THE HEMOPOIETIC MICROENVIRONMENT:

effects of chemotherapy
and irradiation

DE HEMOPOËTISCHE MICRO-OMGEVING:

effecten van chemotherapie
en bestraling

PROEFSCHRIFT

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Eigentlich weiss man nur wenn man wenig weiss;
mit dem Wissen wächst der Zweifel.

J.W. von Goethe
Maximen und Reflexionen,
aus Kunst und Altertum, 1926

Aan mijn ouders

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List of abbreviations

BFU-E	burst-forming unit erythrocytes
BMC	bone marrow cells
BMT	bone marrow transplantation
BSS	buffered saline solution
BPA	burst-promoting activity
BU	busulfan
BW	body weight
CDDP	cis-diamminedichloroplatinum (II), cis-platinum
CFU-c	colony-forming unit in culture
CFU-F	colony-forming unit fibroblasts
CFU-G/M	colony-forming unit granulocytes/macrophages
CFU-GEMMM	colony-forming unit granulocytes, erythrocytes, macrophages, and megakaryocytes
CFU-s	colony-forming unit in the spleen
CTX	cyclophosphamide
DTH	delayed type hypersensitivity
EPO	erythropoietin
G-CSF	granulocyte colony-stimulating factor
G/M-CSF	granulocyte/macrophage colony-stimulating factor
GvH	graft-versus-host
Gy	gray
HGF	hemopoietic growth factor
HM	hemopoietic microenvironment
IL-1	interleukin-1
IL-3	interleukin-3
i.p.	intraperitoneal
i.v.	intravenous
LPS	lipopolysaccharide
LTBMC	long-term bone marrow culture
M-CSF	macrophage colony-stimulating factor
MTX	methotrexate
PBS	phosphate buffered saline
PHSC	pluripotent hemopoietic stem cell
SEM	standard error of the mean
TBI	total body irradiation
TNF	tumor necrosis factor
VCR	vincristine

CHAPTER I

INTRODUCTION

I.1 GENERAL INTRODUCTION

Blood consists of plasma containing cells and cell fragments. Three groups of blood cells can be identified, namely **(a)** red blood cells or erythrocytes, **(b)** white blood cells or leucocytes and **(c)** platelets or thrombocytes. The white blood cells can be separated into neutrophilic-, eosinophilic- and basophilic granulocytes, lymphocytes and monocytes. The life time of these cells is limited. For example, in man life time for normal erythrocytes is about 120 days and for granulocytes a few days. The daily production of these cells required to maintain normal numbers in blood can be calculated from their life span, their concentration in the blood and the total blood volume. Thus, an adult human male produces approximately 2 x 10^{11} erythrocytes and 1 x 10^{10} granulocytes every day. These amounts are necessary for normal maintenance but the production of blood cells can be increased after stress, for example, infection or blood loss.

During embryogenesis blood cell production starts in the yolk sac and subsequently during fetal life it can be found in the liver, spleen and bone marrow (Moore and Metcalf, 1970; Niewisch, 1970). In adult mammals production of hemopoietic cells occurs mainly in the cavities of skeletal bones. The proportion of the bone marrow cavity that is filled with hemopoietic cells depends on species, age and the demand for functional cells. When there is an extra demand for blood cells, hemopoiesis extends to normally inactive bone marrow and sometimes hemopoietic activity can even be found in organs with active hemopoiesis during embryogenesis, e.g. spleen and liver. In mice (the animal used for the investigations described in this thesis) the skeleton is completely filled with active marrow and normally some hemopoiesis is also found in the spleen.

In principle, a single progenitor cell, the pluripotent hemopoietic stem cell (PHSC), has the capacity to produce all mature blood cells. The PHSC can produce daughter cells which have virtually the same capacities as the original cell (self renewal) and cells with a limited proliferative capacity and a commitment to one or more differentiation pathways. Blood cell production (self renewal, proliferation, differentiation and maturation) is regulated by humoral and stromal components that will be discussed in the following chapters. Investigation of the PHSC and early progenitors has been stimulated greatly by the discovery of *in vivo* and *in vitro* clonal assays for these cells. The development of a culture technique to maintain *in vitro* hemopoiesis for several months has greatly facilitated the characterization of the stromal cells of the hemopoietic microenvironment (HM) and their role in supporting hemopoiesis.

The hemopoietic system has a large reserve capacity for increasing the output of mature cells in response to increased demands. There is no evidence of exhaus-

tion with age (Boggs et al., 1984; Botnick et al., 1982; Schofield et al., 1986; Tyan, 1982), although the bone marrow reserve capacity can be diminished (Williams et al., 1986). Exposure to high dose radiation and/or chemotherapeutic agents (e.g. used for cancer treatment, during warfare, or as a consequence of an industrial accident) may result in acute and or long-term damage to the hemopoietic system. Acute damage to the hemopoietic system is often the dose limiting factor for these cancer treatments and higher doses can only be given when they are followed by a bone marrow transplantation (BMT). Usually the hemopoietic system regenerates very quickly, resulting in (almost) normal numbers of mature and progenitor cells in blood and bone marrow. Sometimes, however, persistent defects may be observed in the stem cell compartment and also in the stromal HM. Late complications that may result from chemotherapy or irradiation are obviously of particular relevance in patients with cancer in whom prolonged remission or a cure is achieved. The experiments described in this thesis were performed to study the effects of radiation and chemotherapy upon the hemopoietic tissue, with an emphasis on the stromal cells of the HM.

I.2 HEMOPOIESIS

I.2.1 THE PLURIPOTENT HEMOPOIETIC STEM CELL AND THE EARLY PROGENITORS

The mature, functionally active cells (leucocytes, erythrocytes, etc.) are produced by proliferation and differentiation of pluripotent hemopoietic stem cells (PHSC) and progenitors. Essentially, one PHSC is capable to produce all hemopoietic cells of an individual. Certain tissue cells also originate from the PHSC, like osteoclasts (Ash et al., 1980; Scheven et al., 1986), tissue mast cells (Kitamura et al., 1981), non-lymphoid (dendritic) cells of peripheral lymph nodes (Pugh et al., 1983), epidermal Langerhans cells (Volc-Platzer et al., 1984), antigen-presenting cells in the thymus (Longo and Schwartz, 1980) and Kupffer cells (van Furth, 1980). The capacity of PHSC to produce both lymphoid and myeloid progeny has been demonstrated by Wu et al. (1968) and Abramson et al. (1977) using radiation-induced chromosomal aberrations and recently by Lemischka et al. (1986) using retrovirus-mediated gene transfer to mark hemopoietic stem cells *in vitro*. Using the last method, it has been demonstrated that only a few (1-2) stem cell clones account for the majority of all hemopoietic cells found at any time in the mouse. Furthermore, it is suggested that normal hemopoiesis results from the sequential activation of different stem cell clones rather than from an averaged contribution of the entire stem cell pool (Burton et al., 1982; Lemischka et al., 1986; Mintz et al., 1984). The latter concept introduced by Kay (1965) is supported by studies using long term bone marrow cultures (Chertkov et al., 1985; Reincke et al., 1985). It is suggested from studies by Botnick et al. (1982) and Mauch et al. (1982) that

a selective utilization of hemopoietic stem cells with a lower mean self-renewal capacity occurs *in vivo* to maintain hemopoiesis.

The PHSC and early progenitors committed to one or several hemopoietic lineages occur in very low frequencies in hemopoietic tissue and are very difficult to identify (if at all) in these tissues on pure morphological features. The proliferative and differential capacities of these cells can be examined using *in vivo* or *in vitro* assays. Till and McCulloch (1961) have been the first to describe an *in vivo* method to estimate the number of hemopoietic stem cells (the spleen colony-forming unit or CFU-s). Murine CFU-s are assayed by intravenous injection of a small number of hemopoietic cells from bone marrow, spleen, fetal liver or any other organ containing CFU-s into lethally irradiated recipient mice. The hemopoietic stem cells that home in the spleen proliferate extensively in the irradiated hosts and seven to 15 days after injection macroscopically visible nodules appear on the splenic surface. The number of spleen nodules is during the first 12 days after injection linearly related to the amount of injected normal bone marrow cells (BMC)(Till and McCulloch, 1961). The absolute number of PHSC can be calculated by determination of the fraction of injected cells that seed into the spleen.

Recently it has been shown that the cells determined with the CFU-s assay consist of a heterogeneous population (Magli et al., 1982; Wolf and Priestley, 1986). About half of the day-7 spleen colonies are transient and about half of the day 11/12 colonies are not yet visible on day 8, although colony numbers derived from normal hemopoietic tissue remain constant between 7-15 days. These observations suggest that the early, transient, spleen colonies are derived from stem cells with restricted proliferative potential, though with pluripotent properties, or are derived from the progeny of precursor cells with restricted differential potentials and therefore do not arise from PHSC. Further evidence for the idea of heterogeneity of the CFU-s population is the observation that with fluorescence-activated cell sorting different cell fractions can be obtained which almost exclusively give rise to either day-7 or day-12 spleen colonies (Ploemacher and Brons, 1988a; 1988b; Ploemacher et al., 1987). The occurrence of late appearing colonies may be explained by the assumption that these colonies are derived from the same cell type as the early appearing colonies but originate from a 'younger' stem cell that proliferates in the bone marrow and subsequently migrates to the spleen to give rise to several day 12 colonies (Hodgson and Bradley, 1979; Mulder et al., 1984; Wolf and Priestley, 1986; van Zant, 1984). This hypothesis may also explain the observation that a single cell may give rise to more than one spleen colony (Barnes et al., 1968). There is also some evidence that the spleen colony assay does not detect a type of stem cell with sufficient self-renewal capacity *in vivo* to repopulate an entire individual (Boggs et al., 1982, 1984; Magli et al., 1982; Sharkis et al., 1978). Furthermore, evidence has been presented that day 12 or day 8 CFU-s are not the cells responsible for long-term *in vitro* hemopoiesis but that the latter is supported by a population of younger, resting stem cells (not in cell cycle) (Chertkov et al., 1986; Spooncer et al., 1985). The above men-

tioned observations stress the idea of heterogeneity of the stem cells and the existence of a gradual scale or a continuum of cells with a decreasing self-renewal capacity, increasing commitment (Chertkov et al., 1986; Grossman, 1986; Hellman et al., 1978; Hodgson and Bradley, 1979; Van Zant, 1984) and a different capacity to lodge in the spleen or bone marrow (Ploemacher et al., 1987; Wolf and Priestley, 1986). The youngest progenitor or 'pre CFU-s' is a resting cell, not in cycle and consequently not very sensitive to certain cytostatic agents, e.g. the antimetabolites and other cell cycle dependent cytostatic agents (Chertkov et al., 1986; Rosendaal et al., 1979). The proportion of cells in cycle increases with increasing commitment e.g., day 12 CFU-s are less in cell cycle than day 8 CFU-s and these again less than CFU-C (Chertkov et al., 1986; Hodgson and Bradley, 1979; Pietrzyk et al., 1985).

Pluripotent cells with high self renewal capacity can also be assayed by *in vitro* methods (Keller and Philips, 1982; Nakahata and Ogawa, 1982). The frequency of progenitors, which still have the capacity to differentiate in more than one direction, and cells which are committed to a single line of differentiation (e.g. CFU-GEMM, CFU-GM, BFU-E or CFU-E) can commonly be determined in semi-solid cultures (Bradley and Metcalf, 1966; Johnson and Metcalf, 1977, 1980; Metcalf and MacDonald, 1975). Survival, self-renewal, proliferation and differentiation of PHSC and progenitors *in vivo* and *in vitro* is dependent on specific factors and will be discussed in the next chapters.

I.2.2 THE HEMOPOIETIC MICROENVIRONMENT

The occurrence of effective hemopoietic activity in restricted parts of the body is strong evidence for the existence of a specific HM. In the adult, hemopoiesis is restricted to red bone marrow, whereas in the mouse, the spleen is also an organ with active hemopoiesis.

I.2.2.1 MORPHOLOGY OF THE HEMOPOIETIC MICROENVIRONMENT AND SPATIAL DISTRIBUTION OF HEMOPOIESIS

The hemopoietic cells in bone marrow are surrounded by cells and cell products which form the HM. The HM can be divided in several compartments: **(a)** a vascular compartment of arteries, veins and sinuses, **(b)** a neural compartment of myelinated and unmyelinated nerve fibers and Schwann cells, **(c)** a connective tissue compartment of adventitial reticular cells, (pre)adipocytes, fibroblasts, macrophages and an extracellular matrix of fibers and amorphous ground substance (reviewed by Lichtman, 1981, 1984; Weiss and Sakai, 1984).

In mammals hemopoietic cell proliferation occurs under normal steady state conditions extravascularly. Hemopoiesis is not randomly distributed in the HM but there is a complete or regional segregation of the various lines of differentiation in human spleen and bone marrow. Young granulopoietic cells are preferentially

found close to bone and a gradient of more mature granuloid cells is found towards the axial center of the bone marrow (Lennert, 1952). Megakaryocytes are localized alongside of the sinuses, and large cytoplasmic protrusions from which platelets are derived can be found in the sinus lumen (Becker and De Bruyn, 1976). Clusters of erythroblasts with a central macrophage surrounding them are also found close to the sinus wall (Bessis, 1958). No clear morphologic evidence exists for a regional distribution of hemopoiesis in the mouse bone marrow. However, in some mouse strains the hemopoietic progenitor cells CFU-s, CFU-G/M and BFU-E, are preferentially localized in specific regions of the bone marrow (Lord et al., 1975; Xu and Hendry, 1981). The CFU-s with a high self- renewal capacity and a low cycling rate are found axially and CFU-G/M, BFU-E and cycling CFU-s are found close to the bone. Furthermore, a stromal cell population producing a factor inhibiting CFU-s proliferation is found in axial bone marrow and a cell population of mononuclear phagocytes that produce a stimulator for CFU-s proliferation is found close to the bone (Wright and Lord, 1986; Wright et al., 1982). Further evidence for the spatial distribution of hemopoiesis has been demonstrated by the development of endogenous hemopoietic colonies after irradiation in both spleen and femur. During the first 7-8 days after irradiation, most endogenous colonies are of single lineage with a typical distribution in the marrow. Undifferentiated granulopoietic and megakaryopoietic colonies have been found near bone whereas erythrocytic colonies tend to proliferate in intermediate and central marrow zones (Lambertsen and Weiss, 1983). The spatial distribution of erythropoiesis in central areas and of granulopoiesis and thrombopoiesis concentrated along bone remains for several weeks after irradiation. Hemopoietic colonies developing in spleen after irradiation and BMT also show a spatial distribution, i.e. the large erythroid colonies are subcapsular and do not grow into the stroma, in contrast with the granulocytic colonies growing alongside splenic capsule and trabeculae (Curry et al., 1967; Wolf and Trentin, 1968; Wolf and Rosse, 1982). The distribution of CFU-s in spleen is also not at random, the highest concentration of CFU-s is found subcapsular (Ploemacher and Brons, 1985). Upon infusion of normal BMC into lethally irradiated mice, the ratio of erythroid versus granuloid colonies (E:G ratio) is about 0.7 in the marrow, while it averages 3.5 in the spleen (Wolf and Trentin, 1968). The E:G ratio estimated after injection of normal BMC in both spleen and femur is determined by the microenvironment and does not change after e.g. erythropoietin (EPO) stimulation or repression, cytotoxic treatment or transplantation of the organs to other sides of the body (Curry et al., 1967; Ploemacher et al., 1982). The E:G ratio of marrow stroma even remains unchanged after transplantation into the spleen (Wolf and Trentin, 1968).

I.2.2.2 FUNCTIONAL ASPECTS OF THE HEMOPOIETIC MICRO-ENVIRONMENT

The HM possesses a variety of functions for an optimal production of normally

active hemopoietic cells. The functions of the HM can be divided in **(a)** nourishment of the hemopoietic cells and removal of waste products, **(b)** regulation of self-renewal, proliferation and differentiation of the hemopoietic progenitors, **(c)** storage of functional cells, **(d)** regulation of the release of functional cells into the blood stream, **(e)** lodgement of PHSC and other progenitors from the blood into the HM.

Nourishment, removal of waste products, release of hemopoietic cells from the bone marrow, and lodgement of hemopoietic cells are regulated by the blood vessels and/or the vascular wall that consists of endothelial cells, basement membrane and adventitial reticular cells. The main arterial blood supply of the HM comes from the nutrient artery. It penetrates the cortex through the nutrient canal and bifurcates in ascending and descending medullary arteries from which radial small branches form the cortical capillaries which drain into the marrow sinuses, while the blood leaves the marrow via an emissary vein (Brookes, 1971). The diameter of the blood vessels in the microcirculation of the bone marrow is variable, which permits regulation of the blood flow through the sinuses. The bone marrow microvasculature can be rebuilt to a considerable extent in response to increased demands for hemopoietic activity as has been demonstrated by McClugage et al. (1971) using a device in the tibia of rabbits for *in vivo* microscopy.

Hemopoietic cells enter and leave the HM through the sinuses. The endothelial cells are thought to be responsible for the selective lodgment of hemopoietic progenitors in both spleen and bone marrow during their migration between different parts of the body and after a BMT. The mechanism of the interactions of progenitors and mature blood cells with the endothelial cells is still not understood. However, one of the factors determining homing of progenitors might be the existence of specific surface glycoproteins on the progenitors (Hardy et al., 1986; Ploemacher and Brons, 1981; Ploemacher et al., 1981a, 1981b).

The regulation of the release of functional hemopoietic cells into the blood stream is complex and several cell types, e.g. endothelial cells, adventitial reticular cells and the hemopoietic cells themselves may take part in this regulation (Campbell, 1982). The adventitial reticular cells cover the vascular sinus and are lifted away as maturing hematopoietic cells approach the sinus (Weiss, 1976). The lining of the sinuses with adventitial reticular cells is incomplete and can be changed according to the demand of functional cells. The adventitial cover is decreased after endotoxin-induced leukopenia (Weiss, 1970) or after an EPO injection (Chamberlain et al., 1975a) and is increased after hypertransfusion (Chamberlain et al., 1975b). A reduction of the sinusoidal lining by adventitial reticular cells is not a prerequisite for an increased release of functional cells, other factors (e.g., the complement system) and/or cells are also contributing to the regulation of cell egress (Ploemacher et al., 1980). Furthermore, the egress of hemopoietic cells into the blood stream may be an active process of the contractile adventitial reticular cells by squeezing the cells through the pores in the endothelial cells (Campbell, 1972; Tavassoli, 1977; Weiss, 1970). Changing the blood flow through the marrow sinuses may be another possibility to change cell egress. The blood cells migrate

through pores in the endothelial cells of the sinus wall and not between endothelial cells. The migration is efficiently regulated under normal conditions, i.e. mature blood cells enter the blood stream while immature forms remain in the marrow cords (De Bruyn et al., 1971; Chamberlain and Lichtman, 1978).

The proliferative status of stem cells and the self-renewal capacity is probably under influence of stromal cells. The nature of these cells is not yet fully elucidated, but macrophages are thought to be the cell type producing the stimulator for stem cell proliferation (Lord and Wright, 1982). It is believed that PHSC are quiescent cells with respect to cell cycle activity and that induction of proliferation subsequently decreases the self-renewal capacity and increases differentiation (Grossman, 1986). Schofield (1978) has proposed the existence of special stem cell niches, which are assumed to prevent the stem cell from going into cycle and to mature i.e. to become sensitive to specific hemopoietins and subsequently loose its self-renewal capacity. Using a long-term culture technique, it has been demonstrated that primitive hemopoietic progenitors are adherent to the stromal layer (Coulombel et al., 1983a; Reincke et al., 1985). Furthermore, implantation of the stromal layer of a long-term bone marrow organ culture in conjunction with the adherent primitive hemopoietic progenitors under the kidney capsule of lethally irradiated hosts gave rise to more day 12 and less day 8 spleen colonies than intravenous injection of a single cell suspension derived from similar cultures. Apparently, transplantation of stem cells in close association with stromal cells appears to maintain more primitive stem cells than transplantation without these stromal cells (Sharp et al., 1985). Accordingly, the observation of decreasing self-renewal ability after serial transplantation may be due to the method used (Harrison et al., 1978; Hellman et al., 1978), because the stem cells are transplanted as a single cell suspension and the contact of PHSC with stromal cells is necessary for remaining "primitiveness" of the stem cells.

There are strong indications that the process of commitment is stochastic (Ogawa et al., 1983; Till et al., 1964), but the regulation of proliferation and differentiation of the committed progenitors is thought to be under influence of stromal cells. Evidence for a role of stromal cells regulating hemopoiesis is obtained from *in vitro* culture techniques. The role of each cell group that participates in the HM will be discussed briefly below.

I.2.2.3 THE CELLULAR COMPOSITION OF THE HEMOPOIETIC MICROENVIRONMENT WITH AN EMPHASIS ON REGULATION OF HEMOPOIESIS

A normal function of PHSC and progenitor cells appears to be dependent upon close range interactions with cells or cell products within the hemopoietic organs. The development of an *in vitro* culture system, the long-term bone marrow culture (LTBMC), for maintaining long-term hemopoiesis has facilitated the study of the interactions between the hemopoietic stroma and the hemopoietic progenitors.

The development of a stromal layer composed of endothelial cells, macrophages, fibroblasts, adventitial reticular cells, and adipocytes on the surface of the culture vessel is essential for long-term maintenance of hemopoiesis (Allen and Dexter, 1976; Dexter et al., 1973, 1977a, 1984). Almost all of the following data with respect to the role of stromal cells and their cell products regulating hemopoiesis are derived using this culture technique.

Some of the cell products stimulate hemopoiesis and are called hemopoietic growth factors (HGFs) and some factors inhibit hemopoiesis (reviewed by Broxmeyer, 1984; Moore et al., 1984; Resnitzky et al., 1986). The HGFs are glycoproteins with a molecular weight of 20 to 70 kDalton and can be divided in: **(a)** factors stimulating a variety of hemopoietic progenitor cells to proliferate and differentiate into more than one fully differentiated blood cell type, like interleukin-3 (Il-3) (Ihle et al., 1981, 1983; Iscove et al., 1985; Nicola and Metcalf, 1986; Walker et al., 1985) and granulocyte/macrophage-CSF (GM-CSF) (Burgess and Metcalf, 1977; Metcalf et al., 1980, 1986a); **(b)** lineage-specific growth factors, like EPO (Metcalf and Moore, 1971; Wagemaker et al., 1977), thrombopoietin (Evatt et al., 1976; McDonald, 1976), granulocyte-CSF (G-CSF) and macrophage-CSF (M-CSF) (Metcalf and Burgess, 1982; Metcalf and Nicola, 1983; Metcalf et al., 1986a; Stanley and Heard, 1977) and **(c)** factors which have no intrinsic colony stimulating activity but are synergistic to other HGFs like Hemopoietin-1 (is IL-1) (Stanley et al., 1986) or those changing the cycling rate of CFU-s (Guigon and Frindel, 1978; Guigon et al., 1982; Lord and Wright, 1982). Besides stimulating primitive progenitors to proliferate, some of the HGFs are also essential for survival of these progenitors by increasing glucose transport and ATP generation (Suda et al., 1985; Whetton et al., 1984) and, moreover, have an effect on mature cells. They can increase functional activity, e.g. phagocytosis, oxidative metabolism, inhibition of migration, antibody-dependent cytotoxic killing of tumor cells, expression of function-associated cell surface antigens and stimulation of the synthesis of membrane and nuclear proteins (Fleischmann et al., 1986; Gasson et al., 1984; Lopez et al., 1986; Nicola et al., 1986; Stanley and Burgess, 1983; Weisbart et al., 1985).

Many of the above mentioned HGFs have been purified, biochemically characterized, and produced using recombinant DNA techniques. *In vivo* injection of these purified and recombinant HGFs generates strong evidence for a physiological role of these substances in stimulating and regulating hemopoiesis. Continuous subcutaneous infusion or multiple intraperitoneal injections of IL-3 in normal mice results primarily in an increased hemopoietic activity of all hemopoietic lineages in the spleen (Kindler et al.,1986; Metcalf et al., 1986b). Continuous IL-3 infusion of lethally irradiated mice increases the recovery of injected hemopoietic progenitors (Kindler et al., 1986). Furthermore, 24 hours after a single injection of IL-3 the cycling state of femoral CFU-GEMM, BFU-E and CFU-GM is increased (Broxmeyer et al., 1986). The combined *in vivo* and *in vitro* results suggest that IL-3 production is increased during stress situations when there is a demand for more than one hemopoietic cell lineage. Increased IL-3 production will result in

the production of more early multipotential precursors which in turn produce more functionally active mature cells.

Continuous *in vivo* infusion of recombinant human GM-CSF into primates stimulates femoral hemopoiesis and evokes leucocytosis and reticulocytosis (Donahue et al., 1986). A single injection of murine recombinant GM-CSF into mice increases the cycling rate of CFU-GEMM and CFU-GM but not of progenitors committed to the erythroid lineage (Broxmeyer et al., 1986). Multiple intraperitoneal injections of murine recombinant GM-CSF in mice increases overall numbers of granulocytes and macrophages and their functional activity. Furthermore, an increased splenic and a relatively decreased femoral non-erythroid progenitor content has been found (Metcalf et al.,1987). These observations strongly support the idea that GM-CSF is a regulator of granulocyte-macrophage populations.

Lineage specificity of G-CSF is demonstrated *in vivo* by continuous intravenous infusion of (recombinant human) G-CSF in hamsters (Zsebo et al., 1986) and by multiple subcutaneous injections in monkeys (Welte et al., 1987). G-CSF increases the levels of peripheral neutrophilic granulocytes but has no effect on lymphocytes, monocytes or eosinophils in both hamsters and monkeys. Furthermore, G-CSF is capable to shorten the time period of granulocyte recovery after cyclophosphamide(CTX)-induced myelosuppression (Welte et al., 1987). Injection of EPO (the oldest purified HGF) into mice with suppressed erythropoiesis results in an increased erythroid proliferation and differentiation (Alexanian and Alfrey, 1970; Fried et al., 1970; Miller et al., 1983; Movssaghi et al., 1967) implicating an *in vivo* role for EPO in regulating erythropoiesis.

GM-CSF and other HGFs are detectable in serum of mice and are increased in response to bacterial products, infections and other stress situations (Karp et al., 1983; Metcalf et al., 1967; Ploemacher et al., 1986; Shadduck et al., 1971; Staber et al., 1978). Although no definitive proof is obtained it is very likely that the HGFs play a role in *in vivo* maintenance of hemopoiesis. The relative contributions of the different sources of HGFs (i.e. produced within the hemopoietic organs or outside bone marrow or spleen) are not known, however, it has been demonstrated that granulopoietic proliferation is associated predominantly with intramedullary HGF production (Chan and Metcalf, 1972, 1973). Extramedullary CSF production occurs as a response to infection and tissue damage and attracts and activates granulopoietic and monocytic cells (Metcalf, 1985).

The role of each different stromal cell type participating in the complex hemopoietic regulatory system and its capacity to produce HGFs will be discussed briefly below.

I.2.2.3.1 ENDOTHELIAL CELLS

A definitive role for marrow endothelial cells in the regulation of hemopoiesis has not yet been found. They have been detected in LTBMC from human (Castro-Malaspina et al., 1981a; Keating and Singer, 1983) and murine bone marrow (Zuckerman and Wicha, 1983). Endothelial cell layers (not from bone marrow origin)

can be stimulated *in vitro* to produce specific hemopoietic regulators or colony stimulating factors (GM-CSA and burst- promoting activity) by monocyte/macrophage conditioned media (Bagby et al., 1983a, 1986; Zuckerman et al.,1985), probably interleukin 1 (IL-1) (Segal et al., 1987) and/or tumor necrosis factor (TNF) (Broudy et al., 1986a; Munker et al., 1986). Both, IL-1 and TNF are monokines produced in response to an infection or tissue damage (Beutler et al., 1985; Pettipher et al., 1986) and both agents can stimulate endothelial cells to produce hemopoietic regulator molecules. Furthermore, both factors induce the production of colony stimulating factors after *in vivo* injection (Vogel et al., 1987). Gamma interferon and lipopolysaccharide (LPS) also induce endothelial cells to produce colony-stimulating activity (Adamson, 1986). Combining the above mentioned data, it is very likely that endothelial cells play a role in the local and/or systemic production of hemopoietic factors after an infection or other tissue damage. Whether endothelial cells play a role in regulating hemopoiesis in the bone marrow microenvironment is not known.

I.2.2.3.2 RETICULAR FIBROBLASTS

Long-term *in vitro* hemopoiesis is dependent on the development of a stromal layer. A quantitative and perhaps also qualitative important cell type in this stromal layer is the reticular fibroblast (reviewed by Bentley, 1984). *In vivo* the fibroblasts form a large three dimensional supportive network of cytoplasmic extrusions in association with reticular fibers throughout the marrow parenchyme (Weiss, 1976; Westen and Bainton, 1979). The fibroblastoid cells are not of hemopoietic origin as has been demonstrated using chromosomal markers (Golde et al., 1980; Hentel and Hirschorn, 1971; Lim et al., 1986) and the cultured fibroblastoid cells are different from macrophages and endothelial cells by surface markers (Piersma et al., 1985a), functional activity (phagocytosis and synthesis of certain factors like fibronectin, collagen, laminin, and factor VIII), and morphology (Bentley and Foidart, 1980; Bentley et al., 1981; Castro-Malaspina et al., 1980; Jaffe and Mosher, 1978; Piersma et al., 1985a; Yamada and Olden, 1978).

There is a lot of indirect (morphological) evidence that fibroblasts play a role in hemopoiesis both in mice and man (Chen and Weiss, 1975; Ploemacher et al., 1984a; Westen and Bainton, 1979). A supportive and determinative role for hemopoietic organ derived fibroblasts has been demonstrated after *in vitro* culture and autologous retransplantation of the cultured fibroblasts under the kidney capsule. These fibroblasts can produce an active hemopoietic tissue fragment similar to the place of origin, i.e. spleen or red or yellow bone marrow (Bainton et al., 1986; Friedenstein et al., 1974a; Patt et al.,1982). The fibroblasts derived from red or yellow marrow do not differ morphologically by electron microscopy and both produce the same types of collagen (I and III). However, these fibroblasts differ strikingly in enzymatic content of alpha-naphtylbutyrate esterase, which is abundant only in the yellow marrow derived cells (Bainton et al.,1986). Whether yellow and red marrow fibroblasts display different phenotypes of the same cell type or are

in fact different cell groups is unknown. The first option is more likely since fibroblastoid cells can be induced to produce fat (Greenberger, 1978; Tavassoli, 1984a). A decreasing hemopoietic activity is correlated with an increasing content of adipocytes *in vivo* and perhaps also *in vitro* (Touw and Löwenberg, 1983).

Indirect evidence for an *in vivo* regulating role in hemopoiesis of fibroblastoid cells is demonstrated by the capability of these cells to produce several regulator molecules *in vitro* (Blackburn and Patt, 1977; Brockbank and van Peer, 1983; Harigaya et al., 1981; Wilson et al., 1974; Zipori et al., 1982). However, some groups have failed in detecting CSA in both murine and human fibroblast-conditioned media (Bentley and Foidart, 1980; Castro-Malaspina et al., 1980; Greenberg et al., 1981). This may be due to consumption or degradation of the produced growth factor(s) by contaminating cells e.g. macrophages (Ploemacher et al., 1984; Tushinski et al., 1982). With the help of other cells or cell products, e.g. macrophages, T lymphocytes, endothelial cells, or Il-1, fibroblasts can also produce multilineage growth factors (Broudy et al.,1986b; Zucali et al., 1986) or other regulating factors (Nagao et al., 1986; Piersma et al., 1985a; Zipori et al., 1985). Furthermore, some bone marrow derived fibroblastoid cell lines have been reported to support the continuous growth of early hemopoietic and lymphoid progenitors (Hunt et al., 1987; Quesenberry et al., 1987).

There exists ample evidence for a regulatory role of fibroblastoid cells in *in vitro* culture systems, but their role in regulating hemopoiesis in the marrow HM is not (yet) proved. The relation of fibroblastoid progenitors with disease and the quantitative and qualitative differences during disease and after cytotoxic treatment will be discussed in chapter I.3.3.

I.2.2.3.3 MACROPHAGES

The resident bone marrow macrophages are stromal cells derived from the PHSC and can be distinguished from fibroblasts and endothelial cells by their capacity to phagocytise, by surface membrane antigens (Springer et al., 1979), and by acid phosphatase activity. Many studies have shown intimate associations between macrophages and developing hemopoietic cells and some of the macrophages possess long cytoplasmic protrusions which surround developing hemopoietic cells from both erythroid and myeloid lineages, e.g. the central macrophage in erythroblastic islets in bone marrow and in the adult murine liver after phenylhydrazine-induced hemolytic anemia and the resident bone marrow macrophage (Bessis et al., 1978; Crocker and Gordon, 1985; Hume et al., 1983; de Jong et al., 1987; Ploemacher, 1978). Macrophages are a very heterogeneous cell population with respect to the presence of specific surface antigens and perhaps also with respect to functional activity (Crocker and Gordon, 1985; de Jong et al., 1987).

Macrophages can produce multilineage and lineage specific growth factors, including EPO (Nicola and Vadas, 1984; Rich et al., 1982), and products that stimulate fibroblasts (Zuckerman et al., 1985) and T lymphocytes (Bagby et al., 1981) to produce multilineage and lineage-specific growth factors. The *in vitro*

production of EPO (and other growth factors) by macrophages can be regulated by varying the oxygen tension during culture (Pennathur-Das and Levitt, 1987; Rich, 1986a, 1986b). On the other hand, macrophages can inhibit cell growth by degrading growth factors (Ploemacher et al.,1984a; Tushinski et al., 1982) or by producing inhibiting factors (reviewed by Broxmeyer, 1984; Moore et al., 1984; Resnitzky et al., 1986). Recently it has been demonstrated by Gewirtz et al. (1986) that removal of macrophages from a bone marrow culture from a patient with acquired amegakaryocytic thrombocytopenic purpura (AATP) significantly augments megakaryocyte formation. This megakaryocyte inhibitory activity has been found also in the medium conditioned by the patients macrophages. It has been suggested that in some cases, AATP may be due to a stromal defect, in particular a dysfunction of macrophages regulating megakaryocytopoiesis (Gewirtz et al., 1986). Furthermore, other factors (e.g. lactoferrin, produced by neutrophilic granulocytes) may modulate macrophages to produce regulator molecules (Bagby et al.,1983a). These observations taken together seem to indicate that macrophages in the HM play a role in the regulation of hemopoiesis.

