al., 1972). Polymixin B, which binds to lipid A, can modify or inhibit the toxicity of LPS (Neter et al., 1958; Rifkind 1967, Morrison and Jacobs, 1976). The studies mentioned above suggested that lipid A is responsible for the biologic activity of LPS. This is supported by the observation that endotoxin (or LPS) extracted from the heptose-deficient strain Salmonella minnesota R595, which consists of lipid A and KDO, still shows endotoxic activity (Kim and Watson, 1967). The following list shows a number of endotoxic activities by lipid A complexed to BSA. These activities are the same as those of the much more complex lipopolysaccharide:

- pyrogenicity
- toxicity (lethality)
- Schwartzmann phenomenon
- bone marrow necrosis
- leukopenia/leukocytosis

- mitogenic lymphocyte stimulation
- macrophage activation
- complement activation
- limulus lysate gelation
- adjuvant activity
- colony-stimulating factor induction
- tolerance induction.

Lipid A solubilized with triethylamine showed endotoxin activity comparable with intact LPS (Galanos et al., 1977). These studies firmly established the dominant role of lipid A in endotoxicity.

III.5 Interaction of LPS with cells

The ability of lipopolysaccharides to bind to the cytoplasmic membrane of many mammalian cells is well recognized. The binding characteristics of LPS to erythrocytes, platelets, lymphocytes and granulocytes have been relatively well defined (reviewed by Morrison and Ryan, 1979). The precise target for LPS on the cell membrane has yet to be established unequivocally. LPS can interact with the essential components of biologic membranes: phospholipids and proteins, and can also be associated with and inserted directly into a variety of artificial and natural membranes (reviewed by Morrison and Rudbach, 1981). Binding of LPS to cells does not necessarily provoke a cellular response. This is best demonstrated by the equal binding of LPS to lymphocytes of normal mice and those of LPS-low-responder C3H/HeJ mice (Watson and Riblet, 1975; Gregory et al., 1980). Several investigations in which it was shown that lipids can inhibit binding of LPS to cells, suggest that the lipid portion of the molecule is important for intercalating LPS into the cell membrane (Neter et al., 1956). This was further demonstrated by the fact that lipid A inhibited the attachment of whole LPS molecules to erythrocytes, platelets and lymphocytes (Springer and Adye, 1975; Kabir and Rosenstreich, 1977). Triggering of a cellular response by LPS after binding could be the result of membrane perturbation or interaction with a specific LPS receptor. Many studies have been devoted to the identification of a specific recognition structure (receptor) for LPS or lipid A (reviewed by Morrison and Rudbach, 1981). Springer et al. (1973) extracted a ′LPS receptor′ characterized as a lipoglycoprotein from human erythrocytes. They were unable to obtain receptor-like material specific for LPS from human leukocytes (Springer and Adye, 1975). Yokoyama et al. (1979) in a study to define receptors for LPS on B and T cells reported that histocompatibility-2 complex proteins K and D as well as Ia antigens and IgM and IgD had affinity for LPS. These data and those of Niederhuber et al. (1975) on the inhibition of the proliferative response of B lymphocytes to LPS by anti-Ia sera, suggest a close association between Ia and probably other H-2 antigens on lymphocytes and 'receptors' for LPS. Receptors for the third component of complement (C3) or those for the crystallizable part (Fc) of immunoglobulins seem not to be involved in LPS activation of B lymphocytes (Möller et al., 1975). An antiserum has been described that has a putative specificity for the LPS receptor on murine B lymphocytes (Coutinho et al., 1978; Forni and Coutinho, 1978). This antiserum was raised by immunization of rabbits with spleen cells from LPS responsive mice of the C3H/Tif strain. Antibodies in this antiserum combined only with LPS-reactive cells. Lymphocytes from mice of LPS-nonresponder strains C3H/HeJ and C57BL/10ScCr did not bind this antiserum. Unfortunately, Watson et al. (1980) have not been able to confirm the above mentioned studies, although this may be attributed to minor methodological differences.

III.6 Fate of endotoxin in the body

To gain a better understanding of the mechanisms of endotoxin action, knowledge of the clearance, organ distribution and cellular localization of endotoxin is required. Braude et al. (1955a, 1955b), using a ^{51}Cr labeled E.coli LPS, found a biphasic clearance of LPS from the circulation of rabbits. A rapid initial phase was followed by a slow second phase. The rapid initial phase was shown to be due to the immediate interaction of LPS with plasma high density lipoprotein (Skarnes, 1968; Mathison and Ulevitch, 1979). With the disappearance of LPS from the blood stream there was a rapid uptake of LPS by the liver, which was quantitatively the most important localization of LPS. Although a small amount of LPS was found in the spleen, the LPS concentration was higher than in the liver (Braude et al., 1955b). No essential differences in clearance or distribution of LPS were found between high and low doses or between rabbits and mice, although small doses tended to be cleared more rapidly (Braude et al., 1955b; Carey et al., 1958). Tolerance of LPS led to a more rapid clearance of LPS but had little effect on the organ distribution of LPS (Carey et al., 1958; Herring et al., 1963). Chedid et al. (1966) noted a more rapid clearing of LPS from rough strains than LPS from smooth strains. Most of the investigations mentioned earlier were performed with LPS extracted by the Boivin method and could therefore contain impurities which were also labeled. Ulevitch (1978) labeled highly purified E.coli LPS with ^{125}I, the labeled LPS was indistinguishable from its parent LPS in its physico-chemical and biological properties. Clearance and distribution studies in rabbits of high and low doses gave essentially the same results as those reported by authors using less purified LPS (Mathison and Ulevitch, 1979). After injection of labeled LPS, radioactivity was found in the phagocytic vacuoles of Kupffer cells, in splenic macrophages and leukocytes 5 and 180 minutes after LPS injection. LPS recovered from the liver 3 hours after injection did not appear to have been degraded (Mathison and Ulevitch, 1979). Although endotoxin is taken up primarily by Kupffer cells, it is also taken up by hepatocytes (Willerson et al., 1970; Zlydaszyk and Moon, 1976). Using the highly purified LPS prepared by Ulevitch, Musson et al. (1978) found no differences in the rate of removal from the circulation of an immunological or a toxic dose of LPS between LPS-responsive C3H/St and LPS-low responsive C3H/HeJ mice. However, the absolute amount of LPS accumulated in C3H/St spleens was greater than in C3H/HeJ spleens.

III.7 Interaction of endotoxin with the complement system

The complement system is an enzyme system consisting of a number of proteins all of which are pre-enzymes except for factor D. Activation of the complement system occurs in a stepwise fashion, the first activated factor activates the second and so on. The complement system can be divided into three parts: a terminal phase leading to lysis of bacteria or cells and two activation pathways, the classical and alternative pathway. The terminal phase comprises the factors C5, C6, C7, C8 and C9. The factors C5, C6 and C7 form a complex. This is induced by C5-convertase. Subsequently, C8 is bound to the C567 complex. Upon interaction of C9 with C5678, C9 is polymerised. The complex C56789n is able to penetrate cell membranes and the outer membrane of bacteria. Upon activation of C5, C5a which is an anaphylatoxin and leucotactic factor is split off, the remainder of C5 is C5b.

Activation of complement via the classical pathway is caused mainly by antibody-antigen aggregates or antibody bound to cellular or particulate antigens. In vitro this pathway may also be activated by viruses and acidic polymers such as dextran sulphate. C1 is activated through its subcomponents C1q, C1r and C1s (Fig. 5). Activated C1 activates C4 and C2 yielding C4b2a or C3 convertase. During activation of C4 it is split into C4a and C4b. C3 convertase converts C3 into an activated form; during this process C3a is split off. The remainder, C3b, associates with C4b2a to give the C4b2a3b complex (C5 convertase) which will activate C5.

In experiments involving incubation of Veillonella alcalescens endotoxin with guinea pig serum, it was shown that considerable consumption of C3 took place without significant consumption of the classic pathway components, C1, C4 and C2 (Gewurz et al., 1968b; Frank et al., 1971). From these experiments it was concluded that bacterial endotoxins could activate the complement system at C3. This way of activation has been called the alternative pathway.

Apart from gram negative bacteria, several other substances, such as IgA and polysaccharides of gram positive bacteria and yeast cells can activate complement via the alternative pathway. Activation and control of the alternative pathway are shown in figure 5. Upon activation of C3, C3a is split off, C3a is an anaphylatoxin and has chemotactic activity. The remaining part, C3b complexes with factor B. This complex is activated by factor D to form C3bBb, the C3 convertase of the alternative pathway. C3b is stabilized by factor P(roperdin) and can activate other C3 molecules, thus establishing an amplification loop. There are three inhibitors which control the amplification loop: (1) β1H which has affinity for a strategic site on C3b, (2) C3b inactivator (C3bINA) which cleaves the α chain of C3b and (3) the low molecular weight inhibitor (LMW-INH) which has affinity for factor D. The classical pathway has two controlling proteins: C1 inhibitor and C4b-binding protein.

More than 30 years ago Pillemer et al. (1955) demonstrated complement activation in human serum by many bacterial polysaccharides, in particular bacterial lipopolysaccharides. Complement activation led to consumption of properdin as well as C3. Muschel and associates (1964) and Gewurz et al. (1970) showed that activation of complement was independent of naturally occurring anti-endotoxin antibodies. In addition they found that Boivin preparations of endotoxins were considerably more anti-complementary than Westphal preparations.

The aforementioned experiments of Pillemer et al. (1955) suggested that the polysaccharide component of endotoxin is the dominant chemical moiety of LPS regulating its complement activating activity. Later Mergenhagen et al.

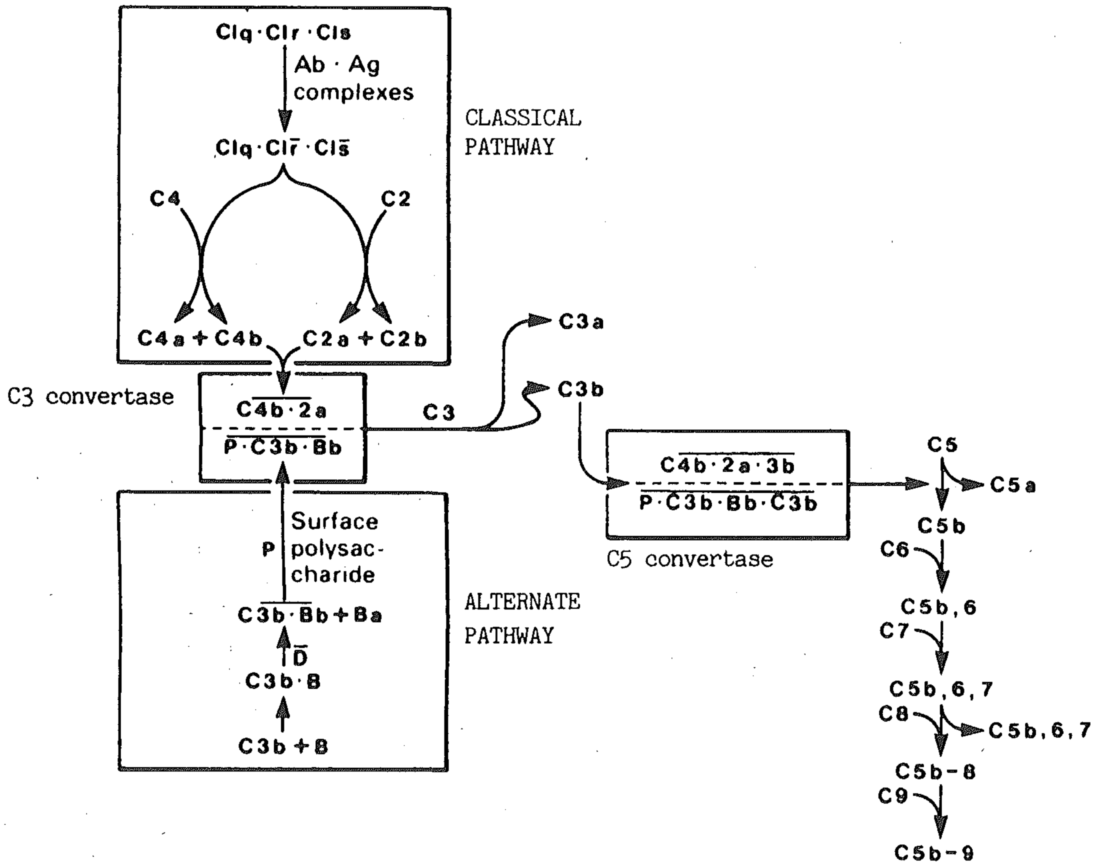

Figure 5. Reaction sequence of the complement system. P:properdin, B:factor B, D:factor D

(1968) observed that endotoxin from a S.Minnesota (Re) mutant was as active as the endotoxin derived from the polysaccharide-containing parental strain. In testing a number of LPS preparations of both smooth and rough strains for anti-complementary activity Galanos found considerable variation in activity for both groups (Galanos et al., 1971). Galanos et al. (1971) and Morrison and Verroust (1973) demonstrated that purified lipid A has the capacity to activate the complement system. In addition both these studies demonstrated that the anti-complementary activity of both lipid A and LPS was highly dependent on its degree of aggregation. This was confirmed by Galanos and Lüderitz (1976) who found that conversion of lipopolysaccharides derived from smooth form bacteria into their low molecular weight triethylamine salt form resulted in a marked reduction of the anti-complementary activity (classical pathway), while conversion into their respective high molecular weight salt resulted in enhanced activity. Activation of the alternative pathway by LPS seemed to be independent of aggregation (Wilson and Morrison, 1982).

Classical pathway activation by lipid A (and endotoxins from rough mutants) was shown by Lachmann and Nicol (1974) and Morrison and Kline (1977). In addition the latter authors showed that alternative pathway activation does not require the active participation of the lipid A region of LPS; it is a function of the O polysaccharide only. It was later demonstrated by Loos et al. (1974) that lipid A and LPS from rough strains can bind to and initiate the activation of purified human C1 via C1q.

It can be concluded that endotoxin can activate the complement system in three ways, i.e. 1. via the classical pathway involving direct interaction of lipid A, or 2. by antibodies to endotoxin and 3. via the alternative pathway involving the polysaccharide moiety.

IV. THE EFFECTS OF LPS ON MURINE HEMOPOIESIS

IV.1. Introduction

Apart from its immediate effect on the circulation of hemopoietic cells the most striking hemopoietic effects of LPS occur in the spleen. Over a period of a few days following i.v. or i.p. injection of LPS the spleen swells due to a greatly increased cellularity and average cell size. There is a dose-dependent increase in the number of CFU-s and GM-CFU with a maximum at 5-7 days after LPS administration (McCulloch et al., 1970; McNeill, 1970; Apte and Pluznik, 1976a). In addition a parallel rise of the numbers of EO-CFU, E-CFU, E-BFU, Meg-CFU and morphologically identifyable hemopoietic cells has been demonstrated (Reissman et al., 1976; Staber and Johnson, 1980). The incidence of B lymphocyte colony forming cells remains unaltered (Staber and Johnson, 1980). LPS is not the only constituent of the cell wall of gram negative bacteria which affects splenic hemopoiesis, since similar changes in the number of hemopoietic cells have been observed after injection of lipoprotein and lipid A-associated protein (LAP) (Staber and Johnson, 1980; Staber et al., 1981). Injection of LPS and lipoprotein triggers splenic CFU-s into cycle (Staber et al., 1978). The same authors demonstrated that GM-CFU accumulation in the spleen did only occur when cycling of CFU-s was induced. The effects of LPS on splenic hemopoiesis described above as well as most other effects of LPS on hemopoiesis are absent in LPS-low-responder C3H/HeJ mice (Apte and Pluznik, 1976a; McVittie and Weinberg, 1980). However, the latter authors described that the administration of a very high dose of LPS in these animals has a small effect on splenic hemopoiesis. The low-responsiveness of C3H/HeJ mice to LPS was first described by Sultzer (1972) who demonstrated that it is due to an autosomal mutation in the C3H strain, that first occurred spontaneously between 1960 and 1965 (Glode and Rosenstreich, 1976). The locus controlling LPS sensitivity has since been mapped to a single gene on the fourth chromosome (Watson et al., 1978). The hyporesponsiveness to LPS of C3H/HeJ mice is thought to be due to the absence of LPS receptors on C3H/HeJ cells. In support of this hypothesis Coutinho et al. (1978) reported binding of a putative anti-LPS receptor antibody to LPS-responder B cells but not to C3H/HeJ B cells.

There is evidence that LPS does not stimulate CFU-s directly, because treatment of bone marrow cells in vitro with serum containing anti LPS-receptor antibodies followed by complement failed to lyse CFU-s (Staber and Metcalf, 1980a). Evidence that LPS stimulates CFU-s indirectly was also obtained by Ploemacher (1984), who demonstrated CFU-s proliferation in diffusion chambers implanted in LPS treated mice. More insight into the mechanism, whereby LPS affects splenic hemopoiesis, was gained when the same authors showed that serum from mice treated with LPS mimicked the effects of LPS on the splenic hemopoiesis (Staber and Metcalf, 1980a, 1980b). The active principle in post-LPS serum has been named splenic hemopoiesis stimulating factor (SHSF). Peak levels of SHSF were found between 30 minutes and 3 hours after i.v. LPS injection (Staber et al., 1984). SHSF has been partially purified. It could be separated from the bulk of GM-CSF and appeared to be a glycoprotein with a molecular weight of approximately 30,000 daltons (Staber et al., 1984). SHSF could not be detected in sera of LPS injected C3H/HeJ mice, while injection of post-LPS serum of closely related LPS-responder C3H/WEHI mice into C3H/HeJ mice was found to increase the number of splenic progenitor cells (Staber and Metcalf, 1980a). This demonstrated that C3H/HeJ mice do not produce SHSF but have a normal sensitivity to this factor. Post-LPS serum had little effect on bone marrow hemopoiesis as measured by progenitor cell numbers (Staber and Metcalf, 1980b). The cellular origin of SHSF has not yet been determined. Whole body irradiation did not affect SHSF generation after LPS injection. This suggests that radioresistant cells are involved. Furthermore, it was observed that whole body irradiation did not induce SHSF (Staber and Metcalf, 1980b). The same authors could not detect SHSF in sera from animals treated with latex or phenylhydrazine, agents which do cause GM-CFU accumulation in the spleen (McNeill, 1970; Hodgson et al., 1972). This would indicate that induction of SHSF is specific for LPS. In vitro, SHSF activity has been found in the supernatant of cultured WEHI-3 cells, a myelomonocytic cell line (Staber and Johnson, 1980). In addition to other hemopoietic regulators this cell line is known to produce IL-3 which stimulates the proliferation of hemopoietic stem cells in vitro and in vivo (Ihle et al., 1982; Garland and Crompton, 1983; Kindler et al., 1985). The relationship between SHSF and IL-3 is not yet clear.

IV.2 LPS and bone marrow hemopoiesis

Changes in the number and type of bone marrow cells have mostly been studied after administration of low doses of LPS. Smith et al. (1961) noted a decline of femoral cellularity up to 24 hours after i.p. injection of 10 µg S.typhosa. Similar findings were reported by Chervenick et al. (1967). Quesenberry et al. (1973a,b) found a decrease of mature hemopoietic cells and GM-CFU with a nadir at 6 hours post-LPS coinciding with mobilization of GM-CFU after a similar dose. Normal cell numbers were attained at 48 hours and at that time GM-CFU proliferation could be demonstrated by an increased hydroxyurea kill. A decrease of tibial CFU-s numbers after LPS injection has been reported by several groups (Hanks and Ainsworth, 1964; Boggs et al., 1968), the magnitude depended on the dose that was used and the mouse strain which was studied. When low doses are injected the apparent decrease may be due to a decreased seeding fraction (Fred and Smith, 1968; Quesenberry et al., 1973b). Proliferation of femoral CFU-s after LPS injection has been demonstrated (Reissman et al., 1970; McCulloch et al., 1970; Quesenberry et al., 1973b). However, high doses of LPS may lead to a severe depletion of nucleated cells including CFU-s in the bone marrow (Vos and Wilschut, 1979). LPS may even cause bone marrow necrosis. Indeed microscopy showed a greatly disordered bone marrow architecture after injection of a sublethal dose of LPS (R.E. Ploemacher, personal communication).

The effect of the absence of endogenous LPS has been studied in germfree mice. In these mice no endotoxin is supposed to leak from the gut into the circulation. A diminished concentration of GM-CFU in the spleen and bone marrow of germfree mice has been reported (Metcalf and Stevens, 1972; Chang and Pollard, 1973). No significant differences between conventional and germfree mice were found by Boggs et al. (1967) and Metcalf and Foster (1969). In studies with dogs, elimination of gram negative bacteria from the gut caused a significant decrease in plasma GM-CSF levels and marrow GM-CFU concentration (McVittie and Walker, 1980). Goris et al. (1985) found that eradication of aerobic gram negative bacteria from the gut flora of mice led to a sharp decrease in the fecal endotoxin concentration. This was paralleled by a decreased sensitivity to hydroxyurea of bone marrow and splenic GM-CFU. These data suggest that gut-derived endotoxin influences myelopoiesis in the bone marrow and spleen.

IV.3 Effects of LPS on hemopoietic mediators

Injection of LPS into mice is followed by a rapid increase in GM-CSF levels in serum and in a variety of tissues (McNeil, 1970; Chervenick, 1972; Quesenberry et al., 1972). A rise in serum GM-CSF after injection of LPS has also been demonstrated in man (Cline and Golde, 1977). Two biochemically separable types of GM-CSF in post-LPS serum were detected by Staber and Burgess (1980). Van den Engh and Bol (1975) described a factor potentiating GM-colony growth in cultures already stimulated by M-CSF. Lipid-A has been implicated as the active part of endotoxin in inducing GM-CSF (Apte et al., 1976). In contrast, Nowotny et al. (1975) reported GM-CSF induction by a non-toxic polysaccharide-rich fraction of endotoxin. This could be due to its action as an antigen, since injection of antigens can induce GM-CSF (Metcalf, 1971). As expected LPS-low-responder C3H/HeJ mice were found to exhibit a low CSF response to LPS (Apte and Pluznik, 1976a; Russo and Lutton, 1977).

Reconstitution of lethally irradiated LPS-low-responder mice with LPS-high-responder bone marrow cells renders these mice sensitive to LPS as appears from a variety of biological effects, e.g. adjuvanticity, lethality, the production of interferon and GM-CSF (Michalek et al., 1980). However, after the reverse transplantation there is no decrease in the enhancement of GM-CSF production (Apte and Pluznik, 1976b; Michalek et al., 1980; Hültner et al., 1982). This has been taken as evidence that two types of cells are involved in the LPS- stimulated GM-CSF production. One appears to be a bone marrow-derived cell and the second one which is either long-lived and bone marrow-derived or a non-hemopoietic cell. Williams et al. (1983) injected normal spleen cells in mice that had been made tolerant to LPS. These spleen cells restored the ability of tolerant mice to increase serum GM-CSF after LPS injection. Spleen cells depleted of adherent cells had a reduced ability to stimulate GM-CSF production, which could be restored by adding thioglycolate-elicited peritoneal macrophages, which themselves did not affect GM-CSF production.

A similar synergism between lymphocytes and macrophages was found to exist for LPS stimulation of GM-CSF production in spleen cell cultures (Apte et al., 1980). These data led these authors to postulate that LPS induces macrophages to release mediators which stimulate lymphocytes to produce and release GM-CSF. No such a requirement has been reported for GM-CSF elaboration by adherent peritoneal cells (Eaves and

Bruce, 1974; Staber et al., 1978). The kinetics of GM-CSF production by macrophages in vitro was similar to that observed in vivo. A maximum response was found at 6 hours with a gradual decline thereafter (Eaves and Bruce, 1974). Lymphocyte cultures produced gradually increasing amounts of GM-CSF until the 5th day of culture (Parker and Metcalf, 1974). Apart from GM-CSF, LPS stimulated-peritoneal macrophages also synthesize and release burst-promoting activity which is probably identical to IL-3 (Kurland et al., 1980). BPA production in cultures of human monocytes stimulated by LPS has been described by Zuckermann et al. (1983). GM-CSF production by spleen cell cultures of C3H/HeJ mice is low, but high LPS concentations may increase GM-CSF in the supernatant to normal i.e. found in spleen cell cultures of LPS-high-responder mice (Apte et al., 1979). Serum from LPS-injected mice could only stimulate the formation of colonies consisting of granulocytes and/or macrophages (Staber and Johnson, 1980). However, the supernatant of LPS-stimulated spleen cells could induce the formation of other colonies among them multilineage colonies, indicating the presence of other HGFs than GM-CSF (Nakahata et al., 1982b; Fabian et al., 1985). The evidence available suggests that macrophages play an important role in the responses of the hemopoietic system to LPS. The absence of some of these responses in LPS-low-responder mice can be explained by the inability of LPS-low-responder macrophages to elaborate HGFs after LPS injection (reviewed by Vogel and Rosenstreich, 1980).

It cannot be excluded that LPS influences hemopoiesis in a non-specific way due to its many interactions with all kinds of cells and the various mediator systems of the body (reviewed by Morrison and Ulevitch, 1978). One group of the non-specific regulators could be the prostaglandins. LPS induces release of PGE2 and PGF by peritoneal macrophages and human monocytes (Kurland and Bockman, 1978). PGE2 has been found to stimulate hemopoietic stem cell proliferation in vitro (Feher and Gidali, 1974).

V. INTRODUCTION TO THE EXPERIMENTAL WORK

In mice hemopoietic stem cells and progenitor cells are almost totally confined to the bone marrow and spleen. Only small numbers can be detected in the peripheral blood. Relatively little is known about the mechanism(s) modulating the circulation and mobilization of stem cells. At present their function and ultimate fate are unknown. Mobilization of hemopoietic stem cells in mice has been achieved by injection of a variety of substances, the most well known being LPS. For some substances, including endotoxin, the transient increase in blood CFU-s immediately following injection (early mobilization) is followed by a second one some days later (delayed mobilization).