I.2.2.3.4 T LYMPHOCYTES

T lymphocytes are derived from the PHSC and usually are not considered to be a part of the hemopoietic stroma. Activated T lymphocytes are thought to be the only cell type capable of producing the multilineage growth factor IL-3 (Ihle et al., 1983; Parker and Metcalf, 1974). However, some cell lines, not derived from T cells, also produce IL-3 (Iscove et al.,1982; Metcalf et al., 1969). Activated T lymphocytes have the ability to produce other factors that stimulate hemopoiesis, e.g. GM-CSF (Cline and Golde, 1974; Gasson et al., 1984; Metcalf and Johnson, 1978; Pluznik et al., 1986; Staber et al., 1982). The production and/or release of these growth factors (IL-3 and GM-CSF) by T lymphocytes may be regulated by different (so far unknown) mechanisms, since IL-3 production is inhibited by cyclosporin-A while GM-CSF production is not affected (Pluznik et al., 1986). Furthermore, T lymphocytes have been implicated in regulation of seeding, proliferation and differentiation of hemopoietic stem cells and early committed progenitors (Goodman et al., 1980; McMillen and Simmons, 1981). These effects of T lymphocytes can be interpreted as effects produced by interactions between lymphocytes and marrow stromal cells (Gordon et al., 1982; Wiktor-Jedrzejczak et al., 1981). Although not essential for *in vitro* bone marrow hemopoiesis, T lymphocytes may play a role in regulating both early and mature stages of erythroid differentiation (Estrov et al., 1986; Goodman and Shinpock, 1972; Keller et al., 1983; Lipton et al., 1983; Lord and Schofield, 1973; Lu et al., 1986; Torok-Storb et al., 1981; Wiktor-Jedrzejczak et al., 1977). In addition, hemopoiesis in long-term cultures with a splenic stromal layer are dependent on the presence of T lymphocytes, while the T lymphocytes appear to play no role in the maintenance of hemopoietic activity in LTBMC (Keller et al., 1983). Recently, two separate thymocyte populations have been isolated, one of which stimulated *in vitro* erythropoiesis, while the other (larger)

population inhibited erythroid colony formation (Sharkis et al., 1986). In humans it has been shown that bone marrow cultures with lymphocytes from patients with Diamond-Blackfan syndrome (Hoffman et al., 1976) or aplastic anemia (Ascensao et al., 1976; Hoffman et al., 1977; Torok-Storb et al., 1980) can inhibit erythroid and/or myeloid colony formation. Lymphocytes from a patient with acquired amegakaryocytic thrombocytopenic purpura are capable to inhibit megakaryocyte formation (Gewirtz et al., 1986). Furthermore, some patients with granulopoietic failure (low blood neutrophil counts and low CFU-GM colonies in bone marrow) had steroid-sensitive T lymphocytes in their bone marrow inhibiting CFU-GM formation (Bagby et al., 1983b), again suggesting a role for T lymphocytes in (dys)regulating hematopoiesis.

In conclusion, within the HM a variety of different cell types participates in the regulation of hemopoiesis by producing HGFs and other stimulating or inhibiting substances. These accessory cells also produce immunoregulatory cytokines (IL-1, IL-2) which stimulate cells in their proximity to produce HGFs (Bagby et al., 1983a; Kasahara et al., 1983). Thus, a complex mechanism of interacting marrow cells and locally produced humoral factors appears to regulate hemopoiesis. The extramedullary produced HGFs also exert their effects locally, as suggested by the observation that in response to an infection, HGFs attract and activate the cells which can eliminate micro-organisms initiating the response. Only in severe infections HGF levels are high enough to stimulate the bone marrow (Metcalf, 1985).

I.3 ASSAYS TO STUDY THE HEMOPOIETIC STROMA

Several assays have been developed to assess the functional activity of the hemopoietic stroma. The functional activity of the hemopoietic stroma, with respect to the assays used, and other factors determining the test results can be separated into: **(a)** the proliferative status of stromal cells; **(b)** the hemopoietic supportive function of stromal cells, e.g. the production of growth factors or **(c)** a combination thereof.

Under normal conditions the hemopoietic stroma shows little proliferative activity (Caffrey et al., 1966; Haas et al., 1969; Kaneko et al., 1982; Meyer-Hamme et al., 1971). However, when there is an extra demand for functional cells (e.g. infection or blood loss) regeneration and remodelling of the hemopoietic stroma does occur, including proliferation of stromal cells, in addition to increased proliferation of hemopoietic progenitors (Da et al., 1986; Hashimoto, 1962; McClugage and McCuskey, 1970; McCuskey et al., 1971). The application of assays in which stromal cells are induced to proliferate can be used to measure proliferative defects and to estimate the capacity of the hemopoietic stroma to respond to stress-induced proliferation or to replace non-functional stromal cells *in vivo*. In some mouse strains this reserve capacity of splenic and femoral stromal cells to proliferate

attenuates with increasing age although these cells do not show evidence of *in situ* failure during the normal life-span of the animal (Hotta et al., 1980; Ploemacher, 1981; Wolf and Arora, 1982). However, this diminution of stromal cell reserve capacity may be expected to become apparent when a destructive agent causing reduction of the stromal cells is followed by a demand for these cells to replicate. Examples for this hypothesis will be discussed later.

Indications to justify separation of the stromal functions are demonstrated by several authors. They all show that a defective proliferative capacity of the stroma is not necessarily accompanied by a defective stromal ability to support hemopoiesis *in vivo* and *in vitro* (Laver et al., 1986; Naparstek et al., 1985; 1986; Ploemacher et al., 1983). The assays used to determine stromal function in the experiments described in this thesis will be discussed briefly below.

I.3.1 QUANTIFICATION OF PERIPHERAL BLOOD CELLS AND HEMOPOIETIC PROGENITORS IN SPLEEN AND BONE MARROW

Quantification of peripheral blood cell numbers (leucocytes and erythrocytes) and hemopoietic progenitor cell numbers (CFU-s, BFU-E and CFU-GM) in the hemopoietic organs (spleen and femur) can be used to determine the *in vivo* stromal capacity to maintain hemopoiesis, on the condition that the quantity and the quality of the PHSC is normal. The usefulness of quantification of peripheral blood cells to estimate stromal function is limited. Changes in the production rate, and compensatory proliferation from committed progenitors do occur (Cronkite et al., 1985) and mask damage to the hemopoietic stroma. However, since immediate survival of the individual is dependent upon the presence of normal functional end cells, determination of these cells after cytotoxic treatment is necessary to control the acute effects of cytotoxic treatment. Quantification of CFU-s or CFU-GM has been used often to determine the damage to the hemopoietic stroma caused by radiation (Kedo et al., 1976; Nelson et al., 1977; Ploemacher et al., 1983), cytostatic agents (Fried and Barone, 1980) or a combination of both (Gregory et al., 1971; Wathen et al., 1981, 1982). This assay gives information about the total functional activity of the *in situ* hemopoietic stroma and in contrast with almost all other stromal assays discussed below, stromal cells are not forced to proliferate. However, any conclusions on possible functional insufficiency of specific stromal cell populations can not be made.

I.3.2 ECTOPIC IMPLANTATION OF BONE MARROW OR SPLEEN

Femurs (with both ends cut off) and spleens, or marrow plugs and spleen fragments, can be implanted in the subcutaneous tissue of the abdomen or under the kidney capsule. The implanted organs show extensive regeneration with accompanying partial restoration of hemopoiesis over a period of a few weeks.

In contrast with other organs, like kidney or liver, the bone marrow tissue does not undergo coagulation necrosis after subcutaneous implantation (Tavassoli, 1976; Tavassoli and Crosby, 1970, 1971). The marrow tissue has an angiogenic potency. Within 12 hours after implantation newly formed host-derived capillaries pierce into the implant and make contact with the donor capillaries and sinusoids. This revascularization is necessary for the survival and regeneration of the hemopoietic stroma. The hemopoietic stroma is sensitive to lack of vascular supply and if this is interrupted for more than 12 hours, e.g. by transferring the implant to a new site during the first day, the implant does not survive (Tavassoli, 1976). If the vascular supply is restored in time, marrow regeneration can be divided into four phases: **(1)** loss of hemopoietic cells and proliferation of the surviving stromal cells to form a monotonous tissue resembling primitive mesenchym within 4-6 days after transplantation; **(2)** formation of primitive sinusoids of donor origin and cancellous bone between 6- 10 days; **(3)** maturation of sinuses and **(4)** active hemopoiesis from 2 weeks after transplantation. The maximal hemopoietic activity is achieved 4 to 6 weeks after implantation (10-25% of the activity in a non-transplanted femur) and this remains constant for the rest of the animal's life (Sahebekhtiari and Tavassoli, 1978; Tavassoli, 1984b).

In contrast to the bone marrow transplant, the spleen transplant undergoes coagulation necrosis like subcutaneous transplants from kidney or liver, but a small peripheral rim of splenic stromal cells survives and subsequently reconstructs the stromal compartment in the necrotic center of the spleen and restoration of hemopoietic activity occurs within a few weeks (Chamberlin et al., 1974; Ploemacher et al., 1982; Tavassoli, 1976; Tavassoli et al., 1973a).

The growth of the implanted organs is under influence of host factors. Implant growth, in particularly that of the splenic implants, is stimulated by irradiation of the host before implantation, or by bleeding or hypertransfusion of the host during growth of the implants (Chamberlin et al., 1974; Molineux et al., 1987; Ploemacher et al., 1982). Furthermore, growth of the implants is dependent on the sex of the recipients. Female mice do not stimulate the growth as well as male recipients. This suppressive effect in female mice is partly strain-dependent and can be reduced considerably by sublethal irradiation of the female recipients before implantation. In addition, radiation chimeras with bone marrow of male origin support the growth of implants better than those recipients in which the bone marrow is of female origin (Schofield, 1986a). An explanation for these observations has not been provided.

The stromal cells (fibroblasts, osteoblasts, endothelial cells, adventitial reticular cells and bone-forming cells) of the implant are mainly from donor origin and the majority of the hemopoietic cells (CFU-s and CFU-G/M) are host derived (Amsel and Dell, 1971; Fried et al., 1973a; Friedenstein et al., 1968; Hotta et al., 1983; Maniatis et al., 1971; Sahebekhtiari and Tavassoli, 1978; Tavassoli and Khademi, 1980). Thus, the total amount of hemopoietic cells and/or hemopoietic progenitors (CFU-s, BFU-E and CFU-G/M) accumulated in the implants 6-8 weeks after

implantation can be used to estimate the stromal regenerative capacity or stromal function of the *in situ* hemopoietic stroma from the implant donors.

The hemopoietic activity in the ectopic implants is qualitatively comparable with the activity at the place of origin. Thus, yellow marrow implants remain yellow and red marrow implants show hemopoietic activity comparable with red marrow (Bigelow and Tavassoli, 1984). As mentioned in a previous chapter, marrow-derived fibroblasts implanted beneath the kidney capsule can also produce hemopoietic active ossicles, with similar hemopoietic activity as seen in the place of origin (Bainton et al., 1986; Patt et al., 1982). Furthermore, when hemopoietic organs from mice with a congenital anemia caused by a hemopoietic stromal defect (mice of the Sl/Sl^{d} genotype with a severe macrocytic anemia (Bernstein et al., 1968; McCulloch et al., 1965; Russell, 1979), and the Sl^{j}/+ mutant mouse strain with a mild macrocytic anemia (Mintz and Cronmiller, 1978), are implanted into normal hosts with normal stem cells, they remain deficient in their capacity to stimulate hemopoiesis (Fried et al., 1973b; Ploemacher and Brons, 1984; Tavassoli et al., 1973b). An excellent demonstration of conservation of an implants functional background is the observation that the stromal defect in Sl/Sl^{d} mice can only be cured following transplantation of normal stroma (Bernstein, 1970; Wolf, 1974) and not after injection of normal BMC (McCulloch et al., 1965; Sutherland et al., 1970). Normal implants do not affect the macrocytic anemia in W/W^{v} mice, which have an inherited stem cell defect (Tavassoli et al., 1973b). There are indications that in some mouse strains the proliferative capacity of hemopoietic stroma decreases during aging, resulting in a decreased hemopoietic activity in the implants (Hotta et al., 1980; Ploemacher, 1981; Wolf and Arora, 1982). Thus, the growth of implants may be considered as a functional test for the hemopoietic stroma. The above mentioned experiments also indicate that the stroma of the implants is from donor origin and that the normal stromal cells or cell products from the host do not migrate to establish normal stromal function in the implants from donors with an inherited stromal defect. However, recently it has been suggested that the recipient may in some way contribute to the development of the stromal microenvironment of the (femoral) implants and may be able to improve hemopoietic activity in the implants derived from mice with the Sl/Sl^{d} genotype (Ebbe et al., 1977; Schofield et al., 1987). The latter observations indicate that measurement of impaired stromal function using the implant assay may be underestimated.

The development of hemopoietic activity in implants derived from mice with a virus-induced leukemia transplanted into normal mice is not impaired but after normal hemopoietic reconstitution the implants are rapidly invaded by leukemic cells transplanted together with the implant (Levy et al., 1976; Ratzan et al., 1973). This observation suggests a normal stroma in this experimentally induced leukemia. The treatment of leukemia with radiation or chemotherapy, however, can damage the regenerative capacity of the subcutaneously implanted organs. Radiation with 5 Gy or more diminishes the regenerative capacity of femoral and splenic implants and they show no sign of recovery up to at least 1 year after irradiation

(Chamberlin et al., 1974; Fried and Adler, 1985; Fried et al., 1976; Piersma et al., 1983a; Ploemacher and van Soest, 1979; Ploemacher et al., 1983). The alkylating agents CTX and busulfan (BU) are also capable to diminish the regenerative capacity (Adler and Kuznetsky, 1984; Fried and Barone, 1980; Fried et al., 1977). Within 24 weeks after CTX but not after BU injection, normalization of the implant regeneration is found (Fried and Adler, 1985). These authors suggest qualitative differences in damage caused by CTX, BU, or irradiation. The question as to which cell population is most vulnerable for these cytotoxic treatments remains unsolved. Some other cytostatic agents, e.g. bleomycin, do not damage the regenerative capacity of the femoral stroma (Adler and Kuznetsky, 1984). These observations make it likely that only radiation and a small group of cytostatic agents, interfering with DNA structure, and perhaps some other drugs like chloramphenicol (Nara et al., 1982), are capable of suppressing the regenerative ability of hemopoietic stroma.

I.3.3 IN VITRO CULTURE OF FIBROBLASTOID STROMAL CELLS

This chapter deals with the quantitative and qualitative differences in fibroblastoid colony formation during disease and following treatment with radiation or drugs in both animals and humans.

In vitro culture of bone marrow-derived fibroblastoid cells is described for the first time by Carrel and Burrows (1910). Populations of cells from hemopoietic organs (spleen and bone marrow) formed adherent colonies in *in vitro* cultures. The predominant cell type in these adherent colonies are fibroblast-like cells. The fibroblastoid nature is further identified by their capacity to synthetize collagen type I and fibronectin (Bentley and Foidart, 1980; Reincke et al., 1982a). The clonal nature of these fibroblastoid cell colonies has been demonstrated by Friedenstein et al. (1974a) and many others (Castro-Malaspina et al., 1980; Gordon and Gordon-Smith, 1981; Greenberg et al., 1981; Nagao et al., 1981). Friedenstein et al. (1974a) also has introduced the concept of the fibroblastoid colony-forming unit or CFU-F, a stromal precursor cell capable of extensive proliferation, and of formation of a stromal tissue characteristic of the organ of origin and capable to produce, in *in vitro* culture, colonies of at least 50 cells.

Hemopoietic organ fibroblasts of several different mouse strains with hemopoietic deficiencies have been studied. The number of CFU-F colonies is normal in the bone marrow of mice with the Sl/Sl^{d} genotype (Brockbank and Ploemacher, 1983; Wiktor-Jedrzejczak, et al., 1983; Wilson and O'Grady, 1976) and the Sl^{J}/+ genotype (Brockbank and Ploemacher, 1983). The increased content in splenic CFU-F found in Sl/Sl^{d} mice is not specific for a stromal defect but related to a general response to chronic anemia (Brockbank and Ploemacher, 1983; Brockbank et al., 1985). So far, the only mouse strain studied which may have a defect in CFU-F quantity (low femoral CFU-F numbers) and quality (interaction with other stromal cells or growth factor production) is the mouse strain with congenital osteopetrosis, the op/op mouse. This defect is characterized by an absence of

functional osteoclasts (Marks and Lane, 1976) and a defective macrophage maturation accompanied with normal granulocyte numbers (Wiktor-Jedrzejczak et al., 1982). The defect in these mice can not be cured after a BMT with normal BMC, but BMC from op/op mice can reconstitute lethally irradiated mice in a normal way. BMC from op/op mice differentiate into mature macrophages *in vitro* when provided with CSA from normal mice and it has been reported that an intraperitoneal injection of op/op mice with conditioned medium from normal fibroblasts results in the production of mature macrophages (Wiktor-Jedrzejczak et al., 1982). Under normal culture conditions the fibroblast colonies are contaminated with macrophages and it has been suggested that macrophage progenitor cells (which are present in the cell suspensions used to culture CFU-F) are stimulated to proliferate by the fibroblastoid cells and overgrow the fibroblastoid colonies (de Jong et al., 1987; Wiktor-Jedrzejczak et al., 1983). The contamination of CFU-F colonies with macrophages (high in normal mouse CFU-F cultures) is very low in op/op mouse, presumably because of the lack of growth factor production (Wiktor-Jedrzejczak et al., 1982). These observations are very suggestive for a functional defect in fibroblastoid cells from op/op mouse resulting in insufficient monocyte/macrophage differentiation.

Femoral CFU-F content has been studied also in leukemic mice. In a murine myeloid leukemia models the CFU-F content has been observed to decrease with increasing tumor load (Ben-Ishay et al., 1984, 1985). This decreased CFU-F colony formation can also have resulted from inhibitory factors produced by leukemic cells during *in vitro* CFU-F colony formation (Ben-Ishay et al., 1984). Furthermore, Nagao et al. (1983a, 1983b) have demonstrated that human leukemia cells also can produce factors that inhibit CFU-F colony formation. Using another murine leukemia model, it was demonstrated that the femoral CFU-F content declines in the early stages of viral leukemogenesis while hemopoietic progenitors were not affected (Zipori and van Bekkum, 1979). In addition, Zipori and Bol (1979) suggested that fibroblastoid cells stimulated leukemic cells *in vitro*.

Sublethal irradiation of mice with 5-9 Gy severely decreases the CFU-F content of hemopoietic organs (Friedenstein et al., 1976, 1981; Greenberger et al., 1982; Piersma et al., 1983a, 1985b; Ploemacher et al., 1983; Xu et al., 1983). Following irradiation recovery of the CFU-F population is very slow and incomplete for a period of 3 upto 18 months after irradiation (Greenberger et al., 1982; Piersma et al., 1983a, 1985b; Ploemacher et al., 1983; Xu et al., 1983). Repopulation of CFU-F in the bone marrow can be enhanced after a second stromal damage e.g. LPS injection (Piersma et al., 1985b). From these observations it has been suggested that the irradiated stroma still contains the majority of viable fibroblastic cells, but that they have lost their proliferative capacity to form colonies *in vitro* due to latent radiation damage. Thus, radiation-damaged CFU-F that are not capable to form colonies *in vitro*, are possibly replaced by surviving CFU-F after LPS-induced CFU-F proliferation. However, a decreased proliferative capacity of stromal cells does not necessarily imply a decreased *in situ* functional activity (e.g. to

produce colony-stimulating activities) of these cells surviving the irradiation as has been shown for femoral stromal cells (Laver et al., 1986; Naparstek et al., 1985; 1986; Song and Quesenberry, 1984; Zuckerman et al., 1986a) and thymic stromal cells (Hirokawa and Sado, 1984).

In addition to irradiation, injection of some cytotoxic agents have also been reported to induce an acute decrease in CFU-F colony formation. A diminished femoral CFU-F content has been found after injecting mice with the alkylating agents BU or CTX. Following this initial decline the CFU-F population size slowly returns to suboptimal levels (McManus and Weiss, 1984; Molineux et al., 1986a, 1986b; Testa et al., 1985). The combination of radiation and either BU (Wathen et al., 1982) or CTX (Wathen et al., 1981) also causes a long-term defect in the marrow CFU-F population size. In contrast, injection of Ara-C and adriamycin in normal or leukemic mice enhances the femoral CFU-F content (Ben-Ishay et al., 1985). Sofar no explanation is available for the lather observation.

In humans CFU-F quantity and to a lesser extent CFU-F quality has been studied in several hematological disorders like myelofibrosis, aplastic anemia, myelodysplastic syndromes and leukemia. The CFU-F colony formation has been studied also after irradiation and/or cytotoxic agent treatment for leukemia and other malignancies.

The CFU-F population size and the functional activity of the cultured CFU-F from almost all patients studied with "idiopathic myelofibrosis" are within normal ranges (Castro-Malaspina and Jharwar, 1984; Castro-Malaspina et al., 1982; Hotta et al., 1985a). In most myelofibrotic patients an increased content of myeloid and/or megakaryocytic progenitors is found (Hotta et al., 1985a; McCarthy, 1985). It has been suggested that abnormal megakaryocytes release platelet-derived growth factor (PDGF) (Castro-Malaspina et al., 1981b; Groopman, 1980). PDGF stimulates collagen production and it is a potent mitogen for fibroblast proliferation. Although, the number of CFU-F in the bone marrow is not affected, PDGF enhances the CFU-F colony size (Hirata et al., 1985). The current opinion is that marrow fibrosis is not the result of an intrinsic CFU-F disturbance but is the response to an abnormal or excessive release of (a) factor(s) that stimulate fibroblasts to proliferate and secrete collagen. Further support for a reactive origin of myelofibrosis are the observations that myelofibrosis occurs in the rare "grey platelet" syndrome with excessive release of PDGF (Coller et al., 1983; Drouet et al., 1981), and that myelofibrosis in leukemia patients disappears after treatment of the leukemia (Manoharan et al., 1979; Mehta et al., 1983; Myers et al., 1974; Oblon et al., 1983; Wolf et al., 1982).

The results of CFU-F quantification in patients with aplastic anemia or leukemia and in patients treated for leukemia with irradiation and/or cytotoxic agents can be seen in Table I.1 CFU-F colony numbers in the bone marrow of patients with aplastic anemia are variable and this may be due to the heterogeneity in origin of aplastic anemia, i.e. a defective PHSC, autoimmune mechanisms (Ascensao et al., 1976) or stromal defects (Ershler et al., 1980). There are some indications

that a small group of patients may have developed aplastic anemia because of a defective HM expressed as a decreased marrow CFU-F content (Reynolds and McCann, 1985; Vladimirskaya et al., 1981). At diagnosis of acute leukemia and after relapse CFU-F colony formation is decreased, in most patients, but increases after chemotherapy treatment and returns to normal after achievement of complete remission (Table I.1). The suppression of CFU-F colony formation during leukemia could be the result of an inhibitory factor produced by leukemic cells (Nagao et al., 1983a). Furthermore, severe treatment of leukemia with chemotherapy and/or irradiation has been reported to reduce the CFU-F content significantly for a period upto 40 months after cessation of treatment (Da et al., 1986; Haworth et al., 1982).

Table I.1. CFU-F quantity in human bone marrow during disease or after leukemia treatment

anemia or leukemia before or after treatment	CFU-F number	Reference
aplastic anemia	normal	Gordon and Gordon-Smith, 1981 Kaneko et al., 1982 Nagao et al., 1981
	increased	Juneja et al., 1984
	decreased	Reynolds and McCann, 1985 Vladimirskaya et al., 1981
leukemia at diagnosis or relapse	normal	Greenberg et al., 1981
	decreased	Bruzzone and Minguell, 1985 Nagao et al., 1983a, 1983b Vladimirskaya et al., 1981
leukemia in complete remission after chemotherapy	(sub)normal	Bruzzone and Minguell, 1985 Nagao et al., 1983a, 1983b Vladimirskaya et al., 1981
	decreased	Da et al., 1986 Haworth et al., 1982

Data on the qualitative aspects of the CFU-F colonies from patients with hematological disorders are limited. In some patients with aplastic anemia it is suggested that they have developed the anemia because of a defective functional activity of the fibroblasts, i.e. the inability of the CFU-F colonies to stimulate hemopoiesis *in vitro* (Gordon and Gordon-Smith, 1983; Hotta et al., 1985b; Juneja and Gardner, 1985; Juneja et al., 1984). In a few patients with a myelodysplastic syndrome the defect may be the result of a disturbed hemopoietic regulatory activity of the fibroblasts (Zipori et al., 1985).

In general, quantitative CFU-F determinations in the individual patient are of limited usefulness due to the large variation in normal values, although in large groups of patients some general conclusions can be made (see above). As has been shown in animal experiments, CFU-F quantification may predict the reserve capacity of the stroma to regenerate and to replace damaged stromal cells after chemotherapy or irradiation-induced damage (Friedenstein et al., 1976; Hotta et al., 1983; Tavassoli et al., 1973a). More important, however, may be the assessment of the ability of CFU-F derived cells (i.e. fibroblasts) to produce hemopoietic growth factors to study the effects of disease and chemotherapy or radiation treatment.

I.3.4 LONG-TERM BONE MARROW CULTURES

The long-term bone marrow culture (LTBMC) system has been introduced by Dexter and Lajtha (1974) and is sofar the best *in vitro* representative for *in vivo* hemopoiesis. For an overview of the technique the reader is referred to excellent reviews by Dexter et al. (1984) and Eastment and Ruscetti (1984). Briefly, murine or human marrow cells are inoculated in flasks and an adherent stromal layer is allowed to develop for 2-4 weeks. This stromal layer is essential for the development of long-term hemopoietic activity. Using optimal culture conditions the hemopoietic progenitors survive during the initial growth phase of the adherent layer and 2-4 weeks after initiation of the culture maintenance and production of hemopoietic progenitors can be measured. This is the so called "one phase" LTBMC (see fig.I.1 A). A serious disadvantage is that only unseparated BMC can be used as these contain both the precursors for development of the stromal layer and for the initiation of hemopoiesis. If the LTBMC is set up from BMC derived from previously cytotoxic agent-treated mice, hemopoietic activity may be diminished due to hemopoietic progenitor damage. This LTBMC system can be supplemented with normal hemopoietic progenitors without stromal cells at the time of inoculation to "overrule" the presumptive cytotoxic agent damaged hemopoietic progenitors. It is assumed that the production of hemopoietic cells in the latter LTBMC system is solely dependent upon stromal integrity (see fig.I.1 B). Using the "two phase" LTBMC system (see fig.I.1 C), a tissue culture flask is inoculated with BMC and after establishment of the stromal layer hemopoietic activity is achieved by addition of a second inoculum of hemopoietic progenitor cells. Before addition of the second inoculum of hemopoietic cells the established stromal layer may be irradiated to

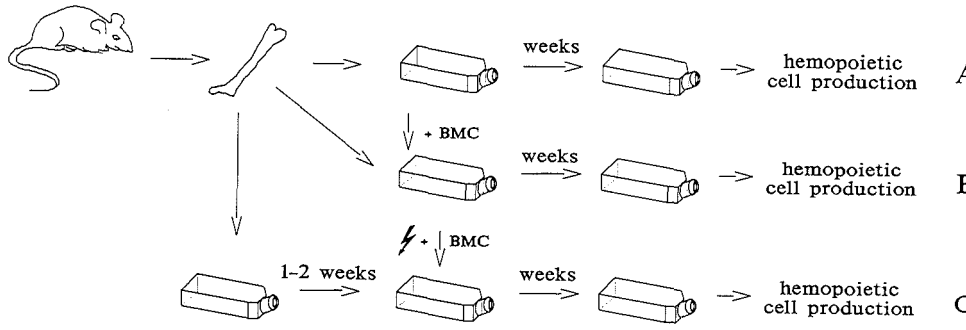

Figure I.1 The long-term bone marrow culture system. See text for explanation

eradicate the hemopoietic progenitors that may have survived during the initial growth phase of the stromal layer. Hemopoietic activity can be observed for at least one year depending on the species used.

The relation between hemopoietic and marrow stromal cells has been studied extensively in this culture system both morphologically and functionally (see also the previous chapter). The stromal elements in the adherent layer of LTBMC include endothelial cells, reticulum cells, adipocytes, fibroblasts, T lymphocytes, macrophages and 'blanket cells' (Allen, 1981; Dexter, 1982; Dexter et al., 1984; Shibata and Inoue, 1986). The existence of endothelial cells in murine LTBMC is still controversial (Allen, 1981, Zuckerman and Wicha, 1983), but they have been demonstrated in human LTBMC (Keating and Singer, 1983; Toogood et al., 1980). The adipocytes in LTBMC are most likely not derived from macrophages as suggested by Tavassoli (1984a), but are derived from other cells, e.g. the fibroblastic reticulum cell (Simmons et al., 1983). The blanket cell, recently characterized by Dexter et al. (1984) and also suggested to be of reticulum cell origin, is a large well-spread polygonal cell, alkaline phosphatase-positive, and capable to produce extracellular fibronectin and laminin (Allen and Dexter, 1984). This cell type owns its name to the association with granulopoietic cobblestone areas, which occur beneath the blanket cell layer (Dexter et al., 1984). Maintenance of hemopoiesis is also possible from spleen cell-derived adherent layers, although hemopoietic activity has not been observed beyond week 6 of culture. Splenic stem cells seeded onto a bone marrow-derived adherent layer, however, maintain hemopoiesis as well as BMC (Keller et al., 1983). These authors have suggested that the long-term maintenance of hemopoiesis in bone marrow-derived adherent layers is the result from a particular BMC class necessary for the regulation of extensive self renewal and maintenance of stem cells. These cells are apparently not present in spleen-derived adherent layers.

The most primitive hemopoietic progenitors in LTBMC are found within the

adherent layer. The self renewal capacity of adherent stem cells is much higher than that displayed by their non-adherent derivatives (Coulombel et al., 1983a; Crouse et al., 1984; Mauch et al., 1980; Reincke et al., 1985; Sharp et al., 1985). Furthermore, maintenance of primitive stem cells is dependent upon close cell-cell interactions with stromal cells and it has been suggested that also *in vitro*, primitive stem cells reside in "niches" (Sharp et al., 1986), as has been proposed for *in vivo* stem cell maintenance (Schofield, 1978). The existence of such niches *in vitro* is stressed by the observations of Sharp et al. (1986), who have been demonstrated that a tissue culture flask can only support a limited number of stem cells, and that saturation of the adherent layer with stem cells occurs after repeated refeedings with fresh bone marrow. The hemopoietic progenitors proliferate and differentiate continuously, resulting in long-term production of all hemopoietic progenitors including CFU-s (Dexter et al., 1977b), CFU-C (Dexter et al., 1977b; Williams et al., 1977), BFU-E (Eaves et al., 1979; Eliason et al., 1979), CFU-Meg (Williams et al.,1978) and lymphoid progenitors (Dexter et al., 1977b; Jones-Villeneuve and Philips, 1980; Schrader and Schrader, 1978).

Hemopoietic activity in LTBMC occurs focally. In murine LTBMC, granulopoiesis is in close association with adipocytes and an increased adipocyte content is associated with increased granulopoiesis (Greenberger, 1978). In contrast, adipocytes in human LTBMC have no stimulatory effect on hemopoiesis and increased fat cell formation is correlated with a decreased hemopoietic activity *in vitro* (Touw and Löwenberg, 1983), as has been observed *in vivo* in humans (Tavassoli, 1984a). Normally, mature erythrocyte production is not observed in LTBMC, but can be induced by supplementing the cultures with anemic mouse serum and EPO (Dexter et al.,1981). Within 2-3 weeks after addition of anemic mouse serum and EPO, erythropoiesis is induced and granulopoiesis inhibited, accompanied with a marked decrease in adipocyte numbers. Erythroid activity is found in areas separate from the previously active granulopoietic regions (Allen, 1981). Long-term bone marrow cultures produce colony stimulating factors, but their role in these cultures has not yet been elucidated (Gualtieri et al., 1984; Lipschitz et al., 1987a; Shadduck et al., 1983).

The LTBMC system has been used to study the effects of aging upon the HM. The results obtained from these investigations are controversial and vary between no effect of aging of the microenvironment upon hemopoietic activity (Schofield et al., 1986; Williams et al., 1986), increased stimulation of hemopoietic activity of the aged HM (Matthews and Crouse, 1981) or decreased stimulatory activity of the aged stroma (Lipschitz et al., 1987b; Mauch et al., 1982). The latter observation is comparable with the results from Hotta et al. (1980) using the subcutaneous implantation technique (see previous chapter).

Some examples in which the LTBMC system has been helpful to monitor the effects of disease and/or treatment with cytotoxic agents upon the HM are discussed below. Long-term cultures established from bone marrow of anemic Sl/Sl^{d} mice with a genetically determined defective HM or from anemic W/W^{v} mice with

a genetically determined stem cell defect, reproduce the anemias *in vitro* (Dexter et al., 1977a; Keller and Philips, 1984; Keller et al., 1983; Petursson and Chervenick, 1985; Zuckerman et al., 1986b). Zuckerman et al. (1986b) have suggested that the stromal defect in Sl/Sl^{d} mice is due to a local inhibitory effect which can be partially eliminated *in vitro* by irradiation of the Sl/Sl^{d} stroma. Ershler et al. (1980) have presented experimental evidence for the existence of a congenital hemopoietic stromal defect in humans. Culture of bone marrow fragments from an anemic patient have resulted in decreased hemopoietic progenitor proliferation and erythropoietic differentiation, despite a normal progenitor content. Long-term bone marrow cultures of some aplastic anemia patients have revealed the existence of a functional defective microenvironment (Hotta et al., 1985b). Establishment of long-term marrow cultures from mice with a spontaneous thymic lymphoma, which heavily infiltrated the bone marrow, results in an almost complete disappearance of the leukemic population (Hays and Hale, 1982). A similar observation has been made in man after establishment of LTBMC from patients with Ph' chromosome positive chronic myeloid leukemia or acute myeloid leukemia (Coulombel et al., 1983b; Eaves et al., 1985). Some LTBMC from acute myeloid leukemia patients, however, have maintained abnormal leukemic cells for a long period of time (Eaves et al., 1985). LTBMC from leukemic patients, sofar, do not present evidence for the existence of a severely abnormal functional HM although some changes in CFU-F quality and/or quantity may occur (see previous chapter). Treatment of leukemias and other malignancies with radio- and/or chemotherapy, however, have the capacity to damage the HM as has been demonstrated by establishment of LTBMC from cytostatic agent-treated mice (Anderson et al., 1982; Hays et al., 1982) or from *in vitro* cytostatic agent-treated or irradiated LTBMC (Greenberger et al., 1984; Horikoshi and Murphy jr., 1985; Laver et al., 1986; Naparstek et al., 1985; Reincke et al., 1982b). It has been observed that the proliferative capacity of stromal progenitors is quite sensitive to *in vitro* irradiation, while the hemopoietic supportive function and growth factor production is relatively resistant to the effects of radiation (Laver et al., 1986; Naparstek et al., 1985; Song and Quesenberry, 1984; Tavassoli, 1982; Zuckerman et al., 1986a). Which cells or cell activities of the hemopoietic stroma are most vulnerable to cytotoxic treatment remains to be determined.

I.3.5 LIPOPOLYSACCHARIDE-INDUCED INCREASED SPLENIC HEMOPOIESIS

LPS injection induces a huge increase in the level of circulating CSF's (Metcalf, 1971; Quesenberry et al., 1972). Various cell types (macrophages and endothelial cells) increase synthesis of CSF after LPS injection (Sheridan and Metcalf, 1972). Following LPS injection an increased splenic hemopoietic activity is found with a massive accumulation of hemopoietic progenitors in the spleen with highest numbers between 3 and 6 days after LPS injection depending on the

amount of LPS injected (Vos et al., 1972). The increased accumulation of splenic CFU-s after LPS injection is both a result of local CFU-s proliferation (McCulloch et al., 1970), and immigration of bone marrow-derived CFU-s (Ploemacher and Brons, 1987). The increase of splenic CFU-s numbers depends on a normal splenic stromal environment, apart from a normal CFU-s proliferative capacity (Ploemacher et al., 1983). Quantification of hemopoietic progenitors in the spleen after LPS injection can be used to estimate stromal integrity of the spleen, provided a normal proliferative capacity of the stem cell pool exists. The increased hemopoietic activity is accompanied with stromal cell proliferation (CFU-F) (Brockbank et al., 1983a) and endothelial cell proliferation (Reidy and Schwartz, 1983). Stromal cell proliferation also occurs in the other above mentioned assays to detect stromal function. This assay demonstrates the existence of a defective hemopoietic stromal compartment in Sl/Sl^{d} mice although these mice have a normal production of humoral regulators (McCulloch et al., 1970; Ploemacher et al., 1986). The other mouse strain with a genetically determined hemopoietic stromal defect, the $Sl^{J}/+$ mice, displays a normal splenic accumulation of hemopoietic progenitors following LPS injection (Ploemacher et al., 1984a; Ploemacher and Brons, 1987). Irradiation induces a severely impaired reaction following LPS injection, which lasts upto one year after irradiation and cannot be restored by a transplantation of normal BMC (Ploemacher et al., 1983).

I.3.6 GROWTH KINETICS OF NORMAL BONE MARROW CELLS IN ANIMALS WITH A NORMAL OR DEFECTIVE MICRO-ENVIRONMENT

A lethal total body irradiation (TBI) which eradicates all hemopoietic stem cells must be followed by a BMT if hemopoiesis is to be re-established. The growth kinetics of intravenously injected normal hemopoietic cells in the different hemopoietic organs can be followed. Because a normal hemopoietic stroma is mandatory for optimal stimulation of hemopoiesis after lethal TBI a comparison of the rate of regeneration of the intravenously injected normal BMC and the maximal hemopoietic activity achieved in normal irradiated mice and in mice with a supposedly defective microenvironment can be used to get an impression of the functional activity of the hemopoietic stroma. Differences in regeneration kinetics are assumed to result from differences in the ability of the hemopoietic stroma to support the injected normal hemopoietic progenitors. In contrast with the other assays mentioned above, the determination of the growth kinetics of normal BMC is assumed to be largely independent of stromal cell proliferation. Under normal conditions and after irradiation of bone marrow (and other organs) some proliferation of stromal cells does occur, e.g. endothelial and reticular cells (Adamson and Bowden, 1983; Caffrey et al., 1966; Meyer-Hamme et al., 1971) or CFU-F (Da et al., 1986), but it is assumed that the regeneration of hemopoietic progenitors during the first weeks is dependent on the existing stroma.

The use of this assay to study stromal function is very sparse. It was applied to study the microenvironmental defect in genetically anemic mice and to study the short-term effects of cytostatic agents and irradiation. The growth kinetics of CFU-s and CFU-GM from normal BMC in genetically anemic SL/Sl^{d} mice has been shown to be delayed in both spleen and femur (McCulloch et al., 1965; Sutherland et al., 1970). Comparison of Sl^{jj}/+ mice (with a mild microenvironmental defect) with normal mice revealed no differences between the two groups concerning the growth of committed progenitors (CFU-GM and BFU-E) although Sl^{jj}/+ mice were deficient in their ability to support macroscopic spleen colony formation (Ploemacher et al., 1984b). Six weeks after CTX treatment a delayed hemopoietic recovery is found after irradiation with 3.0 Gy as compared with non CTX-treated mice and although the proliferative potential of the CFU-s is decreased, the delayed hemopoietic recovery is suggested to be the result of stromal damage (Fried and Barone, 1980). Using a comparable experimental design, Kedo et al. (1976) has demonstrated a delayed recovery of CFU-s in mice exposed to 300 rads after having recovered from 950 rads TBI followed by a BMT.

I.4 LATE EFFECTS OF RADIATION AND CHEMOTHERAPY

Chemotherapy and radiation used in cancer chemotherapy have both the potential for injury, since antineoplastic therapy is not specific for tumor cells but affects normal cells as well. The efficacy of cancer chemotherapy is still improving, which results in increased survival time and cure rates. This encouraging situation, however, permits the occurrence of late therapy-related sequelae, which had heretofore been obscured or precluded by early recurrence of tumor. The late effects of radiation and chemotherapy will be discussed below with special attention to the late effects on the bone marrow compartment.

In general, cell death due to radiation is linked to mitosis and occurs only when the cell divides. Some cell types, e.g. lymphocytes, also show interphase cell death. The effects of radiation can be divided in acute and late effects and depend on the cell renewal characteristics of the irradiated tissue (Rubin, 1984; Wheldon et al., 1982). The different tissues can be separated according to cell renewal characteristics of their parenchymal cell compartment, determined by the lifetime of mature cells. Most epithelial tissues and the bone marrow are examples of tissues with rapid cell renewal characteristics. These tissues have stem cell compartments that rapidly proliferate and differentiate in a mature cell, which can not display mitotic activity. Tissues with slow cell renewal characteristics are described by a parenchymal cell compartment that turns over slowly and is capable to regenerate and/or proliferate to a certain stimulus e.g. microvasculature in wound healing, bone after a fracture or liver after partial resection. In non-proliferative tissues, with very little or no regenerative capacity of parenchymal cells e.g. muscle and the central nervous system, lost cells are replaced by fibrotic

tissue. The slow- or non-proliferative tissue types can be described as cell populations where either all cells are in a long cell cycle and occasionally divide, or the majority of cells are quiescent with others in a short cell cycle.

Regeneration of the different tissues after irradiation is dependent on the type of tissue, radiation dose, and the irradiated organ volume, i.e. the surviving fraction of stem cells or cells capable to proliferate. If the surviving fraction of stem cells is not sufficient to reconstitute the tissue, overt damage will occur. The latency period before the expression of gross tissue injury is determined by the renewal characteristics of the stem cell compartment and it is dose-dependent except after very high doses, when virtually all mitotic activity is abolished. In tissues with rapid renewal characteristics e.g. most epithelial tissues and the bone marrow the latency period will be very short and in slow-, occasional-, or non-proliferating tissues e.g. bone and connective tissue, liver, and the central nervous system the latency period will be much longer (Reinhold, 1984; Rubin, 1984; Wheldon et al., 1982). Another important component of late radiation damage in (almost) all organs is the effect upon the blood vessels, resulting in irregular dilatation and constriction of the vessels and reduction in the size of the capillary bed (Hopewell, 1975; Hopewell et al., 1986; Reinhold, 1984). If these vascular changes always affect the tissues, of which they are part has not been fully elucidated yet (Hopewell et al., 1986; Knospe et al., 1960; 1968).

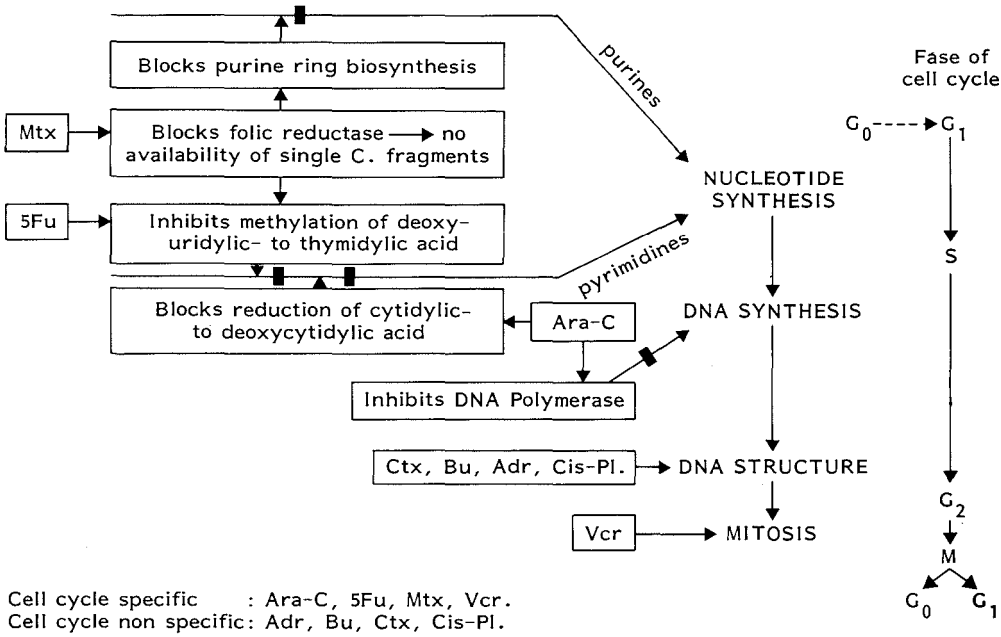

Figure I.2 The mechanism of action of some chemotherapeutic agents

The cytotoxic agents are usually classified according to their mechanism of action during a specific phase in cell cycle (see fig.I.2). Antimetabolites for example are cell cycle specific and react during S phase. Alkylating agents and antitumor antibiotics, however, are not cycle specific and react with DNA irrespective of the cell cycle (among others reviewed by Chabner and Myers, 1982).

During the first decades after the introduction of cytotoxic agents it has been thought that, in contrast with the use of radiation, late effects due to the use of anticancer agents are absent. In the early seventies the first reports have appeared about late effects associated with the prolonged use of anticancer agents in man (reviewed by D'Angio, 1976). It has been believed that tissues composed of mature and non-dividing cells are spared for the damaging effect of chemotherapy, however, this has appeared not to be true. Good examples for the effect of an anticancer drug upon mature cells, are the toxic effect of adriamycin on cardiomyocytes (Bristow et al., 1978) and the effect of cis-platinum on renal tubular cells (Gonzalez-Vitale et al., 1977). The exact nature of how cytotoxic agents exert these late effects is not known but in general the toxic effects of chemotherapeutic agents are dependent upon the cell's capacity to absorb, concentrate, or metabolize these agents and it may be dependent or independent of cell cycle or maturation. In contrast with radiation, chemotherapeutic agents will spare the vascular system (Rubin, 1984), with a few exceptions e.g. bleomycin (Adamson et al., 1974; Lazo, 1986).

There is ample experimental evidence that both radiation and some cytotoxic agents, mainly the alkylating agents, are capable to decrease the regenerative potential of the stem cell compartment. The depletion of stem cell reserve or loss of normal potential of reserve stem cells is described by the term "stem cell senescence" or "accelerated aging" (Botnick et al., 1979; Fried and Adler, 1985; Hellman and Botnick, 1977; Rubin, 1984; Schofield, 1986b). In conclusion, both quantity and quality of the hemopoietic stem cells are affected by the above mentioned treatment modalities. A comparable effect may injure the cells responsible for stromal regeneration after a cytotoxic insult. The stromal compartment, including the vascular system is regenerating slowly (Caffrey et al., 1966; Haas et al., 1969). Apart from normal replacement, remoulding of the hemopoietic stroma can occur after certain stimuli e.g. severe blood loss. This is accomplished by proliferation of the stromal compartment and especially the vascular system (McClugage et al., 1971). Accordingly, the bone marrow can be seen as a slow renewal tissue intermingled with a fast renewal tissue i.e. the stromal compartment and the hemopoietic stem cells, respectively. Irradiation affects fast and slow regenerating tissues in a comparable fashion but at a different time scale. A reduction in stromal stem cell quality and quantity will result in a less optimal reaction towards stress-induced proliferation and in a slackening or incomplete renewal of the stromal compartment. As mentioned in the previous chapters, normal hemopoiesis is impossible without a normal stromal compartment, and a defective stromal compartment may result in permanent aplasia.

Several authors have presented indications that long-term hemopoietic (and stromal) stem cell defects occur in man after irradiation and/or chemotherapy (Haworth et al., 1982; Lohrman and Schreml, 1988; Rubin, 1984; Schreml, 1985; Testa et al., 1985; Vellenga et al., 1987). The decreased stem cell quality becomes overt as a limited reserve capacity after a challenge e.g. another treatment with irradiation or cytotoxic agents or an infection and blood loss (Rubin, 1984). Recently, an impaired reserve capacity of the hemopoietic stem cell compartment in man has been demonstrated also after the injection of recombinant growth factors following chemotherapy treatment for carcinoma (Gabrilova et al., 1988) and following high-dose chemotherapy and autologous BMT for treatment of breast cancer or melanoma (Brandt et al., 1988). The increase in peripheral blood leucocytes is diminished in those patients treated previously with pelvic irradiation (this is approximately 50% of the bone marrow volume with active hemopoiesis in adults) (Gabrilova et al., 1988) or intensive chemotherapy (Brandt et al., 1988).

The experimental data concerning late effects of chemotherapy and radiation are already mentioned in previous chapters. The following paragraph will give some general remarks concerning late effects and regeneration of the bone marrow after treatment with cytotoxic agents. Late effects of radiation and/or chemotherapy in other organs have among others been reviewed by D'Angio (1976), Brady (1981), Byrd (1985), Chessels (1983), Deeg et al. (1984), Hendry (1988), and Rubin (1984) and will not be included here.

The acute marrow aplasia after irradiation is the result of stem cell damage and the second, permanent, aplasia is the result of stromal damage, especially the vascular system (Knospe et al., 1960; 1968). The recovery of hemopoietic activity is preceded by restoration of the stromal integrity (Knospe et al., 1960; 1968; Werts et al., 1980; and previous chapters). With respect to the response of the bone marrow to radiation or cytotoxic agents the marrow must be seen as one functional unit. The bone marrow is a diffuse organ and is found in almost all skeletal bones. The occupation of the bone marrow with active hemopoiesis depends upon species and age. The skeletal bones in mice are completely filled with hemopoietic active marrow and in a normal adult human the bones with active hemopoiesis are the vertebrae, pelvis, and femoral head and neck (Custer and Ahlfeldt, 1932; Hashimoto, 1962). Cytotoxic agents will react with the entire bone marrow organ but radiation will only affect a certain volume of the bone marrow depending on the irradiation fields used. Regeneration of the bone marrow (infield or outfield) is dependent on dose and irradiated bone marrow volume (reviewed by Parmentier et al.,1988; Rubin, 1984; Scarantino et al., 1984). The main regulating factor is the irradiated volume and the radiation dose. Regeneration is almost nonexistent when the irradiated marrow volume remains below 20-30% and using doses above 20 Gy. After irradiation of 40-50% of the bone marrow volume acute reactions are generally more severe and it has been observed that the unirradiated bone marrow may have a decreased hemopoietic activity also (abscopal effect on unirradiated bone marrow), most likely due to an increased rate of differentiation in the unirradiated

marrow (Parmentier et al., 1988). The irradiated field shows no signs of regeneration when less than 50% of the marrow volume is irradiated but the unirradiated hemopoietic marrow may show an increased activity and this may remain active for many years. Subtotal bone marrow irradiation (50-75% of the bone marrow volume) results in a very complex reaction. The unirradiated marrow becomes active and hemopoietic activity is also found in normally not active bone marrow or extramedullary which may lasts for decades. In contrast with irradiation of small fields (20-30%), infield regeneration after subtotal irradiation can be found after use of higher doses upto 40 Gy (fractionated dose regimens). Infield regeneration is particularly evident in younger persons (Sacks et al., 1978). Above mentioned data are from studies in man. The data from mice (or other small rodents) can in general not be used to predict the regeneration patterns of the irradiated bone marrow in man, because of the different dose regimens used (in animal studies usually single high-dose exposures and in humans fractionated protocols) and the different distribution of hemopoietic activity in the bones. However, a comparable regeneration pattern of the bone marrow after irradiation as was observed in man has been demonstrated in a rodent model (Rubin et al., 1977; Scarantino et al., 1984).

Human data about late effects and regeneration of the bone marrow after cytotoxic agents are rare, and usually include reports from combination treatments (see above). For some cytotoxic agents, however, experimental evidence exists that stromal damage can occur (see chapter I.3).

CHAPTER II

INTRODUCTION TO THE EXPERIMENTAL WORK

The objectives of the experimental work as described in the appendix papers can be separated into four main topics: 1. a study to compare the different assays to detect stromal integrity with respect to extent and kinetics of stromal damage and recovery after radiation or chemotherapy treatment (chapter III-VI), 2. a study of the late effects of chemotherapy (especially cis-platinum) and radiation upon the hemopoietic stroma (chapter III-VI), 3. a study to compare the effects of cytotoxic treatment of young or old mice upon the HM (chapter III-V), and 4. a study of the effects of cis-platinum upon the lymphopoietic system (chapter VII).

The assays or parameters used to study hemopoietic integrity in the experimental work of this thesis (chapter III-VI) are: **(a)** the quantification of peripheral blood cells and hemopoietic progenitors in spleen and bone marrow, **(b)** the quantification of hemopoietic progenitor cell recovery in regenerated ectopically implanted bone marrow or spleen, **(c)** quantification of fibroblastic colony-forming units in femur or spleen, **(d)** long-term bone marrow cultures, **(e)** measurement of splenic hemopoietic stem cell accumulation in response to bacterial LPS-induced hemopoietic stress, and **(f)** comparison of growth kinetics of normal hemopoietic progenitor cells following lethal irradiation of mice with a normal or (supposedly) defective microenvironment. The above mentioned assays are discussed in chapter I.3.

Sofar these different assays have not been compared to determine the extent and kinetics of stromal damage. As discussed in chapter I.3 some of these assays determine the proliferative capacity of stromal cells (especially b, c, d, and e) and some determine the hemopoietic supportive function of stromal cells (especially a, b, d, e, and f) or a combination of these two. A comparison of these assays may therefore give a better understanding of which cell (fibroblast or other stromal cell) and cell function (proliferation or the support for hemopoiesis) is most susceptible to chemotherapy or radiation.

The increasing use of effective chemotherapy and irradiation is reflected in increasing cure rates and prolongation of survival after many different types of malignancy, both in adults and children. However, it also increases the appearance of delayed complications after these treatment modalities (chapter I.4). The latter has increased the importance to determine the occurrence of late sequelae of both irradiation and chemotherapy as is described in chapter III-VI.

Data on the late effects after chemotherapy are scarce and during the first decades after the introduction of chemotherapy it has been believed that chemotherapy regimens lack late effects as can be seen from the duration of the studies in the drug developing program in the USA which only last 180 days, prior to allowing the test compound to be introduced to man (DeVita et al., 1979). After clinical indications of long-term effects after chemotherapy treatment, the ex-

perimental research for late sequelae increased (chapter I.4). Cis-platinum is an increasingly and successfully used agent in chemotherapy treatment protocols for many different malignancies (Loehrer and Einhorn, 1984; Schilcher et al., 1984). The mode of action of cis-platinum is not fully understood but it is suggested to be comparable with alkylating agents (Pascoe and Roberts, 1974; Zwelling et al., 1981). It is known that some of the alkylating agents e.g. BU and CTX, exhibit a long-lasting effect on both hemopoietic progenitors and the hemopoietic stroma (chapter I.3 and I.4). It has already been demonstrated by several authors that cis-platinum has an acute toxic effect upon hemopoietic progenitors (Nowrousian and Schmidt, 1982) and induces a long-lasting defect in response to anemic stress produced by bleeding (Braunschweiger et al., 1982). However, whether cis-platinum has an effect upon hemopoietic stromal integrity remains to be solved and this has been the main objective for the studies described in chapter V and VI. The investigations described in chapter VII have been performed to expand knowledge concerning the effects of cis-platinum upon the lymphopoietic system treated during growth.

The late effects after irradiation have been studied extensively (see chapter I.3 and I.4) but the majority of the studies has been carried out in adult experimental animals or man. Since the early and late effects of irradiation of an expanding HM during growth are unknown, such studies have been included (see chapter III). The stromal compartment in murine bone marrow increases during the first 8 weeks of life as determined by the femoral CFU-F content (Brockbank et al., 1983b). The mice have been irradiated at 4 weeks of age, during the expansion of the femoral CFU-F content, and several assays have been used to estimate the stromal integrity upto one year after irradiation which is approximately 1/3 to 1/2 of the life expectancy of mice.

CHAPTER III

RADIATION SENSITIVITY OF HEMOPOIETIC STROMA: LONG-TERM PARTIAL RECOVERY OF HEMOPOIETIC STROMAL DAMAGE IN MICE TREATED DURING GROWTH

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ABSTRACT

We studied the ability of the hemopoietic organ stroma to recover from damage inflicted by 5 or 7 Gy gamma radiation administered during a period of stromal growth in 4-week-old mice. Irradiation resulted in an immediate depletion of femoral colony-forming fibroblastic progenitors (CFU-F) down to 10-20% of age-matched control values. A full recovery to normal numbers occurred between 120 and 240 days after irradiation and was followed by a secondary decrease 1 year after irradiation. This secondary decrease was accompanied by a decrease in the femoral CFU-s and CFU-c content. Femoral CFU-F attained normal numbers and it was demonstrated to occur from surviving CFU-F and could not be enhanced or prolonged following infusion of unirradiated BMC after irradiation. During the transient CFU-F recovery the hemopoietic stroma remained severely damaged as judged by the regenerative capacity of spleen and femur stroma after subcutaneous implantation, and the ability of the spleen to accumulate CFU-s in response to lipopolysaccharide injection. We have reported earlier that in similarly irradiated adult mice, no restoration of femoral CFU-F was observed. This difference between 4-week-old and adult mice could not be explained by a difference in *in vitro* radiosensitivity of CFU-F or in their *in vivo* regeneration kinetics following irradiation and subsequent lipopolysaccharide injection. We conclude from these observations that **(a)** the recovery kinetics of the CFU-F population are different in young and adult irradiated mice, **(b)** infused CFU-F do not contribute to CFU-F regeneration in an irradiated femur, **(c)** CFU-F are not the sole determinants of stromal regeneration in femur and spleen following irradiation.

INTRODUCTION

Radiation-induced damage of hemopoietic stem cells and bone marrow stroma is one of the dose limiting factors in cancer therapy. Stromal injury after radiation has been reported in both animal models and humans (Chamberlin et al., 1974; Fried et al., 1976; Knospe et al., 1960; Rubin et al., 1973; Sacks et al., 1978; Sykes et al., 1964; Testa et al., 1985; Wolf, 1982). Knospe et al. (1960) showed that local irradiation of rat bone marrow with a single dose of 40 Gy or more resulted in permanent aplasia due to loss of sinusoidal structures. Permanent aplasia was also found in human sternal marrow after doses about 30 Gy (Sykes et al., 1964). Sacks et al. (1978) demonstrated, using radioisotope scanning procedures, that the extent of bone marrow regeneration is related to the irradiated bone marrow volume if radiation doses do not exceed 50 Gy. Using a subcutaneous organ implantation technique, which measures the ability of the regenerating donor stroma to support the accumulation and maintenance of host-derived stem cells (Fried et al., 1973a; Hotta et al., 1983), stromal damage was detected at far lower radiation doses, i.e., 4 Gy or more (Chamberlin et al., 1974; Fried et al., 1973a; Fried et al., 1976; Piersma et al., 1983a; Ploemacher and van Soest, 1979; Ploemacher et al., 1983). This stromal insult showed no recovery for at least 9 weeks after treatment (Chamberlin et al., 1974). Quantification of a stromal progenitor cell class (CFU-F) also revealed persistent stromal damage using doses of 5 Gy or more (Piersma et al., 1983a; Ploemacher et al., 1983), with recovery to control levels when lower radiation doses were employed (Xu et al., 1983).

Recently, our laboratory reported that the prolonged depletion of the femoral CFU-F population after lethal TBI could recover to normal numbers following injection with LPS (Piersma et al., 1985b). It was suggested that the irradiated stroma still contained the majority of functionally viable fibroblastic cells, but that they had lost their *in vitro* colony-forming capacity due to latent radiation damage. Thus, the LPS-induced depletion of the CFU-F population in the femur of these mice stimulated repopulation of the stroma with fibroblastic cells still capable to form colonies of at least 50 cells. On the basis of this assumption, we hypothesized that recovery of the CFU-F population in irradiated developing stroma of young mice could be expected without LPS treatment, since the CFU-F population is still expanding in such mice (Brockbank et al., 1983b) and do not have to be forced to proliferate *in situ* to replace latent damaged CFU-F. This led us to study the effects of gamma radiation on the developing hemopoietic stroma in comparison to fully developed adult stroma over a one year period. For this purpose we used the subcutaneous organ implantation technique, quantification of the stromal fibroblastic progenitor cells (CFU-F), hemopoietic progenitor cells (CFU-s and CFU-c) and peripheral blood parameters. We also studied the splenic CFU-s accumulation 5 days after injection of LPS as a measurement of the stromal integrity of the splenic stroma as described previously (Ploemacher et al., 1983). In addition, we investigated whether transplantation of marrow cells was a pre-

requisite of CFU-F recovery in irradiated mice.

MATERIALS AND METHODS

Mice. Female F1(CBA/RijxC57BL/Rij) mice, 4 weeks of age and weighing 14-16 g (young mice), 12 weeks old and weighing 26-30 g (adult mice), and male or female mice 8 to 30 weeks old were used as recipients for CFU-s determination. The animals were purchased from the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands, or from the Radiobiological Institute, TNO, Rijswijk, The Netherlands.

Irradiation. Mice received a dose of 5, 7 or 9 Gy total-body irradiation using a 137Cs gamma source (Gammacell 40 Atomic Energy of Canada Ltd., Ottawa, Canada) with a dose rate of 1.27 Gy/min.

Cell preparation. Single cell suspensions of femoral marrow and spleen were prepared and diluted in a buffered saline solution (BSS). Nucleated cells and red blood cells were counted with a Coulter Counter Model B. Hematocrits were determined using a microcentrifuge.

CFU-s assay. The number of CFU-s was determined by the spleen colony assay of Till and McCulloch (1961). Cell suspensions were injected i.v. into 7-10 lethally irradiated recipients. Seven to eight days later spleens were fixed in Telleyesznizky's solution and macroscopic surface colonies counted.

CFU-c and CFU-F assay. CFU-c and CFU-F were quantified in a semisolid culture medium (10% concanavalin A-stimulated mouse spleen conditioned medium was used as CSF source for CFU-c cultures as described previously (Brockbank et al., 1983b). CFU-c colonies were counted at day 7 of culture and contained at least 50 cells. CFU-F colonies were fixed at day 10 of culture and stained with Giemsa. Only colonies containing at least 50 fibroblastoid cells were counted.

Lipopolysaccharide treatment. Endotoxin of Salmonella typhosa (LPS) (Sigma) prepared by the Boivin method was dissolved in BSS to the appropriate dilution and injected i.v..

Regeneration assay. Spleens and femurs of previously irradiated and control mice were subcutaneously implanted into adult syngeneic hosts as has been described previously (Piersma et al., 1983b). Host mice received 5 Gy TBI 1 day before implantation to stimulate splenic regeneration (Chamberlin et al., 1974). Radiation of host mice had no effect on femoral regeneration. Six to eight weeks later the implants were removed and their CFU-s content was assayed.

Assay of splenic stroma function. Five days after injection of 300 μg LPS the CFU-s content of the spleen was determined. The LPS-induced accumulation of CFU-s in the spleen was used as an indication of the splenic stromal function (Ploemacher et al., 1983).

RESULTS

Survival, body weight, Ht, erythrocyte, and leucocyte counts

During the 12-month study body weight of both 5 and 7 Gy irradiated 4-week-old mice was 10-20% less than that of age-matched nonirradiated controls (data not shown). Following 5 Gy TBI the first mice died 240 days after irradiation, and survival gradually declined to 90% at one year. Seven Gy TBI resulted in a first death after 120 days and a 75% 1-year survival (data not shown). The cause of death remained unexplained since no autopsies were done. Ht and erythrocyte counts fluctuated around 95% (5 Gy) and 85% (7 Gy) of age-matched control values (data not shown). Leucocyte counts were severely decreased 1 week after both 5 and 7 Gy TBI (down to 7-11%), returned slowly to (sub)normal levels (80-100%) at 45 days and remained at this level during the 1-year observation period (data not shown).

Cellularity and hemopoietic and stromal progenitor content in the spleen

One week following 5 Gy TBI, the splenic cellularity decreased down to 12% of unirradiated control mice (Fig.III.1). Splenic cellularity was restored to subnormal levels within 45 days and continued to be (sub)normal during the observation period. After 7 Gy TBI splenic cellularity remained below the 5 Gy TBI data except for the 240-day point. Furthermore, splenic cellularity showed a tendency to a second decrease at 1 year after 7 Gy TBI. The initial decrease of CFU-s numbers (Fig.III. 2A) was followed by a recovery at 45 days up to 90% (5 Gy) and 60% (7 Gy) respectively, and remained at 60 and 40%, respectively, between 120 and 360 days. CFU-c numbers showed a smaller recovery in comparison to CFU-s but reached comparable values 240 and 360 days after irradiation. CFU-F numbers did not show much recovery in the spleen during the 1-year observation time.

Cellularity and hemopoietic and stromal progenitor content in the femur

Following the initial decrease 1 week after irradiation, the femoral cellularity (Fig.III.1) and progenitor content (CFU-s, CFU-c, and CFU-F) (Fig.III.2B) returned to normal or subnormal values. During the first 45 days following irradiation, CFU-s and CFU-c numbers showed a comparable recovery to approximately 60% of age-matched controls after 5 or 7 Gy TBI, while CFU-F numbers showed no sign of recovery. Femoral progenitor cell numbers CFU-s and CFU-c, recovered to (sub)normal and CFU-F numbers to (supra)normal 120 to 240 days after 5 or 7 Gy TBI. However, a secondary severe decrease in the hemopoietic progenitor

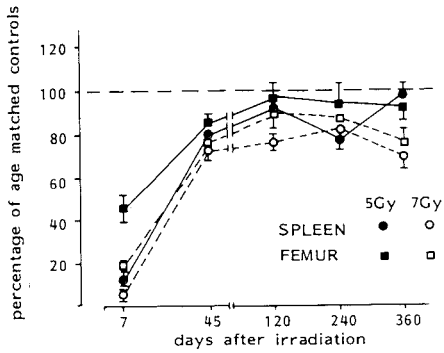

Figure III.1 Nucleated cells of femoral bone marrow and spleen after 5 or 7 Gy TBI. All data are expressed as percentage of age-matched non-irradiated controls ± standard error of 2 or 3 individual experiments. Each experiment consisted of three individual assayed mice per point. Control cellularities averaged 2.38 x 10^8 nucleated cells per spleen and 3.83 x 10^7 nucleated cells per femur.

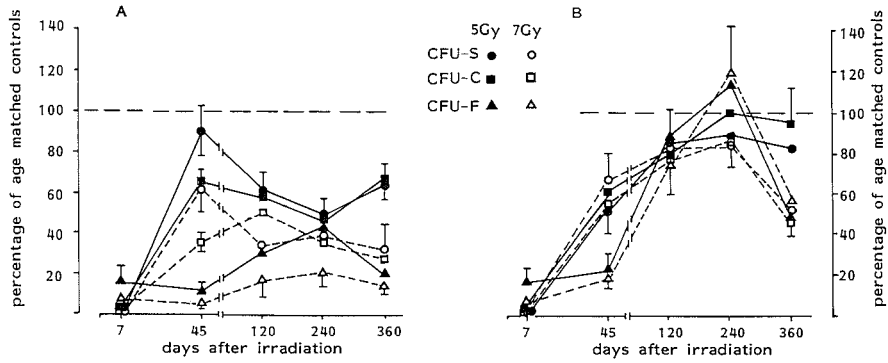

Figure III.2 Hemopoietic and stromal progenitor content in spleen (A) and femur (B). Hemopoietic stem cells (day 8 CFU-s), granulocyte/macrophage colony-forming units (CFU-c) and stromal fibroblastoid colony-forming units (CFU-F) content after 5 or 7 Gy TBI. All data are expressed as percentage of age-matched nonirradiated controls ± standard error of 2-3 individual experiments. Each experiment consisted of three individual assayed mice per point. Averaged control numbers: day 8 CFU-s: 2700 per spleen and 7800 per femur. CFU-c: 7800 per spleen and 37,500 per femur. CFU-F: 340 per spleen and 1270 per femur.

population size was observed at 1 year after 7 Gy TBI, while CFU-F numbers decreased following both 5 and 7 Gy TBI. The data in the figures were selected from mice which showed no sign of overt disease, i.e., a supranormal peripheral blood count and spleen size. One year after 7 Gy TBI approximately 30% of the

surviving mice showed the above mentioned symptoms.

Regeneration assay and splenic CFU-s accumulation after LPS injection

Following irradiation, both spleen and femur showed an immediate and sustained diminished capacity to regenerate and support CFU-s maintenance after subcutaneous implantation into syngeneic hosts (Fig.III.3), while no significant

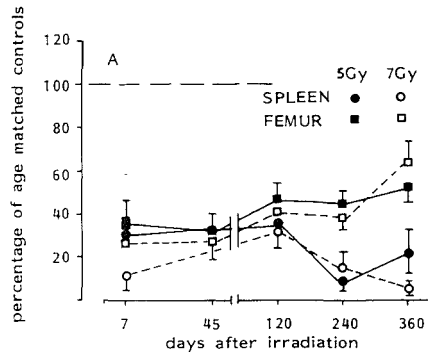

Figure III.3 Assay of CFU-s 6-8 weeks after implantation of femurs or spleens after 5 or 7 Gy TBI. The CFU-s content per organ was used as a quantification of the stromal regenerative capacity after irradiation-induced damage. All data are expressed as a percentage of the CFU-s content per age-matched nonirradiated subcutaneous implanted control organ ± standard error of 2 or 3 individual experiments. Each experiment consisted of 3 or 6 pooled spleens or femurs per point. Averaged control numbers: 183 CFU-s per splenic implant and 1100 CFU-s per femoral implant.

differences were observed between the 5 and 7 Gy irradiated groups. As has been shown previously (Ploemacher et al., 1983), the LPS-induced accumulation of CFU-s in the spleen could be used as an indication of splenic stromal function. The irradiated spleens showed a reduced ability to accumulate CFU-s in response to LPS injection (Fig.III.4). This dose-dependent stromal lesion did not recover during the 1-year observation period.