The purpose of the investigations presented in this thesis was to get insight into the mechanism(s) underlying both early and delayed mobilization of hemopoietic stem cells by LPS and other agents. Additionally, the changes in splenic hemopoiesis seen after administration of LPS have been subject of investigation.

In appendix paper I experiments are described which show that the early mobilization of CFU-s by LPS is dependent on the presence of the complement factor C5 and that for the late mobilization responsiveness to LPS is required. For this purpose mice differing only for the loci controlling C5 and LPS responsiveness were used.

In appendix paper II we further examined the role of certain complement factors, particularly C5, in early CFU-s and progenitor cell mobilization induced by LPS and proteolytic enzymes. This was done indirectly by employing C5-deficient mice, as well as directly by injecting purified C5a or serum containing activated complement. It was demonstrated that mobilization of CFU-s could be achieved by injection of mice with purified rat C5a.

The cellular aspects of the LPS-induced increase in splenic hemopoiesis were investigated by means of bone marrow transplantation between LPS-low and high-responder mouse strains. From data presented in appendix paper III it could be derived that in LPS low-responder mice the deficient late response to LPS could be restored by transplantation of bone marrow cells from LPS-high-responder mice. The transplantation of bone marrow cells from low-responder mice into lethally irradiated responder mice, however, did not completely abolish the late response. This indicates that a long-lived bone marrow-derived cell may be involved.

The presence in post-LPS serum of a factor that stimulates proliferation in vitro of CFU-s was described in appendix paper IV. An in vitro system allowing proliferation of CFU-s and stem cells sorted by a fluorescence activated cell sorter to exclude unwanted effects of other hemopoietic cells were used.

Similar kinetics of SHSF and SAF serum levels in mice after LPS injection were shown in appendix paper V. We could not demonstrate unequivocally the presence of IL-3, a known stimulator of CFU-s proliferation, in post-LPS serum. In order to investigate the specificity of the enhanced serum SHSF and SAF levels following LPS injection, we injected mice with other substances known to increase splenic hemopoiesis. Sera of these mice were tested for the presence of SAF, SHSF and IL-3. None of these substances was found to raise the serum level of SHSF, SAF or IL-3. In the same paper it was demonstrated that the accumulation of CFU-s in the spleen is at least partly caused by migration of CFU-s from the bone marrow to the spleen. To this purpose we made use of parabiotic mice, with one of the parabionts carrying marker chromosomes.

In paper VI a possible relationship between the induction of SHSF and SAF by LPS and the hemopoietic microenvironment was investigated. Microenvironmentally deficient mice of the Sl/Sl^d genotypes appeared to be less sensitive to SHSF in vivo but in vitro their CFU-s had the same sensitivity to SAF as CFU-s from their normal littermates. After LPS injection Sl/Sl^d mice produced normal quantities of SAF and SHSF.

VI. GENERAL DISCUSSION

Hemopoietic stem cells circulate in the blood stream in low numbers (Barnes and Loutit, 1967; Hellman and Grate, 1968). Upon injection a number of substances can cause an immediate and transient increase in circulating CFU-s by forcing stem cells out of the hemopoietic tissues. The experiments described in this thesis were performed to elucidate the mechanism(s) of hemopoietic stem cell mobilization with emphasis on lipopolysaccharide-induced CFU-s mobilization. The early mobilization of CFU-s after LPS injection was absent in C5 deficient mice (Appendix paper I). This confirmed the data of Wilschut et al. (1979) who showed that early CFU-s mobilization by LPS is complement-dependent. The early mobilization of E-BFU and GM-CFU was also absent in C5-deficient mice (Appendix paper I). This confirmed that mobilization by LPS has no specificity for certain cell types and that complement deficiency affects all mobilized cells roughly in a similar way (Ploemacher et al., 1980).

C5a rich serum as well as C5a purified from rat serum was capable of CFU-s mobilization in C5-deficient mice (Appendix paper II). Together these data suggest that the mobilization of CFU-s and other cells by LPS is mediated by C5a. The question remains how C5a exerts its mobilizing properties. One possibility is that cells are actively attracted due to the chemotactic activity of C5a, since it is known that C5a stimulates migration of neutrophils, macrophages, eosinophils and basophils (Snyderman et al., 1969, 1971; Kay, 1970; Kay and Austen, 1972). In contrast to granulocytes it has not been established yet whether stem cells or progenitor cells have a binding site for C5a. Another possibility is that stem cells and progenitor cells move passively together with cells susceptible to the chemotactic activity of C5a. In order to migrate from the medullary cords to the sinusoids of the bone marrow, cells have to pass the sinus lining cells. A reduction of the adventitial covering after injection of endotoxin has been observed by Weiss (1970) and Ploemacher et al. (1980). It was accompanied by a transit of leucocytes towards the sinusoids. A reduction of the adventitial covering without egress of cells was seen after injection of a low, but complement activating dose of cobra venom factor (CoF) (Ploemacher et al., 1980). From these observations it appears that there is no clear involvement of changes of the sinusoid walls in LPS induced mobilization. The observed aspecificity of mobilization could also be explained by assuming that mobilization is the result of a toxic action on the bone marrow of the anaphylatoxin C5a (Hügli and Müller-Eberhard, 1978). However, mobilization by very low doses of LPS cannot be explained in terms of toxicity. It is interesting that Ploemacher et al. (1981) observed that the blood pressure lowering agent diazoxide could diminish the CFU-s mobilization induced by agents of diverse groups such as endotoxin, polyanions and lectins. This could mean that changes in the blood flow through the bone marrow are involved in mobilization. However, the effects of diazoxide on the circulation of mice are not documented. We can conclude that the complement system does not seem to be the final common pathway to CFU-s mobilization. This is further demonstrated by the fact that it is not involved in CFU-s mobilization by polyanions (Wilschut et al., 1979). Trypsin and proteinase in high and low doses mobilized CFU-s in C5 deficient mice (Appendix paper II) but not in normal mice treated with a high dose of CoF (Wilschut et al., 1979). Both proteolytic enzymes cleave C3 directly (Bokisch et al., 1969). Upon cleavage of C3, C3a is released which has albeit limited chemotactic activity (Hügli and Müller-Eberhard, 1978). It is possible that the absence of mobilization in CoF-treated mice is due to exhaustion of C3 and later complement components.

Delayed CFU-s mobilization and the accumulation of CFU-s in the spleen after LPS injection are not C5-dependent (Appendix paper I). They were absent in LPS-low-responder C57BL/10ScCr and C3H/HeJ mice confirming the data of McVittie and Weinberg (1980). It is of interest that the early CFU-s mobilization in these mice was normal. It thus appears that early and delayed mobilization are independent phenomena (Appendix paper I).

The low responsiveness to LPS of C3H/HeJ mice is reflected in all cell types of those mice examined in vitro thus far (Vogel and Rosenstreich, 1980). In order to examine the role of CFU-s derived cells and non CFU-s derived cells in delayed mobilization we injected LPS in low-responder mice at 6 weeks after lethal irradiation and reconstitution with high-responder bone marrow cells. In these mice the response of blood and splenic CFU-s was similar to that in LPS-high-responders (Appendix paper III). This indicates that CFU-s derived cells are required for delayed mobilization. LPS-high responders reconstituted with low-responder bone marrow cells showed a diminished, but still significant response. Apparently the hemopoietic cells involved in delayed mobilization are long-lived and radioresistant. Alternatively these data can be explained by assuming that non-hemopoietic cells are also involved in delayed mobilization. LPS lowers bone marrow CFU-s numbers in LPS-high-responders as well as LPS-low-responder mice (Appendix paper III). This was also observed by McVittie and Weinberg (1980) who noted a 50% decrease in bone marrow CFU-s after i.p. injection of a small (10 µg) dose of LPS. In their experiments LPS increased blood CFU-s numbers in C3H/HeJ mice with a peak on day 3. Since splenic CFU-s numbers did not increase it must be assumed that there was no permanent seeding of bone marrow derived CFU-s in the spleen of those mice. Another possibility is that LPS is toxic for bone marrow CFU-s.

It has been shown that the accumulation of CFU-s and progenitor cells in the spleen after LPS injection is mediated by a serum factor (SHSF). From the results of our experiments it seems that the cells which produce SHSF are radioresistant and long-lived. They appear to be different from the cells which produce GM-CSF after being stimulated by LPS, since no decrease in GM-CSF generation was seen up to 20 weeks after reconstitution of LPS-high-responders with low-responder bone marrow cells (Michalek et al., 1980; Hültner et al., 1982) In our experiments the response to LPS as measured by splenic CFU-s number declined in this period (Appendix paper III), suggesting that the number of cells producing SHSF declined slowly.

The source of SHSF has as yet not been determined. The data from the above mentioned experiments make the macrophage a likely candidate. Macrophages are radioresistant and can be long-lived. They are known to produce other HGFs such as BPA and GM-CSF after LPS stimulation (Staber et al., 1978; Kurland et al., 1980; Zuckerman et al., 1983). In this respect it is interesting that SHSF could be detected in the supernatant of WEHI-3 cells, a cell line of myelomonocytic origin (Staber and Metcalf, 1980b).

Because CFU-s proliferation has been implicated in the increase in splenic CFU-s numbers after LPS injection (Staber and Johnson, 1980), we studied the effect of post-LPS serum on cycling of CFU-s in vitro (Appendix paper IV). In an otherwise serum free culture system post-LPS serum stimulated maintenance of CFU-s in a dose-dependent way. Cycling of CFU-s cultured in the presence of post-LPS serum could be demonstrated by increased kill of stem cells with hydroxyurea. This was also demonstrated with highly purified stem cells, which makes it unlikely that this effect was due to production of a cycling inducing factor, e.g. SAF, in the cultures by contaminating cells. In our experiments CFU-s counts were performed at day 8. These colonies probably not all represent pluripotent stem cells (Magli et al., 1982; Mulder et al., 1985). The effect of post-LPS serum could, however, also be demonstrated with 10 day colonies (unpublished observations). As expected C3H/HeJ post-LPS serum did not contain SAF, but CFU-s derived from C3H/Hej mice were responsive to SAF.

We studied the kinetics of SHSF and SAF in vivo following injection of LPS. SHSF and SAF had similar kinetics, comparable to the kinetics of SHSF described by Staber et al. (1984). Post-LPS serum stimulated proliferation of IL-3 dependent DA-1 cells cultured at high density and for 9 days. A small effect was seen in 4 or 8 day cultures with a low cell density. In the latter assay post-LPS serum synergized with IL-3. The activity of post-lPS serum on DA-1 cells could not completely be abrogated by anti-IL-3. We concluded that post-LPS serum contains an activity stimulating DA-1 cells which is apparently not identical to IL-3. Post-LPS serum was tested in the SAF assay and compared to IL-3, GM-CSF en Hemopoietin-1. The results of these experiments are shown in Table 1 of appendix paper V. Both GM-CSF which is present in high concentrations in post-LPS serum as well as Hemopoietin-1 supported CFU-s maintenance. Post-LPS serum, as well as GM-CSF, synergized with Hemopoietin-1, but not with IL-3. It is unlikely that the effects of post-LPS serum on splenic hemopoiesis are due solely to the presence of GM-CSF. This is concluded from the fact that GM-CSF behaved differently from post-LPS serum in the in vitro assays described. Secondly, induction of GM-CSF in vivo by bacterial cell wall products does not suffice to evoke an increase in splenic CFU-s and progenitor cell content. Indeed, only substances such as lipoprotein and LPS which trigger splenic CFU-s into cycle are capable of stimulating splenic hemopoiesis. Moreover, Staber et al. (1984) could separate SHSF from the major form of GM-CSF on the basis of molecular weight. Although the high molecular weight of GM-CSF in post-LPS serum might have been due to glycosilation (Appendix paper V).

The increase in splenic CFU-s numbers after injection of LPS is not solely the result of CFU-s proliferation since we could demonstrate CFU-s migration from the marrow to the spleen (Appendix paper V). Delayed mobilization by LPS can be summarized as follows. LPS depletes the bone marrow by mobilization of hemopoietic cells and/or through its toxic action on bone marrow. CFU-s enter the circulation and may seed in the spleen. Meanwhile splenic hemopoiesis is stimulated by SHSF/SAF. Splenic CFU-s proliferation and differentiation leads to increased CFU-s and progenitor cell numbers. This process is also stimulated by other HGFs (BPA, GM-CSF, M-CSF) directly or indirectly induced by LPS. Injection of phenylhydrazine (PHZ) or latex particles did not induce SHSF. This may indicate that these agents increase splenic CFU-s primarily through migration of CFU-s. This would agree with the data of Rencricca et al. (1970) who showed that the rise in splenic CFU-s numbers (bone marrow CFU-s decreased) after injection of PHZ was not associated with CFU-s proliferation.

SAF and SHSF levels in Sl/Sl^d mice injected with LPS were not different from the levels found in similarly treated normal littermates. It thus appears not likely that a defect in the formation of those factors is responsible for the lack of response of Sl/Sl^d mice to LPS (Appendix paper VI). Rather, it is suggested that the defective microenvironment does not allow proliferation of splenic CFU-s in response to SAF.

SUMMARY

Hemopoietic stem cells (HSC) are present in the blood in low numbers. Their incidence in the blood can be increased by mobilizing them out of the bone marrow and/or the spleen into the circulation. This thesis describes investigations to elucidate the mechanism of stem cell mobilization.

A number of substances are capable of stem cell mobilization. Peak CFU-s numbers are found minutes to several hours after injection of the particular agent. For some agents this early mobilization is followed by a second rise (delayed mobilization) of blood CFU-s numbers which has its maximum 3-5 days after injection and is accompanied by an increase of the splenic CFU-s population size.

The early mobilization of stem cells, progenitor cells and mature blood cells by lipopolysaccharide (LPS) and zymosan is dependent on an intact complement system. In mice genetically deficient for the complement component C5 no early mobilization of CFU-s and the progenitor cells E-BFU and GM-CFU could be detected. Delayed CFU-s mobilization was not affected by the C5 deficiency. Serum containing activated complement as well as purified rat C5a proved to be capable of early CFU-s mobilization in C5 deficient mice. Together the data from these experiments suggest that the LPS-induced early mobilization of CFU-s and progenitors is mediated by C5a. Whether this is due to its properties as an anaphylatoxin or as a chemotactic substance cannot be said with certainty. Activation of the complement system did not seem to be a final common pathway leading to CFU-s mobilization, since early CFU-s mobilization by trypsin and proteinase was normal in C5 deficient mice.

The requirements for delayed CFU-s mobilization by LPS were studied in mouse strains differing only in the locus which controls LPS sensitivity. In LPS-low-responder mice delayed mobilization was absent while the early CFU-s mobilization proceeded normally. In LPS-high-responder mice the delayed mobilization was accompanied by a large increase in splenic CFU-s numbers and a decrease of bone marrow CFU-s. The first phenomenon was absent in LPS-low-responder mice.

To examine the cellular requirements for delayed mobilization we reconstituted lethally irradiated LPS-low-reponder mice with LPS-high-responder bone marrow cells. These mice responded to LPS with delayed CFU-s mobilization, an

increase in splenic CFU-s numbers and a decrease in femoral CFU-s. When lethally irradiated LPS-high-responder mice were reconstituted with low-responder bone marrow and challenged with LPS a much reduced response of blood, bone marrow and splenic CFU-s was noted, but this response was significantly higher than in untreated LPS-low-responder mice. These studies show that LPS-responsive hemopoietic cells are required for the above mentioned response of the hemopoietic system to LPS. The results of the above mentioned experiments suggest that the hemopoietic cells involved in the stimulation of splenic hemopoiesis by LPS are long-lived and/or radioresistant. Alternatively, the reduced response that still occurred in the reconstituted LPS-high-responders could be due to persistent non-hemopoietic cells.

The accumulation of CFU-s and progenitor cells in the spleen can also be induced by serum from animals injected with LPS. Apparently post-LPS serum contains a mediator of the action of LPS on the spleen. This mediator has been called Splenic Hemopoiesis Stimulating Factor (SHSF). LPS-low-responder mice were found to be unable to produce SHSF. Since LPS causes proliferation of splenic CFU-s in vivo we studied the effect of post-LPS serum on the proliferative behaviour of CFU-s.

Post-LPS serum from LPS-high-responder but not from LPS-low-responder mice stimulated cycling of CFU-s. This was demonstrated by increased kill of CFU-s cultured in the presence of post-LPS serum by hydroxyurea. Cycling could also be demonstrated when highly purified stem cells were used. It could be concluded that post-LPS serum from LPS-high-responder mice contains a stem cell activating factor (SAF).

The identity of stem cell activating factor in post-lPS serum was further investigated in vitro as well as in vivo. We found no evidence for the presence of Hemopoietin-1. Although post-LPS serum could under certain conditions induce proliferation of the IL-3 dependent DA-1 cells, we could not detect significant amounts of IL-3. We cannot exclude that the activity of post-LPS serum in the SAF assay is due to its GM-CSF content since GM-CSF induced cycling of CFU-s in this assay. In vivo, the serum levels of SAF and SHSF showed similar kinetics to those published for GM-CSF after LPS injection. Induction of GM-CSF alone does not stimulate splenic hemopoiesis. This led to the tentative conclusion that the stimulation of splenic hemopoiesis is probably due to the combined actions of IL-3 and GM-CSF.

Although CFU-s proliferation in the spleen after LPS injection has been demonstrated, the role of CFU-s migration in the accumulation of splenic CFU-s had not been investigated sofar.

To study CFU-s migration after LPS injection we made parabionts of CBA/N and CBA/T6T6 mice, the latter carrying two T6 marker chromosomes. After injection of LPS in both parabionts the migration of CFU-s from the bone marrow to the spleen was examined by enumerating the number of T6-marked spleen colonies in the CBA/N spleen. Our experiments indicated that about 36% of splenic CFU-s present six days after LPS injection are of bone marrow origin. In the experimental set up we could not establish quantatively the contribution of immigrant CFU-s proliferation to the CFU-s accumulation.

It is known that Sl/Sl^d mice, which have a defective microenvironment, do not support CFU-s proliferation in their spleens after LPS injection. We investigated whether this could be due to a defective induction of SAF or SHSF. SAF and SHSF generation in these mice was found to be normal. Apparently the defective microenvironment does not allow splenic CFU-s to proliferate despite the presence of CFU-s proliferation.

SAMENVATTING

Een gering aantal hemopoietische stamcellen circuleert in het bloed. Het aantal circulerende stamcellen kan verhoogd worden door ze te mobiliseren uit het beenmerg en/of de milt. In dit proefschrift worden experimenten beschreven die tot doel hadden het mechanisme van stamcelmobilisatie te onderzoeken. Een aantal stoffen heeft het vermogen stamcellen te mobiliseren. Het hoogste aantal stamcellen per milliliter bloed wordt minuten tot uren na inspuiten gevonden, afhankelijk van de aard van de toegediende stof. Sommige stoffen veroorzaken na deze voorbijgaande mobilisatie een tweede, vertraagde, stijging van het aantal circulerende stamcellen waarbij de grootste aantallen 3 tot 5 dagen na injectie worden gevonden.

De vroege mobilisatie van stamcellen, voorlopercellen (E-BFU, GM-CFU) en rijpe hemopoietische cellen na toediening van lipopolysaccharide (LPS) of zymosan is afhankelijk van de complement factor 5 (C5). De vertraagde mobilisatie daarentegen werd niet beinvloed door C5. Zowel injectie van serum waarin het complement was geactiveerd, als gezuiverd ratten C5a kon vroege mobilisatie opwekken bij muizen.

De resultaten van bovengenoemde experimenten suggereerden dat C5a een intermediair is bij de door LPS geinduceerde vroege mobilisatie van stamcellen en voorlopercellen. Het kon niet worden vastgesteld of dit berust op de chemotactische werking van C5a of op het feit dat C5a een anaphylatoxine is. Complement activatie lijkt geen universeel mechanisme van stamcel-mobilisatie te zijn. Zo bleek dat in C5-deficiente muizen de vroege stamcelmobilisatie na inspuiting van trypsine en proteinase normaal verliep.

Bij muizen met een gering reactievermogen op LPS (LPS-low-responders) was de vertraagde mobilisatie afwezig terwijl de vroege mobilisatie normaal verliep. In muizen met een groot reactievermogen op LPS (LPS-high-responders) ging de vertraagde mobilisatie gepaard met een grote toename van het aantal stamcellen in de milt en met een vermindering van het aantal stamcellen in het beenmerg.

Om te onderzoeken of de vertraagde mobilisatie afhankelijk is van (een) bepaalde cel(len) werden LPS-low-responders letaal bestraald en gereconstitueerd met beenmerg van LPS-high-responder. De aldus behandelde muizen vertoonden na inspuiten van LPS de vertraagde mobilisatie van stamcellen en een stijging van het aantal stamcellen in de milt. LPS-high-responders gereconstitueerd met beenmerg van LPS-high-responders vertoonden na inspuiten van LPS een sterk verminderde, maar nog steeds significante stijging van het aantal CFU-s in bloed en milt. De laatstgenoemde resultaten suggereren dat langlevende en/of weinig stralingsgevoelige hemopoietische cellen betrokken zijn bij de LPS geinduceerde vertraagde mobilisatie en stimulatie van de milt hemopoiese. Het is niet uitgesloten dat ook niet-hemopoietische cellen betrokken zijn bij de bovengenoemde activiteiten van LPS.

Niet alleen LPS, maar ook serum van muizen tevoren ingespoten met LPS kan het aantal stamcellen en voorlopercellen in de milt doen stijgen. Post-LPS serum bevat kennelijk een factor die verantwoordelijk is voor het effect van LPS op de milt hemopoiese. Deze factor is "Splenic hemopoiesis stimulator factor" (SHSF) genoemd. omdat LPS in vivo de proliferatie van milt CFU-S stimuleert onderzochten wij het effect van post-LPS serum op de proliferatie van CFU-s in vitro. Post-LPS serum van LPS-high-responders maar niet dat van LPS-low-responders, stimuleerde deling van CFU-s in een verder serumvrij cultuursysteem. Dit kon worden angetoond door een toegenomen hydroxyureum kill van de gekweekte CFU-s. Wij concluderen dat post-lPS serum stem cell activating factor bevat. De aard van SHSF in post-LPS serum werd verder onderzoch in vitro en in vivo. Met het SAF assay werd geen aanwijzing gevonden voor de aanwezigheid van hemopoietin-1 in post-LPS serum. Hoewel post-LPS serum onder bepaalde condities proliferatie kan stimuleren van IL-3 afhankelijke DA-1 cellen lijkt het geen significante hoeveelheden IL-3 te bevatten. Wij kunnen niet uitsluiten dat de activiteit van post-LPS serum in het SAF assay het gevolg is van de GM-CSF erin, daar GM-CSF SAF activiteit bezit. De serumspiegels van SAF en SHSF na LPS injectie toonden eenzelfde kinetiek. Daar aangetoond is dat inductie van GM-CSF niet voldoende is om de milt hemopoiese te stimuleren, concluderen wij dat de stimulatie door LPS van de milt hemopoiese mogelijk het gevolg is van het gecombineerde effect van IL-3 en GM-CSF.

Mogelijke migratie van CFU-s naar de milt na LPS injectie werd bestudeerd in parabionten gevormd door CBA/N en CBA/T6T6 muizen; de laatsten bezitten twee T6 chromosomen. Migratie van beenmerg CFU-s naar de milt werd gemeten door bepaling van het aantal T6 gemerkte miltkolonies in de CBA/N milt. De resultaten van deze experimenten duiden erop dat ongeveer 36% van in de milt aanwezige CFU-s, 6 dagen na LPS injectie afkomstig is uit het beenmerg. Wat de precieze bijdrage is van de uit het beenmerg afkomstige CFU-s aan de accumulatie van CFU-s in de milt was niet vast te stellen in onze experimenten.

Sl/Sl^d muizen hebben een defecte micro-omgeving en vertonen slechts een geringe CFU-s proliferatie in de milt na LPS injectie. Wij onderzochten of dit mogelijk het gevolg is van het ontbreken van SAF of SHSF. De serumspiegels van deze factoren na LPS injectie in deze muizen was normaal. Kennelijk verhindert de microömgeving CFU-s proliferatie, ondanks de aanwezigheid van CFU-s proliferatie-stimulerende factoren.

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CURRICULUM VITAE

Na het behalen van het diploma HBS-B aan de Willem de Zwijger HBS te Rotterdam in 1969 begon de auteur van dit proefschrift in hetzelfde jaar de studie Geneeskunde aan de Faculteit der Geneeskunde van de Rijksuniversiteit te Leiden. Het doctoraalexamen werd afgelegd in 1976 en in 1977 volgde het artsexamen. Hierna was hij een jaar werkzaam als arts-assistent in de Dr. Daniël den Hoed Kliniek te Rotterdam. Van 1978 t/m 1982 was hij in dienst van de Stichting voor Zuiver Wetenschappelijk Onderzoek en werkzaam binnen de vakgroep Celbiologie en Genetica van de Erasmus Universiteit te Rotterdam. Binnen het instituut Celbiologie II werd het in dit proefschrift beschreven onderzoek verricht onder leiding van Prof.Dr. O. Vos. Na een korte periode in het Dr. Bernard Verbeeten Instituut te Tilburg is de auteur thans werkzaam als arts-assistent in opleiding tot internist in het St. Elisabeth Ziekenhuis te Tilburg, opleider Dr. J.H.M. Lockefeer.

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APPENDIX PAPER I

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GENETIC CONTROL OF LIPOPOLYSACCHARIDE-INDUCED MOBILIZATION OF CFUs

DISSOCIATION BETWEEN EARLY AND DELAYED MOBILIZATION OF CFUs IN COMPLEMENT C5-DEFICIENT MICE AND LPS NON-RESPONDER MICE

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ABSTRACT

Lipopolysaccharide (LPS)-induced mobilization of CFUs from haemopoietic tissues into the circulation has a biphasic pattern. The first rise occurs within 30 min of LPS injection, the second 4–7 days later. This second rise coincides with an increase of the CFUs number in the spleen from about 3000 to about 50,000. We have investigated the relationship between the two peaks by making use of complement C5-deficient mouse strains and the LPS non-responder mouse strains C3H/HeJ and C57BL/10ScCr. These latter two strains lack a serologically identifiable structure ('LPS-receptor') which is present in all LPS-responder strains.