CFU-F survival after *in vitro* irradiation of young or adult normal bone marrow cells

From Fig.III.2 it appeared that femoral CFU-F from mice irradiated when 4 weeks old showed a transient recovery to (supra)normal values. This observation was at variance with previous data from adult irradiated mice (Piersma et al., 1985b), in which femoral CFU-F showed almost no recovery during 18 months after irradiation. In an attempt to explain these differences, we compared the *in vitro* radiation sensitivity of CFU-F from 4- and 12-week-old mice and determined the surviving fractions in 4- or 12-week-old mice. As can be seen from Fig.III.5, no

significant difference in CFU-F radiosensitivity was observed.

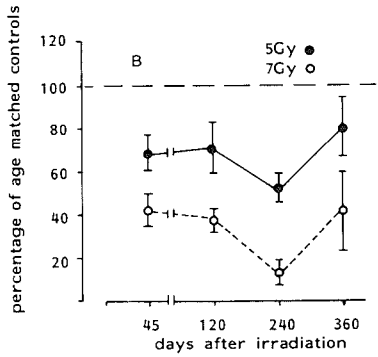

Figure III.4 Splenic CFU-s accumulation at 5 days following injection of 300 μg LPS after 5 or 7 Gy TBI. All data are expressed as percentage of age-matched nonirradiated controls ± standard error of 2 or 3 individual experiments. Each experiment consisted of three individual assayed mice per point. Averaged control number: 150,600 CFU-s per spleen from nonirradiated mice, 5 days after injection of 300 μg LPS.

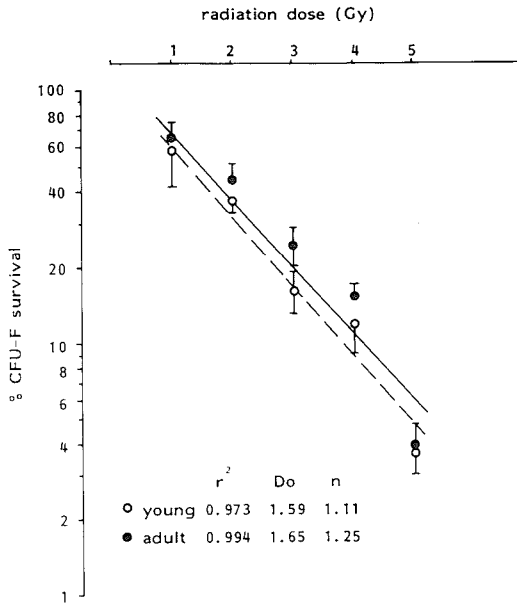

Figure III.5 CFU-F survival after in vitro irradiation of young or adult normal BMC. Each point consisted of 4 individual experiments in triplicate culture dishes ± standard error. All data are expressed in percentage CFU-F survival.

LPS-induced CFU-F recovery in normal and irradiated mice

In a previous observation (Piersma et al., 1985b) we demonstrated that the long-term depletion of CFU-F numbers in 9 Gy TBI mice could be completely restored to non-irradiated control numbers within 4 to 6 weeks following injection of a high dose of LPS. This beneficial effect of LPS was explained by the assumption that after BMT the irradiated stroma still contained viable fibroblastic cells with a limited proliferative capacity to form colonies *in vitro* as a result of latent radiation-induced DNA damage. LPS-induced depletion of CFU-F from the bone marrow presumably stimulated repopulation of the hemopoietic stroma with surviving and/or injected donor-derived colony forming fibroblastic cells. The data in Fig.III.6 indeed demonstrate that LPS injection in normal non-irradiated mice leads to a dose-dependent decrease in CFU-F content and femoral cellularity, which is followed by a regenerative period between 7 and 15 days after the LPS injection.

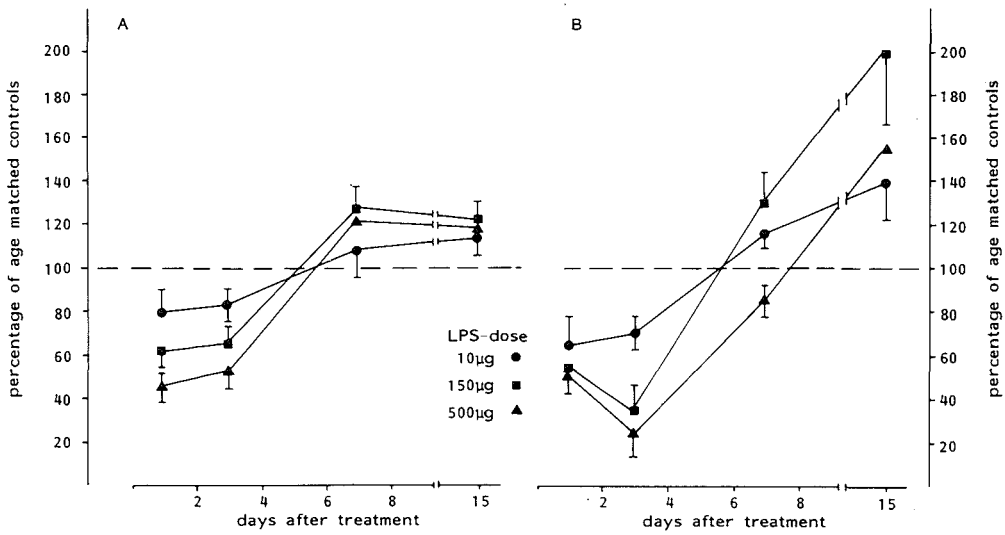

Figure III.6 *Nucleated cells of normal adult femoral bone marrow (A) and femoral CFU-F content (B) following injection of 10 µg LPS, 150 µg LPS or 500 µg LPS. All data are expressed as percentage of age-matched non-LPS-treated controls ± standard error of 3 individual assayed mice. See legends to Figs.III.1 and III.2 for control numbers.*

Subsequently we compared the ability of the CFU-F population to regenerate in mice that had been irradiated at 4 or 12 weeks of age. The mice were injected with either 10 or 150 µg of LPS at 3 months after irradiation. As can be seen from Figs.III.7B,D, LPS treatment induced a recovery of femoral CFU-F in most groups to values exceeding the femoral CFU-F numbers in non-LPS-injected mice. The incomplete CFU-F normalization in the 12 week, 7 Gy + BMC group (Fig.III.7) may

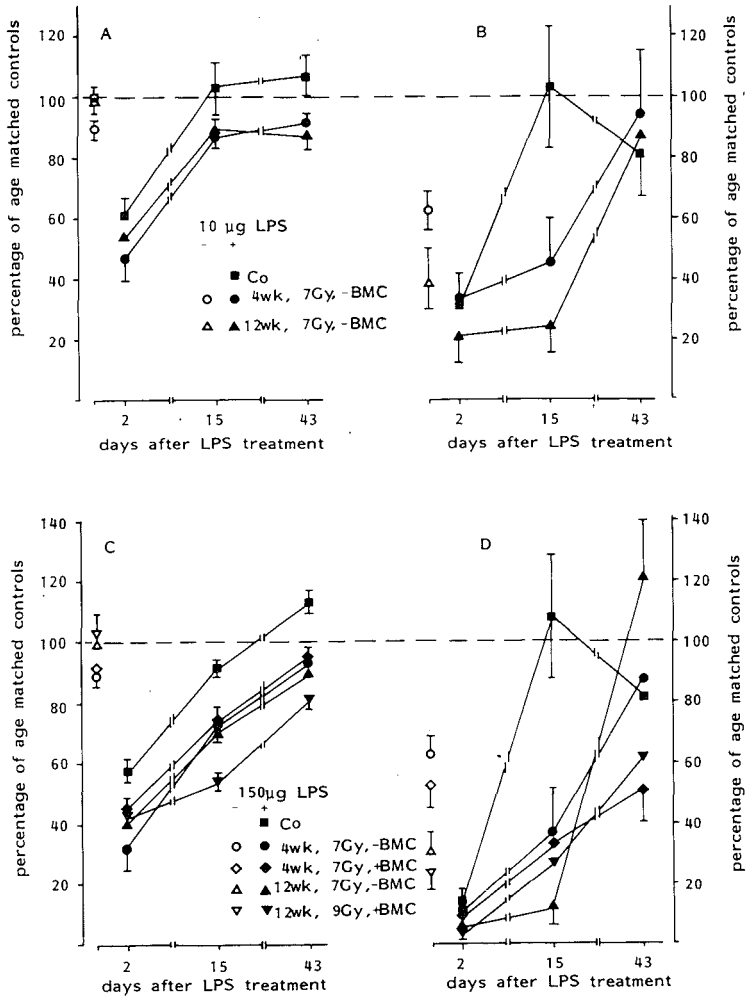

Figure III.7 Nucleated cells of femoral bone marrow (A and C) and femoral CFU-F content (B and D) following injection of 10 µg LPS (A and B) or 150 µg LPS (C and D) 3 months after 7 or 9 Gy irradiation of 4- or 12-week-old mice. All data are expressed as percentage of age-matched non-LPS-treated controls ± standard error of 3 individual assayed mice. See legends to Figs.III.1 and III.2 for control numbers.

have been due to the rather short regeneration time of 4 weeks in this experiment.

It is also evident from Fig.III.7B,D that the recovery of the femoral CFU-F numbers in irradiated mice lagged behind that of control mice, indicating that the

regeneration of the CFU-F in irradiated mice is retarded in comparison to the unirradiated CFU-F. It also appears from Fig.III.7 that transfusion of unirradiated BMC immediately after irradiation did not prevent the postirradiation depletion of the CFU-F population, nor did it facilitate normalization of the CFU-F population following LPS injection. These data demonstrate that transfusion of unirradiated BMC is not a prerequisite for the normalization of the femoral CFU-F population in previously irradiated mice following LPS treatment. Finally, no significant differences were found between the regeneration kinetics of CFU-F following LPS injection in 4- and 12-week-old mice. This strongly suggests that the transient normalization of femoral CFU-F in irradiated 4-week-old mice (Fig.III.2B) results from a CFU-F population with a low regenerative ability similar to that of CFU-F in mice irradiated at 12 weeks of age.

DISCUSSION

It appears from the present study that the femoral but not the splenic CFU-F population size in mice irradiated at 4 weeks of age shows a transient recovery to a normal level following 5 or 7 Gy TBI. Previously it has been reported that irradiation of adult mice leads to a severe and long- term depletion of femoral and splenic CFU-F with doses exceeding 5 Gy (Piersma et al., 1985b; Ploemacher et al., 1983; Xu et al., 1983). These observations suggest that the CFU-F population in young mice is less radiosensitive as compared to that in adult mice. However, when BMC from 4- and 12-week-old mice were irradiated *in vitro* no significant differences in radiosensitivity were measured (Fig.III.5). This indicates that the transient normalization of the CFU-F population in mice irradiated at 4 weeks of age is probably due to a difference in *in vivo* CFU-F kinetics following radiation. To study this possibility, mice were injected with LPS 3 months after 7-9 Gy TBI. Since LPS injection partly depletes the CFU-F population in unirradiated (Fig.III.6) and previously irradiated mice (Figs.III.7B,D), subsequent normalization of the femoral CFU-F number must be accomplished by surviving CFU-F. Such a normalization was indeed observed both in mice irradiated at the age of 4 and 12 weeks. No significant differences in the regeneration kinetics after enforced LPS-induced proliferation were observed between the two age groups. In both groups the proliferative ability of the CFU-F population was similarly delayed as compared to that of LPS-treated unirradiated control mice. These observations can be explained by assuming that latent damaged CFU-F, not able to form colonies *in vitro*, are replaced by surviving CFU-F in young mice, due to normal growth of the femur (Brockbank et al., 1983b). The replacement of latent damaged CFU-F in non growing adult femurs could be accomplished only by forcing CFU-F to proliferate, e.g., by using LPS.

The secondary decrease of the femoral CFU-F numbers following doses exceeding 5 Gy was accompanied by a secondary decrease of hemopoietic progenitor

cell numbers in the femur. These observations are in line with previous reports on the effects of irradiation in mice, rats and dogs (Cowall et al., 1981; Croizat et al., 1979; Knospe et al., 1960; Ploemacher et al., 1983; Xu et al., 1983). In our experiments also approximately 30% of all mice surviving 1 year after 7 Gy TBI had high blood leucocyte counts and occurrence of splenomegaly. Since postirradiation blood abnormalities have been observed to precede the development of leukemia (Cowall et al., 1981), it is suggested that a part of these mice in the present study had developed leukemia. In addition, it might be speculated that the secondary femoral hypoplasia in irradiated mice may represent a preleukemic or myelodysplastic syndrome (Michels et al., 1985). No experimental proof was obtained for these suggestions since no autopsy was performed on these mice.

Transfusion of unirradiated BMC directly following irradiation of young and adult mice did not ameliorate the postirradiation depletion of the CFU-F population, nor did it improve the post-LPS recovery of femoral CFU-F numbers. Previously, it was reported by Piersma et al. (1983b) that infused CFU-F have a remarkably high capacity to lodge into the irradiated femur of mice and are maintained in this site for months. The present data indicate, however, that transfusion of unirradiated BMC is not a prerequisite for the normalization of the irradiated CFU-F population.

The radiosensitivity of CFU-F from 4- and 12-week-old mice (Do was 1.59 and 1.65 Gy, respectively) was comparable with previous reports (Garnett et al., 1982; Werts et al., 1980; Xu et al., 1983), but was greater than was found by Wilson et al. (1974). The radiosensitivity of CFU-F is comparable to or even less than that of the hemopoietic progenitors CFU-s (Do range from 0.8 to 1.2 Gy) and CFU-c (Do range from 1.6 to 2.4 Gy) (Testa et al., 1985; Xu et al., 1983). However, in previously irradiated mice the CFU-F recovery lagged behind that of hemopoietic progenitors (Fig.III.2B), indicating that the recovery of CFU-F following irradiation shows other regeneration kinetics than hemopoietic progenitors.

It is of interest to note, that both the regenerative capacity of implanted femurs and spleen (Fig.III.3) and the LPS-induced CFU-s accumulation in the spleen (Fig.III.4) were severely decreased following irradiation of 4-wk-old mice with 5 or 7 Gy TBI, but, in contrast with femoral CFU-F, did not normalize during the 1-year observation period. Although the immediate radiation toxicity has been reported to be the same for femoral regenerative capacity following implantation and for femoral CFU-F (Piersma et al., 1983a), these assays of stromal integrity (Fried et al., 1973; Fried et al., 1976; Hotta et al., 1983; Ploemacher et al., 1983) apparently measure different stromal components.

The observation of near normal peripheral blood parameters in the presence of a decreased progenitor content in both spleen and femur indicate a compensatory proliferation (Hendry et al., 1983) or an increased cycling rate (Croizat et al., 1979; Hendry and Lajtha, 1972) of the committed progenitors to produce enough functional blood cells. The presented data also indicate that near normal femoral hemopoiesis could occur within a severely (latent) damaged stroma. All stromal

assays used in this study are dependent on proliferation and a decreased proliferative capacity does not necessarily have to imply a decreased function of the stromal cells surviving the irradiation (Laver et al., 1986; Naparstek et al., 1986; Song and Quesenberry, 1984). In line with a previous report (Piersma et al., 1985b) we show here that the diminished CFU-F population after irradiation could be replaced by normal proliferating CFU-F after a second, LPS-induced, stromal damage. This recovery apparently can occur from CFU-F surviving the irradiation, although its kinetics is delayed as compared with that of CFU-F in nonirradiated mice. Furthermore, we found that the kinetics of LPS-induced CFU-F recovery was similar in mice irradiated at 4 or 12 weeks of age and could not be enhanced by a bone marrow transplant. In a previous report we showed that donor-derived stromal cells could be detected in the recipient (Piersma et al., 1983b), and Werts et al. (1980) showed that marrow stromal cells could migrate from an irradiation protected site into an irradiated femoral bone marrow. However, apparently these cells do not contribute to the ultimate CFU-F population size which may depend on other microenvironmental factors, e.g. the endothelial cells from the sinusoids (DeGowin et al., 1976; Knospe et al., 1960).

ACKNOWLEDGMENTS

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CHAPTER IV

LONG-TERM EFFECTS OF CYTOSTATIC AGENTS ON THE HEMOPOIETIC STROMA: A COMPARISON OF FOUR DIFFERENT ASSAYS

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ABSTRACT

We have compared four assays to detect hemopoietic stromal damage induced by various cytostatic agents in young (4-week old) and adult (12-week old) mice. These assays included: **(a)** quantitation of the hemopoietic stem cell content of subcutaneously implanted spleens and femurs, **(b)** quantitation of fibroblastic colony-forming units per femur and spleen, **(c)** quantitation of the growth of normal hemopoietic progenitor cells in irradiated cytostatic drug-treated mice, and **(d)** measurement of splenic hemopoietic stem cell accumulation in response to bacterial lipopolysaccharide-induced hemopoietic stress.

Busulfan caused a short- and long-term hemopoietic stromal defect. However, the four assays used showed different kinetics and severity of the stromal damage. Cyclophosphamide treatment resulted in a short-term stromal damage which was repaired within one week to three months, depending on the assay used. Methotrexate and vincristine did not cause long-term stromal damage as measured by the four assays used, whereas a short-term splenic stromal damage was detected using the subcutaneous implantation technique. No significant differences in stromal sensitivity to drug treatment were observed between young and adult mice. The presented data suggest that the 4 assays used to study stromal integrity measure different components of the HM, and indicate that the use of a single assay may well lead to erroneous interpretations.

INTRODUCTION

With the increasing use of effective cancer chemotherapy it has become important to determine and evaluate the risks of late complications. Recently some

investigators reported a long-term hemopoietic insufficiency after chemotherapy. This long-term insufficiency following antineoplastic agents in adults and children (Haworth et al., 1982; Matsuo et al., 1985; Testa et al., 1985) and experimental animals (Ben-Ishay et al., 1985; Botnick et al., 1981; Braunschweiger et al., 1982; Fried and Barone, 1980; Fried et al., 1977; McManus and Weiss, 1984; Morley et al., 1975; Trainor and Morley, 1976) may be caused by a failure of hemopoietic stem cells or by a defective support capacity of the hemopoietic stroma or a combination thereof.

In order to study the effects of various cytostatic agents on the integrity of the hemopoietic stroma, we employed a series of assays, which are assumed to detect different components of the stroma or combinations thereof, and distinguish between a (latent) proliferative defect or a functional stromal defect. This may be helpful in predicting long-term damage inflicted by cytostatic agents. A functional damage in stromal cells may result in an acute and long-lasting disturbance in hemopoietic regulation. On the other hand, a proliferative defect does not result in an acute stromal defect *in situ*, but may decrease the reserve capacity of the slow regenerating hemopoietic stroma when responding to stress-induced proliferation. In addition it has been reported that late toxicity induced by chemotherapy may not only occur from damage to rapidly proliferating cells, but also may result from damage to non-cycling cells (D'Angio, 1976; Billingham, 1979; Goorin et al., 1981; Weiss and Muggia, 1980). The mechanism by which antineoplastic agents cause damage to the organ stroma are not fully understood. It is also unclear which stromal cells are most vulnerable for cytotoxic treatment.

In the present study we used 2 assays that permit detection of a latent proliferative defect. These assays are the quantitation of the fibroblastoid colony forming unit (CFU-F) and the quantitation of the regenerative capacity of the implanted spleens or femurs. In these assays the stromal cells are forced into mitosis (Fried et al., 1973a; Friedenstein et al., 1974b; Hotta et al., 1983).

In order to detect functional stromal defects under conditions where stromal cell proliferation is limited we quantitated the growth of normal BMC in previously drug-treated mice. In addition, an assay was used to measure the functional activity of the hemopoietic stroma and progenitors at the same time, i.e. quantitation of the CFU-s accumulation in the spleen following lipopolysaccharide (LPS) injection. In the absence of detectable defects in the proliferative activity of the CFU-s compartment, this assay can be used as an indication of the splenic stromal function (Ploemacher et al., 1983). However, defects in the capacity of CFU-s to migrate and/or proliferate in the spleen may also lead to a decreased splenic response to LPS. Finally quantitation of peripheral blood parameters (hematocrit, leucocytes, and erythrocytes) and hemopoietic progenitor content per spleen or femur under normal steady state conditions was used as an indication of the combined functional activity of hemopoietic progenitors and stroma. This study was also undertaken to compare the usefulness of the different assays used to detect stromal integrity.

The cytostatic agents, chosen on the basis of their use in the treatment of malignancies in children, were busulfan (BU), cyclophosphamide (CTX), methotrexate (MTX) and vincristine (VCR). BU was chosen since latent hemopoietic stem cell and stromal damage has been reported in adult treated mice (Fried et al., 1977; McManus and Weiss, 1984; Morley and Blake, 1974) and because of its clinical use in preparation of children with congenital bone marrow disorders for BMT (Parkman et al., 1984). CTX, MTX, and VCR are clinically used in treatment of children with acute lymphoblastic leukemia, the most common childhood malignancy.

MATERIALS AND METHODS

Animals. Female (CBA/Rij x C57BL/Rij)F1 mice, four weeks old (young) and weighing 14-16 g., and female mice, 12 weeks old (adult) and weighing 25-30 g., and male or female mice 8-30 weeks old were used as recipients for CFU-s determination. The animals were purchased from the Laboratory Animals Center of the Erasmus University, Rotterdam, The Netherlands, or from the Radiobiological Institute TNO, Rijswijk, The Netherlands.

Drug treatment. Vincristine (VCR) (Eli-Lilly), cyclophosphamide (CTX) (Asta), and methotrexate (MTX) (Lederle) were dissolved in phosphate-buffered saline (PBS). Ten milligrams of busulfan (BU) (Wellcome) were first dissolved in 1 ml of acetone and further diluted with 67 ml of PBS to the appropriate dilution. The acetone concentration was 15 μl/ml. VCR was injected i.v. twice weekly for 3 consecutive weeks in a total dose of 1.5 mg/kg body weight (BW). BU (total dose 45 mg/kg BW) or MTX (total dose 67.5 mg/kg BW) were injected i.p. three times a week for three consecutive weeks. CTX was injected i.p. once a week (CTX. 3x100mg) (total dose 300 mg/kg BW) or three times a week (CTX. 9x30mg) (total dose 270 mg/kg BW) for three consecutive weeks.

Regeneration assay. The regenerative capacity of spleens and femurs were determined by assaying their CFU-s content at 6 weeks following subcutaneous implantation. Four spleens and 8 femurs derived from 4 experimental donor mice per group were implanted subcutaneously in 4 isologous host mice as has been described previously (Ploemacher et al., 1982). Implants were pooled for CFU-s determination. Adult syngeneic host mice for splenic and femoral implants received 5 Gy TBI 1 day before implantation to stimulate splenic regeneration (Chamberlin et al., 1974). Radiation of host mice had no effect on femoral regeneration.

Irradiation. Whole body irradiation was performed with a cesium-137 source (Gammacell 40, Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.27 Gy/min.

Cell preparation. Single cell suspensions of femoral marrow and spleen were prepared and diluted in alpha medium (alpha modification of Dulbecco's minimum essential medium). Nucleated cells and red blood cells were counted with a Coulter Counter model B. Hematocrits were determined using a microcentrifuge.

CFU-s assay. CFU-s were assayed as described by Till and McCulloch (1961). Cell suspensions were injected i.v. into 7-10 lethally (9 Gy) irradiated recipients. Spleens were fixed 7 days later and macroscopic surface colonies were counted. Radiation control mice contained an average of less than 0.2 colonies per spleen.

CFU-G/M and CFU-F assay. CFU-G/M and CFU-F were quantitated in a semi solid culture medium (0.8% methylcellulose) as described previously (Brockbank et al., 1983b). All colonies counted contained at least 50 cells.

CFU-s accumulation in the spleen. Endotoxin of Salmonella typhosa (LPS) (Sigma) prepared by the Boivin method was used. Five days after i.v. injection of 300 μg of LPS per mouse, the increase in splenic cellularity and CFU-s were determined as an indication of the splenic stromal ability to support proliferation and accumulation of CFU-s (Ploemacher et al., 1983). However, it may also detect defects in the CFU-s compartment with respect to proliferative activity, migration or spleen homing ability.

Growth curves. One year after cessation of cytotoxic treatment, mice received lethal (9 Gy) total body gamma irradiation. Within 2 hours after irradiation, the mice were injected i.v. with 4 x 10^6 syngeneic normal BMC. At 4, 7, 11 and 33 days after irradiation the splenic and femoral cellularity and CFU-G/M content of 3 mice per point were determined. The growth of CFU-G/M was used to determine the stromal ability to support hemopoiesis one year after cytotoxic agent treatment.

Statistics. Values for all parameters in the figures are expressed as arithmetic means in percentage of age-matched controls (± 1 SEM). Each point in figure 1, 3, 4 and 5 was determined in 2-5 separate experiments while each experiment consisted of 3-4 mice per experimental group. At each time point age-matched controls were also assayed. Statistical analysis was done using the Student t test.

RESULTS

Young mice

Busulfan treatment

Immediately after the last BU injection animals showed a transient anemia and neutropenia (data not shown). The cellularity of spleens and femurs remained

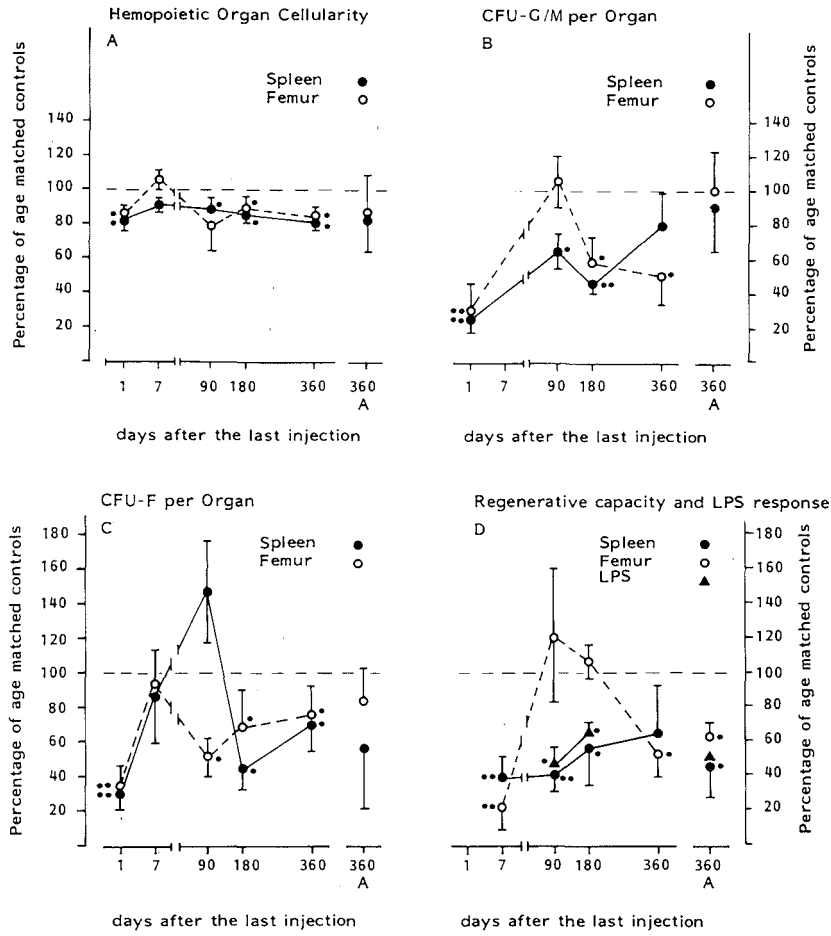

Figure IV.1. *The effect of BU on splenic and femoral cellularity (A), CFU-G/M content (B), CFU-F content (C), regenerative capacity of spleen and femur and CFU-s accumulation in spleens after LPS treatment (D). All data are expressed as percentage of age-matched controls ± SEM of 2-5 experiments. The 1 year data indicated 'A' are from mice treated at 12 weeks of age, the other data are from mice treated at 4 weeks of age. Control cellularities averaged from 1.99 x 10^8 nucleated cells per spleen at the 1 day point to 2.57 x 10^8 at 1 year and from 3.10 x 10^7 nucleated cells per femur at the 1 day point to 4.56 x 10^7 at 1 year. Averaged control numbers: CFU-G/M: 7,600 per spleen and 43,700 per femur. CFU-F: 320 per spleen and 1,570 per femur. Regeneration assay: 115 CFU-s per splenic implant and 930 CFU-s per femoral implant. Splenic CFU-s accumulation after LPS treatment: 110,300 CFU-s per spleen.* * $p<0.05$, ** $p<0.01$.

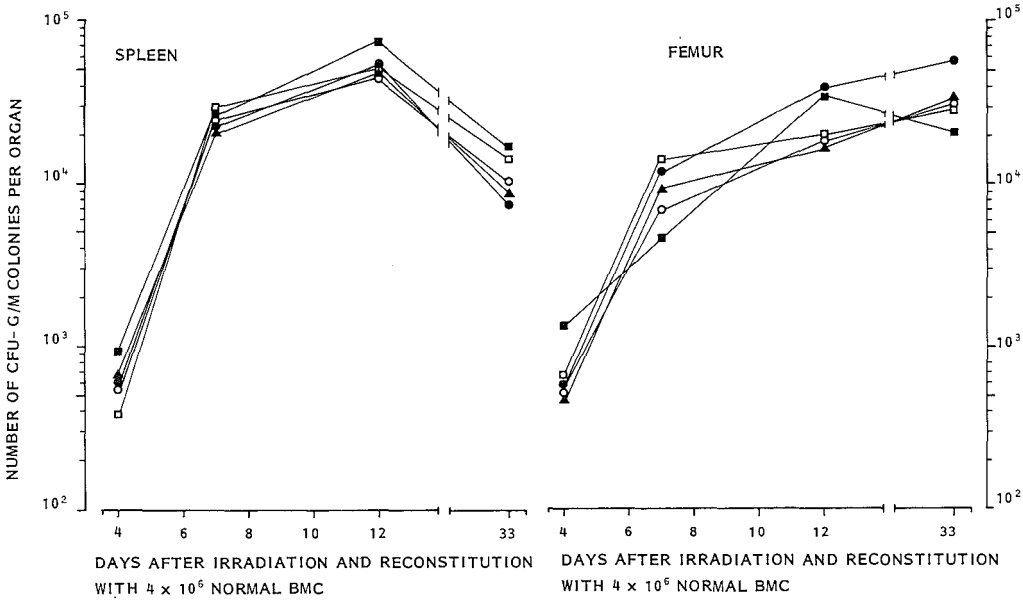

Figure IV.2 Growth kinetics of normal CFU-G/M in spleen and femur of lethally irradiated mice, 1 year after they received the first of a series of cytostatic drug injections at 4 weeks of age. Each point represents the pooled data of 3 mice in 1 experiment. Co (●), BU (■), CTX 3 x 100 (▲), MTX (○) and VCR (□).

below control values during the time period studied, except for a transient restoration in the femur at one week after cessation of treatment (Fig.IV.1A). Both in spleen and femoral marrow hemopoietic (CFU-G/M) (Fig.IV.1B) and stromal parameters (CFU-F, Fig.IV.1C; regenerative capacity, Fig.IV.1D) were severely decreased immediately after treatment.

Femoral and splenic CFU-F numbers (Fig.IV.1C) showed a transient normalization between 1 and 90 days, which was followed by a secondary decrease down to 50-80% of control values. An essentially similar pattern was observed for the femoral regenerative capacity (Fig.IV.1D) and CFU-G/M content (Fig.IV.1B). However, these parameters indicated different levels of BU-induced damage when assayed at comparable time points, indicating that the assessment of a single parameter may yield insufficient information on hemopoietic and stromal integrity. Splenic regenerative capacity and CFU-G/M content did not show a transient return to normal values within this period, but a slow restoration occurred up to 60-80% at the one year time point. One year after cessation of BU treatment all femoral hemopoietic and stromal parameters were significantly below normal.

The post-BU kinetics of the regenerative capacity, the LPS response and the

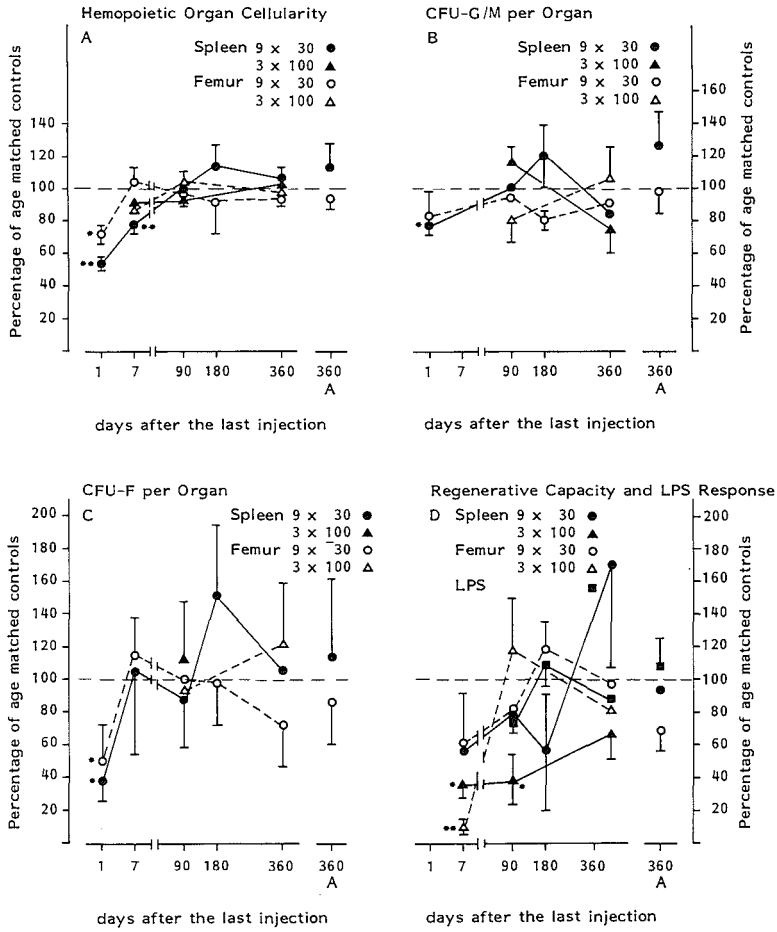

Figure IV.3 *The effect of CTX on splenic and femoral cellularity (A), CFU-G/M content (B), CFU-F content (C) regenerative capacity of spleen and femur and CFU-s accumulation in spleens after LPS treatment (D). All data are expressed as percentage of age matched controls ± SEM of 2-5 experiments. The 1 year data indicated 'A' are from mice treated at 12 weeks of age, the other data are from mice treated at 4 weeks of age. CTX was injected i.p. once a week (CTX (3 x 100mg)) or three times a week (CTX (9 x 30mg)) for three consecutive weeks. For control numbers see legend Fig. 1.* $*p<0.05$. $**p<0.01$.