After injection of eleven different mouse strains with LPS, the numbers of circulating CFUs increased rapidly in all strains, except in the C5-deficient A/J, AKR/J, DBA/2J and B10.D2/oSn mice. On the other hand, the delayed LPS-induced accumulation of CFUs in blood and spleen occurred in all mouse strains tested, including the C5-deficient strains, but not in the LPS non-responder strains C3H/HeJ and C57BL/10ScCr. These results show that (a) early LPS-induced mobilization of CFUs is dependent on the availability of C5, in contrast to the delayed CFUs accumulation in blood and spleen, (b) the presence of the LPS receptor is not required for early CFUs mobilization by LPS and (c) recognition of the mobilizing agent by a specific receptor is required for the delayed accumulation of CFUs in blood and spleen.

A variety of substances can induce mobilization of haemopoietic stem cells (CFUs) and granulocyte-macrophage progenitor cells (CFUc) into the circulation. They include lipopolysaccharides ('endotoxins') (LPS), zymosan, proteolytic enzymes, polyanions and

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lectins (Vos, Buurman & Ploemacher, 1972; Quesenberry *et al.*, 1973; Van der Ham, Benner & Vos, 1977; Cline & Golde, 1977; Vos & Wilschut, 1979; Wilschut *et al.*, 1979). Injection of each of these agents in mice causes a rapid mobilization of CFUs, peaking between 10 min and 6 hr after administration, depending on the substance injected. LPS, zymosan and polyanions have been shown to evoke a delayed rise of blood CFUs numbers as well. This second rise, 4–7 days after the first, is associated with an increase of splenic CFUs numbers from about 3000 to about 50,000 per whole spleen (Vos *et al.*, 1972; Van der Ham *et al.*, 1977; Vos & Wilschut, 1979).

So far, it is unclear what the relationship is between the early and delayed mobilization of CFUs. We have recently shown that decomplementation of mice by the complement activating factor of cobra venom (CoF) can completely prevent the early CFUs mobilization by LPS, zymosan and proteolytic enzymes (Wilschut *et al.*, 1979). This result suggests that the complement system is involved in the early CFUs mobilization evoked by these agents. It has to be established, however, whether only C3 or also the later enzymes of the complement cascade are involved. It has not been reported whether the complement system is also involved in the delayed LPS and zymosan-induced CFUs accumulation in blood and spleen.

Studies on the effects of LPS upon the haemopoietic and immune systems are favoured by the availability of LPS non-responder strains of mice. In recent years two such mutant strains have been identified, namely C3H/HeJ (Sultzer & Nilsson, 1972) and C57BL/10ScCr (Coutinho *et al.*, 1977; McAdam & Ryan, 1978). In both strains the unresponsiveness is inherited as a recessive trait and is determined by an autosomal gene linked to the *Mup-1* locus on chromosome 4 (Watson, Kelly, Largen & Taylor, 1978; Coutinho & Meo, 1979). The locus in charge codes for a serologically detectable LPS receptor on B cells (Forni & Coutinho, 1978) and macrophages (L. Forni, personal communication).

In the present paper we have analysed the relationship between the early and delayed mobilization of CFUs following LPS injection. Therefore, we have made use of mouse strains with a genetically determined deficiency for either complement component C5 (the strains A/J, AKR/J, DBA/2J and B10.D2/oSn) or for the LPS receptor (i.e., C3H/HeJ and C57BL/10ScSr). The results show that C5 is required for early, but not for delayed mobilization of CFUs, while the LPS receptor is required only for the delayed accumulation of CFUs in blood and spleen. Apparently, at least partly different mechanisms underly both phenomena.

MATERIALS AND METHODS

Mice

Male and female mice of the following strains were used: A/J, (C57BL/6J × A/J)F_1, AKR/J, DBA/2J, (C57BL/6J × DBA/2J)F_1, B10.D2/oSn, B10.D2/nSn, C3H/HeJ, C3H/Tif, C57BL/10ScCr and C57BL/10ScCn. The A/J, B10.D2/oSn and B10.D2/nSn mice were purchased from Jackson Laboratories, Bar Harbor, Maine, U.S.A, the (C57BL/6J × A/J)F_1, DBA/2J, (C57BL/6J × DBA/2J)F_1, C3H/HeJ and C3H/Tif mice from the Institut für Biologisch-Medizinische Forschung AG, Füllinsdorf, Switzerland, the AKR mice from OLAC Ltd., Bicester, U.K., the C57BL/10ScCr from Bomholtgard Ltd., Ry, Denmark and the C57BL/10ScSn from Bantin and Kingman, Aldbrough, U.K. The age of all donor and recipient mice ranged between 12 and 20 weeks. For each experiment mice of the same sex were used.

Mobilizing agents

Two different LPS preparations were used in this study: *Salmonella typhosa* 0901 LPS (prepared according to Boivin) from Difco Laboratories, Detroit, Michigan, U.S.A., and *S. abortus equi* LPS, kindly provided by Drs C. Galanos and O. Lüderitz, Max-Planck Institut für Immunobiologie, Freiburg, FRG. Zymosan was purchased from Fleischmann, New York, U.S.A. Each mouse was injected intravenously (i.v.) with the appropriate amount of the mobilizing agent (indicated in the various experiments), dissolved in a volume of 0·5 ml of a balanced salt solution (BSS).

Cell suspensions

Blood samples were collected by cardiac puncture, after killing the mice with carbon dioxide. Blood was immediately heparinized in lithium heparin tubes (Sarstedt, Numbrecht-Rommelsdorf, FRG). Spleen and mesenteric lymph node cell suspensions were prepared as described previously (Benner *et al.*, 1974). Pooled blood samples and pooled spleen cell suspensions from five mice per experimental group were appropriately diluted with BSS and injected intravenously into lethally-irradiated syngeneic recipients in order to determine CFUs numbers.

Irradiation

The recipient mice received lethal dose whole body X-irradiation from a Philips RT 305 X-ray machine. (C57BL/6J × A/J)F_1, AKR/J, (C57BL/6J × DBA/2J)F_1, C3H/HeJ and C3H/Tif mice received 850 cGy (rad), the other mouse strains 800 cGy. Irradiated control mice all died between 8 and 12 days after irradiation.

Spleen colony assay

The spleen colony assay of Till & McCulloch (1961) was used to measure the number of CFUs. The samples to be tested for CFUs content were injected within 6 hr after irradiation. The recipient mice were killed 8 days later and the spleens were fixed in Telleyesnizky's solution. Each group of recipients consisted of at least seven mice.

Cell cultures and reagents

Mesenteric lymph node cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, 100 units penicillin and 100 μg streptomycin/ml (Flow Laboratories, Irvine, Scotland), 5×10^{-5} M 2-mercaptoethanol and 5% human serum. A number of $2{\cdot}5 \times 10^6$ cells/ml was set up in 0·2 ml aliquots in tissue culture plates (No. 3040, Falcon Plastics, Oxnard, California, U.S.A.). Stimulation by LPS was done by adding 50 μg/ml *S. abortus equi* LPS to the medium at the start of the cultures. The cultures were set up in triplicate and incubated in a stationary position at 37°C in a humidified atmosphere of 5% CO_2 in air. Proliferative responses were assayed by pulsing the culture with 2 μCi of tritiated thymidine (specific activity 2 Ci/mM; The Radiochemical Centre, Amersham, England) after 60 hr. The cells were incubated for another 18 hr, after which the cells were harvested in a multiple-cell culture harvester (Skatron A/S, Lierbyen, Norway). The samples were counted in a scintillation spectrometer (Tri-Carb Packard).

LPS responsiveness of the mouse strains used in this study

All the mouse strains which were tested for their capacity for early and delayed mobilization of CFUs following LPS administration, were tested for their *in vitro* mitogenic

TABLE 1. LPS responsiveness *in vitro* by lymph node cells from the mouse strains used in this study

Strain	Ct/min −LPS	Ct/min +LPS	Stimulation index
A/J	3180	23,295	7·3
(C57BL/6J × A/J)F_1	2132	34,559	16·2
AKR/J	7214	71,160	9.9
DBA/2J	9337	68,159	7.3
(C57BL/6J × DBA/2J)F_1	1663	37,604	22·6
B10.D2/oSn	615	4720	7·7
B10.D2/nSn	640	12,785	20·0
C3H/HeJ	1035	718	0·7
C3H/Tif	3489	35,523	10·2
C57BL/10ScCr	368	693	1·9
C57BL/10ScSn	2300	33,552	14·6

The 0·2 ml cultures containing 5 × 10^5 mesenteric lymph node cells were pulsed with 2 μCi tritiated thymidine 60 hr after start. The cells were harvested 18 hr later. Cultures, with or without LPS, were always done in triplicate. Figures represent the arithmetic mean of two independent experiments.

response to LPS. This was done by using mesenteric lymph node cells. It turned out that apart from the already known LPS non-responder strains C3H/HeJ and C57BL/10ScCr all strains responded well to the mitogenic moiety of LPS (Table 1).

RESULTS

Mobilization of CFUs in hereditary complement C5-deficient mice

The capacity of hereditary complement C5-deficient A/J, AKR/J, DBA/2J and B10.D2/oSn (Cinader, Dubiski & Wardlaw, 1964; Rosen, 1975) mice to mobilize CFUs upon i.v. injection of LPS was investigated. Both the early and delayed mobilization of CFUs was assayed. This was done at 30 min and 6 days after LPS administration, respectively, since these are the times of peak response (Vos *et al.*, 1972). At 6 days the spleen was also assayed for CFUs content. As genetically related control strains were used (C57BL/6J × A/J)F_1, (C57BL/6J × DBA/2J)F_1 and B10.D2/nSn mice, which all have normal C5 levels.

All four C5-deficient mouse strains tested were incapable of LPS-induced mobilization of CFUs when tested 30 min after injection (Table 2). All C5 positive control strains, on the other hand, showed an increase of the CFUs numbers from 20–100 to 200–1200 per ml blood. The cell counts in the blood of the various mouse strains followed the same pattern as the CFUs numbers. In the C5 deficient mouse strains the cell counts were not increased 30 min after LPS injection. In the C5 positive control strains, on the other hand, LPS injection caused an up to 2-fold increase of the number of nucleated and red cells per ml blood (data not shown).

When CFUs numbers were assayed in blood and spleen at 6 days after LPS injection, all mouse strains showed a similar increase, independent of whether they were C5 deficient or

TABLE 2. Absence of early LPS-induced mobilization of CFUs in C5-deficient A/J, AKR/J, DBA/2J and B10.D2/oSn mice

Strain	Early mobilization* (CFUs/ml blood)		Delayed mobilization† CFUs/ml blood		Delayed mobilization† CFUs/spleen	
	PBS	LPS	PBS	LPS	PBS	LPS
A/J	17 ± 5‡	43 ± 14	18 ± 3	373 ± 82§	3867 ± 554	30,500 ± 4656§
	24 ± 5	41 ± 6	10 ± 2	625 ± 70§	1332 ± 212	40,727 ± 3183§
(C57BL/6J × A/J)F_1	20 ± 4	1140 ± 154	32 ± 5	688 ± 87	3650 ± 232	44,500 ± 4170
	37 ± 7	1071 ± 102	85 ± 3	1973 ± 96	5360 ± 816	102,000 ± 9728
AKR/J	31 ± 11	35 ± 8	26 ± 7	334 ± 95§	2652 ± 488	19,440 ± 5800§
	42 ± 10	37 ± 11	6 ± 3	367 ± 61§	1332 ± 320	33,160 ± 5360§
DBA/2J	112 ± 17	104 ± 18	111 ± 10	760 ± 110	9920 ± 704	57,500 ± 10,500
	50 ± 14	59 ± 17	67 ± 8	880 ± 77	5995 ± 413	83,200 ± 7631
(C57BL/6J × DBA/2J)F_1	70 ± 6	1100 ± 120	51 ± 7	280 ± 100	8160 ± 1440	78,000 ± 10,200
	34 ± 4	1140 ± 108	68 ± 9	300 ± 41	7378 ± 1063	50,500 ± 7926
B10.D2/oSn	43 ± 6	52 ± 7¶	33 ± 6	480 ± 27	2600 ± 364	40,888 ± 3875
	46 ± 6	50 ± 9¶	38 ± 6	396 ± 52	2460 ± 408	61,777 ± 5966
B10.D2/nSn	73 ± 9	843 ± 167¶	94 ± 12	700 ± 47	3868 ± 196	47,430 ± 3218
	58 ± 16	240 ± 96¶	50 ± 22	320 ± 80	6300 ± 1200	23,000 ± 4100

* Blood samples were taken 30 min after i.v. injection of either PBS or 300 μg *S. typhosa* LPS.

† Blood and spleen were assayed for CFUs content 6 days after i.v. injection of either PBS or 300 μg *S. abortus equi* LPS.

‡ Arithmetic mean ± 1 s.e.m.

§ In this particular experiment the mice were injected with only 100 μg LPS, since higher doses of this LPS preparation were not tolerated for the experimental period.

¶ In this particular experiment the mice were injected with *S. abortus equi* LPS instead of *S. typhosa* LPS.

not. The blood CFUs numbers had increased from 20–100 to 200–2000 per ml, while the number of CFUs per spleen had increased from 1300–10,000 to 20,000–100,000 (Table 2).

Mobilization of CFUs in hereditary LPS non-responder mice

In the same way as in the C5-deficient mice the capacity of early and delayed LPS-induced CFUs mobilization was studied in the LPS non-responder mouse strains C3H/HeJ and C57BL/10ScCr. As genetically related control mice the strains C3H/Tif and C57BL/10ScSn were used. It appeared that the LPS non-responder C3H/HeJ and C57BL/10ScCr mice reacted as good as the control strains to LPS injection with immediate CFUs mobilization (Table 3). However, when CFUs numbers in blood and spleen were assayed at 6 days after LPS injection, the LPS non-responder strains turned out to have normal, not enhanced, CFUs numbers, in contrast to the control mice (Table 3). Thus, the LPS receptor is not required for early mobilization of CFUs, but is necessary for the delayed LPS-induced CFUs accumulation in blood and spleen.

The yeast wall polysaccharide zymosan is, just like LPS, capable of inducing a biphasic rise of blood CFUs numbers (Vos & Wilschut, 1979). This agent also induces a delayed accumulation of CFUs in the spleen, with peak numbers between day 4 and 7 after injection.

TABLE 3. Absence of delayed LPS-induced mobilization of CFUs in LPS non-responder C3H/HeJ and C57BL/10ScCr mice

Strain	Early mobilization* (CFUs/ml blood)		Delayed mobilization†					
			CFUs/ml blood			CFUs/spleen		
	PBS	LPS	PBS	LPS	ZYM	PBS	LPS	ZYM
C3H/HeJ	103 ± 16‡	1634 ± 124	22 ± 5	48 ± 7	50 ± 9	3866 ± 396	3866 ± 420	3600 ± 768
	n.d.§	1592 ± 62	71 ± 12	82 ± 13	33 ± 19	7400 ± 756	7316 ± 904	9600 ± 4120
	43 ± 9	550 ± 112¶						
C3H/Tif	129 ± 13	1440 ± 77	64 ± 8	940 ± 74	n.d.	2680 ± 266	52.850 ± 5750	n.d.
	n.d.	1233 ± 150	140 ± 10	1225 ± 96	283 ± 65	3700 ± 584	54.375 ± 3715	20.520 ± 2320
	133 ± 16	728 ± 74¶						
C57BL/10ScCr	44 ± 7	456 ± 60**	48 ± 6	36 ± 4	71 ± 28	4360 ± 514	4844 ± 590	13.714 ± 1658
	n.d.	1000 ± 90**	72 ± 6	61 ± 13	176 ± 30	5440 ± 614	2867 ± 392	4800 ± 1960
	43 ± 14	190 ± 54¶	n.d.	n.d.	44 ± 3	n.d.	n.d.	3500 ± 500
C57BL/10ScSn	45 ± 7	570 ± 84	23 ± 5	320 ± 39	280 ± 35	4000 ± 575	58.400 ± 8447	55.320 ± 5880
	n.d.	420 ± 70	80 ± 14	881 ± 115	427 ± 40	5280 ± 941	45.500 ± 8974	57.200 ± 4320
	36 ± 6	253 ± 63¶						

* Blood samples were taken 30 min after i.v. injection of either PBS or 300 μg *S. typhosa* LPS.

† Blood and spleen were assayed for CFUs content 6 days after i.v. injection of either PBS or 300 μg *S. abortus equi* LPS and 5 days after i.v. injection of 2 mg zymosan (ZYM).

‡ Arithmetic mean ± 1 s.e.m.

§ n.d., Means not determined.

¶ In this particular experiment the mice were injected with *S. abortus equi* LPS instead of *S. typhosa* LPS.

** In this particular experiment the mice were injected with only 100 μg LPS, since higher doses of this LPS preparation were not tolerated for the experimental period.

As a specificity control of the non-responsiveness of C3H/HeJ and C57BL/10ScCr mice to LPS, we also tested these strains for delayed CFUs mobilization after zymosan injection. It turned out that the LPS non-responder mice responded only weakly to zymosan or not at all to this agent (Table 3), suggesting that the major active component of zymosan is chemically related to LPS.

DISCUSSION

The experiments presented in this paper show that the early and the delayed LPS-induced mobilization of CFUs in mice are under control of two different genes. These genes code for complement factor C5 and the membrane-associated LPS receptor and are both inherited as a dominant trait (Cinader *et al.*, 1964; Rosen, 1975; Coutinho, Möller & Gronowicz, 1975; Coutinho & Meo, 1979). Apparently, at least partly different mechanisms are underlying both phenomena. This gives a genetic basis to the previous observation that an early mobilization of CFUs is not followed *per se* by a second rise of the blood CFUs number some days later (Vos *et al.*, 1972).

Since LPS-non-responder C3H/HeJ and C57BL/10ScCr mice, in spite of the absence of a cellular receptor for LPS, normally increase their number of circulating CFUs immediately after injection of LPS (Table 2), stimulation of the haemopoietic stem cells by the mobilizing agent itself is not required for early CFUs mobilization. All agents that mobilize CFUs also affect the incidence of other blood cells, although not all leukocyte types to the same extent (Ploemacher *et al.*, 1980). Pretreatment of mice with CoF can prevent mobilization of both CFUs and mature leukocytes (Wilschut *et al.*, 1979; Ploemacher *et al.*, 1980). These results suggest that the early mobilization of CFUs and mature leukocytes is for a large part a non-specific process, depending on the availability of complement enzymes. The present study confirms this. It is tempting to hypothesize that the early mobilization is caused by the chemotactic activity of C3a and C5a, for which a variety of blood cells is susceptible (Hugli & Müller-Eberhard, 1978). The relative contribution of the C3a and the C5a components to such an acute inflammatory response might be dependent on the type of mobilizing agent used. Our experiments showing no CFUs mobilization in hereditary C5-deficient (but C3 positive) mice, would imply that C3a contributes hardly or not at all to the chemotactic response induced by LPS. Indeed, there is evidence for this view in the literature. Snyderman *et al.* (1969) have shown that chemotactic activity appeared in guinea-pig serum after addition of LPS. The active factor had an estimated molecular weight of 15,000. The relative activity of this factor was far greater than that associated with any other fraction in activated serum. Snyderman *et al.* (1969) showed that the activity of the factor was inhibited by anti-guinea-pig C5, but not by anti-guinea-pig C3, suggesting that the LPS-induced chemotactic activity resides mainly in the C5a component. Our future studies will be directed to the problem of whether early CFUs mobilization is indeed due directly to the chemotactic activity of complement enzymes or that C3 and C5 are intermediates in a pathway of events finally leading to the mobilization of CFUs.

C3H/HeJ and C57BL/10ScCr mice show lymphoid and non-lymphoid unresponsiveness in a variety of assays (Watson, Largen & McAdam, 1978; McAdam & Ryan, 1978). On the other hand, some authors could establish LPS-induced mitogenesis using C3H/HeJ spleen cells (Skidmore *et al.*, 1975; Sultzer & Goodman, 1976). Later studies have shown that in fact this response was not induced by LPS, but by a contaminating lipoprotein (Morrison, Betz & Jacobs, 1976). Thus, for studies on LPS-induced responses one needs extremely pure LPS. For this reason we made use of the highly purified *S. abortus equi* LPS prepared by Drs Galanos and Lüderitz. In several studies from our laboratory it has been shown that this preparation is unable to induce lymphoid responsiveness in C3H/HeJ and C57BL/10ScCr mice (Coutinho *et al.*, 1975; Coutinho & Meo, 1979). Using this LPS we could not show delayed accumulation of CFUs in blood and spleen of LPS-injected C3H/HeJ and C57BL/10ScCr mice (Table 3). Apparently, the LPS receptor is required for delayed mobilization of CFUs. At present it is impossible to answer the question of whether CFUs themselves have such a receptor.

The initial event causing delayed mobilization of CFUs probably takes place in the bone marrow, since previously splenectomized mice can also exhibit delayed CFUs mobilization (Vos *et al.*, 1972). In mice with spleens, the mobilized CFUs are on transit to the spleen (Monette *et al.*, 1972). There they may become involved in the expanding granulopoiesis in the spleen at the time of delayed CFUs mobilization (Apte *et al.*, 1976; 1977). This splenic granulopoiesis is probably due to the enhanced production of colony-stimulating factors (CSA) after LPS administration (Metcalf, 1974; Apte & Pluznik, 1976), although the role of CSA as an *in vivo* granulopoietin has not been definitely demonstrated. The CSA-induced proliferation and differentiation of some granulocyte and macrophage precursor cells (Bol, 1977; Metcalf, 1978) might account for a temporary positive feed back regulation of the number of splenic CFUs in cycle and upon the number of CFUs released from the bone marrow (Monette *et al.*, 1972). Our present studies are intended to establish such a possible causal relationship and to elucidate the factors (both humoral and microenvironmental) directly involved in the migration of stem cells from bone marrow to spleen.

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APPENDIX PAPER II

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Complement split product C5a mediates the lipopolysaccharide-induced mobilization of CFU-s and haemopoietic progenitor cells, but not the mobilization induced by proteolytic enzymes

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Abstract. Intravenous (i.v.) injection of mice with lipopolysaccharide (LPS), and the proteolytic enzymes trypsin and proteinase, mobilizes pluripotent haemopoietic stem cells (CFU-s) as well as granulocyte-macrophage progenitor cells (GM-CFU) and the early progenitors of the erythroid lineage (E-BFU) from the haemopoietic tissues into the peripheral blood. We investigated the involvement of the complement (C) system in this process. It appeared that the early mobilization induced by LPS and other activators of the alternative complement pathway, such as *Listeria monocytogenes* (*Lm*) and zymosan, but not that induced by the proteolytic enzymes, was absent in C5-deficient mice. The mobilization by C activators in these mice could be restored by injection of C5-sufficient serum, suggesting a critical role for C5.

The manner in which C5 was involved in the C activation-mediated stem cell mobilization was studied using a serum transfer system. C5-sufficient serum, activated *in vitro* by incubation with *Lm* and subsequently liberated from the bacteria, caused mobilization in both C5-sufficient and C5-deficient mice. C5-deficient serum was not able to do so. The resistance of the mobilizing principle to heat treatment (56°C, 30 min) strongly suggests that it is identical with the C5 split product C5a, or an *in vivo* derivative of C5a. This conclusion was reinforced by the observation that a single injection of purified rat C5a into C5-deficient mice also induced mobilization of CFU-s.

Haemopoietic stem cells (CFU's), granulocyte-macrophage progenitor cells (GM-CFU) and the early progenitor cells of erythrocytes (E-BFU) occur in the peripheral blood in extremely low numbers (Goodman & Hodgson, 1962; Hara & Ogawa, 1977; Rickard *et al.*, 1971). Their frequency in the blood can be increased by intravenous (i.v.) injection of certain substances which stimulate their mobilization out of the haemopoietic tissues into the blood

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stream. These substances include lipopolysaccharides (LPS), zymosan, proteolytic enzymes, polyanions and lectins (Vos, Buurman & Ploemacher, 1972; Quesenberry *et al.*, 1973; Ross *et al.*, 1976; van der Ham, Benner & Vos, 1977; Cline & Golde, 1977; Vos & Wilschut, 1979).

In mice, depending on the agent injected, peak CFU-s numbers are found 10 min to 6 hr after injection. LPS and zymosan, in addition, cause a 'delayed' accumulation of CFU-s and haemopoietic progenitor cells in the peripheral blood and spleen, reaching peak numbers about 5 days after injection of the agent (Vos *et al.*,1972; Staber & Johnson, 1980; Benner *et al.*, 1981; Molendijk, Ploemacher & Erkens-Versluis, 1982). Early CFU-s mobilization by LPS, zymosan and proteolytic enzymes, but not by polyanions, can be prevented by prior decomplementation with the complement (C) activating factor of cobra venom (CoF) (Wilschut *et al.*, 1979). LPS and zymosan are also incapable of early mobilization of CFU-s in complement C5-deficient mice (Benner *et al.*, 1981). The delayed CFU-s accumulation in blood and spleen by these agents, on the other hand, was not affected in C5-deficient mice.

The experiments described in this paper were designed to explore the role of the C system in the early mobilization of CFU-s and haemopoietic progenitor cells by LPS and proteolytic enzymes in more detail. The results suggest that C5a is the active principle in the LPS-induced mobilization of CFU-s and haemopoietic progenitor cells. C5a did not appear to be required for mobilization of the various types of colony-forming cells by proteolytic enzymes.