CFU-G/M, but not CFU-F, content showed a good correlation in the spleen. The growth pattern of normal CFU-G/M in the femur of mice at one year after BU treatment (Fig.IV.2) suggests that at this late time point their femoral stroma may

indeed be less capable to stimulate growth of hemopoietic progenitors. Since only 3 mice per experimental point are used in this assay no definite conclusions can be made.

Cyclophosphamide treatment

CTX induced a moderate decrease of the splenic and femoral cellularity (Fig.IV.3A) and CFU-G/M content (Fig.IV.3B) immediately after treatment, followed by almost complete restoration within one week. These data correlate well with the transient damage of the hemopoietic stroma as measured by the CFU-F assay (Fig.IV.3C) and regenerative capacity (Fig.IV.3D), which showed recovery to normal values within 7-90 days. Only splenic regenerative capacity of CTX-treated mice remained below that of age-matched controls. Fractionation of the CTX dose induced a decreased acute stromal toxicity (Fig.IV.3D). The decreased growth of normal CFU-G/M observed beyond day 7 in lethally irradiated recipients previously treated with CTX (Fig.IV.2) suggest that CTX had also decreased the functional capability of the stroma to support hemopoiesis.

Methotrexate and vincristine treatment

Transient increases in splenic cellularity (Fig.IV.4A) and in splenic and femoral CFU-G/M content (Fig.IV.4B) were evident immediately after cessation of MTX treatment. VCR treatment induced a transient increase in splenic CFU-G/M (Fig. IV.5B). In the spleen of both MTX- and VCR- treated mice, CFU-F numbers (Fig.IV. 4C and IV.5C) increased after cessation of treatment and remained above normal from 90-180 days; normal levels were found after one year. In femurs of these mice, CFU-F were transiently increased one week after the last MTX- or VCR-injection. The regenerative capacity of spleens and femurs of the MTX- and VCR-treated mice (Fig.IV.4D and IV.5D) fluctuated around normal values during the observed time period of one year. Due to the large variability of the stromal assays no definite conclusions can be drawn. Only at one week was the splenic regenerative capacity significantly decreased. The ability of these mice to accumulate CFU-s following LPS-injection appeared unchanged over the year following treatment. MTX and VCR treatment resulted in a small stromal defect as judged by the growth kinetics of normal BMC in lethally irradiated MTX- or VCR-treated recipients. At 12 and 33 days a decreased CFU-G/M content was found although the growth during the first 7 days was normal (Fig. IV.2).

Adult mice

At one year following cessation of treatment with BU, CTX, MTX or VCR, adult mice did not show significant differences in sensitivity of the hemopoietic stroma to these agents in comparison to young mice (Fig.IV.1, IV.3-5). In contrast to young BU-treated mice that had a 50% decrease in femoral CFU-G/M, the CFU-G/M content of adult BU-treated mice did not differ from age-matched

controls one year after the last BU injection (Fig.IV.1B). Also adult VCR-treated mice differed from young VCR-treated mice with respect to their splenic CFU-G/M content (Fig.IV.5B).

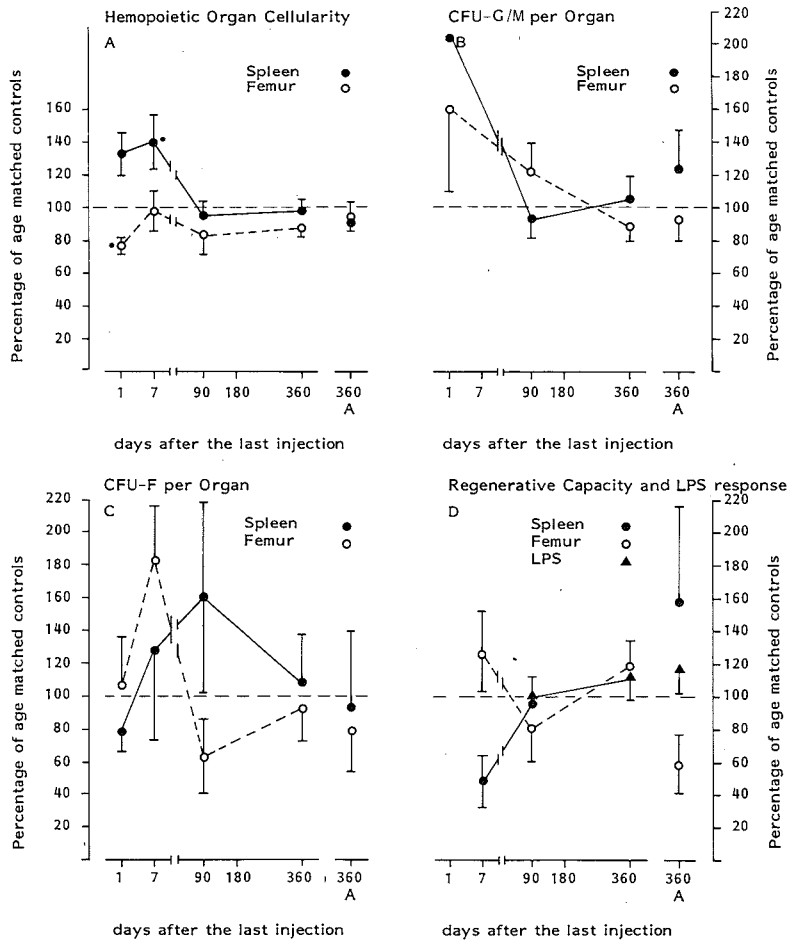

Figure IV.4 *The effect of MTX on splenic and femoral cellularity (A), CFU-G/M content (B), CFU-F content (C) regenerative capacity of spleen and femur and CFU-s accumulation in spleens after LPS treatment (D). All data are expressed as percentage of age matched controls ± SEM of 2-5 experiments. The 1 year data indicated 'A' are from mice treated at 12 weeks of age, the other data are from mice treated at 4 weeks of age. For control numbers see legend Fig. 1. *$p < 0.05$, **$p < 0.01$.*

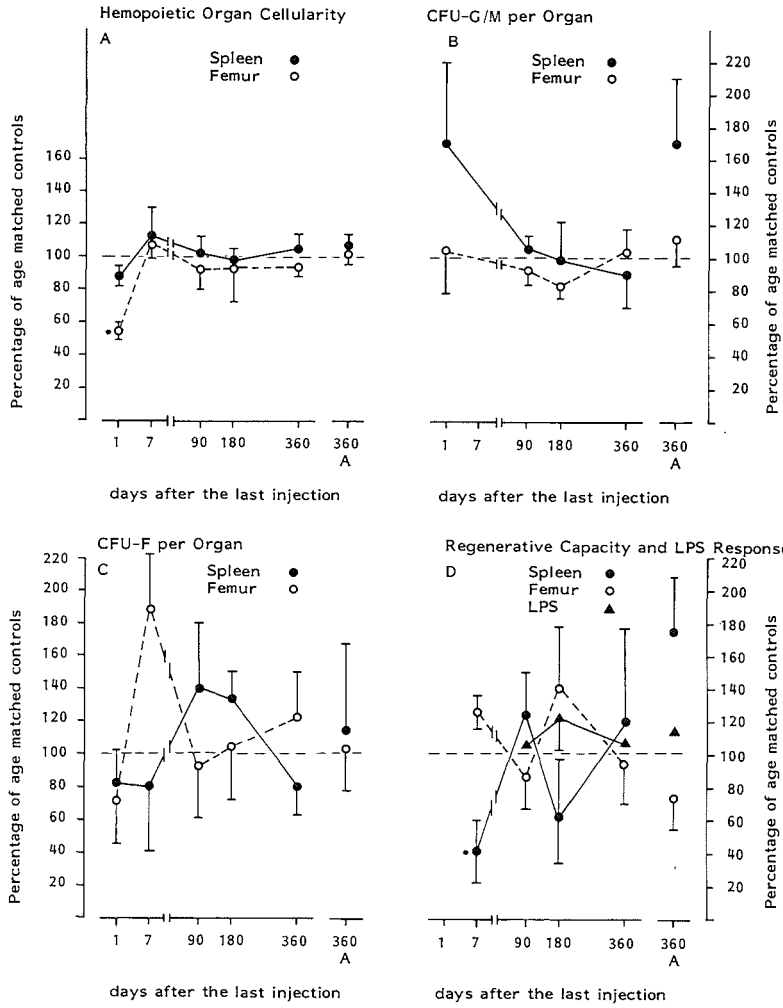

Figure IV.5 *The effect of VCR on splenic and femoral cellularity (A), CU-G/M content (B), CFU-F content (C) regenerative capacity of spleen and femur and CFU-s accumulation in spleens after LPS treatment (D). All data are expressed as percentage of age matched controls ± SEM of 2-5 experiments. The 1 year data indicated 'A' are from mice treated at 12 weeks of age, the other data are from mice treated at 4 weeks of age. For control numbers see legend Fig. 1. *$p < 0.05$ **$p < 0.01$.*

DISCUSSION

Our study clearly demonstrates that the data from 3 stromal assays used, i.e. CFU-F, regenerative capacity, and splenic LPS response, do not correlate with respect to the extent and kinetics of stromal damage inflicted by treatment with cytostatic agents. This notion implies that the use of a single stromal assay can lead to erroneous interpretations of the stromal function after cytotoxic treatment. Moreover, it seems that the stromal defects induced by cytostatic agents are not necessarily accompanied by impaired hemopoiesis and therefore suggest a latent defect in the proliferative capacity of the stroma, rather than a defect in *in situ* stromal function (Fig.IV.1B, IV.1D and IV.2). The proliferative defect that was measured by the implant regeneration assay in the first weeks after BU treatment (Fig.IV.1D) is most likely caused by the small number of surviving stromal cells. Between 90 and 180 days the regenerative capacity of the femoral stroma had reached control levels which suggested a normalization of the number of stromal cells responsible for the maintenance of hemopoietic activity in implanted femoral grafts. It is unlikely that the subsequent secondary decrease observed in the femoral regeneration after BU treatment is also caused by a deficient number of stromal cells. Rather, it may be explained by the assumption that the proliferative potential of the stroma was exhausted during and after BU treatment. This notion of a limited proliferative potential (Hayflick, 1976) seems to be supported by the observation that a decreased regenerative capacity may also occur with ageing of some mouse strains (Hotta et al., 1980; unpublished observations). The existence of a proliferative defect in otherwise normally functioning stromal cells was also observed in long-term bone marrow cultures following *in vitro* irradiation (Laver et al., 1986).

Our laboratory reported recently a similarity between the immediate radiation toxicity for femoral CFU-F and the regenerative capacity of the femur following subcutaneous implantation (Piersma et al., 1983a). In the present study we found that a severely decreased regeneration of spleen and femur implants 7 days after the last BU injection was accompanied by a normal CFU-F content, while the opposite was observed in the femur at 180 days following cessation of BU treatment (Fig.IV.1C and IV.1D). Apparently both assays determine the regenerative ability of different entities of the HM. The data strongly suggest that BU affects regeneration of another stromal component, e.g. endothelial cells, than CFU-F, while gamma radiation at least severely decreases the regenerative ability of CFU-F. These observations indicate that quantitation of CFU-F numbers can not be used to estimate the functional activity of the *in situ* hemopoietic activity or predict long-term effects of cytostatic agents upon hemopoietic stroma. This conclusion is essentially supported by a recent report of Dr. Molineux and his colleagues (1986a), who demonstrated different extent and kinetics of a BU-induced long-term stromal lesion as assayed by the femoral CFU-F numbers and regenerative capacity. This study was performed in adult mice using comparable total doses of BU as were

used in the present report.

Our results on the delayed toxic effects of BU and CTX in young mice are in agreement with and extend previous studies using adult mice (Adler and Kuznetsky, 1984; Fried and Adler, 1985; Fried and Barone, 1980; Fried et al., 1977; McManus and Weiss, 1984). The improvement in regenerative capacity after BU (Fig.IV.1D), which was not observed in adult mice (Fried and Adler, 1985), appears to be at difference with our study. Our data do not show long-term hemopoietic stromal or stem cell damage after MTX or VCR treatment and fully support the previous studies by Trainor and Morley (1976) with respect to the effects of MTX and vinblastine (a VCR-related drug) upon the stem cell compartment.

ACKNOWLEDGMENTS

We would like to thank Professor Dr. O. Vos for critically reviewing the manuscript, and Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript. This investigation is supported by the Netherlands Cancer Foundation (KWF).

CHAPTER V

EFFECTS OF CIS-DIAMMINEDICHLOROPLATINUM (II) UPON HAEMOPOIETIC PROGENITORS AND THE HAEMOPOIETIC MICROENVIRONMENT IN MICE

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ABSTRACT

We studied the short- and long-term effects of a fractionated injection of cis-diamminedichloroplatinum (II) (CDDP) upon the haemopoietic stroma and the haemopoietic precursor cell compartment of young and adult mice. The integrity of the stromal microenvironment was investigated using three different assays including quantification of **(a)** the fibroblastoid progenitor cell compartment, **(b)** the regenerative capacity after subcutaneous implantation of spleen and femur, and **(c)** the growth of normal bone marrow progenitors in lethally irradiated CDDP-treated mice.

CDDP treatment induced a slight anaemia which lasted for the observation period of 1 year, and could not be restored by infusion of normal BMC. The population size of hemopoietic progenitors was severely decreased immediately after CDDP treatment and the CFU-s recovery in the bone marrow was slow and temporary. Stromal function was significantly decreased and normalization occurred within approximately 40 d, depending on the stromal parameter measured. Subsequently, the regenerative capacity of the stroma showed a second decrease which was still detected at 1 year. This pattern of stromal damage has not been reported for any other cytostatic agent. Since the other two assays did not detect a second decrement in stromal integrity it is implied that the three stromal assays used detect different stromal functions. We conclude that CDDP treatment of both young and adult mice results in severe short-term damage and a late occurring secondary regenerative defect of the haemopoietic organ stroma.

INTRODUCTION

It has been demonstrated that cis-diamminedichloroplatinum (II) (CDDP) has significant antineoplastic activity against a variety of solid tumors and lymphomas in both experimental animals (Rosenberg, 1985) and man (Loehrer and Einhorn, 1984; Schilcher et al., 1984). The increase in the percentage of long-term survivors (Loehrer and Einhorn, 1984) emphasized the necessity to study the long-term side effects of CDDP.

Methods to minimize the severe nephrotoxicity (Corden et al., 1985; Jacobs et al., 1984) in order to enable the use of higher doses of CDDP resulted in an increase of response rates, but also enhanced the incidence of myelotoxicity (Khan et al., 1982; Ozols et al., 1983). CDDP treatment has also been reported to increase the sensitivity of the bone marrow for other cytostatics (Haim et al., 1984) or radiotherapy (Keizer et al., 1984). Human and murine studies already indicated a severe short-term CDDP-induced damage of all haemopoietic progenitors, the erythroid progenitor (BFU-E) being most sensitive (Jenkins et al., 1981; Nowrousian and Schmidt, 1982; Rothman and Weick, 1981). Recently Braunschweiger et al. (1982) reported a long lasting defect in the ability of CDDP-treated mice to respond to anaemic stress induced by bleeding.

In view of this we have used three different assays to determine long-term stromal integrity in young (4 weeks old) and adult (12 weeks old) mice, i.e. **(1)** Quantification of the fibroblastoid colony forming unit (CFU-F), a single stromal progenitor class in the haemopoietic stroma (Blackburn and Patt, 1977; Friedenstein et al., 1974a; Tsai et al., 1986). **(2)** Quantification of the regenerative capacity of spleen and femur after subcutaneous implantation. This assay measures the ability of the regenerating donor-derived stroma to accommodate host-derived CFU-s 6-8 weeks after implantation (Fried et al., 1973a; Hotta et al., 1983). **(3)** Quantification of the growth of normal haemopoietic progenitors in irradiated CDDP-treated mice. The first 2 stromal assays listed depend on proliferation of stromal cells (Friedenstein et al., 1974b; Piersma et al., 1985b; Tavassoli et al., 1973a). Recently, it has been shown that a defective proliferative capacity of the stroma is not necessarily accompanied by a defective stromal ability to support haemopoiesis (Laver et al., 1986; Naparstek et al., 1986; Ploemacher et al., 1983). The third assay is assumed to measure the *in situ* stromal integrity under circumstances that do not induce immediate large-scale proliferation of stromal cells.

MATERIALS AND METHODS

Mice. Female (CBA/Rij x C57BL/Rij)F1 mice, 4 weeks old (weighing 14-16 g), and female F1 mice 8 to 35 weeks old (22-34 g), were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and kept under clean conventional conditions.

Drug treatment. Cis-diamminedichloroplatinum(II) (a gift from Bristol-Meyers Company, New York, N.Y.) was dissolved in phosphate-buffered saline (PBS) at a concentration of 0.3 mg/ml and injected i.p. at a dose of 10 mg/kg body weight (BW). This dose is equivalent to a dose of about 30 mg/m^2 in man based on the conversion factors of Freireich et al. (1966). The LD50 of this drug for mice is 14 mg/kg BW (Penta et al., 1979). 4 or 12 weeks old F1 mice were injected three times at weekly intervals. Control mice were injected with the same volume of PBS. There was less than 10% mortality during the observation period.

Colony assays. The CFU-s assay was carried out using the technique of Till and McCulloch (1961). At least 8 recipient mice per point were exposed to a lethal dose of 9.0 Gy total body irradiation (TBI) using a Gammacell 40 ^{137}Cs irradiation unit (Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.27 Gy/min. Irradiated control mice contained an average of 0.2 day-8 colony per spleen. These mice were i.v. injected with appropriate dilutions of the cell suspensions to be tested. Macroscopic surface colonies were counted 8 d later after fixation of the spleen in Telleyesznizky's solution. CFU-c, BFU-E and CFU-F were quantified in a semi solid culture medium as previously described (Brockbank et al., 1983b).

Subcutaneous implantation technique. Spleens and femurs of 4 experimental mice per group were removed after killing the donors with CO_2. Host mice received 5 Gy TBI one day before implantation to stimulate splenic regeneration (Chamberlin et al., 1974). In each recipient mouse two femurs and one spleen were implanted subcutaneously on the ventral abdominal side under Avertin anaesthesia. Six to 8 weeks later the implants were removed and CFU-s per implanted organ assayed.

Growth of normal bone marrow cells in CDDP-treated mice. At different times after cessation of CDDP-treatment, mice received a lethal TBI followed by i.v. injection of 4×10^6 syngeneic normal BMC. The repopulation kinetics of spleen and femur concerning cellularity and CFU-c was determined 4-30 days after TBI and reconstitution as an indication of the stromal ability of spleen and femur to support haemopoiesis.

Growth of bone marrow cells from CDDP-treated mice in normal mice. Normal non-CDDP-treated mice received a lethal TBI and were injected with 4×10^6 BMC from mice at certain times after cessation of CDDP treatment. The femoral cellularity and CFU-c repopulation kinetics in the spleen and femur of these recipients was measured up to 11 days after TBI, as an indication of the regenerative ability of CDDP-treated BMC.

Statistics. Values for all parameters are expressed as arithmetic means, and presented as per cent of age-matched controls unless stated otherwise. SEMs of these data were calculated by converting the absolute SEMs of experimental and control

data in percentages, and taking the square root of their quadrated sum. Thus, the SEMs include both the variance of the means of the control and experimental groups. Determinations for the control and treated group were always performed on the same day to exclude day to day variations. Statistical analysis was done employing the Student t test and using the absolute numbers of both control and treated groups. Statistical difference was assumed at the 95% confidence limits.

RESULTS

Since no significant differences were observed between data from mice treated at 4 or 12 weeks of age, only data from 4-week-old CDDP-treated mice will be presented.

Body weight and peripheral blood

CDDP treatment resulted in a decrease in body weight (BW) with a nadir at 1 week after the last injection (65% of normal). The BW lagged behind that of age-matched control mice until it reached control levels at one year after cessation of treatment (data not shown). Erythrocyte numbers and haematocrit remained significantly below age-matched control numbers between 20 and 360 days after CDDP treatment (Fig.V.1). Further investigation indicated that the anaemia was normochromic and normocytic.

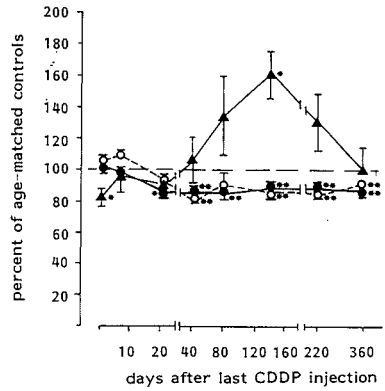

*Figure V.1 Changes in leucocytes (▲), erythrocytes (○) and hematocrit (●) after three CDDP injections of 10 mg/kg given at weekly intervals. Data were converted to per cent of age-matched controls. Data represent the arithmetic means (± 1 SEM).Each point consisted of six to 18 individually assayed mice. Vertical bars indicate SEM. Control values for leucocytes 9.9 X 10^9/l, erythrocytes 8.72 x 10^{12}/l and hematocrit 0.49 l/l. * $p < 0.05$, ** $p < 0.01$.*

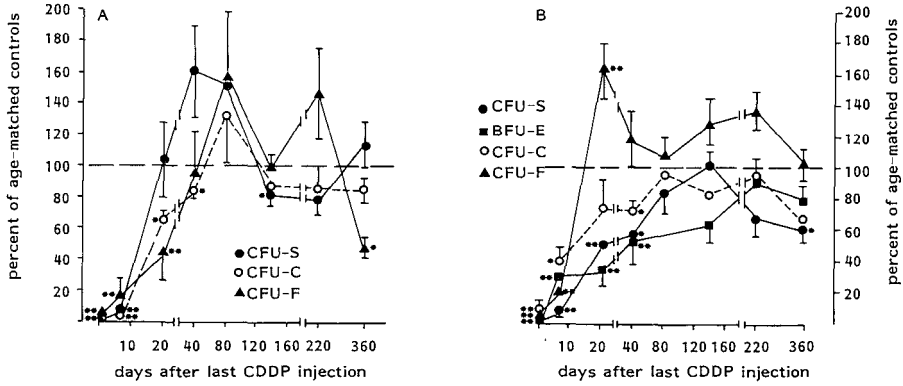

*Figure V.2 Hemopoietic and stromal progenitors in spleen (A) and femur (B) after three CDDP injections of 10 mg/kg given at a weekly interval. Data were converted to per cent of age-matched controls. Each point consisted of six to 18 individually assayed mice. Vertical bars indicate 1 SEM. Control numbers of primitive progenitors in the spleen were: CFU-s 2900, CFU-c 6100, and CFU-F 290 per organ. Control values for the femur; CFU-s 6400, BFU- E 8600, CFU-c 27 200, and CFU-F 1010 per organ. * $p < 0.05$, ** $p < 0.01$.*

Cellularity and haemopoietic and stromal progenitor cell content of spleen and femur

CDDP treatment elicited a large decrease down to 30-40% of control values in the splenic and femoral cellularity, which normalized within 40 days (data not shown). In the spleen the number of the progenitor cells (CFU-s, CFU-c and CFU-F) (Fig.V.2A) were far below control numbers (1-18%) during the first week after cessation of treatment. Subsequently, a fast recovery of CFU-s was observed, followed by restoration of CFU-c and CFU-F. Only at the one year point was the splenic CFU-F content significantly below normal. In the femur the progenitor content (CFU-s, BFU-E, CFU-c and CFU-F) (Fig.V.2B) was also severely decreased immediately after CDDP treatment (1-10% of control). The recovery of the CFU-s population size in the femur was severely delayed as compared with that in the spleen. After day 140 the femoral CFU-s content showed a secondary decline down to 60% of control values. The femoral CFU-F population recovered much faster from the CDDP-induced depletion than the splenic CFU-F population.

Regeneration of spleen and femoral marrow after subcutaneous implantation

The donor-derived femoral marrow capacity to regenerate and support host-derived CFU-s, 6 weeks after subcutaneous implantation into syngeneic hosts (Fig.V. 3), was severely depressed immediately after cessation of treatment but returned to normal at 40 days, followed by a second decrease between 140 and 360 days. The

splenic regenerative capacity was less damaged as compared to that of femoral marrow, and showed a second decrease between 80 and 360 days (Fig.V.3).

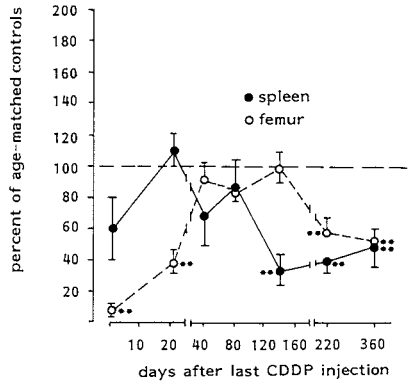

*Figure V.3 Regenerative capacity of spleen and femur after subcutaneous implantation. At different times after cessation of CDDP-treatment femurs or spleen were subcutaneously implanted in syngeneic hosts. Quantification of the CFU-s content per implanted organ 6-8 weeks after implantation (data converted to per cent of age-matched controls) was used to estimate stromal function. Data represent the arithmetic mean of 2-4 individual experiments(± 1 SEM) and each experiment consisted of three to eight pooled spleens or femurs per point. Control spleen implants contained an average of 100 CFU-s. Control femur implants 1100 CFU-s. * $p < 0.05$, ** $p < 0.01$.*

Growth of normal femoral CFU-c in CDDP-treated, lethally irradiated mice

We investigated the *in situ* stromal integrity in CDDP-treated mice by studying the stromal ability to support the growth of injected normal syngeneic BMC after lethal irradiation. Twenty days after the last CDDP injection the femoral and splenic haemopoietic stroma showed a severely decreased capacity to stimulate the growth of normal haemopoietic progenitors (CFU-c) (Fig.V.4A-D), but near normal CFU-c growth occurred in mice at 40 and 220 days after CDDP treatment. At difference with this was the incomplete restoration of nucleated cells in the spleens of these mice at 20 and 40 days after CDDP injection. Femoral cellularity was insufficiently restored at 20, 40 and 220 days. From Table 1 it appears that transplantation of 4×10^6 normal BMC into irradiated CDDP-treated mice (40 days after CDDP) could not restore the normocytic anaemia induced by CDDP as measured at 70 days after BMT.

Growth of CDDP-treated bone marrow cells in normal syngeneic mice

In order to investigate whether CDDP treatment would induce a residual

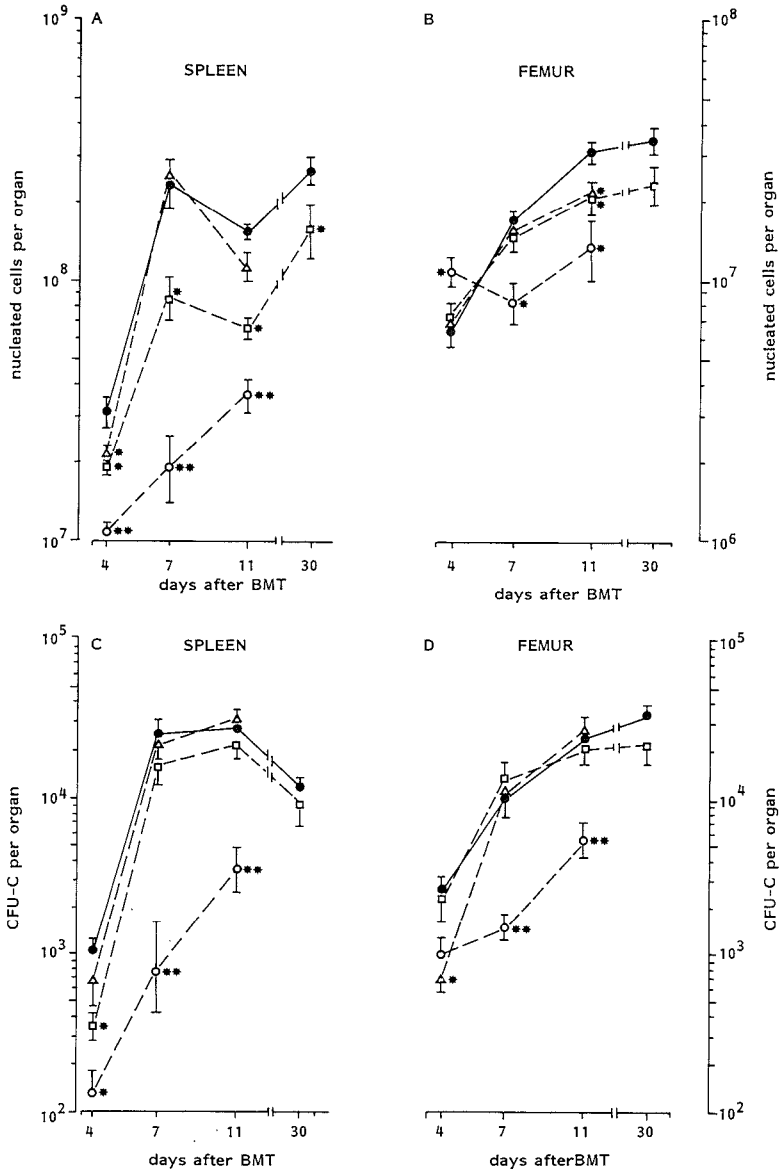

Figure V.4 *Growth kinetics of normal BMC in normal (●) or CDDP-treated mice. At 20 (○), 40 (□) and 220 (△) d after CDDP-treatment, mice were lethally irradiated and reconstituted with 4 x 10^6 normal BMC. Data represent arithmetic means (± 1 SEM) of nine individually assayed mice per point in the control group and three individually assayed mice per point in the CDDP-treated groups. * $p < 0.05$, ** $p < 0.01$.*

stem cell defect we determined the proliferative capacity of CDDP-treated BMC, measured as the number of nucleated cells and CFU-c per femur, and compared this to that of normal BMC in lethally irradiated (9 Gy) but otherwise normal mice. In general, CDDP-treated BMC produced less nucleated cells (Fig.V.5A) and CFU-c (Fig.V.5B) in the femur of the recipients than did BMC from control mice.

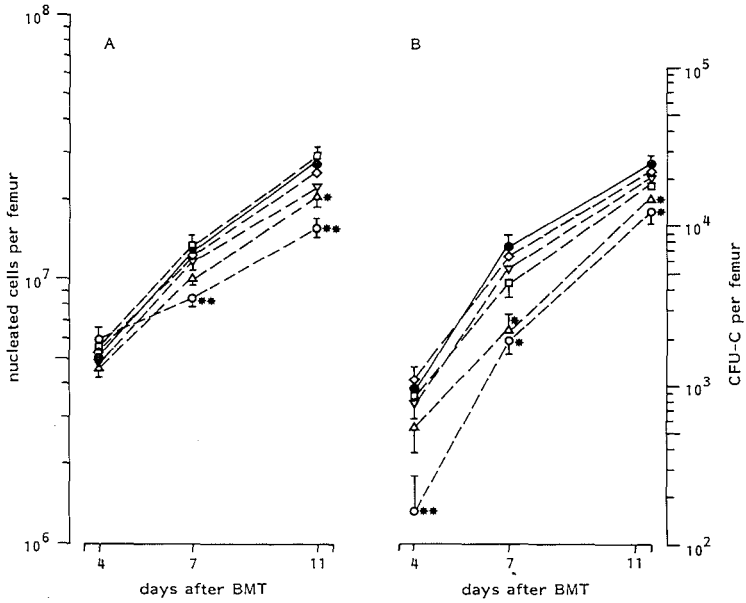

*Figure V.5 Growth kinetics of normal and CDDP-treated BMC in normal recipients expressed as nucleated cells (A) or CFU-c (B) per femur. Normal irradiated mice were grafted with 4 x 10^6 normal BMC (●) or 4 x 10^6 CDDP-treated BMC from mice 20 (○), 40 (△), 80 (▽), 140 (□) or 360 (◇) d after cessation of treatment. Data represent arithmetic means (± 1 SEM) of 15 individually assayed mice per point for the control group and three individually assayed mice per point for the CDDP-treated groups. * $p < 0.05$, ** $p < 0.01$.*

DISCUSSION

Fractionated doses of CDDP induced a long-lasting mild normocytic and normochromic anaemia and transient leucocytosis. Anaemia may be one of the adverse side effects of CDDP treatment in both mice (Nowrousian and Schmidt, 1982) and in man (Getaz et al., 1980; Khan et al., 1982; Ozols et al., 1983; Rothman and Weick, 1981; Wood and Hrushesky, 1984), and may be caused by a decreased erythropoietin production due to nephrotoxicity of CDDP (Wood and Hrushesky,

1984). It has been reported that a comparable fractionated regimen of CDDP as used in the present study induced a long-lasting qualitative and small but significant quantitative defect of the CFU-s population in mice (Dumenil et al., 1982). In difference with this, our observations on the growth potential of CDDP-treated BMC in normal irradiated recipients (Fig.V.5) indicate that CDDP only temporary (day 20) influenced the proliferative ability of the surviving primitive haemopoietic precursors. This suggests that the CDDP-induced anaemia is not the result of an ineffective stem cell compartment but rather is caused by a stromal insult. Supportive evidence for this notion is formed by our observation that infusion of 4 x 10^6 BMC from normal or CDDP-treated mice led to comparable restoration of the radiation-depleted erythroid compartment, in normal mice, while 4 x 10^6 normal BMC could not cure the CDDP-induced anemia (Table V.1).

Table V.1 Haematocrit and erythrocyte numbers in CDDP-treated and normal mice before and after transplantation of CDDP-treated or normal BMC.

			10 weeks after transplantation of:[a]			
	1 hr before BMT		normal BMC		CDDP-BMC	
Recipient	Ht	Ery[c]	Ht	Ery	Ht	Ery
Normal	49.4[d] (0.4)	8.84 (0.15)	49.0 (0.7)	8.88 (0.18)	48.7 (0.9)	8.65 (0.16)
CDDP-treated[b]	42.1 (1.1)	7.42 (0.21)	40.0 (0.9)	7.37 (0.19)	n.d.	n.d.

a. Recipients received 4 x 10^6 BMC from either normal mice, or from mice 40 d after the last CDDP injection.
b. 40 d after cessation of CDDP treatment.
c. Number of erythrocytes x 10^{-9}/ml blood obtained by cardiac puncture under ether anaesthesia.
d. Arithmetic mean (1 SEM) of 10 individually assayed mice.