MATERIALS AND METHODS

Mice and rats

Male and female mice of the following strains were used: AKR (C5-deficient), DBA/2 (C5-deficient), (C3H/Law × DBA/2)F1 (C5-sufficient), B10.D2/oSn (C5-deficient), B10.D2/nSn (C5-sufficient) and (C57BL/Rij × CBA/Rij)F1 (C5-sufficient). The AKR, DBA/2 and (C3H/Law × DBA/2)F1 mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, B10.D2/oSn mice from Jackson Laboratories, Bar Harbor, Maine, U.S.A., B10.D2/nSn mice from Bantin and Kingman, Aldbrough, U.K., and (C57BL/Rij × CBA/Rij)F1 mice from the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands. For the spleen colony assay male mice were used. The age of all donor and recipient mice ranged between 12 and 24 weeks. Lewis rats, 8–12 weeks old, were purchased from the Central Institute for the Breeding of Laboratory Animals TNO, Zeist, The Netherlands.

Mobilizing agents

LPS from *Salmonella abortus equi* was kindly provided by Dr C. Galanos, Max-Planck Institut für Immunobiologie, Freiburg, F.R.G. The properties and source of the proteinase and trypsin used were described previously (Vos *et al.*, 1972; van der Ham *et al.*, 1977; Vos & Wilschut, 1979; Wilschut *et al.*, 1979). Mobilizing agents were dissolved in pyrogen-free phosphate buffered saline (PBS). They were injected i.v. in a volume of 0·5 ml PBS.

Cell suspensions

After killing the mice by exposure to carbon dioxide, blood samples were collected by cardiac puncture and immediately heparinized in plastic tubes containing preservative-free lithium heparin (Sigma, Detroit, Michigan, U.S.A.).

For cell culture, one volume of heparinized blood was mixed with one volume of 0·2% methylcellulose in α-median (α-modification of Dulbecco minimal essential medium). Red blood cells were allowed to sediment for 30 min at room temperature. The leucocyte-rich plasma was pipetted off and the cells were washed twice in α-medium containing 2% fetal calf serum (FCS).

Cell culture
Aliquots of 5 × 10^5 or 10^6 nucleated blood cells were cultured in 35 × 10 mm plastic tissue culture dishes at 37°C in a fully humidified incubator in an atmosphere of 5% CO_2 in air. The culture medium consisted of α-medium supplemented with 0·8% methylcellulose, 1% de-ionized bovine serum albumin (No A-9647; Sigma, St. Louis, Missouri, U.S.A.), 10% FCS, 10^{-4} M 2-mercaptoethanol (Merck-Schuchardt, Hohenbrunn, F.R.G.), 20% mouse spleen Con A conditioned medium and 2·0 U of sheep plasma erythropoietin (Connaught Laboratories Ltd., Willowdale, Ontario, Canada). Granulocyte/macrophage colonies, containing at least 50 cells, were counted on day 7 of culture, erythroid bursts on day 10. All cultures were done in triplicate.

Irradiation
Whole body irradiation was performed with a ^{137}Cs (Gammacell 40, Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1·29 Gy/min. (C57BL/Rij × CBA/Rij)F1 mice received 9 Gy, AKR and (C57BL/6J × DBA/2)F1 mice 8·5 Gy.

Spleen colony assay
The spleen colony assay of Till & McCulloch (1961) was performed as described previously (Molendijk *et al.*, 1982). The spleens were taken out 8 days after irradiation and i.v. injection of the blood samples.

Complement activation *in vitro*
For the *in vitro* activation of C, mouse or rat serum was used from non-heparinized blood that was allowed to clot for 90 min at room temperature. One ml of serum was mixed with 0·5 ml of saline containing 3·2 × 10^9 heat-inactivated (56°; 60 min) *L. monocytogenes* bacteria. This mixture was incubated for 30 min at 37°C. After incubation the bacteria were pelleted by centrifugation at 5000 r.p.m. for 10 min at 4°C. The remaining bacteria were removed from the supernatant by filtration through a 0·22 μm filter (Millipore, Bedford, Massachusetts, U.S.A.). Control serum from the same bleeding was mixed with saline only, but otherwise similarly treated.

Heat inactivation of complement
Mouse serum was heat-decomplemented by incubation in a waterbath for 30 min at 56°C.

Assay for alternative complement pathway activity
The alternative complement pathway activity of the serum samples activated by *L. monocytogenes* bacteria was determined as described previously (van Dijk, Rademaker & Willers, 1980; van Kessel *et al.*, 1981).

Purification of rat C5a
One hundred and seventy five ml of fresh rat plasma collected in 2 mM-EDTA, 1 unit/ml of trasylol, 2 mM benzamidine and 0·5 mM fluryl-methyl-sulphonyl fluoride (PMSF) was precipitated with a final concentration of 6% polyethyleneglycol-6000 during 60 min at 0°C. After centrifugation (15 min, 5000 *g*) the precipitate was dissolved in 35 ml Tris-HCl buffer (0·01 M Trizma base, containing the above mentioned inhibitors and adjusted to a PH of 7·8 with HCl) dialyzed overnight at 4°C against the same buffer, and applied on a 1·5 × 45 cm DEAE Sephacel column which was equilibrated in dialysis buffer. After collection of

50 fractions of 5 ml each, bound C5 was eluted using a linear salt gradient. The C5 haemolytic activity (Daha *et al.*, 1982), which was eluted from the column with a conductivity between 6 and 8 mS, was pooled, dialyzed against 0·01M acetate buffer, pH 6·3 and subjected to cation-exchange chromatography of a 1·5 × 20 cm Sulphopropyl-C50 column. C5 haemolytic activity, eluted from the column with a linear salt gradient and found between 3 and 5 mS, was concentrated to a volume of 5 ml and subjected to gelfiltration on a 2·5 × 90 cm Sephacryl S300 column. C5 haemolytic activity which filtered with an apparent molecular weight of 185,000 daltons was pooled and applied on a column of Sepharose 4B to which anti-rat-C3 and anti-rat-H were coupled (Daha *et al.*, 1979; Daha & van Es, 1982) to remove minor contaminants of rat-C3 and rat-H from the C5 preparation. The final C5 preparation was homogeneous on SDS-PAGE analysis.

To obtain C5a, 3 mg purified C5 an isotonic Veronal buffered saline was reacted during 60 min at 37°C with 1 × 10^9 intermediates bearing the classical pathway C5 convertase EAC1423 (Daha, Hazevoet & Van Es, 1983). The intermediates were then removed by centrifugation and the small molecular weight C5a was obtained by gelfiltration on Sephadex-G75. The final material was dialysed against distilled H_2O, freeze-dried and finally resuspended in 2 ml pyrogen-free 0·15 M NaCl.

RESULTS

Mobilization of CFU-s, GM-CFU and E-BFU in C5-deficient mice by LPS

Complement C5-deficient mice, e.g. DBA/2, B10.D2/oSn and AKR (Cinader, Dubiski & Wardlaw, 1964; Rosen, 1975), are incapable of CFU-s mobilization upon i.v. injection of LPS (Benner *et al.*, 1981). We investigated whether the mobilization of GM-CFU and E-BFU is similarly defective in C5-deficient mice. Therefore, C5-deficient DBA/2, B10.D2/oSn and AKR mice and genetically related, C5-sufficient (C3H × DBA/2)F1 mice and B10.D2/nSn mice were injected with LPS or PBS. In the control mice, injection of LPS led to the mobilization of CFU-s as well as GM-CFU and E-BFU (Table 1). The increase in GM-CFU and E-BFU numbers was proportionally smaller than that of CFU-s. In the C5-deficient mice, mobilization of all three cell types was weak or absent (Table 1). The data from the C5-sufficient and C5-deficient mouse strains differ significantly ($P < 0{\cdot}0001$).

Mobilization of CFU-s in C5-deficient mice by proteolytic enzymes

Injection of C5-deficient mice with the proteolytic enzymes trypsin and proteinase did induce mobilization of CFU-s into the peripheral blood. Both enzymes were found to cause a dose dependent increase of blood CFU-s numbers in normal as well as C5-deficient mice (Table 2).

Mobilization of CFU-s in C5-deficient mice injected with C5-sufficient serum

Injection of C5-deficient DBA/2 mice with 1 ml serum from C5-sufficient (C57BL × CBA)F1 mice enabled these DBA/2 mice to respond to injection of LPS with CFU-s mobilization. DBA/2 mice, injected with 300 μg LPS i.v. 1 hr after infusion of the C5-sufficient serum, had 306 ± 100 CFU-s/ml blood at 30 min after LPS injection, whereas control DBA/2 mice injected with the same amount of LPS had 64 ± 19 CFU-s/ml blood.

CFU-s mobilization by serum-containing activated complement

The fact that LPS is an activator of the alternative C pathway, and the observation that LPS-induced CFU-s mobilization is absent in decomplemented and C5-deficient mice, suggest that activation of C5 via the alternative C pathway may be important for the LPS-induced

Table 1. Mobilization of CFU-s, GM-CFU and E-BFU by LPS in the C5-deficient mouse strains DBA/2, B10.D2/oSn and AKR

Mouse strain	C5†	CFU-s/ml blood*		GM-CFU/ml blood		E-BFU/ml blood	
		PBS	LPS	PBS	LPS	PBS	LPS
DBA/2	−	42 ± 4‡	72 ± 4	43 ± 5	80 ± 6	20 ± 2	19 ± 4
(C3H × DBA/2)F1	+	16 ± 3	730 ± 61	86 ± 9	664 ± 39	104 ± 13	284 ± 15
		21 ± 6	1000 ± 88	50 ± 3	532 ± 28	13 ± 2	93 ± 7
B10.D2/oSn	−	44 ± 5	72 ± 10	78 ± 9	42 ± 13	14 ± 6	14 ± 3
		40 ± 7	92 ± 5	23 ± 2	18 ± 2		
B10.D2/nSn	+	48 ± 5	760 ± 92	75 ± 9	259 ± 7	24 ± 5	61 ± 6
		51 ± 5	594 ± 45	110 ± 19	779 ± 29		
AKR	−	52 ± 7	21 ± 7	14 ± 5	17 ± 3	9 ± 4	8 ± 2
		29 ± 4	80 ± 4	18 ± 8	12 ± 5	16 ± 6	20 ± 4

* Blood was taken 30 min after i.v. injection of 300 μg LPS or 0·5 ml PBS.
† C5-sufficient mouse strains are indicated by +, C5-deficient mouse strains by −.
‡ Arithmetic mean ± SEM.

Table 2. CFU-s mobilization by trypsin and proteinase in the C5-deficient mouse strains DBA/2, B10.D2/oSn and AKR

Mouse strain	C5†	CFU-s/ml blood*				
		PBS	0·4 mg trypsin	2·0 mg trypsin	0·1 mg proteinase	0·5 mg proteinase
DBA/2	−	46 ± 8	119 ± 9	367 ± 97	150 ± 44	640 ± 65
		52 ± 11	71 ± 9	100 ± 7	319 ± 20	594 ± 43
(C3H × DBA/2)F1	+	45 ± 7	89 ± 48	240 ± 112	209 ± 38	570 ± 65
		16 ± 3	45 ± 12	160 ± 32	404 ± 15	547 ± 58
B10.D2/oSn	−	37 ± 9	30 ± 8	143 ± 52	81 ± 8	381 ± 37
		40 ± 7	91 ± 11	160 ± 21	216 ± 18	405 ± 22
B10.D2/nSn	+	53 ± 6	81 ± 11	90 ± 29	186 ± 9	440 ± 40
		48 ± 5	97 ± 6	180 ± 16	187 ± 13	400 ± 32
AKR	−	52 ± 7	61 ± 6	106 ± 14	150 ± 22	586 ± 74
		29 ± 4	142 ± 8	180 ± 16	300 ± 18	686 ± 78

* Blood was taken 30 min after i.v. injection of trypsin, proteinase or PBS.
† C5-sufficient mouse strains are indicated by +, C5-deficient mouse strains by −.
‡ Arithmetic mean ± SEM.

CFU-s mobilization. To investigate this putative mediatory role of C, normal mouse serum (NMS) was incubated *in vitro* with *Listeria monocytogenes* (*Lm*) which has also been shown to activate C via the alternative pathway (Van Kessel *et al.*, 1981). C activation was measured by determining the remaining alternative pathway C activity (van Dijk *et al.*, 1980). Figure 1 shows that *in vitro* incubation of (C57BL × CBA)F1 serum with *Lm* led to a complete C consumption as determined in the haemolytic assay. Neither before nor after incubation with *Lm* did the serum of C5-deficient mice show haemolytic activity.

Injection of mice with *Lm*-treated serum from C5-sufficient mice was found to cause a 4- to 7-fold increase in the blood CFU-s numbers within 30 min (Table 3). Serum which had been heat-inactivated (30 min 56°C) to destroy the C enzymes and subsequently incubated

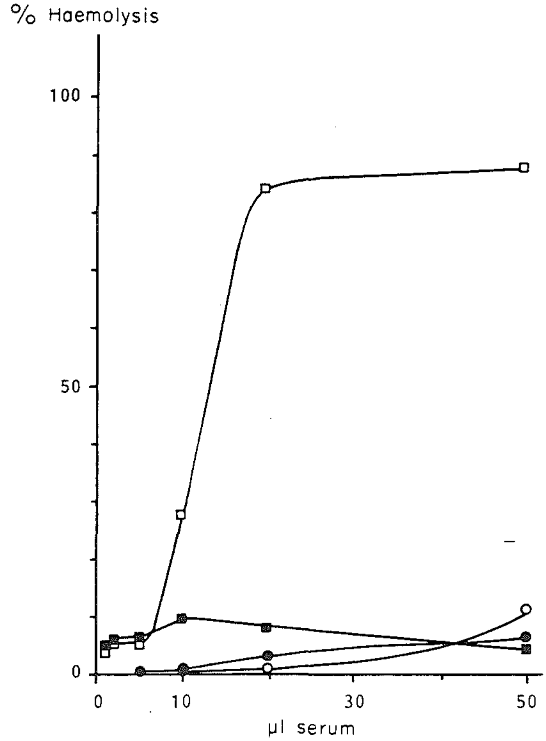

Fig. 1. Haemolytic complement activity of normal, (C57BL × CBA)F1 (□) and DBA/2 (○) serum, and Lm-activated (C57BL × CBA)F1 (■) and DBA/2 (●) serum.

Table 3. CFU-s mobilization by *Listeria*-treated normal mouse serum

Serum donor	C5†	Recipient	C5†	CFU-s/ml blood* NMS‡	*Lm*-NMS	INACT-*Lm*-NMS
(C57BL × CBA)F1	+	(C57BL × CBA)F1	+	87 ± 5§	326 ± 13	85 ± 9
				26 ± 7	304 ± 22	46 ± 6
DBA/2	–	(C57BL × CBA)F1	+	73 ± 8	92 ± 7	92 ± 4
				59 ± 4	61 ± 6	
AKR	–	(C57BL × CBA)F1	+	55 ± 7	56 ± 6	79 ± 6
				78 ± 6	90 ± 5	160 ± 6
(C57BL × CBA)F1	+	DBA/2	–	149 ± 10	669 ± 22	128 ± 4
				105 ± 11	695 ± 48	118 ± 9

* Blood was taken 30 min after i.v. injection of 0·75 ml serum.

† C5-sufficient mouse strains are indicated by +, C5-deficient mouse strains by –.

‡ NMS means normal mouse serum; *Lm*-NMS means NMS incubated with *Listeria* bacteria; INACT-*Lm*-NMS means NMS from which the complement enzymes were inactivated by treatment for 30 min at 56°C and was subsequently incubated with *Listeria* bacteria.

§ Arithmetic mean ± SEM.

with *Lm* had no significant activity. *Lm*-treated serum from C5-deficient DBA/2 and AKR mice could not cause an increase in the blood CFU-s numbers.

Since the observed CFU-s mobilization in C5-sufficient mice injected with *Lm*-treated NMS might be due to activation of the recipients C system, we also injected C5-deficient DBA-2 mice with *Lm*-treated C5-sufficient (C57BL × CBA)F1 serum. Also under these conditions, *Lm*-treated serum caused CFU-s mobilization (Table 3). The somewhat increased numbers of CFU-s in the peripheral blood of the NMS-infused control mice are at the upper limit of the normal range found in untreated control mice. As to the normal variation in background circulating CFU-s numbers, in each experiment a control group was included from the same supplier and delivery.

Not only *Lm*-activated NMS mobilized CFU-s, but also *Lm*-activated normal rat serum was able to do so. This is shown in Table 4 for Lewis rat serum infused in C5-deficient DBA/2 mice.

C5a, in contrast to most other C components, including native C5, is resistent to heat-inactivation for 30 min at 56°C (Hugli & Müller-Eberhard, 1978). We made use of this property to determine whether C5a may be the intermediate in the LPS-induced mobilization of CFU-s. Indeed, NMS from C5-sufficient mice that had been incubated with *Lm*, and subsequently heat-inactivated, was still able to mobilize CFU-s. This was found in C5-sufficient (C57BL × CBA)F1 recipients as well as in C5-deficient DBA/2 recipients (Table 5).

Table 4. CFU-s mobilization by *Listeria*-treated rat serum

				CFU-s/ml blood*		
Serum donor	C5†	Recipient mice	C5†	PBS	NRaS‡	*Lm*-NRaS
Lewis rat	+	DBA/2	−	27 ± 6§	25 ± 6	270 ± 48

* Blood samples were taken 30 min after i.v. injection of 0·75 ml serum.
† Lewis rats are C5-sufficient, DBA/2 mice are C5-deficient.
‡ NRaS means normal rat serum; *Lm*-NRaS means NRaS that has been incubated with *Listeria* bacteria.
§ Arithmetic mean ± SEM.

Table 5. CFU-s mobilization by heat inactivated *Listeria*-treated normal mouse serum

				CFU-s/Ml blood*		
Serum donor	C5†	Recipient	C5†	NMS‡	*Lm*-NMS	*Lm*-NMS-INACT
(C57BL × CBA)F1	+	(C57BL × CBA)F1	+	86 ± 7§	495 ± 21	333 ± 17
				124 ± 8	686 ± 28	630 ± 20
(C57BL × CBA)F1	+	DBA/2	−	24 ± 7	146 ± 18	172 ± 35
				23 ± 5	197 ± 38	172 ± 38
				21 ± 14	368 ± 38	225 ± 74

* Blood samples were taken 30 min after i.v. injection of 0·75 ml serum.
† C5-sufficient mouse strains are indicated by +, C5-deficient mouse strains by −.
‡ NMS means normal mouse serum; *Lm*-NMS means NMS incubated with *Listeria* bacteria; *Lm*-NMS-INACT means NMS that was incubated with *Listeria* bacteria and was subsequently treated for 30 min at 56°C to inactivate the complement enzymes, except C5a.
§ Arithmetic mean ± SEM.

CFU-s mobilization by purified rat C5a

The possible mediatory role of C5a in murine CFU-s mobilization was further analysed by infusing C5-deficient DBA/2 mice with purified rat C5a. Rat C5a was used because no isolation procedures are available for mouse C5a, as yet. It was found that purified rat C5a caused a dose-dependent accumulation of CFU-s in the peripheral blood (data not shown). After i.v. injection of 25 μg purified rat C5a, maximum numbers of circulating CFU-s were found after 15 to 30 min (Table 6). The peak numbers of CFU-s induced by purified rat C5a were of the same order of magnitude as those caused by injection of C5-sufficient mice with LPS or by injection of *Lm*-activated NMS or *Lm*-activated normal rat serum (c.f. Tables 1, 3 and 4).

Table 6. CFU-s mobilization in C5-deficient DBA/2 mice injected with purified rat C5a

	Min after injection of purified rat C5a*				
PBS	5	15	30	60	120
61 ± 11†	160 ± 32	323 ± 60	240 ± 56	218 ± 53	95 ± 15
26 ± 4	68 ± 17	222 ± 39	399 ± 113	230 ± 38	52 ± 8
P value‡	*P* = 0·030	*P* = 0·0024	*P* = 0·014	*P* = 0·011	*P* = 0·035

* Blood samples were taken at the indicated intervals after i.v. injection of 25 μg purified rat C5a.

† Figures represent the arithmetic mean ±1 SEM of the number of CFU-s per ml blood.

‡ *P*-values (calculated by the two-sided Student *t*-test) refer to comparison of the figures found at the various intervals after injection of rat C5a and the figures obtained after injection of PBS.

DISCUSSION

A variety of studies show that i.v. injection of mice with bacterial LPS induces the mobilization of CFU-s from the haemopoietic tissues into the peripheral blood within minutes of injection (Vos *et al.*, 1972; Vos & Wilschut, 1979). This early mobilization is not restricted to CFU-s since more mature cell types are also mobilized, although not all to the same extent (Ploemacher *et al.*, 1980). The present studies extend these observations by showing that GM-CFU and E-BFU are also rapidly mobilized by LPS. It can thus be concluded that the early LPS-induced mobilization of haemopoietic cells neither has cell-type specificity nor is restricted to particular differentiation stages.

In a previous paper we have concluded that the C system is involved in the early LPS-induced mobilization of CFU-s, since LPS-induced CFU-s mobilization is deficient in mice that have been pre-treated with CoF (Wilschut *et al.*, 1979) and also in C5-deficient mice (Benner *et al.*, 1981). In the present paper it appears that this role of the C system is not restricted to the mobilization of CFU-s. The mobilization of GM-CFU, E-BFU (Table 1) and mature cell types (data not shown) is also highly defective in C5-deficient mice. This suggests that C is involved in the LPS-induced mobilization of all types of haemopoietic cells.

In the LPS-non-responder mouse strains C3H/HeJ and C57BL/10.ScCr, which lack a serologically identifiable structure ('LPS-receptor') that is present in all LPS-responder strains (Forni & Coutinho, 1978), the late but not the early mobilization of CFU-s upon LPS injection is absent (Benner *et al.*, 1981). Together with the above data concerning the role of C, this observation suggests that not the LPS-receptor, but one or more C components may be the active principle(s) which account for the rapid mobilization of haemopoietic cells after LPS injection.

In the present paper we show that *in vitro* activation of C5-sufficient NMS and normal rat serum by *Lm* leads to the formation of product(s) that can induce CFU-s mobilization in C5-deficient mice (Table 3 and 4). Such *Lm*-activated serum is still active after heat-inactivation for 30 min at 56°C (Table 3). As C5a and its nonspecific helper factor, in contrast to most other C components, are resistant to heat inactivation (Hugli & Müller-Eberhard, 1978; Perez & Goldstein, 1981), this observation suggests that C5a or an *in vivo* derivative of C5a is the active principle in the LPS-induced early mobilization of CFU-s and haemopoietic progenitor cells. This might be related to the chemotactic activity of C5a (Snyderman *et al.*, 1969; Fernandez *et al.*, 1978), supposing that CFU-s and haemopoietic progenitor cells have a binding site for this fragment.

Recent data suggest that spleen colonies measured at 7–8 days after i.v. injection of CFU-s and colonies measured on days 12–13 are derived from (partly) different subpopulations of CFU-s (Bertoncello, Hodgson & Bradley, 1985). As we determined the spleen colonies on day 8 only, the data presented refer to this particular subpopulation only. It would be of interest to investigate whether CFU-s that give rise to days 12–13 colonies behave similarly.

The mechanism of translation of such a chemotactic stimulus is not entirely known. When the active substance reaches the surface of the cell, an esterase is activated and the hexosemonophosphate shunt is stimulated. Subsequently, calcium enters the cytoplasm and the cytoplasmic contractile proteins assemble (Loor, 1980). Thus, both the energy and the means required for the movement of the cell are provided.

Alternatively, the haemopoietic cells might be sent passively into the circulation, being brought about by other cells which are known to be susceptible to the chemotactic activity of C5a, such as neutrophils and macrophages (Snyderman *et al.*, 1969; Snyderman, Shin & Hauseman, 1971). Furthermore, it cannot be excluded that the mobilization by C5a is mediated by its property to induce contraction of smooth muscle, or the fact that it is an anaphylatoxin inducing the release of histamine from mast cells and basophils (Hugli & Müller-Eberhard, 1978), which may lead to increased vascular permeability.

It is remarkable that the mobilization of CFU-s and haemopoietic progenitor cells by trypsin and proteinase is not decreased in C5-deficient mice, since previous studies have shown that decomplementation of mice by high doses of CoF does inhibit CFU-s mobilization by these enzymes (Wilschut *et al.*, 1979). Proteolytic enzymes, however, have been shown to activate C3 directly (Bokisch, Müller-Eberhard & Cocchrane, 1969; Molenaar *et al.*, 1974), so that the effects of these enzymes on CFU-s mobilization in C5-deficient mice are most likely to be explained by C3-activation and, consequently, release of C3a, which is also an anaphylatoxin, but which has only limited chemotactic activity (Snyderman *et al.*, 1969; Hugli & Müller-Eberhard, 1978). The inability of CoF-treated mice to respond to trypsin and proteinase is probably due to exhaustion of C3 (as well as later components of the C cascade), so that CoF-treated mice cannot generate C3a and C5a, and thus cannot mobilize CFU-s.

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APPENDIX PAPER III

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Mediatory Role of Stem Cell Derived Cells in LPS-Induced Splenic CFU_S Accumulation

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Injection of bacterial lipopolysaccharides (LPS) in LPS-responsive mice produces a transient increase of CFU_S in spleen and blood but not in bone marrow. The cellular aspects of the mechanism underlying this response of the hemopoietic system to LPS were investigated. Bone marrow cells from LPS-high responder mice (BMC-H) of the C3Heb/FeJ and C57BL/10/ScSn strains were transferred to lethally irradiated histocompatible LPS-low responder C3H/HeJ and C57BL/10/ScCr mice and vice versa. Six to ten weeks after reconstitution recipient mice were tested with LPS. Six days after injection of LPS, CFU_S numbers in blood and spleen of low-responders reconstituted with BMC-H showed a 10–17 fold increase compared with PBS-injected controls. Lethally irradiated LPS high-responders reconstituted with low-responder bone marrow cells (BMC-L) still produced a small but significant increase of splenic and blood CFU_S numbers. These results suggest that relatively radioresistant stem cell-derived cells play an important role in the generation of a stimulus inducing the splenic CFU_S accumulation following LPS injection. The decrease of femoral CFU_S numbers was less prominent in mice reconstituted with BMC-L than in those reconstituted with BMC-H. Thus expression of the LPS locus is evident in both medullary and extra-medullary sites.

Key words: lipopolysaccharide – CFU_S – spleen

Injection of bacterial lipopolysaccharides (LPS) produces a profound disturbance of the distribution of hemopoietic stem cells in the body. Within minutes stem cells are mobilized out of the hemopoietic tissues into the bloodstream (1). Four to seven days later there is a second rise of blood CFU_S accompanied by a large accumulation of CFU_S and progenitor cells in the spleen (1,2). Similar changes in splenic hemopoiesis have been described after injecting other bacterial or yeast wall products such as lipoprotein, zymosan or killed bacteria (1,3–8). In recent papers (2,9) it has been described that the stimulation of splenic hemopoiesis by LPS is mediated by a humoral factor which has been named "splenic hemopoiesis stimulating factor (SHSF)."

Previously (10,11) we showed that LPS does not evoke the early rise of blood CFU_S in mice with deficient titers of complement components C3 to C9 or specifically C5. Such a deficiency did not influence the delayed accumulation of CFU_S in blood and spleen. These late effects, but not the early CFU_S mobilization appeared to depend on the LPS responsiveness of the mice being almost absent in LPS low-responder C3H/HeJ and C57BL/10/ScCr strains (11–14). The precise mechanism of LPS unresponsiveness has not yet been resolved. It may involve deficient binding of LPS to receptors or defective triggering after binding (15–17). CBA/N mice have a different defect in LPS responsiveness, the number of LPS reactive B cells is greatly reduced (18). These low-responder mice in combination with histocompatible high responders provide an excellent tool for investigations into the effects of LPS.

To investigate the origin of cells involved in the CFU_S accumulation in spleen and blood we determined CFU_S numbers in blood, spleen and bone marrow following LPS injection into low-responder mice reconstituted with bone marrow from high-responder mice and vice versa.

MATERIALS AND METHODS

Mice. Female C57BL/10/ScSn mice were purchased from TNO, Zeist, The Netherlands, and female C57BL/10/ScCr from Bomholtgård Ltd., Ry, Denmark. Male C3Heb/Fej and C3H/HeJ mice were purchased from Jackson Laboratories, Bar Harbor, Maine, USA. The C3H/HeJ mice were subsequently bred in the animal breeding department of the Erasmus University, Rotterdam, The Netherlands. Male CBA/N and CBA/H-T6 mice were obtained from the Radiobiological Institute TNO, Rijswijk, The Netherlands.

Endotoxin. Westphal-extracted *Salmonella typhosa* 0901 lipopolysaccharide (Difco, Detroit, MI, USA) was diluted in phosphate buffered saline (PBS) and administered iv in 0.5 ml solution.

Cell suspensions. Blood samples were collected by cardiac puncture after killing the mice with carbon dioxide. Blood was immediately heparinized in lithium heparin tubes (Sarstedt, Numbrecht-Rommelsdorf, F.R.G.). Bone marrow cells were collected from femurs and dispersed into a single cell suspension in cold BSS solution according to Mishell and Dutton (29). Spleens were minced with scissors and squeezed through nylon gauze.

Irradiation. Total body X-irradiation was performed with a Philips Mueller 300 X-ray machine operated at 300 kV and 10 mA. C3H/HeJ and C3H/eb/Fej mice received 900 rad, C57BL/10/ScSn and ScCr 800 rad. The doses used were sufficient to suppress endogenous spleen colony formation down to less than 0.1 colony/spleen.

Spleen colony assay. The spleen colony assay of Till and McCulloch (30) was used to measure the number of CFU_S. An appropriate number of cells was injected iv into 8–10 irradiated recipient mice per experimental group. On the 8th day following injection the spleens of the recipients were removed and placed into Telleyesnizky's fixative. The number of macroscopic colonies was counted with the naked eye.

RESULTS

LPS low-responder mice of C3H/HeJ and C57BL/10/ScCr strains were lethally irradiated and reconstituted with 5×10^6 histocompatible bone marrow cells of LPS high-responder mice (BMC-H) of C3Heb/FeJ and C57BL/10/ScSn strains and vice versa. Six to ten weeks after reconstitution recipient mice were injected with LPS. Six days after LPS administration recipient mice were killed and blood, spleen and bone marrow were assayed for CFU_S content. The results are summarized in Table 1. The BMC-H-reconstituted low-responder mice showed a large increase in spleen and blood CFU_S. The effects were of the same magnitude as observed in high-responder mice reconstituted

TABLE 1

Effects of bone marrow transplantation between LPS high and low responder strains on haemopoietic effects of LPS

		CFU_S/spleen		CFU_S/ml blood		CFU_S/femur	
Donor	Recipient	PBS	LPS	PBS	LPS	PBS	LPS
C3Heb/FeJ[a]	C3H/Hej[b]	3491 ± 1496	51,000 ± 12,981	54 ± 4	603 ± 64	7200 ± 1337	1756 ± 320
C57BL/10/ScSn[a]	C57BL/10/ScCr[b]	2800 ± 428	48,713 ± 10,309	45 ± 16	458 ± 153	8389 ± 1405	1286 ± 382
C3H/Hej	C3Heb/FeJ	3081 ± 412	18,621 ± 2194	31 ± 3	75 ± 13	5263 ± 691	2722 ± 537
C57BL/10/ScCr	C57BL/10/ScSn	1877 ± 398	22,007 ± 2584	37 ± 7	132 ± 36	7879 ± 1622	4371 ± 1630
C3Heb/Fej[c]	C3Heb/Fej[d]	2280 ± 449	79,200 ± 17,555	20 ± 6	787 ± 142	4400 ± 693	1566 ± 344
C57BL/10/ScSn[d]	C57BL/10/ScSn	2563 ± 186	38,381 ± 2952	34 ± 5	340 ± 40	7570 ± 429	2000 ± 286
C3H/Hej[d]	C3H/Hej	8700 ± 2300	9000 ± 2147	50 ± 27	72 ± 31	3733 ± 1728	1333 ± 653
C57BL/10/ScCr[d]	C57BL/10/ScCr	3430 ± 710	12,200 ± 1345	43 ± 5	82 ± 9	7382 ± 2016	3825 ± 896

All CFU_S determinations were done 6 days after injection of PBS or 300 μg *S. typhosa* (W) LPS.
Arithmetic mean ± standard deviation of three experiments. Each experiment consisted of two groups of 5 mice.
Lethally irradiated recipients were reconstituted with 5 × 10^6 bone marrow cells.
[a] LPS high-responder.
[b] LPS low-responder.
[c] In these experiments mice were injected with 200 μg LPS since higher doses were not tolerated.
[d] Arithmetic mean ± standard deviation of 2 experiments.

TABLE 2
Splenic CFU_S numbers in CBA/T6 and CBA/N mice after injection of LPS or PBS.

	PBS	LPS
CBA/T6	5247 ± 251	32.325 ± 2530
CBA/N	2900 ± 374	33.486 ± 7602

CFU_S numbers were determined 6 days after iv injection of 100 μg *S. typhosa* (W) LPS.
Arithmetic mean ± standard deviation of three experiments.
Each experiment consisted of two groups of 5 mice.

with BMC-H. Similar experiments were performed with LPS high-responder mice reconstituted with low responder bone marrow (BMC-L). As can be seen in Table 1 their LPS response in blood and spleen was diminished. However, the response was higher than that seen in low-responders reconstituted with BMC-L. Although LPS was able to induce a considerable decrease in the femoral CFU_S content of both high and low-responder mice this decrease tended to be smaller in recipients reconstituted with BMC-L. To study the role of B cells in LPS induced splenic CFU_S accumulation we also injected LPS in B cell defective CBA/N mice, CBA/H-T6 mice were used as controls. There was no significant difference in splenic CFU_S numbers after LPS injection between these strains (Table 2). The increase in CBA/N mice was even larger than in CBA/H-T6 mice.

DISCUSSION

Our experiments show that the responsiveness to LPS in low-responder mice with regard to the accumulation of CFU_S in the spleen and blood, can be largely reversed by transplantation of high-responder bone marrow cells. The reversal of responsiveness in high responders transplanted with low-responder bone marrow was less complete. The inability to completely reverse LPS responsiveness was noted by other investigators during similar transplantation experiments (19–21). Our results show that stimulation of splenic hemopoiesis by LPS is largely determined genetically by the LPS locus in hemopoietic cells. It has been described that LPS effects e.g., lethality, enhancement of CSA production and endogenous spleen colony formation have a hemopoietic cellular basis (19–22).

The precise mechanism of the splenic CFU_S accumulation after LPS is not known. It could be due to local proliferation and/or immigration of CFU_S from the bone marrow (23,24). It has been indicated that a humoral factor present in post-LPS serum (splenic hemopoiesis stimulating factor, SHSF) mediates the LPS-induced increase in splenic hemopoiesis (2,9). Our data favor the hypothesis that cells producing SHSF belong to the progeny of stem cells. The persistence of long-lived radioresistant cells may explain the relative failure of high responders to become low responders following BMT. This notion is supported by the observation that heavily irradiated mice were still able to release SHSF (2). Among the hemopoietic cells which meet the criteria of longevity and radioresistance, B or T cells have to be considered less likely candidates. This is indicated by the observation that nude mice which lack functional T cells show a normal hemopoietic response in the spleen after LPS (6). Our work with B cell defective CBA/N mice described in this paper shows that this deficiency has no effect on the LPS effects. At this moment macrophages seem to be the most likely candidates. They are radioresistant and can be stimulated by LPS to synthesize and release hemopoietic regulators like CSA and BPA (25–28).

In all transplantation combinations a decrease of femoral CFU_S numbers was observed. This decrease was less in animals with low-responder bone marrow than in those with high-responder marrow indicating that the bone marrow response to LPS, similar to that in the spleen is affected by the expression of the LPS locus.

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APPENDIX PAPER IV

Exp. Hematol. 12:794–799 (1984)

Experimental
Hematology

Effect of Serum from Mice Treated with Lipopolysaccharide on Cycling of CFU_s In Vitro

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Abstract. Serum of lipopolysaccharide(LPS)-treated LPS-high-responder C3H/He mice was shown to increase survival of low-responder C3H/HeJ CFU_s in an otherwise serum-free suspension culture by initiating cell cycling. Post-LPS serum of low-responder mice and serum of phosphate-buffered-saline-injected high-responder mice was significantly less effective in this respect. Since prolonged maintenance of CFU_s was also found when cell suspensions highly enriched for stem cells were used, it seems unlikely that assessory cells mediated the effect of the post-LPS serum activity on CFU_s maintenance. The serum activity did not enhance the stimulatory effect of saturating levels of highly purified stem-cell-activating factor (SAF) on CFU_s maintenance in vitro. Upon injection of post-LPS serum from C3H/He mice a relatively small splenic CFU_s accumulation in C3H/HeJ mice was observed.

Key words: CFU_s — Stem-cell-activating factor — Lipopolysaccharide

Intravenous (IV) or intraperitoneal (IP) injection of bacterial lipopolysaccharide (LPS) leads to a dose-dependent increase of the splenic content of hemopoietic stem cells and precursors of granulocytes and macrophages (CFU-GM), erythrocytes (BFU-E), megakaryocytes (CFU-Meg), and eosinophils (CFU-Eo) [1–3]. While the maximum increase in numbers of stem cells and progenitor cells is seen 4–7 days after LPS administration, there is an early (3–6 h) elevation of the level of serum colony-stimulating factor (G/M-CSF) [2, 4–6]. No apparent relationship has been found between LPS-induced serum CSF and the enhancement of splenic hemopoiesis [2]. Increased cycling of CFU_s 24 h after IP injection of LPS and abrogation of the splenic CFU_s accumulation by colcemid suggests that LPS stimulates CFU_s proliferation in the spleen [2, 7]. In vitro, LPS was found to have no direct effect on CFU_s or on hemopoietic progenitor cells [9, 10]. A hemopoietic activity in post-LPS serum that mimics the effect of LPS on splenic hemopoiesis has been described [8, 9]. This activity has been called "splenic hemopoiesis-stimulating factor" (SHSF) [8]. It has been postulated that LPS exerts its effect on splenic hemopoiesis by means of SHSF, and that SHSF has a selective action on spleen hemopoietic populations [9]. Presently, the mode of action of SHSF is unknown; however, it could involve induction of proliferation in splenic CFU_s, migration of CFU_s to the spleen, or both.

We studied the effect of post-LPS serum on the in vitro proliferative behaviour of CFU_s. This has been made possible by a serum-free culture system that allows maintenance and proliferation of CFU_s. It has been shown previously that prolonged maintenance of CFU_s in suspension culture is associated with proliferation of CFU_s [11–13]. The data presented here provide evidence that post-LPS serum contains an activity that initiates DNA synthesis in CFU_s.

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Materials and methods

Mice. Female C3H/He Ola mice were purchased from Olac Ltd. (Bicester, UK). C3H/HeJ mice were obtained originally from Jackson Laboratories (Bar Harbor, MN) and bred subsequently at the Laboratory Animals Department of the Erasmus University (Rotterdam, The Netherlands). BC3(C57BL/Rij × C3H/Law) mice were purchased from the Radiobiological Institute, Rijswijk, The Netherlands.

Lipopolysaccharide. Lipopolysaccharide (LPS) extracted from *Salmonella minnesota* R 595 (Lot RIR 328) was purchased from RIBI Immunochemical Research Inc. (Hamilton, MT). This purified LPS, which is highly enriched for lipid A, free of phospholipids and nucleic acids, and has a protein content of less than 0.3%, was dissolved in phosphate-buffered saline (PBS). Injections were made IP in an injection volume of 0.5 ml.

Cell suspensions. Bone marrow cells (BMC) were flushed from femurs and dispersed into a single-cell suspension in an ice-cold balanced salt solution (BSS).

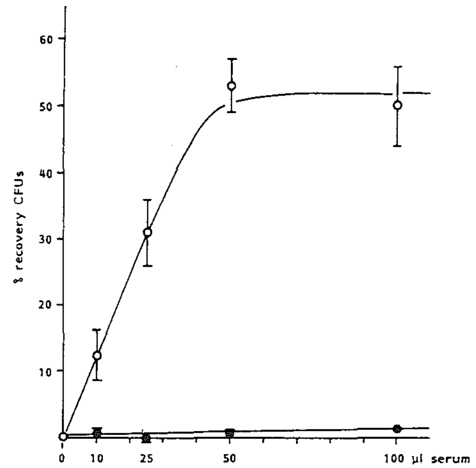

Fig. 1. O, dose effect of post-LPS serum and ●, of post-PBS serum from LPS high-responder C3H/He mice on CFU_s maintenance in vitro. BMC from LPS low-responder C3H/HeJ mice were cultured for four days. Data present the mean survival percentage of three experiments (±SD).

Post-LPS serum. To prepare post-LPS serum, mice were injected IP with 500 µg of LPS; 6 h later they were anesthesized by exposure to carbon dioxide, and blood was collected by cardiac puncture. Clots were allowed to form for 1 h at room temperature and to retract overnight at 4°C. Serum was removed after centrifugation and stored at −20°C until tested.

In vitro hydroxyurea kill. BMC from several cultures were pooled and washed in BSS. Subsequently they were incubated in BSS or 10 mM hydroxyurea (Calbiochem) in BSS for 2 h at 37°C in a shaking water bath. The incubation was stopped by adding ice-cold BSS, and the cells were washed again in BSS and diluted further for CFU_s determination.

Cell sorting. BC3 BMC were enriched for CFU_s by a multistage procedure [14]. In brief, BMC were separated by equilibrium density centrifugation and labeled simultaneously with wheat-germ agglutinin–fluorescein isothiocyanate (FITC) (WGA-FITC). Cells with a buoyant density of below 1.078 g/cm^{-3} were analysed by a light-activated cell sorter (FACS, Becton Dickinson, Sunnyvale, CA) to separate out those with high WGA-FITC fluorescence, low perpendicular light scatter, and medium forward light scatter. The sorted cells were incubated with *N*-acetyl-D-glucosamine to remove WGA–FITC, and the cells were labeled subsequently with anti-H2K-biotin and avidin–FITC, followed by a second sorting by the FACS for the cells with highest H2K density.

In vitro BMC cultures. C3H/HeJ BMC were washed in BSS and resuspended in α medium. Aliquots of 10^5–10^6 nucleated BMC were cultured in a volume of 1 ml in loosely capped round-bottom plastic tubes (Falcon no. 2057) for four days in a fully humidified incubator in an atmosphere of 5% CO_2 in air at 37°C.

Cells counts were performed with a Coulter counter. The culture medium consisted of a α medium supplemented with 10^{-4} *M* 2-mercaptoethanol (Merck), 0.25% (wt/vol) delipidated deionized bovine serum albumin (Sigma), 10^{-6} *M* hydrocortisone-hemisuccinate (Sigma), 4 × 10^{-6} *M* Fe-saturated human transferrin (Behringwerke), 10^{-7} *M* Na_2SeO_3 (Koch-Light), 10^{-6} *M* isoproterenol (Sigma), 1.5 × 10^{-5} *M* linoleic acid (Merck), 1.5 × 10^{-5} *M* cholesterol (Calbiochem), and 10^{-3} g liter^{-1} of a mixture of nucleosides (adenosine, 2-deoxyadenosine, guanosine, 2-deoxyguanosine, cytosine, 2-deoxycytosine, thymidine, and uridine) (Sigma). Cultures were terminated by adding ice-cold BSS. All cultures were done in duplicate.

Table 1. Effect of different sera on the in vitro maintenance of CFU_s

Serum added (50 µl/ml)	% Recovery of CFU_s
Post-LPS C3H/He	59.5 (34–80)[a]
Post-LPS C3H/HeJ	5.5 (0–10)
Post-PBS C3H/He	4.7 (0–9)

[a] Mean (and range) of CFU_s recovery at day 4 of culture; 4–5 batches of each type of serum were tested. Bone marrow from C3H/HeJ mice was used as a source of CFU_s target cells. Each serum batch was derived from at least 12 mice.

Stem-cell-activating factor (SAF). SAF was purified from Con-A-stimulated mouse spleen-cell-conditioned medium (MSCM) [2] by affinity chromatography, gel filtration, and ion exchange chromatography using diethylaminoethyl (DEAE)–sepharose at pH 8.0 (a gift from Dr. G. Wagemaker, Radiobiological Institute TNO, Rijswijk, The Netherlands). This purification procedure increased the specific activity about 82,000-fold, and the preparation contained approximately 40% of the SAF activity of the original MSCM. SAF is a single glycoprotein with a molecular mass of 19,000–20,000 daltons and is effective at concentrations as low as 10^{-11}–10^{-10} *M*. In serum-free suspension cultures of mouse bone marrow cells, high concentrations of SAF result within three days in a doubling of the stem cell population as detected by the macroscopic spleen colony (day-8 CFU_s) assay, while a maintenance of day-10 CFU_s is observed.

Irradiation. Whole body irradiation was performed with a ^{137}Cs gamma source (Gammacell 40 Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.27 Gy/min. C3H/HeJ mice received 8.75 Gy and BC3 mice 9.25 Gy.

CFU_s assay. Spleen CFU were assayed as described by Till and McCulloch [15]. An appropriate number of cells was injected IV into 5–10 lethally irradiated recipients. Colony counts were done on day 8.

Results

Effect of post-LPS serum on CFU_s maintenance in liquid cultures

To test whether post-LPS serum had an effect on the maintenance of CFU_s in bone marrow suspension cultures, various amounts of pooled post-LPS serum from C3H/He mice were added to cultures of C3H/HeJ BMC. Serum from animals injected

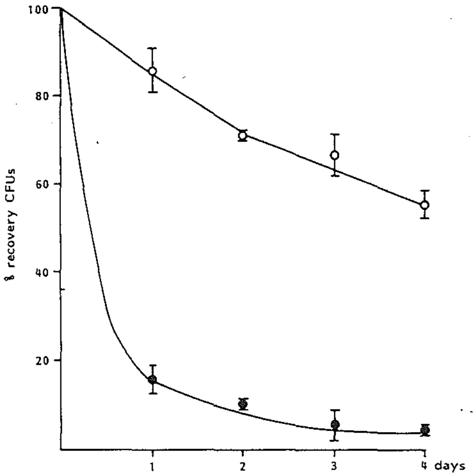

Fig. 2. Time course of CFU$_s$ decline in suspension cultures. Cultures containing 3 × 10^5 BMC were stimulated with ○, 50 μl of post-LPS serum or ●, 50 μl post-PBS serum from C3H/He mice. Data represent the mean ± SD of three experiments. Target cells were of C3H/HeJ origin.

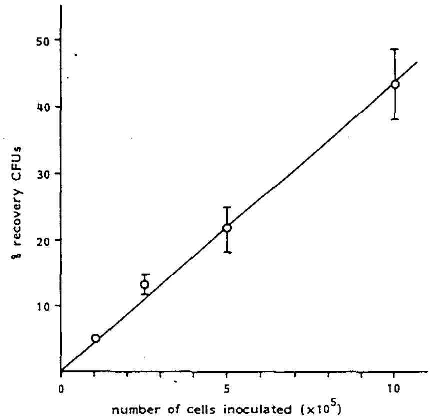

Fig. 3. Effect of bone marrow inoculum size on the day-4 CFU$_s$ maintenance in vitro. Cultures were stimulated with 50 μl post-LPS serum from C3H/He mice. Data present the mean ± SD of three experiments.

with PBS was added to control cultures. C3H/HeJ BMC were used as target cells because this mouse strain is resistant to the effects of LPS [16]. This procedure excluded any effects of possible residual LPS present in post-LPS serum. The results are depicted in Figure 1. Post-LPS serum caused a dose-dependent increase in CFU$_s$ maintenance with a plateau at doses of 50 μl or more. At these doses the recovery of CFU$_s$ was about 50%. No significant effect of post-PBS serum was seen over the range tested.

Subsequently, different batches of post-LPS serum from C3H/He and C3H/HeJ mice were tested. As can be seen in Table 1, there was significantly less effect on CFU$_s$ maintenance of post-LPS serum from LPS low-responder C3H/HeJ mice as compared with post-LPS serum from C3H/He mice. These data also show that the lack of effect of C3H/HeJ post-LPS serum in the maintenance assay is not due to unresponsiveness of C3H/HeJ CFU$_s$ to an activity that promotes maintenance. Rather, C3H/HeJ mice do not seem to produce significant amounts of this activity after LPS injection. Figure 2 illustrates the gradual decline of CFU$_s$ that was seen in post-LPS serum-stimulated cultures during the culture period. Cultures to which post-LPS serum or α medium (not shown) had been added showed a far more rapid decrease of CFU$_s$ with time.

Linearity of the CFU$_s$ maintenance assay was studied by culturing 10^5–10^6 BMC in optimally stimulated cultures (Fig. 3). Over the range of cell numbers tested, a good linearity was found (r^2 = 0.93). The linearity observed and the absence of a lag phase do not support the possibility that cells other than CFU$_s$ are involved in CFU$_s$ maintenance. To investigate this more thoroughly, cell suspensions highly enriched for stem cells by sorting on a FACS were cultured (Table 2). Maintenance observed in cultures containing these sorted BMC matched that of maintenance of CFU$_s$ from non-sorted BMC.

Hydroxyurea kill experiments

Maintenance of CFU$_s$ in suspension cultures could be due to a mere survival of CFU$_s$, or proliferation of CFU$_s$, or both. To investigate these possibilities, cells obtained from three-day-old cultures stimulated with either post-LPS or post-PBS serum were incubated with hydroxyurea, a drug that kills cells in S phase. The results of two experiments are depicted in Table 3. Incubation of cells from post-LPS serum-stimulated cultures with hydroxyurea resulted in a CFU$_s$ kill of 45% and 54%. This suggests that C3H/He post-LPS serum, when added to BMC cultures, initiates DNA synthesis in CFU$_s$.

Specificity of post-LPS serum-derived SAF

Increased maintenance of CFU$_s$ in post-LPS serum-stimulated cultures could be the result of either the

Table 2. Maintenance of FACS-sorted CFU_s in culture

	$CFU_s/10^5$ BMC			% Recovery of CFU_s[a]	
Exp. no.	Nonsorted	Sorted	Enrichment	Sorted	Nonsorted
1	41.8	4375	105×	43	39
2	41.8	4750	114×	49	53

[a] Cultures containing 2.5×10^3 sorted or 2.5×10^5 nonsorted BMC were stimulated with 50 μl of post-LPS serum from C3H/He mice. Target cells were from BC3 mice. All cultures were assayed on day 4. No CFU_s were recovered from cultures to which post-PBS serum was added.

presence of a nonspecific nutrient in the serum or an activity that has specificity for CFU_s. In an attempt to obtain information about the nature of the serum activity, post-LPS serum was added to cultures that were optimally stimulated with highly purified SAF from mouse spleen-conditioned medium. Table 4 shows that post-LPS serum did not significantly enhance CFU_s maintenance in the presence of saturating levels of purified SAF. The results suggest that the activity responsible for prolonged maintenance in post-LPS serum is not additive to the action of SAF but rather acts in a way similar to SAF. These experiments also indicate that CFU_s proliferation is possible in this culture system, resulting in a net increase of CFU_s.

In vivo activity of post-LPS serum

IP injection of 1 ml of post-PBS serum from LPS high-responder C3H/He mice did not induce a significant rise in the splenic CFU_s content of C3H/HeJ mice as measured on day 5 (Table 5). In contrast to post-LPS serum from LPS low-responder C3H/HeJ mice, injection of post-LPS serum from C3H/He mice evoked a doubling of the splenic CFU_s number in C3H/HeJ mice.