It appears that the developing stroma (Brockbank et al., 1983b) in 4-week-old mice has a similar sensitivity for CDDP as the full grown stroma of 12-week-old mice. Both the regeneration assay (Hotta et al., 1983; Tavassoli et al., 1973a) and the CFU-F assay (Friedenstein et al., 1974b) are sensitive measures of latent residual damage, since the stromal cells are forced to proliferate and damaged

cells may consequently not survive. In contrast, the establishment of growth curves of BMC in previously CDDP-treated and irradiated mice is assumed to measure the *in situ* functional stromal integrity under circumstances that do not induce immediate large scale proliferation of stromal cells. All three assays (see Fig.V.3, 2, and 4, respectively) detected an early severe injury of the haemopoietic stroma of both spleen and femur and the subsequent restoration of stromal integrity within approximately 40 d after cessation of CDDP treatment. A late secondary stromal defect was detected by the implant regeneration method, but not by the other two stromal assays. We interpret these findings as follows. CDDP induces an immediate and transient functional and proliferative defect of the haemopoietic stroma. The late secondary loss of the regenerative ability may be explained by assuming that the proliferative potential of the surviving stromal cells was exhausted during and after the early recovery phase. Thus an acceleration may have occurred of the decline in regenerative capacity that has been reported to occur with ageing (Hotta et al., 1980). Apparently, this did not apply to CFU-F, since both in spleen and femur normal levels were found between 80 and 360 days except for the splenic CFU-F content at 1 year. These considerations suggest that a residual damage in other stromal cell types, e.g. endothelial cells, may have caused a loss of stromal regenerative capacity, a situation which is reminiscent of the effect of gamma-radiation on the stroma of 4-week-old mice (chapter III).

The doubling time of the haemopoietic precursors in femur and spleen during recovery after CDDP-induced damage was much longer than was observed for normal or CDDP-treated precursor cells injected in normal lethally irradiated mice (Fig.V.8 and Vos, 1972). In the light of the present data we propose that this slow recovery of haemopoietic precursors in CDDP-treated mice both may have resulted from the presumptive stem cell damage which we observed at day 20, and from the observed dysfunction of the hemopoietic organ stroma before day 40. The decrease in the femoral CFU-s population size between day 220 and 360 suggests a defect in the ability of the stroma to maintain sufficient numbers of early precursors, and remarkably coincides with the secondary loss of the femoral regenerative potential as assessed by the implant method.

Our study on the growth of normal BMC in previously CDDP-treated mice may have clinical relevance with respect to the increased use of high-dose chemotherapy and subsequent autologous bone marrow transplantation in the treatment of solid tumors. We show here that the growth of normal BMC was severely delayed in the femur and spleen of mice, that were treated with CDDP 3 weeks before BMT, as compared to that of non-CDDP-treated mice (Fig.V.5). This may lead to a prolonged and intensified risk of bleeding and infections.

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CHAPTER VI

STROMAL FUNCTION IN LONG-TERM BONE MARROW CULTURES OF CIS-DIAMMINEDICHLOROPLATINUM (II) TREATED MICE

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ABSTRACT

The short- and long-term effects of a fractionated cis-diamminedichloroplatinum (II) (CDDP) administration on the ability of the murine femoral stroma to support hemopoiesis was tested using long-term bone marrow cultures. Furthermore, the femoral content of fibroblastoid colony-forming units, committed hemopoietic progenitors (granulocyte/ macrophage colony-forming units) and pluripotent stem cells (spleen colony-forming units) was studied.

One week after cessation of treatment the stromal and hemopoietic precursors were significantly reduced in number. Normal values were observed at 12 weeks after cessation of the CDDP injections. Long-term bone marrow cultures established from femurs of mice at one week after CDDP administration showed a reduction in their cumulative production of non-adherent cells and granulocyte/macrophage colony-forming units. This reduction was due to a decreased number of hemopoietic progenitor cells present at initiation of culture since it could be ameliorated by the addition of a sufficient number of non-adherent normal hemopoietic progenitor cells. Long-term bone marrow cultures established twelve weeks after CDDP administration produced normal numbers of granulocyte/macrophage colony-forming units when compared with control cultures, but the total cell production was slightly decreased. These results demonstrate that CDDP does not damage the capacity of stromal cells to establish an *in vitro* functional microenvironment.

INTRODUCTION

Since the discovery of the antitumor activity of cis-diamminedichloroplatinum (CDDP) in animals (Rosenberg and Van Camp, 1970; Rosenberg et al., 1969), this

compound is included in several clinical protocols. Its use as a single agent, or in combination with other cytostatic drugs, has markedly improved treatment of head, neck and ovarian cancers and resulted in a high cure rate for advanced testicular cancer (Einhorn and Donahue, 1977; Williams and Whitehouse, 1979; Wiltshaw and Kroner, 1976). The major dose limiting effect of the drug is nephrotoxicity (Madias and Harrington, 1978), but hydration, mannitol and/or furosemide induced diuresis, new administration routes and new dosage schedules have been reported to reduce the degree of renal dysfunction (Chiuten et al., 1983; Hayes et al., 1977; Vogl et al., 1980). Because of this, higher CDDP doses can be used and other side effects like anemia and myelosuppression have become dose-limiting (Blumenreich et al., 1985; Chary et al., 1977; Ozols et al., 1984). Although the exact mode of action is not completely understood, it is suggested that CDDP inhibits DNA synthesis by forming both DNA interstrand and intrastrand crosslinks (Pascoe and Roberts, 1974; Zwelling et al., 1981) comparable with those formed by alkylating agents. It is known that some of these agents, e.g. busulfan and cyclophosphamide, exhibit a long-lasting toxic effect on both hemopoietic progenitor cells and the HM (Fried et al., 1970; Molineux et al., 1986a; Morley and Blake, 1974). The use of CDDP in murine models already demonstrated its dose-related and cumulative toxicity for early progenitors, particularly BFU-E and CFU-s (Jenkins et al., 1970; Nowrousian and Schmidt, 1982). Furthermore, Braunschweiger et al. (1982) observed that CDDP-treated mice have a long lasting defect in response to anemic stress induced by bleeding.

In this study we used a clinically relevant treatment protocol of fractionated CDDP administration to investigate the ability of the stromal compartment to support hemopoiesis in long-term bone marrow cultures derived from CDDP-treated and control mice. In addition, we studied the toxic effects of this drug on hemopoietic and stromal progenitor cell classes.

MATERIALS AND METHODS

Drug treatment. Young (4-wk-old) male (CBA/Rij x C57BL/Rij)F1 mice were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands, and kept under conventional conditions. CDDP (a gift from Bristol-Meyers Company, New York, N.Y.) was dissolved in PBS (8.1 mM $Na_2HPO_4.2H_2O$, 2.2 mM K_2PO_4, 2.7 mM KCl and 0.14 M NaCl) at a concentration of 0.3 mg CDDP/ml. Mice received 10 mg of CDDP/kg body weight weekly for a total of three weeks. The total dose is equivalent to a dose of about 90 mg/m^2 in man based on the conversion factors of Freireich et al. (1966). One or twelve weeks after cessation of drug treatment mice were killed by cervical dislocation and both femurs were removed using a sterile technique.

Preparation of nylon wool filtered non-adherent bone marrow cells. Nylon wool columns were prepared by filling 6 ml syringes with nylon wool from Leuko-pak leukocyte filters (Fenwal). Columns were incubated at 37°C with α-medium (α-modification of Dulbecco's minimal essential medium) supplemented with 20% fetal calf serum (FCS: KC Biological lot no. 300361) for 30 minutes before use. Bone marrow suspensions in α-medium plus 20% FCS (maximal 2 x 10^8 BMC per column) were then layered on the column and allowed to adhere on the nylon wool. After 45 minutes of incubation at 37°C non-adherent cells were eluted with 50 ml of α-medium plus 20% FCS at a flow rate of 1 ml per minute. Nonadherent cells were tested for the presence of fibroblastoid, granulocyte-macrophage and early hemopoietic (day 7 and day 12 CFU-s) progenitor cells.

Long-term bone marrow cultures (LTBMC). Bone marrow was flushed from the femur of control or CDDP-treated mice with a 23 gauge syringe needle using 3 ml of α-medium supplemented with 10% horse serum (Boehringer, lot no. 1309200), 10% FCS, 10^{-5}M hydrocortisone hemi-succinate (Sigma), 10^{-4}M mercaptoethanol (Merck) and transferrin (500 μg/ml; Behringwerke) into 25 cm^2 Costar screw-capped culture tissue flasks. Cultures were maintained at 33°C in a humidified atmosphere consisting of 10% CO_2 in air. At weekly intervals, the total non-adherent cell fraction was removed and 3 ml of fresh medium were added to the cultures. The non-adherent cells were counted with the aid of a Coulter particle counter and assayed for granulocyte-macrophage progenitors.

Spleen colony assay. The CFU-s assay was performed as described by Till and McCulloch (1961). Groups of 8 recipient mice received a lethal dose of 9 Gy total body gamma-radiation using a ^{137}Cs-gamma-source (Atomic Energy of Canada Ltd., Ottawa, Canada) at a dose rate of 1.23 Gy/min, and were subsequently transfused with appropriate dilutions of BMC. After 7 or 12 days spleens were removed and fixed in Telleyesnizky's solution. The number of macroscopically visible spleen colonies was counted.

Fibroblast colony-forming units (CFU-F). BMC were cultured in alpha-medium containing 0.8% methylcellulose and 20% FCS. One ml aliquots of culture medium, containing 5 x 10^5 cells, were plated in 35 mm Costar culture dishes and incubated at 37°C in a humidified atmosphere consisting of 10% CO_2 in air. All determinations were performed in triplicate. On day 10 of culture the cells were washed with PBS, fixed with methanol and stained with 10% Giemsa. Fibroblastoid stromal cell colonies containing at least 50 fibroblastic cells were counted with an inverted microscope.

Granulocyte-macrophage colony-forming units (CFU-G/M). BMC were cultured in alpha-medium (5 x 10^4 nucleated cells/ml medium) containing 0.8% methylcellulose, 10% FCS, 1% BSA and 10% con-A stimulated mouse spleen conditioned medium. Cells

were cultured for 7 days under the same conditions as described for CFU-F. All determinations were performed in duplicate. Granulocyte-macrophage colonies, containing at least 50 cells, were counted with an inverted microscope.

Experimental design. In this study we used the production of non-adherent cells and granulocyte/macrophage precursor cells in LTBMC from femurs of CDDP-treated and control mice as parameters for the toxic effects of CDDP. A CDDP-induced reduction of these parameters could be due to **(a)** a reduction in the number of hemopoietic and/or stromal cells per explanted femoral plug, **(b)** a functional or proliferative defect in hemopoietic and/or stromal cells, or **(c)** a combination of these possibilities. To investigate the first possibility, we compared the production of non- adherent cells and CFU-G/M in control cultures with the production of those cells in cultures set up with half a femoral plug. In order to study the CDDP toxicity for stromal cells, we attempted to reinoculate non-hemopoietic stromal layers from femurs of CDDP-treated and control mice with adherent cell depleted normal BMC. Non-hemopoietic stromal layers were initially obtained using the system of Cohen et al. (1980) in which no horse serum and hydrocortisone was added to the medium. However, re-addition of hydrocortisone and horse serum led to a resumption of the hemopoietic cell production in such cultures indicating that resting stem cells were still present. As a consequence of this, we chose another approach. Cultures derived from whole femurs of treated animals and those derived from halved control femurs were supplemented with an excessive number of adherent cell-depleted normal BMC at initiation of culture. This complement of untreated hemopoietic cells is known to be unable to form an adherent stromal layer. This assay therefore allowed us to study a possible stromal defect induced by CDDP.

RESULTS

Hemopoietic and stromal progenitors

One week after cessation of treatment a reduction of the number of BMC to approximately 50% of control numbers was observed. All progenitor cell types were significantly reduced and the greatest reduction was observed in the CFU-F and CFU-s numbers, which were 17-18% of control values (Table VI.1). Twelve weeks after cessation of treatment all parameters measured had returned to normal values.

Nylon wool filtration

Nylon wool filtration of normal BMC effectively removed CFU-F and resulted in a slight enrichment in the incidence of day 7 and day 12 CFU-s. Relative numbers of BFU-E and CFU-G/M remained normal (Fig.VI.1). The recovery of the total cell number after nylon wool adherence was 26.2 $\pm$ 2.4 percent (mean $\pm$ 1 SE, n=8).

Table VI.1 Stromal and hemopoietic progenitor cell content of the femur after CDDP treatment.

	cells/femur	CFU-GM/femur	CFU-F/femur	CFU-s/femur	
	x 10^7	x10^3	x10^2	x10^2	
				day 7	day 12
			Controls		
1 wk	28.5 ± 1.3[a]	36.6 ± 6.2	15.7 ± 1.4	75.6 ± 16.9	58.7 ± 8.9
	(100)[b]	(100)	(100)	(100)	(100)
	n = 24[c]	n = 24	n = 15	n = 9	n = 9
12 wk	34.0 ± 1.4	33.9 ± 3.3	9.1 ± 4.0	55.9 ± 9.8	n.d.
	(100)	(100)	(100)	(100)	-
	n = 9	n = 5	n = 3	n = 6	-
			CDDP-treated		
1 wk	15.1 ± 1.9	11.9 ± 2.7	2.9 ± 0.7	13.3 ± 5.1	10.1 ± 2.6
	(53)	(33)	(18)	(18)	(17)
	n = 24	n = 24	n = 15	n = 9	n = 9
	p < 0.002[d]	p < 0.002	p < 0.002	p < 0.002	p < 0.002
12 wk	31.9 ± 1.4	32.6 ± 5.0	9.7 ± 4.1	45.7 ± 9.4	n.d.
	(94)	(96)	(107)	(82)	-
	n = 9	n = 6	n = 3	n = 6	-
	n.s.	n.s.	n.s.	n.s.	-

a. Arithmetic mean ± 1 SE.
b. number in parentheses, percentage of control values.
c. n, number of mice assayed.
d. level of significance determined by Mann-Whitney U-test.
n.d. not determined.
n.s. not significant.

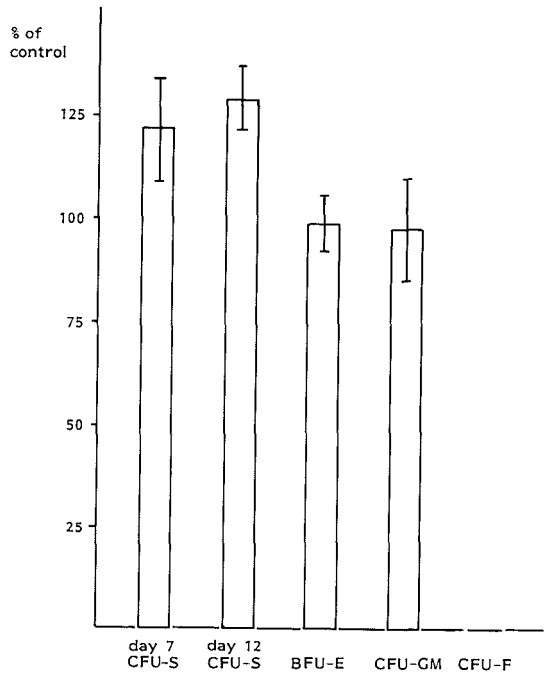

Figure VI.1 Effect of nylon wool adherence on the frequency of hemopoietic and stromal progenitor cells. Data are expressed as progenitor cell numbers present in the non-adherent fraction relative to that of non-filtered normal bone marrow. Each column represents the mean of five replicate experiments (%), except for the CFU-s columns, which are based on three replicate experiments.

Long-term bone marrow cultures.

To determine whether CDDP administration leads to a functional damage of the HM, LTBMC were prepared from whole femurs of CDDP-treated or control mice one week after cessation of treatment. Although no morphological changes in the composition of the stromal layer could be observed between cultures derived from treated or control mice, both the total production of non-adherent cells and CFU-G/M were severely reduced in LTBMC of treated mice (Fig.VI.2A and VI.3A). Two approaches were followed to investigate whether this reduction was due to either a quantitative or qualitative defect of the hemopoietic and/or stromal cells (see Materials and Methods: experimental design). Firstly, we prepared LTBMC of halved control femurs and secondly we supplemented part of the cultures with adherent cell-depleted normal BMC. Such cell suspensions contain all hemopoietic progenitor cell classes but no CFU-F (Fig.VI.1) and were not able to establish

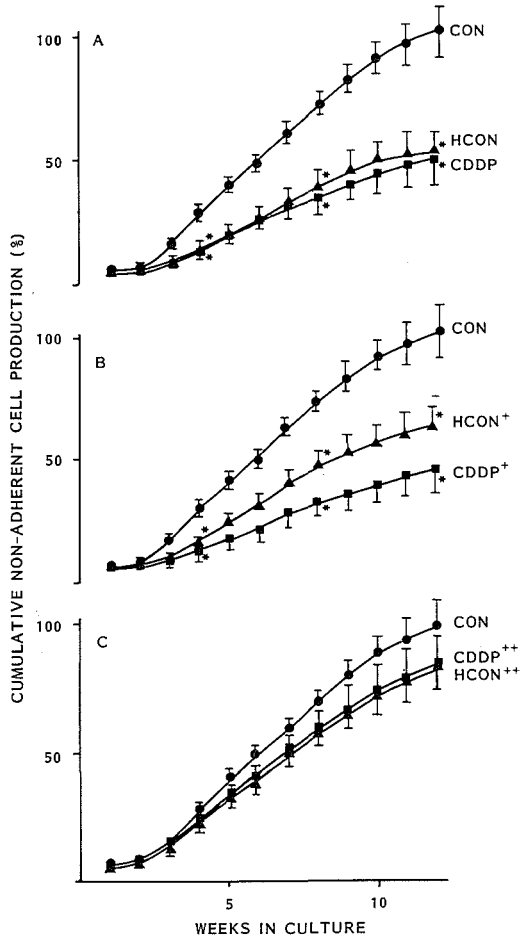

Figure VI.2 *Cumulative non-adherent cell production in LTBMC established one week after CDDP administration from a whole femur of control (●) and CDDP-treated (■) mice, and in LTBMC established from half a control femur (▲). Whole marrow plugs from CDDP-treated mice or halved marrow plugs from control mice were either not (A) or immediately supplemented with 5 x 10^6 (B) or 2 x 10^7 (C) nylon wool-filtered BMC. Data are expressed as the mean of three replicate experiments. Non-adherent cells derived from control LTBMC are expressed in absolute numbers and experimental groups are expressed as a fraction of these control cultures. In each experiment groups contained six cultures derived from six individual mice.*

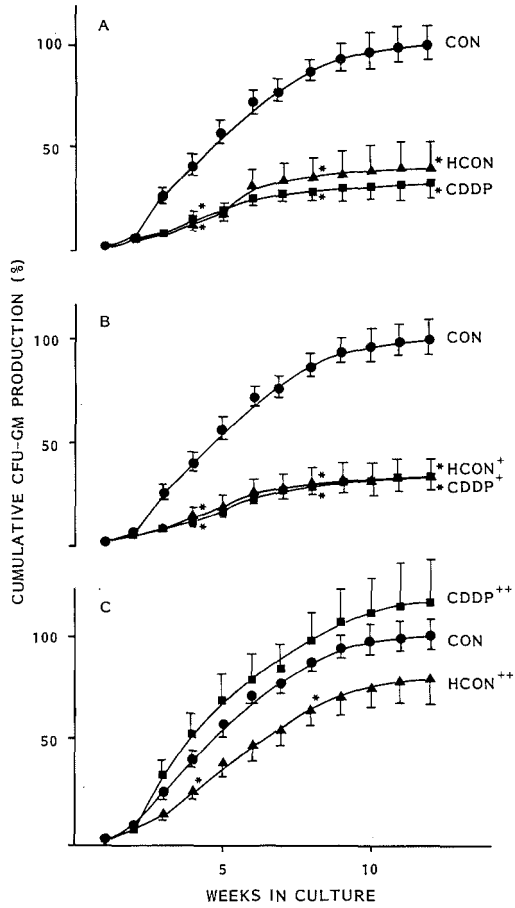

Figure VI.3 The cumulative CFU-G/M production in LTBMC established one week after CDDP administration from a whole femur of control (●) and CDDP-treated (■) mice and in LTBMC established from half a control femur (▲). Whole marrow plugs from CDDP-treated mice or halved marrow plugs from control mice were either not (A) or immediately supplemented with 5 x 10^6 (B) or 2 x 10^7 (C) nylon wool-filtered BMC. Data are presented as indicated in Figure 2.

adherent layers which could maintain hemopoiesis (data not shown).

LTBMC established from half a normal femur plug showed a similar reduction in the cumulative production of non-adherent cells and CFU-G/M as cultures established from whole femurs of treated mice (Fig.VI.2A and VI.3A). Both in cultures established from half a normal femur and a whole CDDP-treated femur,

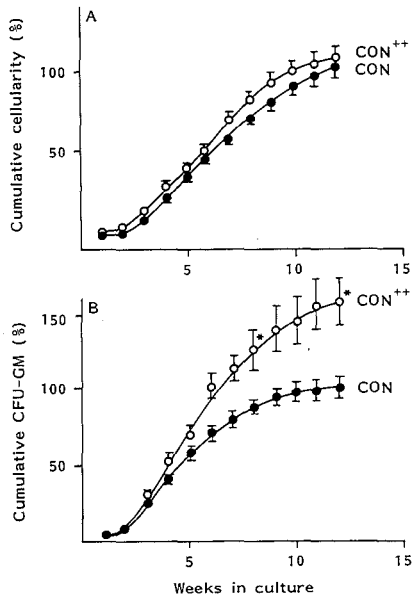

Figure VI.4 The cumulative non-adherent cell (A) and the cumulative CFU-G/M (B) production in LTBMC established from a control femur either not (●) or immediately supplemented with 2 x 10^7 (○) nylon wool-filtered BMC. Data are presented as indicated in Figure 2.

the reduced cell production could not be restored by adding 5 x 10^6 nylon wool filtered cells (Fig.VI.2B and VI.3B). Addition of four times as many nylon wool filtered cells increased the total non-adherent cell numbers, but control values were not achieved (Fig.VI.2C). A comparable effect was seen for the CFU-G/M numbers in marrow cultures derived from the content of half a normal femur (Fig. VI.3C). In difference with this, CFU-G/M numbers in LTBMC of CDDP-treated mice were even increased to 120% of control values (Fig.VI.3C). These observations demonstrated that in LTBMC of treated mice, the ratio between produced CFU-G/M and non-adherent cell numbers was altered. This alteration was not due to the CDDP treatment because it was also seen in cultures of a whole control femur which were inoculated with 2 x 10^7 nylon wool filtered cells (Fig.VI.4). Three months after cessation of treatment the production of CFU-G/M in marrow cultures of CDDP-treated mice was normal compared with control cultures but the cellularity was reduced (Table VI.2).

Table VI.2 Production of non-adherent cells and CFU-GM in LTBMC of CDDP-treated and control mice twelve weeks after cessation of treatment.

	Controls			CDDP-treated		
wk of cult.	cells/ flask $x10^5$	CFU-GM/ flask $x10^2$	CFU-GM/ 5.10^4 cells	cells/ flask $x10^5$	CFU-GM/ flask $x10^2$	CFU-GM/ 5.10^4 cells
1	16.2±1.5[a]	18.9±1.6	62±9	15.4±0.8[a]	10.3±1.3	33±3
2	13.4±1.1	33.3±4.4	122±9	10.4±1.8	19.5±6.9	97±4
3	110.8±15.1	113.2±21.4	57±10	96.8±10.9	84.2±19.7	46±13
4	94.2±5.8	60.4±11.5	32±6	81.6±5.1	68.7±28.5	44±19
5	119.2±13.4[b]	66.4±14.8	27±3	87.6±13.2	61.1±12.0	35±7
6	102.5±14.5[b]	52.7±15.3	25±4	62.9±3.8	71.5±14.2	56±8
7	122.0[c]	58.0	24	64.1±4.8	66.3±19.3	52±16

a. arithmetic mean ± 1 SE of 5 flasks.
b. arithmetic mean ± 1 SE of 3 flasks.
c. one flask

DISCUSSION

One week after cessation of CDDP-treatment all femoral stromal and hemopoietic progenitor cell types were reduced significantly (Table VI.1). CDDP had a greater toxicity for CFU-F and CFU-s than for CFU-G/M. These short-term effects of CDDP are in accordance with other reports (Dumenil et al., 1982; Jenkins et al., 1981; Nowrousian and Schmidt, 1982; Wierda and Pazdernik, 1979a). Twelve weeks after cessation of treatment we observed a normalization of CFU-F and CFU-G/M numbers. At this time day 7 CFU-s numbers were at subnormal values although not significantly different from control numbers. A small and long-lasting reduction of early hemopoietic progenitors was also demonstrated by Dumenil et al (1982). Furthermore, a defective response to anemic or other hemopoietic stress situations after CDDP injections has been reported (Braunschweiger et al., 1982; Kovacs et al., 1986).

Little is known about the effects of CDDP administration on the HM. In this study we demonstrated a considerable reduction of CFU-F one week after cessation of treatment (Table VI.1), which indicates that the proliferative capacity of at least one stromal cell class was reduced. Since it has been shown that a reduced

proliferative capacity of the stroma not necessarily is accompanied by a defective stromal ability to support *in vitro* hemopoiesis (Laver et al., 1986; Naparstek et al., 1986), LTBMC were prepared to investigate a CDDP-induced stromal dysfunction. Other studies already indicated that LTBMC were useful to demonstrate stromal damage in irradiated mice and in mice treated with busulfan or BCNU (Anderson et al., 1982; Cohen et al., 1980; Hays et al., 1982; Tavassoli, 1982). Reinoculation of non-hemopoietic layers with a complement of untreated non-adherent BMC would represent the most direct assessment of stromal integrity. Unfortunately, we did not succeed in the establishment of pure stromal layers. Since irradiation of LTBMC to remove hemopoietic activity as described by others (Quesenberry et al., 1984a) cannot be recommended because this may interfere with CDDP-toxicity, we decided to mask the toxic effects of CDDP on hemopoietic progenitor cells by addition of an excess of nylon wool-filtered cells. Such cell suspensions are depleted of adherent cells and contained normal number of progenitor cells (Fig.VI.1), which are assumed to compete with presumptively CDDP-damaged progenitor cells and thus compensate for CDDP toxicity on hemopoietic cells.

We observed no difference in the production of nonadherent cells and CFU-G/M in LTBMC of CDDP-treated mice and those achieved from half a femur (Fig.VI. 2A and VI.3A). This was unexpected because in CDDP-treated marrow early hemopoietic progenitor cells were reduced to 18% of control numbers, which is three times less than in marrow derived from half a femur. These results can be explained if we assume that a pre-CFU-s exists for which CDDP is less toxic than for day 7 and day 12 CFU-s. The existence of such a cell type has been proposed by Chertkov et al. (1986) who demonstrated that hemopoiesis still occurred in LTBMC from CFU-s-depleted BMC obtained from hydroxyurea-treated mice. In addition, it has been demonstrated that BMC, highly enriched for day 10 CFU-s were not able to maintain hemopoiesis in LTBMC (Spooncer et al., 1985). The assumption of a low incidence of such pre-CFU-s in normal bone marrow would explain why a supplement of 5×10^6 nylon wool-filtered cells did not improve CFU-G/M and cell numbers in LTBMC (Fig.VI.2B and VI.3B). Interestingly, with larger cell numbers inoculated, non-adherent cell production was rather constant regardless of the initial cell number (Fig.VI.2C and VI.4A). Crouse et al. (1984) proposed that this phenomenon, called "flask homeostasis" is caused by regulation of the number of differentiated progeny produced. In contrast, CFU-G/M production showed good correlation with initial cell numbers (Fig.VI.3 and VI.4B). These findings implicate that both the extent of differentiation, expressed as the number of non-adherent cells produced per CFU-G/M, and, with higher cell numbers produced, flask cellularity do not reflect the capacity of the stroma to support hemopoiesis.

We conclude therefore that CDDP does not affect the capacity of the hemopoietic stroma to establish a functional *in vitro* microenvironment. The observed reduction in the cumulative production of non-adherent cells and CFU-G/M in LTBMC of CDDP-treated mice probably is due to the reduced number of hemopoie-

tic progenitor cells at initiation of culture since it could be ameliorated by addition of a sufficient number of non-adherent normal BMC. These results demonstrate that one should be careful to interpret data obtained from LTBMC when including femoral marrow from mice following an ablative protocol. Furthermore, we have demonstrated that a reduction of femoral CFU-F numbers does not correlate with the capacity of the hemopoietic stroma to establish an *in vitro* functional micro-environment. This observation indicates that quantification of CFU-F numbers can not be used as a reliable parameter to estimate the functional capacity of the hemopoietic stroma. This conclusion is in agreement with a previous report from our laboratory (appendix paper 2) demonstrating that regeneration of spleen and femur implants of busulfan-treated mice did not correlate with the initial CFU-F content of these implants.

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CHAPTER VII

SHORT TERM IMMUNOSUPPRESSIVE EFFECTS OF CIS-DIAMMINE-DICHLOROPLATINUM (II) (CDDP) IN MICE

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SUMMARY

The effect of CDDP upon the murine immune system was investigated. CDDP injections dramatically decreased the number of T cells and the functional activity of the cellular immune system as measured in a delayed type hypersensitivity (DTH) assay immediately after cessation. These parameters returned to normal within 3 to 6 weeks after cessation of treatment. The serum Ig levels, RBC and WBC counts, and hematocrit did not show major changes during the observation period.

INTRODUCTION

CDDP has been shown to be an effective antineoplastic agent for solid tumors in experimental animals (Rosenberg and Van Camp, 1970) and man (Prestayko et al., 1979). CDDP represents a new class of cytostatic drugs: it reacts with DNA to form both DNA interstrand and DNA intrastrand cross links as well as DNA protein cross links, thus preventing DNA synthesis and cell division (Roberts and Thompson, 1979). In short-term experiments, a single CDDP injection has been shown to exert immunosuppressive effects (Berenbaum, 1971; Khan and Hill, 1972) and to inhibit T lymphocyte function to a greater extent than B lymphocyte function (Wierda and Pazdernik, 1979b). Recently, CDDP has been reported to induce myelosuppression and anemia when given repeatedly (Corder et al., 1977). The aim of the present study was to investigate the immunosuppressive effects of repeated CDDP injections. Therefore, we determined the effects upon the functional activity

of the cellular and humoral immune system as well as the numbers of T lymphocytes in lymphoid organs.

MATERIALS AND METHODS

Animals. (C57BL/Rij x CBA/Rij)F1 (H-$2^{b/a}$) female mice, 4 weeks old and weighing 14 to 16 g, similar F1 female mice, 8 to 20 weeks old, and DBA/2 (H-2^{d}) female mice, 8 to 25 weeks old, were purchased from the Radiobiological Institute TNO, Rijswijk, The Netherlands. AKR (H-2^{k}) female mice, 10 to 20 weeks old, were purchased from Bomholtgard, Ry, Denmark.

Drug treatment. Cis-diamminedichloroplatinum (II) (Bristol Meyers Company, East Syracuse, N.Y., USA) was dissolved in PBS at a concentration of 0.3 mg/ml and injected i.p. at a dose of 10 mg/kg. The LD50 of this drug for mice is 14 mg/kg BW (Penta et al., 1979). The four week old F1 mice were injected once a week for three consecutive weeks. Control mice were injected with the same volume of PBS. There was less than 2% mortality during the observation period.

Cell suspensions. Cell suspensions were prepared as described in detail elsewhere (Wolters and Benner, 1978). For flow cytometric analyses the cell suspensions were washed and resuspended in PBS with 1% bovine serum albumin (BSA), pH 7.8. Nucleated cells and red blood cells were counted with a Coulter counter model B.

Irradiation. AKR female mice received 7.5 Gy whole body irradiation. Irradiation was performed in a Philips MÜller MG 300 X-ray machine as described previously (Wolters and Benner, 1978). All radiation control mice died between 14 and 21 days after irradiation.

Assay for delayed type hypersensitivity. CDDP-treated and control F1 mice received a subcutaneous immunization in the inguinal area with 1 x 10^{7} DBA/2 spleen cells. Six days later they were challenged in the dorsum of the right hind foot with 2 x 10^{7} DBA/2 spleen cells. The DTH response to this challenge was measured as the difference in thickness of the hind feet 24 hours later. The specific increase in foot thickness was calculated as the relative increase in foot thickness of the CDDP-treated or control F1 mice minus the relative increase in foot thickness of control mice that only received the challenge. The foot swelling of the challenged control mice ranged from 15-25%.

Graft-versus-Host assay. The anti-host DTH reactivity during acute Graft-versus-Host (GvH) reactions was determined as described in detail elsewhere (Wolters and Benner, 1978).

Antisera. Antibodies directed to the Thy-1 cell surface antigen of mouse lymphoid cells was obtained from the tissue culture supernatant of an appropriate hybridoma. The origin of these anti-Thy-1 monoclonal antibodies and their use in immunofluorescence has been described elsewhere (Ledbetter and Herzenberg, 1979). As a second step antiserum, a fluoresceinated rabbit-anti-rat immunoglobulin (RaRa-FITC) was used (Nordic Immunological Laboratories, Tilburg, The Netherlands; lot no. 10-375). This serum was diluted 1:20 v/v in 1% BSA in PBS, pH 7.8.

Flow cytometric analysis. Incubation of 100 µl cell suspensions containing 1 x 10^7 nucleated cells/ml with monoclonal antibodies was performed at 4°C for 30 min in the presence of 1 mM of sodium azide to prevent capping of cell surface antigens. These cells were then centrifuged and washed twice in 1% BSA in PBS, pH 7.8. As a second step antiserum 50 µl RaRa-FITC was applied. Incubation with this antiserum was done for 30 min at 4°C in the presence of 1 mM of sodium azide. Finally the cells were centrifuged and washed twice in 1% BSA in PBS, pH 7.8 and their fluorescence analyzed with a fluorescence-activated cell sorter (FACS II, Becton and Dickinson FACS systems, Sunnyvale, C.A., USA). For each sample 5 x 10^3 cells were analyzed.

Serum Ig levels determination with an enzyme-linked immunosorbent assay (ELISA). A micro ELISA method was adopted to measure quantitatively mouse serum Ig levels of various classes. Details of this method have been described previously (Van Soest et al., 1984). Briefly, diluted serum samples were coated onto the bottom of Terasaki plates. After washing with Dulbecco ` s PBS (DPBS) the plates were incubated for 1 h at 37°C with DPBS-gelatin to coat non-specific binding sites. Subsequently the plates were repeatedly washed with DPBS-Tween-gelatin and incubated for 1 h at room temperature with rabbit-anti-mouse immunoglobulin (RAM-Ig) or isotype-specific antisera of rabbit origin (RAM-IgM, RAM-IgG, RAM-IgA and RAM-IgE) which were affinity-purified and found to be specific for the respective mouse Ig ` s by immunoelectrophoresis. Application of conjugate and substrate and measurement of the fluorescence were described previously (Van Soest et al., 1984). To quantitate the Ig levels, a reference standard of a large pool of normal mouse serum obtained from approximately one-year-old CBA/Rij mice was used on each plate (Mink and Benner, 1979).