Discussion

The data presented in this paper show that addition of post-LPS serum from C3H/He mice to suspension cultures of C3H/HeJ BMC prolongs maintenance of CFU_s in such cultures over a four-day period. In contrast, serum from PBS-injected C3H/He mice was much less active in maintaining CFU_s. The prolonged maintenance of CFU_s was found to coincide with a probable increase in cycling of stem cells as detected by measuring their sensitivity to hydroxyurea. This coincidence was found previously by other investigators [11–13]. The results suggest that post-LPS serum contains an activity that initiates DNA synthesis in CFU_s in vitro. Substances having this activity have been previously termed "stem-cell-activating factor" (SAF) [11]. SAF can be found in media conditioned by human leucocytes, Con-A- or PHA-stimulated mouse spleen cells, and in extracts of hemopoietic tissue [19]. Post-LPS serum also contains G/M-CSF. Although G/M-CSF was found to stimulate the growth of multipotential hemopoietic cells in vitro, it was unable to support maintenance of CFU_s in suspension cultures [18–22]; therefore it seems unlikely that SAF is identical with G/M-CSF. Hemolysate, which is often a contaminant in post-LPS serum, has been reported to increase maintenance of CFU_s, but it did not enhance cycling of CFU_s like SAF did [12]. We observed that saturating levels of SAF and post-LPS serum were not complementary with respect to CFU_s maintenance in vitro. Although the MSCM-derived SAF was not purified to homogeneity and unfractionated serum was used, our observations allow the provisional conclusion that the present activity described in post-LPS serum represents SAF. At present, insufficient information is available to allow comment on the in vivo specificity of SAF for splenic and bone marrow CFU_s populations or to draw conclusions with respect to the site(s) of pro-

Table 3. Proliferative status of CFU_s cultured for three days

Exp. no.	Serum added[a]	Incubated with	Percent CFU_s surviving	% Kill
1	Post-PBS	BSS	100[b]	0
		Hydroxyurea	78	22
	Post-LPS	BSS	100	0
		Hydroxyurea	55	45
2	Post-PBS	BSS	100	0
		Hydroxyurea	95	5
	Post-LPS	BSS	100	0
		Hydroxyurea	46	54

[a] 50 μl of C3H/He serum was added to cultures containing 1×10^6 BMC from C3H/HeJ mice (equally 342 ± 35 CFU_s per culture).
[b] BSS incubation was equated to 100%.

Table 4. Maintenance of CFU_s cultured in the presence of SAF and post-LPS serum

Stimulus added	Percent CFU_s recovered[a]
Post-LPS serum[b]	62 ± 11
SAF[c]	195 ± 7
Post-LPS serum + SAF	217 ± 7

[a] Mean ± 1 SE of three experiments.
[b] Cultures were stimulated with 50 μl post-LPS serum or 10 μl of MSCM-derived SAF or both and assayed at day 4.
[c] Stem-cell-activating factor.

Table 5. Effect of post-LPS serum on splenic hemopoiesis in C3H/HeJ mice

Serum injected	CFU_s/spleen[a]
Post-LPS (C3H/He)	9492 ± 1842[b]
Post-PBS (C3H/He)	5464 ± 2037
Post-LPS (C3H/HeJ)	4065 ± 204
None	4010 ± 320

[a] CFU_s determinations were done five days after IP injection of 1 ml of post-LPS serum or post-PBS serum.
[b] Mean ± SD of three experiments.

duction and the cell type(s) involved. However, relatively radioresistant stem-cell-derived cells have been proposed to play an important role in the generation of a stimulus inducing the splenic CFU_s accumulation following LPS injection [23].

Post-LPS serum from LPS low-responder mice was found to be much less active in promoting in vitro CFU_s maintenance than post-LPS serum from high-responder mice. In contrast to this latter serum, it also did not evoke any splenic CFU_s accumulation in low-responder mice. These observations suggest that the defective increase in splenic CFU_s numbers following LPS injection in these mice [23–25] is due to defective SAF production. At the moment one can only speculate about the relation between SAF and SHSF. The above-mentioned observations suggest that SAF is at least partially responsible for the in vivo effect of post-LPS serum. Proof of this can only be obtained by purification of both activities. It may be of interest to note that normal SAF and SHSF levels exist in Sl/Sl^d and $Sl^j/+$ mice, which suffer from a macrocytic anemia caused by a defect in their hemopoietic stroma [10, 26].

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APPENDIX PAPER V

A SPECTRUM OF HEMOPOIETIC ACTIVITIES IN POST-ENDOTOXIN SERUM AND ITS RELATION TO THE EFFECTS OF ENDOTOXIN ON MURINE HEMOPOIESIS IN VIVO

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ABSTRACT

Injection of lipopolysaccharide from Gram-negative bacteria (LPS) in mice induces accumulation of CFU-s in the spleen, which has been previously shown to be partly mediated by a serum activity (splenic hemopoiesis stimulating factor, SHSF) present in post-LPS serum (PES). PES also induces in vitro cycling and proliferation of CFU-s from LPS low- and high-responder mice in an otherwise serum-free liquid culture assay, which detects stem cell activating factor (SAF).

Evidence is now presented that PES, but not LPS or M-CSF, is a potent stimulator of proliferation in Interleukin-3 (IL-3)-dependent DA-1 cells under specific conditions with respect to culture time and plating density. This activity in PES was suggested to be different from IL-3 since (a) antibodies to IL-3 could only slightly abrogate the PES-induced DA-1 cell proliferation, and (b) the sensitivity of DA-1 cells to IL-3 was remarkably decreased under conditions where PES led to optimal proliferation of DA-1 cells. In a regular DA-1 cell assay, both PES and GM-CSF induced a low level of cell proliferation, but PES contained a synergistic activity in the presence of recombinant IL-3. In serum-free bone marrow cell cultures, both PES, GM-CSF and purified Hemopoietin-1 led to survival of CFU-s, although less as compared to IL-3. In this assay, Hemopoietin-1 synergized significantly with GM-CSF, and less with IL-3 and PES. We conclude from these data that the in vivo effects of PES are likely to be due to non-IL-3 activities.

The in vitro and in vivo activities found in PES are suggested to reflect a functional response of the animals to

LPS, since they were absent in mice following serious perturbation of hemopoietic activity by radiation, induction of anemia or the injection of various mitogens or particulates. These serum activities were also not found in LPS low-responder mice.

INTRODUCTION

There is good evidence that the control of proliferation in the hemopoietic stem cell compartment occurs locally, as the kinetic properties of CFU-s can differ at different sites in the same animal (1,2). Yet, the detection of activities released by adherent bone marrow and spleen cells that can modulate the percentage of cycling CFU-s in vitro has been taken as evidence for the regulation of CFU-s proliferation by humoral factors (3-7). Interestingly, partial body irradiation studies (8) have supported the presence of such humoral factors, since CFU-s in the protected areas are triggered into cycle within 4 hours after irradiation. The stimulation of CFU-s growth in diffusion chambers carried by LPS-injected mice is in agreement with these observations (9).

Lipopolysaccharides from gram-negative bacteria (LPS) have been reported to increase the level of a serum factor called splenic hemopoiesis stimulating factor, SHSF (10,11) that mimics the effect of LPS on splenic hemopoiesis (12), including the large increase of CFU-s numbers (13). However, the unequivocal demonstration of a serum factor, that directly modulates the proliferative status of CFU-s, has hitherto been impossible due to a lack of purified stem cells, serum-free culture systems, and specific assays for stem cell activating factor SAF (14,15).

Recently we reported that serum from mice injected with lipopolysaccharides (PES) contains SAF since it stimulated cycling of CFU-s in vitro (16,17). The use of pure stem cell preparations in these experiments excluded the possibility that SAF was produced by accessory cells during the 4-day incubation period. SAF has been shown to be identical with Interleukin-3 (IL-3) (18), a glycoprotein that has the ability to stimulate in vitro the growth of many kinds of hemopoietic progenitor cells (19-22).

In the present study we have further investigated whether LPS induces a significant release of IL-3 in the systemic blood circulation. To this purpose we assayed the ability of PES to induce in vitro proliferation of IL-3-dependent DA-1 cells (23) and of CFU-S in vitro (16,24). These data were compared with the effects of IL-3, GM-CSF and Hemopoietin-1 in these assays, and with the ability of PES to stimulate a splenic CFU-s accumulation as a measure for serum SHSF concentrations. We also investigated the presence of

IL-3 and SHSF in sera from mice with perturbed hemopoiesis in order to assess a more general in vivo role for IL-3-like activities in the recruitment of CFU-s into DNA synthesis.

MATERIALS AND METHODS

Mice

Female C3H/He and C3Heb/FeJ mice were purchased from Olac Ltd., Bicester, U.K. Male CBA/T6T6, CBA/N and (C57BL/Rij x CBA/Rij)F1, hereafter called BCBA, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. C3H/HeJ mice were originally obtained from Jackson Laboratories, Bar Harbor, Maine, USA and subsequently bred at the Laboratory Animals Centre of the Erasmus University Rotterdam, The Netherlands.

Materials

Westphal-extracted Salmonella typhosa 0901 lipopolysaccharide (LPS-W; Difco, Detroit, MI, USA) and highly purified (protein content less than 0.3%) Salmonella minnesota (R595) lipopolysaccharide (lot no. 328, RIBI Immunochemical Research Inc., Hamilton, Montana, USA) were dissolved in phosphate-buffered saline (PBS) and administered i.v. in a 0.5 ml solution. For perturbation of hemopoiesis the following substances were dissolved or suspended in PBS: latex beads (Serva, Heidelberg, Germany, 0.234μm diameter); polyvinylpyrolidone K30 (PvP; Fluka AG, Switzerland); concanavalin A (Con-A; Pharmacia, Uppsala, Sweden); pokeweed mitogen (PWM; Gibco) and phenylhydrazine-chloride (PHZ; Merck, Darmstadt, West Germany). M-CSF was purified from pregnant mouse uterus extract according to Merchav and Wagemaker (25). Recombinant murine GM-CSF (26) was produced in monkey COS-cells. Recombinant, purified IL-3 was a gift from Dr. G. Wagemaker (Rijswijk, The Netherlands). Hemopoietin-1, defined as a hemopoietic growth factor that promotes IL-3-dependent proliferation of CFU-s, was partially purified from Con-A/TPA-stimulated human leukocyte-conditioned medium by Amicon filtration (3-30 kDa).

Cell suspensions

Spleens were minced with scissors and squeezed through nylon gauze, femurs were flushed with PBS through a 21 gauge needle. Single cell suspensions were then prepared by repeated flushing using a blunt-tipped pipette.

Preparation of sera

Mice were killed by exposure to carbon dioxide and blood was collected by cardiac puncture. Clots were allowed to form for 1 hr at room temperature and to retract overnight at 4oC. Serum was removed after centrifugation and stored at -

20°C until tested. Storage of sera for 4 years under these conditions did not affect the serum activities as measured in the assays described. Before use in in vitro assays sera were sterilized by passage through a 0.22 µm Millipore filter.

SAF assay (24)

Bone marrow cells (BMC) were washed in PBS and resuspended in α-medium. Aliquots of 2.5-3 x 10^5 BMC were cultured in a volume of 1 ml in loosely capped round-bottommed plastic tubes (Falcon no. 2057) for 4 days in a fully humidified atmosphere of 5% CO_2 in air at 37°C. Cell counts were performed with a Coulter counter. The culture medium consisted of α-medium supplemented with 10^{-4}M 2-mercaptoethanol (Merck), 0.25% (w/v) delipidated deionized bovine serum albumin (Sigma), 10^{-6}M hydrocortisone-hemisuccinate (Sigma), 4 x 10^{-6}M Fe-saturated human transferrin (Behringwerke), 10^{-7}M Na_2SeO3 (Koch-Light), 1.5 x 10^{-5}M cholesterol (Calbiochem) and 10^{-3} g/L of a mixture of nucleosides (adenosine, 2-deoxy-adenosine, guanosine, 2-deoxy-guanosine, cytosine, 2-deoxy-cytosine, thymidine and uridine (Sigma). Cultures were set up in duplicate or triplicate. At day 4 the cultures were terminated by adding ice-cold PBS. The cells were assayed for their CFU-s content and CFU-s recovery was calculated.

DA-1 cell culture

DA-1 cells were obtained through Dr. G. Wagemaker (Rijswijk, The Netherlands) and originally provided by Dr. Ihle (23). DA-1 cells, which are considered to be exclusively dependent upon IL-3 for their survival and proliferation, were maintained in α-medium supplemented with 10% fetal calf serum (FCS) and 25% WEHI-3B conditioned medium as a source of IL-3. The WEHI-3B cell line (27) was maintained in α-medium supplemented with 10% FCS. Both the DA-1 and WEHI-3B cell lines were subcultured every 3-5 days by dilution in fresh medium at 10^5 cells/ml. For IL-3 assays, the cells were washed twice in α-medium and 10-20% FCS and either 10^5 cells were added to 1 ml cultures in wells of Costar 24-well plates, or 1-2 x 10^4 cells were cultured in 0.2 ml culture wells. Under these culture conditions no significant cell viability was detected after 4 days of culture using the trypan blue exclusion method. Test sera were added at the beginning of the culture period and DA-1 cell proliferation was determined by counting DA-1 cells after varying periods of culture as stated in the text.

Measurement of splenic hemopoiesis stimulatin factor (SHSF, 10)

Usually, 0.3-1.0 ml of PES or NMS was injected i.p. and spleens were assayed for their CFU-s content five days later.

Spleen colony assay
CFU-s were assayed as described by Till and McCulloch (28). Cells were injected i.v. into 10 lethally irradiated isologous recipient mice. Macroscopic colony counts were made at day 8-10.

Irradiation
A lethal dose of whole body γ-irradiation was given with a two-source ^{137}Cs Gamma cell 40 apparatus (Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.27-1.29 Gy per minute. BCBA mice received 9.4 Gy, CBA mice 9 Gy and C3H/HeJ mice 8.75 Gy. Furthermore, groups of BCBA mice received various doses of radiation to induce a varying hemopoietic insult.

CFU-s migration study
In order to study the possibility that migration of CFU-s from the bone marrow to the spleen contributed to the LPS-induced accumulation of splenic CFU-s, splenectomized CBA/T6T6 mice were joined in parabiosis with spleen-bearing CBA/N mice. Parabionts were joined laterally under Avertin (Merck-Suchardt) anesthesia (29). Mice were united by suturing the peritonea and abdominal wall muscles without leaving a communication between the abdominal cavities. The connective tissue along the thorax was also sutured and the scapulae were joined. Splenectomy was done during surgery for parabiosis. Eight days later, when vascular anastomoses have been shown to be sufficiently developed (29), both mice of each parabiosis pair were injected with either 100 µg of LPS (R595) or PBS. Six days later the CBA/N spleens were suspended and transferred to secondary lethally irradiated CNA/N mice. Again 6 days later individual colonies were dissected from their spleens and studied for the presence of T6T6 marker chromosomes.

RESULTS

Effects of PES on DA-1 cell proliferation
CBA mice were injected i.v. with 300 µg of LPS-W or PBS and their serum (PES) collected 6 hrs later. Using the DA-1 cell assay we compared the effect of PES stimulation to that of IL-3, M-CSF and GM-CSF. Increasing DA-1 cell amplification was observed over 4 days in the presence of increasing concentrations of IL-3 (Fig. 1) when cultures were set up with 10^4 DA-1 cells/0.2 ml culture medium. The PES-induced DA-1 cell proliferation was low, but significantly higher than background levels, where no surviving DA-1 cells were detected. GM-CSF affected DA-1 cell proliferation only at high concentrations (Fig. 5).

After 8 days, both IL-3 and PES induced a larger DA-1 cell amplification than at 4 days. Remarkably, different responses of DA-1 cells were observed when cultures were set up with 2 x 10^4 cells in wells containing 0.2 ml culture medium (Fig. 2) or 10^5 cells in 1 ml cultures. Not only was the background cell survival in part of the experiments 4-6 times the inoculum numbers, but the cells showed a decrease in their responsiveness to IL-3 when the cultures were maintained for 9 days. In contrast, DA-1 cells responded in these conditions well to minute concentrations of PES, whereas the presence of serum inhibitors was evident at doses exceeding 60 µl PES/ML culture medium. The observed activity of PES was not due to residual LPS, since increasing doses of LPS between 0.1 and 10 µg/ml culture medium did not induce any significant increase of cell proliferation (data not shown). Recombinant M-CSF or GM-CSF did not induce increased DA-1 cell proliferation above background levels under these conditions, i.e. 2 x 10^4 cells plated and cultured for 4 or 9 day (data not shown).

When the IL-3 dose response was determined over a culture period of 4 and 8 days in the presence of 50 µl of PES/ml culture medium (Fig. 3,and 4), a significantly increased DA-1 cell proliferation was observed, even at plateau IL-3 concentrations in the 4-day assay. GM-CSF did not have such a synergistic activity for IL-3-induced DA-1 cell proliferation. It should be noted that this was tested using an inoculum of 10^4 DA-1 cells, and that the effect of PES alone (Fig. 1) and GM-CSF (Fig. 5) was low. The synergism of PES for IL-3-induced DA-1 cell proliferation was also studied (Fig. 5) using a dose response for PES in the presence of a 50% response of IL-3 (20 µl/ml culture medium, see fig. 1). Virtually all PES concentrations used, especially those between 2 and 60 µl, increased the IL-3-induced cell proliferation over a 4-day culture period. Again, addition of increasing concentrations of GM-CSF was without effect. However, when tested in an 8-day assay (Fig. 6) it appeared that the benificial effect of PES on IL-3-induced DA-1 cell proliferation was restricted to a very limited concentration range of PES, i.e. between 50 and 100 µl.

In order to test whether the high responsiveness of DA-1 cells (inoculum of 2 x 10^4 cells) to PES in 9-day cultures was due to IL-3 alone, we assayed the DA-1 cell proliferation in the presence of either 0.4 µl of IL-3 or 50 µl of PES per ml culture medium, and increasing concentrations of a conventional rabbit-anti-mouse IL-3 antiserum. As is shown in Fig. 7 anti-IL-3 dramatically inhibited the effect of IL-3, although no full abrogation of DA-1 cell proliferation was observed. Anti-IL-3 only slightly affected the PES activity in this assay when the percentage reduction is compared with that of IL-3. These observations suggest, that the PES-induced DA-1 cell proliferation is mainly caused by a serum activity unlike IL-3. Alternatively, the antibody might have different affinities for recombinant IL-3 and native IL-3 in serum due to possible differences in e.g. glycosylation of the IL-3 molecule. For these reasons, we will further tentatively refer to this activity in PES as DA-1 cell-stimulating activity (DA-SA).

<u>Effects of PES on CFU-s proliferation in serum-free cultures</u>

We tested the activity of PES in relation to that of a series of other hemopoietic growth factors to stimulate CFU-s proliferation in vitro (Table 1). As reported earlier (16), 50 µl of PES/ml culture medium led to a significant CFU-s recovery over a 4-day culture period while no CFU-s were retrieved from these cultures in the absence of PES. Unexpectedly, both GM-CSF and Hemopoietin-1 (H-1) induced a similar CFU-s recovery as PES. H-1 had a synergistic effect on IL-3-induced CFU-s proliferation, and in relative terms it synergized with PES to a similar extend as it did with IL-3. The GM-CSF-induced CFU-s proliferation was greatly enhanced in the presence of H-1. Neither PES nor GM-CSF showed synergism with IL-3. Also, PES or GM-CSF could not further enhance the IL-3-induced CFU-s proliferation in the presence of H-1.

<u>Measurement of SHSF and DA-1 cell-stimulating activity (DA-SA) in PES</u>

Under conditions of an optimal DA-1 cell response to PES, i.e. 10^5 cells inoculated in 1 ml culture wells and a 10-day culture period, we investigated the appearance of DA-SA in sera of mice that had been injected with increasing amounts of LPS-W. DA-SA titers were compared with the SHSF content of the respective sera (Fig. 8). Following injection of only 0.3 µg of LPS-W already a significant concentration of DA-SA was measured in 3 and 6 hr PES. The PES collected at 3 or 6 hr after LPS-W injection was tested in separate experiments. Injection of increasing amounts of LPS-W up to 100 µg per mouse led to an increasing DA-SA concentration in both 3 hr and 6 hr PES. We also used the PES-induced increase in splenic CFU-s numbers as an assay for SHSF. From Fig. 8 it appears that DA-SA, but not SHSF, was detectable in sera from mice that had been injected with low doses of LPS-W. A steep increase of both DA-SA and splenic CFU-s numbers was observed at LPS-W doses exceeding 10-30 µg per mouse.

<u>Kinetics of serum SAF, SHSF and DA-SA following LPS-W injections</u>

Between 1 and 3 hrs following i.v. injection of 100 µg of LPS-W the serum DA-SA level sharply rose (Table 2). At 18 hrs maximum levels of DA-SA were detected when only 1 µl of PES was used as supplement to the DA-1 cell cultures. Seventy hours after LPS-W administration high DA-SA titers could still be detected in the serum. From Table 2 it also appears that the kinetics of SAF in sera from mice injected with 100 µg of LPS-W differed from the DA-SA kinetics in that SAF titers rapidly declined after 18 hrs following LPS-W injection. The SHSF levels following LPS-W administration followed similar kinetics as those of serum SAF.

LPS low-responder mice have a defective SHSF and SAF response, but are responsive to PES from LPS-responder mice

NMS from none of the 4 mouse strains used for these experiments did contain detectable SAF or SHSF activity (Table 3). In contrast to PES from all LPS responder mice used, PES from LPS low-responders showed a defective SAF and SHSF response. The use of C3H/HeJ as donors of bone marrow target cells in the SAF assay and as serum recipients in the SHSF assay demonstrated that residual LPS could not have interfered with the parameters used. Moreover, these data show that LPS low-responder CFU-s both in vitro (SAF assay) and in vivo (SHSF assay) are responsive to factors in PES from LPS responder mice.

Irradiation of mice does not lead to detectable levels of DA-SA and SHSF in serum

Since it has been suggested (4,8) that whole body irradiation of mice rapidly liberates humoral factors that stimulate DNA synthesis in CFU-s in vivo, we investigated whether in vivo γ-irradiation of mice would lead to similarly enhanced serum DA-SA and SHSF concentrations. It is apparent from Table 4 (upper part) that total body irradiation with doses between 0.12 and 10.9 Gy did not induce measurable activities of DA-SA and SHSF within 6 hrs following irradiation. Table 4 (lower part) also shows that neither a previous irradiation of mice nor splenectomy ablated the large increase of serum DA-SA and SHSF in these mice following LPS injection.

Serum DA-SA and SHSF concentrations in mice after various perturbations of steady state hemopoiesis

In order to further define a role for serum DA-SA and SHSF in the humoral control of hemopoiesis, BCBA mice were either made anemic by injection of phenylhydrazine (PHZ), or received various agents, which have been implied in the literature to evoke, by relatively non-destructive means, increased hemopoiesis (30-32).

Table 5 shows that none of the agents used induced a serum DA-SA concentration comparable to that observed after LPS-W administration. The 6 hr sera of mice that received PVP or latex showed a small, but significant rise in serum DA-SA. Serum SHSF data are not included since none of the sera tested induced a splenic CFU-s accumulation upon injection that was significantly different from NMS-injected control spleens.

CFU-s immigration contributes to the LPS-induced splenic CFU-s accumulation

We investigated whether the LPS-W-induced splenic CFU-s increase was merely the result of local (splenic) CFU-s renewal or of accumulation of immigrated bone marrow-derived CFU-s, or both. The experimental set up is described in the chapter Materials and Methods. Assuming that any CFU-s migration from the bone marrow of the splenectomized T6T6 mice would occur at a similar rate as that from the parabiosed CBA/N marrow, a finding of 50% T6T6+ spleen colonies in the second CBA/N recipient´s spleen would indicate that migration fully accounts for the LPS-W-induced increase in spleen CFU-s numbers. From Table 6 it can be seen that bone marrow-derived CFU-s migration occurred following LPS-W administration and that about 18% of all colonies contained the T6T6 marker. These results suggest that at 6 days after injection of LPS-W about 36% of all splenic CFU-s are of marrow origin. It should be realised that such an estimate does not render information about the time of migration, nor does it allow quantitation of the actually immigrated CFU-s numbers, since proliferation of the immigrant CFU-s might have contributed to the total splenic CFU-s content determined at day 6.

DISCUSSION

The data presented here show that serum from mice injected with LPS contains a spectrum of activities, that is reminiscent of IL-3. Thus, PES induced a large increase in the proliferative activity of DA-1 cells, that are generally believed to be solely dependent on IL-3 for their survival. Moreover, PES was able to stimulate proliferation of CFU-s in serum-free liquid bone marrow cultures. In vivo, PES caused an increase of the splenic CFU-s numbers. The kinetics of SHSF and SAF-like activity were similar in PES, but differed from that of DA-SA. However, at the same time the present observations do not support the assumption that the activities of PES are due to IL-3. This notion follows from the following considerations.

With respect to the in vivo effects of PES it is evident that only a single injection of 0.3 ml of PES was able to induce significant increases of CFU-s numbers in the spleen. This is even more obvious when it is realized that only continuous infusion of recombinant IL-3 has been reported to be able to evoke effects in vivo with respect to changes in hemopoietic progenitor cell numbers (33,34). This difference in in vivo activity of PES and IL-3 could be a consequence of differences in glycosylation of the IL-3 molecule, leading to rapid turnover of synthesized IL-3 in vivo. Alternatively, it could also indicate that the in vivo effect of PES is not, or only partly, due to the presence of IL-3 in PES.

In this study injection of a large amount of LPS was required to permit detection of serum activities using the assays for SHSF and SAF. This contrasted sharply with the ability of DA-1 cells to proliferate following stimulation with either minute concentrations of PES (Fig. 2), or in the presence of PES from mice previously injected with small doses of LPS (Fig. 8). Although PES clearly exhibits a SAF-like acitivity in serum-free bone marrow cultures, both GM-CSF and purified H-1 induced a similar CFU-s proliferation in such cultures. This may not be surprising for H-1, because it was not purified to homogeneity and therefore may have contained IL-3. However, the activity of GM-CSF was remarkably high, especially in the presence of H-1. H-1 affected the GM-CSF-induced CFU-s proliferation far more than that of PES, which suggests that the effect of PES may not exclusively be attributed to the high levels of GM-CSF in PES. A possible presence of H-1 in PES was made unlikely by the observation, that PES did not synergize with IL-3 in this assay.

PES was observed to induce a detectable DA-1 cell increase above background levels under all experimental conditions tested, whereas the stimulatory effect of GM-CSF was lower or absent. However, the DA-1 cell response to PES could be modulated to a great extent by changing the culture time and the DA-1 cell density at plating. The data in Figure 2 strongly suggest that the impressive effect of PES on 9-day DA-1 cell proliferation was probably not the exclusive result of the presence of IL-3 in PES, since the effects of IL-3 and PES were remarkably reversed as compared to their effect in 4-day cultures. Furthermore, PES, but not GM-CSF, contained a synergistic activity for IL-3-induced DA-1 cell proliferation (Figs. 3-6). Moreover, the effect of PES on DA-1 cells was not as extensively abrogated as that of IL-3 in the presence of anti-IL-3 (Fig. 7). These observations make it likely that most, if not all, of the PES activity on DA-1 cells was not identical to IL-3. In addition, our data clearly demonstrate that DA-1 cells cannot be considered to be exclusively dependent on IL-3 for their growth and maintenance.

It is unlikely that the high titers of CSF known to be present in PES were fully responsible for the variety of PES effects reported here. Although it has been reported that high concentrations of GM-CSF or G-CSF may stimulate the in vitro proliferation of erythroid, megakaryocytic and multipotential progenitors in addition to CFU-M and/or CFU-G (35,36), PES and GM-CSF had different effects in the three assays used in this study. Secondly, it has been reported (11) that not all substances, which increase serum CSF levels, e.g., mureine, are able to evoke an increase of splenic CFU-s numbers. Indeed, only substances such as lipoprotein and LPS are able to trigger splenic CFU-s into cycle and lead to an increased splenic CFU-s content. Finally, SHSF can be separated from the major form of GM-CSF on the basis of molecular weight (37). We therefore conclude that PES contains a spectrum of activities, including GM-CSF, SAF, SHSF and a thus far unidentified synergistic activity for DA-1 cell proliferation. The level of IL-3, if present, is assumed to be low in PES.

Although PES and LPS have identical effects on splenic hemopoiesis, PES injection does not mimick the depletion of cellularity and CFU-s numbers in the bone marrow following LPS injection (10,11,13). The nadir of bone marrow CFU-s numbers following LPS injection coincides in time with the maximum increase in splenic CFU-s numbers. This prompted us to study the possibility of CFU-s migration from the bone marrow to the spleen. The present study in parabiotic mice indicates that a significant immigration of bone marrow-derived CFU-s may contribute to the large CFU-s accumulation in the spleen of LPS-injected mice. This observation suggests the possibility, that PES injection may not solely induce CFU-s proliferation in the spleen, but similar to LPS, may induce an additional inflow of marrow-derived CFU-s in the spleen.

Of interest are the experiments with LPS low-responder C3H/HeJ mice. These mice are refractory to lipid A due to a mutation in the locus controlling LPS sensitivity (LPS gene on chromosome 4; ref. 38, 39). The absence of increased SAF and SHSF levels in serum of C3H/HeJ mice shortly after LPS injection (Table 2) is reminiscent of their blunted CSF response (40,41). Yet, C3H/HeJ mice responded to injection of LPS responder-derived PES, but not of LPS (42,43), with a large increase in splenic CFU-s numbers, indicating that these mice indeed have a defective induction of SHSF. This conclusion was supported by our observation, that C3H/HeJ CFU-s in serum-free cultures responded well to IL-3 or PES.

We did not find any comparable high IL-3 and SHSF titers in sera of mice following induction of hemolytic anemia, or administration of various mitogens or latex beads. These treatments have been reported to induce an increase of splenic CFU-s numbers (30-32). While irradiation has been shown to elicit the production of humoral factors stimulating CFU-s proliferation (8,41) we could not detect such activities in sera from irradiated mice using the DA-1 cell assay and the SHSF assay. This confirmed the observation of Staber and Metcalf (10) on the lack of a serum SHSF response following whole body irradiation. In view of the specific properties of PES as compared to sera from mice, that had been otherwise treated in order to perturb their hemopoietic steady state, it is tempting to speculate that the serum activities in PES are part of a regulatory mechanism of in vivo hemopoiesis in response to bacterial products. It has been suggested that the immunological status of mice and dogs, especially the number of gram-negative bacteria in the intestine, influences the kinetics of granulocyte precursor cells and stem cells (45-48). This would be effected by endotoxin released from the gut into the systemic circulation. The available data suggest that a signal to the hemopoietic system is given by hemopoietic growth factors (HGF), that may include SHSF, GM-CSF and IL-3, or through different humoral factors of extramedullary origin, which would increase the production of HGF (49) by bone marrow cells. Our observation that splenectomy or whole body irradiation did not prevent the animals to produce high serum titers of DA-SA and SHSF following LPS administration indicates that the cellular sources of these activities are radioresistant cells, which at least can be found in extrasplenic sites. This conclusion is supported by our previous finding, that lethally irradiated LPS high responder mice, following reconstitution with LPS low responder bone marrow cells, were still able to increase their splenic CFU-s numbers after LPS injection (50). These cells are not part of the defective microenvironment in mice carrying the Sl^d or Sl^J alleles (51,52), since these genetically anemic mice respond normally to LPS administration with a large increase of serum SAF and SHSF.

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Table 1
CFU-s proliferation in serum-free cultures

Stimulus	Recovery of CFU-s (no. of CFU-s per culture ± 1 SE)
none	0
IL-3	171.6 ± 19.6
PES	33.6 ± 6.8
GM-CSF	33.6 ± 7.1
H-1	25.2 ± 7.2
H-1 + IL-3	242.4 ± 13.0
H-1 + PES	50.4 ± 5.9
H-1 + GM-CSF	136.0 ± 15.0
IL-3 + PES	182.4 ± 8.4
IL-3 + GM-CSF	186.0 ± 16.4
IL-3 + PES + H-1	199.2 ± 15.6
IL-3 + GM-CSF + H-1	206.4 ± 12.5

3×10^5 bone marrow cells, containing 72.6 (1 SE, 6.36) CFU-s, were cultured for 4 days. The one ml cultures were supplemented with 30 µl of IL-3 (equalling 30 U of IL-3), 50 µl of PES, 150 µl of GM-CSF or 50 µl of H-1, or a combination thereof.

Table 2
SAF, SHSF and DA-SA levels in sera from BCBA mice at various times after treatment with 100 μg of LPS-W

hrs after injection	SAF* (% recovery of CFU-s)	SHSF** (CFU-s/spleen)	DA-SA*** (amplification factor) 1 μl	5 μl
-	4 (2)	2,359	0	0
1	- -	-	0	3.5
3	20 (4)	12,007	25	30
6	62 (11)	28,500	28	28
18	66 (10)	25,510	36	33
42	- -	-	23	31
48	8 (2)	4,845	-	-
70	- -	-	22	30
96	4 (2)	3,333	-	-

* 2.5 x 10 BMC were stimulated with 50 μl of post-LPS serum and assayed at day 4. Data represent the arithmetic mean (1 SE) of 6 tubes in 2 separate experiments.

** 0.3 ml of post-LPS serum was injected ip and spleen CFU-s were assayed at day 5. Data represent the arithmetic mean of 2 separate experiments.

*** 10^5 DA-1 cells were cultured for 10 days in 1 ml of α-medium containing 20% FCS, supplemented with 1 or 5 μl PES. Data represent the arithmetic mean of triplicate wells in a single experiment.

Table 3
Levels of SAF and SHSF in sera from C3H and CBA mice

Serum donor	LPS responder mice	Serum injected or added to cultures	% recovery of CFU-s (SAF-assay)	No. CFU-s per spleen (SHSF-assay)
C3H/HeJ	-	NMS*	0	4,375(596)****
C3H/HeJ	-	PES**	5(2)***	4,714(896)
C3H/He	+	NMS	0	5,464(379)
C3H/He	+	PES	43(8)	9,492(614)
C3Heb/FeJ	+	NMS	0	8,043(554)
C3Heb/FeJ	+	PES	94(2)	14,363(1,117)
CBA	+	NMS	0	9,567(780)
CBA	+	PES	40(7)	36,300(2,535)

For SAF determination 2.5-3 x 10^5 bone marrow cells of LPS low-responder C3H/HeJ mice were cultured for 4 days in the presence of 50 µl of test sera. For SHSF determination recipient mice received 1 ml of NMS or PES, and 5 days later their splenic CFU-s number were determined.

* normal serum from mice 6 hr after injection of 0.5 ml PBS.

** serum from mice 6 hr after injection of 500 µg of LPS-R595.

*** arithmetic mean (1 SE) of 3 replicate experiments.

**** arithmetic mean (1 SE) of 4 replicate experiments, except for the 2 lower lines, which represent data of 3 individually assayed mice.

Table 4
DA-SA and SHSF levels in sera of irradiated or splenectomized BCBA mice

Radiation dose (Gy)	LPS-W (μg)	Sera collected after (hr)	DA-1 cells $x10^5$/well (DA-SA assay)	CFU-s/spleen (SHSF assay)
-	-	1	0*	1,778(354)**
0.12	-	1	0	2,359(462)
0.36	-	1	0.03(0.03)	2,000(333)
1.21	-	1	0.03(0.03)	4,475(787)
3.63	-	1	0.03(0.03)	nd
10.90	-	1	0	2,667(411)
9.6	-	6	0	3,202(338)
-	100	1	2.89(0.33)	4,297(899)
-	100	6	28.01(2.48)	25,100(2,660)
6.00(day 10)	300	6	23.37(1.03)	26,667(4,744)
9.60(day 3)	100	6	16.40(2.71)	25,778(3.437)
9.60(day 10)	100	6	25.33(3.84)	38,571(6,223)
Splenectomy (8 days before)	100	6	18.74(2.96)	37,003(2,444)

10^5 DA-1 cells were cultured for 10 days in 1 ml of α-medium containing 20% FCS. The wells were supplemented with 5 μl of test serum.

* Arithmetic mean (1 SE) of triplicate wells.

** Arithmetic mean (1 SE) of three separate experiments.

Table 5
DA-SA levels in serum from BCBA mice after induction of hemolytic anemia by PHZ or after injection of various mitogens and particulates.

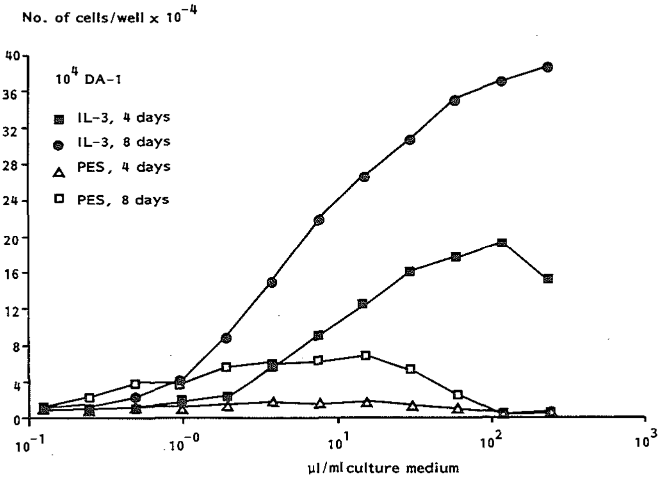
Compound injected	μl test serum in well	Collection of serum after 6 hr	18 hr	42 hr	66 hr
saline	5	0*	0	0	0
(0.5ml ip)	100	0	0	0	0
LPS-W	5	7.34(1.00)	4.41(1.12)	8.06(0.75)	nd
(300μg ip)					
PVP	5	1.45(0.31)	0	0	0
(17.5mg ip)	100	0.82(0.29)	0.15(0.15)	0.18(0.18)	0
latex	5	1.21(0.08)	0	0	0
(0.3mg dry weight)	100	0.18(0.18)	0.06(0.06)	0.04(0.04)	0
PHZ	5	0	0	0	0
(1mg ip)	100	0.07(0.07)	0	0	0
Con-A	5	0	0	0	0
(100μg iv)					
PWM	5	0	0	0	0
(100μg iv)					

10^5 DA-1 cells were cultured for 10 days in 1 ml of α-medium containing 20% FCS, supplemented with 5 μl or 100 μl of test serum.
Figures represent numbers of cells present in wells ($x10^5$).
* Arithmetic mean (1 SE) of DA-1 cell counts ($x10^5$) in triplicate wells.

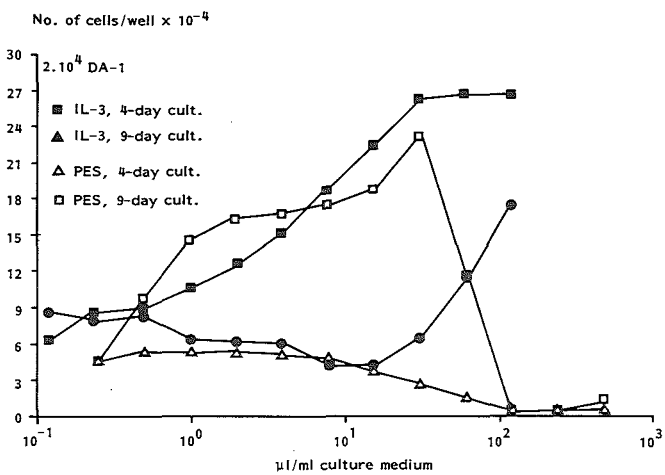
Table 6
Contribution of CFU-s migration to the LPS-induced splenic CFU-s accumulation in parabiosed CBA/N and CBA/T6 mice

Compound injected*	no. of parabionts	no. of colonies examined**	no. of T6T6+ colonies detected
saline	4	50	0
LPS (100 μg)	4	59	11***

* Both mice of any parabiosis pair were injected with either 100 μg of LPS (R595) or 0.5 ml of saline iv at 8 days after the establishment of parabiosis.
** Day-10 CFU-s were assayed 6 days after injection of saline or LPS.
*** Range of % of T6T6+ colonies in individual mice was 12.5-28.5.



<u>Figure 1</u>
Effect of PES and IL-3 on proliferation of DA-1 cells plated in low densities. DA-1 cells (10^4 per 0.2 ml α-medium supplemented with 10% FCS) were cultured for 4 or 9 days.



<u>Figure 2</u>
Effect of PES and IL-3 on proliferation of DA-1 cells plated in high densities. DA-1 cells (2 x 10^4 per 0.2 ml α-medium supplemented with 10% FCS) were cultured for 4 or 9 days.

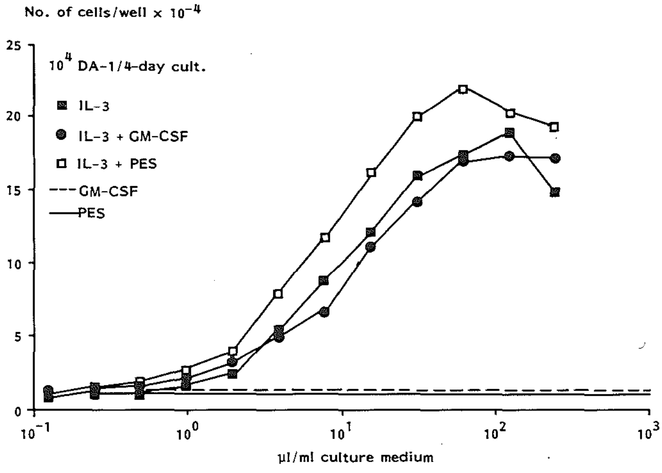


Figure 3
Synergism of PES and IL-3 for stimulation of DA-1 cell proliferation. DA-1 cells (10^4 per 0.2 ml α-medium supplemented with 10% FCS) were cultured for 4 days. Dose responses of IL-3 alone were done in the absence or in the presence of either 50 µl PES/ml or 64 µl GM-CSF/ml culture medium. Full line indicates DA-1 cell proliferation in the presenbce of 50 µl PES/ml alone (equal to about 50% plateau PES response); dashed line, 64 µl GM-CSF/ml alone (equal to about 100% plateau GM-CSF response).

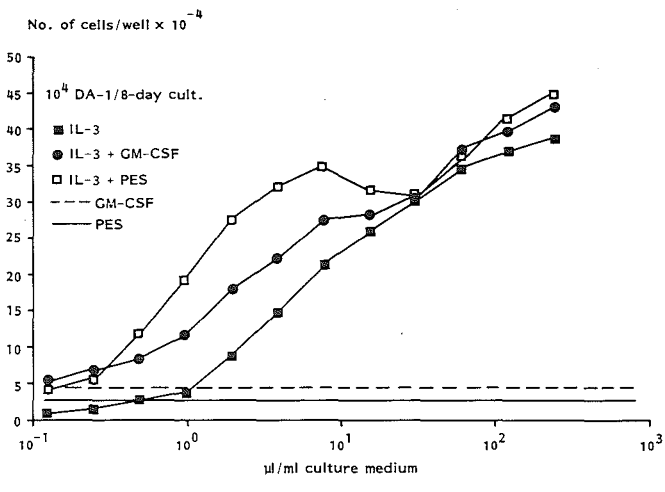


Figure 4
Synergism of PES and IL-3 for stimulation of DA-1 cell proliferation. DA-1 cells (10^4 per 0.2 ml culture medium) were cultured for 8 days. See legends of Fig. 3 for further explanation.

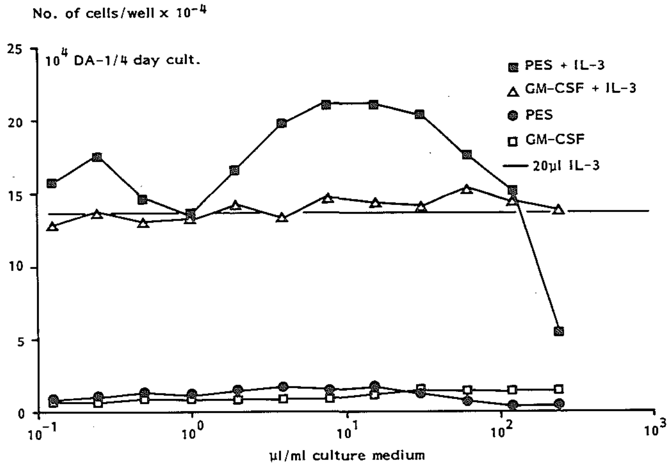


Figure 5

Dose response of DA-1 cells to PES or GM-CSF in the presence of IL-3. DA-1 cells (10^4 per 0.2 ml culture medium) were cultured for 4 days. Dose responses to PES or GM-CSF were done either in the absence or presence of 20 µl IL-3/ml (equal to about 50% plateau IL-3 response).

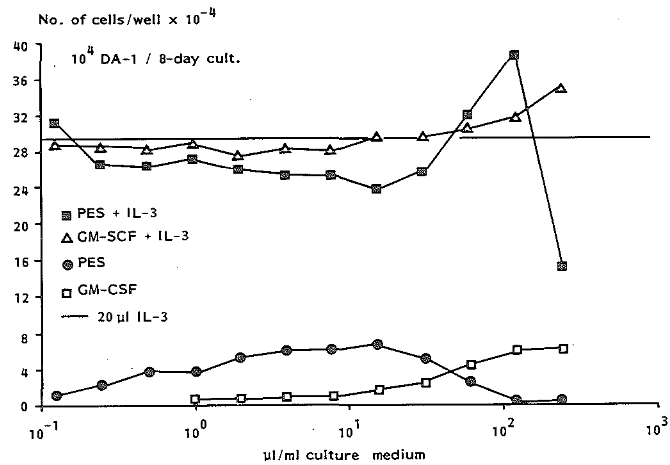


Figure 6

Dose response of DA-1 cells to PES or GM-CSF in the presence of IL-3. DA-1 cells (10^4 per 0.2 ml culture medium) were cltured for 8 days. For further explanation see figure 5.

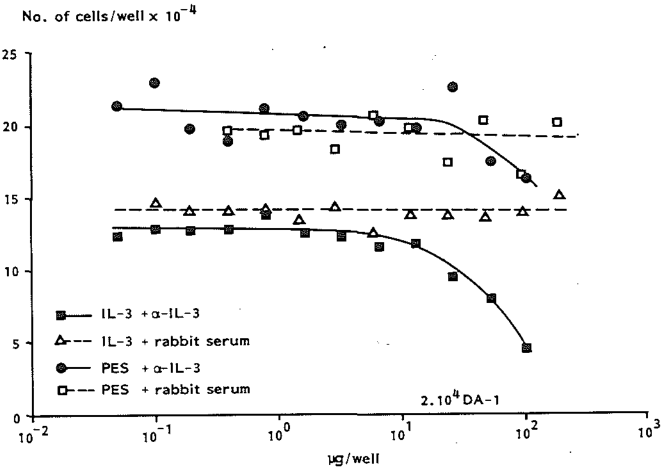


Figure 7
Effect of anti-IL-3 on DA-1 cell proliferation stimulated by 0.4 µl (equalling 0.4 U) of IL-3/ml or 5 µl PES/ml. DA-1 cells (2 x 10^4/0.2 ml culture medium) were cultured for either 4 days (for IL-3 response) or 9 days (for PES response).

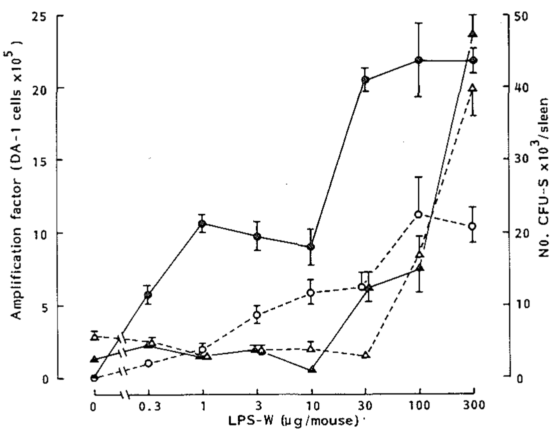


Figure 8
DA-SA and SHSF levels in BCBA sera after injection with varying amounts of LPS-W. DA-1 cell assay: ●—, 3 hr PES: o--, 6 hr PES. SHSF assay: ▲—, 3 hr PES; △--, 6 hr PES. 10^5 DA-1 cells were cultured for 10 days in 1 ml of α-medium containing 20% FCS, supplemented with 5 µl PES. Data represent means of triplicate wells (bars: 1 SE). The 3 hr and 6 hr PES sera were assayed in DA-1 cell cultures set up at different times.

APPENDIX PAPER VI

Exp. Hematol. 14:9–15 (1986)

Experimental
Hematology

Defective Support of Sl/Sl^d Splenic Stroma for Humoral Regulation of Stem Cell Proliferation

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Abstract. Humoral regulation of CFUs proliferation was investigated in Sl/Sl^d mice characterized by a stromal defect, which severely limits in situ proliferation of in vivo colony-forming cells (CFUs). Injection of LPS-W evoked a large enhancement of CFUs numbers in the spleen of normal +/+ mice. Sl/Sl^d mice were found to be refractory to low doses of LPS-W (up to 15 μg/mouse) and to have a diminished response to high doses (up to 150 μg). Serum transfer experiments showed that Sl/Sl^d mice are not defective in the early elaboration (6 h) of a humoral factor (SHSF), which mediates the LPS-induced splenic stem-cell accumulation. In a serum-free in vitro system post-LPS Sl/Sl^d and +/+ sera induced a similar degree of CFUs proliferation, indicating the ability of Sl/Sl^d mice to produce normal levels of stem-cell-activating factor (SAF). Transfer of potent post-LPS serum from normal mice evoked a poorer splenic CFUs accumulation in Sl/Sl^d mice as compared to normal +/+ littermates. The population size of splenic stem cells in Sl/Sl^d mice parabiosed with normal +/+ mice also showed a limited increase in response to LPS-W injection. This diminished in vivo response of Sl/Sl^d mice was not due to a decreased sensitivity of their CFUs for SAF, since Sl/Sl^d and +/+ CFUs showed similar survival rates in vitro in the presence of SAF. We propose that the defective response of Sl/Sl^d mice to LPS-induced humoral regulators is due to a nonmigratory component of the Sl/Sl^d splenic stroma, which limits splenic CFUs proliferation either by a short-range inhibitory activity or by a deficiency of a local stimulatory activity or nutrient unlike SAF or SHSF, which might act in synergy with SAF.

Key words: Hemopoietic stroma — SAF — SHSF — Sl/Sl^d mice — Stem cell regulation

Various findings indicate that the severe macrocytic anemia of Sl/Sl^d (steel) mice results from a genetically determined defect in their hemopoietic stroma rather than in their hemopoietic stem cells or humoral regulatory apparatus [1–3]. It has been suggested that the presence of inhibitory [4] as well as the absence of stimulatory [2] microenvironmental factors might be responsible for the limited erythropoietic activty of the Sl/Sl^d spleen. In an analysis of spleen colony formation of +/+ marrow cells injected into lethally irradiated +/+ and Sl/Sl^d recipients, lodgment of CFUs in the spleens of these congenic mice was found to be similar [5]. The microscopic colonies that developed, however, remained too small to be detected on gross inspections of the spleen [5–7]. The observations that Sl/Sl^d mice show a relative inability to support maintenance and growth of stem cells and progenitor cells in their spleen [6, 8–12] and femurs [13] strongly suggest that the defect in Sl/Sl^d mice is at the level of the regulation of the CFUs population size rather than on the level of the erythron. The specific cellular or biochemical nature of the steel stromal defect on the level of stem cells, however, has not yet been determined.