RESULTS

After repeated CDDP injections the BW of the mice decreased with a nadir at one week after the last injection (Fig.VII.1). At 6 weeks the BW still lagged behind that of age-matched control mice. RBC number and Ht showed a slight increase during 1 week after treatment. From week 3 onwards the RBC and Ht decreased slowly. WBC counts oscillated around control values during the entire

observation period.

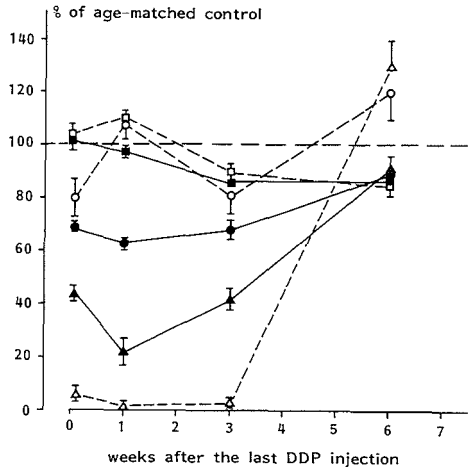

Figure VII.1 Effect of CDDP treatment on various parameters. ●, *body weight (control ± 1 SEM, 17.0 ± 0.5g);*■, *hematocrit (control 49.5 ± 0.5);*□, *RBC number (control 8.3 ± 0.2 x 10^9/ml);*○, *WBC number (control 7.0 ± 1.0 x 10^6/ml);*▲, *spleen cellularity (control 2.0 ± 0.1 x 10^8);*△, *thymus cellularity (wk 0, control 2.6 ± 0.2 x 10^8; wk 6, control 1.5 ± 0.3 x 10^8). Each experimental point represent the arithmetic mean of 6-18 mice. Vertical bars indicate 1 SEM.*

Table VII.1 Numbers of Thy-1^+ cells in spleen and thymus of CDDP treated mice.

Interval after last CDDP injection	Spleen		Thymus	
	control	CDDP	control	CDDP
1 day	14.13 ± 1.26[a]	6.24 ± 1.03	17.95 ± 4.90	0.27 ± 0.08
1 week	10.21 ± 0.84	4.83 ± 1.02	15.36 ± 0.35	0.25 ± 0.10
3 weeks	8.84 ± 0.45	4.09 ± 0.35	13.66 ± 1.62	8.11 ± 5.70
6 weeks	12.47 ± 0.54	11.95 ± 2.05	11.83 ± 0.49	19.80 ± 3.18

a Figures represent the arithmetic mean ± 1 SEM (x 10^7) of the number of Thy-1^+ cells per organ (n = 3-5).

Spleen and thymus cellularity were considerably decreased 1 week after the last CDDP injection. The splenic cellularity returned slowly to control values whereas the thymus cellularity exceeded that of age-matched control values. This decreased cellularity was at least partly due to a decrease of the number of T cells. At 6 weeks the number of T cells in the spleen had returned to control values. In the thymus, on the other hand, the number of T cells exceeded that of age-matched controls (Table VII.1). The influence of CDDP on cell mediated immunity was studied by means of a DTH assay. Thus, CDDP-treated F1 mice and age-matched control F1 mice were subcutaneously immunized with DBA/2 spleen cells 3 and 6 weeks after cessation of treatment. CDDP-treated mice showed a severely impaired DTH response to the DBA/2 alloantigens 3 weeks after the last injection, while the DTH reactivity was normal again at 6 weeks after cessation (Fig.VII.2). One week after the last CDDP injection, spleen cells from F1 (donor) mice displayed decreased anti-host DTH reactivity under GvH conditions. This ability had returned to normal values 3 weeks after cessation of treatment (Fig.VII .2).

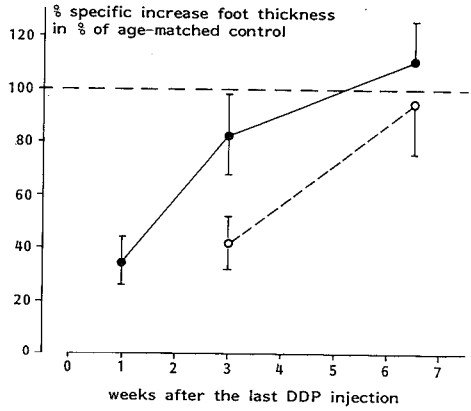

Figure VII.2 *Anti-host DTH reactivity (○) in lethally irradiated AKR (H-2^{k}) mice inoculated with 2 x 10^{7} spleen cells from CDDP-treated or control (C57BL x CBA)F1 (H-2$^{b/d}$) mice. DTH responses were measured 24 h after challenge. Each experimental point represents the arithmetic mean of 6 to 8 mice expressed in % of control. Vertical bars indicate 1 SEM. Primary DTH responsiveness (●) of CDDP-treated and control (C57BL x CBA)F1 (H-2$^{b/d}$) mice sc immunized with 10^{7} spleen cells from DBA/2 (H-2^{d}) mice. DTH responses were measured 24 h after challenge. Each experimental point represents the arithmetic mean of 6 to 8 mice expressed in % of control. Vertical bars indicate 1 SEM.*

The CDDP treatment did not affect the ongoing Ig synthesis as far as could be deduced from the serum Ig levels (Table VII.2).

Table VII.2 Immunoglobulin concentrations in mouse serum at various intervals after the last of 3 weekly CDDP injections.

		Interval after the last CDDP injection			
		1 day	1 week	3 weeks	6 weeks
IgM	Control	32.6 ± 1.6[a]	43.0 ± 5.9	34.3 ± 1.8	33.6 ± 1.3
	CDDP	31.2 ± 2.7	41.7 ± 6.5	25.3 ± 3.2	34.8 ± 0.7
IgG	Control	558.9 ± 40.4	595.6 ± 77.2	622.0 ± 54.5	657.0 ± 108.1
	CDDP	546.1 ± 18.6	606.9 ± 69.8	607.6 ± 127.6	629.5 ± 38.3
IgA	Control	141.4 ± 4.8	139.8 ± 13.6	136.6 ± 5.9	129.7 ± 16.9
	CDDP	147.0 ± 4.2	114.6 ± 14.3	114.3 ± 14.9	117.0 ± 16.3
IgE	Control	357 ± 7	344 ± 13	363 ± 8	315 ± 10
	CDDP	330 ± 6	323 ± 18	372 ± 17	326 ± 10
total Ig	Control	699.9 ± 29.9	678.5 ± 120.5	610.0 ± 43.5	748.8 ± 22.8
	CDDP	789.6 ± 29.7	773.9 ± 83.4	740.6 ± 98.2	737.9 ± 44.5

a. Figures represent the arithmetic mean ± 1 SEM in mg per 100 ml serum. The IgE levels, however, are expressed in μg per 100 ml (n = 3-5).

DISCUSSION

This paper shows that a 3 week long treatment of mice with CDDP has profound effects on the immune system while leaving the hematologic peripheral blood parameters (RBC and WBC counts, and Ht values) almost unchanged. This extends a previous report from Nowrousian and Schmidt (1982) who described minor changes in hematological peripheral blood parameters after a single dose of 12 mg/kg BW.

The effects of CDDP on the immune system seem to be restricted to the T cell compartment: we did not find significant changes in the serum Ig levels. However, determination of serum Ig levels is an insensitive parameter for the functional activity of the humoral immune system (Sabbele et al., 1983).

Table VII.1 shows a markedly decreased T cell content in the spleen one week after cessation of CDDP treatment. As Graft-versus-Host reactions were induced by transplantation of 10^7 nucleated spleen cells, the subsequent DTH response can be related to the T cell content of the inoculum. Such calculations suggest a severely disturbed function of the remaining T cells. The latter might be due to the decreased proliferative capacity caused by the cross linking of DNA by CDDP. Such a decreased proliferative response of T cells has previously been shown in mice 3 days after a single CDDP injection (Wierda and Pazdernik, 1979b). Thus, it seems that proliferation-dependent immune reactions, as occur in DTH and following concanavalin A stimulation (Bloom et al., 1964; Wierda and Pazdernik, 1979b; Wolters and Benner, 1979), are severely but not irreversibly impaired by CDDP.

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CHAPTER VIII

GENERAL DISCUSSION

The general discussion is divided in paragraphs according to the main objectives mentioned in the introduction to the experimental work i.e.: 1. a study to compare the different assays to detect stromal integrity with respect to extent and kinetics of stromal damage and recovery after radiation or chemotherapy (chapters III-VI), 2. a study of the late effects of chemotherapy (especially cis-platinum) and radiation upon the hemopoietic stroma (chapters III-VI), 3. a study to compare the effects of cytotoxic treatment of young or old mice upon the HM (chapters III-V), and 4. a study of the effects of cis-platinum upon the lymphopoietic system (chapter VII).

1. A comparison of the different assays to detect stromal integrity with respect to extent and kinetics of stromal damage and recovery after radiation or chemotherapy treatment.

The parameters or assays used to determine quantitative and/or qualitative defects of the hemopoietic stroma are mentioned in the introduction to the experimental work and have been introduced in chapter I.3. As discussed in chapter I.3 some of these assays determine the proliferative capacity of stromal cells and some determine the hemopoietic supportive function of stromal cells, or a combination of these two. A comparison of these assays may therefore give a better understanding of which cell (fibroblast or other stromal cell) and cell function (proliferation or the support for hemopoiesis) is most susceptible to chemotherapy or radiation. In the chapters III-VI most of the above described assays have been applied and their significance for the measurement of stromal damage has been evaluated. We have shown that the different assays used to estimate stromal integrity do not correlate with respect to extent and kinetics of the cytotoxic treatment-induced stromal damage as will be discussed below. Apparently they detect different stromal functions or regenerating kinetics of different stromal cell populations. This strengthens the notion that the use of a single stromal assay can lead to erroneous interpretations of the stromal function after cytotoxic treatment.

CFU-F

Quantification of the *in vitro* clonable fibroblastoid colony-forming unit, or CFU-F is the only assay solely dependent on the proliferative capacity of a single stromal cell type i.e. the fibroblast. The fibroblasts are an important component of the *in vivo* and *in vitro* HM and there exists ample evidence for a regulatory role for fibroblastoid cells in *in vitro* culture systems, but their role in regulating hemopoiesis in the *in situ* marrow HM is not (yet) proved (chapter I.2.2.3.2). As

has been shown in animal experiments, CFU-F quantification may predict the reserve capacity of the stroma to regenerate and to replace damaged stromal cells after chemotherapy or irradiation-induced damage (chapter I.3.3). This assay and the ectopic implantation technique discussed below are therefore sensitive methods to detect latent residual damage (Friedenstein et al., 1976; Hotta et al., 1983; Tavassoli et al., 1973a). The CFU-F assay, however, does not provide a functional assay of the (supposed) regulatory role of the fibroblasts (chapter I.2.2.3.2 and I.3.3). The effects of both radiation and chemotherapy upon the CFU-F can be separated into acute and late effects. The acute effects after cytotoxic injury are in general determined by dose-response relations i.e. an increasing severity of response with increasing dose. The late effects are determined by the recovery kinetics of the surviving cell population e.g. the CFU-F.

The radiosensitivity of CFU-F (D_o range from 1.59 to 1.65 Gy, chapter III) is comparable to or even less than that of the hemopoietic progenitors CFU-s (D_o range from 0.8 to 1.2 Gy) and CFU-c (D_o range from 1.6 to 2.4 Gy) (Friedenstein et al., 1976; Testa et al., 1985; Xu et al., 1983; Werts et al., 1980). Furthermore, the toxicity of alkylating chemotherapeutic agents (CTX derivatives and cis-platinum) to the fibroblastoid precursor cell is comparable with the toxicity to hemopoietic progenitors (De Jong et al., 1985; and unpublished observations). However, recovery of the CFU-F population in time, is in general much slower than the recovery of hemopoietic progenitor cells after cytotoxic injury. This indicates that the capacity of the stroma to support a (near) normal hemopoietic progenitor content per femur and (near) normal peripheral blood cell numbers, can occur within a stroma in which one stromal cell class has lost its proliferative capacity (chapters III-V). The observation of a decreased proliferative capacity and a normal supportive function for bone marrow stroma *in vivo* and *in vitro* has also been observed by several other authors (Laver et al., 1986; Naparstek et al., 1985; Piersma et al., 1983a; Ploemacher et al., 1983; Song and Quesenberry, 1984) and a comparable effect has been found for thymus stromal cells (Hirokawa and Sado, 1984). This observation can be explained by the assumption that: **1.** the irradiated stroma still contains viable fibroblastic cells which have retained an adequate hemopoietic supportive function but have lost their *in vitro* colony-forming capacity as a result of irradiation-induced damage and **2.** there has been no need for replacement of these fibroblastic cells (see Fig.VIII.1B)(Piersma et al., 1985b). However, after a second insult to the stromal compartment which induces stromal proliferation (LPS injection), recovery of femoral CFU-F numbers has been observed (chapter III; Piersma et al., 1985b). This recovery after irradiation-induced damage has been explained by the assumption that the acute second insult induced by the LPS injection, subsequently stimulates the proliferation of radioresistant CFU-F to replace the latent damaged cells with viable cells, capable to form *in vitro* colonies (see Fig.VIII.1C and D)(chapter III; Piersma et al., 1985b).

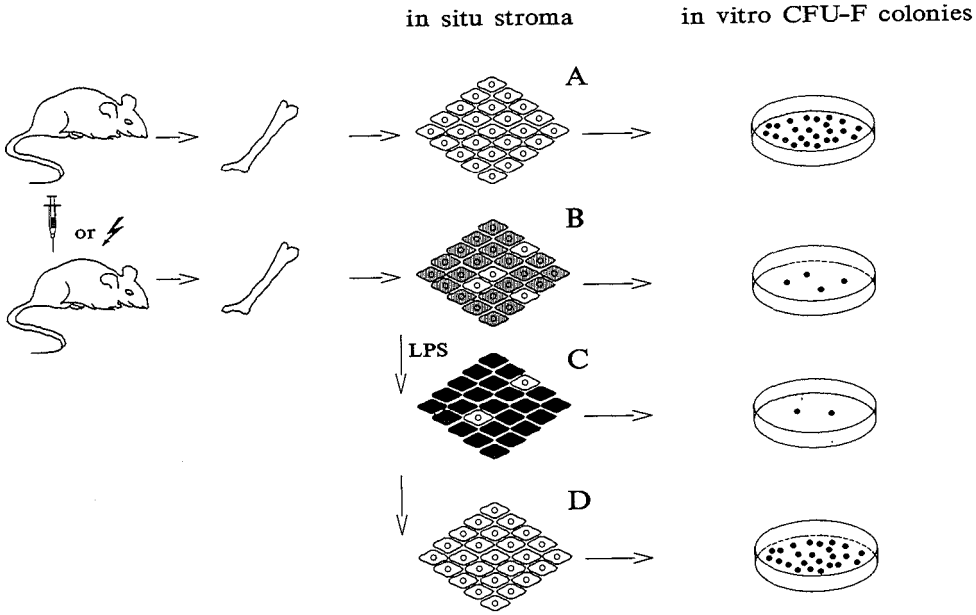


Figure VIII.1 A proposed mechanism for the in situ *recovery of radiation-induced damage and the proliferative capacity of CFU-F in* **adult** *mice. Schematical presentation of the* in situ *hemopoietic stromal compartment and the in vitro growth of fibroblastoid precursors (CFU-F). The* in situ *hemopoietic stroma and the* in vitro *colony forming ability of CFU-F in normal* **adult** *mice is schematically presented in A. Fig. B-D represent the effect of radiation on the stroma of* **adult** *mice. Following irradiation, the stromal compartment shows a good support for* in vivo *hemopoiesis but the CFU-F with latent radiation-induced damage (hatched cells), die after resumption of mitosis* in vitro *(B). After LPS-induced damage stromal cells are forced to proliferate* in vivo *and some radioresistant cells and all the latent damaged (hatched) cells die (black cells) after resumption of mitosis (C), and are replaced by normal (white) cells with a normal capacity to stimulate hemopoiesis* in vivo *and to form colonies* in vitro *(D).*

Ectopic implantation of femur and spleen

The quantification of the hemopoietic repopulation of ectopically transplanted femurs or spleens with donor-derived hemopoietic progenitors is dependent upon the regenerative capacity of host-derived stromal cells and the subsequent ability of these cells to maintain and support hemopoiesis. Differences in hemopoietic activity in ectopic implants are therefore dependent upon the integrity of donor stromal cells (chapter I.3.2). The limiting cell type(s) that determine(s) the regeneration of the microenvironment in ectopic implants of femur or spleen is (are) unknown. The CFU-F or fibroblastoid colony-forming unit is very likely not a

limiting factor because normal CFU-F numbers have been observed in combination with a severely defective hemopoietic activity in ectopic implants after irradiation (chapter III) or after injection with BU (chapter IV), or cis-platinum (chapter V). We conclude therefore that the development of the hemopoietic stroma in the ectopic implants may be more dependent upon another stromal cell, e.g. endothelial cells. The capillaries that pierce into the ectopic femur implant are from host origin but the newly formed marrow sinuses in the implant are from donor origin and are derived from primitive mesenchymal cells or surviving endothelial cells (Sahebekhtiari and Tavassoli, 1978). In the developing marrow stroma of the implants proliferation of stromal cells, probably including endothelial cells, can be detected upto two months after implantation (Sahebekhtiari and Tavassoli, 1978). However, it remains to be elucidated whether endothelial or primitive mesenchymal cell proliferation is the limiting factor in reoccurrence of optimal hemopoietic activity in the implants. In another, comparable system using transplantation of marrow plugs under the renal capsule, it has been suggested that the recipient can contribute in some way to the stromal environment of the grafted marrow (Schofield et al., 1987). A similar observation of a limited host participation in reestablishing hemopoiesis in subcutaneous implanted femur shafts has been reported by Fried et al. (1973b) using mice with the Sl/Sl^{d} genotype as donors and their normal littermates as recipients. However, in both systems the stromal defect has not been ameliorated and it is suggested that the contribution of the host is very limited. With respect to the interpretation of the ectopic transplantation data in the chapters III-V, the contribution of the host will, at most, result in an underestimation of the cytotoxic treatment-induced stromal damage.

The slow recovery of hemopoietic activity in the ectopic implants after chemotherapy-induced damage may be explained by a similar mechanism as is proposed for the CFU-F recovery after irradiation and LPS injection. The stromal cells (apart from CFU-F) not capable to proliferate *in vivo* and in the ectopic implants, due to cytotoxic agent-induced damage, are replaced by cells with a normal proliferative capacity during the slow turnover of the stromal compartment (see Fig.VIII.1 B-D) (Caffrey et al., 1966; Haas et al., 1968; Kaneko et al., 1982; Meyer-Hamme et al., 1971). We suggest that the slow turnover of stromal cells *in vivo* after chemotherapy is comparable with the LPS-induced proliferation of fibroblastic cells after irradiation. The slow hemopoietic recovery in ectopic implants is not observed after irradiation-induced damage. Apparently radiation induces another stromal damage or the *in vivo* recovery of chemotherapy-induced stromal damage follows another pattern as compared with the recovery after radiation, which must be forced by a second insult. The latter suggestion may be verified by ectopic implantation of femurs from irradiated and LPS-injected mice. Enforced regeneration of the stromal cells, responsible for the formation of an adequate implant stroma, has occurred if the hemopoietic activity returns to normal (see Fig.VIII.1 B-D).

The results of the subcutaneous implantation of spleens are in general com-

parable with the results from the femur although differences in extent and kinetics of the stromal damage can be observed (chapters III-V). This may be due to differences in regeneration patterns after subcutaneous implantation (chapter I.3.2), or differences in the stromal composition between spleen and femur. Alternatively, these differences may be due to the different function of the spleen as compared to bone marrow. The mouse spleen is an organ that shows an enormous increase in hemopoietic activity after stress-induced hemopoiesis (e.g. severe blood loss or infection) because the bone marrow of the mouse is completely filled with hemopoietic activity and can not expand. In general, the data concerning splenic transplantation are not as consistent as the femoral subcutaneous implantation data (chapters III-V). This may be due to the less optimal regenerative capacity of the spleen as compared with femur implants and the associated problems to detect low stem cell numbers. Even after stimulation of the splenic regeneration by irradiation of the hosts prior to implantation, splenic implants contain only approximately 3% of the hemopoietic progenitor content of a normal spleen, while femoral implants contain upto 25% of the *in situ* organ content of hemopoietic progenitors (Chamberlin et al., 1974). Pretransplantation radiation of a male host has no effect on the total amount of hemopoietic progenitors per femur implant. However, the less optimal regeneration of femur implants in female hosts can be stimulated by irradiation of the hosts (Schofield, 1986a). These observations have no effect on the interpretation of the data described in chapters III-V as only hosts of the same sex have been used.

LPS-induced increased splenic hemopoiesis

The extent to which hemopoietic stem cells accumulate in the spleen in response to bacterial LPS-induced hemopoietic stress is a measurement of the ability of the splenic stroma to respond to hemopoietic stress, and the proliferative capacity of the hemopoietic stem cells (chapter I.3.5). The injection of LPS results in a very complex reaction of many different cell types. However, the assessment of the hemopoietic activity in the spleen can be used to estimate splenic stromal integrity under the condition that the stem cells have a normal proliferative capacity as has been demonstrated by using irradiated and unirradiated parabiosed mice (Ploemacher et al., 1983). The injection of LPS induces a severe damage of the marrow microenvironment as determined by the decrease in CFU-F and the decreased ability to support normal hemopoiesis (chapter III). In addition, another component of the stroma may be damaged after LPS injection, i.e. the endothelial cells (Reidy and Schwartz, 1983). The splenic hemopoietic activity increases as a compensatory mechanism during the bone marrow recovery phase and is the result of local proliferation (McCulloch et al., 1970) and (some) migration of bone marrow CFU-s to the spleen (Ploemacher and Brons, 1987). Apparently LPS is less toxic to the splenic stroma, the CFU-F numbers are even increased after LPS injection, probably due to migration (Brockbank et al., 1983a). A decreased CFU-s accumulation in the spleen may be the result from **(a)** a decreased support capacity of the

splenic stroma to stimulate local CFU-s proliferation and/or **(b)** a decreased capacity of the splenic stroma to lodge bone marrow-derived CFU-s an/or **(c)** a decreased bone marrow CFU-s content that limits the migration of CFU-s to the spleen. In the experiments described in the chapters III-V, mice treated with chemotherapy or irradiation have not been supplemented with normal BMC after the cytotoxic treatment. This may result in an overestimation of the stromal damage detected using this assay.

Growth kinetics of normal bone marrow cells in animals with a normal or (supposedly) defective microenvironment

This assay determines the growth of injected normal BMC in lethally irradiated mice previously treated with chemotherapeutic agents and compares it with the growth of normal BMC in lethally irradiated mice that have not received chemotherapy (see Fig.VIII.2). This assay may have clinical relevance with respect to the increased use of (autologous) BMT in patients, previously treated with (high-dose) chemotherapy.

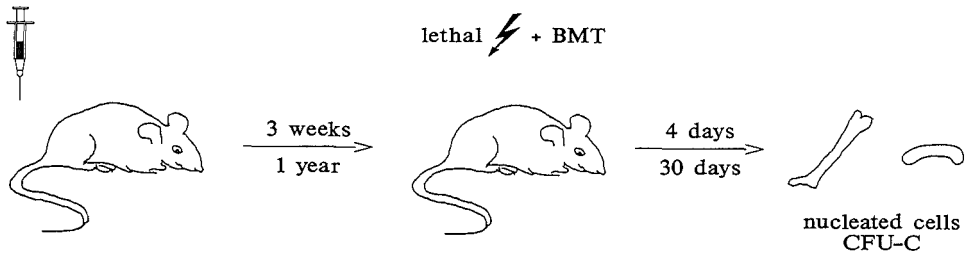


Figure VIII.2 Determination of the growth kinetics of normal bone marrow cells after lethal irradiation and bone marrow transplantation in mice previously treated with cytotoxic agents.

The delayed growth of normal hemopoietic stem cells in the Sl/Sl^{d} mice with a congenital anemia is the result from a marrow stromal defect (McCulloch et al., 1965; Sutherland et al., 1970). We assume that the recovery of the injected normal hemopoietic cells in mice (treated with chemotherapy at least three weeks prior to lethal irradiation and subsequent BMT), is only dependent on the stromal integrity and the capacity of the stroma to stimulate normal BMC (chapters IV and V). Cis-platinum treatment induced a severely delayed and diminished growth of normal BMC upto approximately 6 weeks after its administration (chapter V). The other chemotherapeutic agents tested do not show a significant late defect in the stimulation of hemopoietic cells following BMT one year after their administration. The hypothesis as has been suggested by Blackett (1987), i.e. regulation of the growth of injected BMC via a negative feedback control system from post-mitotic cells in the bone marrow, is not a very likely explanation for the observed

delay in hemopoietic cell recovery after lethal irradiation and BMT in previously cis-platinum treated mice after a comparison with the other assays which also detect a disturbed stromal integrity (chapter V). This strengthens the notion that the use of a single stromal assay can lead to erroneous interpretations of the stromal function after cytotoxic treatment.

The effects of chemotherapy treatment prior to lethal irradiation and subsequent BMT are dependent upon the time between these two cytotoxic treatments. Sublethal chemotherapy treatment (with CTX) followed by lethal irradiation and subsequent BMT within four days after chemotherapy, stimulates the growth of injected normal BMC. This stimulatory effect is no longer detectable seven days after the CTX injection (Gregory et al., 1971). The acute stimulatory effect has been thought to result from the cytotoxic effect of CTX upon the hemopoietic stem cells and not as an effect upon the hemopoietic stroma. It is assumed that the "niches" for the hemopoietic stem cells are liberated by CTX treatment immediately before lethal irradiation and subsequent BMT, and the injected stem cells have more "niches" available, resulting in an increased proliferation. Alternatively, the stimulatory effect may be due to a negative feedback control system from post-mitotic cells in the bone marrow (Blackett, 1987). However, mice that have been treated with CTX six weeks prior to irradiation, demonstrate a delayed endogenous hemopoietic recovery and it has been suggested to result from CTX-induced stromal damage (Fried and Barone, 1980). We suggest that the effects of chemotherapy treatment within a few days prior to irradiation are the result of hemopoietic progenitor cell damage while the late effects are more likely the result of stromal damage.

Long-term bone marrow cultures

Long-term bone marrow cultures (LTBMC) represent an *in vitro* culture system in which a very complex adherent layer composed of different cell types supports hemopoiesis (chapter I.3.4). The analysis of the role of each component in this system is difficult. This complex *in vitro* culture system for hemopoietic organs can be used in several different experimental set ups to estimate stromal integrity after cytotoxic injury i.e. "one-phase" and "two-phase" cultures. The establishment of a LTBMC, derived from mice previously treated with cytotoxic agents, is (see Fig.VIII.3 A-C) dependent upon the proliferative ability of stromal cells, and the support capacity of the stromal layer to maintain hemopoiesis. Production of hemopoietic cells is dependent upon the survival of hemopoietic progenitor cells during the initial growth phase of the stromal layer in a "one-phase" culture system (Fig.VIII.3 A) and in the "two-phase" system, using a supplement of non-adherent (non-stromal, and therefore hemopoietic) cells added at the initiation of the culture (Fig.VIII.3 B). The hemopoietic activity in the other "two-phase" system (see Fig.VIII.3 C) is not dependent upon the survival of hemopoietic cells during the initial growth phase of the stroma because the second inoculum of hemopoietic cells is added after establishment of the stromal layer. The established, stromal

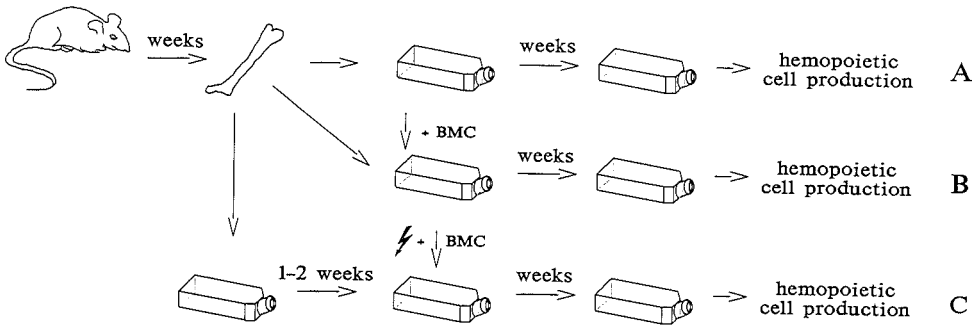


Figure VIII.3 The long-term bone marrow culture system. See text for explanation.

layer may be irradiated to eradicate possible surviving hemopoietic cells, before addition of the second inoculum of normal hemopoietic cells (Quesenberry et al., 1984). It is assumed that irradiation of the established, confluent, non-proliferating stromal layer does not affect its capacity to support and maintain hemopoiesis. However, it has been shown that irradiation of a stromal layer modifies hemopoietic growth factor production (Naparstek et al., 1985). It is not known whether irradiation can interfere with the initial cytotoxic agent treatment of the *in situ* stroma. Initiation of adherent layers without hemopoietic activity from cytotoxic agent-treated marrow cells reinoculated with untreated hemopoietic cells (see Fig.VIII.3 C, without irradiation of the stromal layer) will represent the most direct *in vitro* assessment of stromal integrity (Anderson et al., 1982; Hays et al. 1982). However, we have not succeeded in the production of a stromal layer depleted of hemopoietic activity by manipulating the nutrient composition of the media, therefore, we used the "two-phase" culture system as depicted in Fig.VIII.3 B, i.e. with supplementation of the inoculum with an abundant amount of hemopoietic cells to "overrule" the presumptive cis-platinum damaged hemopoietic progenitors (chapter VI). A reduced production of non-adherent cells in this system may be due to: **(a)** a decreased amount of stromal cells to form an adequate stromal layer, **(b)** a delayed growth of the stromal cells due to cytotoxic agent induced damage and subsequent extinction of the (normal or damaged) hemopoietic progenitors because of an insufficient amount of stromal "niches" for these cells, and/or **(c)** a decreased ability of the stromal cells to stimulate and maintain hemopoiesis. Using this LTBMC system we have not been able to detect a disturbance in stromal integrity after cis-platinum treatment although other stromal assays or parameters (i.e. CFU-F quantification and ectopic implantation) have detected stromal defects (chapter V). However, the proliferative capacity of the CFU-F seemed not to be disturbed much as the CFU-F population size has been observed to return to (supra)normal numbers within three weeks and to remain at this level upto one year after cessation of cis-platinum treatment (chapter V). Fibroblasts are an important component of the

stromal layer of LTBMC (chapter I.3.4) and it seems that the low amount of fibroblast progenitor cells at time of initiation is adequate to form a stromal layer. An explanation for the observed differences between the two assays (i.e. LTBMC and ectopic implantation) may be that the stromal compartment of LTBMC and ectopic implants are different of composition (e.g. the LTBMC stroma lacks a true vascular compartment although endothelial cells are present), or the limiting cell type to establish an adequate stromal compartment may be different for these two assays (e.g. endothelial cells for the ectopic implants and fibroblasts for the LTBMC). Furthermore, it may be possible that the added non-adherent cells change the stromal layer, although the non-adherent cells, derived by nylon wool filtration of normal BMC are depleted of CFU-F and incapable to form an adherent layer on their own. The capacity of added non-adherent cells to change the stromal layer is well known from inocula obtained from LTBMC non-adherent cells, which could even form a new stromal layer (Hays et al., 1982; Halka et al., 1987). Further research is needed to determine the effects of non-adherent bone marrow cell supplementation upon the *in vitro* development of the stromal layer e.g. by the use of non-adherent cells derived from mice with chromosome markers, by using cells with morphologically recognizable differences such as the giant granules seen in the C57Bl/6 Beige mutant mice (Murphy et al., 1973), or by using non-adherent cells from donors with the opposite sex and subsequent identification of morphology and genotype by *in situ* hybridization (Simmons et al., 1987).

Quantification of peripheral blood cells and hemopoietic progenitors in spleen and bone marrow

In combination with other assays and in some specific experiments determination of peripheral blood cell numbers may give information concerning stromal integrity. The observation that transplantation of normal BMC in lethally irradiated and previously cis-platinum treated mice can not correct the anemia (low erythrocyte numbers in the peripheral blood), while the reverse experiment i.e. reconstitution of normal (lethally irradiated) animals with cells from cis-platinum treated mice produce full recovery, strengthens the idea that cis-platinum treatment has the capacity to damage the stromal compartment (chapter V). A similar effect has been observed in mice with a congenital stromal defect, the Sl/Sl^{d} mice. A BMT with normal cells does not cure the anemia in the Sl/Sl^{d} mice but Sl/Sl^{d} BMC produce a full reconstitution in normal lethally irradiated littermates (McCulloch et al., 1965). However, in general, quantification of peripheral blood cells and hemopoietic progenitors is of no use to estimate fundamental damage to the hemopoietic stem cells or stroma (Haworth et al., 1982; Hendry et al., 1983; Ma et al., 1987; Ross et al., 1982; Schofield, 1986b; and appendix paper 1-3). Especially after irradiation but also after exposure to certain chemotherapeutic agents the damage to the hemopoietic stroma can be masked by compensatory proliferation of committed progenitors (Abkowitz et al., 1988; Cronkite et al., 1985; Hendry et al., 1983) or by an increased cycling rate of the stem cells (Croizat et al., 1979; Hendry

and Lajtha, 1972). After irradiation or chemotherapy normal or near normal peripheral blood cell numbers have been found with simultaneously a low hemopoietic progenitor content per femur and spleen (chapters III-V). Which of the two above mentioned factors or both have been responsible for the (near) normal peripheral blood cell numbers has not been explored.

2,3. Late effects of cytotoxic treatment and a comparison of cytotoxic treatment in young versus adult mice.

The data from the experiments described in the chapters III-V concerning late effects of radiation and chemotherapy upon the HM emphasize the necessity for a long follow up of patients treated with cytotoxic agents.