We studied the proliferation of CFUs in the spleen of Sl/Sl^d mice after injection of bacterial lipopolysaccharide-W (LPS). LPS and its biological active moiety, lipid A, have been indicated to be valid tools for this purpose [14, 15] and render results analogous to the growth curves observed when normal CFUs are transplanted into irradiated mice of genotype Sl/Sl^d [6, 8]. Staber and Metcalf [16] hypothesized that the relative inactivity of post-lipid-A serum from Sl/Sl^d mice as compared to that from +/+ mice to enhance the splenic CFU-G/M content in +/+ mice might be the reason why Sl/Sl^d mice are unresponsive to multiple doses of 25 μg LPS [8]. We report here that both Sl/Sl^d and +/+ mice respond to LPS with a similarly increased serum activity, which activates CFUs to proliferate both in vitro and in vivo. It appears, however, that a nonmigratory stromal component of the Sl/Sl^d spleen restricts local CFUs proliferation in response to this serum activity.

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increase in the splenic CFUs upon i.v. injection into LPS-W low-responder C3H/HeJ mice [26], the post-LPS sera induced a similar CFUs accumulation in the spleen of low and high LPS-W responder C3H mice.

Other investigators have demonstrated that media conditioned by marrow adherent cells from Sl/Sl^d mice increased the 24-h survival of CFUs in vitro to about the same extent as medium conditioned by +/+ adherent cells [35]. Such adherent cells showed no deficiency in the ability to produce granulocyte–macrophage colony-stimulating factor [36]. Furthermore, Sl/Sl^d marrow CFUs in vivo were observed to have a higher cycling percentage than +/+ marrow CFUs [11]. These studies are consistent with our observations that Sl/Sl^d mice do not have a deficient humoral CFUs regulation.

The observation that Sl/Sl^d mice are able to mount a normal serum SHSF and SAF response to LPS injection indicates that a factor different from SHSF or SAF is defective in Sl/Sl^d mice. The parabiosis experiments clearly indicate that this defect is a local [1, 31, 37] and probably cellular factor residing at least in the Sl/Sl^d spleen, since joining the anemic Sl/Sl^d mice to normal littermates in parabiosis had no detectable effect on the (in)ability of either member of the parabiotic pair to support the growth of splenic CFUs and CFU-G/M in response to LPS. The limited CFUs accumulation in the spleen obtained by injection of potent PLPSS in Sl/Sl^d mice is in agreement with this conclusion. It is unlikely that the small CFUs increase in the spleen of LPS-injected Sl/Sl^d mice is caused by a diminished sensitivity of their CFUs to SAF, since our experiments did not reveal any difference in the proliferation of Sl/Sl^d and +/+ bone marrow CFUs under optimal SAF stimulation in vitro (Table 3). Therefore, we postulate that the defect in Sl/Sl^d mice is caused by a stromal component, which limits CFUs proliferation in situ either by an inhibitory activity or by a deficient local nutrient level or stimulatory activity unlike SAF, but acting in synergy with SAF. The action of this stromal component might be via cell–cell contact or short-range diffusion and does not limit the serum SAF activity.

In parabiosed Sl/Sl^d mice that were injected with saline, a significant decrease in splenic cellularity and CFUs numbers was observed (Table 5). The most likely explanation is that the presence of the parabiosed +/+ littermate had relieved the pressure for red blood cell formation, which led to an almost hemopoietically inactive spleen in the Sl/Sl^d parabiont. If migration of CFUs from the bone marrow to the spleen would contribute to the massive splenic CFUs accumulation following LPS injection, it is a significant finding that LPS-injected parabiosed Sl/Sl^d mice do not show an increased splenic CFUs accumulation in the presence of a common systemic blood circulation with +/+ mice. This observation supports the conclusion that the poor CFUs accumulation in Sl/Sl^d spleens is due to a local splenic defect.

Injection of PLPSS not only induced accumulation of CFUs but also that of BFU-E and CFU-G/M in the spleen of both +/+ and Sl/Sl^d mice (Tables 2, 4, 5). The simplest hypothesis to explain this observation is to propose that a single factor is elevated by the LPS-W, which activates stem cells into cycle and permits this generation of various types of progenitor cells [16].

There is good evidence that the control of CFUs proliferation occurs locally because the kinetic properties of CFUs differ at different sites in the same animal [38, 39]. On the other hand, we observed that an activity contained in PLPSS, but also in NMS, stimulated CFUs proliferation in vivo and we have measured strongly enhanced SAF levels in PLPSS as compared with NMS. At present we do not know whether this finding implies the existence of a systemic regulation of CFUs proliferation, or the leakage of regulatory factors out of the hemopoietic compartments into the blood that do not essentially contribute to stem-cell homeostasis.

Acknowledgments

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Table 1. Splenic CFUs accumulation following injection of LPS-W in WCB6Fl +/+ and Sl/Sl[d] mice[a]

LPS-W i.v. (μg)	Splenic CFUs content (×10³)				Splenic cellularity (×10⁷)	
	+/+	P[b]	Sl/Sl[d]	P	+/+	Sl/Sl[d]
0	9.1 (4.1)[c]		3.3 (2.4)		28.2	21.0
5	34.5 (11.3)	0.02	3.4 (0.5)	n.s.[d]	36.3	23.3
15	63.0 (7.3)	0.005	2.4 (0.0)	n.s.	39.0	25.6
50	103.7 (4.5)	0.0001	8.4 (0.6)	0.02	47.5	25.5
150	103.7 (6.0)	0.0001	16.2 (1.0)	0.001	36.3	24.4

[a] Mice were either injected with saline or LPS-W and their splenic cellularity and CFUs content measured four days later.
[b] P values were calculated relative to data from non-LPS-injected mice.
[c] Arithmetic mean (±1 SD) of three individually assayed mice.
[d] n.s., no significant difference.

from +/+ mice, induced a significant CFUs accumulation in the spleen of +/+ WCB6F1 mice (Table 2). PLPSS from Sl/Sl[d] as well as +/+ mice induced a large CFUs and CFU-G/M accumulation in the spleen. This indicates that Sl/Sl[d] mice certainly do not have deficient SHSF production. In vitro (Table 3), no stem-cell activating activity (SAF) could be demonstrated in NMS from +/+ or Sl/Sl[d] mice. Addition of 50 μl of PLPSS from Sl/Sl[d] as well as from +/+ mice to this system led to a similar high CFUs recovery after four days of culture. The recovery of CFUs observed after optimal stimulation with highly purified SAF exceeded the inoculum value. CFUs from both congenic mouse strains had a similar survival rate in vitro in the presence of highly purified SAF, indicating that Sl/Sl[d] CFUs did not have a diminished sensitivity for SAF (Table 3). The possibility that the in vivo effects of PLPSS were due to residual LPS was rendered highly unlikely by the observation that PLPSS from +/+ and Sl/Sl[d] mice induced a similar day 4 splenic CFUs accumulation in both the LPS-W low-responder C3H/HeJ mice (+/+ PLPSS, 27,000 CFU/spleen; Sl/Sl[d] PLPSS, 35,000 CFU/spleen), which do not show the splenic CFUs accumulation in response to LPS, and the high-responder C3Heb/FeJ mice (+/+ PLPSS, 29,000 CFU/spleen; Sl/Sl[d] PLPSs, 35,000 CFU/spleen). The sensitive *Limulus* amoebocyte lysate assay indicated that the PLPSS from +/+ and Sl/Sl[d] mice contained less than 0.1 μg of endotoxin per milliliter. In the in vitro assay, the addition of 0.1 μg or 1.0 μg of LPS-W did not induce any CFUs proliferation (Table 3).

Effect of C57BL/Rij PLPSS on splenic CFUs accumulation in WCb6F1/Sl/Sl[d] and +/+ mice

We investigated the ability of Sl/Sl[d] to respond to injection of a potent PLPSS from C57BL/Rij mice. The splenic CFUs, BFU-E, and CFU-G/M content

Table 2. Effects of serum from normal or LPS-W-injected WCB6F1 Sl/Sl[d] or +/+ mice on spleen CFUs and CFU-G/M levels in +/+ mice

Serum injected[a]	CFU-S (×10³) content	P[b]	CFU-G/M (×10³) content	P
—	6.6 (2.4)[d]		21.7 (9.0)	
+/+ NMS[c]	9.8 (2.7)	n.s.[e]	33.9 (12.8)	n.s.
+/+ PLPSS[c]	39.2 (5.6)	0.005	73.5 (8.1)	0.002
Sl/Sl[d] NMS	18.2 (4.9)	0.1	48.7 (15.5)	0.05
Sl/Sl[d] PLPSS	53.5 (17.7)	0.05	88.9 (28.2)	0.02

[a] At 0, 6, and 24 h 0.1 ml serum was injected i.p.
[b] P values were calculated relative to values from non-serum-injected mice.
[c] Normal +/+ mice were injected with either saline or 0.1 ml of normal mouse serum (NMS) or 6 h post-LPS serum (PLPSS) from +/+ or Sl/Sl[d] mice. Four days later their spleens were assayed for the number of CFUs and CFU-G/M.
[d] Arithmetic mean (±1 SE) of three individually assayed mice.
[e] n.s., no significant difference.

and the cellularity in Sl/Sl[d] and +/+ mice is provided in Table 4. It is evident that injection of PLPSS induced a large increase in splenic CFUs numbers of +/+ mice, whereas only a small absolute increase was observed in the spleen of Sl/Sl[d] littermates. When the originally low splenic CFUs number in Sl/Sl[d] mice is taken into consideration, the relative response of these mice was far better than appears from the absolute splenic CFUs figures. Indeed, the most preferable way of expressing the results may be to take the ratio of the splenic CFUs content induced by PLPSS injection versus the untreated control values, since Sl/Sl[d] mice, in contrast to +/+ mice, were observed to be refractory to NMS injection. Thus, the data clearly show that an absolute increase of splenic CFUs numbers in PLPSS-injected Sl/Sl[d] mice, but not in NMS-injected Sl/Sl[d] mice, was present, while the relative response was lower than in similarly treated +/+ littermates. In

Table 3. Effects of serum from normal or LPS-W-injected WCB6F1 Sl/Sl[d] or +/+ mice on the four-day CFUs survival in vitro

Addition to 1 ml culture medium	Origin of target bone marrow cells	CFU-s recovery (3)[a]		
		Exp. 1	Exp. 2	Exp. 3
None	B6 × CBAF1	0.0	0.0	0.0
0.1 μg LPS	B6 × CBAF1	0.0	n.d.	n.d.
1.0 μg LPS	B6 × CBAF1	0.0	n.d.	n.d.
+/+ NMS[b]	B6 × CBAF1	0.0	0.0	0.0
+/+ PLPSS[b]	B6 × CBAF1	59.3 (8.9)	8.5 (4.0)	56.3 (9.6)
Sl/Sl[d] NMS	B6 × CBAF1	0.0	0.8 (0.8)	0.0
Sl/Sl[d] PLPSS	B6 × CBAF1	52.7 (6.4)	10.0 (3.5)	56.3 (6.5)
SAF[c]	B6 × CBAF1	165.0 (30.5)	169.0 (21.3)	n.d.
none	WCB6F1 +/+	n.d.*	n.d.	0.2 (0.2)
SAF	WCB6F1 +/+	n.d.	n.d.	142.6 (22.8)
none	WCB6F1 Sl/Sl[d]	n.d.	n.d.	0.2 (0.2)
SAF	WCB6F1 Sl/Sl[d]	n.d.	n.d.	116.7 (13.7)

[a] Arithmetic mean (±1 SE) of duplicate experiments as assayed in ten irradiated recipient mice. In any one experiment a different pool of each serum type was used. Each serum pool was prepared by the contribution of six mice.
[b] A quantity of 50 μl of normal mouse serum (NMS) or post-LPS serum (PLPSS) was added to 1 ml of otherwise serum-free culture medium.
[c] Instead of serum, 10 μl of stage III MSCM was added.
* n.d., not done.

Table 4. Effect of post-LPS-serum injection on the splenic CFUs, BFU-E, and CFU-G/M content in WCB6F1/J Sl/Sl[d] and +/+ mice[a]

Recipient genotype	Serum	Spleen cellularity ($\times 10^7$)	P[b]	Splenic progenitors					
				CFUs	P	BFU-E	P	CFU-G/M	P
+/+	NMS	29.34[c] (2.96)	n.s.[d]	11,300 (2600)	0.02	7030 (1720)	0.05	5500 (1530)	0.01
	PLPSS	48.63 (1.67)	0.0001	104,450 (7600)	0.0001	91,560 (17,850)	0.001	150,340 (71,030)	0.001
Sl/Sl[d]	NMS	26.41 (2.31)	n.s.	1200 (170)	n.s.	1600 (920)	n.s.	940 (340)	n.s.
	PLPSS	35.00 (8.78)	0.05	8900 (2390)	0.0001	7470 (4500)	0.01	9360 (3570)	0.001
+/+	—	29.29[e] (0.15)		6900 (460)		3800 (100)		2500 (300)	
Sl/Sl[d]	—	25.10 (0.30)		1290 (140)		800 (100)		700 (400)	

[a] Three consecutive i.p. injections with 0.5 ml of C57BL serum were given at 0, 6, and 24 h. Mice were killed at day 4.
[b] *P* values were calculated relative to data from non-serum-injected isologous mice.
[c] Arithmetic mean (±1 SE) of three individually assayed mice.
[d] n.s., no significant difference.
[e] Arithmetic mean (±1 SE) of ten individually assayed mice.

all cases the PLPSS-induced rise in splenic CFU-G/M was considerably more than that of splenic CFUs or cellularity, and in general reflected the CFUs data.

LPS response of Sl/Sl[d] mice during parabiosis with +/+ mice

To investigate the defective splenic CFUs proliferation in LPS-injected Sl/Sl[d] mice in the presence of normal circulating stem cells and regulatory serum components, Sl/Sl[d] and +/+ mice were parabiosed. One week after parabiosis, both mice of any one pair were i.v. injected with either saline or LPS-W. After four days the spleens of saline-injected parabiosed +/+ mice contained twice as many CFUs as individual +/+ mice, whereas the splenic CFUs content in Sl/Sl[d] mice diminished significantly following parabiosis (Table 5). The rise in splenic CFUs numbers following parabiosis is a common observation in normal mice in our laboratory. It is probably related to low-level infections due to the large wound area. LPS-W injection led to comparable differences in splenic CFUs accumulation between parabiosed Sl/Sl^d and +/+ mice as observed between individual mice of either genotype. This observation indicates that CFUs proliferation in the spleen of LPS-injected Sl/Sl^d mice in the presence of +/+ humoral regulators and circulating CFUs is similarly diminished as in the presence of their own humoral regulators and CFUs alone. Moreover, the apparent stromal defect, which prevents optimal CFUs proliferation following LPS injection, apparently has a local nature. This is evident from the observation that the response in normal +/+ littermates was not diminished when they were parabiosed to Sl/Sl^d mice, while the splenic Sl/Sl^d defect was not overruled in such parabionts.

Table 5. CFUs accumulation in the spleen of parabiosed WCB6Fl Sl/Sl^d and +/+ mice after injection of LPS-W

Recipient genotype		µg LPS-W	CFUs (×10^3) content	P[a]	Cellularity (×10^7)	P
+/+	I[b]	0	9.1 (4.1)[c]		28.1	
Sl/Sl^d	I	0	3.3 (2.4)		21.0	
+/+	I	5	34.5 (11.3)	0.005	36.3	0.001
Sl/Sl^d	I	5	3.4 (0.5)	n.s.*	23.3	n.s.
+/+	I	50	103.7 (4.5)	0.0001	47.5	0.0001
Sl/Sl^d	I	50	8.4 (0.6)	0.005	25.5	n.s.
+/+	P[b]	0	18.6 (0.5)	0.005	37.3	0.001
Sl/Sl^d		0	0.6 (0.1)	0.05	6.7	0.0001
+/+	P	5	23.7 (4.1)	n.s.	35.2	n.s.
Sl/Sl^d		5	2.5 (0.3)	0.02	23.0	n.s.
+/+	P	50	100.1 (13.2)	n.s.	68.0	0.001
Sl/Sl^d		50	7.5 (1.3)	n.s.	28.1	n.s.

[a] For unparabiosed mice *P* values were calculated relative to the data from non-LPS-injected isologous mice. For parabiosed mice *P* values were relative to data from similarly treated unparabiosed isologous mice.
[b] Individual (I) and parabiosed (P) mice were injected with either saline or LPS-W and their splenic cellularity and CFUs content were determined four days later.
[c] Arithmetic mean (±1 SD) of four individually assayed mice.
* n.s., no significant difference.

Discussion

LPS and its biological active moiety, lipid A, trigger multipotential hemopoietic stem cells to synthesize DNA in vivo [14, 15] but not in vitro [22 and present data] and increase markedly the splenic content of CFUs [25, 26] and CFU-G/M [27–29]. The increase in splenic progenitor cell numbers has been reported to be mediated by a LPS- or lipid-A-inducible hemopoietic regulator called splenic-hemopoiesis-stimulating factor (SHSF) and possibly not by enhanced levels of GM-CSF [22, 30]. SHSF induces DNA synthesis in CFUs in otherwise serum-free bone marrow cell cultures in vitro [31]. It should be noted here that the massive accumulation of CFUs and early progenitors in the spleen of LPS-injected mice may not exclusively be the result of local multiplication [8]. The biphasic transient increase in the blood content of CFUs following LPS injection [25] suggests that migration from the bone marrow to the spleen might contribute to the splenic CFUs accumulation.

The present observations form the first evidence that Sl/Sl^d mice are not totally unresponsive to LPS, as was suggested by McCulloch et al. [8], but show a refractory response to injection of this bacterial cell wall product reminiscent of their retarded responses to erythropoietic stress [32]. We have demonstrated that Sl/Sl^d mice, in response to LPS injection, are not defective in the production of a factor that increases the four-day CFUs survival in an otherwise serum-free system in vitro. This in vitro assay detects stem-cell-activating factor (SAF), a single glycoprotein with a molecular mass of 19,000–20,000 daltons and an isolelectric point of about 6.3 [21]. SAF initiates cell cycling in cultured hemopoietic stem cells [19, 20] at concentrations as low as 10^{-11} to 10^{-10} *M* [33].

In addition to the observation that Sl/Sl^d PLPSS contained as much SAF as did +/+ PLPSS, PLPSS from Sl/Sl^d was as potent as was +/+ PLPSS with respect to its ability to induce a large CFUs accumulation in the spleen of normal littermates. This observation suggests that Sl/Sl^d mice are also not defective in their SHSF production in response to LPS injection. The present data do not lead to identification of this in vivo activity in PLPSS. Although operationally defined as SHSF [16], this activity might well be equated with SAF, while on the other hand it cannot be excluded that SHSF stimulates certain sites in the body to produce SAF. It is unlikely that residual LPS is responsible for the effects observed in the serum transfer experiments. LPS is removed extremely rapidly from the circulation [34]. Furthermore, the buoyant density of LPS has been observed to decrease after interaction with mouse serum or plasma both in vivo and in vitro, and this density shift is associated with the complete inhibition of important biological activities of LPS. Also, only minimal residual LPS amounts were presently detected in the post-LPS sera by the *Limulus* amoebocyte lysate assay. Although the presently detected LPS-W amounts are not able to evoke a significant

Materials and methods

Mice. Male mice (C57BL/Rij) 48–54 weeks old were purchased from the Dutch Reactor Center (Petten), and four- to ten-week-old male WCB6Fl/J mice (genotype Sl/Sl^d and $+^{sl}/+^{sl}$) were obtained from Jackson Laboratories (Bar Harbor, ME) and maintained at the Erasmus University (Rotterdam, The Netherlands) under clean conventional conditions. The mice aged 12–20 weeks at the time of experimentation. Female C3H/He Ola mice, 17–20 weeks old, were purchased from Olac (Bicester, UK). C3H/HeJ mice were obtained originally from Jackson Laboratories and bred subsequently at the Erasmus University, Rotterdam.

Preparation of post-LPS serum and normal serum. Mice were injected intraperitoneally with 500 μg of *Salmonella typhosa* lipopolysaccharide (prepared according to Westphal, Difco, Detroit, MI) in a buffered saline solution (BSS) or with BSS alone. Cardiac blood was obtained sterilely 6 h later, and clotting was allowed for 1 h at room temperature. After storage at 4°C overnight, serum was collected by two consecutive centrifugations at 500 *g* and stored at −20°C until use. The level of residual endotoxin in the sera was estimated in the *Limulus* amoebocyte lysate (LAL [17]) assay (chromogen substrate Coatest, KabiVitrum, Amsterdam, The Netherlands). In this assay the endotoxin-activated LAL cleaves the *p*-nitroaniline (pNA) from a chromogen substrate (S-2423). Light adsorption by pNA is then measured with a photometer at 405 nm. The sera were tested in LPS-W low-responder C3H/HeJ and compared to high-responder C3H/ebFeJ mice with respect to the capacity of residual endotoxin to increase the splenic CFUs content.

Stem-cell activating factor (SAF) detection system. The assay used to detect SAF is based on the prolonged maintenance of proliferating CFUs in suspension culture as compared to quiescent CFUs [18–21]. In conventional serum-deprived suspension cultures, less than 1% of the CFUs survive for four days of incubation. Suspension cultures that were maximally stimulated with the partly purified supernate of concanavalin-A-stimulated mouse spleen cell cultures (MSCM, stage III), showed after four days a CFUs number between one and three times the initial CFUs number [21]. SAF (MSCM, stage III) was isolated by affinity chromatography, gel filtration, and ion exchange chromatography using DEAE-sepharose at pH 8.0. These procedures increased the specific activity about 82,000-fold, and the preparation contained approximately 40% of the SAF activity of the original MSCM. The SAF activity in the various serum batches was detected by adding 50 μl of a test serum to a 1-ml aliquot of serum-free supplemented α-medium containing 6×10^5 nucleated bone marrow cells (BMC). The BMC were subsequently cultured in duplicate in loosely capped round-bottom Falcon tubes (no. 2057) for a period of four days at 37°C and 5% CO_2 in air in α-medium, supplemented with 0.25% wt/vol delipidated deionized BSA (Sigma), 4×10^{-6} *M* Fe-saturated human transferrin-2Fe (Behringwerke), 10^{-7} *M* Na_2SeO_3 (Koch Light), 10^{-4} *M* β-mercaptoethanol, 10^{-6} *M* isoproterenol (Sigma), 10^{-6} *M* hydrocortisone-hemisuccinate (Sigma), 10^{-3} g/liter nucleosides (adenosine, cytosine, guanosine, uridine, thymidine, 2-deoxyguanosine, 2-deoxyadenosine, and 2-deoxycytosine), 1.125×10^{-5} *M* cholesterol (Calbiochem) and 1.5×10^{-5} *M* linoleic acid (Merck). Cultures were terminated by adding ice-cold BSS.

The activity of splenic-hemopoiesis-stimulating factor (SHSF, reference [22]) in serum was quantitated in vivo by measurement of the splenic CFUs accumulation four to five days after intraperitoneal injection of various volumes of serum batches, which represented serum pools from at least six mice per experiment in the case of Sl/Sl^d and +/+ mice. Serum pools of C57BL/Rij sera were prepared from at least 40 mice.

Colony assays. CFUs were determined as described by Till and McCulloch [23]. A gamma cell 40 cesium 137 radiation unit (Atomic Energy of Canada, Ottawa) was used to irradiate the recipient mice at a dose rate of 1.31 Gy/min with a total dose of 8.5 Gy. The CFUs content of spleens and in vitro cultures was determined by injecting various nucleated cell numbers i.v. into the tail vein of the irradiated mice and measuring the eight-day macroscopic spleen colonies in at least ten recipient mice per experimental point. CFU-GM and BFU-E were quantitated in a semisolid (0.8% methylcellulose, Methocel AP4 Premium, Dow Chemical) culture medium (α-modification of Dulbecco's minimum essential medium) at 37°C and 5% CO_2. Between 1 and 50×10^5 spleen cells were plated per dish. CFU-GM cultures contained 20% con-A-stimulated mouse spleen conditioned medium (MSCM), 10% fetal calf serum (FCS), and 1% bovine serum albumin (BSA, Sigma); BFU-E cultures contained 20% con-A-stimulated MSCM, 10% FCS, 1% BSA, 10^{-4} *M* mercaptoethanol (Merck), 5 μg of egg yolk lecithin (Merck), 10^{-7} *M* $Na_2SeO_3 \cdot 5H_2O$ (Merck), 3.4×10^{-6} *M* transferrin saturated with 1.6×10^6 *M* $FeCl_3 \cdot 6H_2O$, and 0.5–2.0 U of Step III preparation of sheep plasma erythropoietin (Connaught Labs). Granulocyte–macrophage colonies and erythroid bursts were counted on days 7 and 10 of culture, respectively, with an inverted microscope. Under these culture conditions approximately 50% of erythroid bursts were mixed.

Parabiosis experiments. Parabionts of WCB6Fl/J +/+ and Sl/Sl^d mice were established as previously described [24]. Seven days after the operation both mice of each pair were i.v. injected with 5 or 50 μg LPS-W, and four days later the splenic cellularity and CFUs and CFU-G/M content in four individual pairs of each parabiosis group were determined.

Results

The effect of LPS-W on the splenic CFUs content and cellularity

Normal WCB6F1 +/+ mice have a high cellularity and CFUs number in the spleen (Table 1), being two to three times that found in a variety of other mouse strains including C3H, C57BL, AKR, 129, and CBA and their F1 hybrids (unpublished observations). They appeared to be highly sensitive to i.v. injection of small doses of LPS-W as judged by the splenic CFUs accumulation (Table 1). In contrast, Sl/Sl^d mice were refractory to doses of LPS below 15 μg, whereas they showed a diminished response of splenic CFUs numbers following 50 and 150 μg of LPS.

Effect of normal (NMS) and post-LPS (PLPSS) serum on CFUs and CFU-G/M proliferation

Experiments were carried out to measure the activity in Sl/Sl^d and +/+ serum, which induces stem-cell proliferation in vivo (i.e., SHSF) and in vitro (i.e., SAF). In vivo, NMS from Sl/Sl^d mice, but not

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