The observation of normalization of the CFU-F numbers per femur in mice irradiated during growth (chapter III) is not noted in adult irradiated mice from the same strain (Piersma et al., 1983a; Ploemacher et al., 1983). This can be explained by the assumption that during growth replacement occurs of fibroblastic progenitor cells, not capable to proliferate *in vivo* and *in vitro* due to radiation-induced damage, by cells which have the capacity to form *in vitro* colonies of at least 50 cells (see Fig.VIII.4). Thus, normal growth of the femoral stromal compartment during the first weeks of life has a comparable effect as the LPS-induced proliferation of fibroblastic cells after irradiation in adult mice (see Fig.VIII.1 B-D). We have not observed any differences in regeneration between young and adult mice after chemotherapy treatment (chapters IV and V).

From human data it is known that children tolerate the acute adverse effects of chemotherapy better than adults. The maximally tolerated dose expressed on a body weight or surface area basis is nearly always greater in children although the dose-limiting toxicity (e.g. myelosuppression) is in general the same (Bleyer et al., 1985; Glaubiger et al., 1982). The most susceptible tissues or organs for acute adverse effects after chemotherapy are in general those with high cell turnover rates. The precise mechanism(s) leading to less severe acute adverse effects of chemotherapy in children as compared with adults is/are poorly understood. It may be that children have an excess metabolic or excretory rate for these drugs as compared with adults or the children may have different intracellular metabolic rates. Another hypothesis for the increased drug tolerance is that children may have larger numbers of stem cells per organ and per unit body weight or surface area. On the other hand, the growing child may be more vulnerable than the adult patient to the delayed or long-term sequelae of cytotoxic treatment due to the rapidly proliferating state of the different tissues and organs as compared with the mature steady state with slow cell renewal kinetics in adults. In addition, chemotherapeutic agents may cause late effects due to damage of cells that do not replicate or replicate very slowly (chapter I.4). The partial regeneration and recovery of the hemopoietic stroma after the cytotoxic agent-induced damage (radiation, cis-platinum, and BU) is similar or better in mice treated during growth

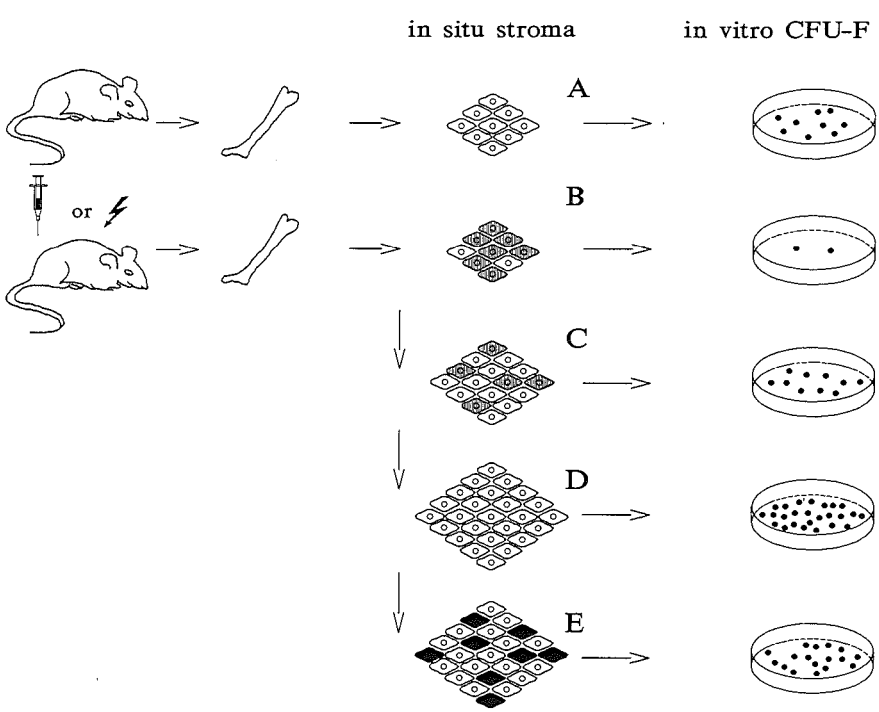


Figure VIII.4 *A proposed mechanism for the* in situ *recovery of radiation-induced damage and the proliferative capacity of CFU-F in* **young** *mice. Schematical presentation of the* in situ *hemopoietic stromal compartment and the* in vitro *growth of fibroblastoid precursors (CFU-F). The* in situ *hemopoietic stroma and the* in vitro *colony forming ability of CFU-F in normal* **young** *mice is schematically presented in A. Fig. B-D represent the effect of radiation on the stroma of* **young** *mice. The stromal cells with a normal capacity to stimulate hemopoiesis* in vivo *are not capable to form* in vitro *colonies and die after resumption of mitosis due to radiation-induced damage (hatched cells) (B). During growth the stromal cells proliferate* in vivo *and the latent damaged (hatched) cells die after resumption of mitosis and are subsequently replaced by normal (white) stromal cells with normal* in vitro *colony forming capacity and a normal capacity to stimulate hemopoiesis* in vivo *(C and D). One year after irradiation (E) the stromal compartment shows a decreased amount of CFU-F and a defective capacity to stimulate* in vivo *hemopoiesis, probably due to the limited proliferative capacity of stromal cells and subsequent slow extinction of the stromal cells (black cells)(see text).*

than in adult mice. However, after the initial recovery a second decrease in stromal function is observed. The CFU-F numbers are again decreased 1 year after irradiation and the hemopoietic activity in ectopic implants is reduced 1/2 to 1 year after BU or cis-platinum treatment. Furthermore, this decreased stromal function is accompanied with a decrease in hemopoietic progenitor content *in situ* after

all three cytotoxic agents (chapters III-V). This may indicate that the proliferative capacity of hemopoietic stromal cells is limited as is observed for skin-derived fibroblasts and other cells (Hayflick, 1965; 1984). Irradiation may induce senescence of the hemopoietic stromal cells. Since the hemopoietic system is very important for survival, there is an enormous proliferative reserve capacity far in excess of a normal individual lifespan. Thus aging of the hemopoietic system (hemopoietic progenitors and stroma) is difficult to observe (if at all) under normal conditions (Harrison et al., 1978; Schofield et al., 1986; Williams et al., 1986). Only after repeated stimulation of proliferation or after cytotoxic agent treatment, a decline in proliferative capacity has been detected in both hemopoietic stem cells and stromal cells (Boggs et al., 1984; Botnick et al., 1982; Hotta et al., 1980; Lipschitz et al., 1987b; MacMillan and Wolf, 1982; Reincke et al., 1982b; Wolf and Arora, 1982; van Zant, 1984). The decline in proliferative capacity can be due to lasting reproductive cell damage and will leave some daughter cells sterile after one or several divisions. Another explanation for the decline in proliferative capacity may be a decrease in the total amount of stem cells with an inherently limited mitotic ability (Hayflick, 1965; 1984). The experiments of Reincke et al., (1982b) support the latter suggestion of residual injury caused by a reduced population size with fully preserved high, but limited, proliferative capacity. In analogy this can be suggested for marrow stromal cells (e.g. CFU-F) as well. Irradiation of young mice results in survival of some CFU-F which repopulate the stromal compartment during normal growth of the organ and the CFU-F (and other stromal cells?), not able to proliferate due to cytotoxic agent-induced damage, are replaced by CFU-F with a normal proliferative capacity. Under normal conditions CFU-F numbers show no decline during aging (Brockbank et al., 1983b) but the presumably extensive proliferation of the surviving CFU-F to replace damaged cells during growth may be responsible for some CFU-F reaching the natural inherently (or cytotoxic agent-induced) limit of their reproductive lifespan one year after irradiation (see Fig.VIII.4 E). Subsequently, this may result in a decreased hemopoietically active bone marrow volume or a less optimal stimulation of hemopoiesis.

A similar mechanism as described above may be responsible for the second decrease observed in the capacity of ectopic implants to stimulate hemopoiesis 1/2 to 1 year after BU and cis-platinum treatment. The decrease in regenerative capacity of subcutaneous implants, derived from cis-platinum treated mice, after initial recovery to normal can not be attributed to a limited proliferative capacity of the CFU-F population because the CFU-F numbers are normal (chapter III). Another stromal cell with limited proliferative capacity must be responsible for this decline e.g. endothelial cells. Endothelial cells are damaged after several different cytotoxic agents and subsequently show an extensive proliferative activity after e.g. radiation, bleomycin, and CTX (Adamson and Bowden, 1974; 1983; Hopewell et al., 1986; Lazo, 1986). Furthermore, endothelial damage is followed by fibroblast proliferation and fibrosis in several different organs (e.g. lung and bone marrow) and will result in further damage of the (hemopoietic) stroma (Adamson and Bowden,

1983; Knospe et al., 1960; 1968).

The significance of animal data to predict effects of cytotoxic agents in human is limited. Although different doses and/or dose regimens are used, in general for both radiation and chemotherapy single high-doses in animal studies and multiple dose regimens in humans, the maximal tolerated doses are comparable on a surface area basis (Freireich et al., 1966) and comparable effects are found.

In conclusion, both radiation and alkylating chemotherapeutic agents (cis-platinum and BU) have the capacity to decrease the regenerative ability or proliferative capacity of hemopoietic stromal cells which may result in latent damage and may become apparent as a hypoplastic syndrome with little tendency to recover to normal. If this decrease is a result of a disturbed remodulation of the stromal compartment, a cytotoxic agent-induced limited proliferative capacity of the stromal cells and a subsequent insufficient remodelling and replacement of the stromal compartment, or a disturbed hemopoietic supportive capacity of individual stromal cells remains to be elucidated. These hypotheses may be answered if we know exactly which cells and/or cell functions are determined by the different stromal assays. Cytotoxic treatment during growth results in similar (chemotherapy) or more severe (radiation) late effects as compared with treatment of adult mice. The data from the experiments described in chapters III-VI concerning late effects of radiation and chemotherapy upon the HM emphasize the necessity for a long-term follow up of patients (children and adults) treated with cytotoxic agents to document the adverse late effects and, to identify the etiologic agents and to alter treatment to give the least toxic therapy with the best quality and duration of survival. Meanwhile, the benefits of current cytotoxic therapy are well worth the potential sequelae upon the hemopoietic system.

4. Study of the effects of cis-platinum upon the lymphopoietic system

The aim of the study described in chapter VII has been to investigate the effects of repeated cis-platinum injections upon the lymphopoietic system. In general chemotherapeutic agents exert immunosuppressive effects. However, some chemotherapeutic agents, including cis-platinum, can augment immune responses (e.g. antibody production and delayed hypersensitivity reactions) at relative short intervals after injection of the cytotoxic agent (Mastrangelo et al., 1986). We have not observed any immunostimulatory effect or any effect upon the immunoglobulin production after cis-platinum injections. The observed immunosuppressive effects of cis-platinum upon the T cell compartment may be caused by a severely decreased amount of T cells capable to proliferate or by a, not irreversibly, decreased proliferative capacity of the T cells. Since the assays used are not specific to determine effects of the lymphopoietic stroma it remains to be seen whether cis-platinum also effects the lymphopoietic microenvironment.

CHAPTER IX

SUMMARY

Blood cell production is normally found in the bone marrow and in mice (the animal used for the investigations described in this thesis) and other rodents to a certain extent also in the spleen. In principle all blood cells can be produced by a single cell i.e. the pluripotent hemopoietic stem cell. This stem cell and its progeny proliferate and differentiate in bone marrow (and spleen) to produce all hemopoietic cells (chapter I.2.1). The hemopoietic organs are composed of specific stromal compartments that support the production of hemopoietic cells. The stromal compartment is not static but can be remodelled in response to certain stimuli e.g. severe blood loss or infection. The different hemopoietic lineages (erythroid, myeloid, and megakaryocytic) are not haphazardly distributed in the bone marrow but are found in specific places and in contact with specific cells or cell products e.g. macrophages and endothelial cells. The hemopoietic stem cell and the progenitor cell proliferation and differentiation, and release of mature functional end cells in the blood stream is thought to be under (partial) control of the stromal cells and cell products (chapter I.2.2).

Bone marrow damage, characterised by stem, progenitor, and/or stromal cell abnormalities has been observed after cytotoxic treatment, especially after radiation and after treatment with alkylating agents. Damage of the hemopoietic system may be latent and only becomes clinically manifest in situations of hemopoietic stress or after a second cytotoxic treatment (chapter I.4). The experiments described in this thesis have been performed to compare differènt assays, used to determine stromal integrity (chapter I.3), and to elucidate the effects of chemotherapy and radiation upon the hemopoietic microenvironment. Furthermore, we compared the effects of cytotoxic treatment of young (growing) and adult mice.

We have clearly demonstrated that the different stromal assays, used in the experiments described in chapters III-VI, do not correlate with respect to the extent and kinetics of stromal damage caused by treatment with chemotherapy or irradiation. Apparently these assays detect different stromal functions or regeneration kinetics of different stromal cell populations. This notion implies that the use of a single stromal assay can lead to erroneous interpretations of the stromal function after cytotoxic treatment.

In the chapters III-V we have demonstrated that both radiation and chemotherapy (cis-platinum, cyclophosphamide, and busulfan) have the capacity to diminish the proliferative capacity of stromal cells or diminish the capacity of the hemopoietic stroma to respond to stress-induced hemopoiesis. However, the *in situ* capacity to stimulate and maintain hemopoiesis by these stromal cells is preserved. Thus, a normal production of peripheral blood cells may be maintained by a stromal compartment with a defective proliferative capacity. The cytotoxic agents used (cis-platinum, cyclophosphamide, busulfan, and radiation) are capable to produce a

temporarily or sustained defect in the CFU-F colony forming ability and in the regenerative capacity of the hemopoietic stroma in ectopic implants. The CFU-F assay determines the proliferative capacity of a single stromal cell class i.e. fibroblastoid progenitor cells (chapter I.3.3). The ectopic implantation assay determines the proliferative capacity of stromal cells and the ability of these cells to stimulate and maintain hemopoietic activity. Ectopic implantation of hemopoietic organs results in regeneration of the stromal compartment, which remains mainly of donor origin while the hemopoietic progenitor cells in these implants are of host origin. The hemopoietic activity found in ectopic implants is therefore dependent upon the regenerative capacity of donor hemopoietic stroma (chapter I.3.2). The limiting stromal cell type(s) necessary for regeneration of hemopoietic stroma in implanted hemopoietic organs (e.g. endothelial cells and/or mesenchymal cells) is/are as yet unknown. The initial severe decrease in stromal integrity after cytotoxic treatment is followed by recovery to sub or (supra)normal values. The recovery of stromal damage is particularly noticeable in mice treated during growth and in adult mice which received a second insult to the hemopoietic stroma. In addition, radiation, busulfan, and cis-platinum treatment induce a second decrease in the CFU-F colony forming ability, in the regenerative capacity of the hemopoietic stroma in ectopic implants, or in both. This second decrease in stromal integrity (1/2 to 1 year after cytotoxic treatment) is accompanied with a defective *in situ* hemopoietic activity, i.e. a low hemopoietic progenitor content per femur and/or spleen.

The above mentioned effects of cytotoxic agents upon the stromal integrity can be explained by one "unifying hypothesis". **(1)** We assume that depending on the cytotoxic dose used, a certain amount of stromal cells die after resumption of mitosis due to the cytotoxic agent-induced damage while simultaneously these cells may demonstrate a normal hemopoietic supportive function i.e. a normal production of peripheral blood cells and a normal amount of hemopoietic progenitors per femur and spleen may be found. The cytotoxic agent-induced damage results in **(a)** low stromal cell colony numbers upon explantation *in vitro* (CFU-F) and/or **(b)** a reduced hemopoietic activity in ectopic implants due to a defective stromal implant regeneration. Whether this decrease is really a result of a disturbed remodulation of the stromal compartment or a disturbed hemopoietic supportive capacity of individual stromal cells remains to be elucidated. **(2)** We assume that recovery to (sub)normal numbers of the stromal integrity after a second insult or during growth is caused by forced *in situ* stromal proliferation (see Fig.VIII.1 and Fig.VIII.4). Cytotoxic agent-induced latent damaged stromal cells (which die after resumption of mitosis) are replaced by cells with a normal proliferative capacity that have survived the initial cytotoxic treatment and subsequently normal CFU-F numbers are found upon explantation *in vitro* and/or a normal hemopoietic activity in ectopic implants is found (see Fig.VIII.1 and Fig.VIII.4). The hemopoietic activity in the ectopic implants remains diminished after irradiation of young mice whereas CFU-F colony numbers return to normal. The fibroblastoid progenitor (CFU-F) is

apparently not the limiting cell type for the restoration of an adequate stromal compartment in the implants. The second decrease in stromal integrity (accompanied with a diminished capacity of the *in situ* stromal compartment to maintain normal hemopoietic progenitor numbers) observed after treatment with cis-platinum, busulfan, and radiation may be explained by the assumption that (3) the extensive proliferation of the surviving stromal cells necessary to replace the cytotoxic agent-damaged cells and the proliferation during normal growth and remodelling of the stroma is responsible for some stromal cells reaching their natural inherently (or cytotoxic-agent induced) limit of their reproductive lifespan. This limit in proliferative capacity subsequently results in a defective (slow) turnover and remodelling capacity of the stromal compartment and ensues in a disturbed stromal compartment with a less optimal maintenance of hemopoiesis (see Fig.VIII.4E). Treatment during growth results in similar (chemotherapy induced) or more severe (radiation induced) late effects as compared with treatment of adult mice.

Extrapolation of the data described in the chapters III-VII to the clinical use of chemotherapy and radiation is limited. However, it can be concluded that the benefits of cytotoxic cancer therapy are well worth the potential late sequelae upon the hemopoietic system but a long-term follow up of both children and adult patients treated with these agents is necessary to document the adverse late effects, to identify the etiologic agents and to alter treatment to give the least toxic therapy with the best quality and duration of survival.

CHAPTER X

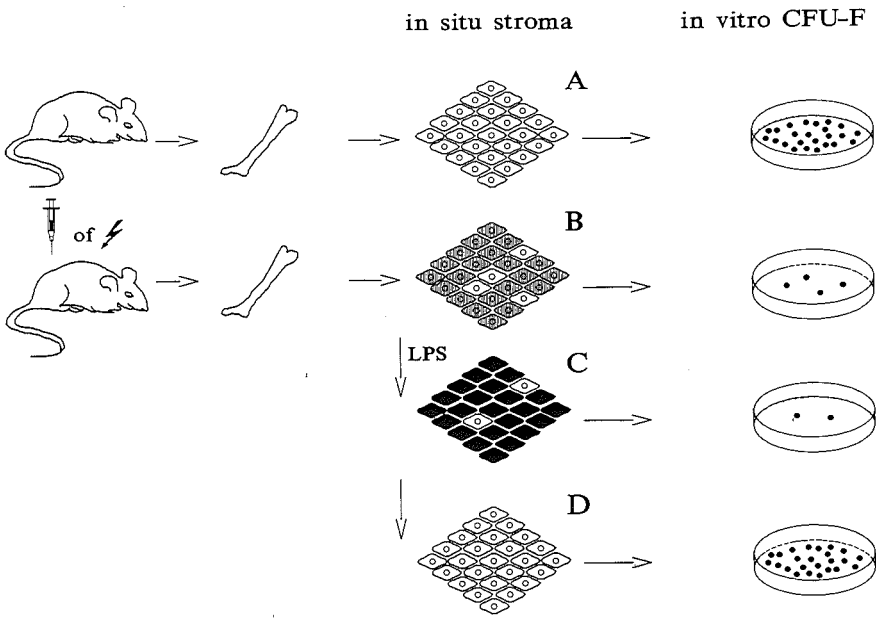
SAMENVATTING

Onder normale omstandigheden wordt bloedcelvorming, of hemopoëse, alleen aangetroffen in het beenmerg. In de muis (het proefdier dat gebruikt is voor de experimenten die in dit proefschrift zijn beschreven) zien we ook in de milt enige hemopoëse. In principe kunnen alle bloedcellen gevormd worden door één enkele cel, de zogenaamde pluripotente hemopoëtische stamcel. Deze stamcellen en hun nakomelingen (progenitor-cellen) prolifereren en differentiëren in het beenmerg (en de milt) en produceren alle bloedcellen. De bloedcelvorming in het beenmerg vindt plaats in een voor dit orgaan karakteristieke micro-omgeving of stroma. Voor een goed functionerende bloedcelvorming is zowel een normale stamcel als een normaal stroma van de hemopoëtische organen nodig. Het beenmerg stroma bestaat uit een dicht netwerk van dunwandige bloedvaten, de sinusoïden, met daartussen een matrix opgebouwd uit vezels en amorfe tussenstof. De matrix wordt gevormd door de stromale cellen, fibroblasten, macrofagen, vetcellen en endotheelcellen. Het beenmergstroma is geen statisch netwerk van vezels, cellen en bloedvaten maar wordt voortdurend geremodelleerd, met name in respons op bepaalde stimuli om de bloedcelvorming te verhogen zoals bij een infectie of bloedverlies. De verschillende differentiatiereeksen voor de bloedcelvorming (de erythroide, myeloide en megakaryocytaire reeks, respectievelijk rode en witte bloedlichaampjes en bloedplaatjes) zijn niet willekeurig verspreid in het beenmerg maar worden op zeer speciale plaatsen, namelijk in specifiek contact met stromale cellen (b.v. macrofagen of endotheelcellen) of celproducten gevonden. Proliferatie en differentiatie van de hemopoëtische stamcellen en progenitorcellen, en het afgeven van volwassen, uitgerijpte bloedcellen aan de bloedbaan staat (geheel of gedeeltelijk) onder controle van de stromale cellen en de verschillende factoren die deze cellen kunnen vormen (hoofdstuk I.2).

Schade aan het beenmerg, gekarakteriseerd door afwijkingen aan de hemopoëtische stamcel, de progenitorcel en/of het stroma, is waargenomen na chemotherapie en/of bestraling (beide middelen worden onder andere gebruikt voor de behandeling van patienten met kanker). De schade aan het hemopoëtische systeem kan latent aanwezig zijn en alleen aanleiding geven tot klinische symptomen indien er b.v. een extra beroep op de bloedcelvorming wordt gedaan (b.v. tijdens een infectie of na bloedverlies) of na een tweede behandeling met cytostatica en/of bestraling (hoofdstuk I.4). De experimenten die zijn beschreven in dit proefschrift zijn enerzijds uitgevoerd om een vergelijking te maken van de verschillende testsystemen, die stromale functie bepalen (behandeld in hoofdstuk I.3), en anderzijds om een beter begrip te krijgen van de effecten die cytostatica en bestraling hebben op de hemopoëtische micro-omgeving. Tevens hebben we de effecten van de behandeling met cytostatica of bestraling van jonge (nog groeiende) muizen vergeleken met die van volwassen muizen.

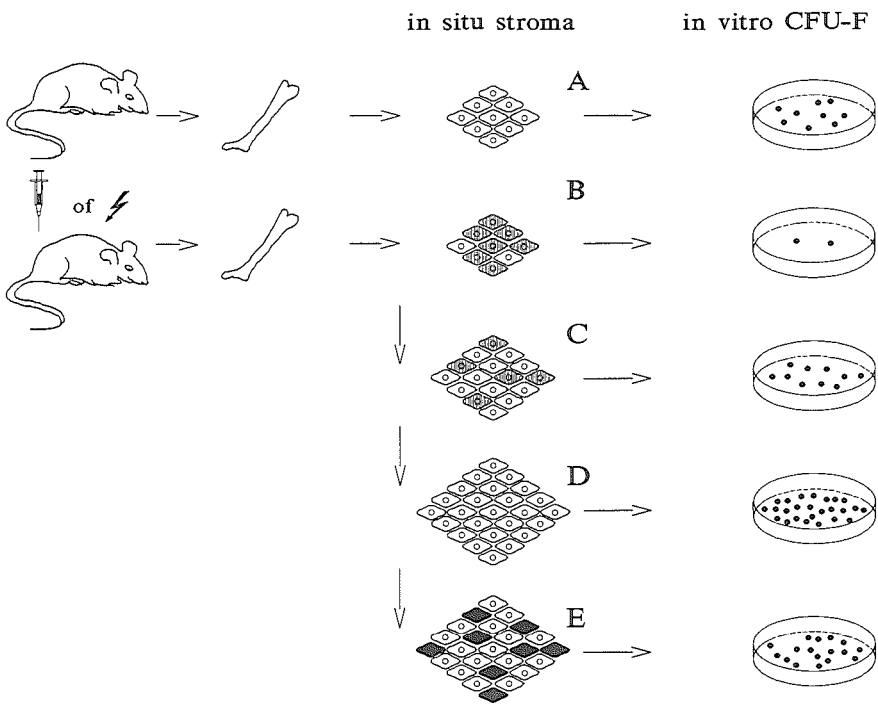
De experimenten beschreven in de hoofdstukken III-VI laten duidelijk zien dat de verschillende testsystemen met betrekking tot de ernst van de gemeten schade en het verloop van herstel van deze schade na behandeling met cytostatica of bestraling niet met elkaar correleren. Blijkbaar bepalen deze testsystemen verschillende stromale functies. Dit impliceert dat het gebruik van één enkele test om de schadelijke effecten van cytostatica of bestraling te bepalen op de hemopoëtische micro-omgeving tot verkeerde conclusies kan leiden.

De experimenten in de hoofdstukken III-V wijzen erop dat zowel bestraling als behandeling met cytostatica (cis-platinum, cyclophosphamide en busulfan) kunnen leiden tot een veminderd vermogen van stromale cellen om te prolifereren *in vitro* en te regenereren *in vivo* en ook kunnen leiden tot een verminderd vermogen van het hemopoëtische systeem om adequaat te reageren op stress (b.v. infectie). Tegelijkertijd echter is het vermogen van de stromale cellen om *in situ* de stam- en progenitorcellen te stimuleren en normale aantallen bloedcellen te vormen niet of nauwelijks aangetast zolang het stroma niet tot deling wordt aangezet. Dit betekent dat een normale productie van bloedcellen kan plaatsvinden in een stroma met een ernstig beschadigde capaciteit om te prolifereren. Cis-platinum, cyclophosphamide, busulfan en bestraling kunnen een tijdelijk of blijvend defect veroorzaken in het vermogen van CFU-F om *in vitro* kolonies te vormen. Ook het regenererend vermogen van het hemopoëtische stroma in ectopisch geimplanteerde organen (beenmerg en milt) is aangetast. Het bepalen van het aantal CFU-F kolonies *in vitro* is een maat voor de proliferatieve capaciteit van één enkel type stromale cel. Deze test geeft echter geen informatie over de (eventuele) regulerende capaciteit van fibroblastaire cellen (hoofdstuk I.2.2.3 en 1.3.3). Ectopisch (subcutaan) implanteren van hemopoëtische organen bepaalt echter het vermogen van meerdere verschillende stromale cellen om te regenereren en om hemopoëtische stam- en progenitorcellen te huisvesten en te stimuleren tot proliferatie en differentiatie. Het ectopisch implanteren van hemopoëtische organen induceert een sterke regeneratie van het stroma, dat vrijwel geheel van donor-origine blijft, terwijl de hemopoëtische stam- en progenitorcellen van gastheer-origine zijn. Deze normaal in het bloed circulerende hemopoëtische voorlopercellen worden aangevoerd via de nieuw gevormde bloedvaten in het subcutane weefsel die contact maken met de geimplanteerde organen. De hemopoëtische activiteit die in de geimplanteerde organen wordt aangetroffen is dus afhankelijk van het regenererende vermogen van het van de donor afkomstige stroma (hoofdstuk I.3.2). De stromale celtypen die de beperkende factor vormen voor een goede regeneratie van de hemopoëtische organen zijn niet bekend. De door de cytotoxische middelen geinduceerde acute stromale schade wordt in het algemeen gevolgd door een (ten opzichte van het herstel van stam- en progenitorcellen) traag herstel tot (sub- of supra)normale waarden. Dit herstel wordt met name gezien in muizen die op jonge leeftijd zijn behandeld. Op langere termijn echter, treed na dit herstel opnieuw een stromaal defect op. Deze late schade (1/2 tot 1 jaar na behandeling) gaat tevens gepaard met een daling van het aantal stam- en progenitor-cellen *in situ*.



Figuur X.1 *Een mogelijke verklaring voor de waargenomen stromale deficiënties na bestraling van het beenmergstroma. In A is het normale* in situ *hemopoëtische stroma in* ***volwassen*** *muizen schematisch weergegeven evenals de normale* in vitro *groei van CFU-F. Fig. B-D tonen schematisch de effecten van bestraling op het stroma van* ***volwassen*** *muizen. Na bestraling is er een goede stimulatie van hemopoëtische stam- en progenitorcellen* in vivo, *maar de beschadigde CFU-F (gearceerde cellen) sterven af nadat ze gaan delen (B). Na het toedienen van een tweede schade die het stroma aanzet tot proliferatie* in vivo *(injectie van lipopolysaccharide, LPS) worden de beschadigde (gearceerde) cellen die afsterven na deling (zwarte cellen) vervangen door normale (witte) cellen met een normale capaciteit om hemopoëse te stimuleren* in vivo *en kolonies te vormen* in vitro *(D).*

De hierboven genoemde effecten van chemotherapeutica en bestraling kunnen als volgt worden verklaard. (1) De stromale cellen worden door de cytotoxische middelen beschadigd maar ze zijn nog wel in staat om de productie van bloedcellen te stimuleren. Als de beschadigde cellen echter gaan delen *(in vitro* of *in vivo)* sterven ze af. Dit gebeurt in bepaalde assays, waarin het stroma tot deling wordt aangezet, zoals in de CFU-F assay en de ectopische orgaan transplantatie (zie Fig.X.1A en B en Fig.X.2A en B). (2) Het herstel van stromale schade tijdens de groei en/of na een tweede beschadiging wordt veroorzaakt door de normale (groei) of geinduceerde (tweede beschadiging) proliferatie van stromale cellen (zie Fig.X.1B-D en Fig.X.2B-D). De door de cytotoxische stoffen beschadigde stromale cellen sterven af nadat ze aan de deling zijn begonnen en worden vervangen door cellen



Figuur X.2 *Een mogelijke verklaring voor de waargenomen stromale deficiënties na bestraling van* ***jonge, nog groeiende*** *muizen. In A is het normale* in situ *hemopoëtische stroma in* ***jonge*** *muizen schematisch weergegeven evenals de normale* in vitro *groei van CFU-F. Fig. B-E geven schematisch de effecten van bestraling weer op het stroma van* ***jonge*** *muizen. De door bestraling beschadigde (gearceerde) stromale cellen, met een normale capaciteit om* in vivo *de hemopoëse te stimuleren, sterven af tijdens de deling en zijn niet in staat om* in vitro *kolonies te vormen (B). Tijdens de groei (waarbij ook de stromale cellen van de hemopoëtische organen delen) worden de beschadigde (gearceerde) cellen vervangen door cellen met een normale proliferatieve capaciteit en een normaal vermogen om stam- en progenitorcellen te stimuleren (witte cellen)(C en D). Een jaar na bestraling (E) is er een tweede daling zichtbaar in de* in vitro *kolonie vorming en in de* in vivo *stimulatie van de hemopoëse, mogelijk als gevolg van een beperkte delingscapaciteit van stromale cellen met als resultaat een langzaam verdwijnen van stromale cellen (zwarte cellen).*

met een normaal proliferatief vermogen die de cytotoxische behandeling hebben overleefd. Dit resulteert tenslotte in een normaal *in vitro* kolonievormend vermogen van de CFU-F en in een normale hemopoëtische activiteit van ectopisch geimplanteerde organen. De hemopoëtische activiteit in vooraf bestraalde organen, die subcutaan worden getransplanteerd, blijft verlaagd, ook de tijdens de groei bestraalde organen tonen geen herstel terwijl de *in vitro* kolonievormende capaciteit

van de CFU-F wel hersteld. De fibroblastaire voorlopercellen zijn dus niet de beperkende factor voor de regeneratie van het stroma van ectopisch geimplanteerde hemopoëtische organen. Een late (tweede) verlaging in de stromale functie wordt gezien na behandeling met cis-platinum, busulfan en bestraling en gaat tevens gepaard met een verlaging van het aantal hemopoëtische stam- en progenitorcellen *in situ*. Deze late stromale schade kan verklaard worden door te veronderstellen dat een percentage van de stromale cellen de limiet van hun proliferatief vermogen hebben bereikt door de voortdurende vervanging van beschadigde cellen. De beperkte delingscapaciteit van stromale cellen kan een natuurlijk aanwezige limiet zijn maar ook geinduceerd zijn door de behandeling met cytotoxische middelen. Deze limiet in de delingscapaciteit zou kunnen leiden tot een verstoorde omvorming en opbouw van de hemopoëtische micro-omgeving met als gevolg een verminderde capaciteit hemopoëtische stam- en progenitorcellen te huisvesten en te stimuleren tot proliferatie en differentiatie (zie Fig.X.2E).

Extrapolatie van de dierexperimentele gegevens zoals beschreven in de hoofdstukken III-VI naar de mogelijke schadelijke bijwerkingen van het klinische gebruik van cytostatica en bestraling is slechts in beperkte mate mogelijk, maar kan wel een indruk geven van de schadelijke effecten die na deze behandelingen op kunnen treden. De voordelen van het gebruik van zowel cytostatica als bestraling voor de behandeling van patienten met kanker wegen zeker op tegen de mogelijke schadelijke bijwerkingen. Het op lange termijn volgen van patienten die behandeld zijn met deze middelen blijft echter noodzakelijk om de eventuele bijwerkingen vast te leggen en om de voor deze bijwerkingen verantwoordelijke stoffen te identificeren. Na evaluatie is het dan mogelijk om de therapie aan te passen door gebruik te maken van die cytotoxische middelen die de minste kans op schadelijke effecten geven met tegelijkertijd de beste kwaliteit en duur van overleving.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd op 9 november 1956 te Vlaardingen geboren. Hij doorliep lagere en middelbare school in Vlaardingen en behaalde het HAVO-b diploma in 1973 en het Atheneum-b diploma in 1975 aan de Christelijke Scholengemeenschap "Westland-Zuid" te Vlaardingen. In datzelfde jaar werd na een gelukkige loting begonnen aan de studie geneeskunde aan de Erasmus Universiteit te Rotterdam. In het derde studiejaar was hij werkzaam als student assistent op de afdeling Celbiologie II, met als onderwerp "leukocyten mobilisatie in leukemische muizen" (onder leiding van Prof.Dr. R. Benner). Het doctoraal examen werd behaald op 18 november 1980 en het artsexamen op 23 juli 1982. Vanaf augustus 1982 tot en met december 1986 was hij werkzaam binnen de vakgroep Celbiologie en Genetica waar onder leiding van Prof.Dr. O. Vos het in dit proefschrift beschreven onderzoek werd verricht. Sinds januari 1987 is hij in opleiding tot patholoog anatoom in het Academisch Ziekenhuis te Groningen (opleider Prof.Dr. J.D. Elema).